Expression of the Sonic Hedgehog pathway components in clear cell renal cell carcinoma

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Abstract. Clear cell renal cell carcinoma (ccRCC) is the most common and the most aggressive histopathological subtype of kidney cancer, with patients exhibiting high mortality rates for metastatic tumors. The Sonic Hedgehog (SHH) pathway serves a crucial role in embryonic development. The abnormal activity of SHH signaling is observed in a broad range of malignancies. However, its role in ccRCC is still undetermined. The aim of the present study was to assess the expression of the SHH pathway genes in ccRCC. Neoplastic and morphologically unchanged kidney tissues were obtained during radical nephrectomy from 37 patients with ccRCC. The *SHH, PTCH1, SMO* and *GLI1* mRNA levels were assessed using the reverse transcription-quantitative PCR. Western blot analysis was used to assess the full-length and C-terminal SHH protein level. The mRNA levels of *SHH, SMO* and *GLI1* were approximately 2-, 2,5- and 7-fold higher in ccRCC tissue compared with control kidney tissue, respectively. Correlational analysis between the mRNA levels of SHH pathway genes and patients' clinicopathological factors revealed decreased and increased mRNA levels of *PTCH1* and *SMO* respectively, in tumor samples derived from older patients (age >62). Furthermore, the level of C-terminal SHH protein in ccRCC samples was significantly lower in a group of males compared with females. No correlation was exhibited between molecular data and patient survival. Western blot analysis indicated a ~3-fold higher level of SHH full-length protein, and a 4-fold lower level of the C-terminal SHH protein domain, in ccRCC tumor tissues compared with normal kidney samples. The current study indicated an involvement of the SHH pathway in ccRCC development.

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Introduction

Kidney cancers are among the 10 most frequently diagnosed malignancies worldwide (1). Clear cell renal cell carcinoma (ccRCC) represents the most common as well as the most aggressive histopathological subtype (2). The 5-year survival rate for systematically spread kidney cancers is approximately 12% (3). Therefore, there is an urgent need for research which may establish new molecular targets responsible for ccRCC initiation and progression (4).

The Sonic Hedgehog (SHH) pathway plays an important role during embryogenesis and in the maintenance of tissue homeostasis during postnatal life (5-7). SHH full-length protein (424aa) is cleaved intracellularly (8) to provide two biologically active products. C-terminal SHH protein (227aa) acts as an autoprocessing domain, while N-terminal SHH protein (174aa) is secreted and may act as a ligand either via auto- or paracrine signaling (9). The binding of N-terminal SHH molecule to the Patched-1 (PTCH1) cell membrane receptor initiates intracellular signal transduction through Smoothened (SMO) co-receptor and GLI zinc finger proteins, which, acting as transcription factors, activate transcription of several target genes, e.g. *MYCN, bcl2* or *VEGF* (10).

Aberrant expression of *SHH*, *PTCH1*, *SMO* and *GLI1* genes associated with cancer progression and patients survival has been reported in a broad range of human malignancies such as basal cell carcinoma (11), breast cancer (12) and other neoplasms (13,14). However, the results of SHH pathway genes expression, both at the mRNA as well as protein level, in ccRCC human tissues are contradictory (15-17).

Therefore, we decided to perform the analysis of the expression of SHH pathway genes at the mRNA level in ccRCC tumor and paired unchanged kidney tissue. Moreover, we assessed the level of full-length SHH protein as well as the C-terminal SHH domain protein in kidney tumor lysates by western blot method. The results were statistically analyzed in terms of clinicopathological features of ccRCC patients and their overall survival (OS).

Materials and methods

Patients and samples. The ccRCC tumor tissue and morphologically unchanged kidney samples were obtained during radical nephrectomy from 37 patients operated in the Department of Urology, Medical University of Gdańsk, Poland. The exclusion criteria for the study were: diagnosis of VHL disease, multifocal or/and bilateral kidney tumors, other than ccRCC histological subtypes of RCC. The study was approved by the local Ethics Committee (decision no. NKEBN/4/2011 and NKBBN/370/2016); written consent was acquired before the surgery from each patient. The clinicopathological features of the patients were presented in Table I.

Material acquisition. Small (ca. 7x2, 7x2, 7x2 mm) pieces of ccRCC and morphologically unchanged tissues (resected from at least 2 cm from the tumor) (18) were placed into test tubes in the operating theater, no longer than 20 min after kidney resection. One of the three sectioned pieces of obtaining material was placed into about 5 volumes of RNA later (Ambion Inc.), and after 24 h placed at -80˚C until analyzed by qPCR and western blotting. The other two samples of tumor tissue were fixed in 4% buffered formalin solution, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for histopathological assessment. The tumor samples were subjected to qPCR and WB analyses only if >60% cells in the respective histological sections in tumor samples presented characteristic features of ccRCC while all cells of unchanged (control) samples presented normal morphology (18,19). If both conditions were not fulfilled, the patient was excluded from the study.

Total RNA isolation. Total RNA from the collected samples was isolated using the ExtractMe Total RNA kit (Blirt) according to the manufacturer's protocol. The collected samples were homogenized in 2 ml tubes with 300 μl lysis buffer and ceramic beads using the MagnaLyser apparatus (Roche Diagnostics) for 40 sec at 6,000 rpm. The obtained RNA was dissolved in 70 μl of nuclease-free water. The quantity and quality of RNA were measured with a spectrophotometer (NanoDrop ND 1000; Thermo Fisher Scientific). RNA samples were stored at -80˚C until further analysis.

First-strand cDNA synthesis. 1 μg RNA was reversibly transcribed using 1 μl RevertAid reverse transcriptase (Fermentas; Thermo Fischer Scientific) and 0,5 μg dT18 primers (Sigma-Aldrich, Munich, Germany) in a total volume of 20 μl. The reaction was performed according to the manufacturer's (Fermentas; Thermo Fischer Scientific) protocol. cDNA samples were collected at -20˚C until further analysis.

Assessment of gene mRNA level. The mRNA assessment was performed by the qPCR technique. Primers' sequences were designed using the Primer-BLAST software; their concentrations, as well as experimentally established reaction conditions, are presented in Table II. The measurements were performed in duplicate using 1 μl of 4x diluted cDNA and SensiFast Sybr™ No-Rox kit (Bioline) chemistry in a total volume of 10 μl. The reaction was conducted on separate PCR plate (4titude) for each gene with negative control (water instead of cDNA) and 10x diluted pooled cDNA as a precision control. StepOne Plus apparatus with accompanying software ver. 2.3 (Life Technologies; Applied Biosystems) was used for the amplification process and data analysis. Geometric mean of Ct (threshold cycle) values for each gene was normalized to the reference gene (*GUSB*), according to our previous normalization study on ccRCC (20), using the Livak's equation (21): $X=2^{\Delta Ct}$, where X stands for expression of gene Y and ΔCt=Ct Gusb-Ct gene Y. Obtained raw expression data for each tumor sample were calibrated to average expression data of control samples (fold change; control sample=1).

Western blot analysis. Renal biopsies were gently fragmented with Mammalian Cell Extraction Kit (Biovision, Inc.) in tissue homogenizer MagnaLyzer (Roche Diagnostics). Measurement of protein concentration in homogenates was performed by Bradford protein assay with Coomasie Brillant Blue dye (Sigma-Aldrich). Bovine serum albumin (BSA; Sigma-Aldrich) was used as a standard for the preparation of the calibration curve. The proteins were next separated by their weight using SDS-PAGE (12%; Mini-Protean Tetra System; Bio-Rad). Electrotransfer from an electrophoretic gel to PVDF membrane was carried out in the Mini-Protean Tetra System apparatus (Bio-Rad). The membrane was next incubated with 3% BSA (Sigma-Aldrich) in TBS (Tris-buffered saline; pH 7.5) at room temperature (RT) for 1 h. To detect SHH protein, PVDF membrane was first incubated with the monoclonal rabbit anti-human SHH antibody [EP1190Y] (dilution 1:1,000; Abcam) overnight at 4˚C, and then with the peroxidase conjugate polyclonal anti-rabbit IgG produced in goat [A6154] (dilution 1:10,000; Sigma-Aldrich) for 2 h at RT. After each incubation step, the TBST solution (0.1% Tween-20 in TBS) was used for washing the membrane. To obtain the electrophoretic bands Chemiluminescent Peroxidase Substrate (Sigma-Aldrich) was used. Afterward, the PVDF membrane was also incubated with a monoclonal anti-GAPDH peroxidase antibody produced in mouse (dilution 1:50,000; Sigma-Aldrich) for 1 h at RT to obtain the signal from the reference protein. Densitometric analysis of electrophoretic bands was conducted through the Quantity One Software (Bio-Rad). The values of band intensity/mm^2 for full-length or C-terminal SHH protein were normalized to those from the GAPDH protein examination. Final semi-quantitative results for tumor samples were obtained as a ratio=mean $units_{Tumor}$/mean $units_{Control}$ for full-length or C-terminal SHH protein.

Statistical analysis. Statistics were performed with the use of GraphPad Prism ver. 5.00 (GraphPad Software, Inc.) and Statistica ver. 13.1 (Statsoft Inc.). To compare clinicopathological and molecular data Wilcoxon signed-rank and Fisher's 2x2 exact tests were used. Any correlation analysis presented in the study was performed by Spearman's test. Kaplan-Meier analysis was performed to verify the associations between obtained molecular data and patients' clinicopathological parameters as well as overall survival. In all statistical analyses, a two-sided $P<0.05$ was considered as statistically significant with a 95% confidence interval.

Results

Clinicopathological characteristics of patients. The study encompassed 37 ccRCC patients, 13 female, and 24 male,

Table I. Clinicopathological characteristics of patients and association between SHH pathway molecular assessments with clinical data.

Patient characteristic n=37	SHH qPCR results (%)			PTCH1 qPCR results (%)			SMO qPCR results (%)			GLI1 qPCR results (%)			Full-length SHH WB results (%)			C-terminal SHH WB results (%)		
	↓ (≤0.117)	↑ (>0.117)	P[a]	↓ (≤0.056)	↑ (>0.056)	P[a]	↓ (≤0.073)	↑ (>0.073)	P[a]	↓ (≤0.004)	↑ (>0.004)	P[a]	↓ (≤0.372)	↑ (>0.372)	P[a]	↓ (≤1.951)	↑ (>1.951)	P[a]
Age [y] mean±SD: 60.70±11.08																		
Range: 33-82																		
≤62 n=19	5 (14.3)[b]	12 (34.3)[b]	0.229	7 (20.6)[b]	11 (32.4)[b]	0.041	7 (24.1)[b]	5 (17.2)[b]	0.046	1 (2.9)[b]	16 (45.7)[b]	0.486	7 (21.9)[b]	10 (31.3)[b]	0.726	13 (43.3)[b]	3 (10.0)[b]	0.675
>62 n=18	2 (5.7)[b]	16 (45.7)[b]		13 (38.2)[b]	4 (11.8)[b]		3 (10.3)[b]	14 (48.3)[b]		0 (0.0)[b]	18 (51.4)[b]		5 (15.6)[b]	10 (31.3)[b]		10 (33.3)[b]	4 (13.3)[b]	
Sex																		
Female n=13	3 (8.6)[b]	8 (22.9)[b]	0.652	5 (14.7)[b]	6 (17.6)[b]	0.458	3 (10.3)[b]	6 (20.7)[b]	1.000	1 (2.9)[b]	11 (31.4)[b]	0.343	5 (15.6)[b]	8 (25.0)[b]	1.000	6 (20.0)[b]	6 (20.0)[b]	0.034
Male n=24	4 (11.4)[b]	20 (57.1)[b]		15 (44.1)[b]	8 (23.5)[b]		7 (24.1)[b]	13 (44.8)[b]		0 (0.0)[b]	23 (65.7)[b]		7 (21.9)[b]	12 (37.5)[b]		16 (53.3)[b]	2 (6.7)[b]	
Tumor size [cm]																		
≤7 cm n=23	4 (11.4)[b]	18 (51.4)[b]	1.000	13 (38.2)[b]	9 (26.5)[b]	1.000	7 (24.1)[b]	12 (41.4)[b]	1.000	0 (0.0)[b]	22 (62.9)[b]	0.371	9 (28.1)[b]	10 (31.3)[b]	0.267	13 (43.3)[b]	5 (16.7)[b]	0.669
>7 cm n=14	3 (8.6)[b]	10 (28.6)[b]		7 (20.6)[b]	5 (14.7)[b]		3 (10.3)[b]	7 (24.1)[b]		1 (2.9)[b]	12 (34.3)[b]		3 (9.4)[b]	10 (31.3)[b]		10 (33.3)[b]	2 (6.7)[b]	
Fuhrman's Histological grade																		
1 + 2 n=16[c]	3 (8.6)[b]	12 (34.3)[b]	1.000	9 (27.3)[b]	6 (18.2)[b]	1.000	3 (10.3)[b]	9 (31.0)[b]	0.450	0 (0.0)[b]	16 (45.7)[b]	1.000	4 (12.9)[b]	10 (32.3)[b]	0.707	10 (34.5)[b]	3 (10.3)[b]	1.000
3 + 4 n=20[c]	4 (11.4)[b]	16 (45.7)[b]		10 (30.3)[b]	8 (24.2)[b]		7 (24.1)[b]	10 (34.5)[b]		1 (2.9)[b]	18 (51.4)[b]		7 (22.6)[b]	10 (32.3)[b]		12 (41.4)[b]	4 (13.8)[b]	
TNM stage																		
Non-metastatic T1-2N0M0 n=20	3 (8.6)[b]	16 (45.7)[b]	0.677	9 (26.5)[b]	11 (32.4)[b]	0.079	6 (20.7)[b]	11 (37.9)[b]	1.000	0 (0.0)[b]	20 (57.1)[b]	0.429	9 (28.1)[b]	9 (28.1)[b]	0.147	12 (40.0)[b]	4 (13.3)[b]	1.000
Metastatic T1-2N1M0 T3N0-2M0 T4N0-2M0 T1-4N0-2M1 n=17	4 (11.4)[b]	12 (34.3)[b]		11 (32.4)[b]	3 (8.8)[b]		4 (13.8)[b]	8 (27.6)[b]		1 (2.9)[b]	14 (40.0)[b]		3 (9.4)[b]	11 (34.4)[b]		11 (36.7)[b]	3 (10.0)[b]	
Sunitinib																		
Yes n=11	1 (2.9)[b]	10 (28.6)[b]	0.392	4 (11.8)[b]	6 (17.6)[b]	0.252	3 (10.3)[b]	6 (20.7)[b]	1.000	0 (0.0)[b]	11 (31.4)[b]	1.000	6 (18.8)[b]	4 (12.5)[b]	0.119	8 (26.7)[b]	2 (6.7)[b]	1.000
No n=26	6 (17.1)[b]	18 (51.4)[b]		16 (47.1)[b]	8 (23.5)[b]		7 (24.1)[b]	13 (44.8)[b]		1 (2.9)[b]	23 (65.7)[b]		6 (18.8)[b]	16 (50.0)[b]		15 (50.0)[b]	5 (16.7)[b]	

[a]P-values were calculated by Fisher's 2x2 test; [b]results were excluded due to negative results (e. g. no amplification or no visible bands); [c]one patient with no grade given; SHH, Sonic Hedgehog Signaling Molecule; PTCH1, Patched 1 Receptor; SMO, Smoothened, Frizzled Class G protein-coupled Receptor; GLI1, glioma-associated oncogene 1; WB, western blot; q, quantitative; SD, standard deviation; TNM, tumor node metastasis.

Table II. Details of qPCR assays.

Gene	Primer sequence and concentration in qPCR reaction	Amplicon size	qPCR efficiency (%)	qPCR conditions
SHH	5'-GCAAAGCAAAAAGACACTCGG-3'	269	95.8	95°C, 2 min; 40x (95°C, 5 sec; 59°C, 10 sec; 72°C, 12 sec; 77°C, 10 sec, sample reading)
mRNA	5'-ATTTAAGGCTCTTGAAGGTCCG-3'200 nM each			Melting curve: 95°C, 15 sec; 60°C, 1 min; 68°C→95°C reading every 0, 3°C
PTCH1	5'-GAATCCCTTTTGAGGACAGGAC-3'	387	103.6	95°C, 2 min; 40x (95°C, 5 sec; 59°C, 10 sec; 72°C, 12 sec; 77°C, 10 sec, sample reading)
mRNA	5'-GCATGGTAATCTGCGTTTCATG-3'200 nM each			Melting curve: 95°C, 15 sec; 60°C, 1 min; 68°C→95°C reading every 0, 3°C
SMO	5'-ACTTCTTCAACCAGGCTGAGT-3'	288	106.7	95°C, 2 min; 40x (95°C, 5 sec; 59°C, 10 sec; 72°C, 12 sec; 77°C, 10 sec, sample reading)
mRNA	5'-TCATCTTGCTCTTCTTGATCCG-3'300 nM each			Melting curve: 95°C, 15 sec; 60°C, 1 min; 68°C→95°C reading every 0, 3°C
GLI1	5'-CTACATCAACTCCGGCCAATAG-3'	151	96.4	95°C, 2min; 40x (95°C, 5 sec; 58°C, 10 sec; 72°C, 12 sec; 77°C, 10 sec, sample reading)
mRNA	5'-TGACAGTATAGGCAGAGCTGAT-3'300 nM each			Melting curve: 95°C, 15 sec; 60°C, 1 min; 68°C→95°C reading every 0, 3°C
GUSB	5'-ATGCAGGTGATGGAAGAAGTGGTG-3'	177	99.6	95°C, 3 min; 35x (95°C, 5 sec; 57°C, 10 sec; 72°C, 10 sec; 75°C, 10 sec, sample reading)
mRNA	5'-AGAGTTGCTCACAAAGGTCACAGG-3'200 nM each			Melting curve: 95°C, 15 sec; 60°C, 1 min; 60°C → 95°C reading every 0.3°C

Q, quantitative; SHH, Sonic Hedgehog Signaling Molecule; PTCH1, Patched 1 Receptor; SMO, Smoothened, Frizzled Class G protein-coupled Receptor; GLI1, glioma-associated oncogene 1; GUSB, Glucuronidase Beta.

with mean age 60.70±11.08 years (Table I). According to AJCC/UICC TNM classification of malignant tumors (1), 17 patients were diagnosed as stage I (T1N0M0), 3 as stage II (T2N0M0), 13 as stage III (T1-2N1M0 or T3N0-2M0) and 4 as stage IV (T4N0-2M0 or T1-4N0-2M1). Histopathological examination of ccRCC tissues indicated 2 patients with grade 1, 14 patients with grade 2, 12 patients with grade 3 and 8 patients with grade 4 (1 patient with no grade given) following to Fuhrman grading system. For some samples, the results of molecular assessments were excluded due to negative results (e.g. no amplification or no visible bands; Table I). The mean follow-up period was 38 months (range, 3-72). All deaths were associated with ccRCC progression. The median overall survival (OS) rate was 24 months. During follow-up, metastases occurred in 11 patients.

Expression of the SHH pathway genes at the mRNA level. qPCR analysis revealed significantly, approximately 2-, 2,5- and 7-fold higher *SHH, SMO* and *GLI1* mRNA levels, respectively, in ccRCC samples, compared to morphologically unchanged kidney tissue (Fig. 1A, C and D). There were no statistically significant differences between the expression of the *PTCH1* gene in cancer and control tissues (Fig. 1B). Correlation analysis between mRNA levels of SHH pathway genes and patients clinicopathological factors revealed lower expression of *PTCH1* as well as higher mRNA level of *SMO* in tumor samples derived from older patients (age >62; P<0,05; Table I). Moreover, the level of C-terminal SHH protein in ccRCC samples was significantly lower in a group of males than females (Table I).

Association between mRNA levels of the analyzed genes. The results of Spearman's test revealed a strong (rs=0.729) positive correlation between *SMO* and *GLI1* expression (Fig. 2F). Moreover, medium positive correlations were observed between the mRNA levels of *SHH* and *SMO* (Fig. 2B) as well as *SHH* and *GLI1* (Fig. 2C) genes (rs=0.561 and rs=0.646, respectively). A negative correlation was found between *PTCH1* and *SMO* (Fig. 2D; rs=-0.579) as well as *PTCH1* and *GLI1* (Fig. 2E; rs=-0.378) expression. There was no statistically significant correlation between the expression of *SHH* and *PTCH1* (Fig. 2A) genes.

Semi-quantitative SHH protein level assessment. Western blot analysis demonstrated some differences in full-length and C-terminal SHH protein levels between tumor and control samples. According to Fig. 3, which presents obtained representative electrophoretic bands, in 5/8 matched tissue pairs the level of full-length SHH protein was higher in tumor samples compared to control. A similar or lower level of full-length SHH protein in ccRCC tissues was observed in 3 cases. We also observed a remarkable difference between the C-terminal SHH protein content, which was much higher in control tissues and very low or undetectable in ccRCC samples. These findings were confirmed by the densitometric analysis which revealed approximately 3-fold higher as well as a 4-fold lower level of full-length and C-terminal SHH proteins respectively, in ccRCC tissues compared to control samples (P<0.05; Fig. 4A and B). Spearman's test did not show any correlation between *SHH* mRNA level and neither full-length nor C-terminal SHH protein levels (data not shown). However, there was a positive correlation

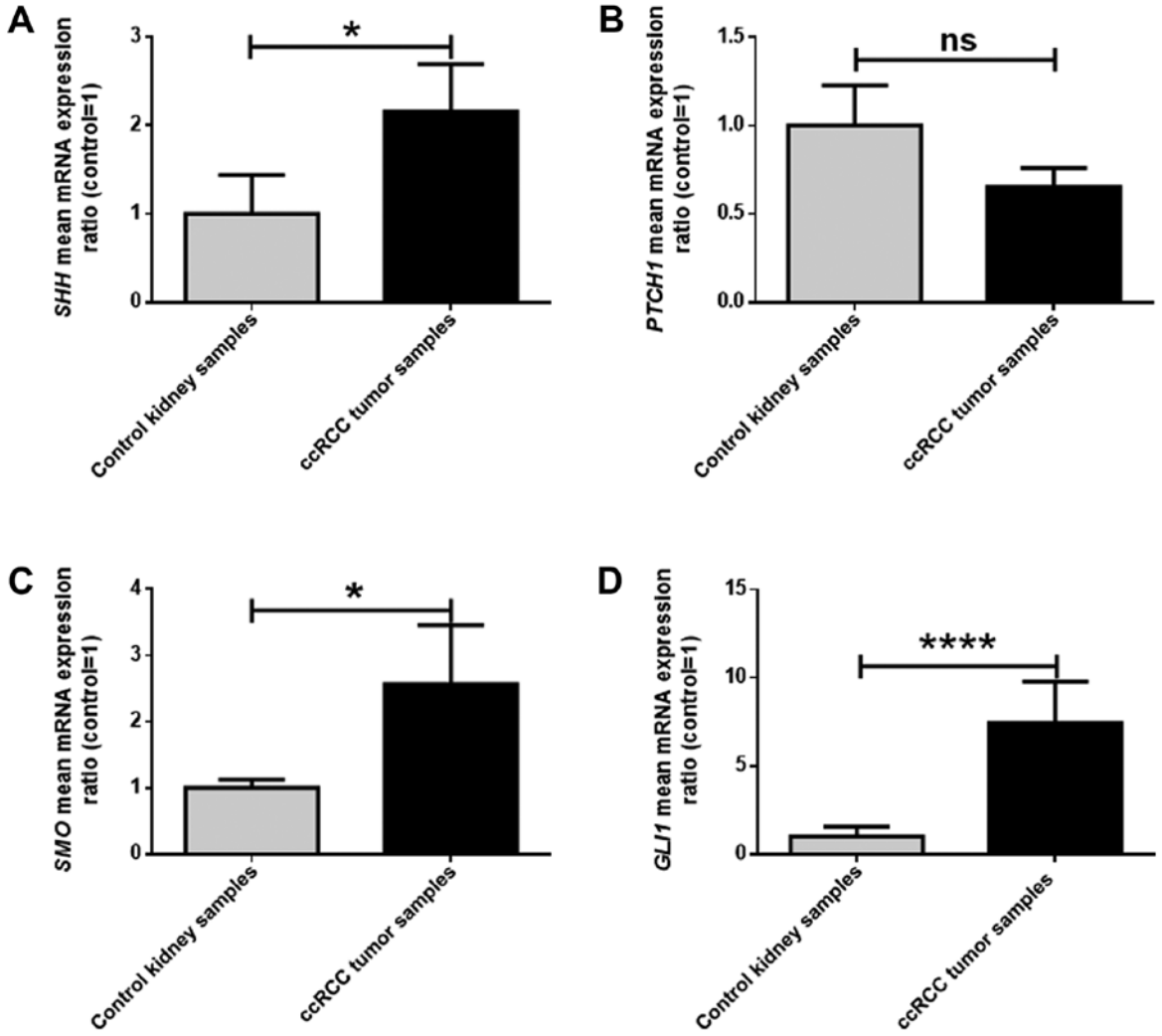

Figure 1. SHH pathway gene mRNA levels in ccRCC tumor samples and morphologically unchanged (control) kidney tissue. (A) *SHH*, (B) *PTCH1*, (C) *SMO*, (D) *GLI1*; mRNA levels in tissue samples were assessed by quantitative PCR. Bars and whiskers represent mean ± SEM normalized to control kidney samples. *P<0.05; ****P<0.0001 between tumor and control samples (Wilcoxon signed-rank test). SHH, Sonic Hedgehog Signaling Molecule; ccRCC, clear cell renal cell carcinoma; PTCH1, Patched 1 Receptor; SMO, Smoothened, Frizzled Class G protein-coupled Receptor; GLI1, glioma-associated oncogene 1.

between the levels of both analyzed SHH protein fragments in cancer tissues, but not in control samples (rs=0,421, P=0,021 and, rs=0,217, P=0.258, respectively; Fig. 5).

Survival analysis. The overall survival of patients with ccRCC was strongly associated with higher Fuhrman grading and male sex (Fig. 6A and B). However, TNM staging, *SHH, PTCH1* and *SMO* mRNA levels as well as full-length and C-terminal SHH protein levels were not correlated with patients' survival (Fig. 6C-H).

Discussion

It has been suggested that the processes of tumorigenesis and embryogenesis display some similar biological features such as increased cell proliferation, differentiation, and migration (22). Indeed, increased activity of the SHH signaling, which is normally limited to the embryonic development, was also observed in basal cell carcinoma (11), breast (12), colon (13) and gastric (14) cancers. However, the contribution of the SHH pathway to ccRCC development remains unclear.

Thirty-seven ccRCC patients were enrolled in the present study. Although the number of participants is relatively small their clinical-pathological data corresponds with characteristic features of ccRCC reported for larger ccRCC cohorts, e.g. mean age of ccRCC manifestation at 61 (19) with our median age of 62 years old. Most of our patients were males, however the M/F ratio (1.83) is comparable M/F ratio (1.56) in the USA in 2011 (23).

To the best of our knowledge, the present study seems to be the first to report increased *SHH* gene expression in ccRCC at the mRNA and protein level. Zhou *et al*, which evaluated the expression of the main SHH pathway components in 58 cases of ccRCC, indicated the lower level of *SHH* mRNA in cancer samples compared to normal kidney tissues (16). Possible explanation concerning the discrepancy between Zhou *et al* and our results may be associated with differences in research methodology, such as different reference gene used in the analysis of the qPCR results. Interestingly, the overexpression of the *SHH* gene at the mRNA level was observed in non-small cell lung cancer compared to matched normal lung samples derived from 83 patients (24). Moreover,

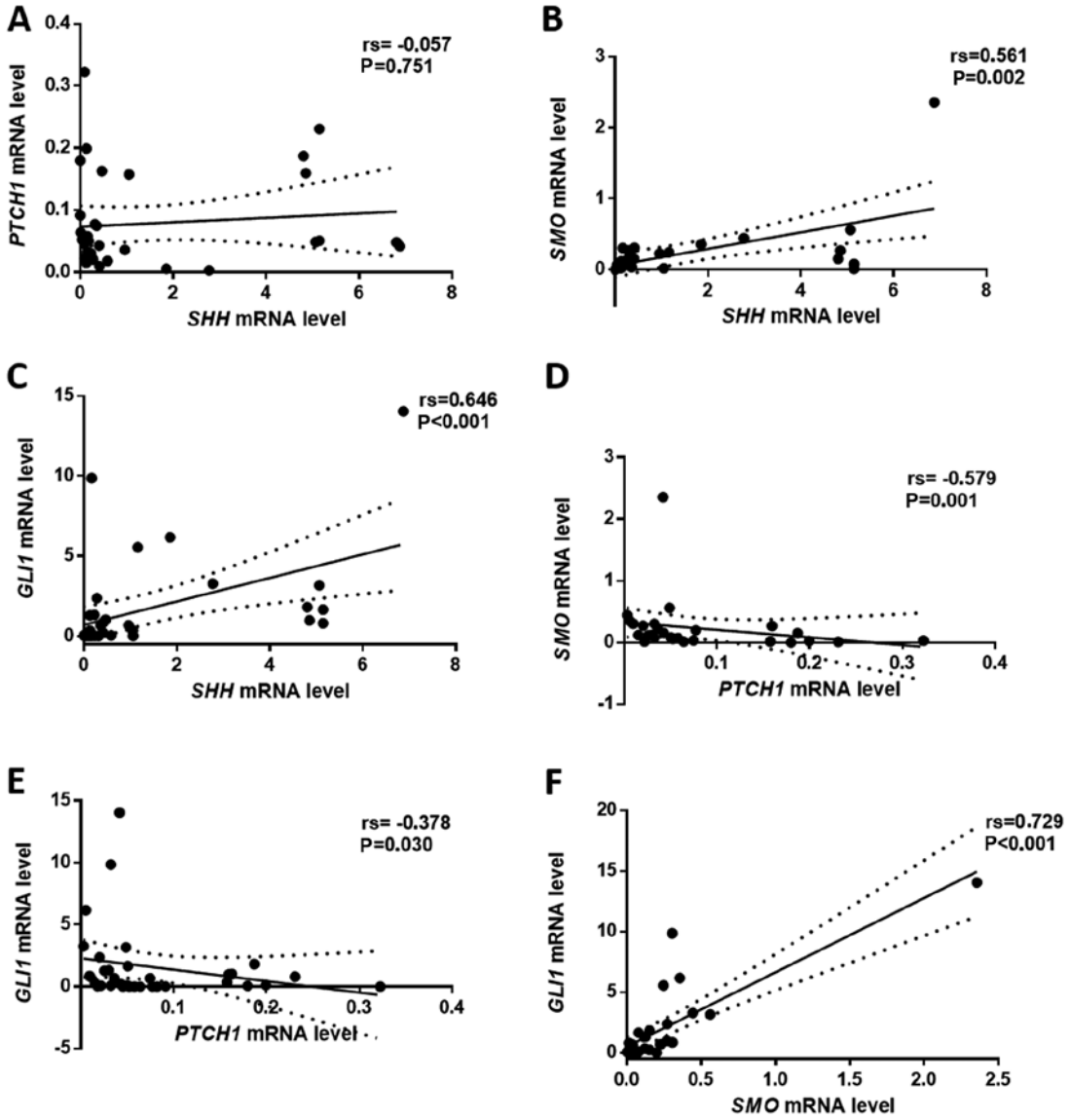

Figure 2. Correlations plots between mRNA levels of the SHH pathway molecules in cancer tissues. P-values were calculated using a Spearman's test. SHH, Sonic Hedgehog Signaling Molecule; PTCH1, Patched 1 Receptor; SMO, Smoothened, Frizzled Class G protein-coupled Receptor; GLI1, glioma-associated oncogene 1.

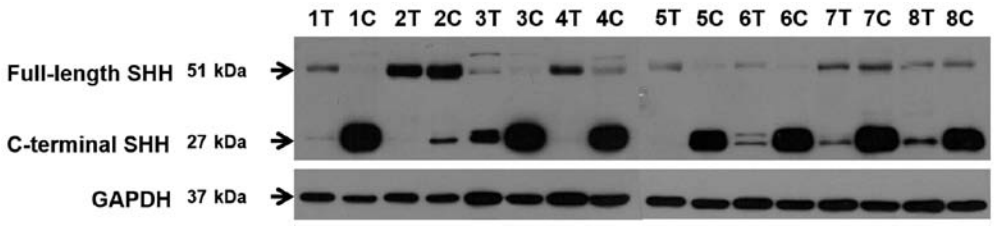

Figure 3. Western blot analysis of full-length and C-terminal SHH protein levels. Representative electrophoretic bands for eight patients (1-8) of full-length (424aa) and C-terminal (227aa) SHH proteins in tumor (T) and control kidney (C) samples. GAPDH was used as a reference protein. In 5 matched tissue pairs, a higher level of full-length SHH protein was indicated in tumor samples compared with the control (patients no. 1, 3, 4, 5 and 6). A similar or lower level of full-length SHH protein in ccRCC tissues was observed in 3 cases (patients no. 2, 7, 8). A marked difference between the C-terminal SHH protein content which was revealed to be increased in control tissues (patients no. 3, 6, 7 and 8) and significantly decreased or undetectable in ccRCC samples (patients no. 1, 2, 4 and 5). SHH, Sonic Hedgehog Signaling Molecule; ccRCC, clear cell renal cell carcinoma.

a higher level of *SHH* mRNA was observed in lung tumors assessed as TNM-2 than TNM-1 and cases in which pleural invasion was presented (24). These findings suggest that *SHH* mRNA level may act as a potential prognostic factor in lung cancer.

To complete our observation of increased *SHH* mRNA level in ccRCC, we performed the measurement of full-length and C-terminal SHH protein contents by the western blot method. Our analysis revealed a considerable increase of the full-length SHH protein level, which confirms the results of

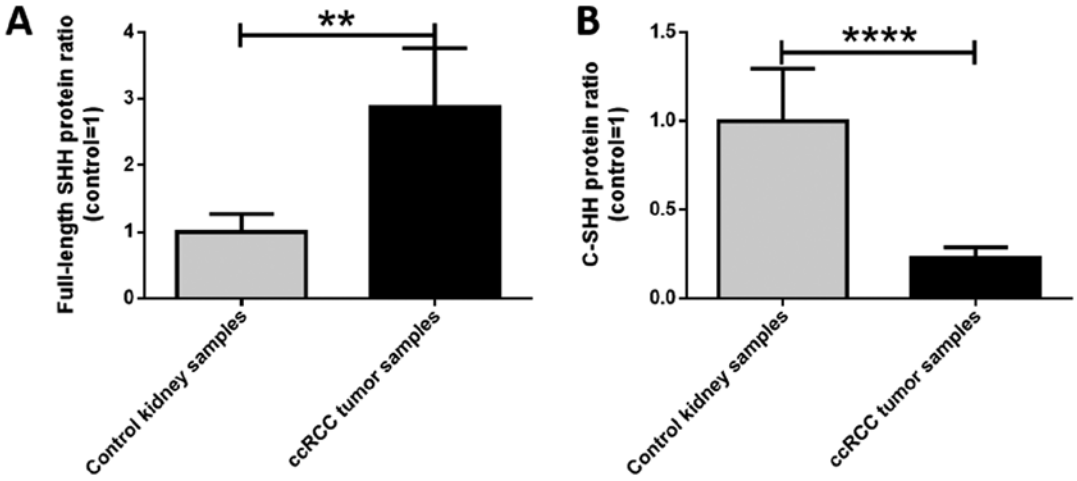

Figure 4. Western blot analysis of (A) full-length SHH and (B) C-terminal SHH protein levels in ccRCC tumor samples and morphologically unchanged (control) kidney tissue. Bars and whiskers represent mean ± SEM normalized to control kidney samples. **P<0.01; ****P<0.0001 between tumor and control samples (Wilcoxon signed-rank test). SHH, Sonic Hedgehog Signaling Molecule; ccRCC, clear cell renal cell carcinoma.

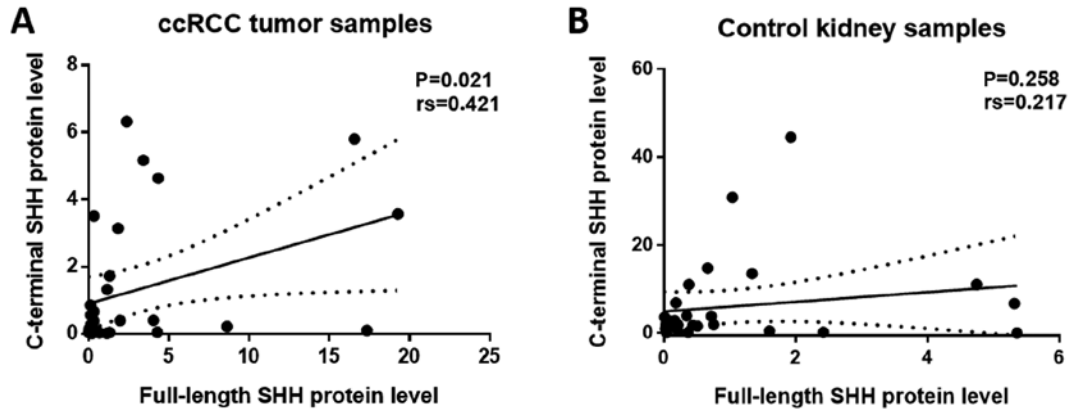

Figure 5. Correlation plots between full-length and C-terminal SHH protein levels in (A) ccRCC tumor samples and (B) morphologically unchanged (control) kidney tissues. P-values were calculated using a Spearman's test. SHH, Sonic Hedgehog Signaling Molecule; ccRCC, clear cell renal cell carcinoma.

the qPCR analysis. We also found a significant decrease of the C-terminal SHH domain in ccRCC tissues, what is the novel observation in cancer tissues. Further experiments are required to find out what is the mechanism of these changes. It has to be mentioned that our analysis did not include the N-terminal SHH domain due to the lack of commercially available highly specific antibodies.

The difference between the level of full-length SHH protein in ccRCC and normal kidney tissue has not been observed so far (9). However, there were some changes at the SHH protein level in other cancer types. Bian *et al* (25) examined 142 papillary thyroid carcinoma samples by immunohistochemical (IHC) method. They demonstrated a statistically higher immunoreactivity of full-length SHH protein in most tumor tissue samples, compared to adjacent non-cancerous thyroid samples as well as the association between SHH protein level and tumor size, clinical staging, and lymph node metastasis (25). Furthermore, aberrant *SHH* gene expression was indicated not only in cancers derived from epithelial cells but also other types of malignancies such as retinoblastoma. IHC staining of 79 retinoblastoma samples revealed that SHH protein was presented in most cases of neoplastic tissues unlike normal retina samples and high SHH immunoreactivity was correlated with advanced disease status including local invasion and metastasis (26).

The Patched1 (PTCH1) receptor is a 12-pass transmembrane protein, which inhibits Sonic Hedgehog signaling when it is unliganded (27). In ccRCC Zhou *et al* found a considerable decrease of *PTCH1* mRNA level (16). Our study also revealed the tendency towards a lower level of *PTCH1* expression at the mRNA level, but the results are not statistically significant.

The SHH pathway signaling transducer, Smoothened protein (SMO), is the main target for several molecular antitumor agents, which are tested in clinical trials (28). To date, two of them, vismodegib and sonidegib, have been approved by the US Food and Drug Administration (FDA) for treating locally advanced and metastatic basal cell carcinoma (BCC) (29). Our results revealed a statistically significant increase of the *SMO* gene expression at the mRNA level in ccRCC samples, compared to control tissues. The previous report about the expression of SHH pathway genes in ccRCC tissues did not indicate differences between *SMO* mRNA level in ccRCC tissues and normal kidney sections (16). However, Dormoy *et al* observed that cyclopamine, the substance that

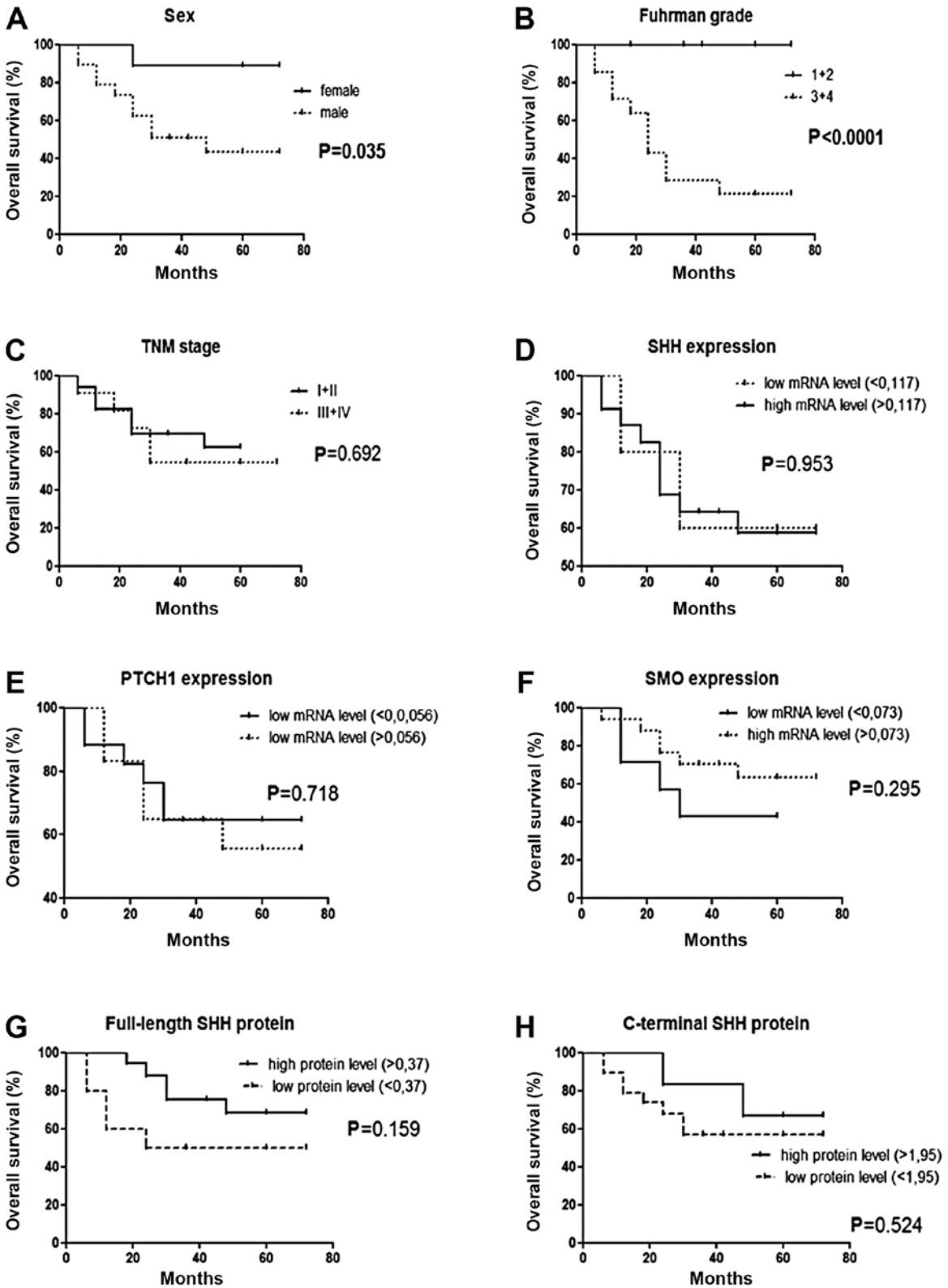

Figure 6. Kaplan-Meier's survival analysis for 28 patients with ccRCC related to (A, B, C) clinicopathological and (D, E, F, G, H) molecular data. The threshold value for each analyzed gene based on the median of (D) *SHH*, (E) *PTCH1* and (F) *SMO* mRNA levels or (G) full-length and (H) C-terminal SHH protein levels in control samples. ccRCC, clear cell renal cell carcinoma; SHH, Sonic Hedgehog Signaling Molecule; PTCH1, Patched 1 Receptor; SMO, Smoothened, Frizzled Class G protein-coupled Receptor; GLI1, glioma-associated oncogene 1.

acts as an SMO protein inhibitor, decreases ccRCC cells proliferation and stimulates their apoptosis *in vitro* as well as *in vivo* (15). These findings suggest that, as in the BCC tumors, SMO may act as a potential drug target for ccRCC.

Among other types of cancers, increased content of SMO protein, assessed by the IHC method, was demonstrated by Ding *et al* in colon cancer tissues, obtained from 96 patients. Moreover, the level of SMO protein was positively related to

the presence of lymph node metastases and higher T stages, which suggested the contribution of this gene in the colon cancer progression (30).

Glioma-Associated Oncogene 1 (GLI1), together with GLI2 and GLI3, are the members of zinc finger transcription factors family (10). Our study indicated a considerable increase of *GLI1* mRNA level in cancer tissues, what is consistent with previous reports regarding ccRCC tissues (15,16). Moreover, Furukawa *et al*, which assessed immunoreactivity of GLI1 and GLI2 proteins in ccRCC tissues derived from 39 patients, observed that strong GLI2 expression, but not GLI1, was correlated with a shorter period of progression free survival (31). Our results also did not indicate an association between *GLI1* mRNA level and patients' survival (data not shown). Elevated GLI1 protein immunoreactivity, assessed by IHC staining of 204 tissue samples, was also observed in breast cancer cells and additionally it was correlated with unfavorable overall survival as well as higher tumor stage (32). Furthermore, increased GLI1 protein immunoreactivity was observed in the other tumor types such as the bladder (33) or ovarian cancers (34).

Our statistical analysis revealed that the expression rates of almost all the SHH pathway components in tumor tissues at the mRNA levels were correlated with each other. These findings suggest that SHH signaling is reactivated in ccRCC through canonical way, dependent on the amount of its upstream regulator, SHH protein (35) The same way of SHH pathway activation has been observed also in breast cancer (36) and non-small cell lung carcinoma cell lines (37), however, such suggestion needs confirmation based on *in vitro* studies with the use of RCC cell lines.

The association between the expression of SHH pathway components and cancer prognostic factors was reported in pancreatic adenocarcinoma (38), glioma (39) and other cancer types (24-26,30,32). Moreover, the expression profiles of SHH signaling genes in some cancer types correlated with the patients' overall survival (14,40). Our statistical analysis did not reveal any relationships between SHH pathway genes mRNA level and ccRCC prognostic factors, however, most of the cited studies based on different techniques (IHC) and semi-quantification of SHH signaling proteins. Due to technical limitations, the IHC method could not be applied to our study, since the selection and antibodies and prior to IHC technique, western blot optimization took too long. Therefore, we plan to perform immunohistochemical studies in a larger cohort of ccRCC patients. There was also no correlation between the level of *SHH*, *PTCH1* and *SMO* mRNA as well as SHH proteins and patients' overall survival. Thus, according to our preliminary findings, observed changes in SHH pathway genes expression in tumor tissues probably are not associated with the ccRCC progression and patients' outcome.

In summary, increased expression of *SHH*, *SMO*, *Gli1* genes and full-length SHH protein level, as well as decrease of C-terminal SHH protein level in tumor ccRCC tissues, suggest the involvement of SHH signaling in ccRCC initiation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AKC performed molecular analysis, performed the statistical tests and prepared the manuscript. JK acquired tissue samples and patient data and revised the manuscript. MM acquired tissue samples and patients' data and revised the manuscript. ZK substantially contributed to the interpretation of the results and revised the manuscript. PMW designed and supervised the study and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study received the approval of the Independent Bioethics Commission at the Medical University of Gdańsk (decision no. NKEBN/4/2011 and NKBBN/370/2016) and written consent was obtained before the surgery from each patient. All experimental procedures were performed according to the regulations and internal biosafety and bioethics guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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