

# DNA methylation of neural EGFL like 1 (*NELL1*) is associated with advanced disease and the metastatic state of renal cell cancer patients

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**Abstract.** Recent studies have shown that *NELL1* expression is silenced epigenetically in human renal cell cancer (RCC) tissues and in RCC cell lines. However, it remains unknown whether *NELL1* promoter methylation observed in clinical specimens might be associated with the clinicopathology or survival of patients with RCC. We analyzed *NELL1* DNA methylation in tissues from patients with RCC and in adjacent normal renal tissues. In addition, we evaluated *NELL1* methylation in cell lines derived from different urogenital tumors (prostate cancer, urothelial cancer and RCC). We performed regression analyses to determine whether *NELL1* methylation is associated with clinicopathological parameters and recurrence-free survival (RFS). This cross-sectional study included 98 patients with RCC and 63 paired tumor and adjacent normal tissue samples. We analyzed a locus in the intron 1 region of *NELL1* with pyrosequencing. We performed *in silico* analysis of *NELL1* methylation in the TCGA Kidney Renal Clear Cell Carcinoma (KIRC) data set (n=284 patients), which served as a validation study. Statistical analyses were performed with the two-sided paired t-test for paired tumor and adjacent normal samples. We used logistic regression for subgroup comparisons and Cox regression for RFS comparisons. The mean methylation level was 6.8% higher in RCC tissues compared to paired adjacent normal tissues (paired t-test,  $P<0.001$ ). Methylation levels in RCC were associated with advanced disease ( $P=0.002$ ), the presence of distant metastases ( $P=0.004$ ), and shorter RFS ( $P=0.035$ , HR: 4.15). *In silico* validation with TCGA KIRC data for adjacent loci also demonstrated that

high relative methylation levels were associated with adverse clinicopathology and shortened RFS. Our results suggest that *NELL1* methylation contributes to RCC disease progression. This finding could provide a clinical marker to complement recent functional analyses in tumor models.

## Introduction

Renal cell cancer (RCC) is the twelfth most common cancer worldwide; in 2012, it accounted for about 338,000 new cases (1). A comprehensive characterization of the molecular alterations associated with RCC was recently carried out by the TCGA Kidney consortium (KIRC) (2). Notably, they described only limited associations between gene mutations and clinical or survival parameters in RCC patients. Thus, it was questionable whether the translation of genetic mutations or variant information would be useful for future personalized diagnoses, prognoses, or therapy predictions. This question was consistent with earlier genetic studies, which demonstrated inconsistent results concerning associations with clinical pathology or patient survival (3). In contrast, the KIRC data, like many previous studies, reported that clear cell (cc) RCC typically showed features of epigenetic alterations, such as hypermethylation of gene promoters and concurrent loss of gene function, due to transcriptional silencing. Indeed, it has been consistently observed, in a substantial number of genes, that DNA hypermethylation occurs with high frequency, and the degree of hypermethylation is correlated with clinical and pathological features of patients. In addition, the tumor suppressors known as RAS-associated domain family 1 (RASSF1A) and secreted frizzled-related protein 1 (SFRP1) were each found in 30% to >70% of tumors. These tumor suppressors were associated with both clinical pathological features and outcomes (4-7). In RCC, a significant incidence of epigenetic alterations has been found in the *COL1A1*, *IGFBP1*, *EDNRB* and *KRT19* genes, in cell lines and primary tumors (8,9). A recent report demonstrated tumor-specific hypermethylation in RCC, which was found to be correlated with adverse pathology and was associated with overall survival among patients undergoing targeted therapy (10).

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In view of the fact that a multitude of hypermethylated genes (11) have been described and that the KIRC data provide a large number of new candidate methylated loci for ccRCC, the question arises, how can we identify the most relevant genes? Based on traditional genetic approaches, we might assume that the most promising candidates for future translational purposes would be genes that are associated with functional losses, high alteration frequencies in tumors, and altered clinical behaviors in patients. Here, we focused on the clinical relevance of DNA methylation in the neural epidermal growth factor-like 1 (*NELL1*) gene. *NELL1* is a multimeric extracellular glycoprotein, originally identified in neural tissues (12). The gene is located on chromosome 11p15.1, and it is involved in osteogenic differentiation in mammals and bone regeneration in defects of the calvarium (13,14).

*NELL1* expression has been detected in normal embryonic kidney, brain and prostate, and in bladder cancer, lymphomas and different cancer cell lines (15-18). A recent study revealed that *NELL1* protein expression was reduced in RCC tissues (19). Considering that promoter hypermethylation and diminished mRNA expression were detected in RCC cell lines, transcriptional silencing was assumed to explain low *NELL1* expression in RCC (19).

No studies have described *NELL1* promoter methylation in RCC tissues or its correlation to clinicopathological parameters or clinical outcome. Here, we investigated *NELL1* promoter methylation in cancerous and adjacent non-cancerous tissues derived from patients with RCC and a subset of patients with ccRCC and in 18 malignant and benign renal cancer cell lines. Results were confirmed *in silico* with the KIRC data set.

## Materials and methods

**Tissue specimens.** Ninety-eight tumor tissue samples were obtained from kidney surgeries carried out between 2001 and 2005 at Eberhard Karls University, Tübingen. Tissue sampling, storage, and processing were performed as described previously (20). Patient characteristics are provided in Table I. Pairs of tissue samples from tumors (TU) and adjacent tumor-free tissue (adN) were obtained from 63 patients.

The Ethics Committee of the institution of Eberhard Karls University approved the study (vote no. 128/2003V and 1213-2011) and informed consent was obtained from each patient. Two pathologists evaluated all specimens, with respect to tumor stage, grade and histological subtypes. Tumor stages were assessed according to the UICC 2002 issue of the TNM system, and nuclear grading was based on the Fuhrman grading system (21). Histological subtypes were assessed according to the consensus classification of RCC. Localized RCC was defined as pT  $\leq$ 2, N0/M0; advanced tumors were defined as pT  $\geq$ 3 and/or N1/M1. Low-stage tumors were defined as the group of pT1 and pT2 tumors; high stage tumors were defined as the group of pT3 and T4 tumors. Follow-up data were available for 24 patients. The duration of the follow-up was calculated from the date of surgery to the date of recurrence, progression, death, or the last follow-up.

**Primary and cancer cell lines.** Prostate cancer (LN-cap, DU-145), urothelial cancer (CLS439, HB-CLS2, HB-CLS1,

Table I. Clinicopathological parameters of the discovery and evaluation cohort.

	Hannover cohort	TCGA cohort
Total cases	98	284
Sex		
Female	35	96
Male	63	188
Age (years)		
Median	64	61
Minimum	35	26
Maximum	91	90
T-classification		
pT1	10	15
pT1a	26	72
pT1b	17	46
pT2	7	32
pT3	3	3
pT3a	9	65
pT3b	22	38
pT3c	1	NA
pT4	1	8
Lymph node status		
N0	85	127
N1	13	9
Nx	NA	148
Distant metastasis		
M0	79	232
M1	19	52
Differentiation		
G1	22	5
G1-2	14	NA
G2	48	117
G2-3	6	NA
G3	8	112
G4	NA	48
Gx	NA	2
Status of disease		
Localized <sup>a</sup>	51	NA
Advanced <sup>b</sup>	46	NA
Paired tissue samples <sup>c</sup>	63	160

<sup>a</sup>pT  $\leq$ 2, N0, M0. <sup>b</sup>pT  $\geq$ 3 and/or N1, M1. <sup>c</sup>Tumor and adjacent normal renal tissue. NA, not available.

5637), and RCC (ACHN, A498, 786-O, RCC-GS, RCC-HS, RCC-MF) cell lines were purchased from Lonza (Basel, Switzerland). Primary normal cell lines (RPTEC, primary renal proximal tubular epithelial cells) were acquired from Cell Lines Services (CLS, Eppelheim, Germany). All cells were cultured according to the manufacturer's protocol and as described previously (20,22).

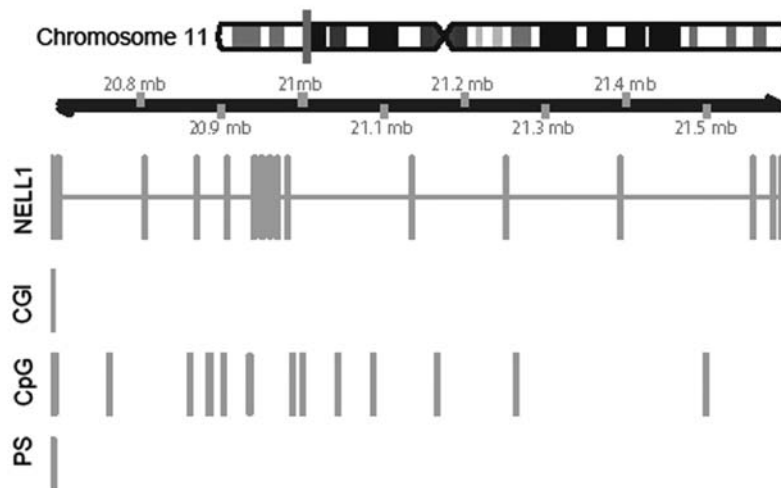


Figure 1. *NELL1* gene information. Schematic Figure of chromosome 11 with information concerning the position of the *NELL1* gene and the *NELL1* transcript used for pyrosequencing (PS), in relation to the CpG-island (CGI) and CpG sites (CpG).

**DNA extraction and bisulfite conversion.** DNA extraction, bisulfite conversion, and control of tumor cell content were performed as reported previously (22). Total DNA was extracted from 20 serial frozen sections (20  $\mu$ m) from each tumoral and normal specimen after proteinase K digestion, with the standard phenol/chloroform extraction method. Each 1  $\mu$ g of extracted DNA was then subject to bisulfite conversion, as described previously, and aliquots were stored at  $-20^{\circ}\text{C}$  (5). Additionally, two serial sections of each tissue sample were stained with hematoxylin-eosin and re-evaluated by pathologists.

**Bisulfite-pyrosequencing for *NELL1* promoter methylation analysis.** We performed methylation analyses of ten *NELL1* CpG sites (base positions: 20,587, ~591, ~596, ~604, ~611, ~615, ~618, ~626, ~634, ~636, ~691) within the CpG island (CGI), which extended over the 5'UTR and exon 1 of the *NELL1* gene (Fig. 1, 'CGI' and 'Pyro'). Analyses were performed with bisulfite-pyrosequencing, according to the manufacturer's protocol (Qiagen, Hilden, Germany) and as described previously (6,23). We used a two-step PCR protocol. For the first step, the primers were: Forward (5'-GGGATGAATTGTGGT AATT-3') and reverse (5'-GGGACACCGCTGATCGTTTAC TACRAAAATCTAACTACAAA-3'). For the second step, the primers were: Forward (5'-GGAAGTAAAGAGGGTAAT ATTG-3') and reverse (Biotin-5'-GGGACACCGCTGATC GTTTA-3'). Measurements were performed with the PyroMark Q24 pyrosequencing system (Qiagen, Hilden, Germany).

**Statistical analysis.** Statistical analyses of *NELL1* methylation levels in paired TU and adN tissue samples were performed with the two-sided paired t-test. Tumor subgroups were assessed with univariate logistic regression analysis and dichotomization, as specified. Visual comparisons of subgroups were performed with notched boxplots, which show the median, the 25% quartiles, and the estimated confidence intervals (notches). Univariate Cox regression analyses were performed to examine recurrence-free survival (RFS). Hazard ratios (HR), 95% confidence intervals (95% CI), and P-values were calculated. Survival was evaluated with the Kaplan-Meier

analysis. All analyses were performed with R version 3.2. The 'maxRank' package was used to calculate the optimum cutoff for the survival analysis (24). Therefore, the corresponding value of 15% is a result of a mathematical calculation in R using the Package 'OptimalCutpoint'.

P-values  $<0.05$  were considered to indicate statistical significance. *In silico* validation was carried out with the subset of level 3 data from the KIRC data set, annotated for *NELL1*. Distributions of the methylation differences obtained for each pair of TU and corresponding adN samples were calculated for each annotated CpG site. We used quality criteria for statistical information on the sites. Consequently, only CpG sites that showed approximately normal or bimodal density distributions were considered for further evaluation. We excluded CpG sites characterized by methylation differences that exhibited more or less discrete and constant values, in the paired tissue group comparisons.

## Results

***NELL1* methylation in tumor cell line models.** Twelve cell lines that represented urological cancer models and one normal tissue cell model (RPTECs) were analyzed for *NELL1* methylation. RPTECs demonstrated low relative methylation values (~3%) and a large number of kidney tumor cell lines, including A498, RCC-HS, and RCC-GS, displayed high relative methylation (30-60%; Fig. 2). Two kidney cancer cell lines (786-O and ACHN) displayed medium relative methylation (10-20% maximum). The prostate cancer cell lines, LN-cap and DU-145, exhibited elevated methylation, and two (HB-CLS2, CLS-439) out of four urothelial cancer cell lines displayed high methylation (~40%, Fig. 2).

***NELL1* DNA hypermethylation in paired tumor and adjacent normal renal tissue samples.** To investigate whether *NELL1* showed tumor-specific DNA hypermethylation, we compared TU paired with adN tissue samples. We found that TU tissues displayed a significantly higher mean methylation level (6.8%) compared to paired adN tissues (paired t-test,  $P=1.64\text{e}^{-5}$ , Fig. 3).

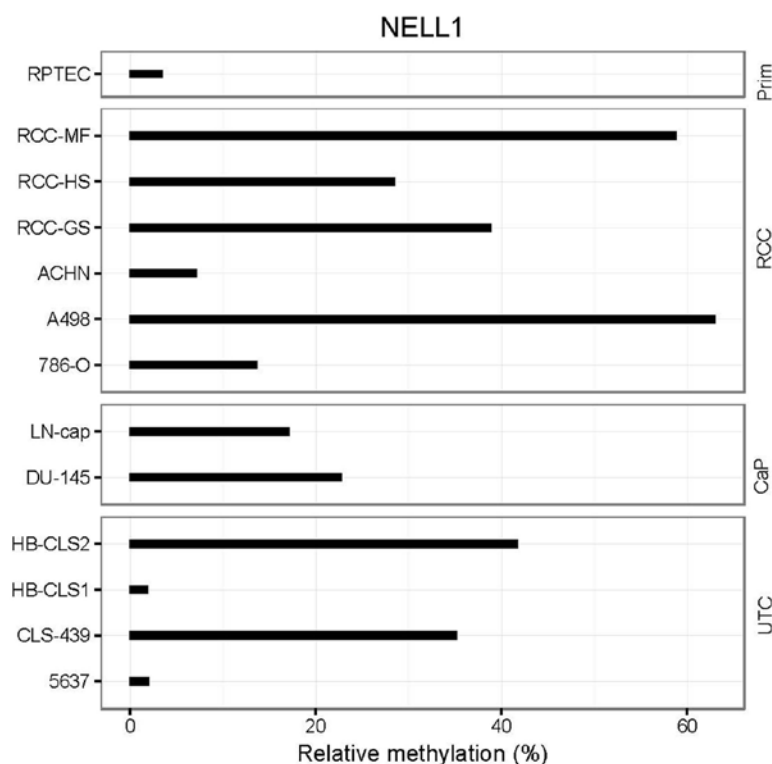


Figure 2. Methylation of *NELL1* in urological cancer cell lines. The diagram shows the relative methylation of *NELL1* measured in six renal cell cancer (RCC), two prostate cancer (CaP), and four urothelial cancer (UTC) cell lines. A normal kidney cell line (RPTEC) was used as a negative control.

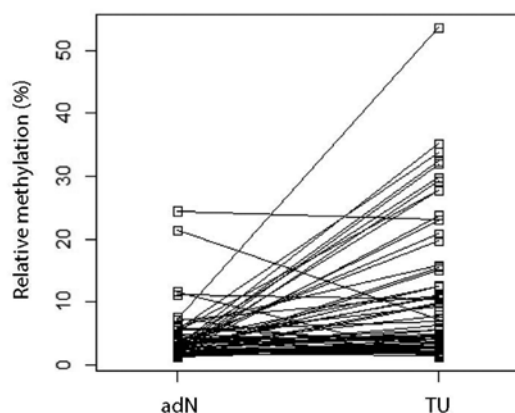


Figure 3. Methylation of *NELL1* in paired tumor and normal tissue samples. Comparison of relative *NELL1* methylation values in tumor (TU) and adjacent normal (adN) tissues acquired from patients with renal cell cancer (RCC). The mean relative methylation level is significantly higher in TU (6.8%) compared to adN ( $P < 0.001$ ).

#### *NELL1* DNA methylation and clinicopathological parameters.

To investigate whether the clinicopathological parameters of the patients might be associated with altered *NELL1* DNA methylation, patients were grouped according to different clinicopathological parameters, and then subjected to univariate logistic regression analyses, following the corresponding dichotomization. We found that methylation was significantly increased in the group with advanced tumors (mean methylation 12.6%) compared to those with localized disease [6.1%;  $P = 0.002$ ; odds ratio (OR)=1.08; Fig. 4A]. Furthermore, the presence of distant metastases in patients was associated with a

higher mean methylation (15.8%) compared to patients without clinically detectable distant metastasis (7.8%,  $P = 0.004$ ; OR: 1.07; Fig. 4B). Low- and high-stage tumor groups had mean methylation values of 7.5 and 11.8%, respectively ( $P = 0.044$ ; OR: 1.05). No significant difference in mean methylation was observed between groups that exhibited low and high histological tumor grades (Table II).

#### *NELL1* promoter methylation and recurrence-free survival.

Kaplan Meier and Cox regression analyses were performed to assess the relationship between *NELL1* DNA methylation and the RFS of patients. We dichotomized patients according to the degree of DNA methylation, based on a cut-off value of 15% relative methylation. The Kaplan Meier analysis indicated that high *NELL1* methylation was associated with a worse RFS (Fig. 5). The univariate Cox regression analysis identified *NELL1* methylation as a significant risk factor for RFS (HR: 4.15, 95% CI: 1.10-15.6;  $P = 0.035$ ).

#### *In silico* validation with KIRC data.

We assessed the KIRC data set for associations between tumor-specific hypermethylation, clinicopathological parameters and survival, in an independent patient cohort. Out of 15 annotated CpG sites selected in a pre-analysis quality check (see Materials and methods), five showed hypomethylation (paired t-test,  $P < 0.05$ , adjusted with Bonferroni-Hochberg for multiple testing) and six showed hypermethylation ( $P < 0.05$ ). We observed significant associations between the methylation state and the state of metastasis in seven out of 15 CpG sites (univariate logistic regression:  $P = 1.8 \times 10^{-2}$  to  $7.3 \times 10^{-7}$ , OR: 12-546). Four of these CpG sites were located within or adjacent to the CGIs

Table II. Overview of *NELL1* DNA methylation analyses in primary RCC tissue samples, and statistical comparison with clinicopathological parameters.

<i>NELL1</i> methylation	Mean relative methylation (%)		P-value <sup>d</sup>	Odds ratio	95% confidence interval
Localized/advanced <sup>a</sup>	6.07	12.62	<b>0.002</b>	1.08	1.03-1.15
Low/high grade <sup>b</sup>	8.60	13.63	0.096	1.04	0.99-1.09
Distant metastasis (M0 vs. M1)	7.75	15.85	<b>0.004</b>	1.07	1.02-1.12
Low/high stage <sup>c</sup>	7.48	11.76	<b>0.044</b>	1.05	1.00-1.09

<sup>a</sup>Localized disease: pT $\leq$ 2, N0, M0; advanced disease: pT $\geq$ 3 and/or N1, M1, <sup>b</sup>Low grade (G1/G1-2); high grade (G2-3/G3). <sup>c</sup>Low stage (pT1/T2), high stage (pT3/T4). <sup>d</sup>Univariate logistic regression. P-values denoted in bold print indicate statistical significance. RCC, renal cell cancer.

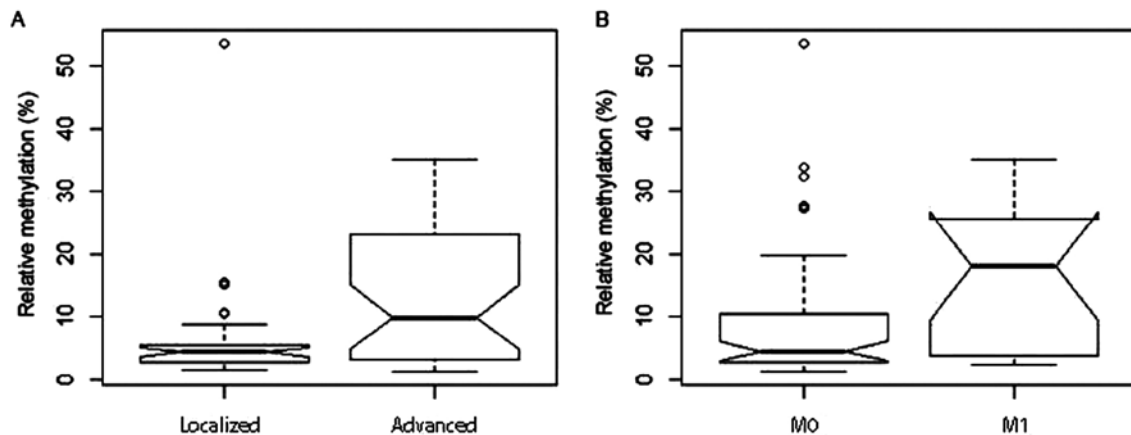


Figure 4. Box plot illustration of *NELL1* methylation in renal cell cancer (RCC). Distribution of relative *NELL1* DNA methylation levels among patients with RCC that exhibited (A) localized or advanced disease, and (B) the presence (M1) or absence (M0) of distant metastases. The notched box plot illustrates the median, the estimated confidence intervals, and the 25% quartiles in both groups. Statistical analyses demonstrated significantly higher mean methylation levels in advanced and M1 groups (see Table II).

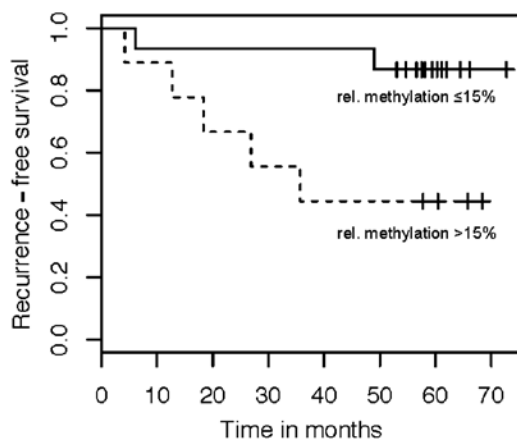


Figure 5. Kaplan-Meier survival analysis. Recurrence-free survival (RFS) in patients with methylation levels above the calculated cut-off point (dotted line) and in patients with methylation levels below the cut-off point (continuous line). The optimal calculated cut-off point was 15% methylation.

presented in Fig. 6. Notably, the corresponding CpG sites were also associated with high-stage tumors, high-grade tumors, and the RFS of patients (Fig. 6).

## Discussion

*NELL1* DNA methylation and epigenetic silencing of gene expression were previously described in RCC cell lines, which suggested that epigenetic alterations were potentially relevant to RCC pathology (19). In the present study, we aimed to ascertain whether *NELL1* DNA methylation was associated with clinicopathological parameters and disease outcome in patients with ccRCC. Comparisons between tumors (TU) and adjacent tumor-free tissue (adN) clearly demonstrated that tumor-specific hypermethylation occurred in the loci analyzed. An interrogation of the KIRC data set showed that several CpG sites in the extended CGI region at exon 1 of the gene also exhibited clear hypermethylation, which confirmed results from former cell line experiments and from the present study. Of note, the DNA methylation observed in cell lines probably reflected both intra-tumoral and inter-patient variability at each locus investigated. Indeed, both baseline methylation and age-dependent methylation show considerable variation among different organ systems. Thus, some of the observed differences in methylation reflected the normal biological variations in methylation expected among different tissues and tissue donors.

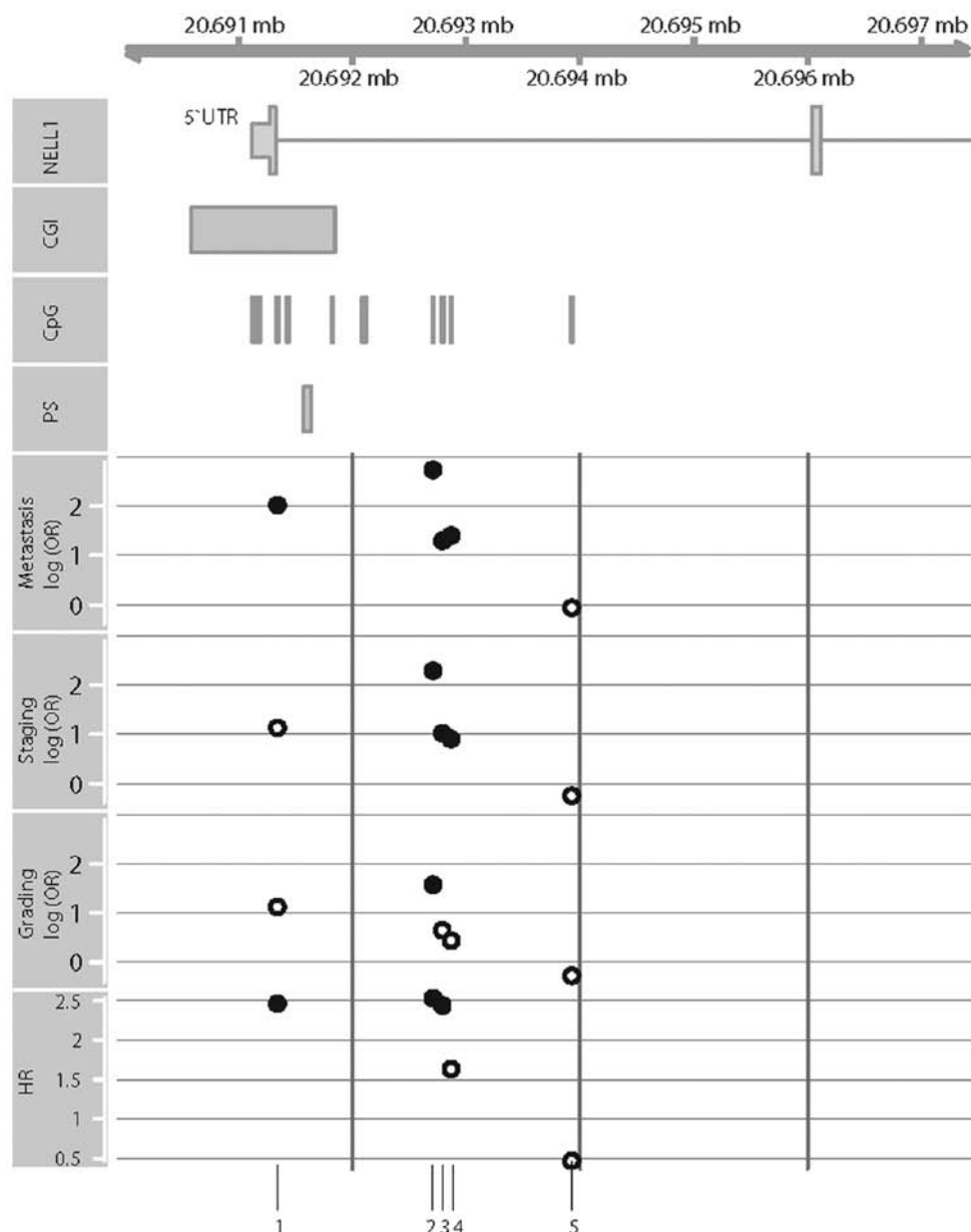


Figure 6. Schematic illustration of the 5'UTR and exon 1 of the *NELL1* gene and the allocated CpG island (CGI) and CpG sites (CpG). The blue bar in row four illustrates our pyrosequencing assay (PS), which was used to determine relative methylation. The second part of the Figure shows the statistical associations between *NELL1* methylation levels in the KIRC cohort and the status of metastasis, tumor stage, tumor grade, and survival. Circles indicated the investigated CpG sites in the TCGA cohort: Filled circles show significant associations between hypermethylation and clinicopathology; open circles indicate statistically non-significant associations. We used *in silico* analyses to investigate these five CpG sites, annotated by the KIRC TCGA consortium (1. cg04261192; 2. cg20764116; 3. cg23549358; 4. cg01461552; 5. cg09150140), among others (described in the Results section). At a majority of the investigated CpG sites in the TCGA cohort, DNA methylation of *NELL1* was highly significantly associated with patient survival (max. HR 2.5; last bar). OR, odds ratio; HR, hazard ratio.

Previous studies indicated that the loss of *NELL1* mRNA and concurrent loss of *NELL1* protein expression are associated with altered tumor cell behavior. Those findings strongly indicated that epigenetic silencing of the *NELL1* gene is functionally relevant (19). Therefore, our finding that high levels of DNA methylation were associated with adverse clinicopathology in patients supported the hypothesis that the loss of *NELL1* function promotes the development of aggressive ccRCC.

DNA methylation of *NELL1* has also been shown in other human cancers. For example, a high frequency of *NELL1* methylation was reported in colon cancer, early-stage esophageal adenocarcinoma (EAC), and gastric cancer (25-27).

Notably, in EAC, *NELL1* methylation was significantly associated with a poor prognosis in patients (25,26). No previous study had investigated whether *NELL1* methylation might also be correlated with disadvantageous clinical parameters, such as advanced disease, presence of distant metastases, or worse survival in RCC. Therefore, our finding that higher methylation was significantly associated with metastatic and advanced disease in patients with RCC extended the number of human malignancies that showed associations between *NELL1* methylation and aggressive tumor biology.

Similarly, our survival analysis indicated that *NELL1* methylation could serve as a prognostic indicator of RFS in patients. However, due to our small survival cohort, a



multivariate analysis was not feasible; due to this limitation, the relevance of this part of the clinical evaluation should be interpreted with caution.

On the other hand, our evaluation of KIRC data confirmed our finding that *NELL1* methylation in the CGI and adjacent CpG sites were associated with the state of distant metastasis, the tumor grade, and the tumor stage. Notably, three of five CpG sites in this region also showed associations with RFS. However, considering that multivariate significance could not be achieved in the *in silico* survival analyses, the association between *NELL1* methylation and patient survival remains to be confirmed in appropriate study cohorts. Moreover, from a technical point of view, only a small fraction (roughly estimated at about 1%) of the totally available CpG sites could be considered for *in silico* TCGA methylation analyses. Thus, the combined number of CpG sites was limited to 25 sites in our experimental and *in silico* approaches. Hence, most likely, in future, it will be necessary to extend both the study cohorts and the number of target CpG sites to determine the relevance of *NELL1* DNA methylation detection for predicting the clinical course of RCC and for translating these findings into individualized therapy and follow-up regimes.

In conclusion, our analyses suggested that *NELL1* DNA methylation is a promising candidate prognostic epigenetic biomarker for more detailed analyses of RCC disease. This finding provides a potential clinical marker to complement previously defined functional data (19). Furthermore, our finding that the prostate and urothelial cancer cell line models demonstrated high relative methylation levels indicated that *NELL1* DNA methylation might be also relevant to investigations of other frequent tumor entities. Thus, *NELL1* methylation might serve as a potential target structure for investigations with molecular therapeutic objectives.

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### Availability of data and materials

All data and materials are available at our local laboratory (Hannover Medical School, 30625 Hannover, Dept. of Urology).

### Authors' contributions

IP wrote the manuscript, prepared the figures, and participated in the study design. ND carried out the methylation analyses and participated in the sequence analyses. JH and IP assembled histopathological, clinicopathological, and survival data. ND and JH isolated and characterized the tissue samples and collected the patients' data. WIA re-evaluated the histopathology, revised the manuscript critically and finally approved the full version. MAK, CAJvK, HT, and AS contributed to the study design, revised the manuscript critically and did the final approval of the version for publication. JS identified the

candidate promoter, conceived the study, developed the study design and analytical assays, constructed and ran the clinical database, performed the statistical analyses (together with IP), and participated in the manuscript preparation. All authors read and approved the final manuscript and provided consent for publication.

### Ethics approval and consent to participate

The Ethics Committee of the Eberhard Karls University approved the study (vote no. 128/2003V and 1213-2011) and informed consent was obtained from each patient.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare no competing interest.

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