

Long intergenic non-coding RNA, regulator of reprogramming (LINC-ROR) over-expression predicts poor prognosis in renal cell carcinoma

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Submitted: 19 April 2018

Accepted: 14 July 2018

Arch Med Sci

DOI: <https://doi.org/10.5114/aoms.2019.85201>

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Abstract

Introduction: Long intergenic non-coding RNA, regulator of reprogramming (LINC-ROR) is a newly identified cytoplasmic long non-coding RNA (lncRNA) implicated in cell longevity and apoptosis. We aimed in the current work for the first time to investigate the association of the expression profiles of LINC-ROR and three stem-related transcriptional factors with clinicopathological data and their impact on renal cell carcinoma (RCC) progression in a sample of RCC patients.

Material and methods: Expression levels of LINC-ROR and stemness-related factors: SOX2, NANOG, and POU5F1 were detected in 60 formalin-fixed, paraffin-embedded tissues, and their paired adjacent non-cancer tissues ($n = 60$) by using real-time qRT-PCR analysis. Additionally, the expression profiles were compared with the available clinicopathological features.

Results: The genes studied were markedly up-regulated in RCC (medians and interquartile ranges were 30.3 (1.84–235.5), 10.2 (1.84–53.9), 5.39 (0.94–23.5), and 12.5 (1.61–43.2) for *LINC-ROR*, *SOX2*, *NANOG*, and *POU5F1*, respectively) relative to paired non-cancer tissue. High expression levels were associated with poor prognosis in terms of tumour undifferentiation (for *LINC-ROR*, *SOX2*, and *NANOG*), lymph node infiltration (for *SOX2*), post-operative recurrence (for *LINC-ROR* and *SOX2*), and shorter overall survival (OS) and progression-free survival (for all genes studied). The best curve for OS prediction was constructed with LINC-ROR data (area under the receiver operating characteristic curve (AUC) = 0.804 at a cut-off value of 72.7, sensitivity 78.9%, and specificity 80.5%).

Conclusions: Collectively, aberrant LINC-ROR and pluripotent gene expression may be recognised as prognostic markers for RCC. Future functional studies are highly recommended to validate the study findings.

Key words: renal cell carcinoma, long intergenic non-coding RNA, regulator of reprogramming, SOX2, NANOG, POU5F1.

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Introduction

Renal cell carcinoma (RCC), which arises from renal tubular epithelial cells, accounts for 2–3% of all cancers worldwide [1]. There has been a global rise in RCC incidence in past decades, and up to 35% of RCC patients present with metastasis [2]. Therefore, discovering biomarkers for early detection and better prognostic stratification of RCC has emerged as a noteworthy target for future investigations.

According to the World Health Organisation (WHO), there are three major histopathological RCC types: clear cell RCC (ccRCC), papillary cell RCC (pcRCC), and chromophobe RCC (chRCC) [3]. Each RCC subtype is known to display unique pathological characteristics, distinct genetic alterations, and different disease outcomes [1]. The interplay of the genetic mechanisms that affect RCC pathogenesis is still poorly understood. The use of integrated molecular analyses enables a deeper understanding of the RCC molecular landscape, resulting in the development of more effective tar-

geted cancer therapies as an extension of existing modalities [4, 5].

Cancer has been found to histologically mimic embryonic tissue [6]. Aggressive cancer cells exhibit phenotypic traits remarkably similar to those of pluripotent stem cells; both have high proliferation rates and a substantial ability to self-renew by bypassing senescence [7]. Accumulating evidence has demonstrated the convergence of tumorigenic signalling and developmental pathways [8]. Since the introduction of induced pluripotent stem cell (iPSC) generation mediated by transcription factor (TF) reactivation, studies have been directed towards identifying the differential expression profiles altered during pathological nuclear reprogramming in cancer [7]. SOX2, NANOG, and POU5F1 (also known as OCT3/4) are three pluripotency-associated TFs that are well known to be up-regulated in various cancer types through modulation of apoptotic signalling pathways [9]. Notably, modifying the expression of pluripotency-associated genes in animal models has generated epithelial-derived tumours in multiple tissues with altered spatial and temporal gene expression [10].

Recent evidence has suggested complex networking between TFs, chromatin organisation, and non-coding RNAs that regulate cell fate and differentiation. Several long intergenic non-coding RNAs (lincRNAs) have also been shown to be associated with pluripotency [11]. LincRNA expression has been shown to be highly correlated with the expression of stem cell-related TFs [12]. Binding sites for pluripotency-related genes have been identified within the promoters of these long non-coding RNAs (lncRNAs) [13]. *In vitro* silencing of lincRNAs has been shown to induce cells to exit the pluripotent state and altered gene expression patterns [14]. In 2010, Loewer *et al.* [11] identified lincRNA, regulator of reprogramming (LINC-ROR or ROR) in human fibroblasts and CD34-positive blood cells during iPSC generation. Furthermore, *in vitro* and *in vivo* studies have determined the critical role of ROR in modulating cell proliferation, apoptosis, and chemosensitivity in cancer [15–17].

To the best of our knowledge, no clinical studies have been conducted exploring the role of ROR in RCC patients to validate the aforementioned *in vitro* and *in vivo* results. In the current study, we aimed to analyse the transcriptomic signature profiling of LINC-ROR and three of the main pluripotency-related genes (*SOX2*, *NANOG*, and *POU5F1*) in RCC patients to assess their clinical utility as prognostic biomarkers.

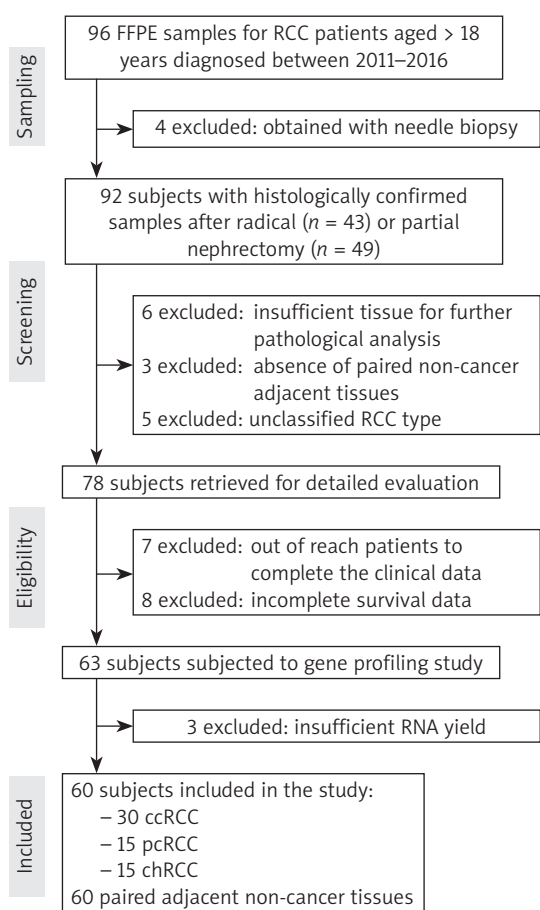


Figure 1. Flow chart of the study population selection.

FFPE – formalin-fixed paraffine-impeded, RCC – renal cell carcinoma, ccRCC – clear cell RCC, pcRCC – papillary cell RCC, chRCC – chromophobe RCC.

Material and methods

Case selection and pathological assessment

In total, 120 formalin-fixed, paraffin-embedded (FFPE) specimens were analysed, including 60 RCC

samples (30 ccRCC, 15 pcRCC, and 15 chRCC) and their paired adjacent non-cancer tissues. Figure 1 shows the flow chart of the current study case selection. All specimens were obtained after radical or partial nephrectomy, and cases with needle biopsies were excluded. All retrieved cases were archived in the Department of Pathology, Suez Canal University between 2011 and 2016. Patient data were obtained from medical records. Missing data were directly retrieved from the patients. Patient follow-ups were performed until December 2016 and ranged from seven to 32 months. Postoperative recurrence was calculated, and survival times were estimated from the date of nephrectomy until patient death or the endpoint of follow-up. The study was conducted following the ethical and legal standards adopted by the Declaration of Helsinki. Approval was obtained from the Medical Research Ethics Committee of the Faculty of Medicine, Suez Canal University. Consent was directly obtained from patients as a routine measure for sample archiving.

Histopathological examination

Histological examination, nuclear grading, and tumour staging of RCC samples were based on haematoxylin and eosin staining. Blind assessments were made without prior knowledge, according to the internationally standardised protocols we mentioned in our previous work [18]. Most of the RCC cases were the clear cell type and contained very little delicate vascular stroma. The papillary and chromophobe types also had only a little amount of stroma, and thus it was unlikely that it affected accurate determination of the expression of the genes assessed in this study.

Gene expression profiling

Total RNA was purified from the FFPE sections using a Qiagen RNeasy FFPE Kit (Cat # 74404, Qiagen, Hilden, Germany) following the manufacturer's protocol. A short incubation of each sample with proteinase K at a higher temperature, as recommended, partially reverses formalin crosslinking of the released nucleic acids, improving RNA yield and quality as well as RNA performance in downstream assays. All samples were subjected to treatment with RNase-free DNase I for two hours at 37°C to eliminate all genomic DNA, including very small DNA fragments that are often present in FFPE samples after prolonged formalin fixation and/or long storage times. At an absorbance ratio of 260/280 nm, RNA concentration and purity were assessed by a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), followed by agarose gel electrophoresis check for RNA integrity. The range of the extracted RNA was (20–65 ng/μl). Reverse transcription was performed with a high-capacity cDNA reverse

transcription kit (Part No. 4374966, Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) using a Mastercycler Gradient Thermocycler (Eppendorf, Hamburg, Germany). Detection of *LINC-ROR*, *SOX2*, *NANOG*, and *POU5F1* gene expression and that of the endogenous control GAPDH was performed using real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). TaqMan assays were used for the pluripotent genes (Applied Biosystems, Thermo Fisher Scientific Inc., assay ID Hs01053049_s1 for *SOX2*, Hs02387400_g1 for *NANOG*, Hs03005111_g for *POU5F1*, and Hs02786624_g1 for *GAPDH*) as previously described in detail [19]. Quantification of LINC-ROR was achieved using SYBR Green with primers for exon 4 (F: GCCTGAGAGTTGGCATGAAT and R: AAAACCTCACTCCCATGTGC) [20]. The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were followed for all PCR reactions. Gene expression in randomly selected study samples (10%) was re-evaluated in separate runs to test the reproducibility of the qPCR analysis, and the results showed very close cycle of quantification (Cq) values with low standard deviations.

Statistical analysis

Data analyses were conducted using the R packages SPSS version 22 and GraphPad Prism version 7. Multivariate analysis was conducted using PCORD version 5.0. The relative expression levels were calculated using the delta-delta threshold cycle method. The Wilcoxon matched-pair signed-rank test was carried out to compare the expression level between cancer samples and their corresponding adjacent non-cancer tissues. Receiver operating characteristic (ROC) curves and the area under the ROC curves (AUCs) were determined to identify the clinical utility of the expressed genes. Ordination analysis and two-way agglomerative hierarchical clustering were performed for data exploration. Spearman's correlation analysis, chi-square (χ^2), Fisher's exact, Mann-Whitney *U* (MW), and Kruskal-Wallis (KW) tests were applied when appropriate. A two-tailed *p*-value was considered significant at values < 0.05. Binary logistic and linear regression methods (Enter and Stepwise models) were applied to determine predictors for recurrence and overall survival (OS). The influence of covariates, such as age, gender, grade, tumour type, size, and gene profile, on OS was analysed. Kaplan-Meier analysis and the Cox proportional hazard model were performed to assess survival rates.

Results

Characteristics of the study patients

The baseline features of RCC patients are shown in Table I. Overall, 21 female and 39 male

Table I. Clinicopathological characteristics of renal cell carcinoma patients (n = 60)

Parameter	N	%	Parameter	N	%
Age:			Tumour size:		
< 60 years	28	46.7	T1	21	35.0
≥ 60 years	32	53.3	T2	25	41.7
Gender:			T3	14	23.3
Female	21	35.0	Pathological grade:		
Male	39	65.0	Grade 1	9	15.0
Histopathological type:			Grade 2	28	46.7
Clear cell RCC	30	50.0	Grade 3	23	38.3
Papillary RCC	15	25.0	Recurrence:		
Chromophobic RCC	15	25.0	Negative	44	73.3
Tumour location:			Positive	16	26.7
Right side	22	36.7	Progression-free survival:		
Left side	38	63.3	≤ 12 months	22	36.7
Number:			≤ 24 months	31	51.6
Solitary	46	76.7	≤ 36 months	7	11.7
Multifocal	14	23.3	Overall survival:		
LN involvement:			≤ 12 months	19	31.7
Negative	39	65.0	≤ 24 months	34	56.6
Positive	21	35.0	≤ 36 months	7	11.7

Table II. Correlation matrix for the clinicopathological characteristics of renal cell carcinoma patients (n = 60)

Parameter	Age	Gender	No.	Grade	T	LN	Rec	PFS	OS
Age	1.000								
Gender	-0.229 (0.078)	1.000							
No	-0.218 (0.095)	-0.008 (0.950)	1.000						
Grade	0.017 (0.897)	-0.213 (0.103)	0.453 (< 0.001)	1.000					
T	-0.074 (0.576)	-0.205 (0.116)	0.491 (< 0.001)	0.447 (< 0.001)	1.000				
LN	0.070 (0.597)	-0.194 (0.137)	0.504 (< 0.001)	0.631 (< 0.001)	0.557 (< 0.001)	1.000			
Rec	-0.180 (0.169)	-0.111 (0.400)	0.202 (0.122)	0.460 (< 0.001)	0.189 (0.149)	0.348 (0.006)	1.000		
PFS	0.260 (0.045)	0.182 (0.164)	-0.154 (0.241)	-0.417 (0.001)	-0.144 (0.271)	-0.243 (0.061)	-0.675 (< 0.001)	1.000	
OS	0.243 (0.061)	0.222 (0.088)	-0.132 (0.314)	-0.347 (0.007)	-0.123 (0.350)	-0.152 (0.248)	-0.523 (< 0.001)	0.968 (< 0.001)	1.000

Data are presented as a correlation coefficient (P value). Spearman's rank correlation was used. No. – number, T – tumour size, LN – lymph node, Rec – recurrence, PFS – progression-free survival, OS – overall survival. Bold values are considered statistically significant at p < 0.05.

RCC patients were included in the study. Their ages ranged from 48 to 79 years (mean ± standard deviation of 58.6 ± 6.31). Notably, in patients with solitary tumours (76.7%), the left kidney was more frequently affected (63.3%). Despite the lack of lymph node metastases in nearly two-thirds of the spec-

imens (65%) and the small tumour sizes (76.7%), most samples showed moderate/poor differentiation (85%). Recurrence occurred in approximately one-fourth of patients (26.7%) after a mean duration of 4 ± 1.7 months. Postoperative disease-free survival ranged from 2 to 32 months. The OS rate

was 68.3% in the first year and 11.7% in the second year, with a mean \pm SD of 11.4 \pm 4.9 months. The correlation matrix showed that tumour grade was positively correlated with tumour number ($r = 0.453$, $p < 0.001$), larger tumour size ($r = 0.447$, $p < 0.001$), lymph node metastasis ($r = 0.631$, $p < 0.001$), and post-operative recurrence ($r = 0.460$, $p < 0.001$) but negatively correlated with progression-free survival (PFS; $r = -0.417$, $p = 0.001$) and OS ($r = -0.347$, $p = 0.007$). Tumour recurrence was moderately correlated with lymphadenopathy ($r = 0.348$, $p = 0.006$). Patient age showed a weak association with PFS ($r = 0.260$, $p = 0.045$) (Table II).

Gene expression analyses

Gene expression profiling demonstrated over-expression of the *LINC-ROR*, *SOX2*, *NANOG*, and *POU5F1* genes in tumour specimens compared with those in their paired non-cancer tissues (all $p < 0.001$). Medians and interquartile ranges of the relative expression levels in RCC were 30.3 (1.84–235.5) for *LINC-ROR*, 10.2 (1.84–53.9) for *SOX2*, 5.39 (0.94–23.5) for *NANOG*, and 12.5 (1.61–43.2) for *POU5F1* (Figure 2). Stratified analyses by age and gender showed similar up-regulation (all p -values < 0.001), with marked over-expression of *LINC-ROR* in female patients less than 60 years of age at disease onset. The correlation matrix showed a significant positive relationship between *LINC-ROR* and both *SOX2* ($r = 0.280$, $p = 0.045$) and *NANOG* ($r = 0.312$, $p = 0.015$).

Prognostic value of gene expression analyses

Univariate analysis revealed marked associations between *LINC-ROR*, *SOX2*, and *NANOG* expression levels and undifferentiated tumours ($p = 0.010$, 0.046, and 0.042, respectively). *SOX2* up-regulation was linked to lymph node infiltration ($p = 0.047$). Moreover, patients with higher *LINC-ROR* and *SOX2* levels experienced post-operative recurrence ($p = 0.003$ and 0.035). The expression of all genes presented significant associations with shorter PFS and low OS (all $p < 0.05$) (Table III). Similar results were found in correlation analyses between gene expression levels and the clinicopathological findings (Table IV).

The prognostic performance of the four genes in predicting OS in RCC patients was assessed using ROC analyses (Figure 3). In single-gene analyses, *LINC-ROR* data elicited the best curve (AUC = 0.804 at a cut-off value of 72.7; sensitivity 78.9%, specificity 80.5%) followed by *NANOG* (AUC = 0.748 at a cut-off value 11.2; sensitivity 68.4%, specificity 75.6%) and *SOX2* (AUC = 0.714 at a cut-off value 25.5; sensitivity 73.3%, specificity 75.6%), whereas *POU5F1* had an AUC of 0.632 at a cut-off value of 15.2, a sensitivity of 52.6%, and a specificity of 56.1%. Despite the good performance of the com-

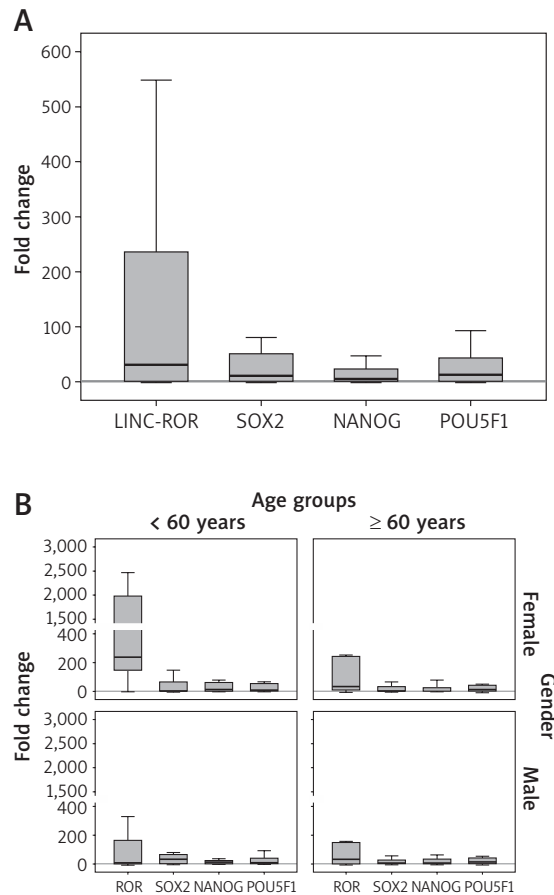


Figure 2. Gene expression of *LINC-ROR* and pluripotent genes in RCC. The data are presented as the median values. The box defines the upper and lower quartiles (25% and 75%, respectively), and the error bars indicate the upper and lower adjacent limits. Gene expression levels in cancer and normal tissues were normalised to GAPDH expression and were calculated using the delta-delta CT method [$= 2^{-\Delta\Delta CT}$] in comparison with normal renal tissues. The red line represents the expression level of normal renal tissues (equivalent to 1). The Wilcoxon matched-pair signed-rank test was used. All p -values were < 0.001 . **A** – Overall analysis. **B** – Stratified analysis by age and gender

combined gene analyses, with AUC values ranging from 0.675 to 0.755, the AUC of *LINC-ROR* remained the best (Table V).

Logistic regression analyses revealed that both lymph node infiltration and *LINC-ROR* expression acted as recurrence predictors in RCC patients ($p = 0.007$ and 0.047, respectively; Table VI). Furthermore, linear regression analyses demonstrated that *LINC-ROR* was an independent predictor for OS ($R = 0.443$, $R^2 = 0.192$, $p < 0.001$).

Survival analysis

Analyses of different influencing factors on the survival time of RCC patients are demonstrated in Table VII. Cox regression showed that *LINC-ROR*,

Table III. Univariate analysis for association between gene profile and the clinicopathological features in RCC patients

Parameter	LINC-ROR		SOX2		NANOG		POU5F1	
	Expression	P-value	Expression	P-value	Expression	P-value	Expression	P-value
Age:		0.450		0.178		0.563		0.947
< 60 years	28	15 (1.3–236)	28.6 (1.8–65)		10 (0.9–23.5)		7.9 (1.9–44.1)	
≥ 60 years	32	33 (4.8–214)	7.19 (1.3–34)		3.9 (0.7–30.5)		14 (1.4–41.3)	
Gender:		0.060		0.62		0.871		
Female	21	39 (11.2–575)	8.6 (1.7–40.9)		4.7 (1.3–38)		14.3 (2.8–46)	0.625
Male	39	12 (1.3–152)	12 (1.7–56.8)		5.6 (0.5–23.1)		12.3 (1.5–42)	
HPD:		0.737		0.818		0.302		
ccRCC	30	18 (1.5–149)	8.0 (1.9–45)		4.8 (0.2–15)		14 (1.5–45)	0.906
pRCC	15	30 (0.5–654)	25 (1.0–65)		15 (1.6–61)		12 (0.7–42)	
chRCC	15	36 (2.8–236)	12 (1.7–61)		4.7 (0.2–14)		10 (1.7–43)	
Side:		0.914		0.742		0.730		
Right	22	21 (2.2–169)	14 (3.1–56)		4.6 (0.4–50)		3.8 (1.2–34)	0.137
Left	38	36 (1.6–239)	10 (1.6–16)		5.9 (1.3–16)		16 (2.3–44)	
Number:		0.442		0.144		0.302		
Unifocal	46	27 (2.2–165)	9.4 (0.4–45)		4.9 (0.4–20.9)		13 (1.5–44)	0.479
Multifocal	14	34 (1.4–985)	17 (3.9–85)		13 (1.8–42.6)		73 (1.6–23)	
Grade:		0.010*		0.046*		0.042*		
Grade 1	9	3.3 (0.6–105)	1.3 (0.16–61)		6.5 (0.004–21)		12.3 (1.7–73)	0.435
Grade 2	28	13 (1.8–112)	7.1 (0.63–30)		2.8 (0.21–9.3)		9.9 (1.4–32.2)	
Grade 3	23	235 (7.8–898)	35.7 (3.9–65)		12.3 (2.0–61)		15 (2.5–44.3)	
T:		0.071		0.063		0.544		
T1	21	35 (2.7–150)	2.1 (0.19–27)		6.5 (0.2–33.6)		12.6 (4.6–45.2)	0.538
T2	25	7.8 (1.1–135)	12 (4.7–45.3)		4.6 (1.1–11.3)		12.3 (1.4–44.2)	
T3	14	244 (16–985)	49 (3.7–86.4)		8.3 (1.1–61.8)		10.5 (1.6–17.9)	
LN:		0.242		0.047*		0.739		
Negative	39	20 (1.6–152)	7.5 (0.2–36.6)		5.1 (0.51–23)		12.6 (1.5–44.3)	0.443
Positive	21	39 (6.9–289)	29.2 (3.8–64)		6.2 (1.0–30.1)		7.9 (1.5–27.7)	
Recurrence:		0.003*		0.035*		0.285		
Negative	44	13 (1.4–130)	6.1 (0.6–45.7)		4.6 (0.3–19)		11.4 (1.4–35)	0.144
Positive	16	236 (34–1708)	36.2 (17–60.4)		12.4 (1.3–38)		23.7 (4–61)	
PFS:		0.001*		0.004*		0.048*		
≤ 12 m	22	281 (55–1168)	37.6 (13.9–64)		14 (3.7–61.8)		17.3 (3.9–50.5)	0.009*
≤ 24 m	31	14.6 (1.6–61)	6.8 (0.2–446)		4.6 (0.19–12)		15.2 (1.3–39)	
≤ 36 m	7	1.5 (0.7–7.8)	3.5 (1.05–4.1)		2.7 (1.5–10.2)		1.4 (0.3–1.5)	
OS:		0.001*		0.011*		0.009*		
≤ 12 m	19	456 (123–1978)	36.6 (12–65)		306 (4.6–61)		15.2 (4.1–49)	0.009*
≤ 24 m	34	22.4 (1.6–69)	7.5 (0.2–48)		3.9 (0.1–12)		16.3 (1.2–40)	
≤ 36 m	7	1.5 (0.7–7.8)	3.5 (1.1–4.1)		2.7 (1.5–10)		1.4 (0.3–1.5)	

Data are presented as median (quartiles). HPD – histopathological diagnosis, ccRCC – clear cell renal cell carcinoma, pRCC – papillary cell RCC, chRCC – chromophobe RCC, T – tumour size, LN – lymph node, PFS – progressive-free survival, OS – overall survival. Mann-Whitney U and Kruskal-Wallis tests were used. *Values are statistically significant at $p < 0.05$.

POU5F1, and recurrence were risk factors that predicted OS (hazard ratio (HR) = 4.21, 95% confidence interval (CI): 1.13–15.6, $p = 0.031$; HR = 3.80, 95% CI: 1.55–9.30, $p = 0.003$; and HR = 4.94, 95% CI: 1.98–12.3, $p = 0.001$; respectively) in RCC

patients. Using Kaplan-Meier analysis, a comparison of survival curves of patients with low versus high *LINC-ROR*, *SOX2*, *NANOG*, and *POU5F1* gene expression revealed significant differences in OS ($p = 0.018$, 0.008, 0.028, and 0.001, respectively),

Table IV. Correlation analysis between gene expression and the clinicopathological characteristics in RCC patients

Parameter	LINC-ROR		SOX2		NANOG		POU5F1	
	r	P-value	r	P-value	r	P-value	r	P-value
Age	-0.002	0.987	-0.189	0.148	0.009	0.949	-0.031	0.812
Gender	-0.245	0.059	0.065	0.624	-0.021	0.872	-0.064	0.629
HPD	0.076	0.563	0.045	0.733	0.031	0.816	-0.053	0.687
Side	0.014	0.916	-0.043	0.745	-0.045	0.733	0.194	0.138
Number	0.100	0.447	0.190	0.146	0.134	0.306	-0.092	0.484
Grade	0.387	0.002	0.314	0.014	0.265	0.041	0.077	0.558
Tumour size	0.185	0.158	0.304	0.018	-0.009	0.946	-0.141	0.282
LN	0.152	0.245	0.258	0.046	0.043	0.742	-0.100	0.448
Recurrence	0.383	0.003	0.274	0.034	0.139	0.289	0.190	0.145
PFS	-0.577	< 0.001	-0.511	< 0.001	-0.339	0.008	-0.419	0.001
OS	-0.576	< 0.001	-0.488	< 0.001	-0.403	0.001	-0.464	< 0.001

Data are shown as a correlation coefficient (r) and its p value. HPD – histopathological diagnosis, LN – lymph node, PFS – progressive-free survival, OS – overall survival. Spearman’s rank correlation test was used. Bold values are statistically significant at p < 0.05.

which suggested that high expression was associated with poor survival (Figure 4). Similar findings were found in samples stratified by the histopathological type.

Multivariate analysis

Bray-Curtis analysis was applied to combine the effects of both clinicopathological characteristics and the transcriptomic signatures of the four tested genes. Samples were scattered along multiple axes; axis 1 represented 30.1% of the variance, whereas axes 2 and 3 accounted for 14.6% and 11% of clustering, respectively. The study population was clustered into three distinct groups according to survival time (Figure 5). Notably, patients with poor survival (i.e. less than 1 year) were most affected by high LINC-ROR levels. The same category of patients was associated with poor differentiation and recurrence.

Discussion

A complex network of TFs, non-coding RNAs, and signalling transducers drives cancer. Unravelling the interactions between these molecular cancer players would pave the road towards a better understanding of cancer development and progression.

Currently, marked up-regulation of *LINC-ROR*, *SOX2*, *NANOG*, and *POU5F1* genes was found in cancer samples compared with the adjacent non-cancer tissues. Additionally, LINC-ROR expression levels were positively correlated with *SOX2* and *NANOG* levels. These results were consistent with previous studies [14, 15]. Pluripotent TFs, which are often epigenetically maintained in a silent state, have been shown to be reactivated in several cancers, and their knockdown or suppression with microRNA or small interfering RNA

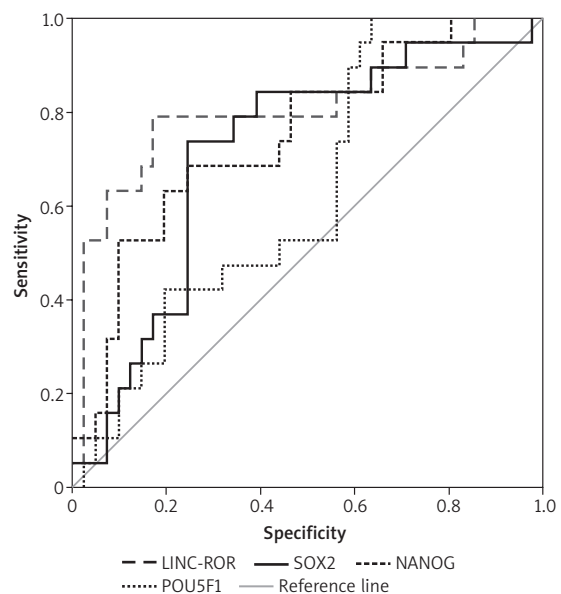


Figure 3. ROC analysis of RCC patients

transfection has been shown to hinder tumour progression, promote apoptosis, and improve chemosensitivity [21]. Interestingly, these TFs are now well known to operate in conjunction with non-coding RNAs. A few human lncRNAs may also be under the direct control of the core pluripotency TFs [22]. A recently identified cytoplasmic lncRNA, LINC-ROR, was reported to play a crucial role in the pluripotency maintenance [23]. The promoter of the *LINC-ROR* gene was found to contain binding sites for *SOX2*, *NANOG*, and *POU5F1* [24]. Upon binding of TFs, *LINC-ROR* transcription is activated, whereas silencing of these proteins suppressed *LINC-ROR* expression through a regulatory feedback loop [25]. Similarly to our findings, *LINC-ROR* over-expression has also been observed

Table V. The prognostic performance of single and joint detection of genes in renal cell carcinoma to predict survival time

Parameter	Area (95% CI)	P-value
Single gene:		
<i>LINC-ROR</i>	0.804 (0.667–0.941)	< 0.001*
<i>SOX2</i>	0.714 (0.573–0.854)	0.008*
<i>NANOG</i>	0.748 (0.615–0.882)	0.002*
<i>POU5F1</i>	0.632 (0.488–0.775)	0.103
Combination:		
<i>LINC-ROR</i> + <i>SOX2</i>	0.748 (0.652–0.845)	< 0.001*
<i>LINC-ROR</i> + <i>NANOG</i>	0.755 (0.659–0.850)	< 0.001*
<i>LINC-ROR</i> + <i>POU5F1</i>	0.715 (0.616–0.814)	< 0.001*
<i>SOX2</i> + <i>NANOG</i>	0.735 (0.640–0.831)	< 0.001*
<i>SOX2</i> + <i>POU5F1</i>	0.675 (0.576–0.773)	0.002*
<i>NANOG</i> + <i>POU5F1</i>	0.695 (0.599–0.791)	0.001*
<i>LINC-ROR</i> + <i>SOX2</i> + <i>NANOG</i>	0.743 (0.665–0.821)	< 0.001*
<i>LINC-ROR</i> + <i>SOX2</i> + <i>POU5F1</i>	0.711 (0.632–0.791)	< 0.001*
<i>LINC-ROR</i> + <i>NANOG</i> + <i>POU5F1</i>	0.720 (0.641–0.798)	< 0.001*
<i>SOX2</i> + <i>NANOG</i> + <i>POU5F1</i>	0.703 (0.624–0.781)	< 0.001*
<i>LINC-ROR</i> + <i>SOX2</i> + <i>NANOG</i> + <i>POU5F1</i>	0.719 (0.651–0.786)	< 0.001*

Under the non-parametric assumption. *Values indicate significance at $p < 0.05$.

Table VI. Multivariable analysis using logistic regression test to predict post-operative recurrence in renal cell carcinoma

Parameter	B	SE	Wald	P-value	OR	95% CI	
						Lower bound	Upper bound
LINC-ROR	0.001	0.000	3.938	0.047*	1.001	1.000	1.002
SOX2	-0.003	0.006	0.263	0.608	0.997	0.986	1.008
NANOG	0.000	0.010	0.002	0.961	1.000	0.981	1.019
POU5F1	0.000	0.000	0.088	0.767	1.000	1.000	1.000
Age	-0.104	0.062	2.781	0.095*	0.901	0.798	1.018
Gender	0.047	0.795	0.004	0.953	1.048	0.221	4.983
LN metastasis	2.137	0.796	7.207	0.007*	8.470	1.780	40.300
Constant	4.016	3.760	1.141	0.285	55.467		

LINC-ROR – long intergenic non-coding RNA-regulator of reprogramming, *SOX2*, *SRY* (sex determining region Y)-box 2, *NANOG* – named after Celtic word *Tír na nÓg* meaning the land of the young, *POU5F1* domain, class 5, transcription factor 1, LN – lymph node, SE – standard error, OR – odds ratio, CI – confidence interval. *Values indicate significance at $p < 0.05$.

in several cancers, including colorectal carcinoma [15] and breast cancer [25].

Another main finding of our study was the association of the four genes with poor prognosis. The Kaplan-Meier curves and ROC analyses showed that the *LINC-ROR*, *SOX2*, *NANOG*, and *POU5F1* expression profiles were associated with shorter survival times. RCC patients with high *LINC-ROR* and *SOX2* levels had a higher recurrence rate after an average of four months post-nephrectomy. Notably, regression analyses confirmed that *LINC-ROR* was an independent predictor for recurrence and poor OS. In addition, *LINC-ROR*, *SOX2*, and *NANOG* expression showed a significant associa-

tion with poor differentiation, whereas *SOX2* was a poor marker for LN metastasis. In accordance with previous studies, melanoma and HCC cells expressing high *POU5F1* and *NANOG* levels exhibited a more aggressive malignant phenotype [26]. *NANOG* expression has also been considered a prognostic biomarker for triple-negative breast cancer (TNBC) [27].

Recently, increasing attention has been focused on the contribution of *LINC-ROR* to cancer progression. *LINC-ROR* has been suggested to be a driving factor in tumorigenesis [24], as depicted in Figure 6. Increased *LINC-ROR* expression levels have been associated with differenti-

Table VII. Survival analysis of renal cell carcinoma patients

Parameter	Survival time, median (95% CI) [months]	Overall comparisons			Cox regression		
		Log rank	Breslow	Tarone-Ware	HR (95% CI)	Wald	P-value
Age:							
< 60 years	13 (9.8–6.1)	0.088	0.054	0.061	Ref.		
≥ 60 years	17 (14.9–19.0)				0.98 (0.94–1.03)	0.317	0.573
Gender:							
Female	13 (8.51–17.4)	0.127	0.077	0.095	Ref.		
Male	16 (13.3–18.6)				0.59 (0.26–1.33)	1.608	0.205
HPD:							
ccRCC	15 (12.3–17.6)	0.103	0.198	0.159	Ref.		
pRCC	17 (13.2–20.7)				0.60 (0.25–1.44)	1.307	0.253
chRCC	15 (7.42–22.5)				1.41 (0.62–3.11)	0.740	0.390
Side:							
Right	16 (12.3–19.6)	0.703	0.489	0.539	Ref.		
Left	15 (12.9–17.0)				1.06 (0.54–2.08)	0.034	0.854
Number:							
Unifocal	16 (14.3–17.6)	0.461	0.291	0.350	Ref.		
Multifocal	13 (11.1–14.8)				0.89 (0.38–2.10)	0.063	0.802
Grade:							
1	16 (13.1–18.9)	0.005*	0.003*	0.003*	Ref.		
2	18 (15.4–20.5)				0.47 (0.17–1.30)	2.101	0.147
3	12 (8.4–15.5)				0.82 (0.22–2.98)	0.089	0.765
T:							
T1	16 (11.5–20.4)	0.709	0.577	0.635	Ref.		
T2	16 (12.7–19.2)				0.84 (0.40–1.77)	0.195	0.659
T3	13 (9.3–16.6)				0.93 (0.26–3.34)	0.011	0.917
LN:							
Negative	16 (13.5–18.4)	0.306	0.235	0.255	Ref.		
Positive	14 (11.3–16.6)				0.83 (0.23–2.89)	0.084	0.772
Recurrence:							
Negative	17 (15.2–18.7)	< 0.001*	< 0.001*	< 0.001*	Ref.		
Positive	10 (9.1–10.9)				4.94 (1.98–12.3)	11.782	0.001*
LINC-ROR:							
Low	22 (13.5–30.4)	0.007*	0.018*	0.012*	Ref.		
High	15 (12.9–17.0)				4.21 (1.13–15.6)	4.641	0.031*
SOX2:							
Low	20 (16.6–23.3)	0.089*	0.008*	0.018*	Ref.		
High	14 (12.1–15.8)				2.57 (0.99–6.6)	3.814	0.051
NANOG:							
Low	18 (16.7–19.2)	0.295	0.028*	0.081	Ref.		
High	14 (11.8–16.1)				1.10 (0.50–2.41)	0.065	0.799
POU5F1:							
Low	22 (19.1–24.8)	0.003*	0.001*	0.001*	Ref.		
High	14 (12.1–15.8)				3.80 (1.55–9.30)	8.544	0.003*

CI – confidence interval, HR – hazard ratio, Ref. – reference, HPD – histopathological diagnosis, ccRCC – clear cell renal cell carcinoma, pRCC – papillary cell RCC, chRCC – chromophobe RCC, T – tumour size, LN – lymph node, PFS – progression-free survival, OS – overall survival. *Values are statistically significant at $p < 0.05$.

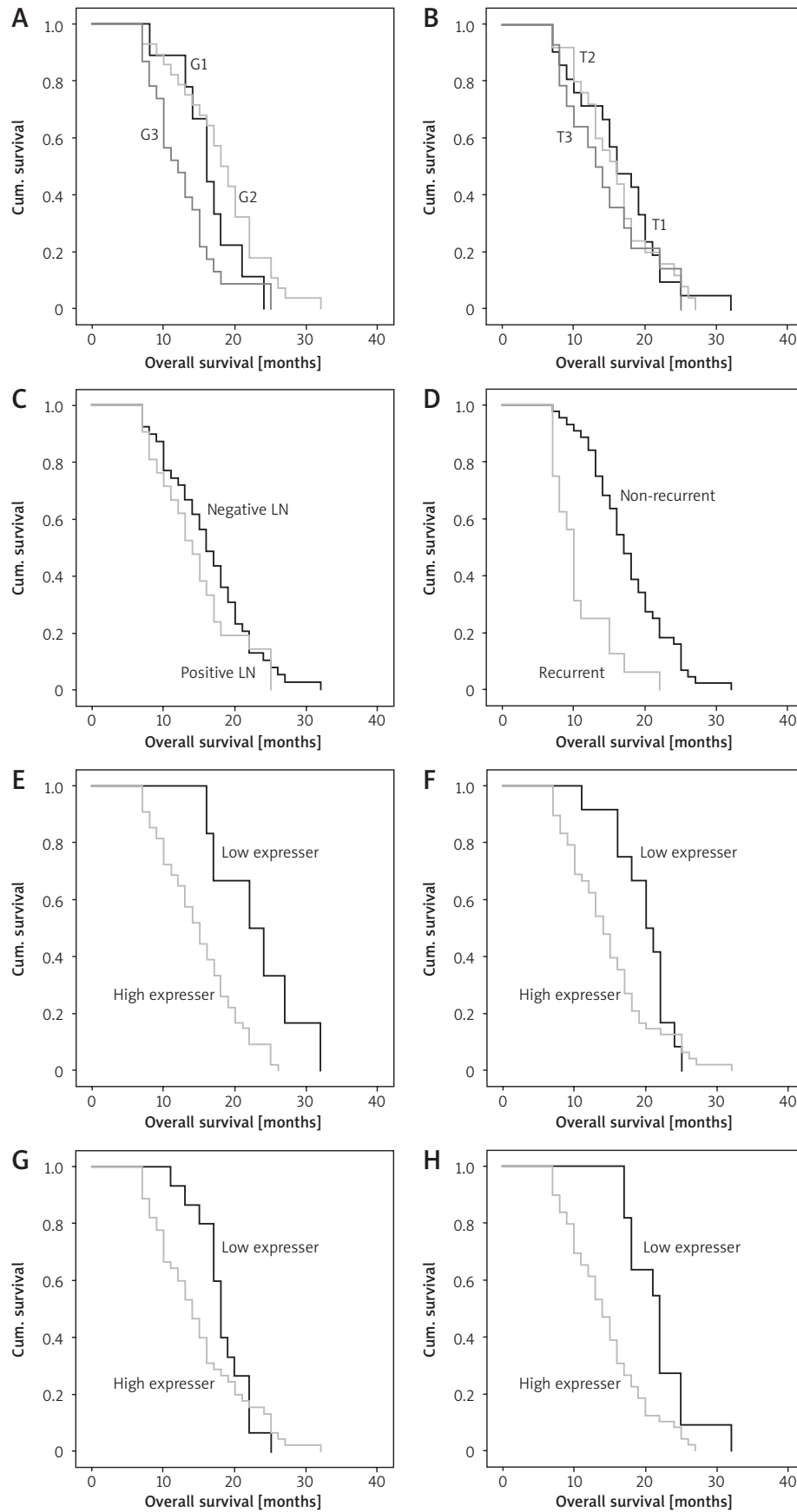


Figure 4. Kaplan-Meier curves comparing patient survival with different clinical variables (A – tumor grade, B – tumor size, C – LN infiltration, D – recurrence, E – LINC-ROR, F – SOX2, G – NANOG, H – POU5F1). Log-rank (Mantel-Cox) test was used for comparisons. The significance level was set at $p < 0.05$

ation, apoptosis, invasion, EMT, and metastasis in cancer [24, 28]. Up-regulation was recognised as a poor prognostic factor in colon cancer [29]. However, LINC-ROR silencing reduced the malignant phenotype of cancer cells [24]. One of the peculiar roles of lncRNAs is acting as competing endogenous RNAs (ceRNAs) by binding and sequestering microRNAs (miRNAs) and thus preventing miRNAs from silencing their target genes [30], a process that has been shown to be relevant to tumourigenesis and differentiation [12]. LINC-ROR has been demonstrated to serve as an miRNA sponge in embryonic stem cell self-renewal [31]. LINC-ROR effectively maintained the levels of the SOX2 TF by binding to miR-145 in human amniotic epithelial stem cells, altering the landscape of pluripotency and differentiation [32]. The tumour-promoting function of LINC-ROR can also be attributed to its influence on transcription frameworks involved in various cancer-related signalling pathways [24]. LINC-ROR can play an oncogenic role by modulating the c-Myc pathway [20]. LINC-ROR has also been shown to elicit re-expression of foetal genes as atrial and brain natriuretic peptides [28]. Additionally, upon DNA damage and expression of the tumour suppressor Tp53, LINC-ROR levels increased and suppressed Tp53 mRNA through a translation repression mechanism [16]. At the DNA level, LINC-ROR has been reported to block the recruitment of a histone-modifying enzyme (G9A methyltransferase),

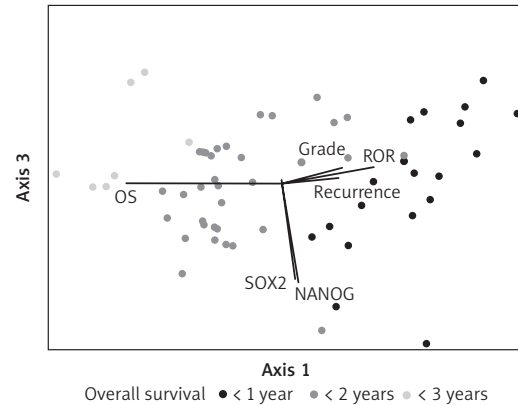


Figure 5. Multivariate analysis cluster of RCC patients according to the combined transcriptomic gene signature and clinicopathological features. PC-ORD v5.0 was used for data exploration. Ordination graph constructed via Bray-Curtis analysis. The following setup parameters were adjusted. Distance method: relative Euclidean method; end-point selection method: variance-regression; axis projection geometry: Euclidean; residual distances: Euclidean; score calculation by weighted averaging. Samples were scattered along multiple axes; axis 1 represents 30.1% of variance, whereas axes 2 and 3 account for 14.6% and 11% of clustering, respectively. The study population was clustered into three distinct groups according to survival time. Notably, most of the patients with poor survival (less than 1 year) manifested high levels of LINC-ROR. The same category of patients was associated with poor differentiation and recurrence

thereby triggering chromatin modification and promoting tumour growth and metastasis [32].

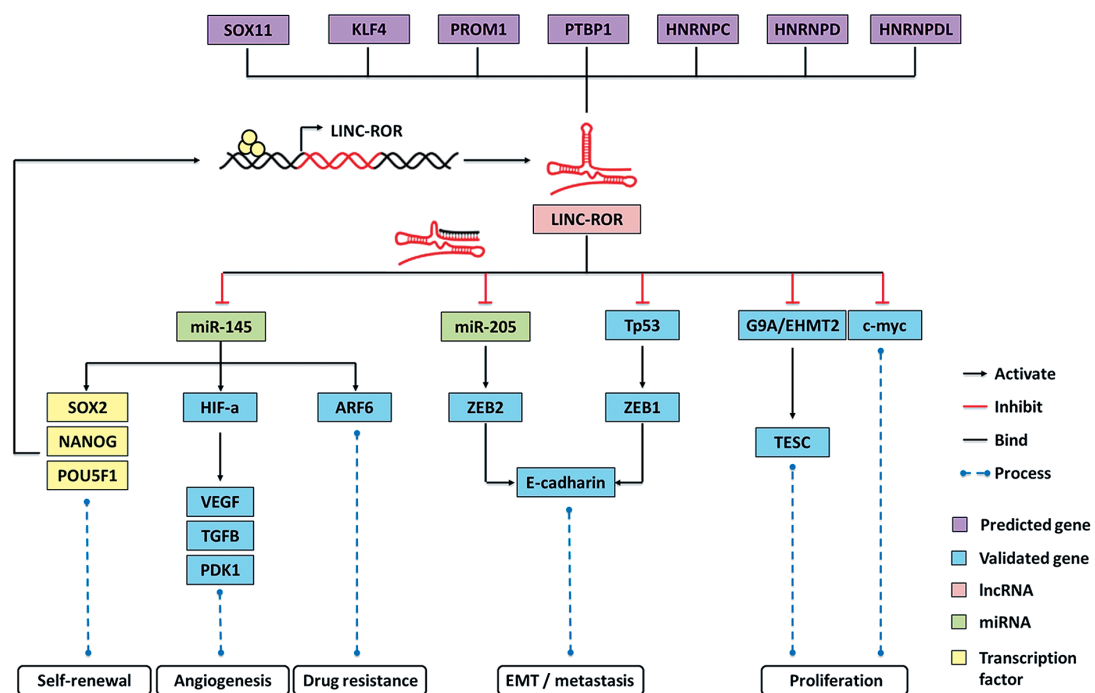


Figure 6. The role of LINC-ROR in tumourigenesis and cancer progression

In conclusion, deregulation of the LINC-ROR/pluripotent gene axis in renal tumours highlights their use as potential prognostic biomarkers. Targeting this RNA directly rather than targeting its regulated miRNAs, tumour suppressor genes, or oncogenes might provide a shortcut for future cancer therapies. However, due to limited clinical studies on LINC-ROR, further studies in other types of tumours are warranted.

Acknowledgments

The authors thank the Centre of Excellence in Molecular and Cellular Medicine and Oncology Diagnostic Unit for providing the facilities for work.

Conflict of interest

The authors declare no conflict of interest.

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