Expression of $p18^{INK4C}$ is down-regulated in human pituitary adenomas

Short Title: Role of $p18^{INK4C}$ in pituitary tumorigenesis

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Abstract

Cyclin-dependent kinase inhibitors represented by the INK4 family comprising $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$ are regulators of the cell cycle shown to be aberrant in many types of cancer. Mice lacking $p18^{Ink4c}$ exhibit a series of phenotypes including the development of widespread organomegaly and pituitary adenomas. The objective of our study is to examine the role of $p18^{INK4C}$ in the pathogenesis of human pituitary tumors. The protein and mRNA levels of $p18^{INK4C}$ were examined by immunohistochemistry and real-time reverse transcription-polymerase chain reaction, respectively. The methylation status of the $p18^{INK4C}$ gene promoter and somatic mutations of the $p18^{INK4C}$ gene were also investigated. $p18^{INK4C}$ protein expression was lost or significantly reduced in 64% of pituitary adenomas compared with levels in normal pituitary glands. $p18^{INK4C}$ mRNA levels were low in all ACTH adenomas and non-functioning (NF)-FSH and in 42%, 70% and 66% of GH, PRL, and subtype 3 adenomas, respectively. $p18^{INK4C}$ mRNA levels were significantly associated with $p18^{INK4C}$ protein levels. Neither methylated promoters in pituitary adenomas, except in 1 NF-FSH adenoma, nor somatic mutations of the $p18^{INK4C}$ gene in any pituitary adenomas were detected. The down-regulation of $p18^{INK4C}$ expression may contribute to the tumorigenesis of pituitary adenomas.

Key words: $p18^{INK4C}$ gene, INK4 family proteins, pituitary adenomas, cell cycle regulators, DNA methylation

Introduction

Pituitary adenomas are common and potentially serious neoplasms that account for 10-15% of all intracranial neoplasms [1]. Although pituitary adenomas arise as benign monoclonal neoplasms, alterations in classic oncogenes and tumor suppressor genes are rarely found in these tumors [2-8]. Recently, germline mutations in the aryl hydrocarbon receptor-interacting protein gene were identified in familial GH adenomas [9,10], whereas somatic mutations of the gene are rarely detected in sporadic GH adenomas [11]. In addition, over-expression of pituitary tumor transforming gene and a truncated form of fibroblast growth factor receptor-4 have been reported in pituitary adenomas [12,13]. Nevertheless, molecular mechanisms involved in the genesis of pituitary adenomas are scarcely known.

A critical point in the cell cycle is the G_1/S transition checkpoint frequently aberrated in cancer cells. Progression of the cell cycle is controlled by cyclins and cyclin-dependent kinase (CDK). Cyclin/CDK complexes induce the progression of cells into the S phase by phosphorylating retinoblastoma protein (pRb) [14]. INK4 comprising $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$ is a family of CDK inhibitors and binds specifically to CDK4 and CDK6, thereby preventing kinase activities [15]. Therefore, inactivation of INK4 leads to activation of cyclin/CDK complexes, resulting in cell cycle progression.

Although expression of $p16^{INK4A}$ is frequently down-regulated in human pituitary adenomas [16-18], the role of other INK4 genes in pituitary tumor suppression has not yet been uncovered in human. Mice lacking of $p18^{Ink4c}$ displayed organomegaly, intermediate-lobe pituitary tumors, lymphomas, testicular tumors, and pheochromocytomas [19]. $p18^{Ink4c-/-}$; $Pten^{+/-}$ or $p18^{Ink4c-/-}$; $Men1^{+/-}$ mice are reported to

develop anterior pituitary tumors with an accelerated rate compared to $Pten^{+/-}$ or $Men1^{+/-}$ mice, respectively [20,21]. Recently, van Veelen *et al.* reported that loss of $p18^{Ink4c}$ in combination with oncogenic Ret increases the risk of medullary thyroid carcinoma [22]. These findings suggest that functional inactivation of the $p18^{Ink4c}$ gene is an important factor in murine endocrine tumors.

To define the role of $p18^{INK4C}$ in human pituitary tumorigenesis, we investigated protein and mRNA levels, promoter methylation status, and somatic mutations of the $p18^{INK4C}$ gene in pituitary adenomas.

Materials and methods

Patients and tumor samples

Tumor samples were collected from unselected patients with sporadic pituitary adenomas. Clinical information on the patients is described in Table 1. In addition to the adenomas in Table 1, another 40 GH adenomas were used for investigating somatic mutations of $p18^{INK4C}$. Tumor size and invasiveness were defined on the basis of preoperative radiological investigations and operative findings and with a modified Hardy's classification [23]. Grade I (microadenomas, <1 cm in diameter) and grade II (enclosed macroadenomas with or without suprasellar extension, ≥1 cm in diameter) tumors were defined as non-invasive. Grade III (local invasion of sphenoid and/or cavernous sinus) and grade IV (diffusive invasion or destruction of sella) tumors were considered to be invasive. All pituitary adenomas were obtained at the time of surgery at Toranomon Hospital (Tokyo, Japan). The study was approved by our internal review board. Fully informed consent was obtained in accordance with institutional guidelines.

Immunohistochemical analysis

$p18^{INK4C}$ expression in 45 pituitary adenomas was investigated by immunohistochemical analysis. To detect $p18^{INK4C}$ protein and Ki-67 antigen, immunolocalization experiments were carried out on sections from representative blocks of paraffin-embedded tissues using the labeled streptavidin-biotin method. After deparaffinization and antigen retrieval using an autoclave oven technique, sections were incubated at 4 °C overnight with mouse monoclonal anti-p18 antibody (1:200; Santa Cruz Biotech, Santa Cruz, CA) or with mouse monoclonal anti-Ki-67 antibody (1:100; DakoCytomation, Glostrup, Denmark). Antigen-antibody complexes were detected using

the cobalt-3, 3'-diaminobenzidine reaction. The slides were counter-stained lightly with hematoxylin or 1% methyl green and mounted for microscopic examination. Sections of human colon carcinoma known to be positive for $p18^{INK4C}$ were used as positive controls. Sections incubated in phosphate-buffered saline without the primary antibody served as negative controls.

Each slide was examined by an observer blinded to the diagnosis and clinicopathologic data and further reviewed and confirmed by a second blinded observer. Any intensity of nuclear staining was considered to present a positive stain for $p18^{INK4C}$ and Ki-67. A total of 500 - 1000 cells were counted and the percentage of $p18^{INK4C}$ -stained tumor cells was scored on a scale of 0 to 4 (0, no staining; 1+, 1 - 5%; 2+, 5 - 30%; 3+, 30 - 50%; 4+, >50%). The Ki-67 antigen labeling index was determined by counting the number of positive cells in a total of 500 - 1000 tumor cells observed in several representative high-power fields (x 400). The results were expressed as a percentage of tumor cells with positive nuclei.

$p18^{INK4C}$ mRNA expression analysis

$p18^{INK4C}$ mRNA levels in 49 pituitary adenomas were investigated. Total RNA from pituitary adenomas was extracted using ISOGEN (Nippongene, Toyama, Japan) according to the manufacturer's instructions. cDNA was synthesized from total RNA using a Prime Script^{TM} RT Reagent Kit (TaKaRa, Kyoto, Japan). Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed at least in duplicate using the Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, CA). The nucleotide sequences of primers were for $p18^{INK4C}$: forward, 5'-GGGGACCTAGAGCAACTTACT-3' and reverse, 5'-GGCAATCTCGGG

ATTTCCAAG-3'; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward, 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse, 5'-GAAGATGGTGATGG GATTTC-3'. The final volume of 20 μl contained 10 μl of 2 x Power SYBR Green PCR Master Mix (Applied Biosystems) and each primer. Genes were amplified with 50°C for 2 min, 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 sec, and annealing and extension at 60°C for 1 min. GAPDH mRNA was simultaneously quantitated as an endogenous control and the $p18^{INK4C}$ gene expression in each sample was normalized to GAPDH expression.

Bisulfite sequencing

Genomic DNA was extracted from pituitary adenomas using the Qiagen DNeasy Tissue Kit (Qiagen, Stanford, CA) according to manufacturer's instruction. The sodium bisulfite modification was performed with genomic DNA using MethylEasy^{TM}Xceed (Human Genetic Signatures, North Ryde, Australia) according to the manufacturer's directions. A primer pair for bisulfite sequencing (forward, 5'-TAGGAATTGGGGTAG TTGGGG-3' and reverse, 5'-TTACCTCTCAAAAAAAATACCARTTT-3') was designed to amplify a region of 425 bp containing 42 CpG dinucleotides.

PCR was carried out in a volume of 20 μl containing about 50 ng of bisulfite-treated genomic DNA using AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR samples were denatured at 95 °C for 10 min and then subjected to 45 cycles of 95 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 1 min. The PCR products were subcloned into pT7Blue T-Vector (Novagen, Madison, WI) and 5 plasmid clones for each sample were sequenced.

Analysis of somatic mutations

To investigate the possibility of inactivating mutations of the $p18^{INK4C}$ gene, genomic DNA from 89 adenomas was subjected to PCR amplification for the coding region in exons 2 and 3 of $p18^{INK4C}$. The nucleotide sequences of primers were, for exon 2 (PCR product size, 280 bp): forward, 5'-AGTCTCCGATGCCATCATGCAGC-3' and reverse, 5'-CACGTAGGCAACATTATTGACTTGTT-3'; and for exon 3 (PCR product size, 515 bp): forward, 5'-GAAGGATTCTACCATTTCTACTTCTTT-3' and reverse, 5'-CTGCTTAACATATGACAGAACTGT-3'.

PCR was carried out in a volume of 20 μl containing 50 ng of genomic DNA using TaKaRa Ex Taq™ (TaKaRa). PCR samples were denatured at 94 °C for 5 min and then subjected to 35 cycles of 94 °C for 30 sec, 50 °C /exon 2 and 55 °C /exon 3 for 30 sec, and 72 °C for 1 min. The PCR products were electrophoresed in a 1% agarose gel to confirm amplification. After treatment of the products with ExoSAP-IT (USB Corporation, Cleveland, OH), samples were subjected to direct sequencing in sense and anti-sense directions using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed on an Applied Biosystems 3700 DNA Analyzer (Applied Biosystems).

Statistical analyses

Using StatView J-4.5 software (Statview, Cary, NC), the Chi-square test and Mann-Whitney U test were performed to determine the significance of associations between different variables. Significant differences were accepted at $P < 0.05$.

Results

$p18^{INK4C}$ protein expression in human pituitary adenomas

We examined $p18^{INK4C}$ protein expression in 4 normal pituitary glands and 45 adenoma specimens. In the 4 normal adenohypophyseal samples, $p18^{INK4C}$ protein exhibited nuclear reactivity with moderate or high density (30% - 60%; 3+ - 4+); no membrane or cytoplasmic localization was identified (Figures 1A and 1B). $p18^{INK4C}$ protein expression was absent (Figure 1C), significantly low ($p18^{INK4C}$ protein expression scale of 1+), and slightly low ($p18^{INK4C}$ protein scale of 2+) in 31%, 33%, and 29% of adenomas, respectively (Table 1). Three tumors showed a normal or high level of $p18^{INK4C}$ protein (Figure 1D and Table 1). The expression of $p18^{INK4C}$ protein in adenomas was not significantly related to patient age, gender, tumor size, or the Ki-67 labeling index (data not shown).

$p18^{INK4C}$ mRNA levels in pituitary adenomas

To reveal the mechanisms responsible for the weak expression of $p18^{INK4C}$ protein, mRNA levels of $p18^{INK4C}$ were measured in the pituitary adenomas used for immunohistochemistry. For comparison, $p18^{INK4C}$ mRNA levels in 3 normal pituitary glands were analyzed. Expression levels that were less than 50% of the mean value for normal pituitary glands were classified arbitrarily as significantly low. $p18^{INK4C}$ mRNA levels were significantly low in all ACTH adenomas and NF-FSH adenomas (Figure 2). $p18^{INK4C}$ mRNA levels were also significantly low in 42%, 70%, and 66% of GH, PRL, and subtype 3 adenomas, respectively (Figure 2). A correlation between mRNA and protein levels of $p18^{INK4C}$ in 42 pituitary adenomas was observed (Table 2).

Methylation status of the $p18^{INK4C}$ gene promoter in pituitary adenomas

The methylation status of the promoter region of $p18^{INK4C}$ was determined by bisulfite genomic sequencing in adenomas with $p18^{INK4C}$ mRNA levels that were less than 30% of the value for normal pituitary glands. Bisulfite sequencing revealed that CpG dinucleotides were methylated in 1 NF-FSH adenoma (sample number 5 in Table 1) (data not shown). Whereas, all 42 CpG dinucleotides analyzed were unmethylated in normal pituitary glands and other adenomas (data not shown).

Mutations of the $p18^{INK4C}$ gene in pituitary adenomas

Sequencing of genomic DNA from 89 pituitary adenomas of various types did not show any mutations within the coding region or splicing junctions of the $p18^{INK4C}$ gene. We detected a known single nucleotide polymorphism, c.342T>C (p.G114G) (rs1043141), in 5 GH adenomas and 1 NF-FSH adenoma.

Discussion

$p18^{INK4C}$ has been implicated as a tumor suppressor gene in a variety of cancers [24-28]. In particular, loss of $p18^{INK4C}$ protein expression has been reported in various human tumors such as testicular cancers, Hodgkin lymphomas, hepatocellular carcinomas, medulloblastomas, and glioblastomas [24-28]. To clarify whether an attenuation of $p18^{INK4C}$ expression is involved in the development of human pituitary adenomas, we investigated the expression of $p18^{INK4C}$ at both the protein and mRNA levels. $p18^{INK4C}$ protein expression was absent or significantly low in 64% tumors, consistent with a recent report of under-expression of $p18^{INK4C}$ in both hormonally active and inactive pituitary adenomas [29]. Although a correlation between the mRNA and protein levels of $p18^{INK4C}$ in most pituitary adenomas was observed, a few pituitary adenomas showed a discrepancy between mRNA and protein levels. The mechanisms responsible for this discrepancy were not clear. Furthermore, low $p18^{INK4C}$ mRNA levels in all NF-FSH adenomas and ACTH adenomas were observed, consistent with a report of significant under-expression of $p18^{INK4C}$ in ACTH adenomas [30]. Based on studies on knock-out mice [19-21], down-regulation of $p18^{INK4C}$ gene expression may be a cause of the tumorigenesis of pituitary adenomas rather than a consequence of adenoma development.

The promoter methylation status of the $p18^{INK4C}$ gene in pituitary adenomas with low mRNA levels was analyzed, because cytosine residues occurring in CpG dinucleotides are targets for DNA methylation, and gene expression is usually reduced when DNA methylation takes place at a promoter [31]. Hypermethylation was detected in only 1 NF-FSH adenoma; the others showed no methylation in the promoter of the $p18^{INK4C}$ gene. Recently, Kirsch *et al*. reported that about 40% of pituitary adenomas displayed $p18^{INK4C}$ promoter methylation by the method of methylation-specific PCR (MSP) [29]. The

contrasting data on promoter methylation may be resulted from different methods. Although MSP is a simple, sensitive and less time consuming method [32], it is a qualitative method and can sometimes lead to false positive results unless the PCR conditions and primers have been designed with stringent criteria [33]. On the other hand, the bisulfite genomic sequencing is the most straightforward means of detecting methylation status, which denotes an accurate map of the position of each methylated cytosine residue [31,34]. Because putative binding sites of Sp1 and E2F in the promoter of the $p18^{INK4C}$ gene are critical for $p18^{INK4C}$ expression [35], we investigated a region of promoter containing binding sites of Sp1 and E2F. Our results demonstrated that aberrant methylation was not a responsible factor for the low levels of $p18^{INK4C}$ in pituitary adenomas.

Alternatively, the down-regulation of $p18^{INK4C}$ expression may be caused by other mechanisms. For example, Tallack *et al.* reported that $p18^{Ink4c}$ expression was down-regulated in erythroid krüppel-like factor (EKLF)-null mice [36]. In addition, expression of $p18^{Ink4c}$ is down-regulated by activated RET or up-regulated by menin [37,38]. However, somatic mutations of MEN1 and RET are rare in human pituitary adenomas [5,6]. Therefore, EKLF or other unknown gene products may be responsible for the down-regulation of $p18^{INK4C}$ expression in human pituitary adenomas.

Furthermore, the mutational status of the $p18^{INK4C}$ gene was investigated in 89 pituitary adenomas. However, no somatic mutations except for a known polymorphism were detected, consisting with the study by Kirsch *et al.* [29]. Genetic alterations of $p18^{INK4C}$ in other tumors as well as in cancer cell lines have also been reported to be rare or absent [39-41]. Therefore, our results suggest that somatic mutational inactivation of the $p18^{INK4C}$ gene does not contribute to the pathogenesis of pituitary tumors.

We conclude that the down-regulation of $p18^{INK4C}$ expression may contribute to the development of pituitary adenomas.

Acknowledgements

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function. Neoplasia 9:533-535, 2007.

Table 1 Clinical features of patients with pituitary adenomas and results of immunohistochemistry for $p18^{INK4C}$

Sample number	Age	Sex	Pathological diagnosis	Hardy's classification[a]	$p18^{INK4C}$ immunohistochemistry[b]
1	50	male	NF-FSH	IV-B	0
2	43	male	NF-FSH	III-C	0
3	71	male	NF-FSH	II-B	0
4	59	male	NF-FSH	II-B	2+
5	35	male	NF-FSH	II-B	3+
6	40	male	NF-FSH	II-B	0
7	67	male	NF-FSH	II-B	1+
8	41	male	NF-FSH	II-B	2+
9	78	male	NF-FSH	II-B	2+
10	68	male	NF-FSH	II-B	1+
11	42	male	NF-FSH	II-A	2+
12	46	female	NF-FSH	III-B	1+
13	71	female	NF-FSH	IV-B	1+
14	43	female	silent ACTH	IV-A	1+
15	57	female	silent ACTH	IV-0	2+
16	36	female	silent ACTH	III-B	1+
17	72	female	silent ACTH	III-C	2+
18	n.a.	female	ACTH	IV-0	0
19	29	female	ACTH	I-0	0
20	55	female	ACTH	II-0	1+
21	45	female	ACTH	II-0	1+
22	23	male	subtype 3	III-B	1+
23	47	female	subtype 3	III-B	2+
24	20	female	subtype 3	IV-C	1+
25	56	male	PRL	III-A	0
26	45	male	PRL	III-0	0
27	86	male	PRL	III-A	not analyzed
28	36	female	PRL	II-0	not analyzed
29	39	female	PRL	III-0	not analyzed
30	27	female	PRL	I-0	1+
31	41	female	PRL	II-A	2+
32	30	female	PRL	I-0	0
33	20	female	PRL	III-B	1+
34	35	female	PRL	I-0	2+
35*	28	female	PRL	II-A	2+
36	28	male	TSH	II-B	3+
37	46	male	TSH	II-B	not analyzed
38	25	female	GH+PRL	III-0	1+

39	63	male	GH	III-A	0
40	48	male	GH	II-A	0
41	55	male	GH	II-0	2+
42	26	male	GH	II-0	2+
43	61	male	GH	II-B	not analyzed
44	33	male	GH	III-B	not analyzed
45*	56	male	GH	III-0	0
46*	33	male	GH	III-B	0
47	39	female	GH	III-B	not analyzed
48	63	female	GH	II-A	4+
49	36	female	GH	II-0	2+
50	56	female	GH	II-0	0
51	74	female	GH	II-0	1+
52	39	female	GH	I-0	1+

[a]Tumor lesions are categorized based on the size of suprasellar expansion as grade A, expanded into the chiasmatic cistern but not up to the anterior third ventricle; B, reached the floor of the third ventricle; C, expanded into the third ventricle up to the foramen of Monroe; 0, no suprasellar expansion. [b]The percentage of $p18^{INK4C}$-stained tumor cells was scored on a scale of 0 to 4 (0, no staining; 1+, 1 - 5%; 2+, 5 - 30%; 3+, 30 - 50%; 4+, > 50%), n.a., not available. *mRNA levels and somatic mutations of the $p18^{INK4C}$ gene were not analyzed.

Table 2 Correlation between $p18^{INK4C}$ mRNA and protein levels in pituitary adenomas

	No. of cases	$p18^{INK4C}$ protein			
		−	±	+	
$p18^{INK4C}$ mRNA levels*	42	(n = 27)	(n = 12)	(n = 3)	*P*
< 50%	33	23	8	2	< 0.05
50 - 99%	6	2	4	0	
100% or over	3	2	0	1	

*mean value for 3normal pituitary glands is considered as 100%

−, no or significantly low levels of $p18^{INK4C}$ protein expression (scales of 0 and 1+); ±, slightly low levels of $p18^{INK4C}$ protein (scale of 2+); +, normal levels of $p18^{INK4C}$ protein (scales of 3+ and 4+). A Chi-square test was used for the statistical analysis and significant differences were accepted at $P < 0.05$.

Figure legends

Figure 1: $p18^{INK4C}$ protein expression in pituitary adenomas. (A) $p18^{INK4C}$ immunostaining in a normal pituitary gland showed a dense nuclear immunoreaction (scale of 3+). Original magnification, x200. (B) Higher magnification of the boxed area in (A) showing positive nuclear $p18^{INK4C}$ staining. Original magnification, x400. (C) $p18^{INK4C}$ immunostaining in a pituitary adenoma (sample number 6) showed no immunoreaction (scale of 0). Original magnification, x100. (D) $p18^{INK4C}$ immunostaining in a pituitary adenoma (sample number 48) showed a strong nuclear immunoreaction (scale of 4+). Original magnification, x200.

Figure 2: $p18^{INK4C}$ mRNA levels in various types of pituitary adenomas

Graphs show mRNA levels of $p18^{INK4C}$ in pituitary adenomas (hatched bars) and 3 normal pituitary glands (blank bars) determined by quantitative real-time RT-PCR. The ratio of $p18^{INK4C}$ mRNA/GAPDH mRNA in each sample (at least duplicate) was calculated and the mean value was presented. The samples were numbered according to the sample numbers in Table 1.

Figure 1

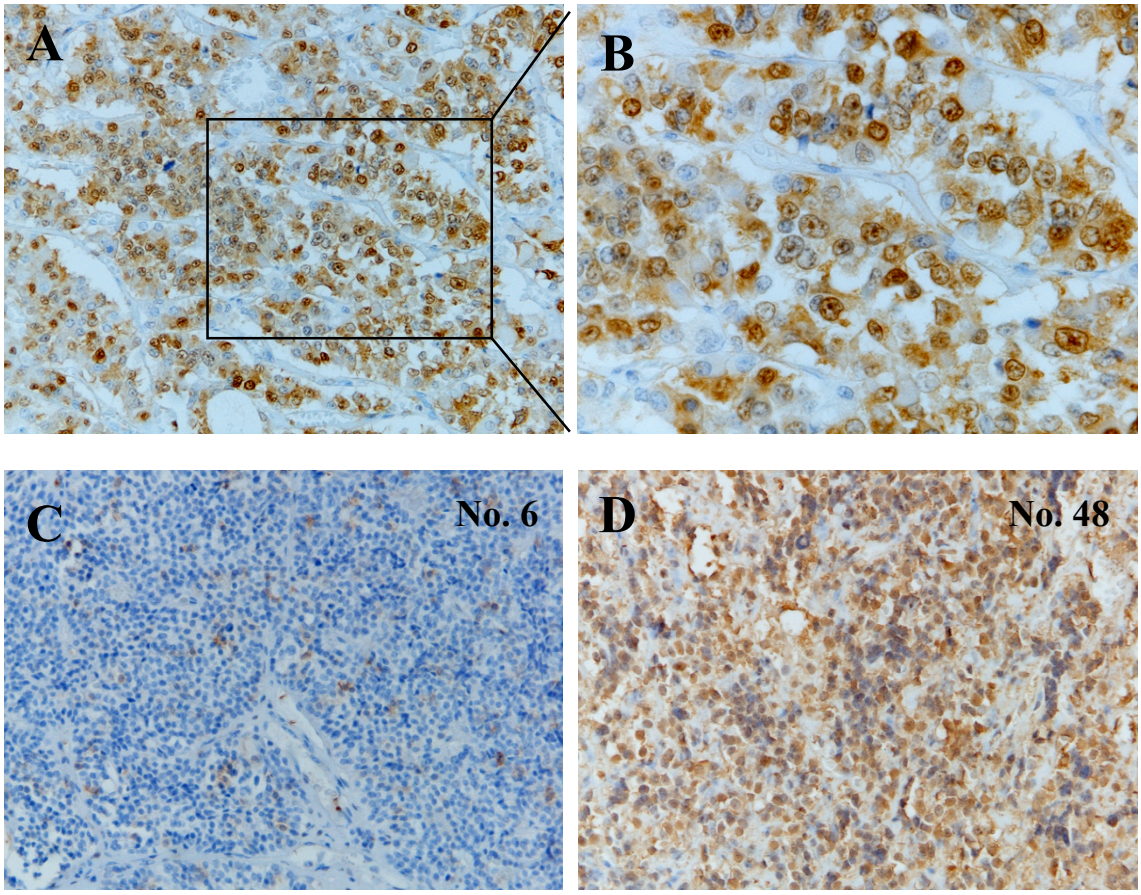
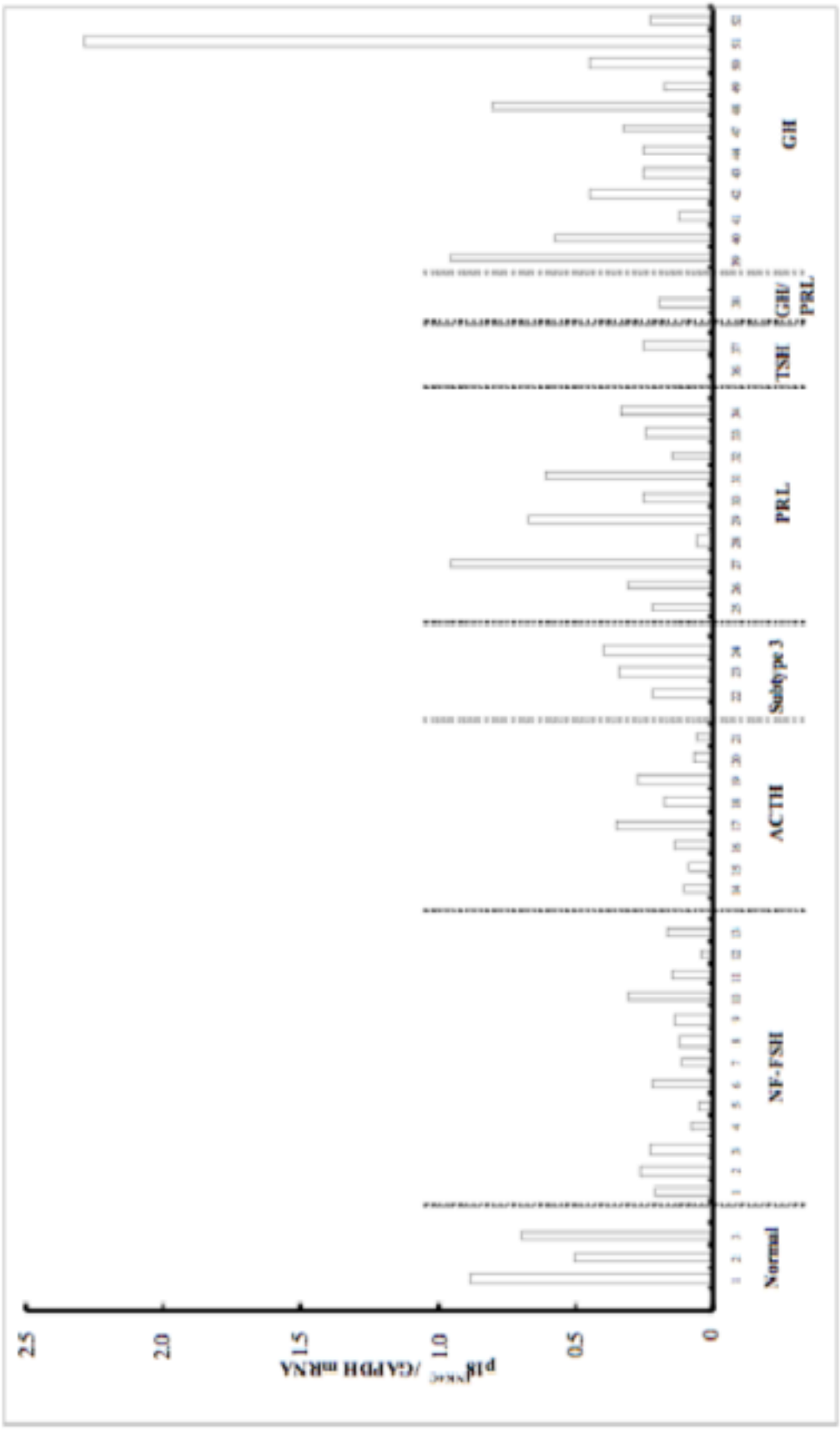


Figure 2


p18^KIP2 /GAPDH mRNA
2.5
2.0
1.5
1.0
0.5
0
Normal
NF-FSH
ACTH
Subtype 3
PRL
TSH
GH/
PRL
GH