Systematic investigation of expression of G2/M transition genes reveals CDC25 alteration in nonfunctioning pituitary adenomas

Henriett Butz[1], Kinga Németh[2], Dóra Czenke[2], István Likó[3], Sándor Czirják[4], Vladimir Zivkovic[5], Kornélia Baghy[6], Márta Korbonits[7], Ilona Kovalszky[6], Péter Igaz[2], Károly Rácz[1,2], Attila Patócs[2,3]

[1]Molecular Medicine Research Group, Hungarian Academy of Sciences and Semmelweis University, Budapest, Hungary

[2]2nd Department of Medicine, Faculty of Medicine, Semmelweis University, Budapest, Hungary

[3]MTA-SE „Lendulet" Hereditary Endocrine Tumors Research Group, Hungarian Academy of Sciences and Semmelweis University, Hungary

[4]National Institute of Neurosurgery, Budapest, Hungary

[5]Institute of Forensic Medicine, University of Belgrade - School of Medicine, Belgrade

[6]1st Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary

[7]Department of Endocrinology, Barts and the London School of Medicine, Queen Mary University of London, London, UK

Keywords: pituitary adenoma, cell cycle, G2/M transition, CDC25A, miRNA

Running title: CDC25A in pituitary adenomas

Please, send correspondence to:

Dr. Butz Henriett MD, PhD

Molecular Medicine Research Group

Hungarian Academy of Sciences and Semmelweis University

46 Szentkirályi str.

Budapest, Hungary H-1088

Fax: +36 1 266 0926

Phone: +36 1 266 0926/55577

E-mail: butz.henriett@med.semmelweis-univ.hu

Acknowledgements

This work has been funded by National Development Agency (KTIA_AIK-2-2012-0010 to Karoly Racz) and by Hungarian Scientific Research Grant (OTKA PD116093 to Henriett Butz). Attila Patocs received the „Lendulet" grant from Hungarian Academy of Sciences.

Competing Interests: "The authors have declared that no competing interests exist."

Abstract

Introduction. Dysregulation of G1/S checkpoint of cell cycle has been reported in pituitary adenomas. In addition, our previous finding showing that deregulation of Wee1 kinase by microRNAs together with other studies demonstrating alteration of G2/M transition in nonfunctioning pituitary adenomas (NFPAs) suggest that G2/M transition may also be important in pituitary tumorigenesis.

Objective. To systematically study the expression of members of the G2/M transition in NFPAs and to investigate potential microRNA (miRNA) involvement.

Methods. Totally, 80 NFPA and 14 normal pituitary (NP) tissues were examined. Expression of 46 genes encoding members of the G2/M transition was profiled on 34 NFPA and 10 NP samples on TaqMan Low Density Array. Expression of CDC25A and two miRNAs targeting CDC25A were validated by individual quantitative real time PCR using TaqMan assays. Protein expression of CDC25A, CDC25C, CDK1 and phospho-CDK1 (Tyr-15) was investigated on tissue microarray and immunohistochemistry.

Results. Several genes' expression alteration were observed in NFPA compared to normal tissues by transcription profiling. On protein level CDC25A and both the total and the phospho-CDK1 were overexpressed in adenoma tissues. CDC25A correlated with nuclear localized CDK1 (nCDK1) and with tumor size and nCDK1 with Ki-67 index. Comparing primary vs. recurrent adenomas we found that Ki-67 proliferation index was higher and phospho-CDK1 (inactive form) was downregulated in recurrent tumors compared to primary adenomas.

Investigating the potential causes behind CDC25A overexpression we could not find copy number variation at the coding region nor expression alteration of CDC25A regulating transcription factors however CDC25A targeting miRNAs were downregulated in NFPA and negatively correlated with CDC25A expression.

Conclusion. Our results suggest that among alterations of G2/M transition of the cell cycle, overexpression of the CDK1 and CDC25A may have a role in the pathogenesis of the NFPA and that CDC25A is potentially regulated by miRNAs.

Introduction

Pituitary adenomas represent the second most frequent (15.3%) central nervous system tumors following meningiomas [1]. Based on recent data their overall prevalence rates varies from 10 to 22%, but clinically relevant pituitary adenomas appear more rarely [2-5]. Also, in recent publications it is described that the prevalence is 3-5 times higher than previously reported [6-7].

Although the familial well-defined hereditary tumor syndromes are linked to a dysfunction of a single gene (*MEN1*, *PRKAR1A*, *AIP*), the molecular mechanisms leading to sporadic pituitary tumor development are still largely unknown particularly for hormonally inactive, nonfunctioning adenomas (NFPA) [8].

In the pituitary, dysregulation of cell cycle has been demonstrated mainly through alteration of genes regulating the G1/S checkpoint [9]. Underexpression of cyclin-dependent inhibitors (CDKIs) (p14, p15, p16, p18, p21, p27), retinoblastoma protein [10-11], and MEG3a (a strong cell growth suppressor that transactivates p53 thereby regulates cell cycle progression) have been found downregulated through hypermethylation in pituitary adenomas [12-13]. Cyclin D and Cyclin E which are involved in G0-G1 phases of cell cycle were overexpressed in 49% (Cyclin D1) and 37% (Cyclin E) of these adenomas as compared to normal tissues [14-16]. Cyclin A labeling index (LI) was also significantly higher in pituitary adenomas [16].

Our group previously identified that Wee1 kinase, a nuclear protein that delays mitosis was downregulated in growth hormone (GH) producing adenomas and NFPAs compared to normal pituitary [17]. Wee1 phosphorylates CDK1 and inhibits its kinase activity, thereby preventing entry into the mitosis at the G2/M checkpoint. Its tumor suppressor function and downregulation showed correlation with prognosis, recurrence and proliferation index in colon cancer and non-small-cell lung cancer [18-19]**.** Additionally, bioinformatics and network analysis of pituitary proteomic data [20] identified dysregulation of cell cycle at the G2/M DNA damage checkpoint, signaling pathways significantly altered in pituitary adenomas. Other clues for involvement of G2/M transition and its promising targeting in pituitary tumors came from the study of Yu et al [21]. They showed that proteosome inhibitors induced apoptosis in pituitary adenoma but not in normal pituitary cells in rat

through blocking the cell cycle at G2/M transition [21]. Furthermore, it was observed that a bioflavonoid was able to inhibit growth through G2/M arrest and inducing apoptosis in lung cancer cell line [22].

Related to pituitary an evaluation of G2/M transition genes in nonfunctioning adenomas is still lacking, therefore our aim was to evaluate the expression of members of G2/M transition and to assess whether any correlation could exist between expression data and clinico-pathological findings.

Materials and methods

Patients

Pituitary adenoma tissues were removed by transsphenoideal surgery at the Hungarian National Institute of Neurosurgery between 2007 and 2011. Totally, 80 NFPAs (36 females, 44 males, mean age±SD: 58.1±13.92 years) and 14 normal pituitary tissue specimens were used for analysis. Ten normal pituitary samples for mRNA expression analysis were obtained by autopsy within 6 h of death from patients with no evidence of any endocrine disease (University Clinical Centre, Belgrade, Serbia). All adenoma samples were consecutively gathered with the permission of the local committee on human research, after written informed consent of each patient. The research protocol was approved by the Scientific and Research Ethics Committee of the Medical Research Council (nr: ad.4457/20121/EKU). RNA extracted from 34 NFPAs and 10 NP fresh frozen specimens were used for initial screening performed with TaqMan Array, 46 NFPA and 4 normal pituitary tissues were used for Tissue microarray (TMA) and 23 NFPAs and 10 NP samples were used for RT-qPCR validation. For TMA adjacent normal pituitary tissues surrounding 4 adenomas (one NFPA and 3 hormone producing adenoma) were used as normal control.

The clinical diagnosis of NFPAs was based on hormone levels measured in serum obtained from patients and on results of immunohistochemistry analysis for SF1 transcription factor specific for gonadotrope origin and six anterior lobe hormones following WHO classification [23]. Immunostaining of Ki-67 proliferation marker in each sample was also performed. All

immunohistochemical studies were carried out at the 1st Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary.

RNA isolation

Removed specimens were stabilized in RNA later (Life Technologies, Grand Island, NY, USA) and then stored at -80 ºC until use. Total RNA was extracted using miRNeasy Mini Kit (Qiagen Inc., Chatsworth, CA). RNA integrity and concentration were measured using Agilent Bioanalyzer 2100 System (Agilent Tech Inc., Santa Clara, USA).

Gene expression profiling using custom TaqMan Low Density Array microfluidic card

Expression profile of 46 genes was analyzed in 34 NFPA and 10 normal pituitary samples using custom made TaqMan Low Density Array (TLDA) (Life Technologies, Grand Island, NY, USA) including assays for POU1F1 and members of the G2/M transition. All procedures were performed according to the manufacturer's instructions. Briefly, reverse transcription of 1 μg of total RNA was performed using the Superscript III First Strand Synthesis Kit (Life Technologies). Then 5 μl undiluted RT product, 55 μl TaqMan Universal PCR Master Mix and 50 μl DEPC treated water were loaded into each channel of TLDA card in a 100 μl final volume. The mRNA expression was determined with RT-qPCR using the 7900 Fast Real-Time PCR System (Life Technologies).

Real-time quantification of miRNAs and CDC25A and CDC25C

RT-qPCR was executed as previously described (17;24). Reverse transcription was performed using Superscript III First Strand Synthesis Kit (Life Technologies) or miRNA specific stem-loop RT primer using TaqMan MicroRNA Reverse Transcription Kit (P/N: 4366596) according to protocols provided by the supplier. For the qPCR specific Taqman probes and TaqMan Universal PCR Master Mix were used (Life Technologies). Reactions were run in triplicates in 384-well plate on 7900 HT RealTime PCR System (Life Technologies). The following Gene Expression and Individual MicroRNA Assays from Applied Biosystems were used: CDC25C (Hs00156411_m1), CDC25A (Hs00947994_m1), CDK1 (Hs00938777_m1), ACTB (Hs99999903_m1), GAPDH (Hs99999905_m1); and hsa-miR-424

(Assay ID: 001149), hsa-miR-503 (Assay ID: 001048), U6 snRNA (Assay ID: 001973), RNU48 (Assay ID: 001006), RNU44 (Assay ID: 001094). Gene expression data were normalized with the use of geometric mean of ACTB and GAPDH in the case of mRNA and geometric mean of RNU44, RNU48 and U6 snRNA in the case of miRNAs as previously those were identified to be stable endogenous controls in pituitary [24]. Expression level was calculated by the ddCt method, and fold changes were obtained using the formula 2^{-ddCt}.

Tissue microarray (TMA), immunohistochemistry, digital microscopy, scoring and image analysis

Formalin-fixed, paraffin-embedded tissues from 46 pituitary samples were selected based on haematoxylin and eosin (H&E) staining and immunostaining for the six anterior lobe hormones. Two-mm cores were collected into a 7x10-sample TMA recipient block. Excel files containing clinicopathological data of 46 samples from 46 patients were linked to recipient block positions using a computer-driven automated instrument TMA-Master (3DHistech Ltd, Budapest, Hungary).

Following antigen retrieval by boiling (15 min, 0.1 mM pH 6 citrate-buffer) and endogenous peroxidase block (1% H_2O_2), tissues were stained with CDC25A mouse monoclonal (Santa Cruz Biotechnology, sc-56264; dilution: 1:100), CDC25C (C20) rabbit polyclonal (Santa Cruz Biotechnology, sc-327; dilution: 1:100), total CDC2 mouse monoclonal (Cell Signaling, Beverly, USA, #9116; dilution: 1:40) and phospho-CDC2 (p-Tyr15) rabbit polyclonal (NBP1-19966, Novus Biologicals, dilution: 1:40) primary antibodies overnight, and biotin conjugated goat anti-rabbit (DakoE0432) and anti-mouse (DakoE0433) secondary antibodies for 60 min. After the addition of ABC reagent for amplification (Vectastain Elute ABC Kit, PK-6101), diaminobenzidine (DAB) chromogen was used for detection. Optimization of each antibody was executed on individual positive and negative control slides.

Immunostained TMA slides were analyzed following full-slide digitalization with the Panoramic Scan and the database-linked TMA Modul software (both 3DHISTECH Kft, Budapest, Hungary). Quantitation was performed using a 12-score system considering both *intensity and frequency* of stained cells. Briefly, weak staining was scored by 1-4, moderate staining was scored by

5-8 and strong staining by 9-12 according to the number of positive cells (<10%, 10-40%, 40-80% and >80%) using TMA Modul Software (3DHISTECH Kft, Budapest, Hungary). Scoring was performed by two independent assessors and average scores were used for statistical analysis. Each score was normalized for the average score of normal samples.

Identification of miRNAs targeting CDC25A and CDC25C by *in silico* target prediction

For target prediction we used four computational target prediction algorithms: microrna.org (http://www.microrna.org/microrna/home.do), Targetscan 4.0 (http://www.targetscan.org), MicroCosm (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) and miRWalk (http://www.ma.uni-heidelberg.de/apps/zmf/mirwalk/index.html)

Statistical analysis

After data normalization using geometric mean of endogenous controls, gene expression levels in NFPA were compared to those found in normal pituitary tissues using T-test or Mann-Whitney U test depending on the results of Shapiro-Wilks normality test. Statistical analysis was performed using Statistica 7.0 software (StatSoft Inc., Tulsa, USA). For correlation analysis between gene expressions and expression of miRNAs and CDC25A and CDC25C or tumor size Pearson's or Spearman rank correlation tests were used. Nonparametric Kruskal-Wallis ANOVA and Spearman rank correlation were used as statistical methods for analyzing TMA results regarding the discrete variables. A value of $p<0.05$ was considered to be significant. Tumor size was calculated using the formula: tumor width x length x height in mm using three direction MRI scans.

Results

1. Expression of genes encoding members of G2/M transition in NFPAs

By analyzing the expression of GH, PRL, FSH, LH, POMC and POU1F1 (Pit-1) genes adenomas expressing POU1F1 transcription factor mRNA were excluded indicating contamination by nontumorous elements [23, 25-26]. Using GAPDH and ACTB as endogenous controls all adenomas

showed decreased expression of GH, PRL and POMC on mRNA level compared to control samples (**Suppl. Figure 1A-1B**).

Expression of CDC25A, CDC25C, Cyclin B, CDCA8, AURKB, BIRC5, YWHAB, BRCA2 and FANCD2 were significantly overexpressed while CDK7, p21, TP53, CHEK2, AURC, GADD45B were underexpressed in NFPA tissue specimens compared to normal pituitary (**Table 1)**. We also identified several correlations among the measured genes which are illustrated on **Suppl. Figure 2**, and showed that CDC25A mRNA expression level correlated with Cyclin B as well as expression of CDC25C with CDK1 in NFPAs but not in normal pituitary tissue.

For validation of the TLDA results expression of CDC25A and CDC25C using individual TaqMan assays was measured on 23 pituitary samples, and the results were similar to those obtained with the TLDA card experiment (**Figure 1**).

2. Increased expression of CDC25A and CDK1 at protein level in NFPAs

After gene expression study we assessed the expression of CDC25A, CDC25C and their target total CDK1 and p-CDK1 on protein level using TMA. CDC25A, total CDK1 and phospho-CDK1 (pCDK1: inactive form) were significantly overexpressed in NFPA samples compared to normal tissues (**Figure 2**). However, the ratio of pCDK1/CDK1 was similar in the two groups of samples suggesting no change in the activity rate only in total amount of protein. In summary, CDC25A was overexpressed in 76% (35/46) of NFPAs (the expression level of CDC25A was higher in all of these adenomas than the highest expression detected in normal tissues). CDC25C was overexpressed in 45.6% (21/46) of NFPAs, but globally the expression of CDC25C at protein level did not differ significantly between NFPA and normal tissues. Elevated level of total CDK1 and pCDK1 were detected in 82.6% (38/46) and 63% (29/46) of NFPAs, respectively. The nuclear CDK1 (nCDK1), staining score did not differ significantly between NFPA and normal tissue.

Correlation between the expression of CDC25A and CDC25C proteins was observed in NFPAs ($p=0.001$) (**Figure 3A**). CDC25A at protein level correlated with tumor size (**Figure 3B**) and with nCDK1 (**Figure 3**C). Nuclear CDK1 the active form of the kinase, showed a positive correlation with

the Ki-67 proliferation index (p=0.0003). Expressions of CDC25C failed to correlate with the nCDK1 scores or tumor size. Neither CDC25A nor CDC25C staining correlated with Ki67 (data not shown).

Comparing primary vs. recurrent adenomas we found that Ki67 proliferation index was higher in recurrent tumours (p=0.001). Also, the inactive form of the CDK1 protein (pCDK1) showed higher expression in primary vs. recurrent adenomas (p=0.008).

3. Potential causes of CDC25 overexpression

In order to reveal potential causes behind CDC25A overexpression we tested copy number variations of chromosomal localizations of *CDC25A*, expressions of transcription factors regulating CDC25A and miRNAs targeting 3'UTR of *CDC25A*.

Copy number variation of chromosomal localizations of *CDC25A* and 3p21 were evaluated in datasets of five independent comparative genome hybridization (CGH) studies performed on pituitary adenoma samples but chromosomal gain for these loci was detected only in a small proportion of NFPAs [27-31] suggesting that no gene amplification occurs in the majority of samples.

For investigating transcription factors regulating CDC25A expression we searched potential binding sites in the *CDC25A* promoter region (2000 bp from start codon 5'upstream) using TFblast (http://www.gene-regulation.com/cgi-bin/pub/programs/tfblast/tfblast.cgi). We identified 7 human transcription factors (data not shown) potentially having binding sites. By reviewing the data obtained in six different high throughput mRNA and/or protein screening studies published earlier (4 mRNA microarray studies [32-35], one study evaluated mRNA microarray parallel with protein array [36] and 1 protein array [37]) we found that none of these transcription factors were differentially expressed in any of the studies (data not shown).

In addition, it has been demonstrated that behind the discordant expression of CDC25 on mRNA and protein level posttranscriptional or posttranslational mechanisms could also stand [38]. Moreover, CDC25A has been described as an experimentally validated target of 8 miRNAs [39-44]. Therefore, we investigated the expression of those 8 miRNAs expression in NFPAs and NPs. We found that miR-449a, -449b, 424 and -503 were significantly downregulated in adenomas compared to NPs and two of

them correlated with tumor size (**Figure 4A**). Of these four miRNAs miR-424 and miR-503 negatively correlated with CDC25A expression (**Figure 4B-C**).

Discussion

Vast literature data suggest the involvement of dysregulation of cell cycle in the pathogenesis of pituitary adenomas but the complex regulation of the G2/M transition has not been comprehensively evaluated. In our current study after evaluation of the expression of members of G2/M transition we further focused on CDC25 family. The CDC25 family members CDC25A, CDC25B and CDC25C have been identified in mammals and each isoform possesses different role in regulation of cell cycle. CDC25A is implicated in the G1/S transition and it is also able to dephosphorylate (activate) the CDK1-Cyclin B complex and, thereby, to promote G2/M transition, while CDC25B and CDC25C were considered as mitotic regulators [45-50]. CDC25A and CDC25B have been considered as oncogenes, their overexpression has been frequently demonstrated in several types of cancer [51].

We found that CDC25A was overexpressed in NFPAs both at mRNA and protein levels, and CDC25C showed overexpression only at mRNA. This finding is in line with other observations showing a discordant expression of CDC25 phosphatases at mRNA transcript and protein levels [38]. By reviewing data of five different CGH studies performed on pituitary adenoma samples and all available published mRNA microarray datasets no chromosomal gain or overexpression of CDC25A regulating transcription factors have been revealed. These results suggested that behind of overexpression of CDC25A posttranscriptional or posttranslational mechanisms stand.

Our results showed that the whole G2/M transition was over-activated in NFPA compared to normal tissues. Overexpression of genes promoting mitosis (chromosomal passenger complex (CPC) members CDC8A (Borealin), BIRC5 and AURKB) further supports this observation. AURKB was also reported to be expressed in 80% (8/10) of benign prolactiomas and showed higher expression in aggressive prolactinomas versus non-aggressive ones and it was also associated with recurrence or progression [52-53].

CDC25A and CDC25C dephosphorylate CDK1 on Tyrosine-15 (Tyr-15). CDK1 forms a complex with Cyclin B. In NFPAs, we found elevated CDK1 at protein level but not at mRNA level suggesting that posttranscriptional regulation of the CDK1 expression occurred. Among miRNAs targeting CDK1 [54-56] miR-410 and miR-24 were underexpressed in NFPAs compared to NPs [24]. CDC25A targeting miR-424 and miR-503 were downregulated in nonfunctioning and gonadotrope adenomas but not in GH-producing tumors, and their expression correlated with pituitary adenoma size [24, 57-61]. These two miRNAs are encoded at Xq26.3 in a miRNA cluster together with hsa-miR-450a, miR-450b and hsa-miR-542. Importantly, the latter three miRNAs of the cluster were also found to be underexpressed in our previous analysis in NFPA samples compared to normal pituitary [24] and their expression level also showed a strong negative correlation with tumor size [24]. Because CDC25A protein but not mRNA expression positively correlated with adenoma size, we conclude that miR-424 and miR-503 may have a role in the regulation of CDC25A expression in NFPAs and they function as tumor suppressor miRNAs. Our study revealed an inverse correlation between miR-424, miR-503 and tumor size further supporting their role in the pathogenesis of NFPA.

In summary, to our best knowledge this is the first report systematically investigating G2/M transition and reporting CDC25 alteration in NFPA using a relative large sample size. Our results suggest that overexpression of CDK1 and CDC25A have a role in tumor growth of NFPA via dysregulating G2/M transition of cell cycle. We assume that the CDC25A-CDK1 pathway is a potential therapeutic target in NFPA. Our results presented that the overexpression of CDC25A is at least partly related to the decreased expression of tumor suppressor miRNAs (miR-424 and miR-503).

Reference List

(1) Dolecek TA, Propp JM, Stroup NE, Kruchko C. CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2005-2009. (2012) Neuro Oncol; Suppl 5:v1-49

(2) McDowell BD, Wallace RB, Carnahan RM, Chrischilles EA, Lynch CF, Schlechte JA Demographic differences in incidence for pituitary adenoma. (2011) Pituitary 14: 23-30

(3) Daly AF, Tichomirowa MA, Beckers A. The epidemiology and genetics of pituitary adenomas. (2009) Best Pract Res Clin Endocrinol Metab 23: 543-54

(4) Ezzat S, Asa SL, Couldwell WT, Barr CE, Dodge WE, Vance ML, McCutcheon IE. (2004) The prevalence of pituitary adenomas: a systematic review. Cancer 101: 613-9

(5) Tjörnstrand A, Gunnarsson K, Evert M, Holmberg E, Ragnarsson O, Rosén T, Filipsson Nyström H. (2014) The incidence rate of pituitary adenomas in western Sweden for the period 2001-2011. Eur J Endocrinol 171: 519-26

(6) Beckers A. (2010) Higher prevalence of clinically relevant pituitary adenomas confirmed. Clin Endocrinol (Oxf) 72: 290-1

(7) Fernandez A, Karavitaki N, Wass JA. Prevalence of pituitary adenomas: a community-based, cross-sectional study in Banbury (Oxfordshire, UK). (2010) Clin Endocrinol (Oxf) 72: 377-82

(8) Heaney AP. (2005) Pituitary tumour pathogenesis. Br Med Bull 75-76: 81-97

(9) Musat M, Morris DG, Korbonits M, Grossman AB. (2010) Cyclins and their related proteins in pituitary tumourigenesis. Mol Cell Endocrinol 326: 25-9

(10) Ogino A, Yoshino A, Katayama Y, Watanabe T, Ota T, Komine C, Yokoyama T, Fukushima T. (2005) The p15(INK4b)/p16(INK4a)/RB1 pathway is frequently deregulated in human pituitary adenomas. J Neuropathol Exp Neurol 64: 398-403

(11) Yoshino A, Katayama Y, Ogino A, Watanabe T, Yachi K, Ohta T, Komine C, Yokoyama T, Fukushima T. (2007) Promoter hypermethylation profile of cell cycle regulator genes in pituitary adenomas. J Neurooncol 83: 153-62

(12) Zhao J, Dahle D, Zhou Y, Zhang X, Klibanski A. (2005) Hypermethylation of the promoter region is associated with the loss of MEG3 gene expression in human pituitary tumors. J Clin Endocrinol Metab 90: 2179-86

(13) Gejman R, Batista DL, Zhong Y, Zhou Y, Zhang X, Swearingen B, Stratakis CA, Hedley-Whyte ET, Klibanski A. (2008) Selective loss of MEG3 expression and intergenic differentially methylated region hypermethylation in the MEG3/DLK1 locus in human clinically nonfunctioning pituitary adenomas. J Clin Endocrinol Metab 93: 4119-25

(14) Simpson DJ, Frost SJ, Bicknell JE, Broome JC, McNicol AM, Clayton RN, Farrell WE (2001) Aberrant expression of G(1)/S regulators is a frequent event in sporadic pituitary adenomas. Carcinogenesis 2001;22: 1149-54

(15) Jordan S, Lidhar K, Korbonits M, Lowe DG, Grossman AB. (2000) Cyclin D and cyclin E expression in normal and adenomatous pituitary. Eur J Endocrinol 2000;143: R1-R6.

(16) Turner HE, Nagy Z, Sullivan N, Esiri MM, Wass JA. Expression analysis of cyclins in pituitary adenomas and the normal pituitary gland. (2000) Clin Endocrinol (Oxf) 53: 337-44

(17) Butz H, Liko I, Czirjak S, Igaz P, Khan MM, Zivkovic V, Balint K, Korbonits M, Racz K, Patocs A. (2010) Down-regulation of Wee1 kinase by a specific subset of microRNA in human sporadic pituitary adenomas. J Clin Endocrinol Metab 95: E181-E191

(18) Backert S, Gelos M, Kobalz U, Hanski ML, Bohm C, Mann B, Lovin N, Gratchev A, Mansmann U, Moyer MP, Riecken EO, Hanski C. Differential gene expression in colon carcinoma cells and tissues detected with a cDNA array. (1999) Int J Cancer 82: 868-74

(19) Yoshida T, Tanaka S, Mogi A, Shitara Y, Kuwano H. The clinical significance of Cyclin B1 and Wee1 expression in non-small-cell lung cancer. (2004) Ann Oncol 15: 252-6

(20) Zhan X, Desiderio DM. (2010) Signaling pathway networks mined from human pituitary adenoma proteomics data. BMC Med Genomics 3: 13

(21) Yu R, Ren SG, Melmed S. (2002) Proteasome inhibitors induce apoptosis in growth hormone- and prolactin-secreting rat pituitary tumor cells. J Endocrinol 174: 379-86

(22) Yang JH, Hsia TC, Kuo HM, Chao PD, Chou CC, Wei YH, Chung JG. (2006) Inhibition of lung cancer cell growth by quercetin glucuronides via G2/M arrest and induction of apoptosis. Drug Metab Dispos 34: 296-304

(23) Al-Shraim M, Asa SL. (2006) The 2004 World Health Organization classification of pituitary tumors: what is new? Acta Neuropathol 111: 1-7

(24) Butz H, Likó I, Czirják S, Igaz P, Korbonits M, Rácz K, Patócs A. (2011) MicroRNA profile indicates downregulation of the TGFbeta pathway in sporadic non-functioning pituitary adenomas. Pituitary 14: 112-24

(25) Kobayashi I, Oka H, Naritaka H, Sato Y, Fujii K, Kameya T. (2002) Expression of Pit-1 and growth hormone-releasing hormone receptor mRNA in human pituitary adenomas: difference among functioning, silent, and other nonfunctioning adenomas. Endocr Pathol 13: 83-98

(26) Asa SL, Bamberger AM, Cao B, Wong M, Parker KL, Ezzat S. (1996) The transcription activator steroidogenic factor-1 is preferentially expressed in the human pituitary gonadotroph. J Clin Endocrinol Metab 81: 2165-70

(27) Daniely M, Aviram A, Adams EF, Buchfelder M, Barkai G, Fahlbusch R, Goldman B, Friedman E. (1998) Comparative genomic hybridization analysis of nonfunctioning pituitary tumors. J Clin Endocrinol Metab 83: 1801-5

(28) Metzger AK, Mohapatra G, Minn YA, Bollen AW, Lamborn K, Waldman FM, Wilson CB, Feuerstein BG. (1999) Multiple genetic aberrations including evidence of chromosome 11q13 rearrangement detected in pituitary adenomas by comparative genomic hybridization. J Neurosurg 90: 306-14

(29) Harada K, Nishizaki T, Ozaki S, Kubota H, Harada K, Okamura T, Ito H, Sasaki K. (1999) Cytogenetic alterations in pituitary adenomas detected by comparative genomic hybridization. Cancer Genet Cytogenet 112: 38-41

(30) Trautmann K, Thakker RV, Ellison DW, Ibrahim A, Lees PD, Harding B, Fischer C, Popp S, Bartram CR, Jauch A. (2001) Chromosomal aberrations in sporadic pituitary tumors. Int J Cancer 91: 809-14

(31) Szymas J, Schluens K, Liebert W, Petersen I. (2002) Genomic instability in pituitary adenomas. Pituitary 2002;5: 211-9

(32) Michaelis KA, Knox AJ, Xu M, Kiseljak-Vassiliades K, Edwards MG, Geraci M, Kleinschmidt-DeMasters BK, Lillehei KO, Wierman ME. Identification of growth arrest and DNA-damage-inducible gene beta (GADD45beta) as a novel tumor suppressor in pituitary gonadotrope tumors. (2011) Endocrinology 152: 3603-13

(33) Elston MS, Gill AJ, Conaglen JV, Clarkson A, Shaw JM, Law AJ, Cook RJ, Little NS, Clifton-Bligh RJ, Robinson BG, McDonald KL. (2008) Wnt pathway inhibitors are strongly down-regulated in pituitary tumors. Endocrinology 149: 1235-1242

(34) Evans CO, Young AN, Brown MR, Brat DJ, Parks JS, Neish AS, Oyesiku NM. (2001) Novel patterns of gene expression in pituitary adenomas identified by complementary deoxyribonucleic acid microarrays and quantitative reverse transcription-polymerase chain reaction. J Clin Endocrinol Metab 86: 3097-3107

(35) Morris DG, Musat M, Czirják S, Hanzély Z, Lillington DM, Korbonits M, Grossman AB. (2005) Differential gene expression in pituitary adenomas by oligonucleotide array analysis. Eur J Endocrinol 153: 143-151

(36) Moreno CS, Evans CO, Zhan X, Okor M, Desiderio DM, Oyesiku NM. (2005) Novel molecular signaling and classification of human clinically nonfunctional pituitary adenomas identified by gene expression profiling and proteomic analyses. Cancer Res 65: 10214-10222

(37) Zhan X, Desiderio DM. (2010) Signaling pathway networks mined from human pituitary adenoma proteomics data. BMC Med Genomics. 3:13

(38) Boutros R, Lobjois V, Ducommun B. (2007) CDC25 phosphatases in cancer cells: key players? Good targets? Nat Rev Cancer 7: 495-507

(39) Huang JC1, Babak T, Corson TW, Chua G, Khan S, Gallie BL, Hughes TR, Blencowe BJ, Frey BJ, Morris QD. (2007) Using expression profiling data to identify human microRNA targets. Nat Methods. 4(12):1045-9

(40) Shi L1, Zhang J, Pan T, Zhou J, Gong W, Liu N, Fu Z, You Y. MiR-125b is critical for the suppression of human U251 glioma stem cell proliferation. (2010) Brain Res 1312:120-126

(41) Lee SO, Masyuk T, Splinter P, Banales JM, Masyuk A, Stroope A, Larusso N (2008) MicroRNA15a modulates expression of the cell-cycle regulator Cdc25A and affects hepatic cystogenesis in a rat model of polycystic kidney disease. J Clin Invest. 118:3714-24

(42) de Oliveira PE, Zhang L, Wang Z, Lazo JS. (2009) Hypoxia-mediated regulation of Cdc25A phosphatase by p21 and miR-21. Cell Cycle 8:3157-64

(43) Sarkar S, Dey BK, Dutta A. (2010) MiR-322/424 and -503 are induced during muscle differentiation and promote cell cycle quiescence and differentiation by down-regulation of Cdc25A. Mol Biol Cell 21:2138-49

(44) Yang X, Feng M, Jiang X, Wu Z, Li Z, Aau M, Yu Q. (2009) miR-449a and miR-449b are direct transcriptional targets of E2F1 and negatively regulate pRb-E2F1 activity through a feedback loop by targeting CDK6 and CDC25A. Genes Dev 23:2388-93

(45) Galaktionov K, Beach D. (1991) Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. Cell 67:1181-94

(46) Sadhu K, Reed SI, Richardson H, Russell P. (1990) Human homolog of fission yeast cdc25 mitotic inducer is predominantly expressed in G2. Proc Natl Acad Sci U S A 87: 5139-43

(47) Fernandez-Vidal A, Mazars A, Manenti S. (2008) CDC25A: a rebel within the CDC25 phosphatases family? Anticancer Agents Med Chem 8:825-31

(48) Mailand N, Podtelejnikov AV, Groth A, Mann M, Bartek J, Lukas J. (2002) Regulation of G(2)/M events by Cdc25A through phosphorylation-dependent modulation of its stability. EMBO J 21:5911-20

(49) Li M, Yin S, Yuan J, Wei L, Ai JS, Hou Y, Chen DY, Sun QY. (2008) Cdc25A promotes G2/M transition in oocytes. Cell Cycle 7:1301-2

(50) Melixetian M, Klein DK, Sørensen CS, Helin K. NEK11 regulates CDC25A degradation and the IR-induced G2/M checkpoint. (2009) Nat Cell Biol 11:1247-53

(51) Kristjansdottir K, Rudolph J. (2004) Cdc25 phosphatases and cancer. Chem Biol 11:1043-51

(52) Wierinckx A, Auger C, Devauchelle P, Reynaud A, Chevallier P, Jan M, Perrin G, Fèvre-Montange M, Rey C, Figarella-Branger D, Raverot G, Belin MF, Lachuer J, Trouillas J. (2007) A

diagnostic marker set for invasion, proliferation, and aggressiveness of prolactin pituitary tumors. Endocr Relat Cancer 14:887-900

(53) Raverot G, Wierinckx A, Dantony E, Auger C, Chapas G, Villeneuve L, Brue T, Figarella-Branger D, Roy P, Jouanneau E, Jan M, Lachuer J, Trouillas J. (2010) Prognostic factors in prolactin pituitary tumors: clinical, histological, and molecular data from a series of 94 patients with a long postoperative follow-up. J Clin Endocrinol Metab 95:1708-16

(54) Mraz M, Dolezalova D, Plevova K, Stano Kozubik K, Mayerova V, Cerna K, Musilova K, Tichy B, Pavlova S, Borsky M, Verner J, Doubek M, Brychtova Y, Trbusek M, Hampl A, Mayer J, Pospisilova S. (2012) MicroRNA-650 expression is influenced by immunoglobulin gene rearrangement and affects the biology of chronic lymphocytic leukemia. Blood 119:2110-3

(55) Chien WW, Domenech C, Catallo R, Kaddar T, Magaud JP, Salles G, Ffrench M. (2011) Cyclin-dependent kinase 1 expression is inhibited by p16(INK4a) at the post-transcriptional level through the microRNA pathway. Oncogene 30:1880-91

(56) Takeshita F, Patrawala L, Osaki M, Takahashi RU, Yamamoto Y, Kosaka N, Kawamata M, Kelnar K, Bader AG, Brown D, Ochiya T. (2010) Systemic delivery of synthetic microRNA-16 inhibits the growth of metastatic prostate tumors via downregulation of multiple cell-cycle genes. Mol Ther 18:181-7

(57) Li XH, Wang EL, Zhou HM, Yoshimoto K, Qian ZR. (2014) MicroRNAs in Human Pituitary Adenomas. Int J Endocrinol 2014:435171

(58) Sivapragasam M, Rotondo F, Lloyd RV, Scheithauer BW, Cusimano M, Syro LV, Kovacs K. (2011) MicroRNAs in the human pituitary. Endocr Pathol 22:134-43

(59) Liang S, Chen L, Huang H, Zhi D. (2013) The experimental study of miRNA in pituitary adenomas. Turk Neurosurg 23:721-7

(60) Bottoni A, Zatelli MC, Ferracin M, Tagliati F, Piccin D, Vignali C, Calin GA, Negrini M, Croce CM, Degli Uberti EC. (2007) Identification of differentially expressed microRNAs by microarray: a possible role for microRNA genes in pituitary adenomas. J Cell Physiol 210:370-7

(61) D'Angelo D, Palmieri D, Mussnich P, Roche M, Wierinckx A, Raverot G, Fedele M, Croce CM, Trouillas J, Fusco A. (2012) Altered microRNA expression profile in human pituitary GH adenomas:

down-regulation of miRNA targeting HMGA1, HMGA2, and E2F1. J Clin Endocrinol Metab 97:E1128-E1138

Tables

Table 1. Expression of genes involved in the G2/M transition in NFPA tumors compared to normal tissue

Gene	fold change	p value
GADD45B	0.11	0.0001
CDKN1A	0.17	0.0000
AURKC	0.49	0.0029
CDK7	0.61	0.0005
CHEK2	0.64	0.0082
TP53	0.68	0.0026
YWHAB	1.47	0.0233
CCNB1	1.67	0.0167
FANCD2	1.89	0.0075
CDC25A	2.16	0.0039
CDCA8	2.52	0.0002
CDC25C	2.75	0.0106
BRCA2	2.80	0.0002
BIRC5	5.30	0.0011
AURKB	6.09	0.0000

Figure Legends

Figure 1. Overexpression of CDC25A and CDC25C was validated by individual Taqman assays on real time-quantitative PCR. CDC25A: fold change: 2.8, p=0.001; CDC25C: fold change: 7.0, p<0.001). (Y axis represents log2RQ).

Figure 2. Expression of CDC25A, CDC25C, CDK1 and p-CDK1 in normal pituitary and NFPA. Mean and SE of semi-quantitative immunohistochemistry scores are indicated by the ranges. *, p<0.05; **, p=0.05.

Figure 3. Correlation between the expression of CDC25A, CDC25C proteins, nuclear CDK1, Ki67 and tumor size represented by immunostaining scores. Significant correlation was observed between CDC25A and CDC25C (R=0.45, p=0.001; (**A**)), CDC25A and tumour size (R=0.66, p=0.01; (**B**)), CDC25A and nCDK1 (R=0.34 and p= 0.01; ((**C**)). Spearman correlation analysis was performed and p <0.05 was accepted as statistically significant.

Figure 4. (**A**) Expression of CDC25A targeting miRNAs. All miRNAs were downregulated in NFPA samples compared to normal pituitary (Y axis represents log2RQ). miR-424: fold change: -24.89, p=0.0021; miR449a: fold change:-3.92, p=0.0014; miR-449b: fold change:-15.46, p<0.0001; miR-503: fold change:-53.22, p=0.0016. (**B-C**) Correlation between the expression of CDC25A gene and miR-424 miRNA and CDC25A gene and miR-503 miRNA measured by individual quantitative real-time PCR. Significant correlation was observed between The expression of CDC25A gene and miR-424 miRNA (R=-0.51, p=0.02), and CDC25 A gene and miR-503 miRNA (R=-0.55, p=0.01). Spearman correlation analysis was performed and p<0.05 was accepted as statistically significant.

SF1 Supplementary Figure 1. (**A**) Expression of anterior pituitary hormones' transcripts and POU1F1 transcription factor. (**B**) Endogenous controls in normal (NP) and nonfunctioning pituitary adenoma (NFPA) tissues.

SF2 Supplementary Figure 2. Correlations among the G2/M mRNA expression levels. Red indicates positive, green indicates negative correlations (in all cases $p<0.05$). Black represents no correlation.