Pathol. Oncol. Res. (2008) 14:9–13
DOI 10.1007/s12253-008-9006-3

ORIGINAL PAPER

BMI-1 Expression is Inversely Correlated with the Grading of Renal Clear Cell Carcinoma

Nicolas Kozakowski · Afschin Soleiman · Johannes Pammer

Received: 26 October 2007 / Accepted: 3 December 2007 / Published online: 15 March 2008

Abstract BMI-1 regulates cell proliferation and differentiation, is involved in stem cell maintenance and can act as an oncogene. We investigated BMI-1 expression in healthy normal kidney and in 77 renal tumours by immunohistochemistry, and correlated it with tumour differentiation. BMI-1 could regularly be demonstrated in distal tubules and in Bowman's capsule, whereas it was mostly lacking in proximal tubules, indicating that it may rather be a differentiation marker of different renal cell populations than a stem cell marker. In contrast to previous studies demonstrating a correlation between BMI-1 expression and malignancy, we showed that its expression was inversely correlated with the differentiation grade of clear cell carcinoma. Furthermore, despite their different biologies, BMI-1 was strongly expressed in both papillary carcinomas and oncocytomas. Thus, in renal clear cell carcinomas BMI-1 is rather a differentiation marker lost in carcinomas with high malignancy than an oncogene involved in tumour progression.

Keywords BMI-1 · Renal cell carcinoma · Differentiation · Stem cell

Abbreviations

PcG	polycomb group
CCRCC	clear cell renal cell carcinoma
PRCC	papillary renal cell carcinoma
CRCC	chromophobe renal cell carcinoma

N. Kozakowski · A. Soleiman · J. Pammer (✉)
Institute of Clinical Pathology, Medical University of Vienna,
Währinger Gürtel 18-20,
1097 Vienna, Austria
e-mail: johannes.pammer@meduniwien.ac.at

Introduction

Genes of the Polycomb group (PcG) regulate cell differentiation and proliferation [1]. They are involved in oncogenesis [2, 3] and the maintenance of a variety of stem cells [4, 5]. One member of this family of genes is *BMI-1*, that has been extensively studied in fly and mouse models [6], in human development [7] and lymphoid diseases [5, 8]. In humans, *BMI-1* was initially shown to regulate haematopoiesis and differentiation of lymphocytes [7] and to be involved in cerebral development [9]. The BMI-1 protein, as well as other proteins from the PcG family, modify the three-dimensional structure of chromatin [10, 11] and thereby block the transcription of some genes such as $p16^{Ink4a}$ and $p19^{Arf}$, which are involved in tumour suppression, resulting in oncogenic effects [3]. Subsequently the proto-oncogene *BMI-1* has been shown to be upregulated in a large number of neoplasias, namely in lymphomas [12], cerebral tumours [9], breast cancer [13] and other epithelial tumours [14, 15] and to be an oncogene associated with poor prognosis in various tumours [8, 16].

The fact that renal cell tumours derive from epithelial cells of the nephron is commonly accepted. However, exact cell populations giving rise to the different subtypes of renal tumours are only incompletely known: clear cell renal cell carcinomas (CCRCC) are thought to arise from the epithelium of the proximal renal tubules and papillary renal cell carcinomas (PRCC) from the distal part of it, whereas chromophobe renal cell carcinomas (CRCC) may be derived from cells of the collecting duct, similar to oncocytomas [17, 18].

A variety of neoplasias may be derived from stem cells [19, 20]. Both a renal stem cell as well as a renal tumour stem cell [21–23] have already been proposed, but their existence has not yet been definitely confirmed. In the present study we have examined the expression of BMI-1

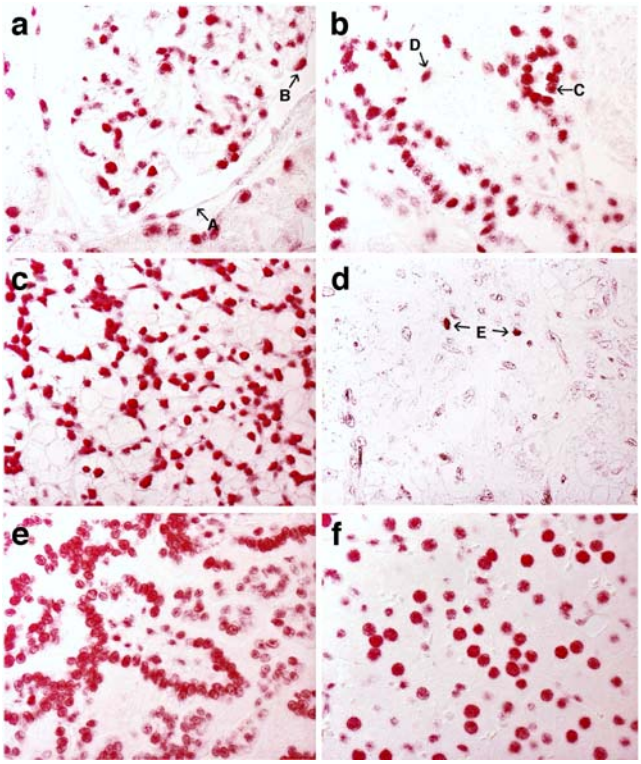

Fig. 1 Expression pattern of BMI-1in renal parenchyma and tumours. **a** BMI-1 is regularly expressed in mesangial cells and the capsule of the glomeruli (*A*: Bowman's capsule; *B*: podocyte) as well as **b** in tubules (*C*: distal tubule; *D*: proximal tubule). Whereas low grade clear cell carcinomas were typically distinctly positive for BMI-1 (**c**), poorly differentiated carcinomas were partly negative for BMI-1 (**d**, *E*: intratumoral lymphocytes). Papillary renal cell carcinomas (**e**) and oncocytomas (**f**) show a high expression of BMI-1 (alkaline phosphatase, original magnification ×400)

in healthy renal tissue and compared this expression with that in oncocytomas and a variety of renal cell carcinomas. In addition, we investigated the relationship between BMI-1 expression and tumour grade. Since *BMI-1* has already been linked to cancer stem cell regulation [4], we also looked for a potential renal cancer stem cell, which could conceivably be identified by means of its BMI-1 expression.

Materials and Methods

Tissue Samples

Formalin-fixed, paraffin embedded tissue of healthy renal tissue (n=77), CCRCC (n=40), PRCC (n=16, five type 1 and 11 type 2), CRCC (n=15) and oncocytomas (n=6) were derived from the files of the Institute of Clinical Pathology, Medical University of Vienna. RCC were graded according to Fuhrman [24]. PRCC were additionally graded considering the nucleolar grade [25]. A recent study argued that grading CRCC would not be appropriate [26]. However, no consensus for the grading of CRCC exists and therefore CRCC were graded like CCRCC.

Immunohistochemistry

Samples were immunostained for BMI-1 using monoclonal antibody clone 229F6 (Upstate Biotechnology, Lake Placid, NY). Antigen retrieval was performed by boiling sections in a microwave oven (600 W) in 1 mM EDTA pH 8.0 buffer for 20 min. Primary antibody was applied overnight at 1:300 dilution, followed by an APAAP kit (1:50, Dako, Glostrup, Denmark). The enzyme reaction was developed with an alkaline phosphatase substrate kit (Biogenex, San Ramon, CA). Negative controls were carried out on consecutive tissue sections using isotype-matched control reagents (IgG_1, Coulter, Hialeah, FL and IgG_1, PharMingen, San Diego, CA, 1:250). A semi-quantitative assessment of expression was performed independently by two clinical pathologists: no staining = 0; weakly positive = 1; moderately positive = 2; strongly positive = 3.

Western Blotting

Renal tubular cells HK2 and erythroleukaemia cells K562 (serving as a positive control) were grown to 80% confluence. Cells 1×10^6 were scraped and lysed in 500 µl reducing Laemmli sample buffer at 95°C for 5 min. A 5–15% gradient SDS-PAGE was performed using the Protean II electrophoresis system (BioRad, Richmond, CA). Proteins were transferred onto nitrocellulose (Schleicher&Schuell BioScience, Dassel, Germany). Immunoblotting was done with a monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) and a rabbit polyclonal antibody (IgG) raised against BMI-1 (Santa Cruz Biotech., Santa Cruz, CA, each one diluted 1:100), followed by alkaline phosphatase conjugated

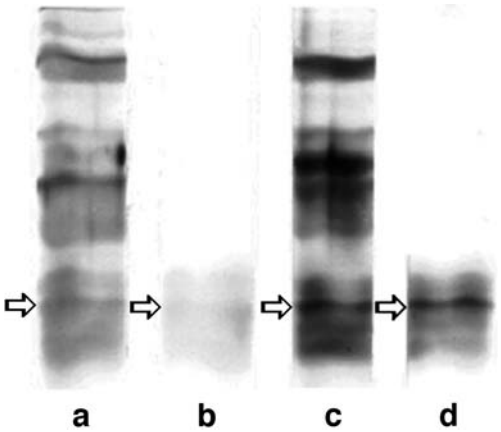

Fig. 2 Renal tubular cells HK2 are positive for BMI-1. Renal tubular cells HK2 (*lanes a* and *b*) and erythroleukaemia cells K562 (*lanes c* and *d*) express BMI-1 as assessed by Western blotting with polyclonal (*lanes a* and *c*) and monoclonal (*lanes b* and *d*) antibodies

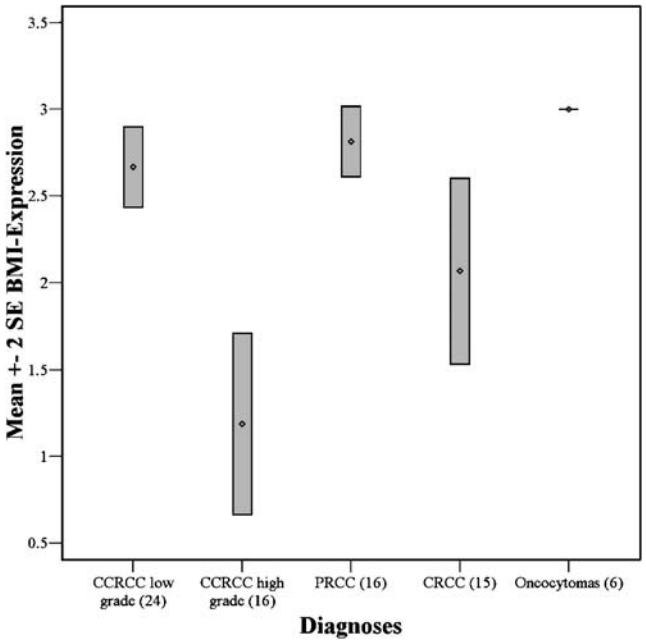

Fig. 3 BMI-1 expression is differently expressed in renal tumours. Whereas BMI-1 is distinctly expressed in low grade CCRCC, its expression is significantly lower in high grade carcinomas (Mann–Whitney: $p=0.007$). By comparison, both PRCC and oncocytomas express BMI-1 constitutively. *SE* standard error of mean; *open diamond*: mean value: 2.67 for low grade CCRCC, 1.19 for high grade CCRCC, 2.81 for PRCC, 2.07 for CRCC and 3 for oncocytomas. No staining = 0; weakly positive = 1; moderately positive = 2; strongly positive = 3

anti-rabbit Fc (diluted 1:7,500; Promega, Madison, WI) as a secondary antibody and Fast Red (ID Labs, London, ON, Canada) as a chromogen.

Statistics

The statistical significance of inter-group differences was evaluated by univariate analysis of variance (ANOVA) with subsequent Tukey-tests using SPSS 10.0.7 (SPSS Inc., Chicago, IL). As a control, Mann–Whitney *U* test and Spearman's rank correlation coefficient were performed for additional comparison of groups.

Results

BMI-1 is Regularly Expressed in Normal Renal Parenchyma

In normal renal tissue, BMI-1 was exclusively expressed in the nuclei of cells (Fig. 1). We could observe a homogeneous and moderate nuclear staining in mesangial cells (Fig. 1a) and a weak to moderate staining in cells of Bowman's capsule (Fig. 1a, A). Cells of proximal convoluted tubules showed a negative to weak staining (Fig. 1b, D), cells of Henle's loops only a focal moderate staining (not shown), distal tubule cells a strong homogeneous nuclear staining and cells of collecting ducts expressed BMI-1 heterogeneously and weakly (Fig. 1b, C). Endothelial cells were mostly negative for BMI-1 and vascular smooth muscle cells showed a homogenous moderate nuclear staining, whereas fibroblasts were variably positive. Lymphocytes stained moderately to strongly for BMI-1 and served as an internal positive control.

BMI-1 is Expressed in the Renal Tubular Cell Line HK2

In order to demonstrate the specificity of BMI-1 staining in renal tubular cells by immunohistochemistry, we performed Western blotting using the renal tubular cell line HK2. The specificity was demonstrated with both monoclonal and polyclonal antibodies. BMI-1 expression of HK2 cells expression was weaker than that of our control erythroleukaemia cells, but still distinct (Fig. 2).

BMI-1 Expression is Strong in PRCC and Oncocytomas Whereas it is Heterogeneous in CCRCC and CRCC

Similar to healthy renal tissue, BMI-1 staining of tumour cells was confined to nuclei and nucleoli generally stained stronger than the rest of the nucleus. The expression of BMI-1 was significantly different between all different types of renal tumours (ANOVA, $p=0.012$). Variable BMI-1 expression was found in CCRCC and in CRCC ranging from negative (four cases of CCRCC and one case of CRCC, score=0) to strongly positive (20 cases of CCRCC and five cases of CRCC, score=3; Fig. 3).

For statistical analysis, Fuhrman's grades 1 and 2 and also grades 3 and 4 were grouped together in a low- (grade 1+2) and a high-grade group (grade 3+4). The expression of BMI-1 was distinctly stronger in low grade (mean

Table 1 Statistical analysis

	BMI-1 expression (score)[a]			
	1	2	3	4
Clear cell renal cell carcinoma ($n=40$)[b]	4	6	10	20
Papillary renal cell carcinoma ($n=16$)[c]	0	0	1	15
Chromophobe renal cell carcinoma ($n=15$)	1	3	6	5
Oncocytoma ($n=6$)[d]	0	0	0	6

[a] BMI-1 expression is significantly different between various renal tumor types (ANOVA, $p=0.012$).
[b] BMI-1 expression is significantly lower in high grade carcinomas (Mann–Whitney: $p=0.007$).
[c] In addition, BMI-1 expression is significantly different between CCRCC and PRCC (Tukey test, $p=0.042$; Mann–Whitney: $p=0.012$)
[d] In oncocytomas BMI-1 is constantly highly expressed.

value=2.67) than in high grade (mean value=1.19) CCRCC (Fig. 3) and this difference was significant (Mann–Whitney: p=0.007). In addition, BMI-1 expression and Fuhrman's grade in CCRCC were inversely correlated (Spearman's p<0.001).

In contrast, oncocytomas and PRCC essentially stained strongly for BMI-1 (Table 1). Only one case of PRCC was moderately positive. The staining of most PRCC was homogeneous, whereas oncocytomas showed a strong but more heterogeneous staining pattern in various tumour regions. The constant strong staining for BMI-1 in PRCC was remarkable in comparison with the variable staining in CCRCC and CRCC. This difference was significant between PRCC and CCRCC (Mann–Whitney: p=0.012, Table 1). There was no significant difference of BMI-1 expression between type 1 and type 2 PRCC (Mann–Whitney: p=0.74).

Discussion

The pathogenesis of renal cell tumours has only been partially investigated and some authors have suggested the existence of renal cancer stem cells [21], similar to stem cells in leukaemia [5, 27] or solid tumours [15, 28] which were shown to be positive for BMI-1. Although the expression of BMI-1 in both normal renal parenchyma and renal cell carcinomas has already been demonstrated [29], no detailed study of the expression of this oncoprotein in different renal tumours has been performed yet.

BMI-1 is indispensable for stem cell maintenance in a variety of tissues. In the kidney, putative stem cells have been identified in proximal and distal tubules [30] or in Bowman's capsule [31], where they expressed CD133 and other stem cell markers. In our study a moderate homogenous staining for BMI-1 was observed in cells of Bowman's capsule, possibly indicating stem cell properties. In addition, no or very weak expression of BMI-1 was encountered in cells of proximal tubules, whereas distal tubules displayed a strong homogenous expression for BMI-1. Since BMI-1 was regularly and extensively expressed in several renal compartments, our data suggest that BMI-1 may rather be a differentiation marker of these compartments than a stem cell marker in the kidney.

We found BMI-1 to be dissimilarly expressed in different types of renal tumours. In contrast to other studies indicating association of BMI-1 upregulation with malignancy of tumours such as lymphoma, breast or prostate cancer [8, 13, 15, 16], our results show that BMI-1 expression is lost in highly malignant CCRCC as compared to low grade carcinomas, and its expression inversely correlated with Fuhrman's grading of renal cell cancer. Accordingly BMI-1 expression may be a sign of less malignant behaviour in CCRCC. Remarkably some tumours seem to express BMI-1 regularly: oncocytomas and PRCC show a characteristically strong expression. Since those two types of tumours exhibit differing biological characteristics, our data suggest that BMI-1 is rather an unspecific marker of renal tumours that is lost in CCRCC with low differentiation than an oncogene indicating high malignancy. Consequently, the widespread expression of BMI-1 did not enable the identification of putative renal cancer stem cells.

In summary, BMI-1 is distinctly expressed in mesangial cells and cells of the distal part of the tubules as detected by immunohistochemistry. It is also weakly and inconsistently expressed in other renal cells. In addition, this study shows variable expression of BMI-1 in benign and malignant tumours of the kidney. Benign oncocytomas and PRCC show constant strong staining for BMI-1, whereas poorly differentiated CCRCC show loss of BMI-1 staining. Moreover BMI-1 is inversely correlated with the grading in this tumour type. Thus BMI-1 is rather a differentiation marker for different cell types in renal parenchyma than a stem cell marker in these tissues, and does not seem to indicate a higher malignancy in CCRCC.

Acknowledgements We wish to thank Heidemarie Rossiter for critically reading the manuscript.

References

1. Jacobs JJ, Kieboom K, Marino S et al (1999) The oncogene and Polycomb-group gene BMI-1 regulates cell proliferation and senescence through the ink4a locus. Nature 397:164–168
2. Jacobs JJ, Scheijen B, Voncken JW et al (1999) BMI-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. Genes Dev 13:2678–2690
3. Kim JH, Yoon S, Kim CN et al (2004) The BMI-1 oncoprotein is overexpressed in human colorectal cancer and correlates with the reduced p16INK4a/p14ARF proteins. Cancer Lett 203:217–224
4. Liu S, Dontu G, Mantle ID et al (2006) Hedgehog signaling and BMI-1 regulate self-renewal of normal and malignant human mammary stem cells. Cancer Res 66:6063–6071
5. Raaphorst F (2003) Self-renewal of hematopoietic and leukemic stem cells: a central role for the Polycomb-group gene BMI-1. Trends Immunol 24:522–524
6. Muller J, Gaunt S, Lawrence PA (1995) Function of the Polycomb protein is conserved in mice and flies. Development 121:2847–2852
7. Lessard J, Schumacher A, Thorsteinsdottir U et al (1999) Functional antagonism of the Polycomb-Group genes eed and BMI1 in hemopoietic cell proliferation. Genes Dev 13:2691–2703
8. Van Kemenade FJ, Raaphorst FM, Blokzijl T et al (2001) Coexpression of BMI-1 and EZH2 polycomb-group proteins is associated with cycling cells and degree of malignancy in B-cell non-Hodgkin lymphoma. Blood 7:3896–3901
9. Leung C, Lingbeek M, Shakhova O et al (2004) BMI1 is essential for cerebellar development and is overexpressed in human medulloblastomas. Nature 428:337–341
10. Pirrotta V (1997) PcG complexes and chromatin silencing. Curr Opin Genet Dev 2:249–258

11. Saurin AJ, Shiels C, Williamson J et al (1998) The human Polycomb group complex associates with pericentromeric heterochromatin to form a novel nuclear domain. J Cell Biol 142:887–898
12. Bea S, Tort F, Pinyol M et al (2001) BMI-1 gene amplification and overexpression in hematological malignancies occur mainly in mantle cell lymphomas. Cancer Res 61:2409–2412
13. Kim JH, Yoon S, Jeong SH et al (2004) Overexpression of BMI-1 oncoprotein correlates with axillary lymph node metastases in invasive ductal breast cancer. Breast 13:383–388
14. Breuer RH, Snijders P, Sutedja GT et al (2005) Expression of the p16(INK4a) gene product, methylation of the p16(INK4a) promoter region and expression of the polycomb-group gene BMI-1 in squamous cell lung carcinoma and premalignant endobronchial lesions. Lung Cancer 48(3):299–306
15. Van Leenders GJ, Dukers D, Hessels D et al (2007) Polycomb-group oncogenes EZH2, BMI1, and RING1 are overexpressed in prostate cancer with adverse pathologic and clinical features. Eur Urol 52(2):455–63
16. Van Galen JC, Muris J, Oudejans JJ et al (2007) Expression of the polycomb-group gene BMI1 is related to an unfavourable prognosis in primary nodal DLBCL. J Clin Pathol 60:167–172
17. Cohen HT, Macgovern FJ (2005) Renal-cell carcinoma. N Engl J Med 353:2477–2490
18. Martel CL, Lara PN (2003) Renal cell carcinoma: current status and future directions. Crit Rev Oncol Hematol 45:177–190
19. Reya T, Morrison S, Clarke MF et al (2001) Stem cells, cancer, and cancer stem cells. Nature 414:105–111
20. Valk-Lingbeek ME, Bruggeman S, Van Lohuizen M (2004) Stem cells and cancer: the Polycomb connection. Cell 118:409–418
21. Al-Awqati Q, Oliver J (2002) Stem cells in the kidney. Kidney Int 61:387–395
22. Oliver JA, Maarouf O, Cheema FH et al (2004) The renal papilla is a niche for adult kidney stem cells. J Clin Invest 114:795–804
23. Gupta S, Verfaillie C, Chmielewski D et al (2006) Isolation and characterization of kidney-derived stem cells. J Am Soc Nephrol 17(11):3028–40
24. Fuhrman SA, Lasky L, Limas C (1982) Prognostic significance of morphologic parameters in renal cell carcinoma. Am J Surg Pathol 6:655–663
25. Sika-Paotonu D, Bethwaite P, McCredie MRE et al (2006) Nucleolar grade but not Fuhrman grade is applicable to papillary renal cell carcinoma. Am J Surg Pathol 30:1091–1096
26. Delahunt B, Sika-Paotonu D, Bethwaite PB et al (2007) Fuhrman grading is not appropriate for chromophobe renal cell carcinoma. Am J Surg Pathol 31:957–960
27. Lessard J, Sauvageau G (2003) BMI-1 determines the proliferative capacity of normal and leukaemic stem cells. Nature 423:255–260
28. Collins AT, Maitland NJ (2006) Prostate cancer stem cells. Eur J Cancer 42:1213–1218
29. Sanchez-Beato M, Sanchez E, Gonzalez-Carrero J et al (2006) Variability in the expression of polycomb proteins in different normal and tumoral tissues. A pilot study using tissue microarrays. Mod Pathol 19:684–694
30. Bussolati B, Bruno S, Grange C et al (2005) Isolation of renal progenitor cells from adult human kidney. Am J Pathol 166:545–555
31. Sagrinati C, Netti G, Mazzinghi B et al (2006) Isolation and characterization of multipotent progenitor cells from the Bowman's capsule of adult human kidneys. J Am Soc Nephrol 17: 2443–2456