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Decreased of CDK Inhibitor *p18^{INK4C}* mRNA Expression in Sporadic Clinically Non-functioning Pituitary Adenomas

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Abstract

The cyclin-dependent kinase inhibitor *p18^{ink4c}*, a cell cycle negative regulator, has been linked as a tumor suppressor gene in a variety of cancers, including endocrine tumors, and recent evidences have been demonstrated that the down-regulation of this gene may contribute to the pituitary tumorigenesis. The purpose of this study was to confirm this initial report and investigate this gene expression in sporadic pituitary adenomas, and this association with clinicopathological features as age, gender and tumor size. Thirty-eight pituitary tumor specimens extracted from patients who had undergone hypophysectomy, previously classified by immunohistochemistry, were included. Tumor samples were submitted to total RNA isolation, and *p18^{ink4c}* mRNA relative expression profile was evaluated. Among the patients included, 21 were male (55.26%) while 17 were female (44.74%), and the patients' ages ranged from 18 to 73 years old. The tumor samples consisted in 14 clinically non-functioning adenomas, and 24 functioning adenomas, which were: 5 ACTH-secreting, 11 GH-secreting and 8 PRL-secreting. This report confirms the initial analysis exposing the loss of CDK inhibitor *p18^{ink4c}* expression levels in most pituitary adenomas and a statistically significant decrease of *p18^{INK4C}* relative expression in non-functioning adenomas compared to functioning. These results altogether propose that *p18^{INK4C}* gene down-regulation might be involved in pituitary tumorigenesis; furthermore, its role seems to be more significant in non-functioning pituitary adenomas.

Keywords: *p18(INK4C); CDKN2C;* Pituitary adenomas; Cell cycle; Tumor suppressor

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Introduction

Pituitary adenomas represent one of the most frequent intracranial tumors. Recent data published suggest that they comprise about 15 to 20% of all diagnosed primary brain neoplasms [1,2]. Although these tumors demonstrate a high infiltrative capability into nearby tissues, which confers the main cause of morbidity on this pathology, they are considered benign neoplasms due to their metastatic inability [3]. Clinical signs can be neurological due to tumor mass growth and subsequent compression of structures around the pituitary gland, or systemic due to abnormal hormone secretion [4,5]. For this reason, pituitary adenomas are clinically classified as non-functioning (NFAs), which are the hormonally inactive tumors, or functioning (FAs), when there is a difference in hormone secretion [6,7].

Although the pathogenesis of pituitary adenomas is still poorly understood, considerable evidence indicates that the pituitary tumorigenesis is a complex process involving multiple factors, including genetic and epigenetic ones [8-10]. Somatic mutations and alterations in tumor suppressor genes or in oncogenes known in other types of tumors, like *ras, p53, PKC, c-erbB2* are not involved in the pituitary tumorigenesis [11-14]. However, a number of oncogenes, tumor suppressor genes and cell cycle mediators have been identified to be functionally involved in the initiation and progression of pituitary adenomas [9,15-18].

These tumors are believed to have a monoclonal origin, where the deregulation in the division process of one unique cell generates the tumor [11,14,19]. 80% of pituitary adenomas show some alteration in the expression of at least one gene related to G1 to S phase of cell cycle progression [20], a critical transition checkpoint frequently found in cancer cells. These abnormalities are frequently related to overexpression of cyclin dependent kinases (CDKs) due to down-regulation of the CDKs

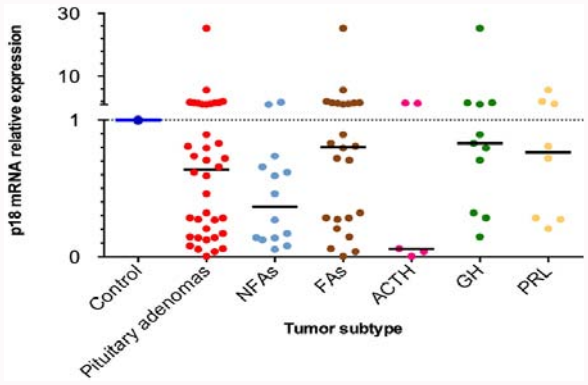

Figure 1: Real time quantitative PCR analysis of *p18*^{INK4C} relative gene expression in pituitary adenomas.
Representative image showing *p18*^{INK4C} relative expression in all pituitary adenomas subtypes. The medians of the quantified expression values are represented by the horizontal bar, while the relative gene expressions in the normal tissue are defined as 1.0.
Median of relative gene expression, normalized by constitutive gene PGK1, determined by $2^{-\Delta\Delta CT}$: (a) Control: normal pituitary tissue: 1.00; (b) All pituitary adenomas tested: 0.63; (c) NFAs: 0.36; (d) FAs: 0.80; (e) ACTH-secreting: 0.059; (f) GH-secreting: 0.83; (g) PRL-secreting: 0.76. (NFAs: non-functioning adenomas; FAs: functioning adenomas; ACTH: ACTH-secreting; GH: GH-secreting; PRL: PRL-secreting).

inhibitors (CDKIs) genes through epigenetic influences [21,22].

CDKN2C gene, also known as *p18*^{INK4C} or *INK4C,* belongs to INK4 family, one of the two main families of CDKIs, together with *p15*^{INK4C}, *p16*^{INK4C}, and *p19*^{INK4C} which are implicated in mediating a wide range of cell growth control signals. *P18* acts inhibiting G1 to S phase progression of cell cycle by blocking retinoblastoma protein (pRB) phosphorylation by CDKs, what precludes the E2F transcription factor release [23,24], resulting in cell cycle progression. Functional inactivation of this pathway is a common event in the development of most types of cancer [25].

More recently, *p16*^{INK4C}*mRNA* down-regulation of expression was reported in pituitary adenomas[26,27], showing that abnormalities in cell cycle regulatory proteins are increase recognized as crucial factors in pituitary tumorigenesis. P18 is highly expressed in the pituitary tissues, and the specificity of this gene function is further underscored by the fact that loss of *p18*^{INK4C} as well as *p27* (another member of CDKIs family) expression resulted in spontaneous development of pituitary tumor in mice [24]. The down-regulation of both *p18*^{INK4C} and *p27*^{KIP1} genes developed tumors also in thyroid, parathyroid, adrenals, pancreas and testicles [24,28,29]. In humans, loss of p18 protein was detected by immunohistochemistry (IHC) technique in pituitary adenomas, probably due to damage to the promoter region [30]. A recent study using real time quantitative PCR (qRT-PCR) technique showed that *p18*^{INK4C} gene expression is significant down-regulated in pituitary adenomas when compared with the normal pituitary gland tissue, and this loss of expression may contribute to the development of pituitary adenomas [17]. In this sense, as *p18*^{INK4C} plays an important role in cell cycle regulation, it is suggested that the absence of this gene activity might be involved in the pituitary gland tumorigenesis, although little is known about its expression pattern in different types of pituitary tumors. To gain more insight into the molecular pathogenesis of pituitary adenomas, we purpose to investigate *p18*^{INK4C} mRNA gene expression in human sporadic pituitary benign tumors, and this association with tumor subtype, in relation to available clinicopathological parameters in these tumors.

Materials and Methods

Control, patient samples and clinicopathological parameters

Thirty-eight fresh sporadic pituitary adenoma samples were collected at the time of surgery from patients who had undergone to transphenoidal hypophysectomy at São José Hospital from Irmandade Santa Casa de Misericórdia (ISCMPA) in Porto Alegre, Brazil. After surgical resection, the fresh tumor tissue was split into two parts, being one half delivered to histological examination. The other half of the samples collected were immediately frozen in liquid nitrogen for RNA stabilization and stored at -80°C until they were processed.

All tumors were histologically examined to confirm the diagnosis, and a portion of each specimen was fixed and embedded in paraffin for immunohistological studies. Formalin-fixed specimens section was subjected to Haematoxilin and Eosin (H&E) staining allowing tumor identification, and the diagnosis was confirmed by immunohistochemistry analysis for FSH, LH, GH, TSH, ACTH and PRL hormones after surgery. In cases of clinically non-functioning adenomas, hormone staining was completely absent or the staining pattern did not align clearly with an adenoma subtype. The neuropathological diagnosis employing the current WHO classification was established by clinical, biochemical and radiological findings. The following clinicopathological features were obtained from medical records: age, gender, tumor subtype and tumor size.

In addition, a pool of 88 normal human pituitary glands obtained from autopsies was obtained commercially (Clontech Laboratories Inc., Palo Alto, CA, USA).

Ethics

The Committee of Ethics in Research from Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) approved this study, and written consent was obtained before the surgery from each patient who provided samples.

Total RNA extraction and cDNA syntesis

Total RNA form pituitary adenomas was isolated using Trizol® reagent (Invitrogen, Carlsbad, EUA) according to manufacturer's instructions and its quantification and purification was measured by spectrophotometry at 260nm using BioSpec Nano (Shimadzu Corp, JPN). Samples with relation 260nm/280nm equal or higher than 1.7 were included. After optimized RNA concentration, all samples and controls were reversely transcribed using ImProm-II™ Reverse Transcription System (Promega, Madison, WI, EUA), according to

the manufacturer's protocol. The final volume of each reaction was 20µL.

Quantitative RT-PCR analysis

The target gene expression was measured by qPCR using SYBR-Green Master Mix 2X reagent (Applied Biossystems, Foster City, CA, EUA) and specific primers to each gene. The reactions were performed using 7500 Real-Time PCR System (Applied Biossystems, Foster City, CA, USA), according manufacturer's recommendations. Relative quantification of each sample was done at least in duplicate. To confirm cDNA integrity and to perform normalization, the housekeeping gene *PGK1* (phosphoglycerate kinase 1) was used as endogenous control. Oligonucleotide sequences are follow:. A RNA pool of 88 normal pituitary glands was used as a PCR reaction calibrator. Samples from RT reactions in the absence of cDNA were also used as negative control. At the end of the PCR cycles, melting curve analyses were performed to validate the generation of the specific PCR product expected. The relative expression of $p18^{INK4C}$ mRNA level was quantified by Threshold Cycle (Ct) method. In this method, results are given as the number of PCR cycles that passed a certain fluorescence threshold and so, data were expressed as Ct values [31]. Relative cDNA expression (the normalized target concentration related to the endogenous reference) was given by the formula: $2^{-\Delta\Delta Ct}$, where ΔΔCt=Ct (Target gene-PGK1 tumor) - (Normal pituitary target gene-PGK1 normal pituitary pool). The relative efficiency was determined by evaluating ΔCt against serial dilutions of target genes.

Statistical analysis

All the statistical analysis was performed using SPSS 16.0 software package (SPSS Inc., IBM Company, Chicago, USA) and GraphPad prism version 6.0 (GraphPad Software, San Diego, CA, USA). Significant differences were accepted at the $p \leq 0.05$. Descriptive statistical analysis and normality test were carried out to determine the distribution of data. Analysis of variance was performed using Kruskal–Wallis followed by post-hoc Dunn's test. Mann–Whitney test was used to compare medians between groups. Expression status and clinicopathological features were analyzed using Chi-square (χ2) and Fisher's exact test. Student's test analysis was performed to verify association between age and tumor subtype.

Results

Association between *p18ink4c* mRNA expression and clinicopathological parameters

Among all 38 samples of pituitary adenomas included in this study, 14 were classified as clinically non-functioning adenomas (NFAs) (36.85%) and 24 as functioning adenomas (FAs) (63.15%) which were: 5 ACTH-secreting (13.15%), 11 GH-secreting (28.95%) and 8 PRL-secreting (21.05%). The patients' genders were: 21 male (55.26%) and 17 female (44.74%).

The patients' ages ranged from 18 to 73 years old, and the mean age (mean ± SD) was 46.71 ± 15.21. Student's *t* test showed that patients with FAs were significantly younger (40.21 ± 13.13 years; mean ± SD) than patients with NFAs (57.86 ± 11.90 years; mean ± SD) (*P*=0.002).

The tumor size showed variability only in the ACTH-secreting adenomas, in which 4 were microadenomas (≤1cm) while one remaining ACTH-secreting adenomas was classified as macroadenomas (≥1cm). In this sense, χ2 test showed a significant association between tumor size and subtype (*P*=0.001) in the subset of ACTH-secreting adenomas. These data are shown in Table 2.

Table 1: Oligonucleotides sequences.

Gene	Primer Sense	Primer Anti-sense
$p18^{INK4C}$ [1]	5'- GGGGACCTAGAGCAACTTACT-3'	5'- GGCAATCTCGGGATTTCCAAG-3'
PGK1 [2]	5'- GAACAAGGTTAAAGCCGAGCC-3'	5'- GTGGCAGATTGACTCCTACCA-3'

[1]Hossain, *et al.*, 2009 [17]; [2]Harvard Medical School Primer Bank.

There was no statistically significant difference in $p18^{INK4C}$ gene expression in relation to gender (*P*=0.1812), age (*P*= 0.4481), or tumor size (*P*= 0.2759) between NFAs and FAs.

$p18^{INK4C}$ mRNA is under expressed in pituitary adenomas

There was a positive expression of constitutive gene in all samples included, and the quantified $p18^{INK4C}$ relative gene expression in the pool of normal pituitary tissue received the arbitrary value of 1.0. Overall, 23 examined samples showed loss of mRNA $p18^{INK4C}$ relative expression, representing 60.5% of all pituitary adenomas tested, when compared with the pool of normal pituitary tissue mRNA.

Interestingly, there was a statistically significant decrease of the quantified $p18^{INK4C}$ relative gene expression in NFAs compared to the FAs group (*P*<0.0359). Though NFAs showed a significantly down-regulation of $p18^{INK4C}$ expression to all FAs combined, NFAs presented significantly reduced levels compared to somatotropiomas (*P*=0.0093). Neverheless, the comparisons between $p18^{INK4C}$ relative expression between NFAs *vs.* ACTH e NFAs *vs.* PRL showed no significant difference (*P*=0.4313 and *P*=0.1423 respectively). There was no statistical difference when we compared $p18^{INK4C}$ mRNA expression between FAs subtypes (*P*=0.3683). Since $p18^{INK4C}$ gene expression was significantly lower in the NFAs, we explored whether there were differences in its expression among the subtypes of NFAs. We found that $p18^{INK4C}$ was significant higher in somatotropinomas, but not in the other functioning adenoma subtype (Figure 1).

Discussion

Abnormalities in cell cycle regulatory genes and proteins are increasingly recognized as crucial factors in various types of tumors, including pituitary tumorigenesis, and have been shown as contributors to tumor development and progression [32,33]. $p18^{INK4C}$ belongs to INK4 family and acts inhibiting CDK4 and 6 activation by the type D cyclins [34], which is responsible for the progression of G1 to S phase of cell cycle transition [20].

A number of cell cycle regulators have been supposed to play a role in pituitary tumorigenesis, like *p27* and *p16*[9,16], and in this sense, loss of $p18^{INK4C}$ gene expression together with others CDKIs was already linked to endocrine tumorigenesis [24,29]. In mice, the isolated down-regulation of $p18^{INK4C}$ gene expression resulted in pituitary tumor development [28]. Due to the pivotal roles of CDKs in tumor transformation, the inhibition of CDK family members has attracted particular attention in the development of antitumor therapy. Studies have shown that inhibition of CDK is an effective option for suppressing abnormal tumor cell proliferation [35]. Considering that about 80% of human pituitary adenomas present some alteration in, at least, one gene responsible for the progression of G1 to S phase of cell cycle transition [20], plus the limited information about pituitary tumorigenesis as well as $p18^{INK4C}$ role in pituitary adenoma transformation, we aimed to evaluate the $p18^{INK4C}$ relative expression in pituitary adenomas and investigate its association with each clinical tumor subtype.

Table 2: Clinicopathological classification of pituitary adenomas.

Patient's ID	Age (Years)	Gender	Tumor Size Classification*	Hormone Staining (IHQ)	Clinical Diagnosis
1	55	M	Macro	LH	Non-functioning
2	72	F	Macro	LH, FSH	Non-functioning
3	73	M	Macro	FSH	Non-functioning
4	69	M	Macro	LH, FSH	Non-functioning
5	45	M	Macro	GH,PRL	Non-functioning
6	53	M	Macro	LH, FSH	Non-functioning
7	65	M	Macro	Negative	Non-functioning
8	58	M	Macro	GH,PRL	Non-functioning
9	33	F	Macro	GH	Non-functioning
10	45	M	Macro	PRL	Non-functioning
11	70	F	Macro	GH, PRL	Non-functioning
12	60	M	Macro	GH	Non-functioning
13	63	F	Macro	Negative	Non-functioning
14	49	M	Macro	FSH	Non-functioning
15	34	F	Macro	GH,PRL	Acromegaly
16	66	F	Macro	GH	Acromegaly
17	42	F	Macro	GH	Acromegaly
18	58	M	Macro	GH,PRL	Acromegaly
19	40	F	Macro	GH, PRL	Acromegaly
20	40	M	Macro	GH, PRL	Acromegaly
21	38	F	Macro	GH, PRL	Acromegaly
22	48	M	Macro	GH, ACTH, PRL	Acromegaly
23	46	M	Macro	GH	Acromegaly
24	46	M	Macro	GH, ACTH, TSH, FSH, LH, PRL	Acromegaly
25	18	M	Macro	GH, LH, FSH, PRL	Acromegaly
26	43	F	Micro	ACTH	Cushing Disease
27	63	M	Micro	GH,PRL,ACTH	Cushing Disease
28	20	M	Macro	ACTH, GH, PRL	Cushing Disease
29	40	F	Micro	ACTH, TSH, PRL, FSH, LH, GH	Cushing Disease
30	22	F	Micro	ACTH	Cushing Disease
31	37	F	Macro	PRL	Prolactinoma
32	45	F	Macro	PRL	Prolactinoma
33	28	F	Macro	GH,PRL	Prolactinoma
34	22	M	Macro	PRL	Prolactinoma
35	30	F	Macro	GH,PRL	Prolactinoma
36	58	M	Macro	PRL	Prolactinoma
37	48	M	Macro	PRL	Prolactinoma
38	33	F	Macro	GH, PRL	Prolactinoma

M: male; F: female; Macro: macroadenomas (≥1cm), Micro: microadenomas (≤1cm); IHQ: Imunohistochemical staining; Non-functioning: no clinical evidence of hormone secretion; Acromegaly: growth hormone producing; Cushing disease: Adrenocorticotroph-hormone producing; Prolactinoma: prolactin-hormone producing, Negative: null cell.

*Determined by imaging and surgical parameters.

This study showed no significant relationship between the tumor prevalence and the patients' gender, in agreement with previous publications [5,15,36]. Although, patients diagnosed with FAs were statistically younger than the patients diagnosed with NFAs. This data could be explained by the fact that the FAs are diagnosed sooner than the NFAs ones, mainly due to the earlier presentation of its symptoms. These data are in agreement with recent ones published by CBTRUS, pointing that these tumors are not common in patients until 19 years old, representing 2% of all brain tumors diagnosed. According some publishing by CBTRUS, in which the incidence of these tumors is low until 55-65 years old, and increases progressively with age, with a highest incidence among 65-74 years old [2].

Among the tumors in the FAs group, 83.3% were classified as

macroadenomas and 16.6% as microadenomas, whereas in the NFAs group 100% of the tumors were macroadenomas. The ACTH-secreting adenomas presented statistically significant variance in tumor size compared with the other samples included in this study. It was found an association between tumor size and subtype (P=0.001) in the subset of ACTH-secreting, while four of five ACTH-secreting adenomas were microadenomas. These data are in agreement with previous publications that show high frequency among corticotrophinomas as microadenomas. In some cases, the small tumor size can even hamper its location through radiological exams [37,38].

It was found a significant down-regulation of $p18^{INK4C}$ relative gene expression in NFAs when compared with FAs group. These data are in agreement with Hossain *et al.*, who had previously observed that $p18^{INK4C}$ gene expression is down-regulated in pituitary adenomas when compared with the normal gland tissue [17]. Considering that $p18^{INK4C}$ gene is a CDK 4 and 6 families inhibitor, which acts regulating the cell cycle progression, the loss of gene expression would be one of the causes of the high cell proliferative potential in the NFAs, and highlights contribution of failures in the regulatory mechanisms of the progression in this pathology. We speculate that these failures would be an explanation to the tumor growth in NFAs [34,39,40]. Thus, in the present work, we were able to suppose that $p18^{INK4C}$ was differently expressed in NFAs compared to FAs. These data once more corroborate the findings published by Hossain *et al.* who had observed that $p18^{INK4C}$ relative gene expression value in the NFAs and the ACTH-secreting adenomas corresponds to 0.5 or less, what is 50% of the gene expression arbitrary value compared with the normal pituitary tissue [17]. Therefore, in this study, we demonstrate that $p18^{INK4}$ is under-expressed at the gene level mainly in NFAs, and this finding indirectly supports the candidacy of $p18^{INK4}$ as a pituitary related marker.

A limitation of our study is the small sample of functioning adenomas subtype, and no samples of thyrotroph and gonadotroph tumors, but these reflect the rarity of these tumors and the generally small size of these tumors, which can make it difficult to obtain workable amount of material in some cases.

Evidences that tumorigenesis in NFAs and FAs occurs through different molecular pathways had already been described. *Meg3 e Gadd45y* negative cell cycle regulators were also significantly down-regulated in NFAs when compared to FAs [15]. Therefore, based in the present results, the loss of expression of $p18^{INK4C}$ gene might be a contribution mainly to the non-functioning sporadic pituitary adenomas tumorigenesis. It´s already known that control of the cell cycle is a vital part of the cell's replication machinery. Disruption of this process is commonly seen in pituitary tumors and we are now beginning to identify regulatory elements, which are likely to play a major role in pituitary oncogenesis. Complementary studies are required to understand CDK4 and 6 families and their inhibitors contributions to pituitary tumorigenesis, to elucidate the intracellular signaling pathways involved in those tumors.

Elucidation of the mechanisms underlying tumorigenesis and transformation from benign to invasive/atypical pituitary adenomas represent a major challenge to the current understanding of these tumors, and may have important implications for diagnosing molecular classification, prognosis regarding tumor progression and recurrence, guiding adjuvant treatments, such as radiation therapy, and identifying potential targets for future therapies. In this sense, a better understanding of the gene expression and of the mechanisms that regulate it may facilitate the development of therapeutic agents affecting the phenotypical behavior of these tumors.

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