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Patterns of Gene Expression in Pituitary Carcinomas and Adenomas Analyzed by High-Density Oligonucleotide Arrays, Reverse Transcriptase-Quantitative PCR, and Protein Expression

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Very few of the genes that are important in pituitary tumor initiation, progression, and metastasis have been identified to date. To identify potential genes that may be important in pituitary tumor progression and carcinoma development, we used Affymetrix™ GeneChip HGU-133A-oligonucleotide arrays, which contain more than 15,000 characterized genes from the human genome to study gene expression in an ACTH pituitary carcinoma metastatic to the liver and four pituitary adenomas. Reverse-transcriptase real-time quantitative-PCR (RT-qPCR) was then used to analyze 4 non-neoplastic pituitaries, 19 adenomas, and the ACTH carcinoma. A larger series of pituitary adenomas and carcinomas were also analyzed for protein expression using tissue microarrays (TMA) (*n* = 233) and by Western blotting (*n* = 18). There were 4298 genes that were differentially expressed among the adenomas compared to the carcinoma, with 2057 genes overexpressed and 2241 genes underexpressed in the adenomas. The beta-galactoside binding protein galactin-3 was underexpressed in some adenomas compared to the carcinomas. Prolactin (PRL) and ACTH tumors had the highest levels of expression of galectin-3. The human achaete-scute homolog-1 ASCL1 (hASH-1) gene was also underexpressed in some adenomas compared to the carcinoma. Prolactin and ACTH tumors had the highest levels of expression of hASH-1. ID2, which has an important role in cell development and tumorigenesis, was underexpressed in some adenomas compared to the carcinomas. Transducin-like enhancer of split four/Groucho (TLE-4) was over-expressed in adenomas compared to the ACTH carcinoma. The differential expression of these genes was validated by RT-qPCR, by immunohistochemistry using TMA and by Western blotting. These results indicate that the LGALS3, hASH1, ID2, and TLE-4 genes may have important roles in the development of pituitary carcinomas.

Key Words: DNA microarray; pituitary; carcinoma; galectin-3.

Introduction

Pituitary adenomas constitute approx 10–15% of intracranial neoplasms, while pituitary carcinomas are extremely uncommon malignancies. However, recurrence of pituitary tumors is present in up to a third of adenomas, and some of these tumors represent aggressive or atypical adenomas. Recent studies have provided molecular insights into the pathogenesis of some pituitary tumors by identifying and characterizing specific genes that are altered during pituitary tumorigenesis *(1–16)*. Tumor progression to the development of pituitary carcinoma, usually from a preexisting adenoma, is less well understood, because pituitary carcinomas are extremely uncommon with a small number of reported cases *(17–19)*, Only a few studies have analyzed the genetic alterations in these malignant tumors *(20–22)*.

The identification of candidate genes important in pituitary tumorigenesis has been assisted by the use of expression-profiling techniques such as differential display and microarray analyses. Several normal genes involved in pituitary tumorigenesis have been identified by differential display *(3–5)*. Microarray analyses have been used in early studies of rat and mouse pituitaries *(23–25)*. Several recent studies have used DNA microarrays to identify putative candidate genes that may be important in pituitary tumorigenesis in humans *(26–29)*. The study of Evans et al. used c-DNA array with 7075 genes to compare normal pituitary

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and several types of adenomas and identified a few genes that were overexpressed in various types of tumors compared to normal pituitary *(26)*. Tanaka et al. used expression profiling to identify pituitary-specific genes that encoded normal pituitary-specific transcripts *(27)*. Morris et al. used the Affymetrix HG-U133A GeneChip oligonucleotide array and pooled RNA from normal pituitary and pituitary adenomas to characterize genes that may be important in pituitary tumorigenesis including lysosome-associated protein transmembrane-4-B, BCL-2 associated athanogene, and the CDK inhibitor p18 *(28)*.

Because of the lack of concordance between different microarray platforms, validation of the microarray data is required *(29,30)*. Analysis of pituitary carcinomas by DNA array has not previously been reported mainly because these cancers are extremely uncommon. In this study, we used the Affymetrix GeneChip HGU-133A array to study an ACTH-secreting pituitary carcinoma that had metastasized to the liver and compared the carcinoma to four pituitary adenomas in order to characterize genes involved in the development of pituitary carcinomas. The validation of individual gene expression was performed using groups of normal pituitaries, pituitary adenomas, and pituitary carcinomas by RT-qPCR, immunohistochemistry with TMA, and Western blot analyses.

Results

The expression profiles of the ACTH carcinoma were compared to four adenomas including a PRL adenoma, a GH adenoma, an ACTH adenoma, and a FSH/LH adenoma with the Affymetrix GeneChip Hu95A. There were a total of 4298 genes that were differentially expressed among the pituitary adenomas compared to the carcinoma. There were 2057 genes overexpressed in the adenomas and 2241 genes underexpressed in the adenomas compared to the ACTH carcinoma.

Expression Profiles of PRL Adenoma

In the PRL adenoma array, 602 genes were overexpressed ≥ 2.0-fold, while 533 genes were underexpressed. PRL was over-expressed >200-fold, while angiopoietin-1 was overexpressed by 23-fold, and TLE-4 was overexpressed by 48-fold. Other overexpressed genes included VGF by 9-fold, ATM by 4.6-fold, and RAB-25 by fivefold. Underexpressed genes included PTTG-1 by 2.5-fold, LGALS8 by fourfold, IL-6R by fourfold, VEGF by fivefold, and CHGA by sixfold. GADD-45 gamma was underexpressed by 80-fold, ID2 by ninefold, while POMC was underexpressed by 100-fold.

Expression Profile of GH Adenoma

In the GH adenoma array 449 genes were overexpressed and 566 genes were underexpressed by greater than twofold in each category. Overexpressed genes included GH by 1162-fold, PRL by 132-fold, FOS by 85-fold, DAPK-1 by 4.5-fold, while underexpressed genes included FOXO-1A by threefold, ASH-1 by fourfold, and HMG-2L1 by 3.1-fold.

Expression Profile of ACTH Adenoma

In the ACTH adenoma array, there were 406 overexpressed and 528 underexpressed genes greater than twofold in each group. Overexpressed genes included galanin by 9.3-fold, GADD45 beta by 8.7-fold, ATM by 4.6-fold, AR by 4.6-fold, RAB-25 by 4.2-fold, hASH-1 by 2.9-fold, and PRKAR-1A by 2.3-fold. Underexpressed genes included PTTG-1 by 2.1-fold, TP53 by 2.5-fold, MEG3 by 2.5-fold, CHGA by 6.2-fold, and LGALS-3 by 10-fold.

Expression Profile of FSH/LH Adenoma

In the FSH/LH adenoma array, there were 600 overexpressed and 614 underexpressed genes by more than twofold each. The overexpressed genes included TLE-4 by 13.2-fold, DAPK-1 by 6.5-fold, ATM by 4.7-fold, AAMP by 2.8-fold, while underexpressed genes included VEGF by ninefold, IL-6R by ninefold, hASH-1 by 19.5-fold, POMC by 100-fold, galanin by 33-fold, ID2 by 9-fold, and LGALS-8 by fourfold.

Identification of Candidate Genes

Because of the large number of putative candidate genes generated by the DNA microarray analysis, in order to determine some of the possible genes important for tumor progression in pituitary tumors, we selected a small number of genes to validate by RT-qPCR, immunohistochemistry, and Western blotting. This approach included examining specific genes that were detected as being overexpressed or underexpressed by twofold or more in pituitary adenomas compared to pituitary carcinoma. The genes selected for validation included LGALS-3, ASH-1, ID2, and TLE-4. The genes were selected based on a twofold or greater increase expression in the adenomas or carcinomas, and the detection of the genes of another member of the same family in two or more adenomas compared to the carcinoma. For example, ID2 was detected in all four adenomas, ASH-1 in three tumors, TLE-4 in two tumors, while LGALS-3 was present in one tumor, but LGALS-8 was present in two additional tumors.

Validation Studies

RT-real time quantitative PCR for LGALS-3 showed the highest level of expression in PRL and ACTH adenomas and the ACTH carcinoma with low levels of expression in GH, gonadotrophin, and null cell adenomas (Fig. 1A). TMA and Western blot also showed the highest levels of protein expression in PRL and ACTH tumors (Figs. 1B–D). Examples of immunohistochemical staining for galactin-3, ID2, and TLE-4 are shown in Fig. 2. RT-qPCR analysis of hASH-1 also showed fivefold greater expression in the PRL and ACTH adenoma groups and the ACTH carcinoma compared to the GH, gonadotrophin, and null cell

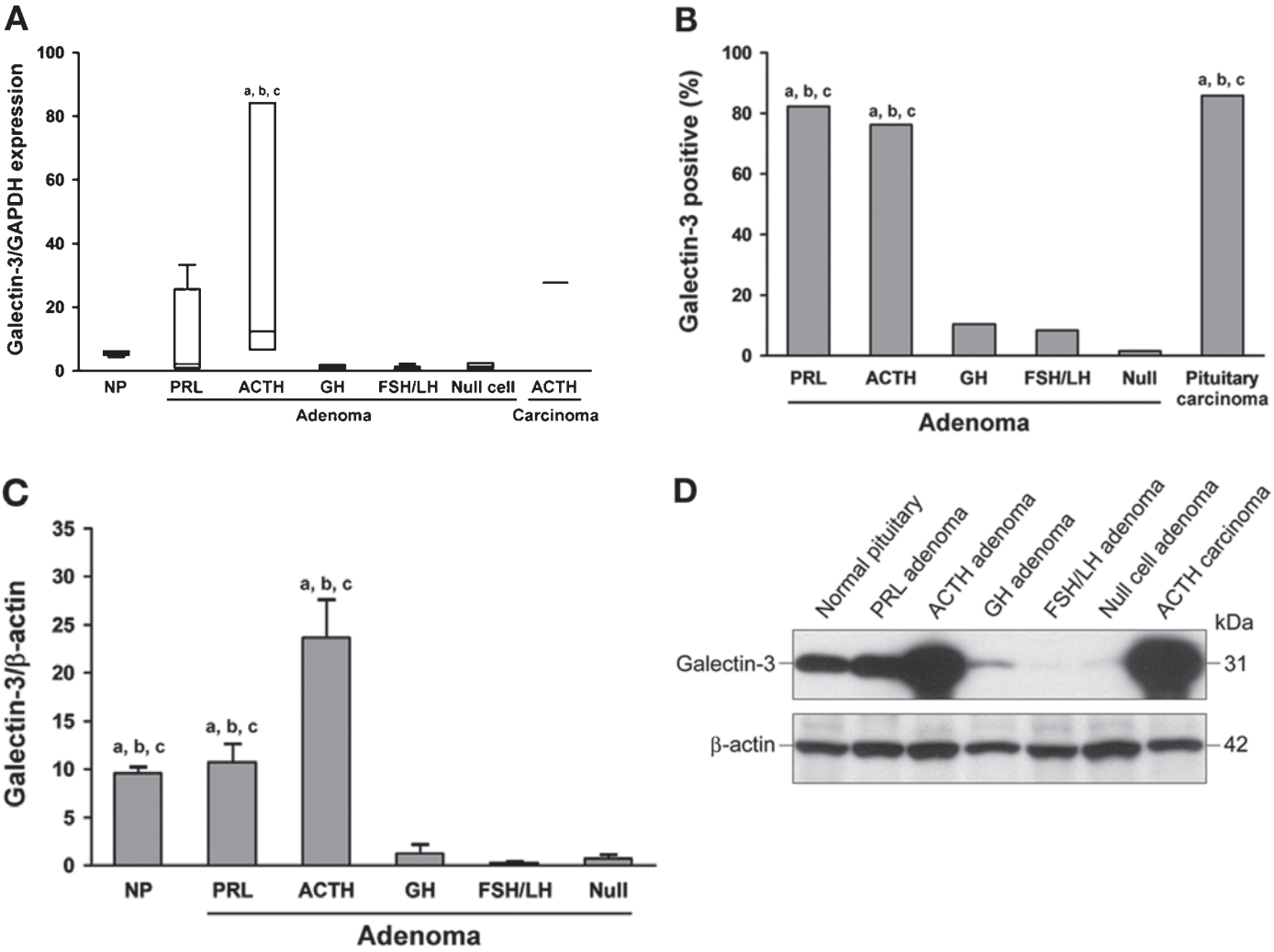

Fig. 1. (**A**) RT-qPCR graphs showing the mRNA expression levels of galectin-3 in normal pituitary (NP), pituitary adenomas, and an ACTH pituitary carcinoma. Samples were from four different normal or tumor tissues except for three ACTH adenomas and one ACTH carcinoma. The whiskers represent the maximum and minimum values, the boxes represent the 25th and 75th percentile ranges of values, and the bar represents the median value. Galectin-3 in ACTH adenomas was significantly overexpressed compared to the GH (a), FSH/LH (b), and null cell (c) adenomas ($p < 0.05$). The Kruskal-Wallis analysis of variance and *t*-test were used to calculate the differences in expression. (**B**) TMA analysis of galactin-3 expression in 226 pituitary adenomas and 7 pituitary carcinomas. The positive percentage of each tumor type is shown. The ACTH and PRL adenomas and the ACTH carcinomas had significantly higher levels of expression of galactin-3 compared to the GH (a), FSH/LH (b), and null cell (c) adenomas. (**C**) Western blot analysis of galactin-3 expression in normal pituitary and pituitary adenomas. The ACTH carcinoma, ACTH adenoma, and PRL adenoma have significantly higher levels of galactin-3 compared to the GH (a), FSH/LH (b), and null cell (c) adenomas ($p < 0.05$). $n = 3$ for each group. The results were normalized relative to β-actin. (**D**) Representative Western blot showing expression of galactin-3 by a normal pituitary, pituitary adenomas and an ACTH pituitary carcinoma. β-actin was used to normalize for gel loading.

group (Fig. 3A). Western blots showed the highest levels of expression in PRL and ACTH tumors (Figs. 3B,C). RT-qPCR analysis of ID2 showed variable distribution in all adenoma types with a slightly higher level in the ACTH carcinoma and in one growth hormone adenoma compared to other tumor groups (Fig. 4A). TMA analysis showed the highest level of ID2 expression in the pituitary carcinoma groups, which was significantly higher than the ACTH, FSH/LH, and null cell adenoma groups ($p < 0.05$) (Fig. 4B). RT-qPCR analysis of TLEL4 showed that PRL adenomas had the highest levels, while the carcinoma had the lowest level of expression (Fig. 5A). TMA analysis also showed that PRL adenomas had the highest level of TLE4 expression (Fig. 5B). PRL carcinomas ($n = 4$) also expressed high levels of TLE4, but ACTH carcinomas ($n = 3$) showed very low levels of TLE4 expression. Similar findings were seen in the Western blot analysis (Fig. 5C).

Discussion

The gene expression profiling of pituitary adenomas and ACTH pituitary carcinoma identified 2057 genes that were overexpressed and 2241 genes that were underexpressed in the adenomas. The use of the Affymetrix HGU-133A array

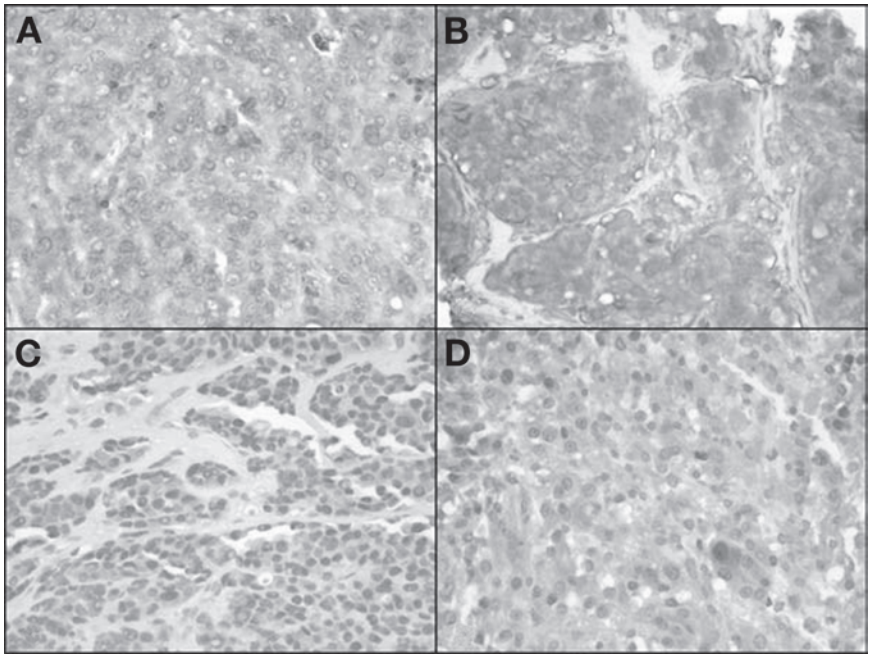

Fig. 2. Examples of immunohistochemical staining of TMA showing cytoplasmic staining for galactin-3 in an ACTH carcinoma (**A**). Cytoplasmic and focal nuclear staining for ID2 is present in a PRL carcinoma (**B**). Strong nuclear staining for TLE-4 is present in a PRL adenoma (**C**), and focal staining for TLE-4 is seen in an ACTH carcinoma (**D**).

with over 15,000 genes was used to identify candidate genes. The goal of this experiment was to identify specific genes that were overexpressed or underexpressed in pituitary carcinomas and aggressive adenomas that could (a) serve as markers to predict tumor progression or recurrence and (b) identify genes that could have therapeutic potential for treating pituitary adenomas or carcinomas.

Because of the variability reported in microarray analyses *(29,30)*, we used RT-qPCR, immunohistochemistry with TMA and Western blotting analyses to validate some of the potential candidate genes obtained from the DNA array studies. We selected four genes to validate which were overexpressed in the carcinomas including LGALS-3, hASH-1, and ID2, while TLE-4 was more commonly overexpressed in adenomas, compared to the ACTH carcinoma. The RT-qPCR analysis with four samples from each tumor type and non-neoplastic pituitaries provided further insight into the potential biological significance of some of the candidate genes. For example, PRL and ACTH adenomas expressed high levels of LGALS-3 and hASH-1 compared to GH, FHS/LH, and null cell adenomas. Because PRL and ACTH adenomas are the two adenoma types giving rise to most pituitary carcinomas *(17,18)*, these results appear to provide insight into the role of these genes in pituitary carcinoma development. However, our analyses also indicated that overexpression of some genes in pituitary carcinomas such as TLE-4 was dependent on the specific type of carcinoma. For example, PRL carcinomas had high levels of TLE-4, compared to ACTH carcinomas.

Galectin-3 is a β-galactoside binding protein involved in a variety of biological functions including cell proliferation and adhesion, angiogenesis, apoptosis, tumor progression, and metastasis *(31–39)*. There are over 13 members of the galectin family. A recent study from our laboratory showed that galectin-3 was expressed mainly in PRL and ACTH cells *(11)*. Absence of galectin-3 expression in some pituitary adenoma subtypes was due to epigenetic methylation of the LGALS-3 gene in some tumors *(12)*. The DNA array analysis found galectin-3 as well as galectin-8 overexpression in various tumors. A recent study detected galectin-8 in pituitary adenomas and cell lines by RT-PCR *(39)*. Because of the overexpression of galectin-3 only in specific subtypes of pituitary adenomas, galectin-3 may be a potential therapeutic target for patients with recurrent or metastatic pituitary tumors. Medical therapy with drugs that are stable inhibitors of galectin-3 such as UJ-2005 has been reported *(40)*.

We observed that hASH-1 was overexpressed in the ACTH tumors compared to the other tumors, and the RT-qPCR analysis showed much higher expression in the PRL and ACTH tumors compared to the other tumor types. The human achaete-scute homolog-1 (hASH-1) is a member of the basic helix–loop–helix (bHLH) family of transcription factors, which plays a critical role in neuronal/endocrine determination and differentiation during normal development of the nervous system and the endodermal endocrine cells *(41–46)*. hASH-1 has been detected in neuroendocrine tumors from the lung, gastrointestinal tract, and pituitary

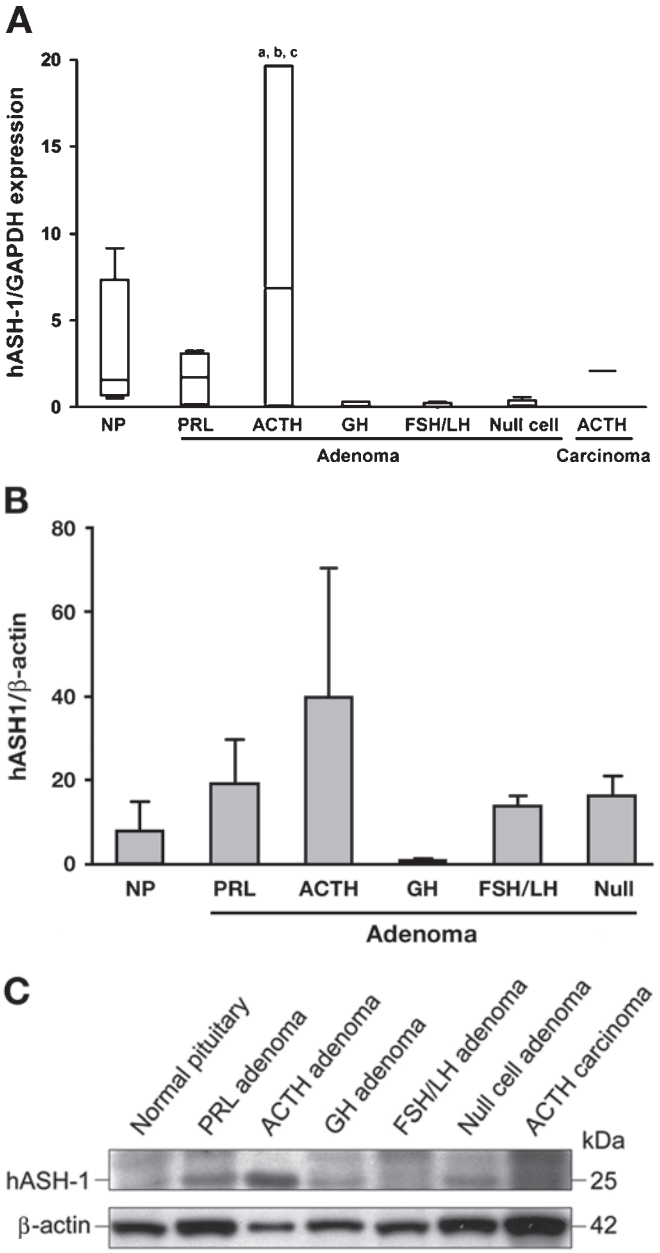

Fig. 3. (**A**) RT-qPCR graph showing the mRNA expression levels of hASH-1 in normal pituitary (NP), pituitary adenomas, and an ACTH pituitary carcinoma. Samples were from four different normal and tumor tissues except for three ACTH adenomas and an ACTH carcinoma. The whiskers represent the maximum and minimum values, the boxes represent the 25th and 75th percentile ranges of values, and the bar represents the median value. ACTH adenomas were significantly overexpressed compared to the GH (a), FSH/ (b), and null cell (c) adenomas ($p < 0.01$) and PRL adenomas ($p < 0.05$). (**B**) Western blot analysis of hASH-1 protein expression in pituitary adenomas and normal pituitary ($n = 3$ for each group). The results were normalized relative to β-actin. (**C**) Representative Western blot showing hASH-1 protein expression by a normal pituitary, pituitary adenomas and a pituitary carcinoma. β-actin was used to normalize for gel loading.

(44–46). In a study of 33 pituitary adenomas and pituitary cell lines, hASH-1 mRNA was detected in normal pituitaries, in corticotrophs and clinically nonsecreting adenomas, and in some adenomas derived from pit-1–dependent lineages *(45)*. However, these investigators did not separate pit-1–dependent PRL from pit-1–dependent GH adenomas *(45)*. Our analysis shows that hASH-1 is most commonly expressed in PRL and ACTH tumors, the two tumor subtypes that frequently progress to carcinoma. In other neuroendocrine tumors such as pulmonary neuroendocrine carcinoids and carcinomas, hASH-1 expression was correlated with a significantly shortened survival in small-cell carcinoma patients and was virtually absent in low-grade typical carcinoid tumors *(46)*.

ID2 was expressed by all groups of pituitary tumors but was expressed at a higher level in the pituitary carcinoma. The ID proteins (ID1 to ID4) have important roles in animal development and cancer because they antagonize HLH transcription factors and mediate mitogenic signals as well as inhibiting differentiation *(48–50)*. ID1 expression is mediated by TGF-beta, while ID2 and ID3 expression is induced by bone morphogenetic proteins (BMP). Knockdown of endogenous ID2 or ID3 sensitizes epithelial cells to BMP that leads to growth inhibition and induction of transdifferentiation, which is mediated by SMAD signal *(51)*. Because TGF-beta and BMP have significant regulatory roles in pituitary cell growth, ID2 may be an important molecule that may mediate these effects. So targeting ID2 may have potential benefits in the treatment of aggressive or metastatic pituitary tumors.

The transducin-like enhancer of split-4 (TLE-4) gene in humans, a mammalian homolog of the Groucho gene-related protein in mice, and the co-repressor protein Groucho in *Drosophila (52,53)* was upregulated in various pituitary adenomas compared to the ACTH pituitary carcinoma by DNA microarray analysis. The TLE family consists of four proteins of similar molecular weight and structure, which are broadly but specifically expressed in several developing organs in mammals and do not bind DNA directly but modulate the process of transcription by physically interacting with transcription factors. In the developing mouse pituitary, TLE-1 is important for pituitary organogenesis with interactions with HESX-1 and PROP-1 *(54–56)*. TLE co-repressors interact with multiple transcription factors including RUNT domain proteins, which have recently been identified in pituitary tumors in our laboratory (unpublished data). The TLE proteins, by binding to different transcription factors, can be docked to specific DNA regions and can form multimeric complexes, which influences histone acetylation and chromatin structure including recruitment of histone deacetylases *(54)*. Histone deacetylases could remove acetyl residues from histone, making the chromatin more compact and transcriptionally inactive *(54)*. In our TMA studies, which had seven carcinomas for analysis, TLE-4 was highly expressed in all four PRL carcinomas but was expressed only focally in the ACTH carcinomas. This observation highlights the importance of lineage-specific expression of pituitary genes and emphasized the need

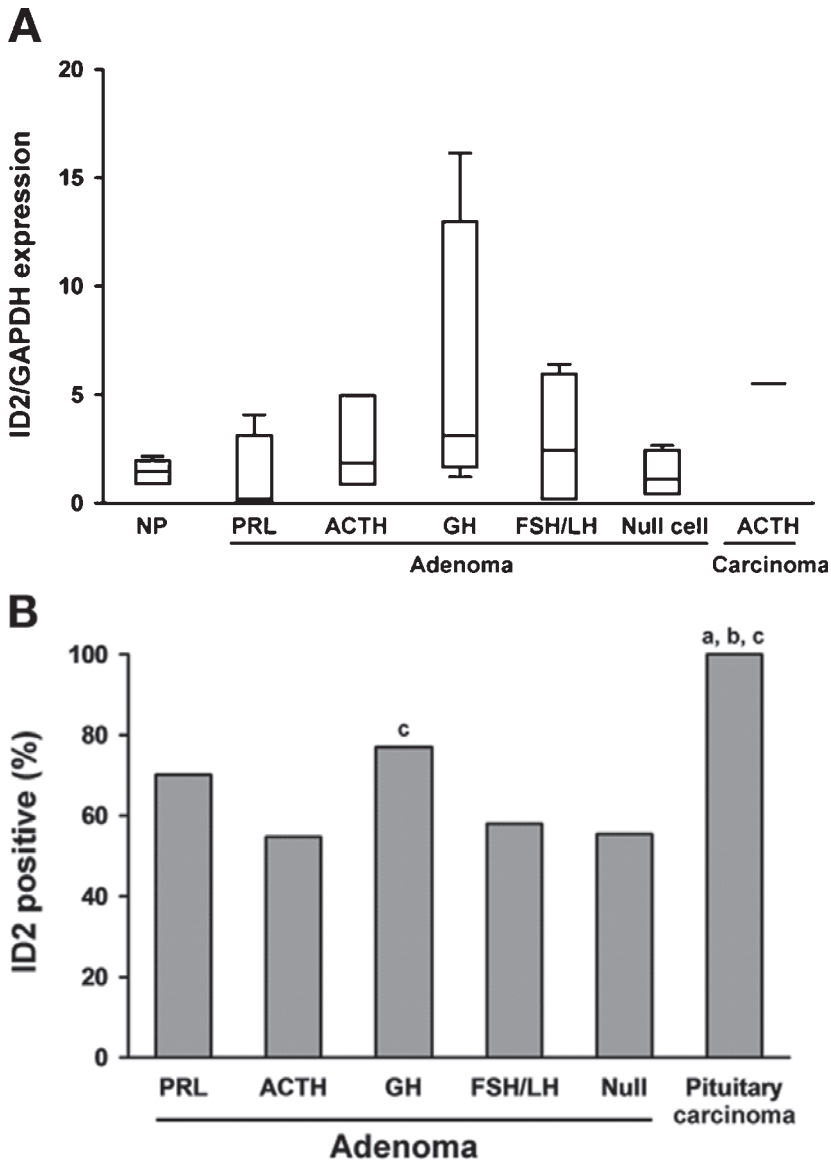

Fig. 4. (**A**) RT-qPCR graph showing the mRNA expression levels of ID2 in normal pituitary (NP), pituitary adenomas, and an ACTH pituitary carcinoma. Samples were from four different normal or tumor tissues except for three ACTH adenomas and an ACTH carcinoma. The whiskers represent the maximum or minimum values, the boxes representing the 25th and 75th percentile ranges of values, and the bar represents median value. Kruskal–Wallis analysis of variance and *t* test were used to calculate the differences in expression. (**B**) TMA analysis of ID2 protein expression in 226 pituitary adenomas and 7 pituitary carcinomas. The positive percentage of each tumor type is shown. The pituitary carcinomas were significantly greater than the ACTH (a), FSH/LH (b), and null cell (c) adenomas ($p < 0.05$).

to validate DNA array studies with different methods. More work will be required to elucidate the role of TLE in pituitary tumor progression and metastasis.

The identification of some of the genes that have been previously reported to be important in pituitary tumorigenesis in our DNA microarray analyses such as PTTG-1 *(3,15)*, GADD45 gamma *(4)*, TP53 *(17)*, MEG3 *(14)*, and DAPK-1 *(9)* supports the utility of DNA microarray in identifying putative candidate genes that may have a role in pituitary tumorigenesis. A recent report analyzing clinically nonfunctional pituitary adenomas also identified several of the genes or related genes detected in our studies, including TLE-2 and hASH-1, which were upregulated in adenomas compared to normal pituitaries *(56)*.

In summary, we have analyzed the expression profile of more than 15,000 genes comparing pituitary adenomas to a case of pituitary ACTH carcinoma by DNA microarray analysis. Validation by RT-qPCR, immunohistochemical analysis, using TMA, and Western blotting supported the differential distribution of some of these gene and protein products in pituitary tumors. The importance of galectin-3 in pituitary tumor progression has previously been demonstrated *(11,12)*. However, more work is needed to characterize the importance of the other candidate genes identified

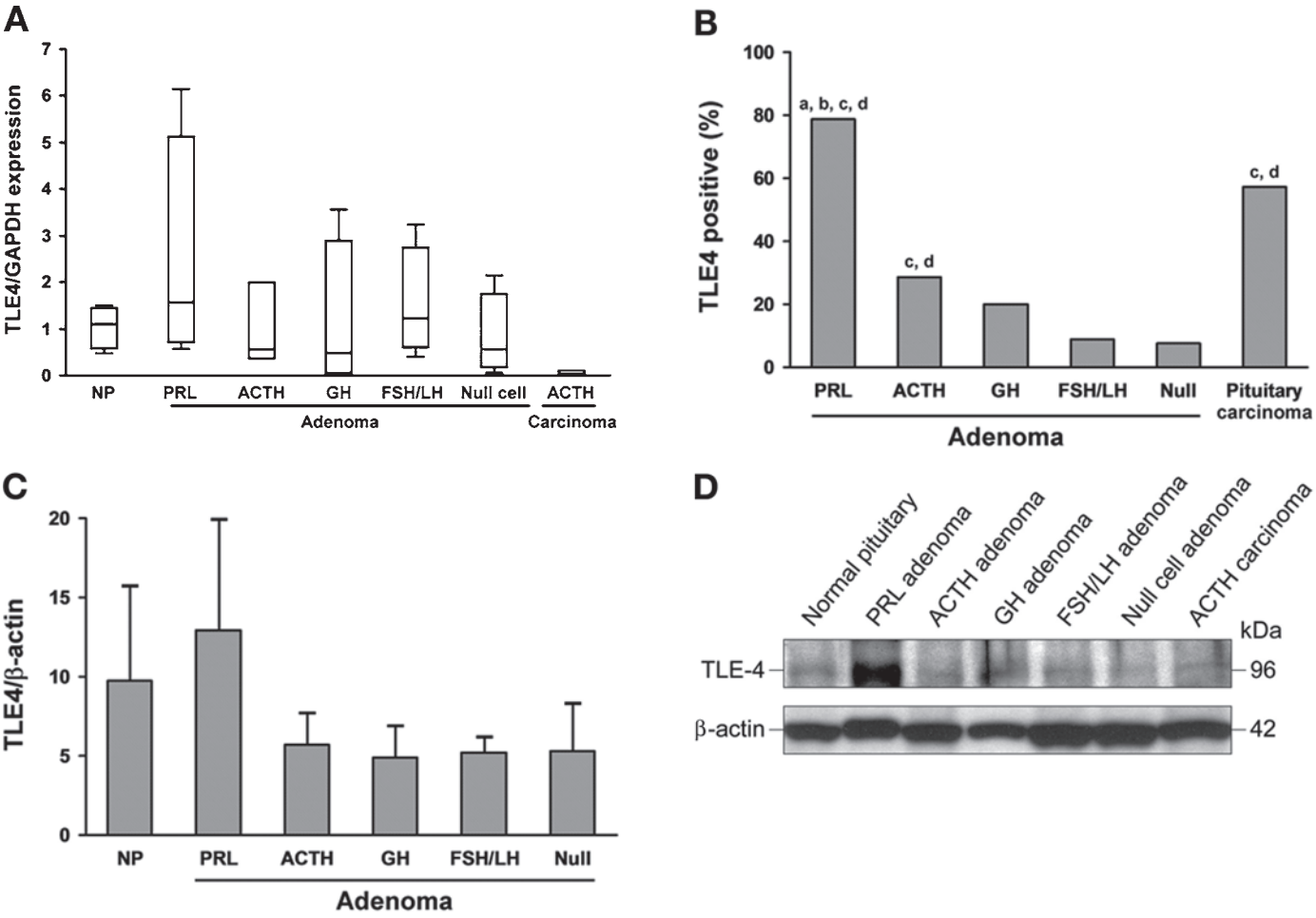

Fig. 5. (**A**) RT-qPCR graph showing mRNA expression levels of TLE-4 in normal pituitary (NP), pituitary adenomas, and one ACTH pituitary carcinoma. Samples were from four different normal or tumor tissues except for three ACTH adenomas and one ACTH carcinoma. The whiskers represent the maximum and minimum values, the boxes represent the 25th and 75th percentile ranges of values, and the bar represents the median value. Kruskal–Wallis analysis of variance and *t* test were used to calculate the differences in expression. (**B**) TMA analysis of TLE-4 protein expression in 226 pituitary adenomas and 7 pituitary carcinomas. The positive percentage of each tumor type is shown. Among the carcinomas, the PRL ($n = 4$) tumors were strongly positive, while the ACTH carcinomas ($n = 3$) were only focally positive or negative. Staining in the PRL adenomas and carcinomas was significantly greater than other tumor types. Statistical comparisons to ACTH adenoma (a), GH adenoma (b), FHS/LH adenoma (c), and null cell adenoma (d) are indicated ($p < 0.01$). (**C**) Western blot analysis of TLE-4 expression in normal pituitaries and pituitary adenomas ($n = 3$ cases for each group). The results were normalized relative to β-actin. (**D**) Representative Western blot showing TLE-4 expression in a normal pituitary, pituitary adenomas and an ACTH pituitary carcinoma. β- actin was used to normalize for gel loading.

in this study for pituitary tumor progression and metastasis. Our studies highlight the unique nature of each type of pituitary tumor, which is dependent on the hormone produced and emphasizes the unique molecular alterations in different types of pituitary tumors.

Materials and Methods

Patients and Tumor Characterization

Four pituitary adenomas (PRL, GH, ACTH, and FSH/LH) and an ACTH pituitary carcinoma metastatic to the liver were used for microarray analysis (Table 1). Institutional Review Board permission was obtained for this study. DNA microarray analysis was used to compare the gene expression profile of the pituitary carcinoma using duplicate samples of each case. The duplicate samples were made after RNA extraction. The carcinoma was compared to cases of prolactin, growth hormone, ACTH, and an FSH/LH adenoma. For RT-qPCR studies, we examined four normal pituitaries obtained within 8 h postmortem from patients without endocrine diseases. Samples for the normal pituitaries were taken from sections including the lateral wings and mucoid wedge to include all cell types in the anterior pituitary: nineteen adenomas and an ACTH carcinoma which was used for high-density oligonucleotide arrays (HDAs) analysis. TMAs were made up of 233 samples of PRL, GH,

Table 1
Clinicopathologic Features of Pituitary Tumors Used for DNA Microarray Analyses

Diagnosis	Age/Sex	Immunohistochemical Findings	Follow-up
PRL adenoma	46/F	Positive for PRL	No recurrent tumor (4 yr)
GH adenoma	56/M	Positive for GH diffusely, focally positive for PRL	No recurrent tumor (6 yr)
ACTH adenoma	44/M	Positive for ACTH	No recurrent tumor (5 yr)
FSH/LH adenoma	44/M	Positive for FSH, LH, and alpha subunit	No recurrent tumor (6 yr)
ACTH carcinoma	47/F	Liver metastasis positive for ACTH and chromogranin A	Died of metastatic pituitary carcinoma 13 yr after surgery

ACTH, and FSH/LH adenomas as well as four PRL and three ACTH carcinomas. Tumor were determined by clinical, biochemical, immunohistochemical findings, and electron microscopy. A section of tumor was examined after hematoxylin and eosin staining to determine that only pituitary tumor tissue was included.

RNA Extraction

Total RNA was extracted with the TRIzol reagent (Invitrogen, Carlsbad, CA) as previously reported *(11,12)*. The RNA was re-suspended in diethyl pyrocarbonate (DEPC)–treated water and purified with the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The concentration and quality of each RNA sample was calculated using the A260 and A280 spectrophotometric reading. RNA was stored at –70°C until used. Integrity of the RNA was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). A total of 8 μg of total RNA for each sample was used for the Affymetrix assay.

Gene Array Sample Preparation, Hybridization, and Scanning

The purified cDNA was used as a template for in vitro transcription reaction for the synthesis of biotinylated complementary RNA (cRNA) using an RNA transcript labeling reagent (Affymetrix). Labeled cRNAs were then fragmented and hybridized onto the Affymetrix GeneChip HU95A oligonucleotide array. Briefly, appropriate amounts of fragmented cRNA and control oligonucleotide B2 were added along with control cRNA (BioB, BioC, BioD), herring sperm DNA, and bovine serum albumin to the hybridization buffer. The hybridization mixture was heated to 99°C for 5 min followed by incubation at 45°C for 5 min before injecting the sample into the GeneChip. Hybridization was then performed at 45°C for 16 h and then mixing on a rotisserie at 60 rpm. After hybridization, the solution was removed and arrays were washed and stained with streptavidin–phycoerythrin (Molecular Probes, Portland, OR).

After washing and staining, probe arrays were scanned using the Affymetrix Microarray Suite 5.0 and confocal scanner. The quality of the fragmented biotin-labeled cRNA in each experiment was evaluated before hybridizing onto the HGU-133A expression array by both Agilent Bioanalyzer and hybridizing onto a Test-3 microarray as a measure of quality control. Hybridization and confocal scanning were performed at an institutional microarray core facility.

Gene Array Data Analysis

Affymetrix probe expression values were scaled to target signal of 500 with GCOS 1.2 software (Affymetrix, Santa Clara, CA). Scaled expression values as well as detection calls and change calls were uploaded in Genespring 7 software (Slicon Genetics Inc., Redwood City, CA) to perform data analysis. Signal values were normalized to 50th percentile per chip and to median per gene. A gene was identified as differentially expressed in samples being compared if each of three of the following conditions was satisfied. (1) Change call for the gene was I of D (increase or decrease). (2) Detection call was P (present) in the sample where the gene was upregulated. (3) Fold change was equal or greater than the cutoff value (twofold). When replicates of one type of sample were compared to replicates of another type of sample, then multiple comparisons were made. Each sample of the first type was compared to each sample of the second type. For example, if samples A and B were replicates of one type and X and Y or the other, the four comparisons were made: A to X, A to Y, B to X, and B to Y. A gene was considered differentially expressed in the two groups of samples if it satisfied all three criteria listed above in all four comparisons.

Standard Curve cDNA Synthesis

Each cDNA standard was generated for RT-qPCR assay as previously described *(57,58)*. Briefly, 1 μg of previously extracted total RNA from a normal pituitary was reverse-transcribed using the Stratagene First-Strand RT-PCR Kit according to the manufacturer's instruction. Conventional PCR was performed for each primer set using 2 μL of the cDNA from the RT reaction. Each product resolved as a single band on an agarose gel and was excised and purified. The PCR product for each gene (GAPDH, GLALS3, ASH1, ID2, and TLE-4) was then ligated into the pGEM-T vector using the pGEM-T Easy Vector System (Promega) and transformed into XL-1 Blue Competent Cells (Stratagene). Transformed cells were cultured overnight in LB medium containing ampicillin (100 μg/mL) and the plasmid was extracted and purified using the Qiafilter Plasmid Midi Kit (Qiagen). The plasmid was digested with *Eco*RI

followed by agarose gel electrophoresis. The insert was excised from the gel and purified, and the concentration determined by OD readings. The resulting purified cDNA standard was stored at –70°C in single-use aliquots until used.

Reverse Transcriptase Real-Time Quantitative PCR (RT-qPCR)

RT-qPCR was performed by the LightCycler System (Roche) using the FastStart DNA Master Sybr Green I Kit (Roche) as previously reported *(57,58)*. Each PCR reaction contained a total volume of 20 μL and the qPCR cycling conditions were performed according the manufacturer's instruction; 2 μL of a 1:10 dilution of template cDNA, 0.5 m*M* each primer and 4 m*M* $MgCl_2$ were used for each primer set unless otherwise stated. GAPDH was amplified as the internal housekeeping standard. The standard curve samples, ranging from 10^2 to 10^7 copies, were included in the same run as the experimental samples. A negative control in which water was substituted for cDNA was included in each run, and the sample identity was confirmed for each gene by the expected PCR product size on the agarose gel electrophoresis.

qPCR was performed for GAPDH with an annealing temperature of 60°C using the sense primer 5'-aaggtgaaggtcgga gtcaacg-3' and antisense primer 5'-gttgtcatggatgaaccttggcc-3' *(58)* resulting in a 495 bp product. Primers used for galectin-3 were sense 5'-atggcagacaatttttcgctcc-3' and antisense 5'-atgtcaccagaaattcccagtt-3' *(12)* with an annealing temperature of 60°C and a 719 bp product *(2)*. hASH1 primers were designed using the software Oligo 6.7 (Molecular Biology Insights, Inc. Cascade, CO). hASH1 primer sequences used were sense 5'-gagcaggagcttctcgacttca-3' and antisense 5'-gatgcaggttgtgcgatcac-3' with a 61°C annealing temperature and a 104 bp product. ID2 primers included sense 5'-actcgc atcccactattgtc-3' and antisense 5'-ggtccattcaacttgtcctc-3' *(59)* at 58°C, which amplified a 290 bp product. The TLE4 primers used were sense 5'-gtttgtaagcactggaaagg-3' and antisense 5'-aaaaaggatgacagagcaaa-3' *(60)* at an annealing temperature of 52°C and a product size of 241 bp.

Relative Quantification

To normalize the expression level of each experimental sample, the target copy number was divided by the GAPDH housekeeping copy number resulting in a relative ratio. A minimum of three experiments were performed for each gene studied. Results were expressed as the mean ± SEM.

Tissue Microarray Analysis

Tissues from 233 formalin-fixed paraffin-embedded human pituitary tumors were constructed for tissue microarray analysis (TMA), as previously reported *(35)*. Each type of pituitary tumors was grouped into one microarray block, including PRL adenomas ($n = 45$), ACTH adenomas ($n = 38$), GH adenomas ($n = 29$), FSH/LH adenomas ($n = 48$), null cell adenomas ($n = 66$), and pituitary carcinomas (total seven cases including three ACTH and four PRL carcinomas). Three cores of each specimen were used, and a grid pattern 12 holes wide and 18 holes long for up to a total of 216 tissue cores per block was constructed. Normal liver tissue was interspersed between the pituitary tissues for orientation. Sections were cut at 5 microns onto positive charged slides and used for immunohistochemical analysis. The same samples of TMAs were used for immunostaining with all four antibodies.

Antibodies used for TMA included Gal-3 (1/250) from Vector Labs, Burlingame, CA; TLE4 (1/50) from Santa Cruz Biotechnology, Santa Cruz, CA; and ID2 (1/200) from Santa Cruz Biotechnology. For immunohistochemistry, all tissue microarray slides were microwaved for 10 min in an 800-W microwave oven in 0.1 m*M* citrate buffer, pH 6.0, as previously described *(61)*. Immunostaining was performed with the avidin–biotin complex peroxidase method (Vector, Burlingame, CA). Incubation with the primary antibodies was done overnight at room temperature. Negative controls consisted of substituting normal serum for the primary antibodies.

Western Blotting

Western blotting was done using 15 pituitary tumors and 3 non-neoplastic pituitaries as previously described *(11)* with antibodies to galactin-3 (1/500), ASH 1 (1/500), and TLE-4 (1/500). The same samples were used for Western blotting with all four antibodies. Densitometric analysis was done with GelDoc system and Quantity One Software (BioRad Laboratories).

Statistical Analysis

The RNA expression levels in different groups of normal pituitary and pituitary tumors detected by RT-qPCR were evaluated by Wilcoxon/Kruskal–Wallis analysis and *t* test. The protein expression levels in different groups of normal pituitary and pituitary adenomas detected by Western blotting were evaluated by the *t* test. The immunohistochemical results of tissue microarray were analyzed by Fisher's exact test. $p < 0.05$ was considered statistically significant.

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