miR-23b and miR-130b expression is downregulated in pituitary adenomas

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1. Introduction

Pituitary adenomas (PAs) are one of the most frequent intracranial tumors with a prevalence of clinically apparent tumors close to one in 1000 of the general population and are the third most common intracranial tumor type after meningiomas and gliomas (Scheithauer et al., 2006). GH- or ACTH-secreting PAs mostly composed by the so called “null cell” PAs (devoided of pituitary hormone immunoreactivity) and gonadotroph adenomas (defined by FSH and/or LH immunoreactivity) and gonadotroph adenomas (defined by FSH and/or LH immunoreactivity) and gonadotroph adenomas (defined by FSH and/or LH immunoreactivity) and gonadotroph adenomas (defined by FSH and/or LH immunoreactivity) and gonadotroph adenomas (defined by FSH and/or LH immunoreactivity) and gonadotroph adenomas (defined by FSH and/or LH immunoreactivity) and gonadotroph adenomas (defined by FSH and/or LH immunoreactivity) and gonadotroph adenomas (defined by FSH and/or LH immunoreactivity) and gonadotroph adenomas (defined by FSH and/or LH immunoreactivity) and gonadotroph adenomas (defined by FSH and/or LH immunoreactivity) and gonadotroph adenomas (defined by FSH and/or LH immunoreactivity) and gonadotroph adenomas (defined by FSH and/or LH immunoreactivity) and gonadotroph adenomas (defined by FSH and/or LH immunoreactivity) and gonadotroph adenomas (defined by FSH and/or LH immunoreactivity) and gonadotroph adenomas (defined by FSH and/or LH immunoreactivity) and oncogenic and/or hyperplastic cell proliferation (Mol, 2003; Asa and Ezzat, 2004). However, the molecular events leading to pituitary tumor development are still unclear. Epigenetic events, such as hypermethylation and/or microRNA (miRNA)-dependent...
MiRNAs are a class of small non-coding RNAs which regulate gene expression at post-transcriptional level. They bind to 3'-untranslated (3'-UTR) regions of target mRNAs, causing block of translation or mRNA degradation (Bartel, 2004). They play important roles in essential cellular processes such as differentiation, cell growth and cell death (Miska, 2005). Several studies have demonstrated altered expression of specific miRNAs in different types of human neoplasias suggesting that they play a key role in tumorigenesis (Fabbrì et al., 2008). Many studies have shown alterations of miRNA expression in pituitary adenomas. Several of these deregulated miRNAs may be involved in cell proliferation, apoptosis, cancer development and progression (Bottoni et al., 2007; Amaral et al., 2009; Butz et al., 2011; D’Angelo et al., 2012; Palmieri et al., 2012).

We have recently reported the identification of specific miRNAs, miR-23b, miR-130a and miR-130b, whose upregulation by TSH is required for thyroid cell growth and is protein kinase A-CREB dependent (Leone et al., 2012). We also identified SMAD3, a member of TGF-β pathway that has an inhibitor role in thyroid follicular cell proliferation, as target of miR-23b. Functional studies demonstrate that the over-expression of miR-23b promotes thyroid cell growth (Leone et al., 2012).

The aim of our work was to verify whether miR-23b and miR-130b misexpression occurs in non-thyroid cell system, such as pituitary adenomas, where alterations of the cAMP pathway are frequent. Indeed, cAMP signaling is hyperactivated in GH-secreting adenomas, with an increased phosphorylation of the cAMP response element-binding protein (p-CREB) (Nishizawa et al., 2013; Gadelha et al., 2013). Moreover, it has been demonstrated that the arylhydrocarbon receptor-interacting protein (AIP), whose mutations have been linked to predisposition to pituitary adenomas, acts as a tumor suppressor by maintaining a low concentration of the AIP required for thyroid cell growth and is protein kinase A-CREB dependent (Leone et al., 2012).

The expression of miR-23b promotes thyroid cell growth (Leone et al., 2012).

2. Materials and methods

2.1. Tissue collection and RNA isolation

PAs were obtained from patients operated on for medical reasons. For each tumor, some fragments were either snap-frozen in liquid nitrogen or collected in RNA Later (Ambion) at surgery and stored at −80 °C until RNA extraction. Diagnostic immunohistochemistry for pituitary hormones was performed in all cases on paraffin-embedded sections and tumors were classified into somatotroph (n = 15) and NFPa (n = 21) including gonadotroph (n = 16) and null cell (n = 5) PA (Lloyd et al., 2004). We declare that in accordance with the scientific use of biological material was obtained from all patients.

Total RNA isolation from PAs was performed with TRIzol reagent (Invitrogen) according to manufacturer’s instructions. RNAs from human normal pituitary glands were used as control. Normal pituitaries were obtained from autopsies of two females and three males, aged between 50 and 60 years, devoid of endocrine diseases.

2.2. Bioinformatic prediction of miRNA target genes

Genes potentially targeted by the selected miRNAs were identified by using different on-line available tools such as TargetScan (www.targetscan.org), miRandA (www.microrna.org) or miRWalk (www.umm.uni-heidelberg.de/apps/zmf/mirwalk). For more details see Supplemental Information.

2.3. Cell lines and transfection

GH3 rat pituitary tumor cells secreting PRL and GH and Human Embryonic Kidney HEK-293 cells were cultured in DMEM containing 10% FBS.

For transfection of miRNA oligonucleotides, cells were transfected with 50 nmol/ml pre-miRNA precursors or control no-targeting scrambled oligonucleotides (Ambion) using siPORT neoFX Transfection Agent (Ambion).

2.4. Reverse Transcription and quantitative Real Time (qRT)-PCR

Reverse Transcription and qRT-PCR for mature miRNA were performed according to manufacturer’s instructions of miScript System Kits (Qiagen) (for more details see Supplemental Information). qRT-PCR analyses for HMGA2 and CCNA2 expression were performed as previously described (De Martino et al., 2009). To calculate the relative expression levels we used the 2−ΔΔCT method (Livak and Schmittgen, 2001). Primers for glucose-6-phosphate dehydrogenase (G6PD) were used for miRNA normalization and primers for RNU6 (Qiagen) were used for miRNA normalization. The primers used to amplify the above mentioned genes are reported in Supplemental Information.

2.5. Western blotting and antibodies

Western blot analysis was performed as previously described (Lloyd et al., 2004) and the membranes were incubated with antibodies against CCNA2 (sc-751, Santa Cruz), α-tubulin (SC-8035, Santa Cruz), α-actin (sc-1616, Santa Cruz), Vinculin (sc-7649, Santa Cruz), anti-HMGA2 antibody previously described (Finelli et al., 2002).

2.6. Luciferase target assays

Cells were co-transfected with the modified Firefly luciferase vectors described in Supplemental Information, along with the Renilla luciferase reporter plasmid and the miRNA oligonucleotides. Firefly and Renilla luciferase activities were measured 36 h after transfection with the Dual-Luciferase Reporter Assay System (Promega). Firefly activity was normalized to Renilla activity as control of transfection efficiency.

2.7. Growth curve assay

Exponentially growing GH3 and HEK-293 cells were plated in 6-well plates and transfected with 50 nmol/ml of pre-miR miRNA precursor or scrambled oligonucleotide using siPORT neoFX. Cells were counted in triplicate at 24, 48, 72 and 96 h after transfection with a Burker hemocytometer chamber.

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2.8. Flow cytometry

HEK-293 cells were plated and synchronized by serum deprivation for 48 h. Then, the cells were transfected with 50 nmol/ml of pre-miRNA precursor or scrambled oligonucleotide using siPORT neoFX and collected after 72 h.

After trypsinization, cells were washed in phosphate-buffered saline and fixed in 70% ethanol. Staining for DNA content was performed with 2 μg/ml propidium iodide and 20 μg/ml RNase A for 30 min. For each measure 20,000 events have been analyzed. We used a FACScan flow cytometer (Becton Dickinson) that was interfaced with a Hewlett-Packard computer (Palo Alto, CA). Cell cycle data were analyzed with the CELL-FIT program (Becton Dickinson).

2.9. Statistical analysis

Student’s t-test was used to determine the differences between two population of samples. Data are presented in the text as mean ± SEM and p < 0.05 was accepted as statistically significant.

To compare the differences between groups, one-way analysis of variance was used. The significance of differences was determined by Analysis of Variances (ANOVA) followed by Dunnett’s test as the post hoc test using Graph Pad Prism 5.0. Data are presented in the text as mean ± SEM and p < 0.05 was accepted as statistically significant.

3. Results

3.1. miR-23b and miR-130b are downregulated in pituitary adenomas

To evaluate the possible role of miR-23b and miR-130b in pituitary tumorigenesis, we analyzed the expression of these miRNAs in a panel of 15 somatotroph PA and 21 NFPA in comparison with 5 normal pituitary glands. As shown in Fig. 1, miR-23b was downregulated in all tumors, except one GH (Panel A). MiR-130b was also downregulated in all tumors, apart from one NFPA (Panel B).

In order to understand the mechanism by which the downregulation of miR-23b and miR-130b might be involved in pituitary tumorigenesis, we identified, using bioinformatic tools (miRanda, TargetScan and MiRwalk), several potential target genes. Interestingly, we found HMGA2 and CCNA2 genes as predicted targets for miR-23b and miR-130b, respectively. Therefore, we focused our attention on these target genes since HMGA proteins are involved in cell cycle dysregulation, which plays an important role in the development of PA (Fedele et al., 2006). Moreover, we have previously demonstrated that HMGA2 plays a critical role in pituitary tumorigenesis. Indeed, the HMGA2 gene was found overexpressed in most human PAs (De Martino et al., 2009; Qian et al., 2009) and transgenic mice overexpressing HMGA2 developed mixed PRL/GH PAs (Fedele et al., 2002). A-type cyclins also have a critical role on the cell cycle regulation. In fact, by coupling successively to CDK2 and CDK1, they participate in the S/G2 transition and progression through G2 (Yam et al., 2002).

In Fig. 2A we report the miRNA-targeting sites of miR-23b on HMGA2 3’-UTR. To validate its influence on the expression of the HMGA2 gene, we have performed a time course, collecting HEK-293 cells transfected with miR-23b and relative scrambled, at 24, 48, 72 and 96 h. As shown in Fig. 2B, transfection of miR-23b decreased HMGA2 protein levels at 72 h as compared to the scrambled oligonucleotide-transfected cells. Interestingly, as shown in Fig. 2C, this was accompanied by a decrease in HMGA2 transcripts, even though there is not a perfect complementarity between the miR-23b and its target sequences. This result strongly suggests that miR-23b reduces HMGA2 protein level also by affecting HMGA2 mRNA stability. In Fig. 3A we report the miRNA-targeting sites of miR-130b on CCNA2 3’-UTR. Similarly, we looked at time course of CCNA2 protein levels in cells transfected with miR-130b. As shown in Fig. 3B, transfection of miR-130b decreased CCNA2 protein levels at 48 and 72 h as compared to the scrambled oligonucleotide-transfected cells. In contrast, no significant

![Fig. 1. Analysis of miR-23b and miR-130b expression in pituitary adenomas by qRT-PCR. The relative expression values indicate the relative change in the expression levels between somatotroph PA and NFPA (including gonadotroph (n = 16) and null cell (n = 5) PA) versus normal pituitary glands, assuming that the mean value of the normal samples was equal to 1. The range of variability of the expression of these miRNAs in normal pituitary tissues is less than 10%. Each bar represents the mean value ± s.e. from three independent experiments performed in triplicate. *p < 0.05 compared to normal pituitary.](http://dx.doi.org/10.1016/j.mce.2014.03.002)
257 changes in the CCNA2 transcripts were observed (Fig. 3C). These data are consistent with post-transcriptional regulation of the CCNA2 protein by miR-130b without effects on CCNA2 mRNA degradation.

In order to verify that a direct interaction between miRNAs and the CCNA2 and HMGA2 mRNAs was responsible for protein level decrease, the 3′-UTRs of CCNA2 and HMGA2 mRNA were inserted downstream of the luciferase ORF, either in sense (3′-UTR-CCNA2 and 3′-UTR-HMGA2) or in antisense (3′-UTR-CCNA2 MUT and 3′-UTR-HMGA2 MUT) contexts.
3.3. The miR-23b and miR-130b inhibit cell proliferation

To understand the role of miRNA downregulation in pituitary tumorigenesis we analyzed their effects on cell proliferation. The effect of miR-23b expression on cell growth was studied on human HEK-293 cells, we did not use the rat GH3 cells for this experiment since HMGA2 is not expressed in these cells. As shown in Fig. 4A (left panel), a significant reduction of cell number was observed 96 h after transient transfection with miR-23b compared with scrambled oligonucleotide-transfected cells. Even a more drastic reduction of the proliferation was obtained when the HMGA2 protein expression was silenced by shRNA interference (data not shown). Conversely, the role of miR-130b was studied on GH3 cells transiently transfected with miR-130b or the scrambled oligonucleotide. As shown in Fig. 4A (right panel), a significant reduction of cell number was also observed 96 h after transfection with miR-130b compared with scrambled-oligonucleotide transfected cells. We have also performed the same experiments on the HEK-293 cell line achieving almost identical results (Supplementary Fig. 1A).

Finally, to better characterize the effects of the analyzed miRNAs on cell cycle progression, miRNA precursors or scrambled oligonucleotide were transfected in HEK-293 (Fig. 4B, left panel) and GH3 cells (Fig. 4B, right panel), and analyzed by flow cytometry. Interestingly, miR-23b-transfected cells displayed an increase in the G1 phase population and a decrease in the S-phase, compared to scrambled-transfected cells. These results indicate that the overexpression of this miRNA affects the G1-S transition of the cell cycle progression, as expected by its target (HMGA2). In contrast, miR-130b-transfected cells displayed a decreased number of cells in the G1-phase, whereas the cell population in the G2-phase was increased, compared with scrambled-oligonucleotide transfected cells. These findings are consistent with downregulation of its target gene (CCNA2), which is involved in the regulation of S/G2 transition and progression through G2 phase of the cell cycle (Yam et al., 2002). We have also performed the same experiments on the HEK-293 cell line achieving almost identical results (Supplementary Fig. 1B).

3.4. miR-23b and miR-130b downregulation is associated with an increase in HMGA2 and CCNA2, respectively, in human PAs

In order to further analyse the potential role of miRNA downregulation in human pituitary tumorigenesis, the expression of HMGA2 was studied by western blot in a subset of GH-PAs showing a marked downregulation of miR-130b. As shown in Fig. 5A, an increased CCNA2 protein expression was observed in all cases, in comparison with normal pituitary. Similarly, we previously reported increased HMGA2 protein levels in GH-PAs, in comparison with normal pituitary (D'Angelo et al., 2012). In this study, we found that miR-23b downregulation was associated with overexpression of HMGA2 mRNA evaluated by qRT-PCR in GH, gonadotrophin and null cell PAs (Fig. 5B).

4. Discussion

In this study, we have analyzed the expression of miR-23b and miR-130b in pituitary adenomas. Both were found to be downregulated in GH, gonadotrophin and null cell PA in comparison with
In this study, we demonstrate that miR-130b interaction with Q4 showing a marked downregulation of miR-130b. Functional studies suggest a role for the downregulation of these miRNAs in tumor progression. Indeed, overexpression of both miR-23b and miR-130b was found to inhibit cell growth. Moreover, FACS analysis demonstrated that, consistently with their identified target genes, miR-23b retains the cells in the G1 phase of the cell cycle, whereas miR-130b retains the cells in the G2 phase. These findings further support the hypothesis that downregulation of miR-23b and miR-130b may contribute to development of human PAs through an overexpression of HMG2 and CCNA2, respectively. However, we have to consider that downregulation of these miRNAs may contribute to pituitary tumorigenesis also by targeting other genes coding for proteins involved in cell proliferation as SMAD3, a member of TGF-β pathway that we identified as target of miR-23b.

Interestingly, recent studies have demonstrated that miR-23b and miR-130b are also downregulated in prolactinomas (Chen et al., 2012a,b), suggesting this might represent a general event in pituitary tumorigenesis. It is noteworthy, to observe that both miRNAs were not reported in our previous study where the miRNA expression profile of 12 GH-adenomas was analyzed. Unfortunately, these techniques are not completely precise yet, and this also accounts for different results obtained by different groups analyzing the same tumor histotypes. Moreover, we did not analyze miR-23b in the study by Palmieri et al., where we focused just on a limited number of miRNAs able to target the genes coding for the HMG proteins.

Notably, both miR-23b and miR-130b have been found deregulated in several human neoplasias. Indeed, miR-23b is overexpressed in bladder (Gottardo et al., 2007) and oral squamous cell carcinoma (Scapoli et al., 2010) in breast cancer (Paris et al., 2012) where it acts as a potential oncomir. Conversely, it is downregulated in prostate cancer (Tong et al., 2009), hepatocellular carcinoma (Salvi et al., 2009) where it acts as a tumor suppressor by targeting the uroki- type plasminogen activator (uPA) and c-met (Salvi et al., 2009). Also miR-130 is upregulated in several normal pituitary gland, in contrast with the results obtained in thyroid cells stimulated by TSH (Leone et al., 2012) and thyroid adenomas (Leone, manuscript in preparation) showing upregulation of these miRNAs. We also identified the HMG2 gene as target of miR-23b. Overexpression of HMG2 is a frequent event in PAs and high levels of HMG2 protein have been correlated with tumor size, invasiveness and higher Ki-67 index in PRL, silent ACTH- and gonadotroph adenomas (Qian et al., 2009). We have previously shown that HMG2 overexpression in prolactinomas was frequently associated with alterations of the HMG2 gene, located in chromosome 12, including amplification, rearrangements and trisomy of chromosome 12 (Finelli et al., 2002). In this study, we demonstrate that miR-23b interaction with HMG2 mRNA 3' UTR in vitro is directly responsible for HMG2 protein decrease. In addition, miR-23b was found to affect HMG2 mRNA stability in vitro and an opposite behavior between HMG2 transcripts and the expression of miR-23b was observed in human PAs. Therefore, miR-23b downregulation can, at least partially, account for HMG2 overexpression in NFPAs where genomic HMG2 alterations have been rarely observed (Pierantoni et al., 2005). Then, downregulation of miRNAs targeting HMG2 represents an alternative mechanism of HMG2 overexpression in PA and may potentially synergize with HMG2 gene alterations to induce high HMG2 protein levels. Moreover, we identified the CCNA2 gene as target of miR-130b. A-type cyclins exert a critical role on the cell cycle regulation. In fact, Cyclin A is the only cyclin able to regulate multiple steps of the cell cycle, since it participates in the G1/S transition and progression through G2 phase of the cell cycle (Yam et al., 2002).

In this study, we demonstrate that miR-130b interaction with CCNA2 mRNA 3' UTR in vitro is directly responsible for CCNA2 protein decrease, with no effect on CCNA2 mRNA stability. Accordingly, CCNA2 was found to be overexpressed in a subset of GH-secreting PAs showing a marked downregulation of miR-130b. Functional studies suggest a role for the downregulation of these miRNAs in
In conclusion, we report the identification of miRNAs drastically and constantly downregulated in PAs. Since these miRNAs target genes, such as HMGA2 and CNN2, whose overexpression plays a critical role in pituitary tumorigenesis, it is reasonable to retain that their downregulation might contribute to this process suggesting an approach to the therapy of PAs based on the restoration of the downregulated miRNAs.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mce.2014.03.002.

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Types of cancer, such as non-small cell lung cancer (Yang et al., 2010), pancreatic (Chen et al., 2012a,b) and breast cancer (Shi et al., 2011), whereas is down-regulated in chronic lymphocytic leukemia (Kovalova et al., 2012).

In conclusion, we report the identification of miRNAs drastically and constantly downregulated in PAs. Since these miRNAs target genes, such as HMGA2 and CNN2, whose overexpression plays a critical role in pituitary tumorigenesis, it is reasonable to retain that their downregulation might contribute to this process suggesting an approach to the therapy of PAs based on the restoration of the downregulated miRNAs.

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