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MicroRNA-23b* targets proline oxidase, a mitochondrial tumor suppressor protein in renal cancer

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Abstract

Proline oxidase (POX) is a novel mitochondrial tumor suppressor, which can suppress proliferation and induce apoptosis through the generation of reactive oxygen species (ROS) and decreasing hypoxia inducible factor (HIF) signaling. Recent studies have demonstrated the absent expression of POX in human cancer tissues, including renal cancer. However, the mechanism for the loss of POX remains obscure. No genetic or epigenetic variation of POX gene was found. Here, we identified the up-regulated miR-23b* in renal cancer as an important regulator of POX. Ectopic overexpression of miR-23b* in normal renal cells resulted in striking down-regulation of POX, while POX expression increased markedly when endogenous miR-23b* was knocked down by its antagomirs in renal cancer cells. Consistent with POX-mediated tumor suppression pathway, these antagomirs induced ROS, inhibited HIF signaling and increased apoptosis. Furthermore, we confirmed the regulation of miR-23b* on POX and its function in the DLD1 Tet-off POX cell system. Using a luciferase reporter system, we verified the direct binding of miR-23b* to POX mRNA 3'UTR. In addition, pairs of human renal carcinoma and normal tissues showed the negative correlation between miR-23b* and POX protein expression, providing its clinical corroboration. Taken together, our results suggested miR-23b*, by targeting POX, could function as an oncogene; decreasing miR-23b* expression may prove to be an effective way of inhibiting kidney tumor growth.

Keywords

proline oxidase; miR-23b*; renal cancer; ROS; tumor suppressor

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Conflict of Interest

No potential conflicts of interest were disclosed.

Introduction

During the last decade, the important functions of proline metabolism in human health and disease have received increasing attention. Proline, together with hydroxyproline, constitutes more than 25% of incorporated residues of collagen, the most abundant protein in the human body. Proline oxidase (POX) is a mitochondrial inner-membrane enzyme involved in the metabolism of proline to pyrroline-5-carboxylate (P5C). This reaction, when coupled with the conversion of P5C to proline by P5C reductase, mediates the proline cycle to shuttle redox equivalents between mitochondria and the cytosol (Liu *et al.*, 2005; Phang, 1985; Phang *et al.*, 2008). P5C provides a direct carbon bridge connecting proline metabolism with the TCA cycle through glutamate and α -ketoglutarate (α -KG) (Phang, 1985). Changes in the cycling of proline through POX may alter the redox balance critical for regulation of cell growth and apoptosis.

POX has been identified as one of a few mitochondrial tumor suppressors. It can induce apoptosis through the generation of reactive oxygen species (ROS) and also reduce hypoxia inducible factor (HIF) signaling by elevating cellular α -KG, a critical substrate for prolyl hydroxylase-catalyzed hydroxylation of HIF-1 α and degradation (Liu *et al.*, 2009; Liu *et al.*, 2005; Liu *et al.*, 2006; Liu *et al.*, 2008). POX participates in the apoptosis induced by p53, cytotoxic agent adriamycin, and peroxisome proliferator-activated receptor (PPAR)- γ ligands, in a variety of cancer cell types (Donald *et al.*, 2001; Liu *et al.*, 2005; Liu *et al.*, 2008; Pandhare *et al.*, 2006; Polyak *et al.*, 1997). Its upregulation inhibits growth of various cultured tumor cells and suppresses tumor formation in a xenograft model (Liu *et al.*, 2009; Liu *et al.*, 2008). Recent studies have demonstrated the absence or reduction of POX in a variety of human tumor tissues as compared with their normal tissue counterparts, including kidney, colon, stomach, liver and pancreas (Liu *et al.*, 2009; Liu *et al.*, 2008; Maxwell and Rivera, 2003). However, we did not find any genetic or epigenetic variation of the POX gene and its promoter in human tumor panels (COSMIC database and our unpublished data). The mechanism for differential POX expression and its contribution to tumor development is still not clearly defined.

Recently, with the discovery of microRNAs (miRNAs), a new mechanism to regulate protein expression has been revealed. miRNAs are a class of conserved, endogenously expressed, noncoding small RNAs (18~25 nucleotides). They can negatively regulate gene expression at the posttranscriptional level by cleavage and/or translational repression of their mRNA targets through specific, albeit imperfect base pairing with the 3'-untranslated region (3' UTR) of target mRNAs (Bartel, 2004; Chen and Rajewsky, 2007). This imperfect and limited complementarity to target sites enables individual miRNA to target a large number of mRNAs and also hampers the identification of the specific genes it regulates. Early analyses estimated that ~30% of known human genes are under miRNA control (Lewis *et al.*, 2005), whereas later reports increased this number to >90% (Miranda *et al.*, 2006). However, though hundreds of miRNAs have been identified to date, the elucidation of their specific targets and functions has been limited.

miRNAs have been shown to modulate a variety of biological processes, including cellular differentiation and proliferation, metabolic signaling and apoptosis. In recent years, growing evidence has strongly implicated the involvement of miRNAs in carcinogenesis (Iorio *et al.*, 2005; Ma *et al.*, 2007). Dysregulated miRNAs may function as oncogenes, such as miR-17-92 cluster and miR-214 (Northcott *et al.*, 2009; Yang *et al.*, 2008), or tumor suppressor genes, such as miR-205 and let-7 (Gandellini *et al.*, 2009; Johnson *et al.*, 2005), depending on the targets they regulate. Although some targets of these miRNAs have been identified, miRNA regulators of critical cancer proteins and pathways remain largely unknown, including POX-mediated pathways described above. Therefore, we proposed that the dysregulation of candidate miRNAs could be a possible mechanism for POX downregulation in tumors. Here we examined miRNAs to identify a novel mechanism resulting in POX down-regulation in renal cancer.

Results

POX expression is markedly reduced in renal cancer cell lines and human renal carcinoma tissue samples

We measured POX mRNA and protein expression by real-time RT-PCR and western blot in several renal cancer and normal cell lines. Figure 1a and 1b showed that POX mRNA levels in the clear cell renal carcinoma cell lines including 786-0, TK10 and UO31 were about 50% of those in normal renal cell lines HREpC and HRCEpC, while POX protein levels were only 4% of those in normal renal cells.

To confirm the expression levels of POX in vivo, we obtained 16 paired renal cell carcinoma (clear cell subtype) and corresponding normal renal tissues to analyze its protein expression by immunohistochemical (IHC) staining. We found that in 13 out of 16 pairs of renal tissues, the expression of POX was strikingly decreased in carcinoma tissues compared with their normal counterparts. The representative images from 5 pairs of normal/cancer tissues are shown in Figure 1c.

Overexpressed miRNAs potentially related to POX in renal cancer cell lines

To identify possible miRNAs negatively regulating POX expression, we performed a bioinformatic analysis using three public databases, miRBase, TargetScan and Microinspector, integrated the projected results from these three databases, and identified 91 potential miRNAs targeting POX mRNA 3' UTR totally (data not shown).

To investigate the involvement of miRNAs in the reduced expression of POX in renal cancer, we compared miRNAs expression profiles of three renal cancer cell lines (786-0, TK10 and UO31) with two normal renal cell lines (HREpC and HRCEpC) by miRNA microarrays. We analyzed the changes of 91 putative miRNAs related to POX from 723 human miRNAs tested. 10 miRNAs displayed increased expression in renal cancer cells relative to normal cells (Table 1). Among the 10 miRNAs, miR-23b* (predicted by Microinspector), miR-151-5p (predicted by miRBase and Microinspector), miR-30b* (predicted by miRBase and Microinspector), miR-595 (by miRBase, TargetScan and Microinspector) and miR-766 (by TargetScan) showed over 1.5 fold (0.58 in log₂

transformed value) increase in cancer cells compared to non-transformed cell lines, which were selected for further study.

Negative regulation of miR-23b* on POX expression

To assess whether the identified miRNAs could modulate the expression of POX, we transfected normal renal cells HREpC, which expressed relatively low levels of those miRNAs, with 100nM synthesized mimic miRNAs. The overexpression of each miRNA after transfection was confirmed by real-time RT-PCR using U6 as an internal control (Supplementary Figure S1a). POX mRNA and protein levels were observed by real-time RT-PCR and western blot, respectively. As shown in the Figure 2a and 2b, ectopic expression of miR-151-5p and miR-23b*, especially the latter decreased POX protein expression significantly (about 65% calculated by densitometry), while POX mRNA level was decreased only 19% ($p<0.05$).

We further demonstrated the inhibitory effect of miR-23b* on POX protein level in DLD1 Tet-off POX cells, a colon cancer cell line stably transfected with full-length POX cDNA containing 3' UTR. POX is overexpressed when doxycycline (DOX) is removed. Mimic miR-151-5p and miR-23b* were transfected into those cells overexpressing POX. The scrambled negative siRNA had no effects either on POX mRNA or protein expression. MiR-23b* but not miR-151-5p inhibited POX mRNA (15% decrease, $p<0.05$) and especially POX protein expression significantly (53% decrease calculated by densitometry), comparable to those transfected with a specific POX siRNA (78% decrease by densitometry) (Figure 2d). Therefore, miR-23b* was chosen for additional studies.

Since the amount of miRNAs transfected above (100nM) lead to very high levels of miRNAs in cells, to verify that miR-23b* plays a physiologic role in controlling POX expression, we titrated down the amount of transfected mimic miR-23b* close to the level of renal cancer cells in the normal renal cells HREpC. Transfection with 2.5nM to 100nM mimic miR-23b* resulted in 10 to 100 fold increase in miR-23b* expression. Even at the lowest concentration (2.5nM), POX protein inhibition was apparent (Supplementary Figure S2). On the other hand, we transfected the renal cancer cells TK10 and 786-0, which have a relatively high level of miR-23b*, with antagomirs against miR-23b* to inhibit the expression of endogenous miR-23b*. Real-time RT-PCR confirmed miR-23b* was down-regulated 64.8% and 93.4% by 100nM and 200nM antagomirs, respectively, which was in the physiologic range of renal normal cells (Supplementary Figure S1b). In the Figure 2e and 2f, an obvious increase in POX protein (2.2 ~ 2.9 fold), but not mRNA expression (only 12% increase, no statistical significance), was observed in TK10 cells upon transfection with those two concentrations of antagomirs, suggesting that the identified miRNA physiologically regulates POX expression. Similar changes were obtained in the 786-0 cell line (data not shown).

miR-23b* directly targets POX mRNA 3' UTR

Because miRNAs regulate gene expression by binding to target mRNA 3' UTR, we tested whether miR-23b* could bind directly to the 3' UTR of POX mRNA to inhibit POX expression. To this end, we cloned the full length 3' UTR of POX mRNA into the luciferase

expressing vector pMIR-REPORT just downstream of the luciferase stop codon. We then transiently expressed this construct or original reporter in TK10 renal cancer cells. As shown in Figure 3b, compared with the pMIR-REPORT, we observed a significant decrease of luciferase activity in the cells transfected with pPOX 3' UTR reporter ($p < 0.05$), which suggested repressive effects from the endogenous miR-23b*. Then we cotransfected with pPOX 3' UTR reporter and two concentrations of mimic miR-23b* to examine if the reporter activity could be further attenuated. Luciferase activity was decreased 40% and 60% by 100nM and 200nM mimic miR-23b*, respectively, compared with the negative siRNA control ($p < 0.01$). Similar results were obtained in 786-0 cell line (data not shown). These data indicate that miR-23b* impairs POX mRNA translation by directly binding to its mRNA 3' UTR.

To further confirm the above conclusion, we obtained the plasmid pPOX 3'UTR-MUT containing the mutated POX 3'UTR in the miR-23b* binding site, and as well as synthesized mutant miR-23b* sequence (Figure 3a). As shown in Figure 3c, the luciferase activity of pPOX 3'UTR increased significantly when the binding site was mutated (pPOX 3'UTR-MUT vs pPOX 3'UTR group), although it was still lower than the original pMIR-REPORT, which may be due to the effect of yet unidentified miRNAs affecting POX. As expected, exogenous mimic miR-23b* also had no effect on the luciferase activity of the pPOX 3'UTR-MUT group. For the pPOX 3'UTR group, exogenous mimic miR-23b* decreased its luciferase activity, whereas mutant miR-23b* did not (Figure 3d).

Effects of ectopic expression of miR-23b* on cell growth status and the involvement of POX

As mentioned earlier, POX has been shown to induce apoptosis through generation of ROS and inhibit HIF signaling by increasing α -KG. To prove that down-regulation of POX mediated by miR-23b* could attenuate the functional effects of POX in DLD1 Tet-off POX cells, we monitored proliferation assay, ROS generation and HIF-1 α expression. As shown in the Figure 4a, overexpression of POX significantly impaired survival of cells when DOX was removed, whereas this impairment was partially reversed in the presence of miR-23b*. Figure 4b showed that the changes in ROS production were comparable with removal of DOX when POX expression was regulated by either POX siRNA or miR-23b*. These results further confirmed the role of miR-23b* in cell growth and apoptosis through the regulation of POX. Furthermore, we checked the expression of HIF-1 α , a key factor in tumor formation. As expected, miR-23b* could significantly reverse POX-induced HIF-1 α (Figure 4c).

On the other hand, renal cancer cells expressed a relatively high level of miR-23b* as described above. Considering the above function of POX, we wondered if the viability of renal cancer cells could be impaired by up-regulating POX expression through miR-23b* knockdown. Figure 5a showed that the apoptotic percentage increased from 6.6% to 16.0% when we transfected TK10 cells with miR-23b* antagonists. As expected, if we simultaneously down-regulated POX expression using POX siRNA, the apoptotic percentage decreased to 10.4%. The viability of cells transfected with negative control siRNA remained unchanged. This result confirmed that the biological effects promoted by

miR-23b* are mainly mediated by down-regulation of POX. Using a DCF assay, we showed that ROS changes were similar to those in the apoptosis assay (Figure 5b), which is consistent with a POX-dependent pro-apoptotic mechanism mediated by ROS production. The same result was also confirmed in the 786-0 cell line. In addition, we further examined the expression of HIF-1 α in these renal cancer cells. Consistent with the previous reports (Sowter *et al.*, 2003), in the VHL-defective 786-0 renal cancer cell line, native HIF-1 α gene is not expressed (data not shown). But in the VHL wild type TK10 cell line, HIF-1 α showed a significant decrease after miR-23b* was knocked down, again implying that POX-mediated roles on HIF-1 α was regulated by miR-23b* (Figure 5c).

Expression of miR-23b* is frequently increased in human renal cell carcinoma tissues and negatively correlates with POX protein expression

To examine the clinical relevance of miR-23b* and its negative relationship with POX, we analyzed the expression of miR-23b* and POX in the aforementioned 16 paired clear cell renal cell carcinoma tissues and normal counterparts by real-time RT-PCR and western blot, respectively. Most tumor tissues (11 of 16; 68.8%) revealed 1.6~12.7 fold higher levels of miR-23b* expression relative to corresponding normal tissues (Figure 6a). By contrast, only 7 of 16 tumor tissues showed a decrease in POX mRNA levels with respect to normal counterparts (Figure 6b). There was no significant negative correlation between miR-23b* and POX mRNA levels by Spearman's correlation analysis (correlation coefficient = -0.11, $p = 0.68$). However, when we tested POX protein expression by western blot, 13 of 16 tumor tissues showed 24.8%~98.6% reduction with respect to normal counterparts (Figure 6c and 6d). By using \log_{10} transformed data, paired t-tests indicated the significant differences in both miR23b* ($t = -2.58$, $p = 0.02$) and POX protein expression ($t = 3.56$, $p = 0.003$) between the tumor and corresponding normal tissues. Spearman's correlation analysis showed a significantly negative correlation between miR-23b* and POX protein (correlation coefficient = -0.75, $p = 0.00082$).

Furthermore, we validated the miR-23b* expression by *in situ* hybridization (ISH). The results showed a content consistent with those from real-time RT-PCR. Figure 6e shows the representative images of the miR-23b* expression by ISH and POX protein expression by IHC staining from paired cancer and normal tissues in the same patient. These results suggest that increased miR-23b* expression is a frequent event in human renal carcinoma and may be involved in renal carcinogenesis.

Discussion

In this study, we robustly confirmed the reduced expression of POX in renal cancer *in vitro* and *in vivo*, strongly suggesting its tumor suppressor role. The functional loss of POX may contribute to the resistance of tumor cells to apoptosis, and consequently favors tumor progression. However, no genetic or epigenetic basis for its reduced expression has been found in human tumors. POX was initially identified as a p53-induced gene (Polyak *et al.*, 1997) and Maxwell SA et al suggested that the reduced expression of POX mRNA in renal cancer was due to mutated p53 (Maxwell and Rivera, 2003). But p53 mutation is not a frequent event in clear cell renal carcinoma (Girgin *et al.*, 2001; Suzuki *et al.*, 1992) which

accounts for most of kidney cancer. Furthermore, our results showed POX expression was reduced in both p53-mutant (786-0 and TK10) and p53 wild-type cell lines (UO31). Therefore, we considered other unknown factors may result in the reduction of POX. Noticeably, we found that renal tumor cells had much lower POX protein expression than normal renal cells in the face of similar mRNA level; this prompted us to seek its possible regulation through miRNAs, which plays a prominent role in inhibition of translation in mammals.

We examined miRNA effects on POX down-regulation in renal carcinoma cells. By comparing clear cell renal cell carcinoma cell lines with primary normal renal cells, we generated a miRNA over-expression profile. Several human miRNAs (miR-23b*, miR-151-5p, miR-30b*, miR-595 and miR-766) putatively related to POX were shown to be significantly increased in those cancer cells. Some of these have not been reported in previous studies (Gottardo *et al.*, 2007; Nakada *et al.*, 2008), which may be due to biological differences as well as technical differences, including different analytic platforms or number of miRNA probes used. We tested 723 probes using Agilent microarray chips, whereas some previous studies used fewer probes. In addition, the focus of the present study limited our exploration of the entire miRNAs misexpression profile in renal cancer. Despite these differences, our data has overlaps with previous reports, such as up-regulated miR-151-5p and miR-342-3p (Chow TF, 2009), down-regulated miR-141, miR-200c and miR-34a in clear cell subtype renal cell carcinoma cells (data not shown) (Lodygin *et al.*, 2008; Nakada *et al.*, 2008).

Growing numbers of miRNAs have been implicated in apoptosis and carcinogenesis by regulating the expression of specific target genes. In the current study, we have identified miR-23b* as a potential oncomir candidate capable of targeting the POX tumor suppressor gene. Firstly, we provided evidence about inverse relationship of miR-23b* with POX expression. The decrease of POX mainly in protein by miR-23b*, but not mRNA level, was consistent with many previous reports; in mammals, miRNAs more often inhibit protein translation of the target mRNA, not inducing its degradation (Williams, 2008). Furthermore, we validated that POX indeed is a direct target of miR-23b* by using a luciferase reporter system. More importantly, functional analysis showed that the silencing of POX by exogenous miR-23b* altered the growth status of DLD1 Tet-off POX cells overexpressing POX. The apoptotic percentage of renal cancer cells increased significantly after restoration of POX expression by the knockdown of miR-23b*. And the effects on growth inhibition and apoptosis corresponded to the production of ROS induced by POX. These results indicate that renal cancer cell lines may require high levels of miR-23b* to inhibit the expression of the pro-apoptotic gene POX to evade apoptosis.

The clinical relevance of these in vitro findings is substantiated by the data obtained in human renal carcinoma tissues. We examined POX and miR-23b* expression in the same tissues. Contrary to decreased POX protein but not mRNA, miR-23b* was frequently increased in renal carcinoma, strongly suggesting that the increased miR-23b* might contribute to renal oncogenesis and progression by down-regulating POX. This provides a possible strategic opening to inhibit tumor growth by decreasing the levels of miR-23b* or

blocking its function. However, the potential of miR-23b* and POX as diagnostic indicators or therapeutic targets in renal carcinoma remains to be investigated.

In fact, due to the identification of the von Hippel Lindau (VHL) tumor suppressor as a negative regulator of HIF- α in renal cancer, the treatment of renal cell carcinoma by targeting HIF- α or its downstream genes has shown great promise (Kaelin, 2009; Smaldone and Maranchie, 2009). However, the development of more effective management strategies remains necessary. Since POX has been found to down-regulate HIF-1 signaling by increasing α -KG, a substrate of PHD (Liu *et al.*, 2009), we examined the possible effects on HIF-1 α by miR-23b* inhibition of POX expression. In DLD1 Tet-off POX cells, miR-23b* did induce the expression of HIF-1 α by inhibiting POX. Although in 786-0 cell line with VHL-negative background, HIF-2 α , but not HIF-1 α exhibits the tumorigenic potential (Kondo *et al.*, 2002; Maranchie *et al.*, 2002), HIF-1 α was inhibited after miR-23b* antagomirs increased POX expression in TK10 renal cancer cells without VHL mutation. This finding provides a broader prospect for miR-23b* application in renal cancer therapeutics based on POX-dependent HIF-1 α signaling.

As mentioned above, POX is encoded by a p53-induced gene (Polyak *et al.*, 1997). In 2003, Maxwell SA et al reported reduced expression of POX mRNA in renal cancer was due to p53 mutation (Maxwell and Rivera, 2003). But, since p53 mutation is not a frequent event in clear cell renal carcinoma, we could not exclude the possibility that wild-type p53 regulates the final expression of POX by miR-23b*-dependent mechanism in renal cancer. To verify this view, we expressed exogenous p53 in p53 mutant renal cancer cell line TK10 to investigate the effect of p53 on miR-23b*. The results showed that p53 significantly increased the expression of miR-23b* (Supplementary Figure S3), suggesting that the upregulation of miR-23b* by p53 may counteract the direct role of p53 on POX gene expression in clear cell renal carcinoma. This interaction might also account for discrepancies between POX mRNA and protein expression.

In addition, current evidence suggests that miR-23b* could be regulated by factors other than p53. For example, several reports have shown the link between upregulation of miR-23b and hypoxia (Guimbellot *et al.*, 2009; Kulshreshtha *et al.*, 2008; Kulshreshtha *et al.*, 2007). Since miR-23b and miR-23b* share the same precursor, miR-23b* also could be regulated by HIF. In renal cell carcinoma, the constitutive expression of HIF due to VHL deficiency may link this regulation of miR-23b* with VHL. In lymphoma cells, the oncogenic transcription factor c-Myc transcriptionally suppresses miR-23b to enhance mitochondrial glutaminase expression and glutamine metabolism (Gao *et al.*, 2009). Thus, c-Myc effects on miR-23b* is a likely possibility. The fact that HIF-1 negatively regulates mitochondrial biogenesis by inhibiting c-Myc activity in VHL-deficient renal carcinoma cells (Zhang *et al.*, 2007) further increases the possibility that miR-23b* could be regulated by VHL, HIF and c-Myc, thereby affecting the expression of POX. These regulatory interactions are of great interest and are being pursued.

An additional point worth mentioning concerns the definition of miRNA*. As previously documented, pre-miRNAs are processed by the endonuclease Dicer into a miRNA duplex: one strand becomes mature miRNA, whereas the other strand commonly named as

miRNA*, usually was thought to be easily degraded and thus short-lived (Denli *et al.*, 2004; Gregory *et al.*, 2004). Recent evidence indicates that some of the miRNA* are also functional, such as miR-9* and miR-199* (Kim *et al.*, 2008; Migliore *et al.*, 2008; Nass *et al.*, 2009; Yang *et al.*, 2008). Our identification of miR-23b* provides another example.

In summary, we demonstrate for the first time that the loss of POX expression in cancer is mediated by miRNAs, and provide evidence supporting the oncogenic role of miRNAs by regulating a tumor suppressor gene. Knockdown of miR-23b* not only increased apoptosis by up-regulating POX through ROS generation, but also decreased HIF-1 α signaling in renal cancer cells. These findings suggest that miR-23b* might be a novel therapeutic target for renal cell carcinoma. This potential role is being intensively investigated.

Materials and Methods

Cell lines and tissue specimens

The human renal cancer cell lines (786-0, TK10 and UO31) were provided by the NCI cell line repository and were cultured in RPMI-1640 supplemented with 10% fetal bovine serum. Two normal human renal/renal cortical epithelial cells (HREpC and HRCEpC) were obtained from PromoCell, and were cultured in renal epithelial cell medium provided by PromoCell. In addition, DLD1 Tet-off POX cells were cultured as described (Donald *et al.*, 2001).

Sixteen pairs of human clear cell renal cell carcinomas and corresponding normal renal tissues with appropriate informed consent were obtained from the Cooperative Human Tissue Network. Their histologic characteristics were confirmed by the pathologist.

miRNA microarray expression profiling

Total RNA from each cell line was harvested using a RNeasy Mini Kit (Qiagen). miRNA profiling was performed using human miRNA microarray chips (V2) from Agilent Technologies, which contains 723 human miRNA probes. Briefly, 100 ng of mRNA from each sample were labeled with the Cy3 fluorescent dye followed by dephosphorylation and denaturation. Hybridization and washing of the microarray slides were performed as recommended. Scanning was performed using a G2505B Microarray Scanner (Agilent Technologies). Following scanning of the microarrays, we used Agilent Feature Extraction Software to generate raw intensity data. Average log₂ ratios were calculated based on the two measurements of each miRNA.

Real-time RT-PCR analysis of miRNAs and POX mRNA expression

Total RNA was harvested using a RNeasy Mini Kit (Qiagen). For POX mRNA analysis, the cDNA was synthesized using the SuperScript II Reverse Transcriptase Kit (Invitrogen). SYBR Green Supermix (Bio-Rad) was used for real-time PCR applications. The primer set for POX was 5'-GCCATTAAGCTCACAGCACTGGG (forward) and 5'-CTGATGGCCGGCTGGAAGTAG (reverse). β -actin served as an internal control using the following primers: 5'-ATCCACGAAACTACCTTCAACTC (forward) and 5'-GAGGAGCAATGATCTTGATCTTC (reverse). Stem-loop real-time RT-PCR for mature

miRNAs was done using the Taqman MicroRNA assay (Applied Biosystems) as described (Ma *et al.*, 2007), using U6 as an internal control.

RNA oligoribonucleotides and cell transfections

Mimic miRNAs were designed as described previously (Lim *et al.*, 2005). Antagomir against miR-23b* (anti-23b*) was anti-sense sequence of mature miR-23b*. The small interference RNA (siRNA) targeting POX mRNA was designated as POX siRNA. The control RNA duplex for both mimic miRNAs and POX siRNA (designated as negative siRNA), and single-strand RNA control for anti-miR-23b* (designated as negative RNA), were scrambled and nonhomologous to any human genome sequences. All of the RNA oligoribonucleotides were synthesized by Invitrogen. Renal cancer cells, normal renal cells or DLD1 Tet-off POX cells were transfected with above RNA molecules by using Lipofectamine 2000TM (Invitrogen) after 24 h of seeding. Briefly, Lipofectamine 2000TM was mixed with Opti MEM medium and incubated for 5 min. Meanwhile, RNA molecules were diluted in Opti MEM reduced serum medium (Gibco). Then, the diluted miRNA and Lipofectamine 2000 were mixed and incubated for 20 min at room temperature, and was added to each well/flask.

Plasmid vector construction

The 3' UTR of POX (393bp) containing the putative miR-23b* binding sequence was amplified by PCR from cDNA synthesized from total RNA. The used primers contained restriction sites (lowercase): forward, 5'-AGCactagtCAGCACACCCTTAGCC -3' (SpeI); reverse, 5'-GCAGaagcttTGGTTTATTGACCAG -3' (HindIII). The PCR product was cloned into the SpeI and HindIII restriction sites downstream of the ORF of luciferase in pMIR-REPORT Vector (Ambion) to generate the pPOX 3' UTR reporter.

Mutation of the miR-23b* binding site in the 3'UTR of POX mRNA was created using site-directed mutagenesis by the megaprimer PCR method (Figure 3a). The primers include the forward and reverse primers shown above, and the mutated primer: 5'-ACACAGCCCGAaaaCTTGGGGAG -3' (mutation sites are shown in lowercase). The mutated PCR product was cloned into the cloning site of pMIR-REPORT to generate the pPOX 3'UTR-MUT reporter. All cloned products were verified by sequencing before use.

The p53 expression construct was made by amplifying p53 cDNA and was subsequently cloned into pTarget as described before (Pandhare *et al.*, 2006). The pCi control vector was obtained from Promega.

The following procedures are included in the supplementary materials: western blot analysis, luciferase reporter assay, apoptosis assay, proliferation assay, measurement of intracellular ROS, POX immunohistochemical (IHC) staining and miR-23b* in situ hybridization (ISH).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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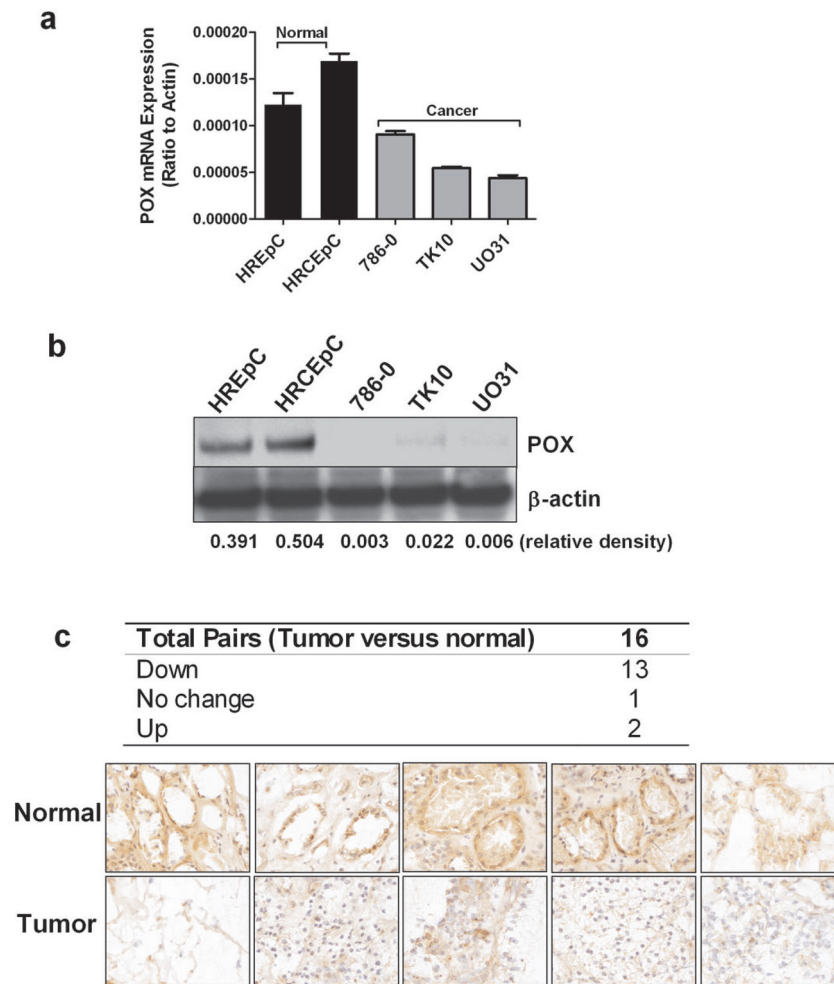
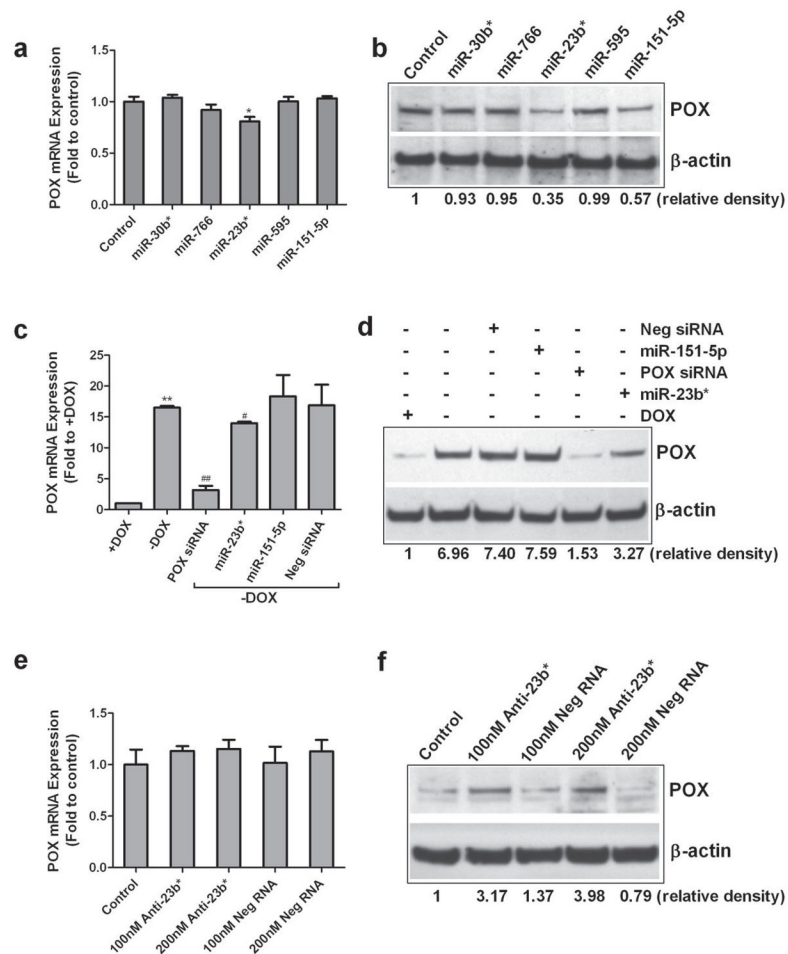


Figure 1. Reduced POX expression in renal cancer cell lines and human renal carcinoma tissue samples. (a) POX mRNA levels were evaluated by real-time RT-PCR in normal renal cell lines (HREpC and HRCEpC) and renal cancer cell lines (786-0, TK10 and UO31). β -actin was used as an internal control. Data are reported as mean \pm SEM. (b) POX protein expression was tested by western blot. β -actin served as loading control. All results are representative of three independent experiments. (c) 16-paired renal carcinoma/normal tissues were stained by immunohistochemistry for the expression of POX. The representative images from 5 pairs of normal/cancer tissues are shown (40 \times).

**Figure 2.**

Inverse Relationship of miR-23b* with POX Expression. HREpC normal renal cells were transfected for 24 hr with 100nM mimic miRNAs. POX mRNA and protein expression were tested by real-time RT-PCR (a) and western blot (b), respectively. DLD1 tet-off POX cells were transfected with 100nM miR-23b* and miR-151-5p. POX siRNA served as a positive control and scrambled siRNA as a negative control. POX mRNA and protein expression were tested by real-time RT-PCR (c) and western blot (d), respectively. In TK10 renal cancer cells, miR-23b* was knocked down by transfection with antagonists (Anti-23b*). Total RNA and protein was harvested after 48 hr. POX mRNA and protein expression were tested by real-time RT-PCR (e) and western blot (f), respectively. All results are representative of three independent experiments. Real-time PCR data are reported as mean \pm SEM. * p <0.05, ** p <0.001 compared with control or “+DOX” group, # p <0.05, ## p <0.001 compared with “-DOX” group.

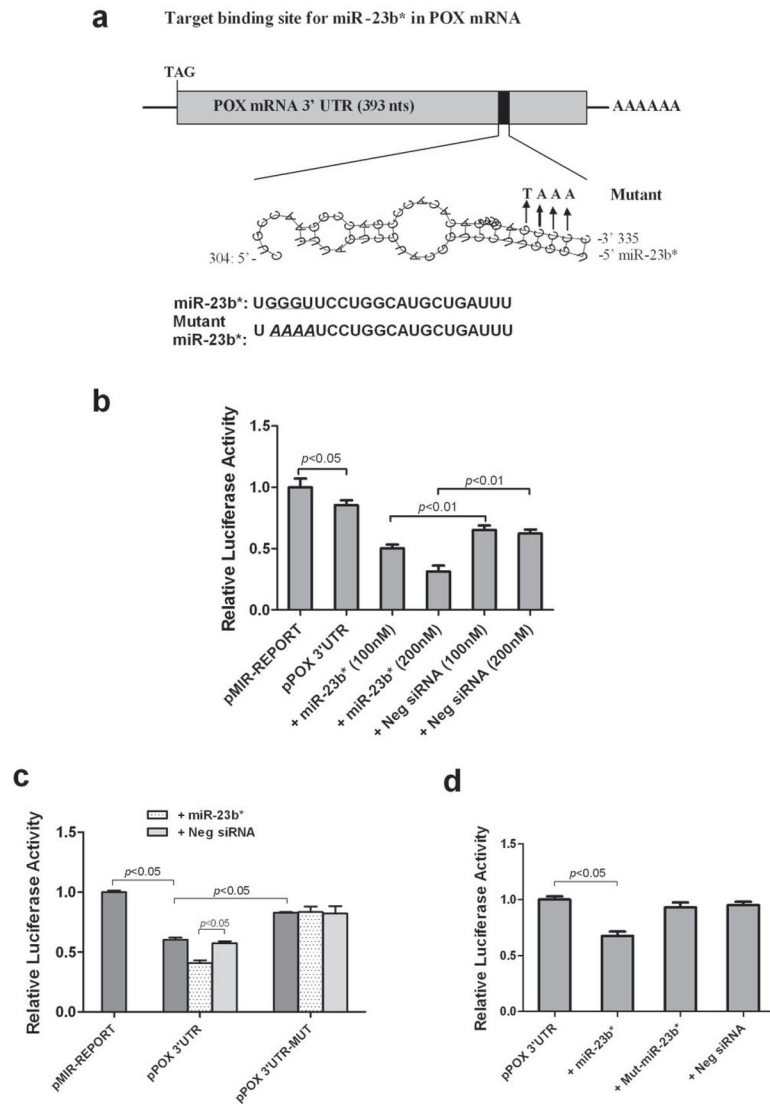


Figure 3. miR-23b* Directly Targets 3' UTR of POX mRNA. (a) Schematic representation of miR-23b* target binding site in the POX mRNA 3' UTR identified by the Microinspector prediction program. Mutations of 3'UTR and miR-23b* in the seed sequences were indicated. (b) The 3'UTR of POX mRNA was amplified by PCR and cloned downstream of a firefly luciferase gene of pMIR-REPORT to construct the pPOX 3' UTR vector. TK10 renal cancer cells were transfected with pMIR-REPORT or pPOX 3' UTR reporter with or without mimic miR-23b* or negative control siRNA. (c) The POX 3'UTR with mutations was constructed as the pPOX 3'UTR-MUT plasmid. TK10 renal cancer cells were transfected with pMIR-REPORT, pPOX 3' UTR or pPOX 3'UTR-MUT reporter with or without 100nM mimic miR-23b* or negative control siRNA. (d) TK10 renal cancer cells were transfected with pPOX 3' UTR reporter with 100nM mimic miR-23b*, mutant miR-23b* or negative control siRNA. All transfections used pRL-null renilla luciferase reporter as an internal control. Data are reported as relative luciferase activity normalized to

that of pMIR-REPORT group (3b and 3c) and that of pPOX 3' UTR (3d). All results were done in triplicates and repeated in two independent experiments. Values represent means \pm SEM. *p* value was obtained in one-way ANOVA analysis.

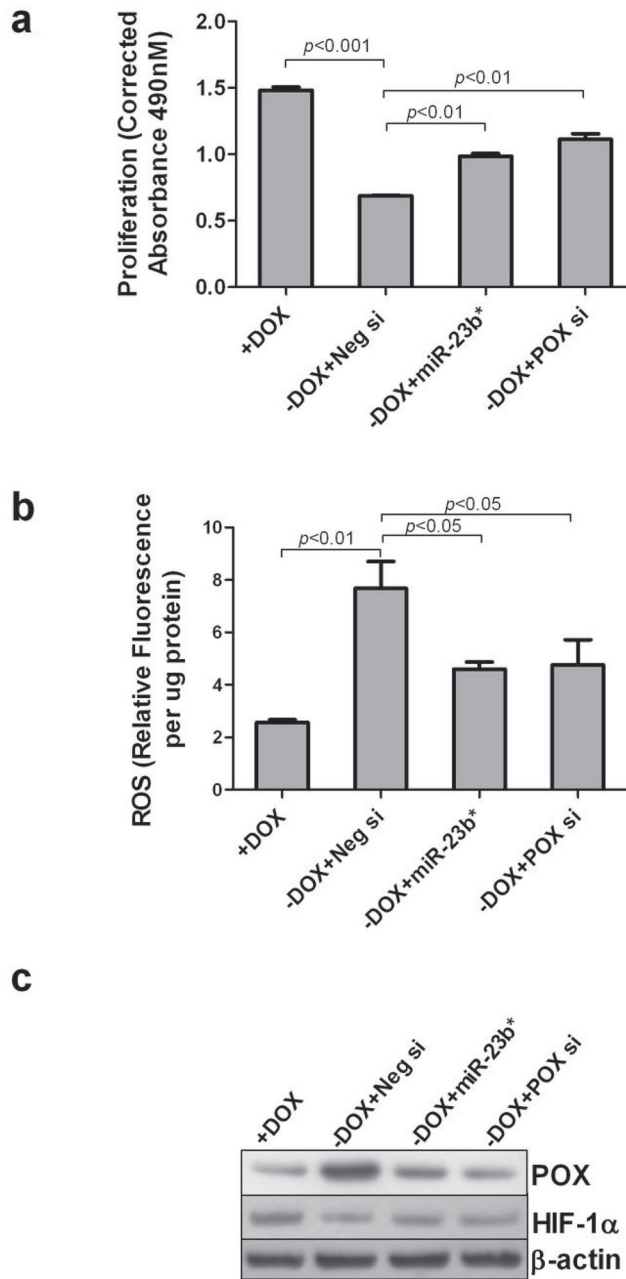


Figure 4.

miR-23b* Inhibits ROS Production and Promotes the Proliferation of DLD1 Tet-off POX Cells by Decreasing POX Expression. (a) DLD1Tet-off POX cells were transfected with 100nM mimic miR-23b*, POX siRNA or negative siRNA. After 6 hr, the medium was replaced with new medium without DOX for 48 hr. Proliferation assay was performed by MTS method. (b) ROS production was performed by DCF assay after cells were treated as above. All values represent means \pm SEM (n = 3). p value was obtained in one-way ANOVA analysis. (c) HIF-1 α expression was shown by western blot after cells were treated as above.

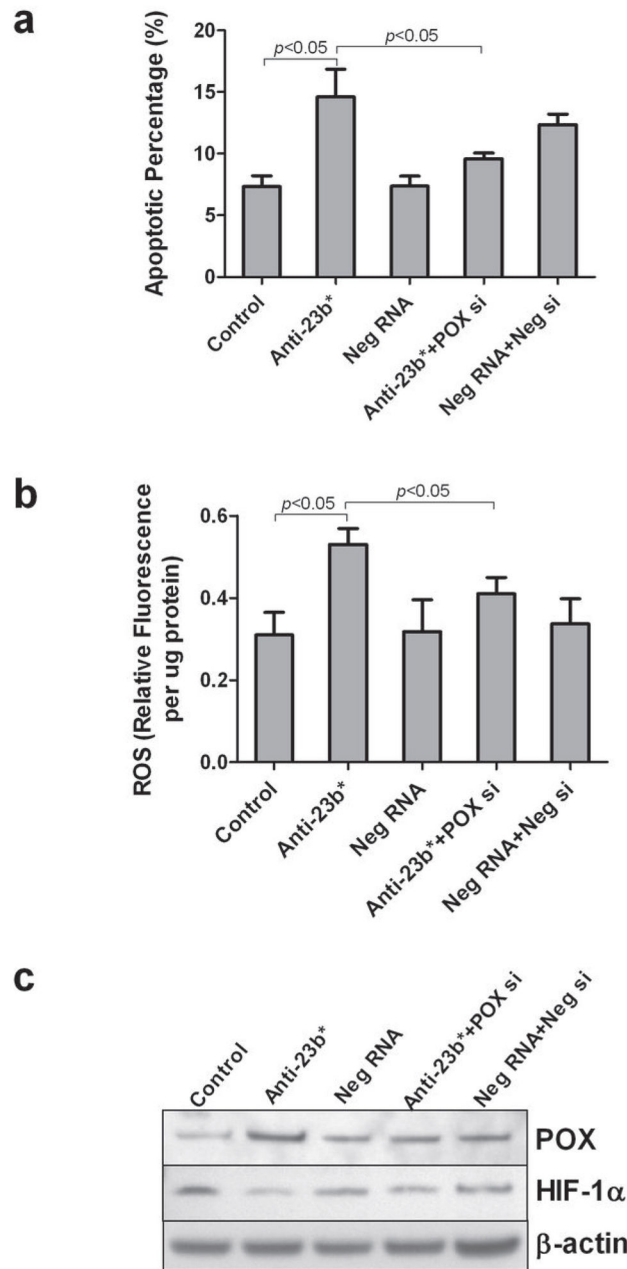
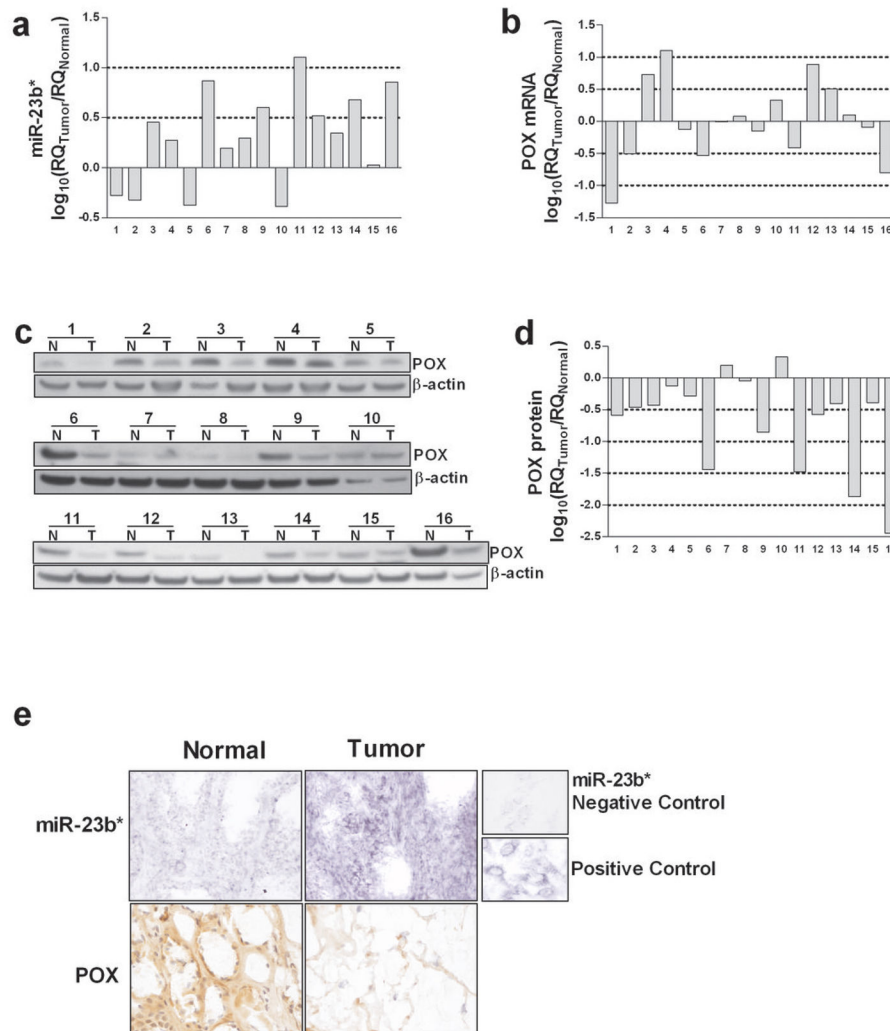


Figure 5. Knockdown of miR-23b* Sensitizes Renal Cancer Cells to Apoptosis

(a) TK10 cells were transfected with 100nM antagonims of miR-23b* (anti-23b*) with or without 100nM POX siRNA (POX si). Scrambled RNAs served as negative controls.

Apoptosis assays were performed by flow cytometry analysis. Transfections were done in triplicates and repeated twice in independent experiments. (b) TK10 cells were treated as described above. ROS production was measured by DCF assay. Values represent means \pm SEM of the mean (n = 3). p value was obtained in one-way ANOVA analysis. (c) HIF-1 α expression was shown by western blot after TK10 cells were treated as above.

**Figure 6.**

Expression of miR-23b* and POX protein in human renal cell carcinoma and corresponding normal tissues. (a) The expression of miR-23b* and POX mRNA in 16 paired human clear cell renal cell carcinoma and normal tissues were examined by real-time RT-PCR, using U6 and β -actin as the internal control, respectively. POX protein was measured by western blot. The relative expression values of POX protein were obtained by scanning the density of western blot bands (c). The expression of miR-23b* (a) and POX mRNA (b) and protein (d) in renal carcinoma versus matched normal tissue are reported as \log_{10} transformed relative quantity (RQ). (e) POX protein and miR-23b* expression were verified by immunohistochemistry and in situ hybridization, respectively. HREpC cells with and without mimic miR-23b* transfection were used as positive and negative control, respectively. The representative images from one pair of normal/tumor tissues which corresponds to case 6 in figure 6a, c and d are shown (40 \times).

Table 1

Upregulated miRNAs related to POX in renal cancer cells

Upregulated miRNA potentially targeting POX	Sequence of target POX mRNA 3' UTR	Log ₂ (Tumor/Nor mal cells)	<i>p</i> (<i>t</i> test)
Hsa-miR-23b* (uggguuccugcugcugauuu)	5'...CTGCCAAGGCCAGCCACACAGCCCGAGCCCC	1.79	<0.01
Hsa-miR-151-5p (ucgaggagcucacagucuagu)	5'...ACTTTTGGGAACTCTCCTCGA	1.75	<0.01
Hsa-miR-30b* (cugggagguggauguuuacuuc)	5'...GAGGTGAGGTCAGGTGCCTCCCAG	1.20	<0.01
Hsa-miR-595 (gaagugugccguggugugucu)	5'...CACCUUUUUACCCCCACACUUG	1.00	<0.01
Hsa-miR-766 (acuccagccccacagccucagc)	5'...CACACUUGCAGAGCUGCUGGAGG	0.58	<0.05
Hsa-miR-296-5p (agggcccccucuaucuccgu)	5'...UCUCCUCGAAUGUGUGGGCCCAA	0.48	<0.05
Hsa-miR-299-5p (ugguuuaccguccacauacau)	5'...GGCCUGCCUGGUCAAUAACCAC	0.36	<0.05
Hsa-miR-342-3p (ucucacacagaaucgacccgu)	5'...AACTTTTGGGAACTCTCCTCGAATGTGTGGGC	0.34	<0.05
Hsa-miR-940 (aagcaggccccccgucuccc)	5'...GGCACTCAGGTGTGGGCCGAACCTGATACCTG	0.30	<0.05
Hsa-miR-30e* (cuuucagucggauguuuacagc)	5'...CCTGGGACAGCCACTGGAAA	0.26	<0.05

miRNA expression profiling was performed using human miRNA microarray chips in renal tumor cell lines (786-0, TK10 and UO31) and normal renal cell lines (HREpC and HRCEpC). 91 putative miRNAs related to POX were analyzed through the average log₂ ratios (tumor to normal cells) calculated based on the two measurements of each miRNA and t-test. Upregulated miRNAs potentially related to POX are shown in the table.