ZNF492 and *GPR149* methylation patterns as prognostic markers for clear cell renal cell carcinoma: Array-based DNA methylation profiling

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Abstract. The present study aimed to identify novel methylation markers of clear cell renal cell carcinoma (ccRCC) using microarray methylation analysis and evaluate their prognostic relevance in patient samples. To identify cancer-specific methylated biomarkers, microarray profiling of ccRCC samples from our institute (n=12) and The Cancer Genome Atlas (TCGA) database (n=160) were utilized, and the prognostic relevance of candidate genes were investigated in another TCGA dataset (n=153). For validation, pyrosequencing analyses with ccRCC samples from our institute (n=164) and another (n=117) were performed and the potential clinical application of selected biomarkers was examined. We identified 22 CpG island loci that were commonly hypermethylated in ccRCC. Kaplan-Meier analysis of TCGA data indicated that only 4/22 loci were significantly associated with disease progression. In the internal validation set, Kaplan-Meier analysis revealed that hypermethylation of two loci, zinc finger protein 492 (*ZNF492*) and G protein-coupled receptor 149 (*GPR149*), was significantly associated with shorter time-to-progression. Multivariate Cox regression models revealed that hypermethylation of *ZNF492* [hazard ratio (HR), 5.44; P=0.001] and *GPR149* (HR, 7.07; P<0.001) may be independent predictors of tumor progression. Similarly, the methylation status of these two genes was significantly associated with poor outcomes in the independent external validation cohort. Collectively, the present study proposed that the novel methylation markers *ZNF492* and *GPR149* could be independent prognostic indicators in patients with ccRCC.

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Introduction

Renal cell carcinoma (RCC) is a common malignancy of the genitourinary system, and is classified into various subtypes based on histopathological characteristics and each subtype has a diverse prognosis (1,2). From a clinical viewpoint, three main RCC subtypes are important in research: Clear cell RCC (ccRCC; 80-90% cases), papillary RCC (10-15% cases), and chromophobe RCC (4-5% cases) (1,2). The development of RCC is complex, and numerous parameters, including anatomical, histological, clinical and molecular factors contribute to disease outcome. Despite great improvements in understanding the major molecular mechanisms involved

in RCC, none of the conventional or molecular markers have demonstrated satisfactory sensitivity and specificity to be considered as a prognostic indicator of RCC (1,2). Thus, the majority of studies have focused on the development of suitable biomarkers to improve diagnosis and prognostication in RCC.

As a result of recent advancements in understanding of epigenetic modifications, DNA methylation is now regarded as one of the key findings in the development and progression of cancer. In RCC, several DNA methylation biomarkers associated with tumor development, histological subtypes, prognosis and response to therapy have been reported (3-13); however, the majority of these markers were identified in cohorts examined in single-center studies with variable results. Therefore, their use in routine clinical practice is limited. The development of high-throughput bioinformatics technology, such as genome-wide methylation pattern analysis, now enables tumor-specific novel molecular markers to be identified from numerous samples that can readily be compared with other cohorts via database sharing.

Establishing the association between biomarkers and clinical outcomes in RCC is likely to prove useful for developing effective treatment strategies and improving clinical management. The aim of the present study was to identify novel biomarkers that could predict the outcomes of RCC using genome-wide DNA methylation microarray data derived from patients analyzed in our institute and from The Cancer Genome Atlas (TCGA), and to evaluate their clinical application in ccRCC cases with long-term follow-up.

Materials and methods

Subjects and sample collection. A total of 355 human kidney specimens were obtained from patients (age range: 30-83 years, sample collection: February 1999 to September 2015) with primary, histologically-confirmed ccRCC who underwent radical or partial nephrectomy from two independent institutes including: i) 201 human kidney specimens [164 ccRCC and 37 matched normal control (NC)] from Chungbuk National University Hospital (CBNUH); and ii) 154 human kidney specimens (117 ccRCC and 37 matched NC) from Kyungpook National University Hospital (KPNUH). To enhance the homogeneity of the study population, patients were enrolled into the study according to the following criteria: i) clinical and pathological stage T1-T4 without lymph node or distant metastasis; and ii) a minimum follow-up period of 3 months. Patients whose samples had a positive surgical margin at final pathology were not included to avoid bias in survival analysis. The pathology of samples was independently reviewed by a pathologist unaware of the intended use of the clinical data. The specimens were obtained from the CBNUH and KPNUH, members of the National Biobank of Korea, which is supported by the Ministry of Health, Welfare and Family Affairs. The collection and analysis of all samples were approved by the Institutional Review Board of the CBNUH (approval no. 2010-01-001) and KPNUH (approval no. KNUMC 2016-05-021). Informed consent was obtained from each patient. The study design and validation strategies were presented in Fig. 1. Tumors were categorized based on pathological stage and histological subtype, as previously described (14,15). Nuclear differentiation was graded according to the Fuhrman Nuclear Grade system (16). All patients were followed-up and managed according to standard recommendations (1). Progression was defined as lymph node involvement and/or metastatic disease as determined by a computed tomography scan or bone scan.

DNA methylation profiling. Genomic DNA (gDNA) from patient's samples was extracted according to the manufacture's protocol by using the Wizard Genomic DNA Purification System (Promega Corporation, Madison, WI, USA). Bisulfite-modified gDNA was prepared using the EZ DNA Methylation-Lightning kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The methylation status was assayed using the Infinium HumanMethylation450 BeadChip array (Infinium Methylation 450K; Illumina, Inc., San Diego, CA, USA), which assesses the methylation status of >480,000 CpG sites distributed across the whole genome. A total of 24 matched DNA samples (pairs of NC and ccRCC from 12 patients) were used for DNA methylation profiling. Fluorescence signals corresponding to C- or T-nucleotides were measured (GenomeStudio Methylation Module, V1.8, Illumina, Inc., San Diego, CA, USA), and the data were used to assign a quantitative measure of methylation of specific CpG islands (β value): Ranging from 0 (complete unmethylation) to 1 (complete methylation). The complete sets of microarray methylation data are available online (http://www.ncbi.nlm.nih.gov/geo/; accession no. GSE92482.

Array profiling data from TCGA. For comparisons with the array data from the selected CBNUH patient samples and to investigate the prognostic relevance of the identified methylated CpG islands, the TCGA kidney renal clear cell carcinoma (KIRC) level 3 data set, also obtained using the Infinium HumanMethylation450 BeadChip, was investigated (https://cancergenome.nih.gov/). Methylation profiles of 313 ccRCC patient samples were available within the KIRC dataset. The dataset was divided into two groups: i) Training set, 160 pairs of ccRCC and NC matched samples; and ii) test set, an addition 153 ccRCC samples without matched NC samples. The training set data were used for comparisons with the candidate CpG sites identified with the CBNUH samples, and the test set data were used for assessing the prognostic relevance of those candidates. Baseline characteristics of the KIRC patients are provided in Table SI.

Pyrosequencing (PSQ) analysis. The DNA methylation status of the candidate CpG island loci identified using the Infinium HumanMethylation450 array were specifically assessed by PSQ analysis of 331 human kidney samples (CBNUH, 152 ccRCC and 25 matched NCs; KPNUH, 117 ccRCC and 37 matched NCs) using a PyroMark Q96 ID instrument (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. Each primer was designed using Pyrosequencing Assay Design Software v2.0 (Qiagen, Inc.). PSQ primers were designed to encompass the CpG island loci determined from the Illumina Infinium array. The primer sequences and amplification conditions are described in Table SII.
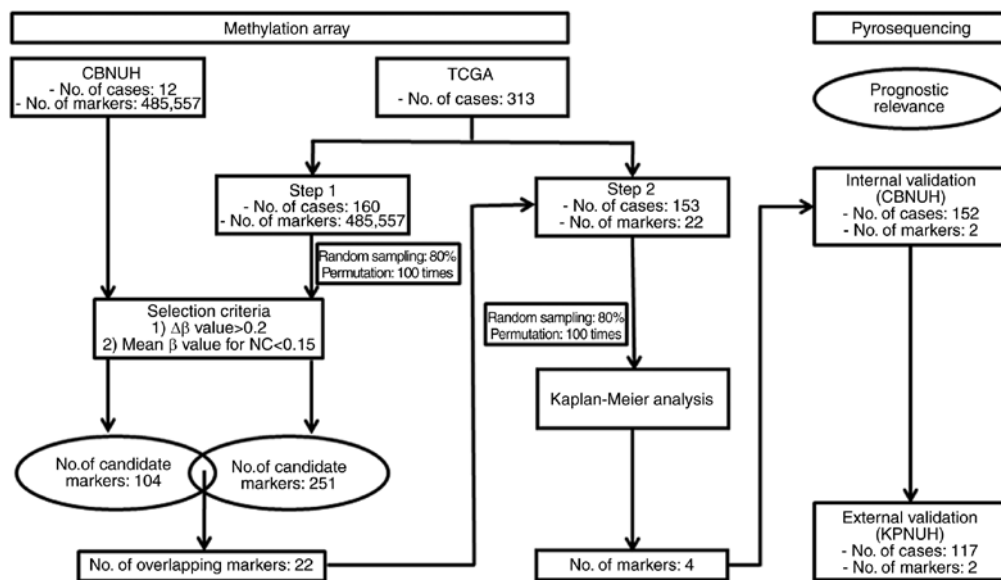
Figure 1. Study design and validation strategies. Genome-wide microarray methylation profiles of ccRCC and corresponding NC samples from CBNUH and TCGA cases were compared to identify ccRCC-specific methylated genes. CBNUH cases: pairs of ccRCC and normal tissue from 12 patients; TCGA cases: pairs of ccRCC and normal control samples from 160 patients. Step 1, TCGA training set; step 2, TCGA test set. Random sampling: 80%; Permutation: 100 times. Pyrosequencing assays used to validate the candidate markers in CBNUH and KPNUH cases. ccRCC, clear cell renal cell carcinoma; CBNUH, Chungbuk National University Hospital; TCGA, The Cancer Genome Atlas; KPNUH, Kyungpook National University Hospital.

Statistical analysis. The levels of DNA methylation were normalized using quantile normalization in R (v2.10.0, http://www.r-project.org/). The criteria applied for the selection of ccRCC-specific methylated CpG island loci were as follows: i) a difference in the levels of DNA methylation between ccRCC and NC ($\Delta\beta$ value) >0.2; and ii) a mean β-value for NC<0.15. Using the same selection criteria, the training set from the TCGA data was used for comparisons with genes identified from CBNUH ccRCC samples. With the same selection criteria of CBNUH samples, a permutation test (80% random sampling; 100 times) was conducted to reduce unduly identified CpG island loci. Only CpG island loci that possessed >50-times difference were selected for further analysis. Among the candidate CpG island loci identified, those common to the CBNUH ccRCC samples and the TCGA training set were selected as candidate prognostic indicators of ccRCC. The prognostic relevance of candidate CpG island loci was explored with the TCGA test dataset using Kaplan-Meier analysis (100 times permutation, 80% sampling) and those loci that had statistical significance >50 times were regarded as targets for further validation with ccRCC samples by PSQ.

The differences in continuous variables between groups were assessed using a two-sample t-test or ANOVA trend analyses using polynomial contrasts and data were presented as the mean ± standard deviation. Receiver operating characteristic (ROC) curves were used to estimate the capability of candidate markers in predicting the of progression of disease, and to determine the optimal cut-off point for dividing patients into subgroups (hypomethylation or hypermethylation) with the highest combined sensitivity and specificity. A multivariate Cox proportional hazards regression model was used to evaluate the prognostic value of candidate markers adjusted for well-known clinicopathological factors (sex, age, body mass index, grade and stage). Statistical analysis was performed using SPSS software v21.0 (IBM Corp. Armonk, NY, USA) and R v2.12.1 software. P<0.05 was considered to indicate a statistically significant difference.

Table I. Baseline characteristics of the patients enrolled in the present study.

Variables	Microarray (n=12)	Pyrosequencing (n=152)
Age (years, mean ± SD)	57.3±11.3	64.3±13.8
Gender [n (%)]		
Male	8 (66.7)	110 (72.4)
Female	4 (33.3)	42 (27.6)
BMI kg/m^2 (mean ± SD)	25.9±3.3	24.6±3.6
Fuhrman grade, [n (%)]		
G1	2 (16.7)	34 (22.4)
G2	7 (58.3)	68 (44.7)
G3	2 (16.7)	45 (29.6)
G4	1 (8.3)	5 (3.3)
T stage, [n (%)]		
T1	4 (33.3)	112 (73.7)
T2	4 (33.3)	14 (9.2)
T3	4 (33.3)	26 (15.1)
T4	-	3 (2.0)
Progression-free survival (months) Median (range)		27.2 (3.0-188.9)
Progression [n (%)]		
No		109 (71.8)
Yes		43 (28.2)

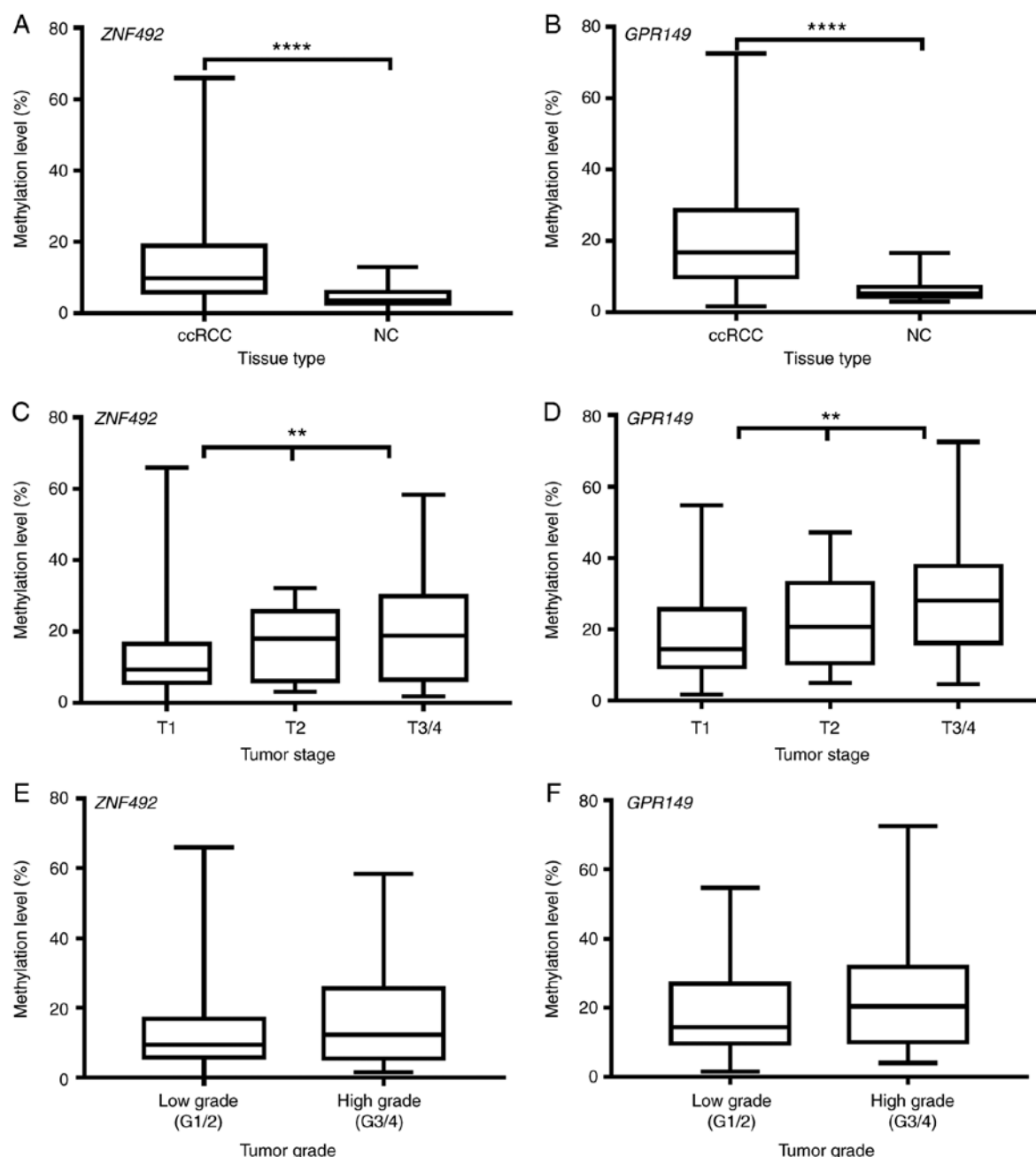BMI, body mass index; SD, standard deviation.

Figure 2. Association between methylation markers and clinicopathological characteristics. (A and B) The methylation levels of *ZNF492* and *GPR149* were significantly increased in the ccRCC specimens than in the NCs ($^{****}P<0.05$). (C and D) Increased methylation levels of *ZNF492* and *GPR149* were significantly associated with advanced pathological T stage ($^{**}P<0.05$). Methylation levels of (E) *ZNF492* and (F) *GPR149* exhibited a tendency to increase with higher grade, but the differences in methylation were not statistically significant ($P=0.059$, 0.052, respectively). ccRCC, clear cell renal cell carcinoma; GPR149, G protein-coupled receptor 149; NC, normal control; ZNF492, zinc finger protein 492.

Results

Baseline characteristics. The baseline characteristics of the 164 CBNUH patients with ccRCC were presented in Table I. The mean age and median progression-free survival period of the patients for PSQ were 64.3±13.8 years and 27.2 months (ranging 3.0-188.9 months), respectively; 43 patients (28.2%) experienced disease progression.

Identification of differentially methylated genes in ccRCC and NC. Genome-wide microarray methylation profiles of ccRCC samples and corresponding NC samples from 12 CBNUH cases

were compared to identify ccRCC-specific methylated genes. After applying selection criteria ($\Delta\beta$ value >0.2 and mean β-value in NC<0.15), 104 unique CpG island loci that were hypermethylated in ccRCC compared with the NCs were identified (Table SIII). The 104 selected candidate markers were then validated by comparing with the TCGA training data set from 160 pairs of ccRCC and matched NC samples subjected to the aforementioned selection criteria. In the TCGA training set, 251 CpG island loci were hypermethylated in ccRCC compared with the NCs (Table SIV). Of these, 22 CpG island loci overlapped with those identified as ccRCC-specific in the 12 CBNUH cases (Table SV). The prognostic importance of
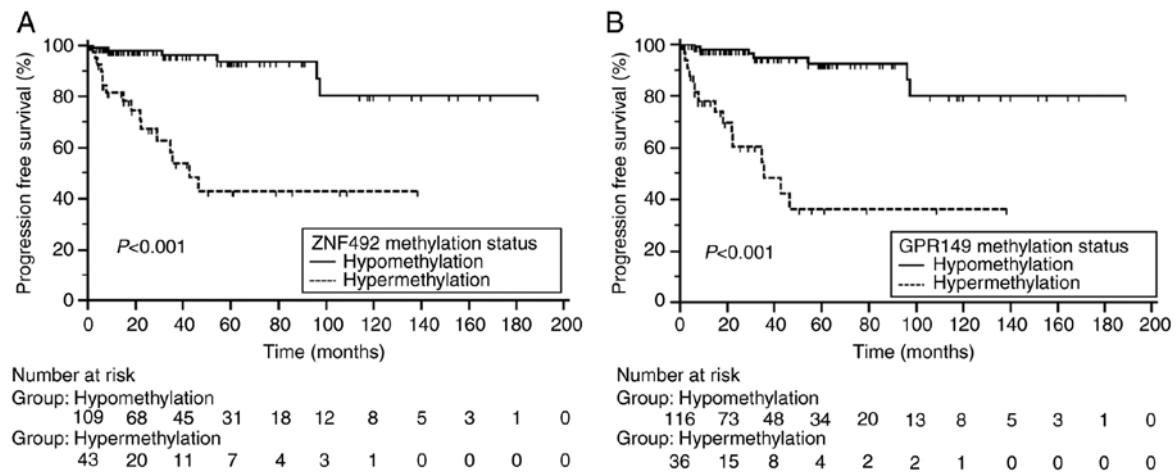
Figure 3. Kaplan-Meier analysis of the Chungbuk National University Hospital cohort. Kaplan-Meier estimates of the probability of progression according to methylation status (identified by pyrosequencing) in 152 patients with clear cell renal cell carcinoma. (A) ZNF492. (B) GPR149. GPR149, G protein-coupled receptor 149; ZNF492, zinc finger protein 492.

the 22 candidate CpG island loci were then examined with the TCGA test dataset. In the 100-times permutation test of the Kaplan-Meier analysis, 4/22 CpG island loci were selected as targets for validation by PSQ using 152 CBNUH ccRCC samples. The candidate genes at the four loci were as follows: Zinc finger protein 492 *(ZNF492;* at two CpG island loci), twist family bHLH transcription factor 1 *(TWIST1)*, and G protein-coupled receptor 149 *(GPR149)*.

PSQ analysis and internal validation of prognostic relevance in the clinical samples. To verify the prognostic relevance of candidate genes using a different methodology in a larger, different set of samples, we performed PSQ analyses on bisulfite-modified gDNA obtained from 177 CBNUH human kidney specimens (152 ccRCC and 25 matched NC). PSQ analysis was performed on two of the four candidate CpG island regions in the present study, including one of the two *ZNF492* loci (Infinium HumanMethylation450 target ID, cg01485075) and *GPR149* (cg00046499). Analysis of the candidate gene TWIST1 (cg26818735) was not possible due to technical variability across multiple PSQ assays of complex sequences of the homopolymer for PSQ.

To examine the association between methylation patterns and clinicopathological factors, methylation values for each gene were compared between samples. The methylation levels of *ZNF492* and *GPR149* were significantly higher in the ccRCC specimens than in the NCs (P<0.05). Increased methylation levels of *ZNF492* and *GPR149* were significantly associated with advanced pathological T stage (P<0.05). Decreased methylation levels of *ZNF492* were associated with higher body mass index; however, no significant association was observed with *GPR149*. The methylation levels of *ZNF492* and *GPR149* exhibited significantly increased methylation compared with control samples and had a tendency to increase with higher grade; however, differences between low and high grade tumors in *GPR149* methylation were not statistically significant (Fig. 2, Table SVI).

The value of candidate methylation markers for the prediction of disease progression was determined using the area under the ROC curve (AUC). The AUC values for

ZNF492 and *GPR149* were 0.716 [95% confidence interval (CI), 0.636-0.787; P<0.001] and 0.781 (95% CI, 0.707-0.842; P<0.001), respectively (Fig. S1). To validate the prognostic relevance of candidate methylation markers, the methylation values of *ZNF492* and *GPR149* were dichotomized (hypomethylation or hypermethylation) using optimal cut-off points (*ZNF492:* 18.11 and *GPR149*: 29.31), and survival analysis was performed. Kaplan-Meier estimates identified significant differences in time-to-progression according to the methylation status of *ZNF492* and *GPR149* (log-rank test, P<0.05; Fig. 3). In the multivariate Cox regression analyses, the methylation status of *ZNF492* [hazard ratio (HR), 5.44; 95% CI, 2.04-14.53; P=0.001] and of *GPR149* (HR, 7.07; 95% CI, 2.65-18.89; P<0.001) was suggested as independent predictors of disease progression (Table II).

External validation of prognostic relevance in the clinical samples. The validity of two genes as methylation markers in ccRCC was also evaluated using an independent set of PQS data from KPNUH samples, which included ccRCC (n=117) and matched NC (n=37) tissues (Table SVII). The association between the two methylation markers and clinicopathological characteristics revealed similar results to those of the CBNUH dataset (Table SVIII). The AUC values for *ZNF492* and *GPR149* were 0.717 [95% CI, 0.542-0.891; P=0.014] and 0.866 (95% CI, 0.746-0.986; P<0.001), respectively (Fig. S1). Patients with hypermethylated genes had shorter durations of progression than those of hypomethylated status (log-rank test, P<0.05, respectively; Fig. 4). According to the Cox multivariate regression analysis, the methylation status of *ZNF492* (HR), 3.89; 95% CI, 1.04-14.61; P=0.044] and of *GPR149* (HR, 33.67; 95% CI, 5.87-192.88; P<0.001) were significantly associated with disease progression (Table SIX).

Discussion

DNA methylation is a crucial mechanism for the development and progression of cancer. The methylation status of various genes can be used as a biomarker for risk assessment, early detection, prediction of prognosis, and therapy response in

Table II. Univariate and Multivariate Cox regression analyses for determining disease outcome based on the methylation status of each gene.

Variables[a]	Model 1[b]				Model 2[c]			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)[a]	P-value	HR (95% CI)[b]	P-value	HR (95% CI)[a]	P-value	HR (95% CI)[b]	P-value
Age	1.02 (0.98-1.05)	0.373	-	-	1.02 (0.98-1.05)	0.373	-	-
Sex	0.779 (0.30-2.00)	0.603	-	-	0.779 (0.30-2.00)	0.603		
BMI	0.37 (0.16-0.86)	0.021	0.35 (0.12-1.04)	0.058	0.37 (0.16-0.86)	0.021	0.22 (0.07-0.65)	0.007
Grade	5.39 (2.19-13.23)	<0.001	5.39 (2.19-13.23)	<0.001	5.39 (2.19-13.23)	<0.001	1.85 (0.55-6.29)	0.323
Stage		<0.001		0.001		<0.001		0.003
T1	Reference	-	Reference		Reference	-	Reference	
T2	5.65 (1.71-18.67)	-	6.37 (1.56-25.71)		5.65 (1.71-18.67)	-	7.43 (1.89-29.28)	
T3/4	10.33 (3.81-28.01)	-	7.75 (2.56-23.47)		10.33 (3.81-28.01)	-	5.78 (1.77-18.94)	
ZNF492	9.32 (3.62-23.91)	<0.001	5.44 (2.04-14.53)	0.001	-	-	-	-
GPR149	-	-	-	-	10.51 (4.26-25.93)	<0.001	7.07 (2.65-18.89)	<0.001

[a]Variables were categorized as follows: Median age (low vs. high), sex (male vs. female), BMI (<22.9 vs. ≥23 kg/m^2), tumor grade (G1/2 vs. G3/4), and methylation status (hypomethylation vs. hypermethylation). [b]Multivariate analysis included clinicopathological variables and the methylation status of *ZNF492*. [c]Multivariate analysis included clinicopathological variables and the methylation status of *GPR149*. BMI, body mass index; CI, confidence interval; GPR149, G protein-coupled receptor 149; HR, hazard ratio; ZNF492, zinc finger protein 492.
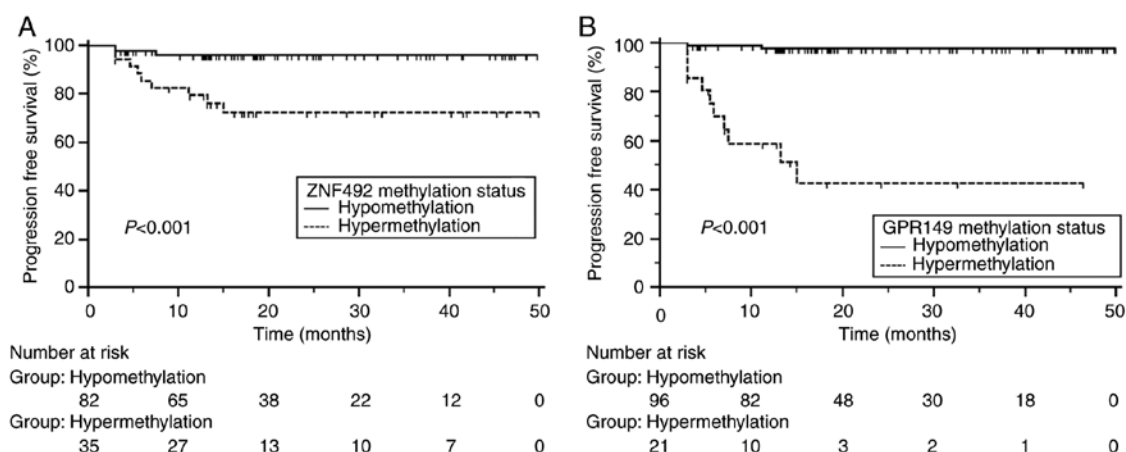

Figure 4. Kaplan-Meier analysis of the Kyungpook National University Hospital cohort. Kaplan-Meier estimates of the probability of progression according to methylation status (identified by pyrosequencing) in 117 patients with clear cell renal cell carcinoma. (A) ZNF492. (B) GPR149. GPR149, G protein-coupled receptor 149; ZNF492, zinc finger protein 492.

numerous types of cancer (17,18). In the present study, novel methylation markers that were able to predict the likelihood of disease progression were identified based on genome-wide DNA methylation array datasets of clinical samples from our own institute and from TCGA. Close associations with unfavorable characteristics of disease, including advanced stage were noted. Furthermore, the methylation status of each of these markers was revealed to be an independent indicator of progression in two cohorts of patients with ccRCC.

　　Increasing evidence has indicated that the methylation status of individual or combinations of genes are associated with different outcomes in patients with RCC and may serve as independent indicators of prognosis (3-6). In a study of 179 patients who underwent radical or partial nephrectomy

for ccRCC, Ras association domain-containing protein 1 methylation status was significantly associated with higher grade and advanced stage. Additionally, this gene served as an independent variable for the prediction of cancer-specific survival in patients with ccRCC (6). Similarly, another study revealed that the combined methylation status of collagen α-1 (XIV) and basonuclin was independently associated with a poorer prognosis, and served as a better prognostic indicator than tumor stage or grade (4).

　　Previously, microarray-based clinical studies have sought novel molecules that could enhance diagnostic and prognostic ability alone or with conventional biomarkers (7-11). In a genome-wide study with kidney tissues from 96 patients, a set of DNA methylation markers was demonstrated to reliably

discriminate between malignant and benign lesions, and classical histological subtypes (7). In addition, these findings were validated using a microarray data set from TCGA comprising >1,000 kidney samples. In addition, other studies have suggested the possible roles of methylation signatures for the detection and prognostic prediction of RCC (8-11). Microarray-based DNA methylation profiling may therefore provide insight into the disease and, most importantly, reveal biomarkers for the detection and prognostication of RCC. In the present study, using methylation profiles of our clinical samples and those in TCGA, we selected CpG island loci commonly hypermethylated in ccRCC compared with NCs. The potential of these candidate loci as indicators of prognosis was first explored in a test set of TCGA data and independently validated with a different technique (PSQ) in two independent ccRCC cohorts with long-term follow-up. Our methodological approach for gene selection and prognostic validation using multiple steps with different independent data sets was selected to enhance the reliability of our results as much as possible. Furthermore, limiting patient enrollment to those with ccRCC increased the homogeneity of the samples, which may enhance the likelihood of identifying differences. Our candidate markers are likely to be applicable across various ethnicities as they were determined from data derived from Western and Eastern populations. The results of the present study are therefore promising; however, further investigation is required to determine the false prediction rate of these markers in particular.

Limitations of the present study must be addressed. Only 12 samples were used for genome-wide microarray methylation profiling to identify candidate markers. To overcome the limited sample number, a large TCGA dataset was utilized simultaneously. Additionally, validation was performed using PSQ in a larger set of different samples from our institute and others. These types of multiple validation strategies with different datasets may improve the reliability of our results. Additionally, the effects of methylation status on mRNA and protein expression should have been evaluated. Gene expression and immunohistochemical analysis could not be performed due to the lack of available tissue samples. Although the association between alterations in methylation and gene expression of selected markers was not determined in the present study, preliminary data analysis with TCGA data revealed that the expression of *ZNF492* was significantly lower in cancer tissues than in NC samples (Fig. S2). Furthermore, our study investigated hypermethylation genes instead those hypomethylated. Global hypomethylation or locus-specific hypermethylation in CpG island-rich promoters is a common phenomenon of cancer. Numerous sites of hypomethylation that vary between normal and RCC tissues could be identified with a microarray dataset. In the present study, we aimed to analyze 2-5 genes at most with validation in clinical samples. Providing that too many candidate genes are selected, it would be difficult to validate their clinical relevance in clinical samples. In this regard, we focused on the hypermethylation rather than the hypomethylation of genes.

To the best of our knowledge, this is the first report to demonstrate that *ZNF492* and *GPR149* act as methylation-induced prognostic indicators in RCC. Currently, limited information regarding the specific function of these genes is available in the literature. *ZNF492*, also known as *ZNF115*, is a member of the zinc finger protein family and is located at 19p12. There are eight different classes of zinc finger motifs and each class has a distinct biological role (19). Evidence accumulated over several decades has demonstrated crucial roles for zinc finger proteins in the initiation and progression of cancer in numerous type of cancers (20-24). As an example of the role of methylation, one zinc finger family member *ZNF545* was reported to serve as a tumor suppressor by inducing cell apoptosis, inhibiting ribosome biogenesis, and suppressing nuclear factor-ϰB and activator protein-1 signaling in nasopharyngeal, esophageal, lung, gastric, colon, and breast cancer upon methylation (21). In addition, a combination of methylated CpG island sites corresponding to those of zinc finger genes was able to predict survival outcome in patients with gastric cancer (22). *GPR149* is a member of the G protein-coupled receptor (GPCR) family, which is located at 3q25.2. GPCRs comprise a large family of cell-surface receptors and control various features of tumorigenesis, as well as many cancer-associated signaling pathways (25,26). With aid of genome-wide approaches, numerous biomolecules have recently emerged as novel biomarkers in several human tumors; however, in the majority of cases, the association between a specific disease and a putative biomarker has not been yet determined. Similarly, we have not investigated the functional roles of *ZNF492* and *GPR149* in the tumorigenesis and progression of RCC, which poses as a limitation of the present study. However, establishing novel biomarkers, even of unknown function, and the potential application in the detection, prognostic prediction and use as therapeutic targets is likely to prove valuable in various types of human disease. Therefore, functional studies are required to determine the full potential of the putative biomarkers identified in the present study.

Availability of data and materials

The datasets generated and analyzed during the current study are available in the GEO repository, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=azovkgyunvcdrij&acc=GSE92482.

Authors' contributions

YJK, SCL, TGK and WJK made substantial contributions to the concept of the present study. YJK, WJ, TGK and WJK conducted the experiments. Validation of the data was

conducted by YJB, JSK and SPS. YJK, WJ, HYY, SMK, SKL, SPS, HWK, WTK, SJY, HSS, KHR, SWK, YSH and GSY performed data analysis. YJK, WJ, YJB and JSK collected data. YJK drafted the manuscript. YJK, WJ, XMP, HYH, SMK, SKL, HWK, WTK, SJY, HSS, KHR, SWK, YSH, GSY, SCL, TGK and WJK reviewed the manuscript. SCL, TGK and WJK provided guidance and supervision. YJK acquired funding for the study.

Ethics approval and consent to participate

The collection and analysis of all samples were approved by the CBNUH (approval no. 2010-01-001) and KPNUH (approval no. KNUMC 2016-05-021). Institutional Review Board and informed consent was obtained from each subject. The study methodologies conformed with the standards set by the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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