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ORIGINAL ARTICLE

Systematic Investigation of Expression of G2/M Transition Genes Reveals CDC25 Alteration in Nonfunctioning Pituitary Adenomas

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

Keywords Pituitary adenoma · Cell cycle · G2/M transition · CDC25A · miRNA

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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 clinicopathological 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 ^{o}C 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

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–b).

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 (Fig. 1).

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 (Fig. 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) (Fig. 3a). CDC25A at protein level correlated with tumor size (Fig. 3b) and with nCDK1 (Fig. 3c). 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 ($p = 0.008$).

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

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

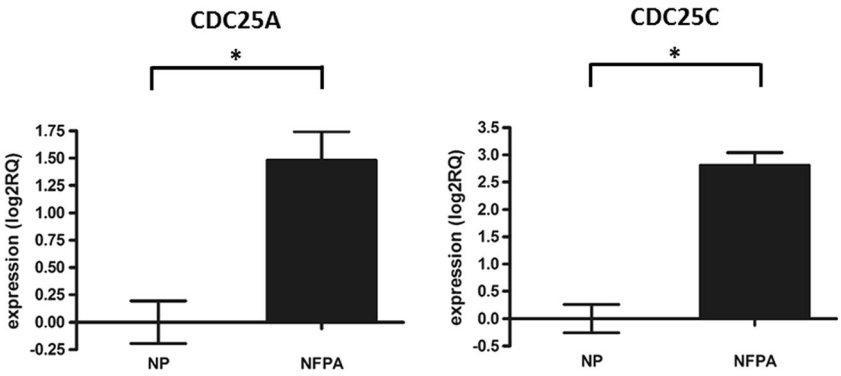

Fig. 1 Overexpression of CDC25A and CDC25C was validated by individual Taqman assays on real time-quantitative PCR. CDC25A: fold change: 2.8, $p = 0.001$; CDC25C: fold change: 7.0, $p < 0.001$). (Y axis represents log2RQ)

(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).

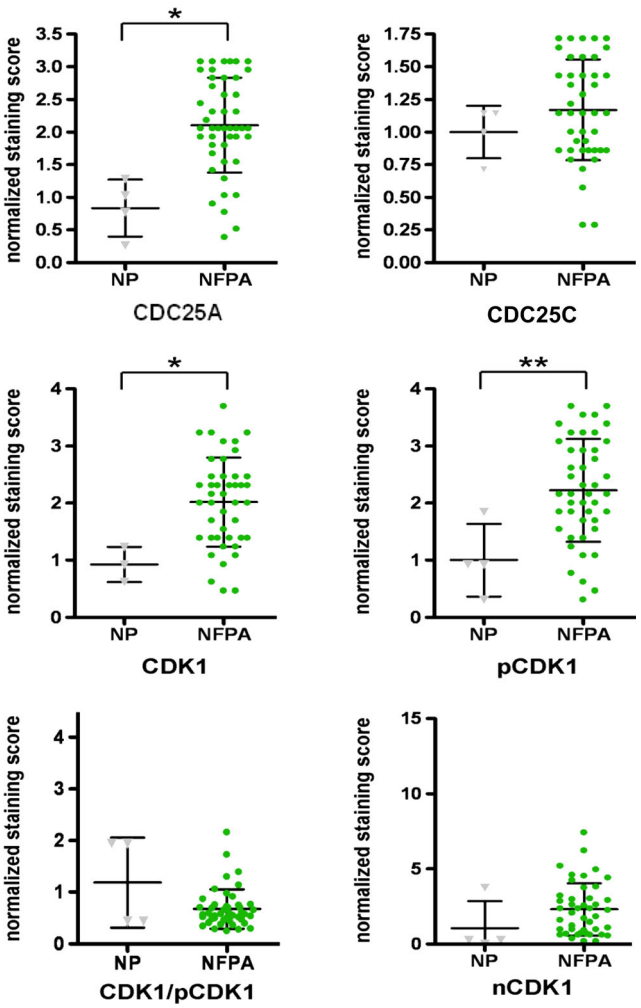

Fig. 2 Expression of CDC25A, CDC25C, CDK1 and p-CDK1 in normal pituitary and NFPA. Mean and SE of semi-quantitative immunohistochemistry scores are indicated by the ranges. *, $p < 0.05$; **, $p = 0.05$

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 (Fig. 4a). Of these four miRNAs miR-424 and miR-503 negatively correlated with CDC25A expression (Fig. 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

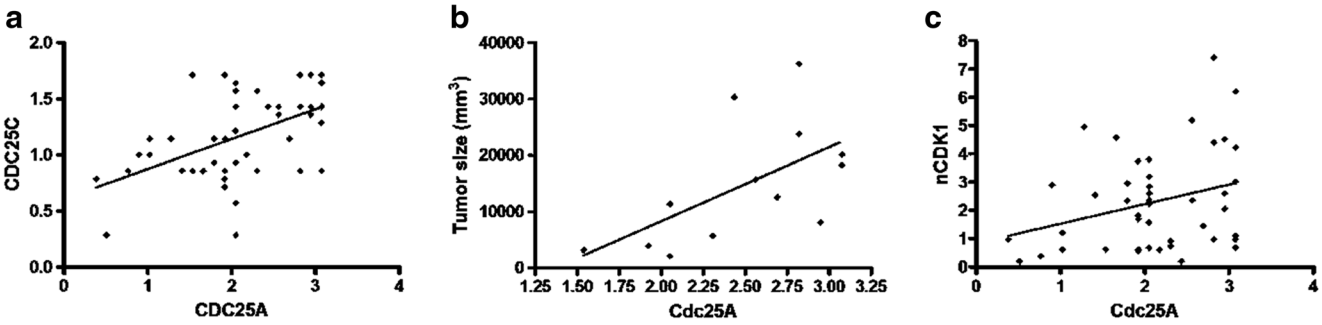

Fig. 3 Correlation between the expression of CDC25A, CDC25C proteins, nuclear CDK1, Ki67 and tumor size represented by immunostaining scores. Significant correlation was observed between CDC25A and CDC25C (R = 0.45, $p = 0.001$; **a**), CDC25A and tumour size (R=0.66, $p = 0.01$; **b**), CDC25A and nCDK1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

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

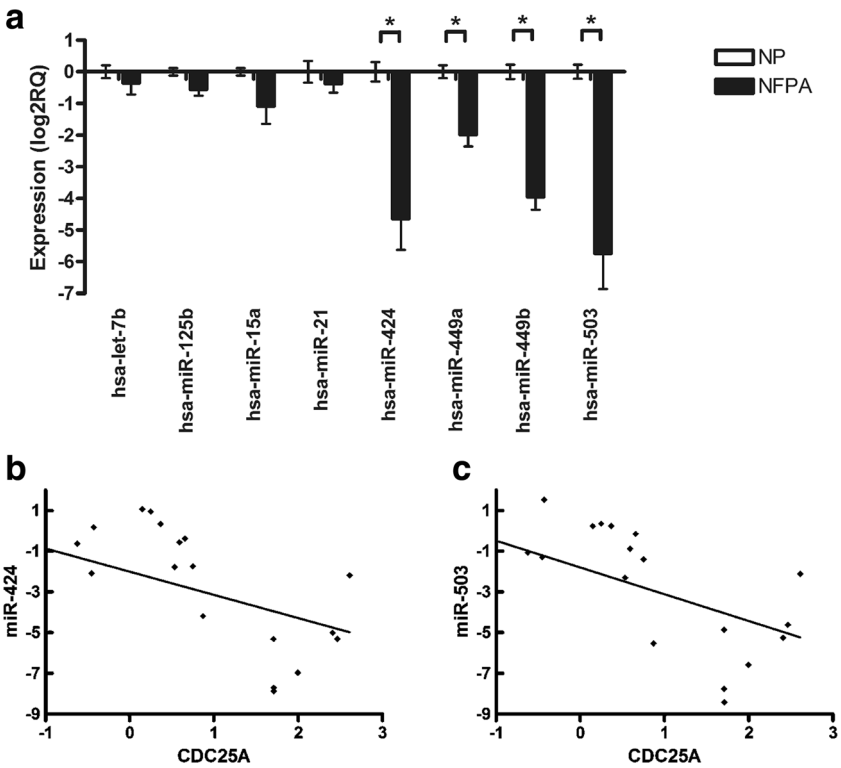

Fig. 4 **a** Expression of CDC25A targeting miRNAs. All miRNAs were downregulated in NFPA samples compared to normal pituitary (Y axis represents log2RQ). miR-424: fold change: -24.89, $p = 0.0021$; miR449a: fold change:-3.92, $p = 0.0014$; miR-449b: fold change:-15.46, $p < 0.0001$; miR-503: fold change:-53.22, $p = 0.0016$. (**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

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).

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Compliance with ethical standards

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

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