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Original Article

Evaluation of the role of downregulation of SNF5/INI1 core subunit of SWI/SNF complex in clear cell renal cell carcinoma development

Elzbieta Sarnowska[1*], Michal Szymanski[2*], Nataliia Rusetska[1], Marcin Ligaj[3], Iga Jancewicz[1], Pawel Cwiek[4], Marta Skrodzka[5], Marcin Leszczynski[1], Joanna Szarkowska[1], Alicja Chrzan[3], Malgorzata Stachowiak[1], Jaroslaw Steciuk[4], Anna Maassen[4], Lech Galek[5], Tomasz Demkow[2], Janusz A Siedlecki[1*], Tomasz J Sarnowski[4*]

[1]Department of Molecular and Translational Oncology, M. Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland; [2]Department of Uro-oncology, M. Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland; [3]Department of Pathology, M. Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland; [4]Institute of Biochemistry and Biophysics Polish Academy of Sciences, Warsaw, Poland; [5]Department of Urology, Hospital of Ministry of Interior, Bialystok, Poland. [*]Equal contributors.

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Abstract: Clear cell renal cell carcinoma (ccRCC) is characterized by stabilization of hypoxia-inducible factor (HIF1), and mutations in *von Hippel-Lindau* (*VHL*) gene. Additionally, in about 40% of ccRCC cases the mutation in *PBRM1* (*POLYBROMO1*) gene coding for a non-core subunit of SWI/SNF chromatin remodeling complex was found suggesting potential impairment of this complex function in ccRCC. In this study we assessed the extent to which the core SWI/SNF complex subunit - INI1 (hSNF5/SMARCB1) is affected in ccRCC and whether it has any consequences on the development of this type of cancer. The evaluation of INI1 protein level in samples from 50 patients with diagnosed ccRCC, including three displaying rhabdoid features, showed the INI1 positive staining in rhabdoid cells while the conventional ccRCC cells exhibited reduced INI1 level. This indicated the rhabdoid component of ccRCC as distinct from other known rhabdoid tumors. The reduced INI1 protein level observed in all conventional ccRCC cases used in this study correlated with decreased *SMARCB1* gene expression at the transcript level. Consistently, the overexpression of INI1 protein in A498 ccRCC cell line resulted in the elevation of endogenous *SMARCB1* transcript level indicating that the INI1-dependent regulatory feedback loop controlling expression of this gene is affected in ccRCC Moreover, the set of INI1 target genes including i.e. CXCL12/CXCR7/CXCR4 chemokine axis was identified to be affected in ccRCC. In summary, we demonstrated that the inactivation of INI1 may be of high importance for ccRCC development and aggressiveness.

Keywords: Clear cell renal cell carcinoma (ccRCC), SWI/SNF CRC, SNF5/BAF47/INI1, SMARCB1

Introduction

Renal cell carcinoma (RCC) is the most common type of malignant adult kidney tumor [1]. Approximately 75% of RCC cases represents the clear cell renal cell carcinoma subtype (ccRCC) [2]. ccRCC is characterized by glycogen and fatty acid accummulation in tumour cells [3]. These metabolic changes are associated with stabilization of hypoxia-inducible factor (HIF1) and mutations in *von Hippel-Lindau* (*VHL*) gene which occur in about 90% of ccRCC tumors [4, 5]. Further study showed that about 40% of ccRCCs demonstrate mutations of *PBRM1* (*POLYBROMO1*) a gene coding for the non-core subunit of SWI/SNF chromatin remodeling complex (CRC) [6]. Therefore, the *PBRM1* is the second most frequently mutated gene in ccRCC after *VHL* [7].

About 30% of primary ccRCC patients develop metastases, and further 20 to 40% of patients show recurrence after cancer resection [8]. This type of cancer is additionally highly resistant to classical chemotherapy and shows modest response to targeted therapy, therefore understanding molecular mechanism of ccRCC tumorigenesis, progression and metastases

formation is vital and may lead to the development of new forms of therapy.

The occurrence of mutation in *PBRM1* gene in ccRCC suggests that in this type of cancer the function of the whole SWI/SNF chromatin remodeling complex may be affected, which is commonly observed for other cancer types. It has been reported that genes coding for SWI/SNF subunits are frequently mutated in various types of cancer including lung cancer, ovarian cancer, synovial sarcoma, breast cancer, melanoma, pancreatic cancer, Burkitt's lymphoma, etc. [9]. The SWI/SNF is an evolutionarily conserved multisubunit protein complex, involved in transcriptional control of gene expression in the ATP-dependent manner. In most tissue types this complex is composed of the functional core: SWI2/SNF2 type ATPase - BRM or BRG1, one SNF5-type subunit - the SNF5/BAF47/INI1 protein (called INI1 in this work) and two SWI3 type subunits BAF155 and BAF170 along with several non-core accessory subunits [10].

Two classes of SWI/SNF CRC exist in human cells: BAF and PBAF. Both BAF and PBAF are multisubunit molecular complexes which carry a set of common subunits and show differences in composition of their accessory subunits. The most important subunits distinguishing BAF and PBAF SWI/SNF CRCs are: BAF250a and b encoded by *ARID1A* and *ARID1B* genes (for BAF complex), and BAF180 encoded by *PBRM1* gene frequently mutated in ccRCC (for PBAF complex) [6, 9]. Lack of BRM - an ATPase subunit of SWI/SNF complex was found in poorly differentiated ccRCC [11], while the *ARID1A* gene coding for BAF250a SWI/SNF subunit has recently been described as new prognostic marker for ccRCC [12]. Biallelic loss of *SMARCB1* gene coding for INI1 was observed in pediatric malignant rhabdoid tumors (MRT) [13, 14]. Additionally, loss of INI1 was identified in epithelioid sarcoma, schwannomatosis, multiple meningiomas and renal medullary carcinoma [9, 15]. The available data on INI1 protein abundance in ccRCC is limited and contradictory. A case of rhabdoid RCC with minute focus ccRCC component featured by the loss of INI1 protein has been recently reported [16]. In another report Agaimy et al. [17] found loss of SWI/SNF core subunits other than INI1 in RCC with rhabdoid features, while INI1 protein level was mostly not altered.

In this study we investigated the expression of INI1 protein in 50 ccRCC samples. Three of them showed a rhabdoid component representing 10-20% of tumor tissue. Our analysis indicated that the INI1 protein level was not decreased in the rhabdoid cells of ccRCC with rhabdoid component. Simultaneously we found decreased level of INI1 in non-rhabdoid ccRCC cells when compared to normal kidney tube epithelial cells. Further investigation of the remaining 47 cases with conventional ccRCC indicated substantial decrease of INI1 protein in cancer cells. Moreover, the reanalysis of transcriptomic data available for ccRCC indicated significant correlation between disturbances in *SMARCB1* gene expression and poor prognosis. Comparative analysis of transcriptomic datasets of ccRCC samples available in public sources and datasets from genome-wide INI1 distribution study resulted in the identification of INI1-dependent genes with misregulated expression in ccRCC. Among them the subsequent Gene Ontology analysis identified genes belonging to carcinogenesis related GO terms. The overexpression of INI1 in A498 renal cell line resulted in the substantially decreased expression of *CXCR4* and *CXCR7* genes belonging to CXCL12/CXCR7/CXCR4 axis. Moreover, the A498 line overexpressing INI1 protein exhibited elevated expression of the endogenous *SMARCB1* transcript suggesting the impairment of INI1-dependent regulatory feedback loop controlling *SMARCB1* expression in ccRCC. Collectively, our findings suggest important role of INI1 in ccRCC development, progression and metastasis.

Materials and methods

Patients

We retrospectively analyzed formalin-fixed, paraffin-embedded as well as snap frozen samples from 50 patients with ccRCC diagnosed between January 2013 and December 2016 in two Polish hospitals: The Maria Sklodowska-Curie Memorial Cancer Center in Warsaw and Hospital of Ministry of Interior in Bialystok. All samples were re-reviewed by a pathologist for Fuhrman grade, tumor stage and the presence of additional histological components.

Tissue microarray and immunohistochemistry

Tissue array was prepared using Quick-Ray Tissue Microarrayer according to producer instruction. Hematoxylin and eosin stained slides from paraffin embedded ccRCC samples were used to identify ccRCC and normal kidney tissue, later submitted for tissue microarray.

Immunohistochemical analysis was performed on 4-μm tissue sections of paraffin embedded tumor and reference tissue using the EnVision FLEX+, Mouse, High pH Detection System (Dako, Glostrup, Denmark). Sections were deparaffinized with xylene and rehydrated in ethanol solutions. Heat-induced epitope retrieval was performed in Target Retrieval Solution (Dako) for 20 minutes at 96°C. After cooling, the slides were treated for 5 minutes with an endogenous peroxidase blocker (Dako), followed by incubation with monoclonal antibody against INI1 [monoclonal antibody SNF5 (D9C2), Cell Signalling Technology] for 30 minutes at room temperature, and then labeled with the EnVision FLEX+, Mouse, High pH Detection System (Dako). The color reaction product was developed with 3,3'-diaminobenzidine tetrahydrochloride (Dako) as a substrate. Nuclear contrast was achieved with hematoxylin counterstaining by 1 minute.

Protein isolation and western blot

Total protein extract was isolated from 30 mg of snap frozen ccRCC and normal kidney tissue samples using 8 M urea buffer. On each lane 30 μg of total protein extracts were loaded. Proteins were separated on 12% SDS-PAGE containing 0.5% TCE and visualized on UV to assess proper loading in each lane. Subsequently, separated proteins were transferred on PVDF membrane (Millipore). TBS with 5% BSA was used to block non-specific antibody binding. After blocking, membranes were incubated overnight at 4°C with anti-FLAG antibody (Cell Signaling Technology) or anti-INI1 antibody (Cell Signaling Technology) in 1:1000 dilution in TBS containing 5% BSA. Samples were then washed twice and incubated 60 minutes with anti-rabbit secondary antibody (Bio-Rad) diluted 1:10000. The signal was detected by WesternBright Quantum chemiluminescent detection kit (Advansta) using X-ray films (Amersham) or chemiluminescent documentation system (depending on the necessity).

RNA extraction and qRT-PCR

Total RNA was isolated from 30 mg of fresh frozen material or cell culture using RNA Isolation Kit (Qiagen) according to protocol. The reverse transcriptase reaction was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche). Expression of *SMARCB1* gene was measured by SybrGreen (BioRad) with *UBIQUITIN* as reference gene using following primers: 5'GACCAGGACAGGAACACGAG3', 5'CAAATGGAATGTGTGCCGG3' for S*MARCB1*, and 5'ATTTGGGTCGCGGTTCTTG3', 5'TGCCTTGACATTCTCGATGGT3' for UBI. Primers for CXCR4 was obtained from qPrimerDepot database [18] 5'CTTGTCCGTCATGCTTCTCA3', 5'CTTGTCCGTCATGCTTCTCA and for CXCR7 from Berahovich [19] 5'AGCACAGCCAGGAAGGCGAG3', 5'TCATAGCCTGTGGGTCTTCCG3'.

Cell culture and transfection

A498 human renal cancer cell line (obtained from ATCC) was grown in RPMI 1640 (Biowest) with 10% Fetal Bovine Serum (FBS) (Biowest) and 1% penicillin/streptomycin (Gibco) in humidifier incubator (37°C and 5% CO_2) according to manufacturer instruction.

Cloning and overexpression of INI1 protein in A498 cell line

cDNA coding for INI1 protein with FLAG-tag obtained from Addgene collection (Plasmid #1953) in vector pFastBac1 INI1 provided by Robert Kingston [20] was moved on *Eco*RI restriction site into pcDNA3.1 mammalian expression vector. A498 cell transfection was performed using TurboFect reagent (Thermo Fisher) according to protocol. Cells were collected 48 h post transfection. As mock control empty pcDNA3.1 was used.

Microarray analysis

The microarray datasets obtained from Gene Omnibus Database (GEO; GSE36895) from 29 ccRCC samples and 24 normal kidney cortex samples [21] were reanalyzed using GeneSpring GX software (Agilent) according to guided workflow with 1.45 fold change. All 24 normal kidney cortex samples were normalized and treated as normal, and all 29 ccRCC samples were normalized and treated as cancer sample.

Table 1. Clinical and histopathological characteristic of the patients. N/A, data not available

Characteristics	n (%)
Gender	
Female	19 (38%)
Male	31 (62%)
Age	
<60	16 (32%)
≥60	34 (68%)
Fuhrman grade[a]	
G1	6 (12.2%)
G2	22 (44.9%)
G3	18 (36.8%)
G4	3 (6.1%)
pT category[b]	
pT1	26 (53%)
pT2	4 (8.2%)
pT3	9 (18.4%)
pT4	1
NA	9 (18.4%)
Tumor max diameter (cm)	
<7	32 (64%)
≥7	18 (36%)
Type	
ccRCC	47 (94%)
ccRCC with rhabdoid features	3 (6%)
Metastasis	
N/A	20 (40%)
No metastasis	20 (40%)
Metastasis	10 (20%)
Female	2 (20% of total metastasis)
Male	8 (80% of total metastasis)

[a]based on Fuhrman grade classification; [b]according to pTNM classification.

Statistics

Statistical analysis was performed using MedCalc software and GraphPad Prism 5.0 by Shapiro-Wilk normality test, paired t-test, Mann-Whitney rank test for independent samples and multiple regression. *P* value <0.05 was considerated as statistically significant. Cox Proportional Hazard regression was used for estimation the relationship between the gene expression and survival rate using SurvExpress platform [22] and data obtained from GEO database GSE3538 containing additional clinical information: grade, performance status, gender, age and stage of the disease [23]. Two risk groups were generated using the prognostic index median. Log-rank test was used to evaluate the equality of survival curves [22].

Results

Clinical features of patients

The clinicopathological features of patients enrolled in this study are detailed in **Table 1**. A total of 50 cases were analyzed. Patients were 19 females and 31 males aged 34 to 83 years (mean, 69). There were statistical differences in the gender (male 62% and woman 38%) but no differences in distribution of the age in these groups. More than 80% of all patients had G2 and G3 Fuhrman grade tumors while 53% of studied cases were stage pT1 according to pTNM/AJCC. Three samples of ccRCC showed a rhabdoid component representing 10-20% of tumor tissue. Ten patients had metastatic disease at the time of diagnosis, 2 (20%) of them were female and 8 (80%) were male.

INI1 positive staining in rhabdoid cells in ccRCC with rhabdoid component

Typical rhabdoid tumors are INI1 negative due to mutation in *SMARCB1* - INI1 encoding gene [13, 14]. The case report published for rhabdoid RCC with minute ccRCC component [16] showed loss of INI1 protein in the rhabdoid cells while another report [17] showed that the loss of INI1 protein is rarely observed in RCC with rhabdoid component including ccRCC. With three ccRCC tumors with rhabdoid component in our study group we decided to assess INI1 protein level. Interestingly, we found specific INI1 positive staining in rhabdoid cells of all 3 cases (**Figure 1A**), which confirmed the suggestion of Kryvenko and collages [24] that rhabdoid RCC is different from malignant rhabdoid tumors. Observed INI1 staining was even stronger in rhabdoid cells compared to normal kidney proximal tube epithelial cells. Strikingly, we found significant decrease of INI1 protein abundance in conventional ccRCC cells. This observation was an indication that the rhabdoid component of ccRCC differs from conventional ccRCC cell also at the INI1 protein level.

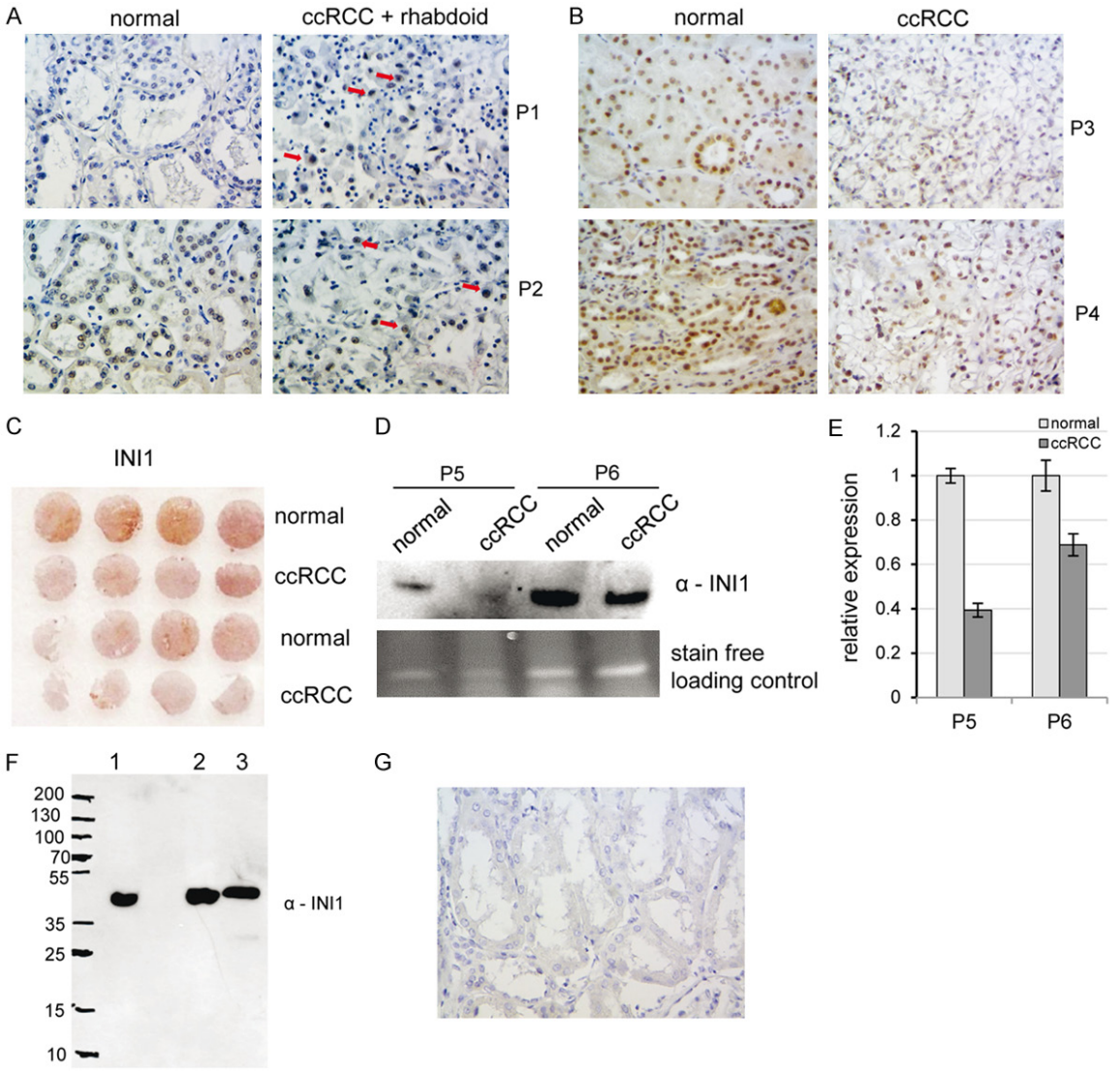


Figure 1. Downregulation of INI1 in ccRCC cells but not in rhabdoid cells in ccRCC with rhabdoid component. A. Immunohistochemistry for INI1 protein in ccRCC with rhabdoid features (10-20%), and corresponding normal kidney tissue from independent patients (P1 and P2), magnification 40×. Arrows indicated rhabdoid cells. B. INI1 immunostaining in ccRCC and corresponding normal kidney tissue from independent patients (P3 and P4), magnification 40×. C. Tissue microarray (TMA) from ccRCC and corresponding normal kidney sample. D. Western blot using anti-INI1 antibody in patients samples - ccRCC and normal kidney tissue. As a loading control membrane stained with TCE (stain free) was used. E. qRT-PCR measurement of the *SMARCB1* expression level on the same patient samples as in 1D. F. Western blot analysis using anti-INI1 antibody on three different human cancer cell lines serving as positive control. 1-MCF7, 2-MDA-MB231, 3-positive control provided by the manufacturer. G. Negative control for immunohistochemistry (without anti-INI1 antibody).

However, this finding did not provide information if the decrease of INI1 protein level in conventional component of ccRCC is a general feature for all ccRCC cases or just restricted to cases with rhabdoid features.

INI1 is downregulated in ccRCC cells

Given the observed decreased INI1 protein abundance in the conventional component of ccRCC with rhabdoid features, we decided to examine further 47 ccRCC cases for INI1 expression using immunostaining on paraffin embedded samples with INI1 specific antibody. We found significant decrease of INI1 staining in ccRCC cancer cells in comparison to normal kidney tube epithelial cells (**Figure 1B** and **1C**) which suggests the INI1 protein decrease may be a general feature of ccRCC. To confirm the IHC results the western blot analysis was per-

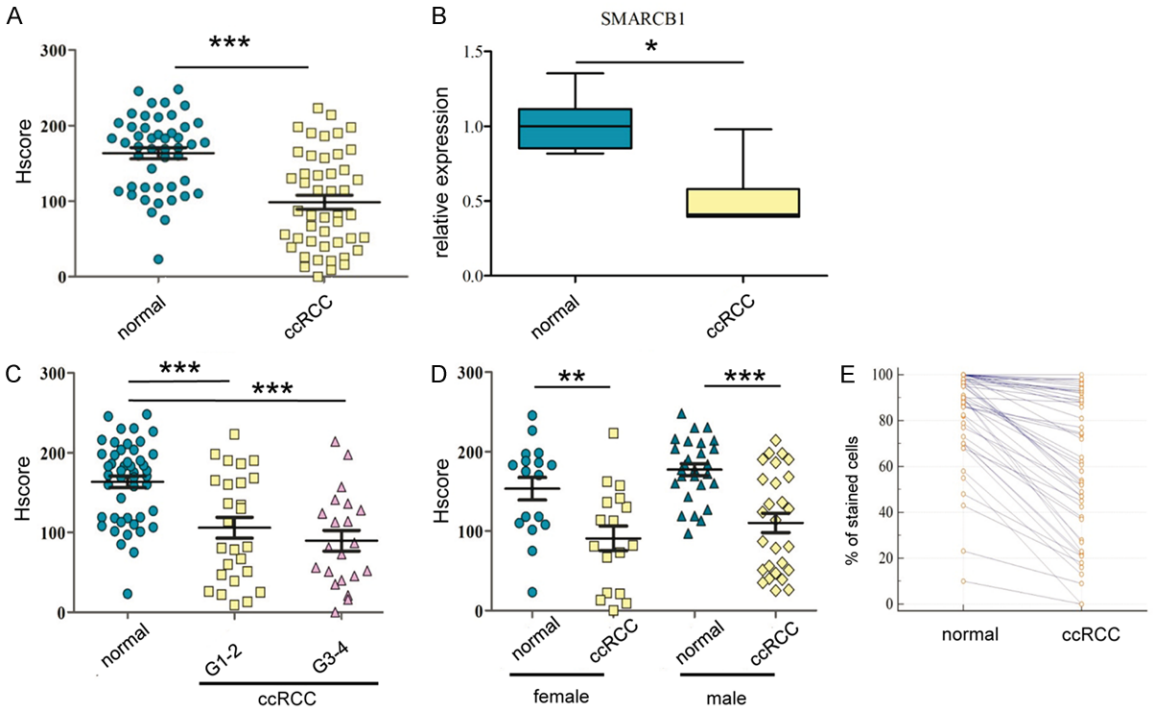


Figure 2. Association of INI1 abundance with clincopathological component. A. Hscore of INI1 stained cells diagram in ccRCC samples and corresponding normal kidney tissue. B. *SMARCB1* gene expression in ccRCC tumors and normal tissue as a control (*P* value <0.05). C. Hscore of INI1 stained cells in different Fuhrman grade (G1-2 and G3-4) of ccRCC samples vs normal kidney tissue. D. Hscore of INI1 stained cells in ccRCC samples and corresponding control according to gender (*P* value <0.001 for women and *P* value <0.0001 for men). E. Dot and line diagram of INI1 stained ccRCC cells and corresponding normal kidney epithelial tube cells.

formed on snap frozen ccRCC and corresponding normal kidney samples. This confirmatory analysis showed that indeed the INI1 is less abundant in cancer samples when compared to control (**Figure 1D**). This observation was fully consistent with the results of *SMARCB1* transcript level measurements in samples from the same patients as in **Figure 1D** using qRT-PCR (quantitative Real-Time PCR) method with *SMARCB1* specific primers (**Figure 1E**). As a reference, primers specific to the *UBI* (*UBIQUITIN*) cDNA were used. The specificity of IHC was confirmed by both negative and positive controls (**Figure 1F** and **1G**).

Given the above observation, we decided to assess the extent of INI1 loss in ccRCC cancer cells and calculated the percentage of stained cells combined with the intensity of staining (Hscore) [25] for tumor tissue and normal kidney samples. The Hscore calculation was performed on 1000 of both cancer and proximal tube epithelial cells. This analysis showed statistically significant decrease of INI1 staining ($p<0.0001$) in ccRCC samples comparing to normal kidney tissue, (**Figure 2A**), similar results were obtained when only percentage of INI1 stained cells was counted (data not shown). To verify identified potential correlation between the reduced INI1 protein abundance on western blot and *SMARCB1* transcription level (**Figure 1D** and **1E**) in ccRCC samples we measured *SMARCB1* (INI1) transcript level in a higher number of snap frozen ccRCC samples using qRT-PCR method with *SMARCB1* specific primers. Statistically significant downregulation ($p<0.05$) of *SMARCB1* mRNA was observed in cancer cells comparing to control samples (**Figure 2B**). This suggests that decrease of INI1 protein may indeed result from reduced transcription levels of *SMARCB1* gene in ccRCC, however the mutations inactivating *SMARCB1* have not been reported for ccRCC.

We then checked for potential correlation between INI1 staining and the ccRCC related features i.e. Fuhrman grade, tumor size, lymphocyte infiltration as well as other parameters

Table 2. Correlation between the INI1 staining and clinicopathological factors in ccRCC. The statistically significant value indicated by asterisk and bold (*P* value <0.05)

Factors	r	P	95% CI	
			Lower limit	Upper limit
Lymphocyte infiltration	0.2900	**0.0432***	0.009609	0.5282
Tumor size	0.04085	0.7805	-0.2431	0.3184
Fuhrman grade	-0.07826	0.5930	-0.3517	0.2075
Gender	0.1004	0.4927	-0.1861	0.3711
Age	-0.1825	0.2096	-0.4410	0.1041

*significant *P* value.

like age, gender, etc. We observed discrete differences in the extent of INI1 staining between ccRCC and corresponding control samples in Fuhrman grades G1-2 ($p<0.0001$) and G3-4 ($p<0.001$) groups (data not shown), when percentage of staining cells was calculated, although, no differences was observed in Hscore between G1-2 with G3-4 (**Figure 2C**). Weak differences in statistical significance were observed between males and females. In males the differences in INI1 staining between ccRCC cells and control samples were more statistically significant ($p<0.0001$) than in females group ($p<0.001$) (**Figure 2D**). The comparative analysis of cancer samples and corresponding normal kidney epithelial cells indicated decreased level of INI1 in all pairs of examined samples (**Figure 2E**).

Interestingly, the correlation coefficient analysis showed statistically significant correlation between higher INI1 protein level in ccRCC cells and lymphocyte infiltration of the tumor, but for other factors like tumor size, grade, age and gender the INI1 level did not correlate (**Table 2**). Similarly, the multivariate analysis of all factors revealed statistically significant correlation between INI1 protein level in ccRCC and lymphocyte infiltration. This data strongly suggest the involvement of INI1 protein and most likely the whole SWI/SNF complex in ccRCC development.

Disturbances in SMARCB1 expression contribute to poor survival

Our study of ccRCC samples showed decrease of INI1 protein abundance as well as *SMARCB1* gene expression downregulation in tumor cells. Therefore, we decided to check if the *SMARCB1* transcript level in tumor samples correlates with survival rate of ccRCC patients. Therefore we performed Cox proportional hazard regression on independent 177 ccRCC patients cohort (GSE3538) [23] according to performance status, gender, grade and stage of the disease. The statistically significant associations were found for *SMARCB1* gene expression for examined cohort (HR=1.91 [95% C.I 1.24-2.93], p=0.003303), (**Figure 3A**) and grade 3 (HR=2.2 [95% C.I 1.19-4.08], p=0.01245) (**Figure 3B**), however no differences were found for grade 2 and 4. Interestingly, most significant differences in survival were found for *SMARCB1* according to performance status 1 (HR=4.65 [95% C.I 2.21-9.77], p=4.944e-05) (**Figure 3C**) but no significant associations were found for performance status 0, 2 and 3. Moreover, this study also indicated that *SMARCB1* expression level may have a predictive value only in male patients (HR=2.19 [95% C.I 1.18-4.05], p=0.0126) but not in female (**Figure 3D** and **3E**). This observation together with recently reported important role of androgen receptor in ccRCC development and progression [26] as well as direct interactions of SWI/SNF complexes with androgen receptor may help address the question why ccRCC most commonly affects male patients [27]. Consistently with the observed variabilities in the *SMARCB1* expression level, in our IHC study we also found distinct intensity of the INI1 staining among all analyzed ccRCC samples (**Figure 3F**) further confirming the importance of INI1in ccRCC.

INI1 controls genes involved in cancer progression and metastasis

Given the observation that the disturbances in the *SMARCB1* expression contribute to poor survival we decided to check the extent to which transcriptomic alteration in ccRCC are caused by observed INI1 alterations. Comparative analysis of ChIP seq data for INI1 direct target genes (i.e. INI1 protein has been found on their *loci* [28]) and genes with altered expression in ccRCC (GE036895) [21] resulted in identification of 849 common genes (Supplementary File 1). Among INI1 target genes with altered expression 478 were upregulated in ccRCC in this analysis (**Figure 4A**). The Gene Ontology analysis identified class of

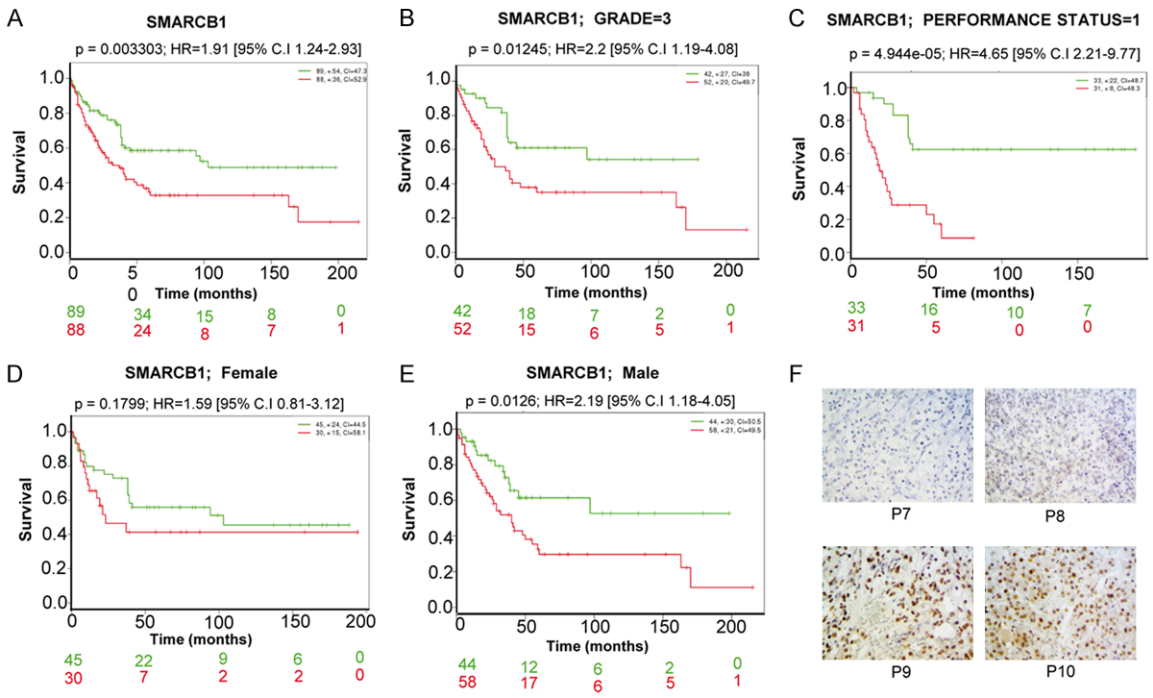
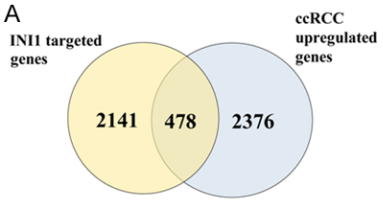


Figure 3. Kaplan-Meier survival curves for ccRCC patient according to high and low *SMARCB1* expression. A. Survival analysis using Cox proportional hazard model for 177 patients with diagnosed ccRCC. B. Fuhrman grade. C. Performance status. D and E. Gender. F. Examples of low (P7 and P8) or high (P9 and P10) intensity of IHC-staining for INI1 in ccRCC samples used in this study. The green and red color lines indicated patients with low and high risk. Numbers of patients in each group and log-rank *p* values, hazard ratio (HR) and confidence intervals (CI) are shown below the graph.



B

Gene Ontology term	Pvalue	Genes annotation
Programmed cell death	2.23e-05	SERPINB9, FIGNL1, CRIP1, TNFAIP8, GCLM, ARHGEF6, IFI16, PN MA2, ANO6, TNFRSF10B, ERN1, JUN, DIP2A, PDK1, CDCA7, NCK2 , CEBPB, ANGPTL4, VEGFB, CXCR7, NSMAF, DDIT4, TMEM214, I D3, DUSP1, JAG2, PERP, PYCARD
Cell proliferation	1.87e-07	N4BP2L2, FIGNL1, CEP120, CRIP1, SOX11, JARID2, IFI16, MARCK SL1, S100A11, TNFRSF10B, PELI1, EFNB2, ERN1, JUN, PDK1, CDC A7, NCK2, CEBPB, TYMS, VEGFB, ID2, DDIT4, JAK1, FABP6, ADA RB1, CD1D, KIAA0922, BTG3, BST2, JAG2, PYCARD
Blood vessel development	0.00047	MESP1, LOX, ANGPTL4, JMJD6, COL4A1, VEGFB, CXCR7, WASF2, EFNA1, COL4A2, JAK1, FAM105B, EFNB2, JUN
Cell motility	0.00190	MESP1, PREX1, ANO6, EFNB2, JUN, NCK2, PLXNA3, VEGFB, WAS F2, TMEFF2, DDIT4, EFNA1, JAK1, ADARB1, SIRPA, BST2, JAG2, R CC2, CXCL16, PYCARD

D

Gene Ontology term	Pvalue	Genes annotation
Cell differentiation	2.25e-06	HOXD1, BHLHB9, IL11, FST, CA2, BIN1, CACNA2D2, CXADR, CTGF, SMAD6, NEFH, TUBB2B, EPB41L5, RAB7L1, DYNLL2, CYB5D2, EGR1, PRDX3, PLEKHB1, MAL, DSP, N4BP2L2, NAGLU, BEX1, CXCL12, MYCN, LGR4, DAB2, KLF15, TOB1, NTNG1, NTN4, PPP1R16B, GATA5, CLASP2, UST, TGFBR3
Epithelium development	0.00061	PSMA5, DSP, MYCN, GPC4, FST, CA2, IRX2, LGR4, SMAD6, DAB2, KLF15, NTN4, PPP1R16B, EPB41L5, GATA5, CLASP2
Response to chemical	0.00766	PSMA5, AGL, CGN, CA2, GNG12, POLR2L, CXADR, CTGF, SMAD6, NEFH, CDO1, ATP5G3, EPB41L5, BCAR3, PINK1, EGR1, PRDX3, NDUFA10, SESN2, SSTR2, CXCL12, SLC31A1, DAB2, KLF15, TOB1, NTN4, RASL11B, GGH, QDPR, GATA5, PDE3B, GCH1, SH3BP4, TGFBR3
Morphogenesis of an epithelium	0.00983	PSMA5, NTN4, MYCN, GPC4, CA2, EPB41L5, IRX2, CLASP2, LGR4, DAB2

Figure 4. Set of genes misregulated in ccRCC and identified as direct INI1 target genes. A. Venn diagram of INI1 target genes and genes upregulated in ccRCC samples. B. Selected Gene Ontology terms enriched in genes upregulated in ccRCC and INI1 target. C. Venn diagram of INI1 target genes and genes downregulated in ccRCC. D. Selected Gene Ontology terms enriched in genes downregulated in ccRCC and INI1 target.

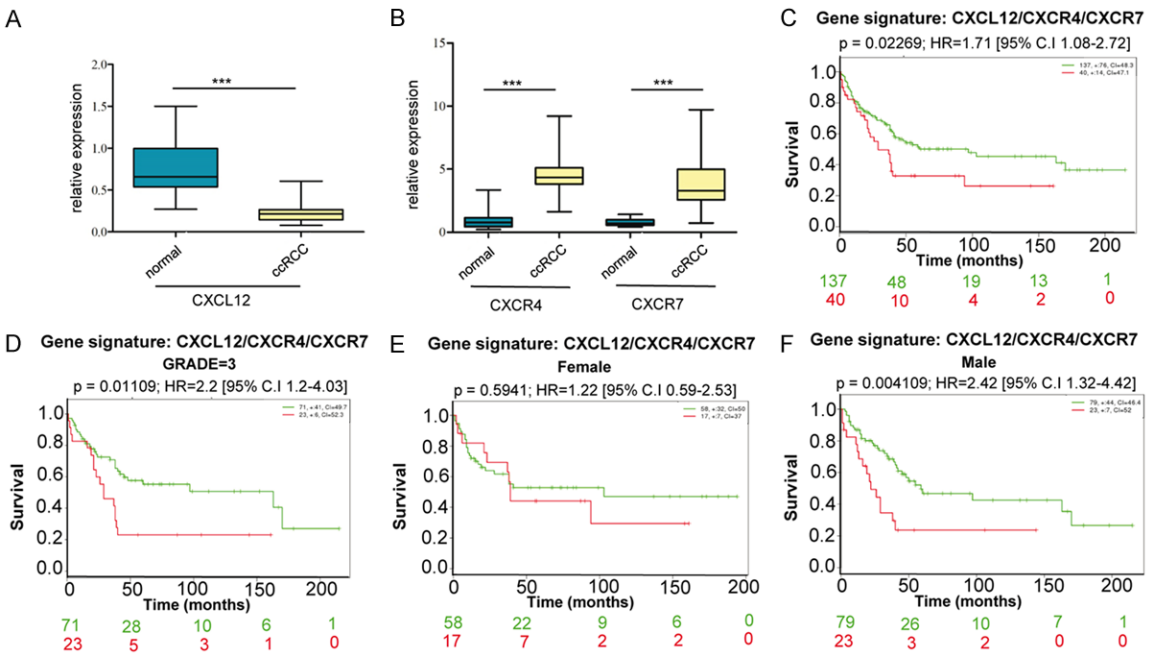


Figure 5. CXCL12/CXCR4/CXCR7 axis in ccRCC samples. A. CXCL12. B. CXCR4 and CXCR7 expression levels in ccRCC patients samples compared to normal kidney tissue. C. Survival analysis using Cox proportional hazard model for 177 patients with diagnosed ccRCC depending on *CXCL12/CXCR4/CXCR7* genes expression. D. Fuhrman grade. E, F. Gender. The green and red color lines indicated patients with low and high risk, respectively. Below the graph are shown numbers of patients in each group and log-rank *p* values, hazard ratio (HR) and confidence intervals (CI).

genes involved in various cellular processes including these related to cancer progression and metastasis formation such as: cell motility, blood vessels development, cell proliferation and programmed cell death (**Figure 4B**). Using Gene Ontology analysis we found that among 371 genes downregulated in ccRCC and targeted by INI1 (**Figure 4C**) the following GO terms were enriched: cell differentiation, epithelium development, morphogenesis of epithelium, response to chemical (**Figure 4D**). Detailed analysis for genes directly targeted by INI1 with misregulated expression in ccRCC resulted in identification of genes encoding CXCL12 and CXCR7 proteins, which are involved in metastases formation in various cancers by CXCL12/CXCR4/CXCR7 axis [29] (**Figure 5A** and **5B**). Additionally, ccRCC is also characterized by the misexpression of *CXCR4*, however this gene was not found as INI1 direct target gene (**Figure 5B**). The Cox proportional hazard regression analysis showed that the *CXCL12/CXCR4/CXCR7* axis transcript alterations correlate with poor prognosis (survival) for ccRCC grade 3 and male patients (**Figure 5C-F**). This indicates that INI1 may be directly involved in cancer progression and metastases formation. Subsequently, we found that overexpression of INI1 protein in A498 renal cancer cells (**Figure 6A** and **6B**) caused downregulated expression of *CXCR4* and *CXCR7* genes (**Figure 6C**), which provides evidence that INI1 loss observed in ccRCC may promote cancer metastases formation by direct regulation of CXCL12/CXCR4/CXCR7 axis in this type of cancer (**Figure 6D**). Moreover, we found that the overexpression of INI1 in A498 line caused elevated expression of *SMARCB1* gene which has already been reported as the direct target for the INI1 protein [28]. This suggests INI1-dependent regulation of *SMARCB1* gene.

Discussion

Clear cell renal cell carcinoma is the most common renal cancer in adults. When limited to kidney it can be successfully cured by surgery. Around 30% of patients relapse after nephrectomy or has metastatic disease at the time of diagnosis [30] which corresponds to poor prognosis. Therefore, the understanding of mechanism of ccRCC development and metastases

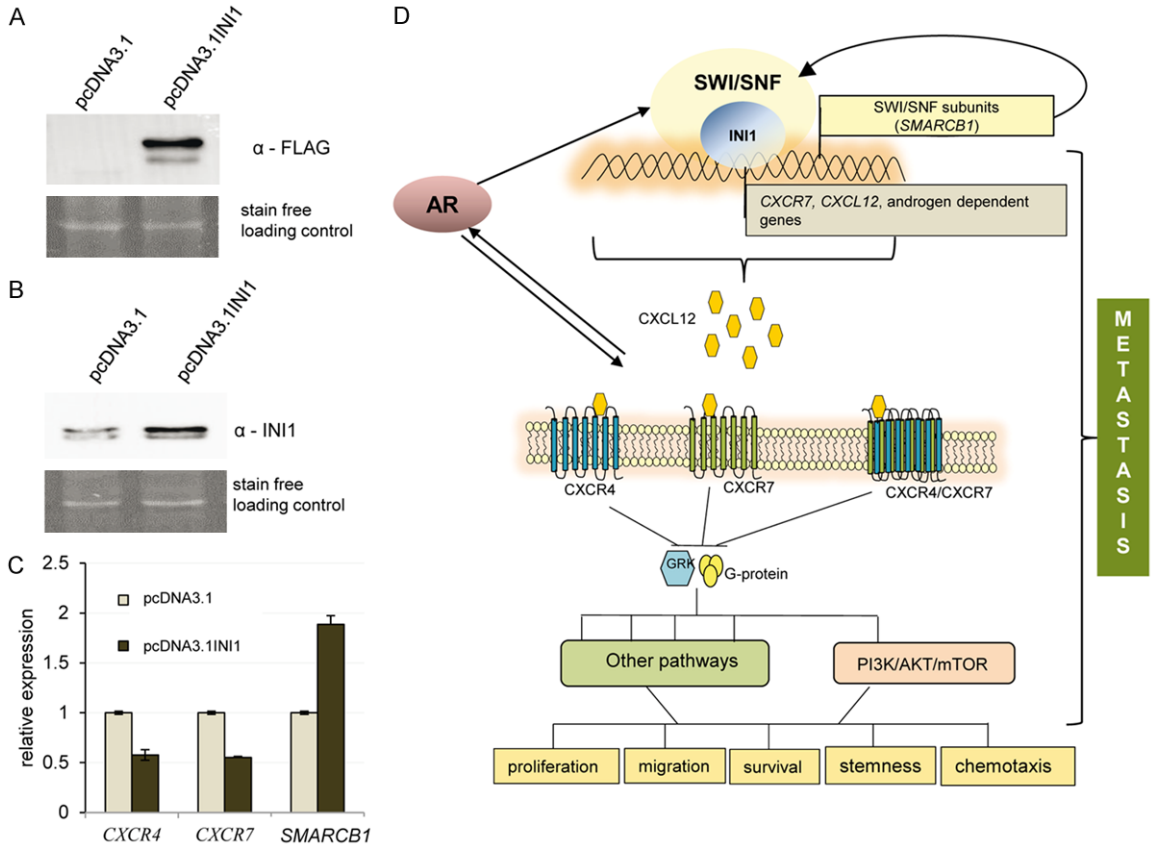


Figure 6. INI1 influences CXCL12/CXCR4/CXCR7 chemokine axis. A. INI1-FLAG overexpression in A498 cells, which correspond with ccRCC grade 2. As a loading control membrane stained with TCE (stain free) was used. B. Western blot with anti-INI1 antibody demonstrating increase of INI1 abundance in A498 cell line overexpressing INI1 protein. As a loading control membrane stained with TCE (stain free) was used. C. qRT-PCR analysis for *CXCR4*, *CXCR7* and *SMARCB1* (endogenous transcript) genes expression in A498 cell lines overexpressing INI1 protein. D. Model describing self-regulation of the *SMARCB1* gene by INI1 protein and the interdependence of INI1, AR pathway and CXCL12/CXCR4/CXCR7 axis.

formation is very important and potentially may lead to development of new forms of therapy. The TCGA study [31] as well as some other reports provided evidence that the aberrations of the SWI/SNF chromatin remodeling complex (CRC) functions may play important role in ccRCC.

In this study we show, that INI1 - the core subunit of SWI/SNF chromatin remodeling complex may play an important role in ccRCC development. INI1 has been already reported as a tumor suppressor [32]. Mutations inactivating INI1 were found in various types of cancer in adults as well as in highly malignant pediatric rhabdoid tumors (MRT) [13, 14, 33-35]. In mouse model, the INI1 deficient xenografts resembles these observed in MRT, which corresponds with rhabdoid phenotype [36]. Moreover, the INI1 loss was detected in rhabdoid tumor with minute focus of clear cell renal cell carcinoma in 65-year-old patient [16]. On the other hand, the kidney MRT in children is recognized as high-grade malignancy composed completely of rhabdoid cells [37]. In contrast, another report shows that in most ccRCC with rhabdoid component the INI1 protein level was intact [17]. To clarify this contradiction we analyzed the INI1 protein level in three cases of ccRCC with rhabdoid component. All three cases showed strong INI1 staining and high-grade differentiation (Fuhrman grade 4). This observation is in line with Kryvenko statement that rhabdoid component of ccRCC is probably not a distinct type of cancer but rather dedifferentiated form of high-grade ccRCC, not related to pediatric rhabdoid tumors [24]. This conclusion is further supported by the study of

Agaimy and colleagues, who analyzed 32 cases of undifferentiated and rhabdoid RCC and found that 4 were INI1 negative, 10 showed reduced INI1 and 18 exhibited intact INI1 protein level [17].

Interestingly, in contrast to the rhabdoid component of ccRCC, statistically significant downregulation of INI1 protein in conventional ccRCC cells was found in our study. Moreover, we found the decrease of INI1 protein level in all ccRCC cases included in this study. We also checked the relationships between INI1 protein and mRNA in some of ccRCC cases and found that the decrease of protein level correlated with significantly lower *SMARCB1* transcript level in ccRCC cancer cells when compared to normal kidney tube epithelial cells. The decrease of INI1 protein level was independent of Fuhrman grade, but discrete differences in statistical significance were found between grade 1-2 and 3-4 and gender. Using Cox proportional hazard model analysis we found that the higher expression level of *SMARCB1* in the tumor correlates with poor prognosis in the male group, however there was no correlation in female patients. This analysis also showed correlation with poor prognosis for patients with Fuhrman grade 3 interestingly the strongest correlation was found for patients in relative good condition.

Correlation between higher expression of *SMARCB1* gene in some ccRCC tumor samples (however still decreased when compared to the control tissue) and poor prognosis for male patients may be likely explained by the involvement of SWI/SNF CRC in androgen biosynthesis pathway according to ENCODE database [38, 39] and regulation of androgen dependent genes by direct interacting with androgen receptor (AR) [40], and AR association with higher tumor stage and promotion of ccRCC migration and invasion [26]. However further confirmatory study is required.

Our comparative analysis of INI1 target genes and genes misregulated in ccRCC samples followed by the Gene Ontology classification resulted in identification of GO terms statistically enriched for INI1-target genes upregulated in ccRCC, related to cancer progression and metastasis formation. They were: programmed cell death e.g. PDK1 (3-phosphoinositide-dependent protein kinase-1) which suppresses apoptosis when overactive [41]; cell proliferation, blood vessels development, leukocyte activation and cell motility. GO terms statistically enriched for INI1-target genes downregulated in ccRCC were related to cell differentiation, epithelium development and morphogenesis, and response to chemicals. Close inspection of the INI1-target genes misregulated in ccRCC resulted in identification of *CXCR7* (upregulated) and *CXCL12* (downregulated) both belonging to the CXCR4/CXCR7/CXCL12 chemokine axis, well known regulators of tumor growth and metastasis formation in many types of cancer [29]. The Cox analysis showed that affected expression of these genes correlates with poor prognosis for ccRCC patients. Noteworthy, this correlation was significant for ccRCC Fuhrman grade 3 and male group only, analogous to what was observed for *SMARCB1* expression. It has already been reported that CXCR4/CXCR7/CXCL12 axis and AR interplay to promote cancer progression and metastasis formation, as CXCL12/CXCR4 promote ligand independent activation of androgen receptor [42]. AR together with CXCR4 and CXCR7 regulate the CXCL12 dependent cell motility in prostate cancer [43]. Consistently, the overexpression of INI1 in A498 cancer cells which corresponds to clear cell renal cell carcinoma grade 2, caused downregulated expression of *CXCR4* and *CXCR7* genes. Our observation regarding the INI1-dependent *CXCR4* and *CXCR7* regulation is in line with the recently reported strong effect of INI1 reexpression in MRT cell lines, where the restored expression of INI1 protein induced G_1 cell cycle arrest and lead to the modification of p21 or p16 *loci* expression [44]. In summary, our data strongly suggest close relation of INI1 and ccRCC development, tumor progression and metastases formation. So far the knowledge about transcriptional control of *SMARCB1* gene expression is strongly limited, however there are some evidences indicating that among other mechanisms the SWI/SNF complex may be directly involved in this process [28, 45]. In fact, during our study we found that the INI1 overexpression in A498 cell line indeed caused elevated expression of the *SMARCB1* gene. This finding clearly indicated that the alterations of self-control of *SMARCB1* expression by the INI1 (and likely INI1-containing SWI/SNF complex) may be one of the possible explanations of observed INI1 loss in ccRCC. On the other hand,

we can't exclude the existence of other mechanisms controlling *SMARCB1* expression level like i.e. the SWI/SNF and Polycomb (PcG, Polycomb Repressive Group) antagonism (SWI/SNF complex has been reported as the important factor in the eviction of Polycomb proteins [46]) which may result in altered DNA or histone methylation on *SMARCB1* promoter or the existence of a long non-coding RNA which may interfere with *SMARCB1* transcript, or other unrecognized yet mechanisms. However, the complete elucidation of the mechanism underlying decreased expression of *SMARCB1* gene in ccRCC requires further examination.

Proper SWI/SNF complex function usually requires INI1 protein [10, 47, 48, 49]. However there is emerging evidence that in some tumors the SWI/SNF complex activity may act independently of INI1 [50] and INI1 protein deficiency in cancer cells redefines the SWI/SNF subunits composition [51].

Alteration in stoichiometry of complex composition is likely to lead to loss of proper SWI/SNF function or gaining of a novel, cancer related function, which may cause dramatic consequences in global gene expression by transcriptional regulation, epigenetic and higher order chromatin structure disturbances. It has been already shown, that during pancreatic cancer development the BRG1 ATPase (another core subunit of SWI/SNF CRC) acts as either tumor suppressor or oncogene depending on stage of the disease [52]. This may explain the correlation between higher (although decreased when compared to control kidney tissue) *SMARCB1* gene expression and poor prognosis observed in our study. Additionally, the identified correlation of stronger INI1 staining in ccRCC cells with lymphocyte infiltration supports this hypothesis as consistently immune infiltration in ccRCC associates with poor prognosis in male patients, higher tumor grade and stage [53]. Taken together our findings support the hypothesis that downregulation of INI1, the core subunit of SWI/SNF CRC may occur on a very early stage of carcinogenesis, and can promote ccRCC development and poor prognosis. On the other hand, as the ccRCC with rhabdoid component shows higher aggressiveness, poor prognosis and usually intact INI1 protein level, the involvement of INI1 in cancer progression and metastases still requires further careful investigation.

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Disclosure of conflict of interest

None.

Abbreviations

ccRCC, clear cell renal cell carcinoma; SWI/SNF, Switch/Sucrose Non Fermentable; CRC, Chromatin Remodeling Complex; AR, Androgen Receptor; GEO, Gene Expression Omnibus database; CXCR4 and CXCR7, chemokine receptors; CXCL12, chemokine.

Address correspondence to: Tomasz J Sarnowski, Institute of Biochemistry and Biophysics Polish Academy of Sciences, Pawinskiego 5A, 02-106 Warsaw, Poland. Tel: +48 22 5925781; Fax: +48-226584636; E-mail: tsarn@ibb.waw.pl; Janusz A Siedlecki, Department of Molecular and Translational Oncology, M. Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Roentgena 5 Str., 02-781 Warsaw, Poland. Tel: +48225422093; Fax: +48225422093; E-mail: jas@coi.pl

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