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Original Article

NBR1 and KIF14 Downstream of the Mammarian Target of Rapamycin Pathway Predict Recurrence in Nonmuscle Invasive Low Grade Urothelial Carcinoma of the Bladder

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Purpose: The lack of identified mammalian target of rapamycin (mTOR) pathway downstream genes that overcome cross-talk in nonmuscle invasive low grade (LG)-urothelial carcinoma (UC) of the bladder is a clinical limitation in the use of mTOR inhibitor for the treatment of UC.

Materials and Methods: Presently, gene expression patterns, gene ontology, and gene clustering by dual (p70S6K and S6K) siRNAs or rapamycin in 253J and TR4 cell lines were investigated by microarray analysis. mTOR/S6K pathway downstream genes suppressed to siRNAs, and rapamycin up-regulated or rapamycin down-regulated genes were identified. The mTOR downstream genes examined using a tissue microarray of 90 nonmuscle invasive LG-UC patients to assess whether any of these genes predicted clinical outcomes. A knockout study evaluated the synergistic effect with rapamycin.

Results: In the microarray analysis, mTOR pathway downstream genes selected consisted of 4 rapamycin down-regulated (FOXM1, KIF14, MYBL2, and UHRF1), and 4 rapamycin up-regulated (GPR87, NBR1, VASH1, and PRIMA1). In the tissue microarray, FOXM1, KIF14, and NBR1 were more expressed at T1, and MYBL2, and PRIMA1 were more expressed in tumors exceeding 3 cm. In a multivariate Cox regression model, KIF14 and NBR1 were significant predictors of recurrence in nonmuscle invasive LG-UC of the bladder. In a NBR1 knock out model, rapamycin treatment synergistically inhibited cell viability and colony forming ability compared to rapamycin only.

Conclusions: The results implicate KIF14 and NBR1 as mTOR/S6K pathway downstream genes that predict recurrence in nonmuscle invasive LG-UC of the bladder and demonstrate that NBR1 knockout overcomes rapamycin cross-talk. **(Korean J Urol Oncol 2017;15:28-37)**

Key Words: Urinary bladder neoplasms · mTOR · Biomarkers · Microarray analysis · Recurrence

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INTRODUCTION

Cell-signaling pathways have a critical role in tumorigenesis. Underlying gene or protein alterations in these pathways can influence cell cycle control, DNA repair, and carcinogen metabolism.[1] In particular, a subset of mammalian target of rapamycin (mTOR) pathway alterations occur in urothelial carcinoma

(UC) of the bladder and appear to influence tumor behavior.[2]

In a previous study, we investigated how inhibition of phosphorylated p70S6K (p-p70S6K) suppresses the proliferation and growth of nonmuscle invasive low grade (LG)-UC *in vitro* and whether p-p70S6K can serve as a predictive biomarker for the recurrence of nonmuscle invasive LG-UC of the bladder in patients.[3] Despite such promising preclinical observations, clinical trials with mTOR inhibitors have not proved encouraging.[4,5] This might be due to cross-talk of mTOR pathways leading to multiple sites of regulation and the diversity of genetic aberrations activating this pathway.

In the postgenome era, high-throughput methods have enabled comprehensive analyses of molecular activities in different cell types, which may lead to a greater understanding of cross-talk. In this context, the DNA microarray has become the preferred method for large-scale analysis of gene expression.[6] The DNA microarray is a molecular technology that enables the identification of gene interactions from experimental data through computational analysis.[7] The explanations of the molecular events led directly to the discovery of novel predictive biomarkers for LG-UC of the bladder recurrence and progression, and overcome the clinical limitation of mTOR inhibitor.

Our aim was to identify mTOR pathway downstream genes that are transcriptionally altered due to mTOR pathway inhibition in UC cells using between RNAi and small molecule inhibitor. p70S6K, was knocked down using 2 different siRNAs in LG-UC cell lines (253J, RT4), since these cell lines show high-level of amplification and overexpression of mTOR downstreams and rapamycin is known to target mTOR pathway. This study might be to search mTOR pathway downstream genes to overcome cross-talk at non muscle invasive LG-UC of the bladder.

MATERIALS AND METHODS

1. Cell Cultures and Reagents

Human LG-UC cell lines (253J and RT4) were purchased from the American Type Culture Collection (Manassas, VA, USA). The 253J cell line was cultured in Dulbecco's modified Eagle's medium and the RT4 cell line was cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum and 100-μ/mL penicillin/spectromycin. Rapamycin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies to p70 S6 kinase (gene name; RPS6KB1), phosphorylated-p70 S6 kinase (p-p70 S6 kinase, Ser371), and β-actin were purchased from Cell Signaling Technologies (Beverley, MA, USA). To generate stable knockdown cell lines, plasmid short-hairpin RNA (shRNA) constructs and a non-targeting shRNA control were purchased from Sigma-Aldrich. The 253J cell line was transfected with either 5 μg of each shRNA plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Puromycin (0.1−1 μg/mL) was initiated 2 days after shRNA transfection.

2. Cell Viability Analysis and Colony Formation Assay

Human LG-UC NBR1 and FABP4 knockdown 253J cell lines were plated in 96-well plates in complete medium and treated with the various concentrations of rapamycin. After 48 hours, cell viability was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay according to the manufacturer's instructions (Sigma-Aldrich). Colony formation assays were performed following treatment with 0.1-μM and 1-μM rapamycin for 48 hours, at which time the cells were replated in 12-well plates at low density ($1x10^3$ cells per well) in complete medium for 2 weeks. Colonies were fixed with methanol and stained with 0.1% crystal violet (Sigma-Aldrich). Colony numbers were assessed visually and colonies measuring at least 50 μm were counted.

3. Western Blot Analysis

Cells were washed with ice-cold phosphate-buffered saline and trypsinized using lysis buffer (Intron, Seoul, Korea). The lysates were stored at -20°C until analysis. The amount of protein was quantified by the Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were loaded onto Readygels (4%−20% Tris-HCL; Bio-Rad) and electrophoresis was performed according to the manufacturer's instructions. Proteins were blotted onto polyvinylidene fluoride membranes (Invitrogen) and incubated for 1 hour at room temperature in 5% skim milk for blocking. Blots were incubated with primary antibodies overnight at 4°C and with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. The membranes were developed using enhanced chemiluminescence.

4. siRNA Transfection

p70S6K siRNA (ID#: 6566, sense strand: 5'-GUGCCAAUC AGGUCUUUCU-3', antisense strand: 5'-AGAAAGACCUGA

UUGGCAC-3') was purchased from Cell Signaling Technologies, and S6K siRNA (ID#: sc92312, sense strand 5'-CCUUCAACC ACUAUCAGAAUU-3', antisense strand: 5'-UUCUGAUAGU GGUUGAAGGUU-3') was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Transient transfections into 253J and RT4 cells were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Cells treated with 10 μM of the mTOR inhibitor rapamycin and siRNAs were harvested at 48 hours for subsequent experiments. 253J and RT4 cells were grown in 6-well plates until they reached 85%–90% confluence. Five microliters of Lipofectamine 2000 reagent was used for transfection in the absence of serum. The cells were harvested for Western blot analysis at 24 hours after transfection.

5. Microarray Analysis

Total RNA was isolated from cells that were grown to approximately 70% confluence using TRI reagent (Gibco BRL/Life Technologies, Grand Island, NY, USA) for the extraction of total RNA using the RNeasy protocol (Qiagen, Valencia, CA, USA). The quality of the RNA was assessed by 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) to control the integrity of the samples before microarray hybridizations. For the control, dual siRNAs (p70S6K, and S6K) and the rapamycin group in the 253J and RT4 LG-UC cell lines, synthesis of target cRNA probes and hybridization were performed using a Low RNA Input Linear Amplification kit (Agilent Technology, Santa Clara, CA, USA) according to the manufacturer's instructions. Amplified and labeled cRNA was purified on cRNA Cleanup Module (Agilent Technology) according to the manufacturer's protocol. The absolute value of the fold-change (*R/G* and *G/R*) had to be >2.0 in at least one experiment, and the average intensity (*A*) had to be >300. This filter reduced the number of genes from 8976 to 234. Data were filtered to select genes that had both a fold-change to remove the background of mostly unchanging genes, and an average intensity distinguishable from the noise of the microchip hybridization. The hybridized images were scanned using a DNA microarray scanner and quantified with Feature Extraction Software (Agilent Technology). All data normalization and the selection of 2-fold-changed genes were performed using GeneSpringGX 7.3 (Agilent Technology).

6. Patient Cohort and Tissue Microarray Construction and Immunohistochemistry

We retrieved 100 nonmuscle invasive LG-UC bladder specimens collected at Chung-Ang University Hospital between 2005 and 2013 after obtaining Institutional Review Board approval (approval number: C2012082). We excluded samples from patients who had a history of preoperative treatment including radiotherapy, systemic chemotherapy, or intravesical therapy. All patients' clinicopathological data were retrieved from electronic medical records. These included patient demographics and preoperative information, such as diagnostic procedures and clinical stage. Follow-up data on disease recurrence and progression were obtained by review of patient's medical records. Because it was a retrospective study of anonymous patients, we could not get informed patient consent. However, the study was carried out in agreement with the Declaration of Helsinki.

For the immunohistochemical procedures, 4-μm-thick sections were deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval with a buffer solution using a streamer autoclave. Sections were then incubated with the appropriate primary antibody. After the application of a secondary antibody, slides were developed using 3-3'-diaminobenzidine chromogen and counterstained with hematoxylin. Tissue microarray (TMA) spots with artificial folds or those lacking target tissue representation were omitted from further analyses. Antibodies, condition, positive control, and staining localization are summarized in Supplementary Table 1. Staining conditions were optimized on sections from formalin-fixed, paraffin-embedded tissue controls for each antibody as specified by the manufacturers.

Antibody reactivity was detected and counterstaining was done as described above. The primary antibody was omitted for negative controls. Tumor TMA spots stained with each marker were evaluated for extent (percent of positive cells), and intensity (0 to 3+ score). The standard H-score (scale of 0–300) was calculated based on the product of the percentage of cells showing staining (0–100) multiplied by the intensity of staining (1, weak; 2, moderate, and 3, strong).[8] A final H-score was generated by taking the average of triplicate tissue samples. The H-scores of markers were used in the statistical analyses. In Kaplan-Meier curve and Cox regression model, a cutoff value of expression of each marker was used according to the median

tumor H-score. Strong expression of a marker was defined as expression equal to or greater than the cutoff value, and weak expression as no expression or expression less than the cutoff value.

7. Statistical Analyses

IBM SPSS Statistics ver. 21.0 (IBM Co., Armonk, NY, USA) was used for all statistical analyses. The analysis of variance test and Student t-test were performed at $p<0.05$ to find genes that differentially expressed across conditions. Hierarchical clustering was performed by similarity measurements based on Pearson correlations around zero. Recurrence-free and progression-free survival curves were estimated by using the Kaplan-Meier method, and any differences in the survival curves were compared by log-rank tests. A Cox regression model was used to investigate predictive factors for the recurrence of LG-UC in a multivariate analysis.

RESULTS

1. Western Blot Analysis for mTOR Pathway Expression After Dual siRNAs or Rapamycin Treatment in the LG-UC Cell Lines

Expression of the p70S6K proteins in the 253J and RT4 cell lines was assessed. To study the downstream targets of mTOR/S6K pathway, 253J and RT4 cells were treated with dual (p70S6K, and S6K) siRNAs and rapamycin (Fig. 1A) to validate the inhibition of p70S6K gene expression with dual

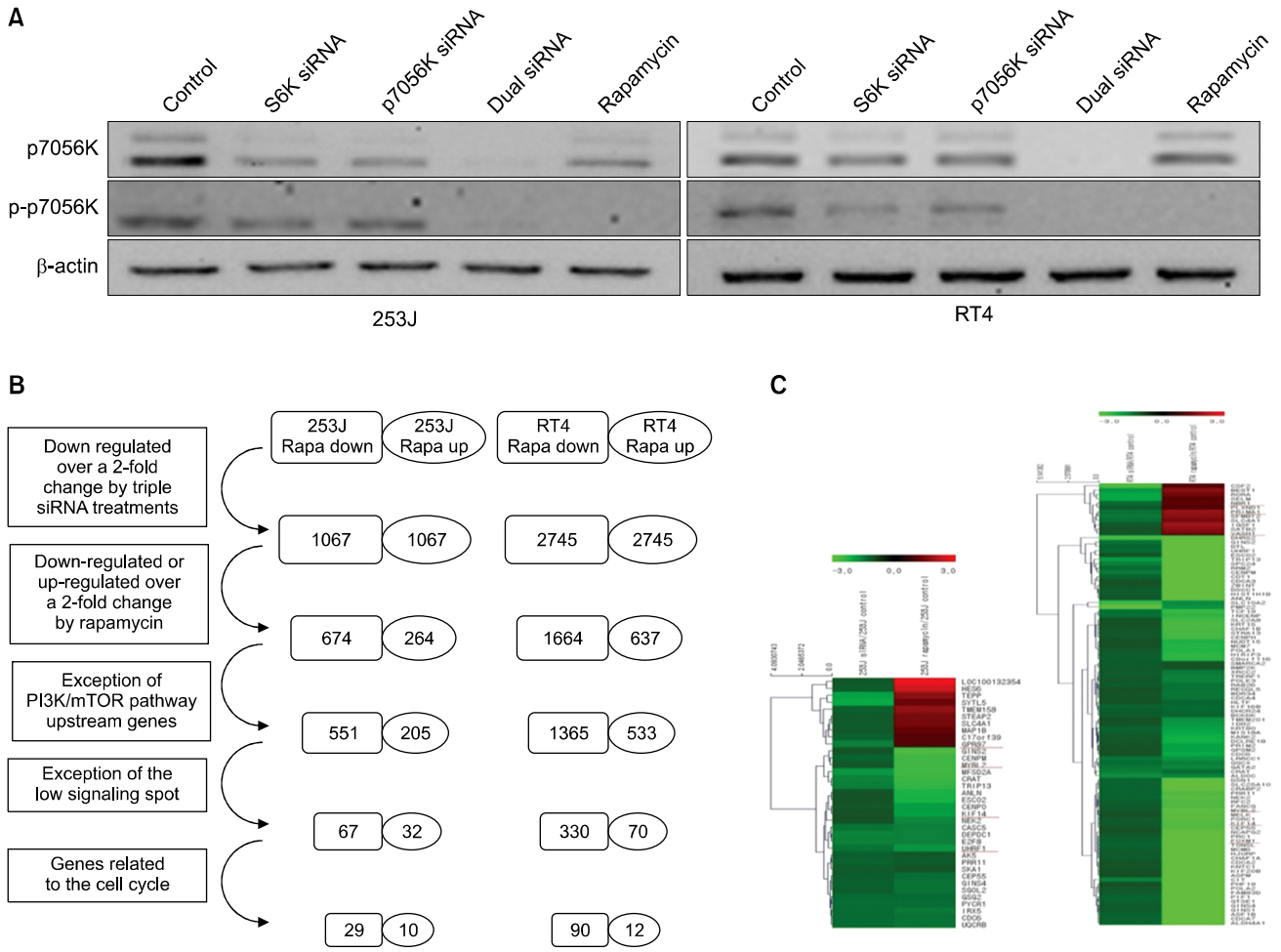

Fig. 1. Protein-level validation of p70S6K suppression after p70S6K and S6K siRNA treatments, gene filtering, and gene expression profiling in low grade-urothelial carcinoma cell lines. (A) Reduced gene expression was observed in 253J and RT4 cell lines after transfection of siRNAs against p70S6K and S6K, (B) Gene filtering process in 253J and RT4 cell lines. (C) Hierarchical clustering analysis from 253J and RT4 cell lines: the red spots indicate up regulation, green spots indicate down regulation, black spots indicate an absence of modulation, and gray spots indicate the absence of values. mTOR: mammarian target of rapamycin.

siRNAs before performing cDNA microarray analysis. The dual siRNAs blocked the protein expression of p70S6K in 253J and RT4 cells, and rapamycin inhibited the phosphorylation of p70S6K.

2. Gene Expression Patterns After Treatment With siRNAs or Rapamycin

In 253J cells, 1,067 genes were down-regulated by dual siRNAs compared to control. In RT4 cells, 2,745 genes were down-regulated by the dual siRNAs. We filtered reliable genes along with objective criteria (Fig. 1B). The first filtering genes were down-regulated 2 fold by the dual siRNAs compared to control. The second filtering genes were down-regulated or up-regulated >2 fold by rapamycin compared to the control. Third filtering genes, with the exception of phosphoinositol-3-kinase/mTOR pathway-related upstream genes were selected. Fourth filtering genes, with the exception of the low signaling spot (flag A, M), were selected. Fifth, we filtered genes related to the cell cycle.

Hierarchical cluster analysis was used to profile gene expression patterns (Fig. 1C). In 253J cells, 29 of 39 final filtered genes were rapamycin down-regulated genes, and 10 genes were rapamycin up-regulated genes. Ninety of 102 genes were rapamycin down-regulated genes and 12 genes were rapamycin up-regulated genes in RT4 cells. Thirteen rapamycin down-regulated genes (*CEP5, GINS2, UHRF1, NEK2, ANLN, CENPM, CDC6, KIF14, ESCO2, TRIP13, GINS4, FOXM1, MYBL2*) were simultaneously filtered at 5637 and T24, but no rapamycin up-regulated gene was simultaneously filtered.

3. Selection of mTOR Pathway Downstream Genes According to the Gene Expression Patterns After siRNAs or Rapamycin Treatment in LG-UC Cell Lines

Of 219 genes filtered in 253J and RT4 cells lines, four genes were simultaneously filtered at 5637 and T24, and 4 genes were associated with cancers as determined by literature scrutiny (Table 1). FOXM1, KIF4, MYBL2, and UHRF1 were rapamycin down-regulated and GPR87, NBR1, VASH1, and PRIMA1 were rapamycin up-regulated. Gene ontology (GO) classes enriched in the gene expression profiles of RNAs and rapamycin-treated UC cell lines were investigated using a GO categorizer (Table 1).[9] In UC cell lines, representative enriched GO classes were functional categories involved in cell cycle (FOXMa, MYBL2, UHRF1), microtubule-based process (KIF14), cyclase activity by G-protein signalling (GPR87), mitogen (NBR1), immune response (VASH1), and protein anchor (PRIMA1).

4. Patient and Tumor Characteristics and Expression of mTOR Pathway Downstream Genes in Relation to Clinicopathological Variables

FOXM1, KIF14, MYBL2, UHRF1, GPR87, NBR1, VASH1, and PRIMA1 expression was detected in 89 (98.9%), 87 (96.7%), 88 (97.8%), 79 (87.8%), 88 (97.8%), 90 (100%), 88 (97.8), and 82 of 90 LG-UC samples (91.1%), respectively (Fig. 2A). The median H-score of FOXM1, KIF14, MYBL2, UHRF1, GPR87, NBR1, VASH1, and PRIMA1 was 43.3, 43.3, 50, 15.6, 65.9, 53.3, 50, and 30, respectively, in LG-UC samples.

The basic characteristics of the patients are provided in

Table 1. Selection of mTOR pathway downstream genes according to the gene expression patterns after siRNAs or rapamycin treatment in LG-UC cell lines

Gene name	Description	GO term	Expression by rapamycin
FOXM1	forkhead box M1	Cell cycle	D
KIF14	kinesin family member 14	Microtubule-based process	D
MYBL2	v-myb myeloblastosis viral oncogene homolog	Cell cycle	D
UHRF1	ubiquitin-like with PHD and ring finger domains 1	Cell cycle	D
GPR87	G protein-coupled receptor 87	Cyclase activity by G-protein signaling	U
NBR1	Next to BRCA1 gene 1 protein	mitogen	U
VASH1	vasohibin 1	Immune response	U
PRIMA1	proline rich membrane anchor 1	Protein anchor	U

mTOR: mammarian target of rapamycin, LG-UC: low grade-urothelial carcinoma, GO: gene ontology, D: downregulated expression, U: upregulated expression.

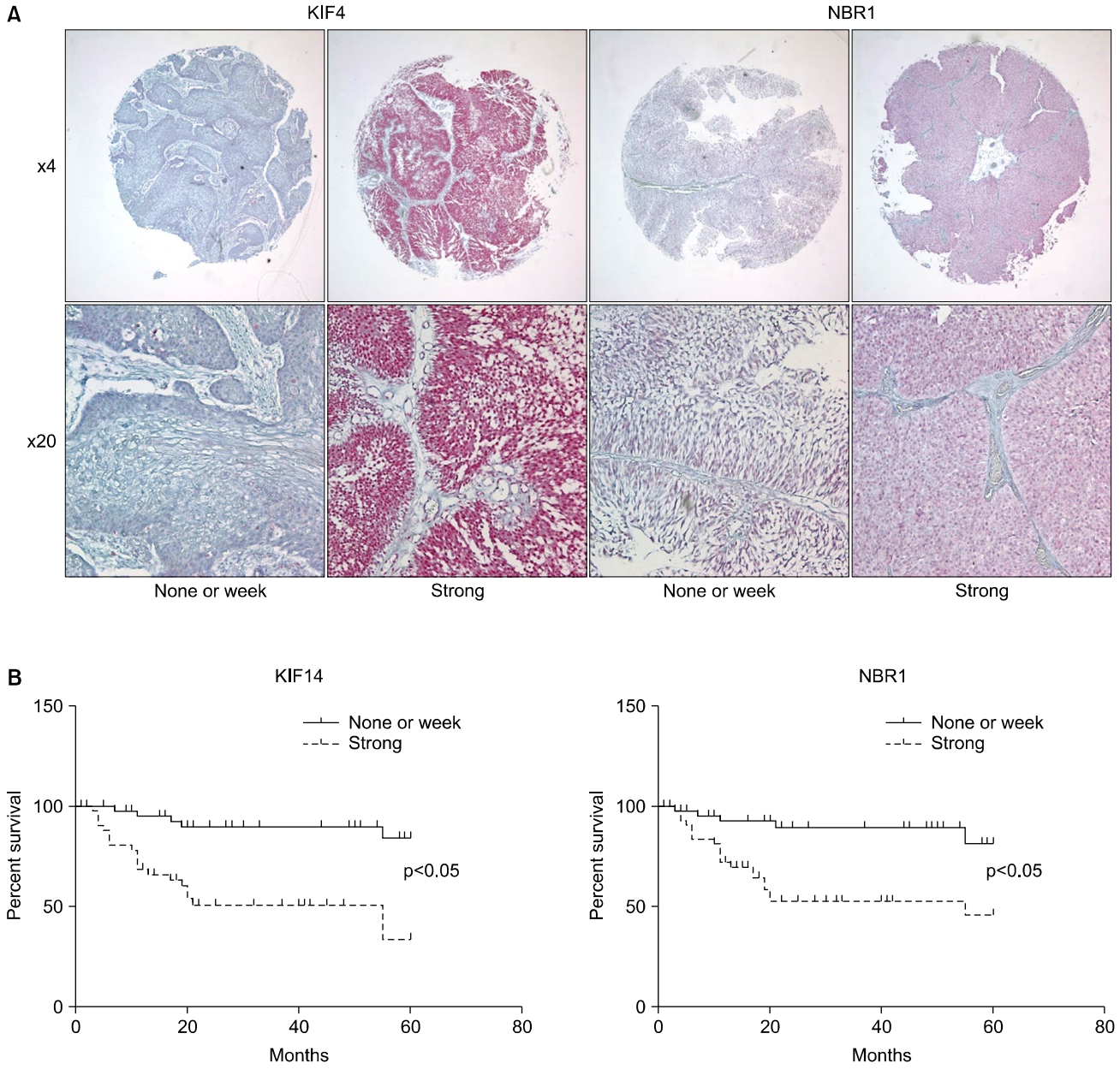

Fig. 2. Expression of *KIF14* and *NBR1* genes in LG-UC of bladder tissues and Kaplan-Meier curves based results in LG-UC patients. (A) Immunohistochemical staining of KIF14 and NBR1 in paraffin-embedded sections of LG-UC tissues (x40, x200). (B) In the recurrence-free survival curves, nonmuscle invasive LG-UC patients with strong KIF14, and NBR1 immunohistochemical staining exhibited decreased recurrence-free survival. LG-UC: low grade-urothelial carcinoma.

Supplementary Table 2. The mean age at the time of diagnosis of UC was 66.0 years (range, 36.0–93.0 years) and the median follow-up duration was 27 months (range, 3–60 months). Twenty-three patients (25.6%) had recurrence of UC within a mean follow-up of 11.1 months (range, 3–21 months). Three patients (3.3%) developed a nonmuscle invasive high grade UC of bladder 4.0, 13.0, and 20.0 months later. Those aged 60–70 years comprised the largest age fraction (41.4%) and 87.8% of the patients were male. Primary tumors accounted for 17.8% of the prior recurrence rate. Single tumors were observed most frequently (66.7%), and a tumor size <3 cm (76.7%) was observed more frequently than tumors >3 cm. Eighty-four patients (93.3%) had Ta cancer.

Among rapamycin down-regulated genes, FOXM1 (H-score: 42.0±21.2 vs. 23.6±17.6, $p<0.05$) and KIF14 (43.0±23.1 vs. 21.1±16.2, $p<0.05$) showed stronger expression at T1 than Ta

of pathologic T stage and MYBL2 showed stronger expression >3 cm than other tumor sizes (H-score: 46.8±21.7 vs. 32.4±22.1, $p<0.05$), (Supplementary Table 2). Of the rapamycin up-regulated genes, NBR1 (H-score: 54.7±22.6 vs. 28.6±31.0, $p<0.05$) was more strongly expressed at T1 than Ta of pathologic T stage, and PRIMA1 was more strongly expressed in tumors >3 cm in size (H-score: 38.2±26.6 vs. 22.7±24.2, $p<0.05$), (Supplementary Table 3).

In Cox regression model, tumor multiplicity, strong *KIF14*, strong *MYBL2*, and strong *NBR1* gene expression predicted shorter recurrence-free survival in LG-UC in univariate analysis (Table 2). In multivariate analysis, tumor multiplicity ($p<0.05$; hazard ratio [HR], 2.746), strong *KIF14* ($p<0.05$; HR, 3.573), and strong *NBR1* gene expression ($p<0.05$; HR, 4.275) were independent factors predicting recurrence (Table 2). In immunohistochemical staining, KIF14 was expressed in the nucleus of tumor cells and NBR1 was expressed in the cytoplasm of tumor cells, and non-muscle invasive LG-UC patients with strong KIF14, and NBR1 immunohistochemical staining exhibited decreased recurrence-free survival in Kaplan-Meier survival curve (Fig. 2A, B).

5. Cell Proliferation and Colony Growth Inhibition Effect of Rapamycin in NBR1 Knockout 253J Cells

Knockout of NBR1 (a rapamycin up-regulated gene) in 253J cells was done and used to evaluate the synergistic effects of rapamycin and NBR1 inhibition. We selected 2 stable shNBR RNA transfected 253J cells (shNBR#1 and shNBR1#2) by Western blot analysis. Cell proliferation-inhibition effects of a low dose (1 μM) of rapamycin were increased in shNBR#1 and shNBR#2 compared with control in low concentration ($p<0.01$) (Fig. 3A). Colony growth-inhibition by the same low dose of rapamycin was aggravated in NBR knockout 253J cells compared with control (Fig. 3B) ($p<0.01$).

DISCUSSION

Since the mTOR pathway is associated with recurrence of LG-UC, molecular gene profiling by downstream suppression of the mTOR pathway, which is associated with the recurrence of LG-UC, may be a candidate novel molecular target therapy. Gene expression patterns, GO, and gene clustering by dual (p70S6K and S6K) siRNAs or rapamycin in 253J and RT4 cells were investigated by microarray analysis. mTOR pathway downstream genes suppressed >2 fold by the siRNAs, and rapamycin up-regulated or down-regulated by >2 fold were selected. These genes were validated mTOR with immunohistochemistry using TMA of 90 nonmuscle invasive LG-UC patients to assess whether the genes could predict clinical aggressiveness and long-term outcomes. In cDNA microarray analysis and TMA analysis, 2 rapamycin up regulated genes (KIF14 and MYBL2) and one rapamycin down regulated gene (*NBR1*) were significant predictors of recurrence in non-muscle invasive LG-UC in the bladder. These results re-

Table 2. Cox proportional hazards ratio to identify predictive clinicopathologic factors for recurrence in LG-UC of the bladder

Clinicopathologic factor	Cox proportional hazards ratio (HR with 95% CI)	
	Univariate	Multivariate
Age (>70 yr)	0.541 (0.239-1.224)	1.860 (0.761-4.547)
Male sex	1.708 (0.402-7.257)	0.701 (0.157-3.316)
No. of tumors, multiple	2.479 (1.128-5.446)*	2.746 (1.174-6.422)
Tumor size, >3 cm	0.968 (0.386-2.426)	1.651 (0.589-4.629)
FOXM1, >43.3 H-score	1.709 (0.767-3.812)	-
KIF14, >43.3 H-score	6.122 (2.250-16.657)*	3.573 (1.158-11.023)*
MYBL2, >50 H-score	3.661 (1.516-8.839)*	1.626 (0.608-4.349)
UHRF1, >15.6 H-score	1.066 (0.486-2.341)	-
GPR87, >65.9 H-score	1.353 (0.607-3.018)	-
NBR1, >53.3 H-score	4.818 (1.803-12.876)*	4.275 (1.494-12.232)*
VASH1, >50 H-score	1.732 (0.777-3.862)	-
PRIMA1, >30.0 H-score	1.437 (0.651-3.172)	-

LG-UC: low grade-urothelial carcinoma, HR: hazard ratio, CI: confidential interval.
*$p<0.05$.

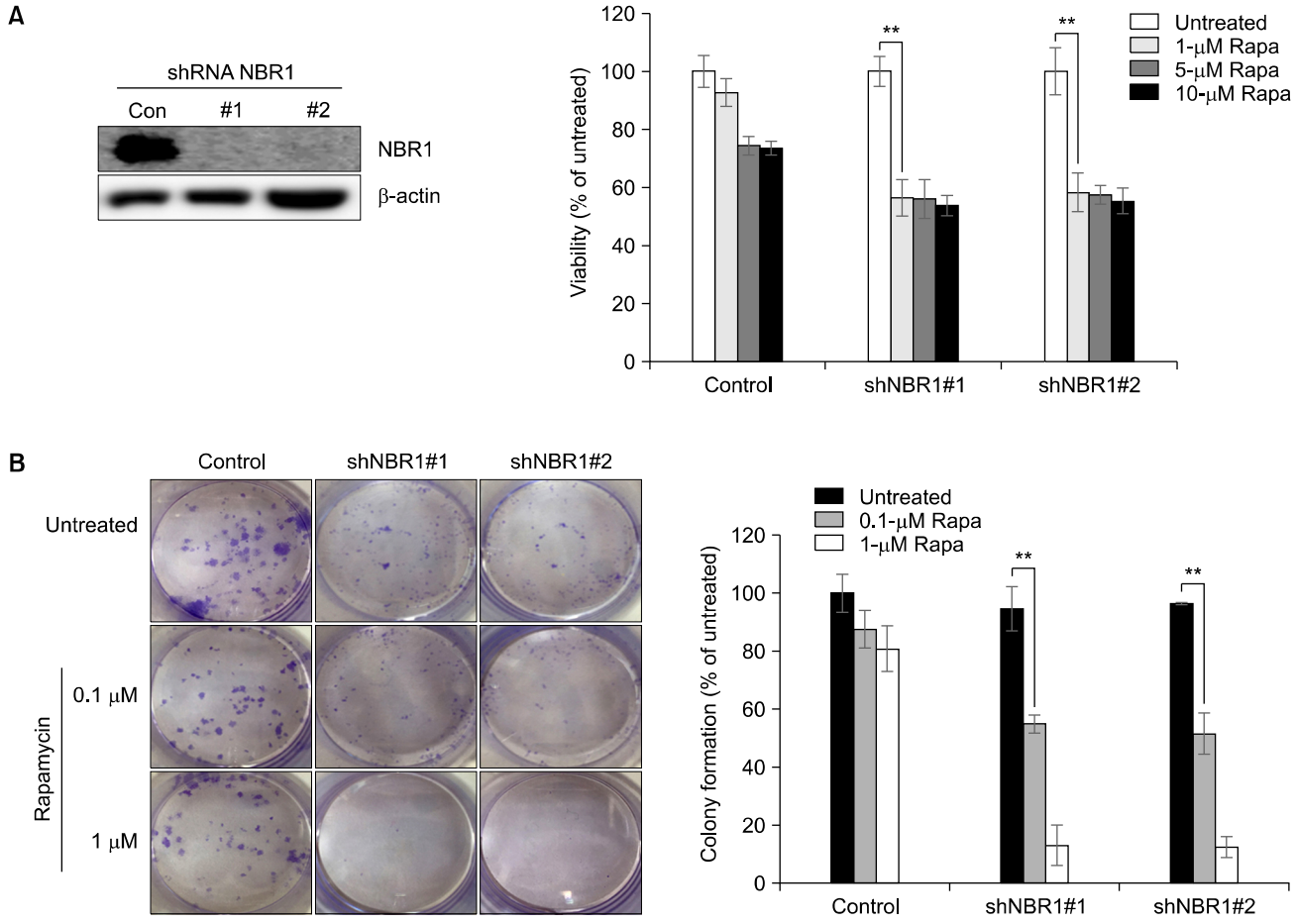

Fig. 3. Downregulation or upregulated NBR1 expression after shRNA and rapamycin treatment in low grade-urothelial carcinoma 253J cell line. (A) Western blots of whole-cell lysates from NBR1 stable knockdown and nontargeted shRNA control 253J cell line after puromycin selection: control, pLKO.1 control vector; shNBR1#1 and shNBR1#2, NBR1 shRNA vectors. (B) Cell viability determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are mean±standard deviation (SD) (n=6). (C) Colony-forming ability of NBR1 stable knockdown 253J cell lines. After 48-hour treatment with 0.1-, 1-μM rapamycin, cells were replated in 12-well plates at low density. Colonies were counted after 2 weeks of growth. Data are mean±SD (n=3). **$p < 0.01$.

vealed that molecular profiling clustered UC of the bladder on the basis of histopathogenesis and on biological criteria. Additionally, novel molecular targets identified were associated with histopathologic criteria and might become biomarkers of clinical utility in the management of patient with nonmuscle invasive LG-UC of the bladder.

KIF14 is involved in hepatocarcinogenesis, and KIF14 overexpression can be a genetic marker of hepatocellular carcinoma.[10] Elevated cell proliferation after KIF14 overexpression in SNU-761 cells and repressed after KIF14 knockdown in SNU-449 cells consistently support the oncogenic potential of KIF14. In addition, KIF14 is a mitotic kinesin that promotes the completion of cytokinesis.[11] Our data show that the increased expression of KIF14, a rapamyicn down-regulated gene, is related with higher recurrence of LG-UC of bladder. This means that KIF14 might be mTOR pathway downstream gene that predicts the recurrence at nonmuscle invasive LG-UC of the bladder. mTOR inhibitor be germane for patients with KIF14 expression.

Autophage has been generally thought to be a nonselective degradation system, but NBR1 (neighbor of *BRCA1* gene), the adaptor protein, has been shown to be involved in selective authophagy.[12] These scaffolding proteins selectively target some ubiquitinated proteins to autophagosomes for their degradation. Proteins like NBR1 are cytoprotective and help in scaffolding of misfolded or mutated proteins to form aggregates and ultimately to permit their autophagic degradation. Gambogic acid induced oxidative stress dependent caspase activation regulates

both apoptosis and autophagy by targeting various key molecules (NF-κB, Beclin-1, p62, and NBR1) in human bladder cancer cells.[13] In our study, higher expression of NBR1, rapamycin up-regulated gene was related to higher recurrence in non-muscle invasive LG-UC of the bladder, and NBR1 knockout potentiated the cell proliferation and growth inhibition effect of rapamycin. These findings support the views that up-regulated NBR1 could inhibit autophagy of bladder cancer cells by rapamycin and that NBR1 may be a novel target to predict the effect of mTOR inhibitor and to overcome the cross-talk of mTOR pathway.

Rapamycin could have clinical utility to prevent recurrence in LG-UC when rapamycin down-regulated genes are expressed in nonmuscle invasive LG-UC of the bladder. Rapamycin up-regulated gene expression may predict recurrence after rapamycin treatment. We need adjuvant new targeted agents to inhibit these genes combined with rapamycin. Moreover, this gene may be a useful target gene for novel targeted agents. Individual therapies for non-muscle invasive LG-UC of the bladder even in the face of based of cancer heterogeneity could be possible, even with the present limitation concerning gene screening of LG-UC. Further *in vitro* and *in vivo* studies are needed to evaluate the mechanisms and functions of the suspect genes, and clinical trials will be necessary to validate the candidate genes.

CONCLUSIONS

From microarray and clinical characteristics, our results revealed that KIF14 (rapamycin up-regulated gene), and NBR1 (rapamycin down-regulated gene) were the mTOR-related biomarker to predict recurrence in LG-UC of the bladder and the identification and validation of putative molecular targets have been facilitated by the combination of cDNA microarray and TMA, and NBR1 knockout overcomes the rapamycin cross-talk. Since mTOR expression is associated with the recurrence of LG-UC, molecular gene profiling by suppressing the mTOR pathway downstream, which is associated with the recurrence of LG-UC, may be a candidate for novel molecular target therapies.

CONFLICT OF INTEREST

The authors claim no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary Tables 1–3 can be found via https://doi.org/10.22465/kjuo.2017.15.1.28.

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Supplementary Table 1. Antibodies used for immunohistochemical staining

Antigen	Species	Source	Dilution	Positive control	Staining
FOXM1	Rabbit	Monoclonal Ab	1:50	Breat	Nucleus
KIF14	Rabbit	Monoclonal Ab	1:50	HeLa cell	Nucleus
MYBL2	Rabbit	Monoclonal Ab	1:50	Bladder	Nucleus
UHRF1	Rabbit	Monoclonal Ab	1:250	Heart	Nucleus
GPR87	Rabbit	Monoclonal Ab	1:50	Prostate	Cell membrane
NBR1	Rabbit	Monoclonal Ab	1:50	Heart	Cytoplasm
VASH1	Rabbit	Monoclonal Ab	1:50	Placenta	Secreted
PRIMA1	Rabbit	Monoclonal Ab	1:50	Bladder	Cell membrane

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Supplementary Table 2. Clinicopathological characteristics of patients and rapamycin down-regulated gene expression (mean H-score) with respect to categorized pathological parameters in nonmuscle invasive LG-UC of the bladder

Clinical pathologic feature	No. of patients (%)	H-score, mean±SD			
		FOXM1	KIF14	MYBL2	UHRF1
Age (yr)					
＜60	28 (31.1)	43.8±20.4	47.7±24.0	51.0±20.8	22.9±24.3
60–70	40 (44.4)	43.1±21.7	41.8±24.0	41.7±23.8	27.3±28.8
＞70	22 (24.4)	32.5±20.6	33.4±19.0	36.9±20.5	20.8±22.8
Sex					
Male	79 (87.8)	40.9±21.0	42.3±22.6	44.0±22.4	25.0±24.4
Female	11 (12.2)	39.8±24.8	36.2±28.0	39.1±24.0	20.1±36.6
Prior recurrence rate					
Primary	74 (82.2)	40.9±21.1	41.3±22.8	42.9±22.9	24.2±25.0
≤1 rec/yr	13 (14.4)	41.3±22.3	41.8±26.6	45.9±21.6	24.6±29.9
＞1 rec/yr	3 (3.3)	34.4±31.5	47.8±26.7	45.0±26.8	26.4±43.5
No. of tumors					
1	60 (66.7)	41.4±20.9	43.0±23.7	43.1±23.0	21.1±22.2
2–7	24 (26.7)	39.0±23.2	37.2±23.8	44.0±22.8	29.9±33.3
≥8	6 (6.7)	40.7±20.9	45.1±15.7	45.0±20.7	34.3±26.5
Tumor size (cm)					
≤3	69 (76.7)	40.9±22.0	42.7±24.2	32.4±22.1**	26.4±27.6
＞3	21 (23.3)	40.0±19.2	38.0±20.0	46.8±21.7	17.8±18.9
Cancer stage					
Ta	84 (93.3)	23.6±17.6*	21.1±16.2*	44.4±22.5	25.1±26.4
T1	6 (6.7)	42.0±21.1	43.0±23.1	30.4±20.8	14.2±17.7

LG-UC: low grade-urothelial carcinoma.
*$p < 0.05$. **$p < 0.01$.

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Supplementary Table 3. Clinicopathological characteristics of patients and rapamycin up-regulated gene expression (mean H-score) with respect to categorized pathological parameters in LG-UC

Clinical pathologic features	No. of patients (%)	H-score, mean±SD			
		GPR87	NBR1	VASH1	PRIMA1
Age (yr)					
<60	28 (31.1)	61.7±21.8	50.9±22.5	48.6±18.8	35.0±27.7
60–70	40 (44.4)	64.8±21.3	54.6±23.6	50.6±20.2	35.8±27.6
>70	22 (24.4)	53.0±22.0	48.9±26.7	41.4±20.8	31.8±24.8
Sex					
Male	79 (87.8)	60.0±22.5	52.5±24.1	48.5±19.6	34.3±26.5
Female	11 (12.2)	68.0±16.2	48.9±23.1	42.1±23.4	36.5±29.5
Prior recurrence rate					
Primary	74 (82.2)	60.5±21.6	51.8±23.5	46.4±20.0	33.4±26.3
≤1 rec/yr	13 (14.4)	62.6±23.8	50.9±27.6	53.0±20.1	43.5±26.8
>1 rec/yr	3 (3.3)	63.7±30.3	64.1±21.6	56.7±20.8	24.5±39.4
No. of tumors					
1	60 (66.7)	62.2±21.8	54.6±23.9	46.9±20.9	34.4±27.3
2–7	24 (26.7)	55.8±20.8	43.8±21.5	48.6±19.6	33.8±25.4
≥8	6 (6.7)	68.6±27.1	59.6±29.0	52.5±14.4	39.8±30.0
Tumor size (cm)					
≤3	69 (76.7)	60.6±23.2	53.0±24.4	47.8±20.7	22.7±24.2*
>3	21 (23.3)	62.1±17.2	49.1±18.7	47.4±18.1	38.2±26.6
Cancer stage					
Ta	84 (93.3)	62.1±20.6	28.6±31.0*	48.6±20.1	35.8±26.7
T1	6 (6.7)	45.5±35.0	53.7±22.6	34.7±15.2	17.6±23.7

LG-UC: low grade-urothelial carcinoma.
*$p<0.05$. **$p<0.01$.