Biochemical and Biophysical Research Communications 422 (2012) 607–614

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The enhancer of zeste homolog 2 (EZH2), a potential therapeutic target, is regulated by miR-101 in renal cancer cells

Toshihiko Sakurai [a], Vladimir N. Bilim [a], Andrey V. Ugolkov [c], Kaori Yuuki [a], Masaaki Tsukigi [a], Teiichi Motoyama [b], Yoshihiko Tomita [a,*]

[a] Laboratory of Molecular Oncology, Department of Urology, Yamagata University School of Medicine, Iida-nishi 2-2-2, Yamagata 990-9585, Japan
[b] Department of Human Pathology (Second Department of Pathology), Yamagata University School of Medicine, Iida-nishi 2-2-2, Yamagata 990-9585, Japan
[c] Tumor Biology Core, Center for Developmental Therapeutics, Chemistry of Life Processes Institute, Northwestern University, Silverman Hall B733, Evanston, IL, USA

ARTICLE INFO

Article history:
Received 8 May 2012
Available online 15 May 2012

Keywords:
Renal cell carcinoma
Enhancer of zeste homolog 2
MicroRNA-101

ABSTRACT

We investigated a prognostic significance and the mechanism of aberrant nuclear expression of EZH2, a histone methyltransferase, in human renal cell carcinoma (RCC). We found nuclear EZH2 in 48 of 100 RCCs and it was significantly correlated with worse survival in RCC patients. We detected a decreased expression of miR-101 in 15 of 54 RCCs. We found that re-expression of miR-101 resulted in EZH2 depletion and decreased renal cancer cell proliferation. Our results show nuclear EZH2 as a prognostic marker of worse survival in human RCC, and identify miR-101 as a negative regulator of EZH2 expression and renal cancer cell proliferation.

1. Introduction

Kidney cancer is the sixth and eighth most common cause of cancer for men and women, respectively [1]. About 30% of patients with renal cell carcinoma (RCC) are diagnosed with metastatic disease at initial presentation and up to 50% of RCC patients develop metastases after surgical resection of tumor [2]. Currently, immunotherapy is the only available systemic therapy for metastatic RCC showing some effect in less than 20% of metastatic RCC patients [3,4]. Recently, molecular targeted drugs emerged as the treatment option for advanced RCC [5], which have been shown to improve RCC patients survival [5]. However, the response to these drugs is only partial [6] and is associated with severe toxicity in RCC patients [7]. Thus, the identification of new therapeutic targets and development of new approaches for the treatment of advanced RCC is urgently needed.

Enhancer of zeste homolog 2 (EZH2), a histone methyltransferase, is a catalytic subunit of a Polycomb Repressive Complex 2 (PRC2) which methylates histone H3 on lysine 27 [8]. EZH2 is involved in epigenetic silencing of large number of genes involved in differentiation and proliferation and is essential for embryonic development [9]. Recent studies suggest EZH2 overexpression as an important positive regulator of cancer cell growth in multiple human malignancies [8] including prostate [10], bladder [11], breast [12], colorectal [13] and pancreatic cancer [14]. However, the role of EZH2 in RCC remains controversial. Whereas Wagener et al. [15] study showed EZH2 nuclear overexpression as an independent unfavorable marker of cancer specific survival in RCC patients, Hinz et al. [16] reported that high mRNA levels of EZH2 in RCCs indicate less aggressive tumor phenotypes with a favorable prognosis in RCC patients.

Here, our objective was to investigate a prognostic significance and the mechanism of aberrant nuclear expression of EZH2 in RCC. We found nuclear accumulation of EZH2 as a prognostic marker of worse survival in RCC, identified EZH2 as a positive regulator of renal cancer cell proliferation, showed miR-101 as a negative regulator of EZH2 expression in renal cancer cells, and demonstrated p27Kip1 tumor suppressor as EZH2 target gene. Our study suggests EZH2 as a potential therapeutic target and miR-101 overexpression as a new approach to target EZH2 in the treatment of RCC.

2. Materials and methods

2.1. Patients

The study was approved by the Ethical Committee of Yamagata University and all patients signed an informed consent form. Surgical specimens from 110 patients who underwent surgery from 2003 to 2008 at the Yamagata University Hospital were included in the study. RCC patients' clinical characteristics are presented in the Table 1. The longest followed up was 105.4 months (median 39.7 months). Survival analysis was done for patients without metastases at the time of operation.

* Corresponding author. Fax: +81 23 628 5370.
E-mail addresses: ugolkov@northwestern.edu (A.V. Ugolkov), ytomita@med.id.yamagata-u.ac.jp (Y. Tomita).

0006-291X/
http://dx.doi.org/10.1016/j.bbrc.2012.05.035

Table 1
Patients' clinical characteristics.

Median age (range) years	63 (25–87)
Male/female	73/37
Affected side (right/left)	60/50
Surgery	
Laparoscopic/open	48/62
Radical/partial nephrectomy	73/37

2.2. Immunohistochemical staining

Monoclonal mouse antibody against EZH2 and p27Kip1 (BD Transduction, San Diego, CA) were used for immunohistochemical analysis. The staining was performed as described previously [17]. Detection was performed by peroxidase method using Histofine simple stain MAX-PO MULTY (Nichirei, Tokyo, Japan) and DAB in the presence of H_2O_2. EZH2 nuclear accumulation was defined as positive staining of more than 50% of cancer cell nuclei throughout the tumor regardless of cytoplasmic staining.

2.3. Cell culture and RNA interference

Renal cancer cell lines ACHN, KRC/Y, Caki1, Caki2, A704, A498, and KH39 were obtained from ATCC. KU19-20 was kindly provided by Dr. Mototsugu Oya (Department of Urology, School of Medicine, Keio University, Tokyo, Japan). The cells were cultured as described

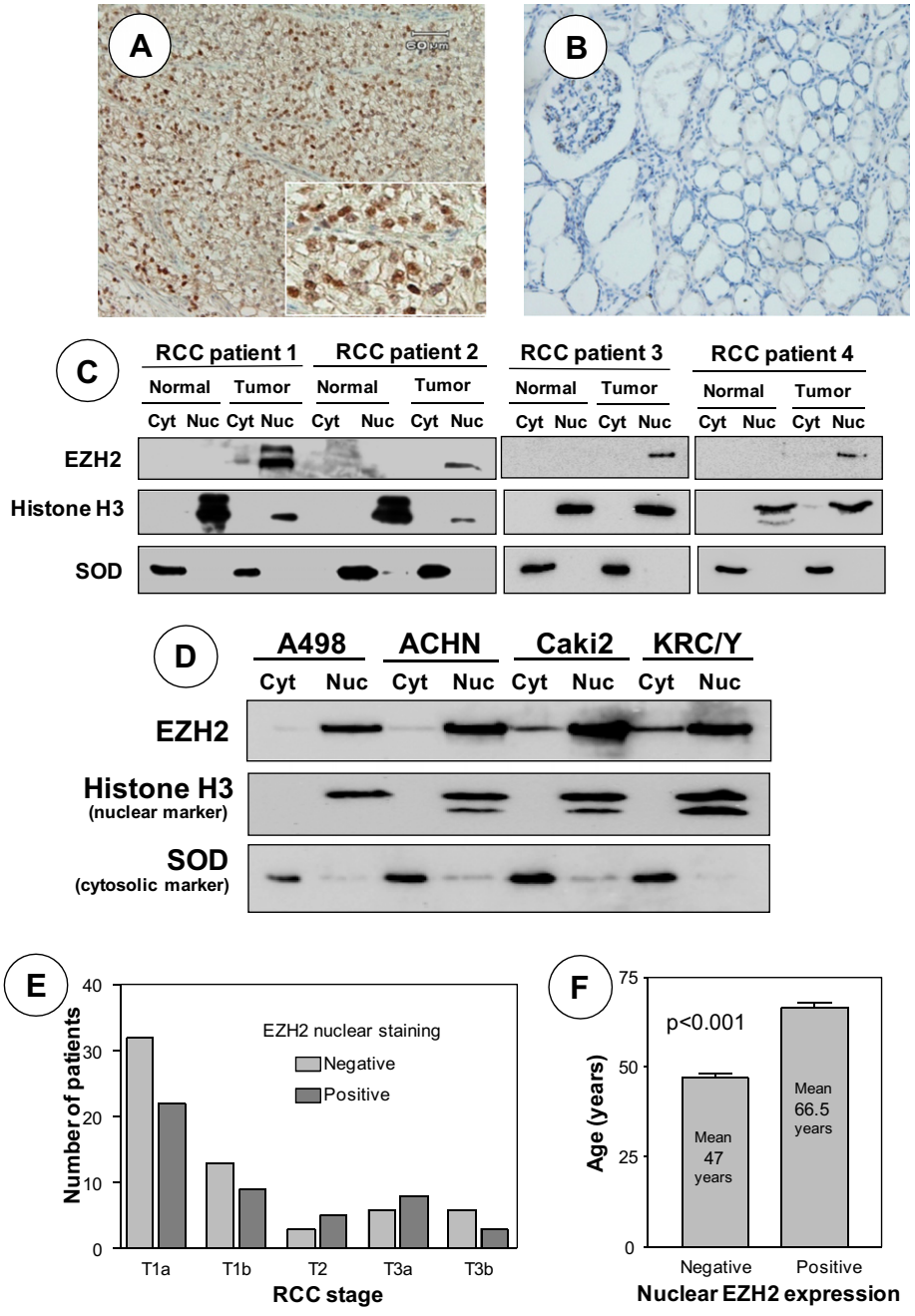

Fig. 1. EZH2 is overexpressed in the nuclei of renal cancer cells. (A–B) Immunohistochemical analysis of EZH2 expression and localization in RCC specimen (A) and normal kidney (B). (C–D) Equivalent amount (50 mcg) of nuclear and cytosolic proteins from RCC tissues and normal kidney (C), and from RCC cell lines (D) were analyzed with Western immunoblotting. (E) Distribution of EZH2 nuclear expression in different stages of RCC. (F) Nuclear accumulation of EZH2 is associated with older age in RCC patients.

Table 2
Analysis of EZH2 expression in renal tumors.

		Total	EZH2 positive
Histological type	Clear cell	92	37 (40%)
	Papillary	6	3 (50%)
	Chromophobe	1	1 (100%)
	Cystic	5	3 (60%)
	Unclassified	6	4 (67%)
pT stage	1a	57	23 (40%)
	1b	22	9 (41%)
	2	8	5 (63%)
	3a	14	8 (57%)
	3b	9	3 (33%)
pN status	N(−)	107	45 (42%)
	N(+)	3	3 (100%)
cM	M(−)	100	46 (43%)
	M(+)	10	2 (20%)
Fuhrman grade[a]	1	57	14 (25%)
	2	34	20 (59%)
	3	12	9 (75%)
	4	7	5 (71%)
Total		110	48 (44%)

[a] Fuhrman grade, Fisher's exact test $p < 0.001$, chi square 18.619. Fuhrman grade 1 vs. 2–4 Fisher's exact test $p < 0.001$, chi square 15.929. Fuhrman grades 1–2 vs. 3–4 Fisher's exact test $p = 0.004$, chi square 7.

previously [17]. Cell viability was estimated using MTS cell proliferation assay as described previously [17]. Anti-EZH2 siRNA (Dharmacon, Thermo Fisher Scientific K.K, Yokohama, Japan) and unrelated control siRNA (Invitrogen, Life Technologies Corporation, Tokyo, Japan) were used for genetic knockdown experiments. Precursor miRNA, pre-miR-101 (Applied Biosystems Japan, Tokyo, Japan) was used for cell transfection using Lipofectamine 2000 (Invitrogen).

2.4. Immunoblot analysis

Western blotting analysis was performed as described previously [17]. Nuclear/cytoplasmic protein separation was performed by Dignam method as described previously [14]. The following antibodies were used: anti-EZH2 and anti-p27Kip1 (BD Transduction); anti-Histone H3 (SIGMA, Saint Louis, Missouri); anti-trimethyl-Histone H3(Lys27) (Upstate Cell Signaling Solutions, Temecula, CA); anti-Cu/Zn SOD (Stressgen, Ann Arbor, MI); anti-β-actin (Abcam, Cambridge, MA).

2.5. MicroRNA extraction and real-time qRT-PCR

Total cellular RNA (including microRNA) from surgical specimens immersed in RNAlater (Applied Biosystems, Tokyo, Japan) tissue storage solution and stored at −80 °C and cultured cell lines was extracted using mirVanaTM miRNA Isolation Kit (Applied Biosystems Japan, Tokyo, Japan) and the first strand DNA was synthesized using a Taqman MicroRNA RT Kit (Applied Biosystems Japan, Tokyo, Japan). For real-time quantitative RT-PCR we used TaqMan® MicroRNA Assays (Applied Biosystems) targeting human *microRNA101* and *RNU6B*(U6) small nuclear RNA as an endogenous control. The expression of miRNA was calculated using the comparative Ct (2-delta-delta Ct) method [18]. Each reaction was run in triplicate and mean with SD were calculated. If the normalized value was significantly ($p < 0.05$) lower than normal kidney counterpart, the miR-101 expression in tumor was considered decreased.

2.6. Statistical analysis

All continuous variables in the present study met the criteria for a normal distribution and were analyzed using a two tailed *t*-test or one-way ANOVA. Categorical data were analyzed using Fisher's exact test. For survival analysis of the patients Kaplan–Meier curves for EZH2 negative and positive tumors were generated and compared using Logrank test. Data were analyzed using SPSS for Windows.

3. Results

3.1. EZH2 nuclear accumulation is a strong predictor of worse survival in RCC patients

By immunohistochemical analysis, we found EZH2 nuclear expression selectively in cancer cells in surgically resected RCCs, whereas EZH2 expression was not detectable in normal renal tissues (Fig 1A–C). EZH2 expression was found in the nucleus with no detectable cytoplasmic staining in cancer cells within surgically resected RCCs (Fig 1A and C). We detected positive EZH2 staining in 48 of 110 (44%) examined RCCs. We found that positive EZH2 staining correlated with higher Fuhrman nuclear grade of RCC (Table 2, Fisher's exact test $p < 0.001$, chi square 18.619). We found nuclear expression of EZH2 in RCC cell lines by nuclear/cytosol fractionation (Fig. 1D). We found that the percentage of EZH2-positive RCCs increased from stage T1a to T2 and T3. Positive EZH2 staining was significantly associated with older age of RCC patients (Fig. 1F, $p < 0.001$). We found that EZH2 nuclear expression predicted a shorter overall (Fig. 2A, $p = 0.036$) and recurrence-free (Fig. 2B, $p = 0.022$) survival of RCC patients. These results suggest EZH2 nuclear accumulation as a feature of renal cancer cells and an indicator of worse survival in RCC patients.

3.2. Depletion of EZH2 leads to re-expression of p27Kip1 and suppression of renal cancer cell proliferation

Although our results show nuclear accumulation of EZH2 as a feature of renal cancer cells, its role in renal cancer cell proliferation and survival remains unclear. To investigate the role of EZH2 nuclear accumulation in RCC, we knock-down EZH2 in human renal cancer cells by transfection with EZH2 targeting siRNA (Fig. 3A–C). We found that depletion of EZH2 resulted in a decreased proliferation in ACHN, Caki1 and A498 renal cancer cell lines (Fig. 3A). We did not find an induction of apoptosis upon depletion of EZH2 in renal cancer cells (data not shown). Because EZH2 is a putative repressor of gene expression, we searched for tumor suppressor genes which could be re-expressed upon depletion of EZH2 in RCC. It has been reported that EZH2 can downregulate E-cadherin, p16(Ink4A), p21(CIP1,WAF1), p27Kip1, and APAF1 tumor suppressor genes [14,19]. After screening for potential EZH2-regulated tumor suppressor genes, we found that only p27Kip1 was re-expressed in ACHN and other renal cancer cell lines upon depletion of EZH2 (Fig. 3C and data not shown). Consistent with the known role of EZH2 in posttranslational histone modifications, we found that depletion of EZH2 resulted in a decrease of histone H3 methylation in ACHN cancer cells (Fig. 3C). These results suggest a positive role of EZH2 in renal cancer proliferation and identify the tumor suppressor p27Kip1 as a new target of EZH2 in RCC.

3.3. Re-expression of miR-101 depletes EZH2 expression in RCC cells leading to decreased cancer cell proliferation

miR-101, one of microRNAs, has been reported as a negative regulator of EZH2 [20,21]. Our search of TargetScanHuman [22] database confirmed two highly conserved target sequences of miR-101 at position 59–65 and position 114–121 of EZH2 3′ UTR. Using TaqMan MicroRNA assay, we found very low expression of

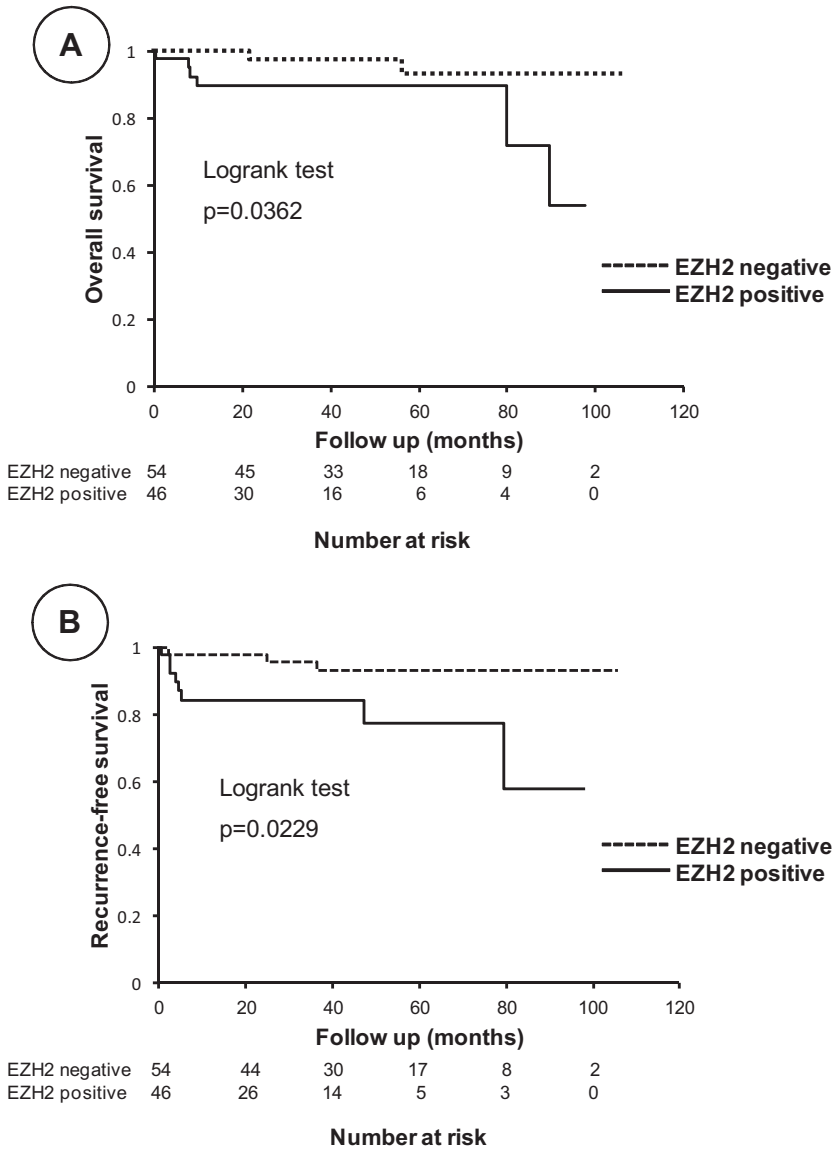

Fig. 2. Nuclear EZH2 is associated with worse survival of RCC patients. (A–B) Overall (A) and recurrence-free (B) survival of RCC patients without metastases at the time of operation is shown with negative (dotted line) or positive (solid line) nuclear EZH2 expression as detected by immunohistochemical staining of tumor tissues. Patients' survival was compared using Logrank test.

miR-101 in 8 RCC cell lines ranging from 0.3 to 0.02-fold as compared to expression of miR-101 in normal kidney (Fig. 4A). Relative expression of miR-101 in clinical RCC samples ranged from 0.02 to 588 fold as compared to its normal counterpart (Fig. 4B). We found a significant decrease of miR-101 expression in 15 of 54 (28%) surgically resected RCCs (Fig. 4B). Although we found lower expression of miR-101 in tumors than in its normal counterparts in 8 of 24 (33%) RCCs with EZH2 nuclear expression, these data were not statistically significant (Fig. 4C). Decreased miR-101 was significantly associated with younger patients' age (Fig. 4D, $p < 0.05$) and non-clear cell histology in RCC patients (Table 3, Fisher's exact test $p = 0.0137$, chi square 5.343).

To investigate the role of miR-101 in regulation of EZH2 aberrant expression in RCC, we transfected renal cancer cells with miR-101 precursor. Re-expression of miR-101 led to a depletion of EZH2, re-expression of EZH2-regulated p27Kip1 tumor suppressor and suppression of renal cancer cell proliferation (Fig. 4E–G). It is known that microRNA may affect cellular protein levels by either mRNA degradation or translational repression [23]. Using quantitative RT-PCR, we found no changes in EZH2 mRNA expression levels after re-expression of miR-101 in ACHN and Caki1 renal cancer cells (Fig. 4H) whereas EZH2 protein expression was decreased (Fig. 4E). These results suggest that miR-101 might downregulate EZH2 expression through translational repression. Our results suggest miR-101 as a negative regulator of EZH2 expression in renal cancer cells.

4. Discussion

Epigenetic repression of tumor-suppressor genes emerged as an important mechanism of carcinogenesis [24]. Recent studies showed that EZH2 is involved in epigenetic silencing of large number of genes via posttranslational modification of histone H3 [8] and direct control of DNA methylation [25]. EZH2 has been reported to be involved in pathogenesis of multiple human malignancies [8]. However, the role of EZH2 in human RCC remains controversial [15,16,26,27].

In the present study, we demonstrated nuclear overexpression of EZH2 in 48 of 110 (44%) surgically resected RCCs and in eight

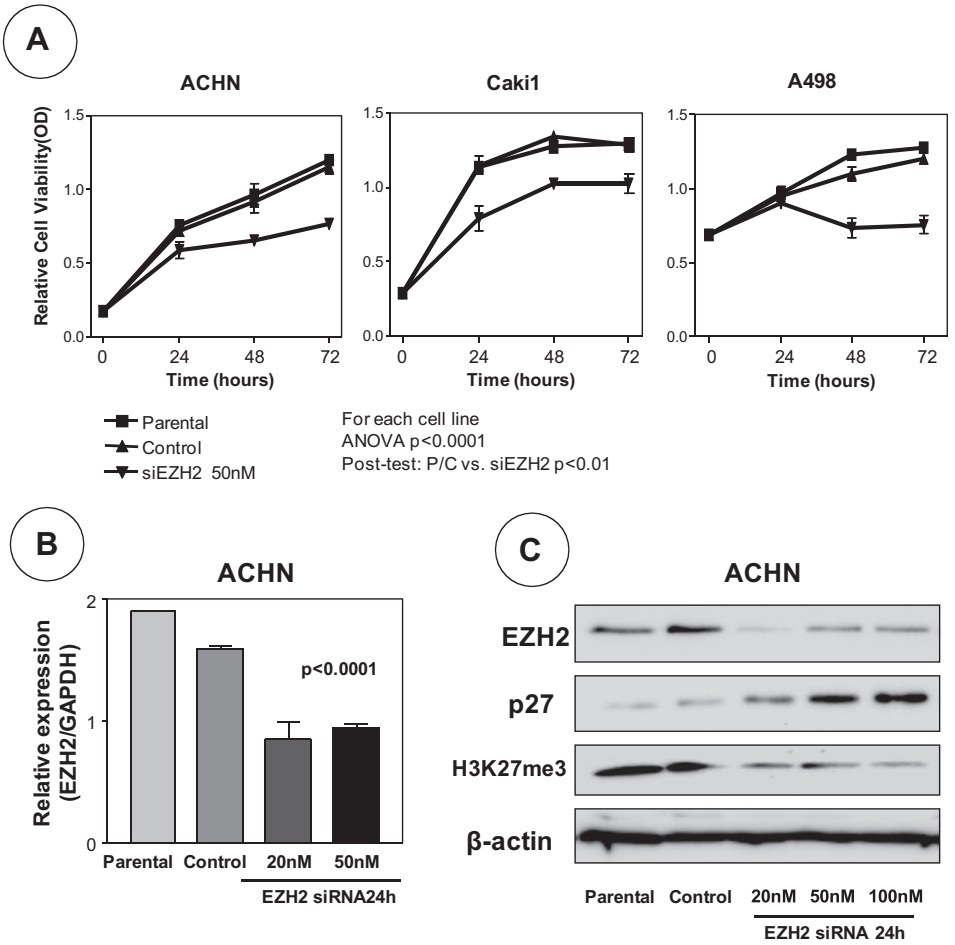

Fig. 3. Depletion of EZH2 leads to re-expression of p27Kip1 and suppression of renal cancer cell proliferation. (A) A relative cell viability was measured by MTS assay in the cell lines transfected with EZH2 siRNA, negative control siRNA or left untreated. (B-C) EZH2 siRNA transfection of ACHN cancer cells resulted in suppression of EZH2 mRNA expression as shown by qRT-PCR (B), downregulation of EZH2 protein and re-expression of p27Kip1 as demonstrated by Western immunoblotting (C).

RCC cell lines, thus identifying EZH2 nuclear accumulation as a potential marker of renal cancer cells. Our study is the first to show aberrant nuclear expression of EZH2 in clinical RCC tissues by nuclear/cytosolic fractionation and Western immunoblotting. Our findings are supported by Wagener et al. study showing overexpression of EZH2 by qRT-PCR at mRNA levels in 21 human RCCs as compared to its normal counterparts [26] and immunohistochemical staining of RCC tissue microarrays [15]. Together, our studies suggest an overexpression of EZH2 at mRNA and protein levels in RCCs. Our findings are further supported by other studies demonstrating overexpression of EZH2 in human breast, prostate and pancreatic cancers [10,12,14]. Consistent with our Western blot findings of nuclear EZH2 in surgically resected RCCs, we found nuclear accumulation of EZH2 selectively in cancer cells in clinical RCC samples by immunohistochemical staining.

It is still unclear how EZH2 expression is regulated in human cancer cells. EZH2 could be potentially regulated by miRNAs, small RNA molecules which act as post-transcriptional regulators of a gene expression. We are unaware of any previous report assessing the role of miR-101 in the regulation of EZH2 expression in RCC. Here, we provided evidences that re-expression of miR-101 downregulates EZH2 expression in renal cancer cells resulting in inhibition of cellular proliferation. We demonstrated that miR-101 expression is significantly decreased in eight RCC cell lines and in 15 of 54 (28%) of surgical RCC tissues as compared to normal kidney tissue. Although we found lower expression of miR-101 in tumors than in its normal counterparts in 8 of 24 (33%) RCCs with EZH2 nuclear expression, this data could not reach statistical significance. These results demonstrate a complexity of regulatory network in which both miR-101 and EZH2 are involved suggesting that miR-101 is probably not a sole negative regulator of EZH2 expression. There is another potential explanation of miR-101 positive expression in RCC tissues showing EZH2 nuclear accumulation. RCC is notorious for the frequent presence of tumor infiltrating immune cells [28]. Lymphocytes [29] and endothelial cells [30] have been shown to express miR-101. Other miR-101-positive benign cells might contaminate surgically resected tumor samples. There is a possibility that these normal cells in tumor sample might bias the results of miR-101 expression due to high sensitivity of PCR reaction which we used to analyze miR-101 levels.

In our study, we demonstrated that EZH2 nuclear accumulation was strongly associated with increased tumor nuclear grade (Fuhrman grade), an indicator of high malignant potential in human RCC. We showed that EZH2 nuclear staining is a marker of worse overall and disease-specific survival in RCC patients. These data is in agreement with Wagener et al. [15] study showing EZH2 nuclear overexpression as an independent unfavorable marker of cancer-specific survival in RCC patients. Contrary to our and Wagener et al. [15] findings, Hinz et al. [16] reported that high mRNA levels of EZH2 in human RCCs indicate less aggressive tumor phenotypes with a favorable prognosis in RCC patients. This discrepancy in the

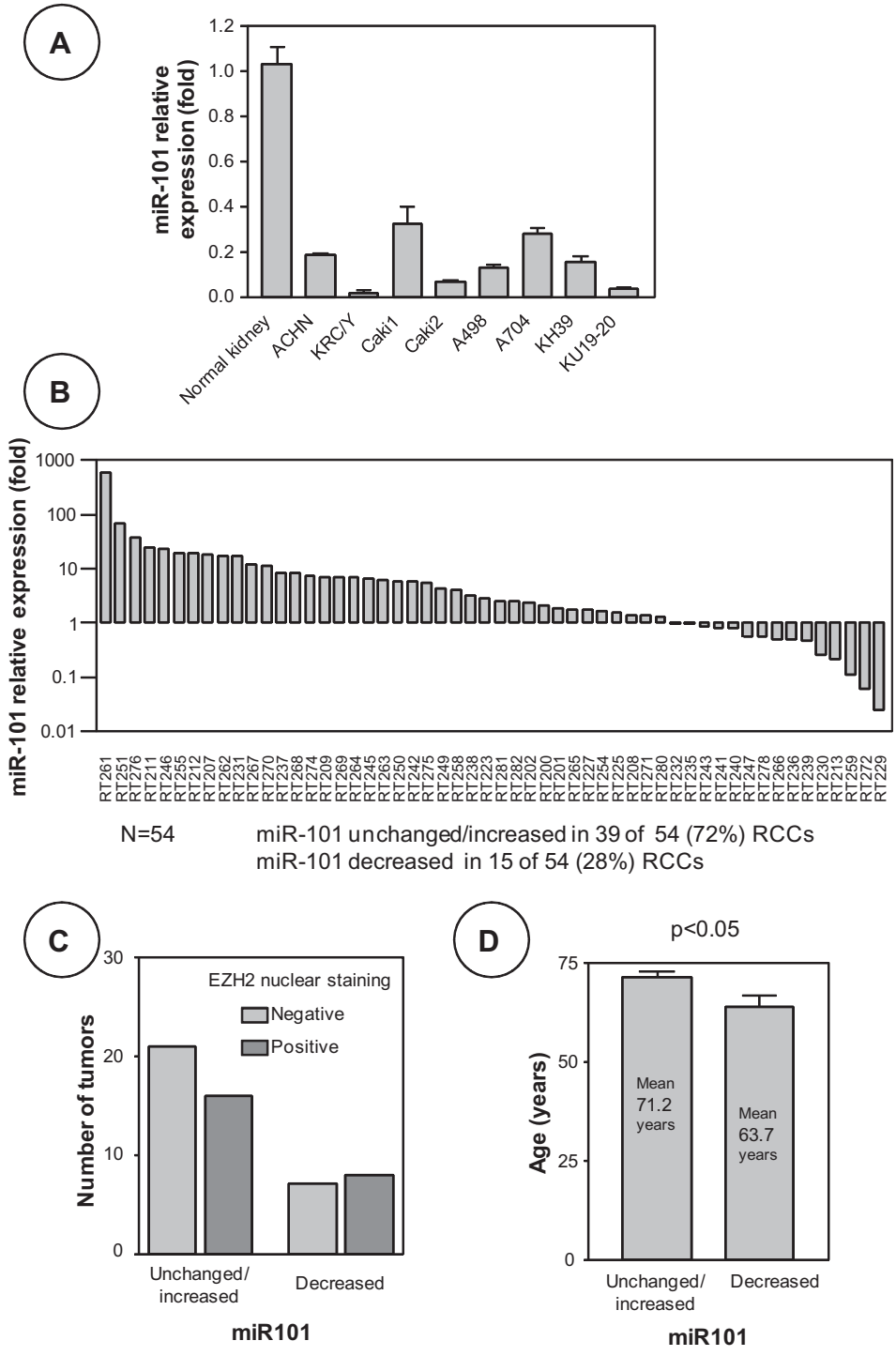

Fig. 4. EZH2 is negatively regulated by miR-101 in renal cancer cells. Relative expression of miR-101 was measured by TaqMan® MicroRNA Assay in RCC cell lines (A) and in 54 RCCs (B). RT-PCR data was normalized to RNU6B and compared with normal kidney using the comparative delta-delta C(t) method. Mean values SD error bars are presented. (C) Analysis of miR-101 expression with EZH2 immunohistochemical staining in RCCs. (D) Decreased miR-101 expression in RCCs was associated with younger patients' age ($p < 0.05$). ACHN and Caki1 renal cancer cells were transfected with pre-miR-101, control or left untreated (parental). Western immunoblotting and qRT-PCR were performed (E). Cellular viability was measured by MTS cell proliferation assay (F). Relative expression of p27 (G) or EZH2 (H) was analyzed using real time quantitative RT-PCR. The bars indicate relative expression (normalized to GAPDH). P, parental cells. C, control.

evaluation of EZH2 as prognostic marker in RCC patients can be explained by different approaches (mRNA vs. protein expression) applied to analysis of EZH2 expression in different studies. It is known that microRNA may affect cellular protein levels by either mRNA degradation or translational repression [23]. Here we demonstrated that re-expression of miR-101 downregulates EZH2 protein expression through translational repression while EZH2 mRNA levels were unchanged in renal cancer cells. Our findings suggest that EZH2 protein expression could be suppressed by miR-101 through translational repression even in case of EZH2 mRNA overexpression in the same RCC tumor in a certain cohort of RCC patients. Although several studies utilized an analysis of

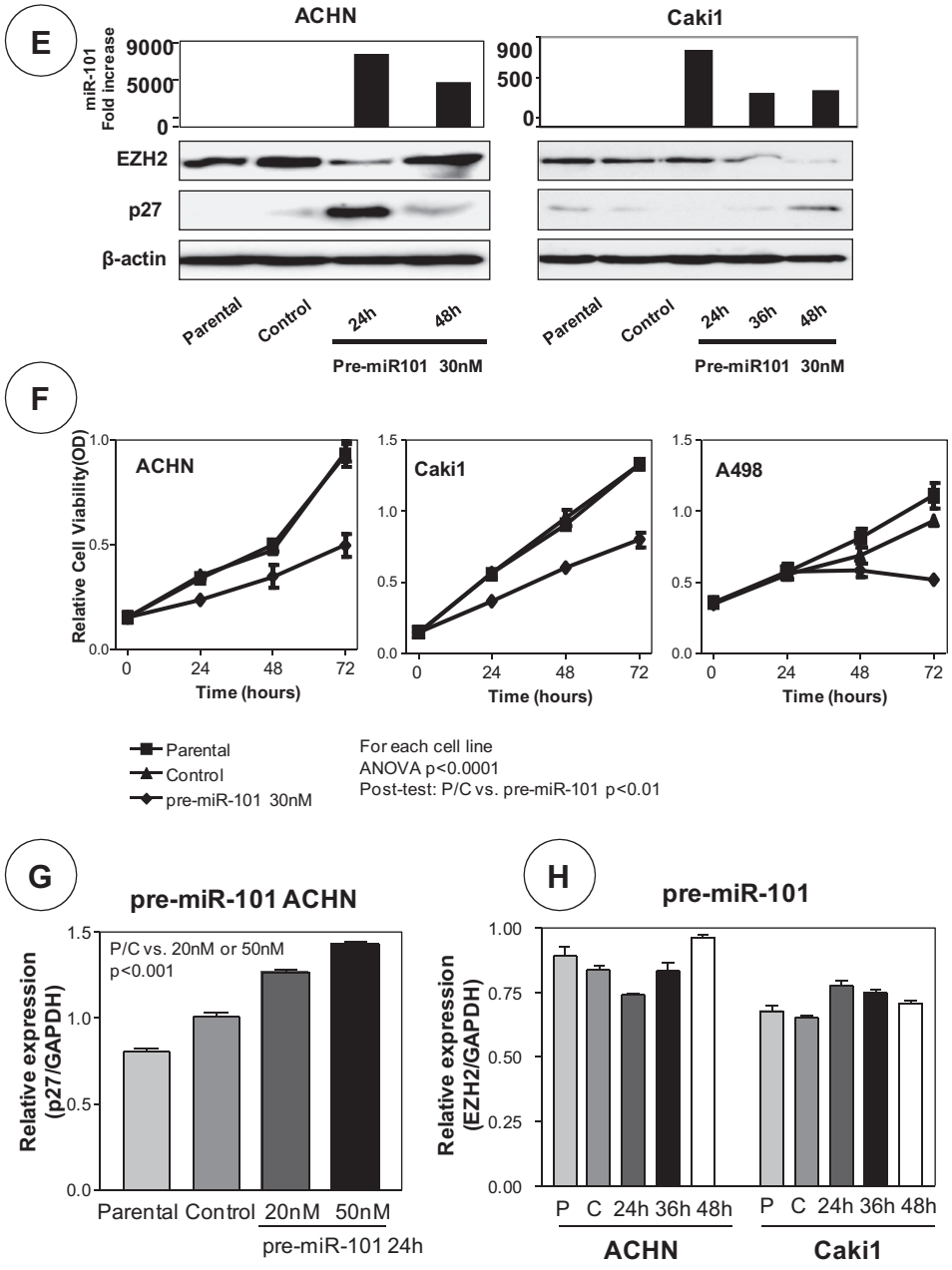

Fig. 4 (*continued*)

Table 3
miR-101 decreased expression was associated with non-clear cell histology in RCC patients.

	miR-101 unchanged/ increased	miR-101 decreased
Clear cell histology	37	10
Non-clear cell histology	2	5

Fisher's exact test $p = 0.0137$, chi square 5.343.

EZH2 mRNA expression in human RCCs [16,26,27], our results suggest that analysis of EZH2 protein expression is more reliable in human RCCs and probably in other types of human cancer.

In our study, we demonstrated that EZH2 positively regulates proliferation but not survival of renal cancer cells. These findings are supported by our previous study of pancreatic cancer where we found that EZH2 depletion suppressed proliferation with no effect on pancreatic cancer cell survival [14]. Our data are in agreement with another study where EZH2 knockdown resulted in decreased proliferation and cell cycle arrest without apoptosis in prostate cancer cells [10]. Another study by Wagener et al. [26] supports a speculation that EZH2 depletion might have an effect on cancer cell survival in some RCC cell lines under certain conditions, although the exact mode of such an effect remains to be determined.

To investigate the mechanism of EZH2-mediated cancer cell proliferation, we analyzed expression of tumor suppressor genes which could be negatively regulated by EZH2 in renal cancer cells. We identified p27Kip1, a cyclin-dependent kinase inhibitor, as a new target gene of EZH2 in renal cancer cells. Moreover, re-expression of miR-101 resulted in EZH2 depletion, upregulation of p27Kip1 and decreased renal cancer cell proliferation. Our results

suggest that EZH2 might contribute to renal cancer cell proliferation in part through epigenetic silencing of p27Kip1.

In summary, our study shows nuclear accumulation of EZH2 as a prognostic marker of worse survival in human RCC, identifies EZH2 as a positive regulator of renal cancer cell proliferation, indicates miR-101 as a negative regulator of EZH2 expression in renal cancer cells, demonstrates p27Kip1 tumor suppressor as EZH2 target gene, and suggests EZH2 as a potential therapeutic target in the treatment of human renal cancer. Future clinical approaches to inhibit EZH2 might include gene therapy using RNA interference and reintroduction of EZH2-suppressing miRNAs in human RCC. In view of EZH2 positive regulation of renal cancer cell proliferation, development of EZH2 pharmacological inhibitors is an attractive goal in future therapy of human RCC.

References

[1] R. Siegel, D. Naishadham, A. Jemal, Cancer statistics, CA: A Cancer Journal for Clinicians 62 (2012) 10.

[2] R.M. Bukowski, Natural history and therapy of metastatic renal cell carcinoma: the role of interleukin-2, Cancer 80 (1997) 1198–1220.

[3] R.M. Bukowski, Cytokine therapy for metastatic renal cell carcinoma, Seminars in Urology Oncology 19 (2001) 148–154.

[4] R.J. Motzer, J. Bacik, B.A. Murphy, P. Russo, M. Mazumdar, Interferon-alfa as a comparative treatment for clinical trials of new therapies against advanced renal cell carcinoma, Journal of Clinical Oncology 20 (2002) 289–296.

[5] R.M. Bukowski, Systemic therapy for metastatic renal cell carcinoma in treatment naïve patients: a risk-based approach, Expert Opinion on Pharmacotherapy 11 (2010) 2351–2362.

[6] T. Yuasa, N. Tsuchiya, S. Urakami, Y. Horikawa, S. Narita, T. Inoue, M. Saito, S. Yamamoto, J. Yonese, I. Fukui, K. Nakano, S. Takahashi, K. Hatake, T. Habuchi, Clinical efficacy and prognostic factors for overall survival in Japanese patients with metastatic renal cell cancer treated with sunitinib, BJU International 105 (2011) 1811–1813.

[7] F. Di Fiore, O. Rigal, C. Menager, P. Michel, C. Pfister, Severe clinical toxicities are correlated with survival in patients with advanced renal cell carcinoma treated with sunitinib and sorafenib, British Journal of Cancer 109 (2012) 1349–1354.

[8] J.A. Simon, C.A. Lange, Roles of the EZH2 histone methyltransferase in cancer epigenetics, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 647 (2008) 21.

[9] A.P. Bracken, N. Dietrich, D. Pasini, K.H. Hansen, K. Helin, Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions, Genes & Development 20 (2006) 1123–1136.

[10] S. Varambally, S.M. Dhanasekaran, M. Zhou, T.R. Barrette, C. Kumar-Sinha, M.G. Sanda, D. Ghosh, K.J. Pienta, R.G.A.B. Sewalt, A.P. Otte, M.A. Rubin, A.M. Chinnaiyan, The polycomb group protein EZH2 is involved in progression of prostate cancer, Nature 419 (2002) 624.

[11] J.D. Raman, N.P. Mongan, S.K. Tickoo, S.A. Boorjian, D.S. Scherr, L.J. Gudas, Increased expression of the polycomb group gene, EZH2, in transitional cell carcinoma of the bladder, Clinical Cancer Research 11 (2005) 8570–8576.

[12] C.G. Kleer, Q. Cao, S. Varambally, R. Shen, I. Ota, S.A. Tomlins, D. Ghosh, R.G.A.B. Sewalt, A.P. Otte, D.F. Hayes, M.S. Sabel, D. Livant, S.J. Weiss, M.A. Rubin, A.M. Chinnaiyan, EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells, Proceedings of the National Academy of Sciences 100 (2003) 11606–11611.

[13] O. Fluge, K. Gravdal, E. Carlsen, B. Vonen, K. Kjellevold, S. Refsum, R. Lilleng, T.J. Eide, T.B. Halvorsen, K.M. Tveit, A.P. Otte, L.A. Akslen, O. Dahl, Expression of EZH2 and Ki-67 in colorectal cancer and associations with treatment response and prognosis, British Journal of Cancer 101 (2009) 1282.

[14] A.V. Ougolkov, V.N. Bilim, D.D. Billadeau, Regulation of pancreatic tumor cell proliferation and chemoresistance by the histone methyltransferase enhancer of zeste homologue 2, Clinical Cancer Research 14 (2008) 6790–6796.

[15] N. Wagener, S. Macher-Goeppinger, M. Pritsch, J. Husing, K. Hoppe-Seyler, P. Schirmacher, J. Pfitzenmaier, A. Haferkamp, F. Hoppe-Seyler, M. Hohenfellner, Enhancer of zeste homolog 2 (EZH2) expression is an independent prognostic factor in renal cell carcinoma, BMC Cancer 10 (2010) 524.

[16] S. Hinz, S. Weikert, A. Magheli, M. Hoffmann, R. Engers, K. Miller, C. Kempkensteffen, Expression profile of the polycomb group protein enhancer of zeste homologue 2 and its prognostic relevance in renal cell carcinoma, The Journal of Urology 182 (2009) 2920.

[17] M. Tsukigi, V. Bilim, K. Yuuki, A. Ugolkov, S. Naito, A. Nagaoka, T. Kato, T. Motoyama, Y. Tomita, Re-expression of miR-199a suppresses renal cancer cell proliferation and survival by targeting GSK-3beta, Cancer Letters 315 (2012) 189–197.

[18] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods 25 (2001) 402.

[19] C.J. Chang, M.C. Hung, The role of EZH2 in tumour progression, British Journal of Cancer 106 (2012) 243.

[20] S. Varambally, Q. Cao, R.-S. Mani, S. Shankar, X. Wang, B. Ateeq, B. Laxman, X. Cao, X. Jing, K. Ramnarayanan, J.C. Brenner, J. Yu, J.H. Kim, B. Han, P. Tan, C. Kumar-Sinha, R.J. Lonigro, N. Palanisamy, C.A. Maher, A.M. Chinnaiyan, Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer, Science 322 (2008) 1695–1699.

[21] J.M. Friedman, G. Liang, C.-C. Liu, E.M. Wolff, Y.C. Tsai, W. Ye, X. Zhou, P.A. Jones, The putative tumor suppressor microRNA-101 modulates the cancer epigenome by repressing the polycomb group protein EZH2, Cancer Research 69 (2009) 2623–2629.

[22] R.C. Friedman, K.K.-H. Farh, C.B. Burge, D.P. Bartel, Most mammalian mRNAs are conserved targets of microRNAs, Genome Research 19 (2009) 92–105.

[23] R.S. Pillai, MicroRNA function: multiple mechanisms for a tiny RNA?, RNA 11 (2005) 1753–1761

[24] R. Franco, O. Schoneveld, A.G. Georgakilas, M.I. Panayiotidis, Oxidative stress, DNA methylation and carcinogenesis, Cancer Letters 266 (2008) 6.

[25] E. Vire, C. Brenner, R. Deplus, L. Blanchon, M. Fraga, C. Didelot, L. Morey, A. Van Eynde, D. Bernard, J.-M. Vanderwinden, M. Bollen, M. Esteller, L. Di Croce, Y. de Launoit, F. Fuks, The polycomb group protein EZH2 directly controls DNA methylation, Nature 439 (2006) 871.

[26] N. Wagener, D. Holland, J. Bulkescher, I. Crnković-Mertens, K. Hoppe-Seyler, H. Zentgraf, M. Pritsch, S. Buse, J. Pfitzenmaier, A. Haferkamp, M. Hohenfellner, F. Hoppe-Seyler, The enhancer of zeste homolog 2 gene contributes to cell proliferation and apoptosis resistance in renal cell carcinoma cells, International Journal of Cancer 123 (2008) 1545–1550.

[27] M. Avissar-Whiting, D.C. Koestler, E.A. Houseman, B.C. Christensen, K.T. Kelsey, C.J. Marsit, Polycomb group genes are targets of aberrant DNA methylation in renal cell carcinoma, Epigenetics 6 (2011) 703–709.

[28] Y. Tomita, T. Nishiyama, M. Fujiwara, S. Sato, Immunohistochemical detection of major histocompatibility complex antigens and quantitative analysis of tumour-infiltrating mononuclear cells in renal cell cancer, British Journal of Cancer 62 (1990) 354–359.

[29] D. Sasaki, Y. Imaizumi, H. Hasegawa, A. Osaka, K. Tsukasaki, Y.L. Choi, H. Mano, V.E. Marquez, T. Hayashi, K. Yanagihara, Y. Moriwaki, Y. Miyazaki, S. Kamihira, Y. Yamada, Overexpression of enhancer of zeste homolog 2 with trimethylation of lysine 27 on histone H3 in adult T-cell leukemia/lymphoma as a target for epigenetic therapy, Haematologica 96 (2011) 712–719.

[30] M. Smits, S.E. Mir, R.J.A. Nilsson, P.M. van der Stoop, J.M. Niers, V.E. Marquez, J. Cloos, X.O. Breakefield, A.M. Krichevsky, D.P. Noske, B.A. Tannous, T. Würdinger, Down-regulation of miR-101 in endothelial cells promotes blood vessel formation through reduced repression of EZH2, PLoS ONE 6 (2011) e16282.