Target Molecule Expression Profiles in Metastatic Renal Cell Carcinoma: Development of Individual Targeted Therapy

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The aim of this study is to analyze the level of target molecule expression in metastatic renal cell carcinoma (RCC) to determine whether there is a correlation between molecular marker expression and clinical response. Ten patients with metastatic RCC, who received receptor tyrosine kinase (RTK) targeted therapy after cytoreductive or radical nephrectomy, were included. The expression of target molecules relating to the RTK, mammalian target of rapamycin, hypoxia inducible factor, mitogen activated protein kinase, and adenosine monophosphate-activated protein kinase pathways were analyzed using real-time polymerase chain reaction and immunohistochemistry. We correlated the level of target molecule expression with clinical response, including efficacy and adverse events experience during RTK targeted therapy. All patients showed similar histological subtype and grade on pathological examination; however, the expression of RCC target molecules was very different among the patients. The expression of molecules related to the RTK pathway in RCC tissue as well as relative expression of molecules in RCC tissue compared to normal kidney tissue, were higher in patients who showed a good response to RTK targeted therapy compared to those that showed a poor response. Target molecule expression in normal kidney tissue was higher in patients who experienced high-grade adverse events than in patients who experienced low-grade events. Target molecule expression in metastatic RCC correlates with targeted therapy clinical response including efficacy and adverse events. Personalized target molecule expression profiles could be used to predict clinical response to different targeted therapies, thus helping optimization of targeted therapies for patients with metastatic RCC.

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INTRODUCTION

A thorough understanding of the molecular biology of renal cell carcinoma (RCC) has led to the development of novel targeted therapies for the disease. These targeted therapies have revolutionized the treatment of metastatic RCC, improving progression-free and overall patient survival. However, these novel therapies have also brought about new challenges in the man-

agement of toxicity [1-3]. In recent years, several targeted therapies have become available for the first- and second-line use in metastatic RCC (Fig. 1). Despite having a clear scientific rationale and promising preclinical results, clinical trials of these therapies have revealed limited benefits [4]. As such, their specific advantage as presurgical or postsurgical systemic therapy remains unclear.

While tumor cell type is predictive of the overall prognosis for patients with RCC, there remains great heterogeneity in clinical outcome, even for tumors of the same histological classification and stage [5]. The selection of optimal therapies based on biomarkers may enhance clinical outcomes, and delay the emergence of resistance [6]. However, at present, drugs continue to be prescribed according to the RCC histological subtype, without consideration of interpersonal genetic variation. In the future, it is likely that the molecular characteristics of tumors will be increasingly used to predict clinical response and ad-

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Figure 1. Diagram of pathways related to renal cell carcinoma (RCC) development, including key target molecules for each pathway, and developed (or developing) inhibitors for these target molecules. RCC signaling is divided into five pathways: receptor tyrosine kinase (RTK), mammalian target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK), hypoxia-inducible factor (HIF), and AMP-activated protein kinase (AMPK). These pathways are closely related to each other and several proteins participate in more than one pathway.

verse events related to targeted therapies. In order to increase drug efficacy and minimize adverse responses to targeted therapy, the expression of target molecules in individual patients should be investigated. Several studies have attempted to identify predictive molecular factors in RCC, and suggest that further efforts in this direction may bring success [7-10]. Ultimately, the use of genetic profiles to identify cancers at risk of progression or serious morbidity under targeted therapy, may allow us to personalize and optimize treatment.

We hypothesized that the levels of target molecule expression in metastatic RCC would correlate with a patient's clinical response to targeted therapy. In this study, we analyzed the expression of target molecules in metastatic RCC, and examined the correlation between molecular marker expression and clinical manifestation.

MATERIALS AND METHODS

Patients and samples

This study was approved by the Ethics Committee of Kyungpook National University School of Medicine. Between January 2010 and December 2012, ten patients with metastatic RCC (synchronous; 8 patients and metachronous; 2 patients), which was pathologically confirmed after cytoreductive or radical nephrectomy, were enrolled. All patients initially received a standard schedule and dosage of targeted therapy after nephrectomy. The targeted therapy dose was modified on the basis of adverse events, in accordance with the manufacturer's recommendations. The patient clinicopathologic characteristics including prognostic model using Heng criteria [11] are shown in Table 1. There were eight patients of pathological stage T3 or higher, and two patients of T1b and T2b. There were eight male

and two female patients, all with histologically proven high-grade clear cell RCC. One of the patients presented with a mixed type tumor composed of clear cell and chromophobe RCC. There were eight patients with synchronous metastasis and two patients with metachronous metastasis, for whom lung metastasis was observed at 12 and 6 months. The most common site of metastasis was the lung. The RCC and normal kidney tissue samples were obtained for each patient, from the Department of Urology, Kyungpook National University Medical Center, after cytoreductive or radical nephrectomy. Samples were analyzed using histology and standard molecular biology techniques. RCC tissue was obtained the fresh tissue rather than others and was mostly located in the periphery of the tumor. Normal kidney tissue was excised from the part of the specimen that was furthest from the cancer tissue.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from approximately 50 mg of tissue sample using the RNeasy Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. A total 2 µg of RNA was used for complementary DNA (cDNA) synthesis using the cDNA Reverse Transcription Kit (Applied Biosystems, Paisley, UK). Polymerase chain reaction (PCR) primers were designed using Primer Express Software (Applied Biosystems, Paisley, UK); primer sequences are listed in Table 2. PCR reactions were performed using an ABI Prism Sequence Detection System 7500 and SYBR Green PCR Master Mix (Applied Biosystems, Paisley, UK). For data analysis, the $2^{-\Delta\Delta C_t}$ method of relative quantification was used to estimate copy number. All samples were determined in duplicate. Negative controls that lacked cDNA were performed for each reaction. All expression data was normalized to beta-actin.

Immunohistochemical analysis

Immunohistochemical (IHC) analysis was performed in RCC and normal kidney tissue to verify the expression of proteins corresponding to each gene analyzed by PCR. A series of 5-µm sections from selected paraffin-embedded tissues were dewaxed and rehydrated. After blocking endogenous peroxidase and non-specific reactivity, the slides were incubated with various primary antibodies at 4°C overnight. After the primary antibody reaction, the slides were incubated with secondary antibody for 30 min prior to application of Vectastain® Elite ABC Reagent (Vector Laboratories, Burlingame, CA, USA) for a further 30 min. Liquid diaminobenzidine (DAB; Dako, Glostrup, Denmark) was used as a chromogenic agent for 5 min and sections were counterstained with Mayer's hematoxylin. For each antibody, a control reaction to normal kidney tissue was performed for each patient.

Outcomes of assessment

The messenger RNA expression of target molecules related to the receptor tyrosine kinase (RTK), mammalian target of rapamycin (mTOR), hypoxia inducible factor (HIF), mitogen activated protein kinase (MAPK), and 5' adenosine monophosphate-activated protein kinase (AMPK) pathways were analyzed in RCC and normal kidney tissue using real-time PCR analysis. We used IHC analysis to confirm the protein expression of VEGFR1, VEGFR2, VEGFR3, PDGFRα, and PDGFRβ. Based on their clinical response to RTK targeted therapy after six months of treatment, we divided the patients into good and poor response groups, and analyzed differences in target molecule expression between the two groups. We also performed analyses based on the severity of adverse events experienced during RTK targeted therapy and target gene expression in normal kidney tissue. Patients who received mTOR inhibitors as sec-

Table 1. Patient clinicopathological characteristics

Number	Sex/ Age	Initial stage	Fuhrman grade	Cell type	Kind of metastasis	Initial site of metastasis	Risk category using the Heng model
1	M/53	T3aN0M1	3	Clear cell	Synchronous	Lung	Favorable-risk group
2	F/64	T2bN0M1	4	Clear cell	Synchronous	Lung	Favorable-risk group
3	M/43	T3aN0M0	3	Clear cell	Metachronous	Lung	Favorable-risk group
4	M/38	T3bN1M1	3	Clear cell	Synchronous	Lung	Intermediate-risk group
5	M/56	T3bN1M1	4	Clear cell	Synchronous	Lung	Intermediate-risk group
6	M/37	T3bN1M1	4	Clear cell	Synchronous	Bone	Intermediate-risk group
7	M/63	T1bN0M0	3	Clear cell	Metachronous	Lung	Favorable-risk group
8	M/64	T4N1M1	3	Clear cell	Synchronous	Lung	Intermediate-risk group
9	F/55	T3aN0M1	4	Clear cell	Synchronous	Lung & bone	Poor-risk group
10	M/39	T3aN0M1	3	Mixed type (clear cell with chromophobe)	Synchronous	Bone	Poor-risk group

Table 2. Real-time polymerase chain reaction primer sequences

Gene	Sequence
RTK pathway	
<i>VEGFR1</i>	5'-TCCTTTATCCTGGATGCTGACA-3' 5'-CAAGGTGCTAGCCATCTTATTCTTT-3'
<i>VEGFR2</i>	5'-CTGGTTTTAGAAGGTTCGCTGTT-3' 5'-ACACGTAACGGTCTGGAAGGA-3'
<i>VEGFR3</i>	5'-ACGACCTGGGCTCGTATGTG-3' 5'-TCGACGCTGATGAAGGGATT-3'
<i>PDGFRA</i>	5'-AAAAAGCGAAGGCGCAATC-3' 5'-AATAAGCCCCCTCAACCT-3'
<i>PDGFRB</i>	5'-TGGGCTAGACACGGGAGAATACT-3' 5'-GGTGGGATCTGGCACAAGA-3'
<i>FLT3</i>	5'-AGTTCACGAACCCCTGAAAT-3' 5'-GCTGCCTACACATTCCTTGATCTT-3'
<i>KIT</i>	5'-AAACACGGCTTAAGCAATCCA-3' 5'-GCGTGTGCTGTCTTCTTCC-3'
<i>RET</i>	5'-CGCAACCGCGGCTTT-3' 5'-TGGGTGACAGGAAGACCTTGA-3'
mTOR pathway	
<i>PIK3</i>	5'-TCCCCGCTCCTAATTCATT-3' 5'-TGAGTACTCTGGGCTGGAATTC-3'
<i>MTOR</i>	5'-CTGCATCAGGACCTCTTCTCCT-3' 5'-CGACTGTAACCTCTCTCCTGCCA-3'
<i>p70</i>	5'-CTGAAGAAGCAGCGTACCAAGA-3' 5'-GTTCTGGCGCTTCTCCTTA-3'
MAPK pathway	
<i>RAS</i>	5'-ATCTTGCCTCCCTACCTTCCA-3' 5'-TGGAGATATCCACAGCAGCAGTA-3'
<i>RAF</i>	5'-TGGAATACTGATGCTGCGTCTT-3' 5'-GGAACGTCTTCCGAGCAAAG-3'
<i>MAPK</i>	5'-TGCATATACGCCCTTGAGCTACT-3' 5'-CTCAGGCATGGAACGTGGTT-3'
HIF pathway	
<i>HIF1A</i>	5'-GTAGTGCTGACCCTGCACTCAA-3' 5'-TCCATCGGAAGGACTAGGTGTC-3'
<i>CAIX</i>	5'-CGGAAGAAAACAGTGCCTATGA-3' 5'-CTTCCTCAGCGATTCTTCCA-3'
<i>26S</i>	5'-GAAAGCCCGAGCTGCCTTA-3' 5'-GGGCAGTAGATGGCATTTCG-3'
AMPK pathway	
<i>FYNK</i>	5'-AATGGCTTACATCGAGCGCAT-3' 5'-GCCAATCCGAAGTCAGCAATC-3'
<i>PDK4</i>	5'-GGAACCAAGAACCTGGCAAA-3' 5'-CACAAACATTGAGGAAGCAGCA-3'
<i>β-ACTIN</i>	5'-ATCGTCCACCGCAAATGCT-3' 5'-AAGCCATGCCAATCTCATCTTG-3'

RTK: receptor tyrosine kinase, mTOR: mammalian target of rapamycin, MAPK: mitogen activated protein kinase, HIF: hypoxia inducible factor, AMPK: adenosine monophosphate-activated protein kinase

ond-line targeted therapy were analyzed using similar methods.

RESULTS

Eight patients (cases 1, 2, 4, 5, 6, 8, 9, and 10) who had synchronous metastasis underwent cytoreductive nephrectomy as initial management. After surgery, they received the RTK inhibitor sunitinib. Two patients (cases 3 and 7) developed meta-chronous metastasis at 12 months and 6 months after radical nephrectomy. After confirming metastasis, these patients also received RTK targeted therapy using pazopanib and sunitinib, respectively. In point six months after the administration of targeted agents, a partial response was observed in four patients (cases 1, 3, 4, and 5) and stable disease was identified in one case (case 2), as determined using Response Evaluation Criteria in Solid Tumors. The remaining five patients showed progressive disease on follow-up study within 6 months. These patients were all subsequently administered the mTOR inhibitor everolimus as a second-line targeted therapy, however, disease progression continued in all five patients. In comparison with Heng model [11], the good response group after targeted therapy was composed of three favorable-risk patients and two intermediate-risk patients. In addition, the poor response group after targeted therapy was composed of one favorable-risk patient, two intermediate-risk patients, and two poor-risk patients. Adverse events to the first-line targeted therapy occurred in eight patients. Severe adverse events ($\geq$ grade 3) were observed in three patients (Table 3).

H&E staining was performed in order to observe the RCC and normal kidney tissue morphology. Target molecule gene expression was evaluated using real-time PCR. Although the majority of patients showed a similar histological subtype and grade in the pathological examinations, the expression of RTK pathway-related target molecules was highly variable in both the RCC and normal kidney tissue (Fig. 2). Differences in the expression of target molecules related to the mTOR, MAPK, HIF, and AMPK pathways were also observed between the RCC and normal kidney tissue of individual patients (Fig. 3). IHC was performed to confirm gene expression data using antibodies against VEGFR1, VEGFR2, VEGFR3, PDGFR α , and PDGFR β . The results obtained by IHC were similar to those obtained using real-time PCR. Representative images of tissue morphology staining are shown in Figure 4.

In the patients who showed a good response after first-line RTK targeted therapy, the average mRNA expression of the RTK pathway molecular targets (VEGFR1, VEGFR2, VEGFR3, PDGFR α , PDGFR β , FLT3, KIT, and RET) in RCC tissue appeared higher than that in patients who showed a poor response (Fig. 5A). Using the Mann-Whitney test, the expression levels

of VEGFR3, FLT3, and RET were shown to be significantly different between good response group and poor response group. The mean ratio of gene expression in RCC tissue proportional to normal kidney tissue was higher in the patients that showed a good response compared to those that showed a poor response (Fig. 5B). The relative values of VEGFR3 and FLT3 expression in RCC tissue compare to those in normal kidney tissue were significantly different depending on the clinical response. In four of five patients (cases 1, 2, 3, 4, but not 5) who showed a good response to first-line RTK targeted therapy, the expression of most of the RTK pathway target molecules was higher in RCC tissue than in the normal kidney tissue, unlike the poor response group (Fig. 2). In three patients (cases 6, 7, and 10) of the poor response group, most of target molecule expression in normal kidney tissue was higher than that in RCC tissue. In the remaining two patients (cases 8 and 9) of the poor response group, there was a mixed pattern of target molecule expression in RCC and normal tissue (Fig. 2). All of the patients who showed a poor response to RTK targeted therapy received second-line targeted therapy using an mTOR inhibitor. However, all of the patients showed a poor clinical response to the mTOR inhibitor

(Table 3). Using real-time PCR analysis it was shown that all of these patients had a lower expression of the target molecule (mTOR) in RCC tissue than that in normal kidney tissue (Fig. 6). The expression of VEGFR1, VEGFR2, VEGFR3, PDGFRA, and PDGFRB in normal kidney tissue was significantly different between patients who experienced low-grade ($\leq$ grade 2) and high-grade ($\geq$ grade 3) adverse events (Fig. 5C).

DISCUSSION

RCC is categorized according to cell morphology, grade, and stage, and a patient's prognosis is different depending upon these classifications. Localized RCC is best treated by surgical resection; however, systemic treatment is necessary for metastatic cases. Immunotherapy, chemotherapy, and combined immunotherapy have been used for systemic treatment of advanced RCC, but these treatments are associated with significant cytotoxicity, nonspecific behavior, and a low response rate [12].

Completion of the human genome project and technological advances has helped to identify pivotal molecules within cer-

Table 3. Clinical responses after targeted therapy

Number	Surgical treatment	First-line targeted therapy	Response after first-line therapy	Side effects after first-line therapy (grade)	Second-line targeted therapy	Response after second-line therapy
1	Cytoreductive nephrectomy	RTK inh. (Sunitinib)	PR	None	-	-
2	Cytoreductive nephrectomy	RTK inh. (Sunitinib)	SD	Thrombocytopenia (grade 1)	-	-
3	Radical nephrectomy	RTK inh. (Pazopanib)	PR	Hair color change (grade 1), gastritis & diarrhea (grade 1)	-	-
4	Cytoreductive nephrectomy	RTK inh. (Sunitinib)	PR	HFS (grade 2), anorexia (grade 2)	-	-
5	Cytoreductive nephrectomy	RTK inh. (Sunitinib)	PR	HFS (grade 3), elevated LFT (grade 3), renal failure (grade 3), anorexia (grade 2)	-	-
6	Cytoreductive nephrectomy	RTK inh. (Sunitinib)	PD	HFS (grade 2), gastritis (grade 3)	mTOR inh. (Everolimus)	PD
7	Radical nephrectomy	RTK inh. (Sunitinib)	PD	None	mTOR inh. (Everolimus)	PD
8	Cytoreductive nephrectomy with both adrenalectomy	RTK inh. (Sunitinib)	PD	Mucositis (grade 1)	mTOR inh. (Everolimus)	PD
9	Cytoreductive nephrectomy	RTK inh. (Sunitinib)	PD	Hypertension (grade 2), elevated LFT (grade 2), gastritis (grade 1), hair color change (grade 1)	mTOR inh. (Everolimus)	PD
10	Cytoreductive nephrectomy	RTK inh. (Sunitinib)	PD	Perianal abscess (grade 3), elevated LFT (grade 3)	mTOR inh. (Everolimus)	PD

RTK inh: receptor tyrosine kinase inhibitor; mTOR inh: mammalian target of rapamycin inhibitor; PR: partial response, SD: stable disease, PD: progressive disease, -: not treated, HFS: hand foot syndrome, LFT: liver function test

Figure 2. Expression of RTK-related target molecules (VEGFR PDGFR, FLT3, C-KIT, RET) in RCC and normal kidney tissue (Ctrl) of patients 1–10, quantified using real-time polymerase chain reaction (PCR). *Y-axis: relative change fold to beta-actin. RTK: receptor tyrosine kinase, RCC: renal cell carcinoma.

Figure 3. Expression of target molecules relating to the mTOR, MAPK, HIF, and AMPK pathways in RCC and normal kidney tissue of patients 1–10, quantified using real-time PCR. *Y-axis: relative change fold to beta-actin. mTOR: mammalian target of rapamycin, MAPK: mitogen-activated protein kinase, HIF: hypoxia-inducible factor, AMPK: adenosine monophosphate-activated protein kinase, RCC: renal cell carcinoma, PCR: polymerase chain reaction.

tain RCC patient populations, and therapy specifically aimed at these molecules has demonstrated a robust clinical efficacy. The drugs currently approved as targeted agents for the treatment of RCC can be divided into two main classes depending on whether they target the RTK or mTOR pathway [13]. The

most well-defined targets are the RTK growth factor signaling pathways, including their ligands and downstream cytosolic kinases. Clinical trials have demonstrated the feasibility and safety of inhibiting RTKs [14]. Current RCC targeted therapies include RTK inhibitors such as sorafenib, sunitinib, and pazopanib

Figure 4. Representative images of immunohistochemical (IHC) staining for the target molecules VEGFR1, VEGFR2, VEGFR3, PDGFR α , and PDGFR β in RCC and normal kidney tissue. IHC analysis of protein expression was used to confirm the gene expression data obtained by real-time PCR. RCC: renal cell carcinoma, PCR: polymerase chain reaction.

Figure 5. (A) The mean RTK-related target molecule expression in RCC tissue of patients who showed a good or a poor response after RTK targeted therapy, quantified using real-time PCR. (B) The relative ratio of RTK-related target molecule expression in RCC tissue proportional to normal kidney tissue in patients who showed a good or a poor response after RTK targeted therapy, quantified using real-time PCR. (C) The mean RTK-related target molecule expression in normal kidney tissue of patients who showed low grade or high grade morbidity during RTK targeted therapy, quantified using real-time PCR. RTK: receptor tyrosine kinase, RCC: renal cell carcinoma, PCR: polymerase chain reaction.

Figure 6. mTOR expression in RCC and normal kidney tissue (Ctrl) of patients 1–10, quantified using real-time PCR. mTOR: mammalian target of rapamycin, RCC: renal cell carcinoma, PCR: polymerase chain reaction.

and mTOR inhibitors such as temsirolimus and everolimus [15]. Furthermore, recent advances have identified the MAPK, HIF, and AMPK pathways as potential therapeutic targets.

Hypoxia, resulting from tumor growth beyond a critical size, has been found to regulate a complex cascade of genes, including the proangiogenic growth factors vascular epithelial growth factor (VEGF) and PDGF [16]. VEGF and its receptors play a pivotal role in physiologic and pathologic angiogenesis, which is essential for tumor progression and the development of metastasis [17]. Generally, RCCs are characterized as having abundant blood vessels, with extensive hemorrhage, necrosis, and tumor thrombi associated with angiogenic activity [18]. VEGF is a potent promoter of tumor angiogenesis, with VEGF mRNA and protein levels higher in RCC than in normal kidney cortex [19,20]. PDGF is a growth factor extensively involved in multi-dimensional cellular dynamics and in tumorigenesis [21]. Recent study identified that the cytoplasmic expression of VEGF, VEGFR, PDGF-B, and PDGFR- β in tumor cells was dependent on the pathologic stage and cellular type of RCC [22]. Molecules directed against VEGF/PDGF protein and VEGF/

PDGF receptor signaling are available for RCC treatment.

Although clinical trials of targeted therapies have demonstrated improved outcomes, the degree of response to targeted therapies varies considerably between patients. In addition, targeted therapies have been associated with various toxicities, including fatal adverse events [23,24]. It is therefore important to select patients with metastatic RCC, who would be expected to benefit from targeted agents, before the administration of these agents. This would help to avoid unnecessary treatment and spare metastatic RCC patients from toxic effects of targeted therapy if they are unlikely to show a good response. In the era of molecularly targeted therapies, it is important to identify reliable predictors of efficacy and adverse events in order to provide the optimal treatment for individual patients with metastatic RCC. At present, conventional clinical practice provides treatment based on tumor histology (grade and TNM stages). However, this does not give sufficient information to enable optimized treatment for individual patients [25]. The identification of biomarkers of efficacy and safety is necessary to select the appropriate treatment for patients and to improve their

clinical outcome [26]. Molecules related to the biology of RCC have been investigated as potential biomarkers for predicting therapeutic efficacy. In this study, we analyzed the correlation between the expression of target molecules in RCC and normal kidney tissue with clinical outcomes, including drug efficacy and adverse events, in order to examine the potential for personalized therapy based on these molecular markers. Although our study was conducted in a small number of patients, the expression of some molecules related to the RTK pathway was significantly different depending on the drug efficacy and adverse events to the targeted therapy. Prospective studies in larger patient populations with long-term follow-up will improve our understanding of the molecules related to the RTK pathway. In these regards, our results in the present study suggest an excellent example for the many researchers engaged in a personalized therapy in mRCC.

Recently, the variability in patients' responses to tumor therapeutic agents has been recognized. Molecular investigations have demonstrated that this variability is a result of differential target gene expression [27]. Therefore, clinicians have now begun to appreciate the concept of "personalized" therapy, which aims to improve therapeutic efficacy, reduce side effects, and increase patient survival rates [27,28]. In this study, we compared the expression of several target molecules related to the RTK pathway in RCC tissue and normal kidney tissue. Although patients showed similar histological subtype and grade in pathological examinations, the gene expression of the target molecules varied widely between individuals. This suggests that use of the same targeted agent for all patients will be ineffective because of RCC heterogeneity.

Several molecular markers have been assessed for their use as prognostic predictors in patients with RCC who are treated with RTK-pathway inhibitors [8,9,29,30]. In recent studies, the expression of angiogenesis related molecular markers, such as vascular epithelial growth factor (VEGF), VEGFR, and PDGFR, in tumor specimens has been assessed. These studies found that the expression of RTK pathway-related factors, such as VEGFA, VEGFR, VEGFR3, PDGFR α , and PDGFR β , were prognostic indicators for clinical response and progression free survival [7,9,10,31]. However, the value of molecules in the RTK pathway as prognostic biomarkers for RCC patients treated with targeted therapy remains controversial because RTK is act as multi-kinase inhibitors. In our study, the expression of RTK pathway-related molecules in RCC and normal kidney tissue generally appeared higher in patients that showed a good response to RTK-targeted therapy compared to those that showed a poor response. Although statistical significance was only observed for some of the target molecules, we found that the global expression of molecules involved in the RTK

pathway showed a similar pattern for each individual (Fig. 2). It is possible that the indications of targeted therapy efficacy can be gained from either patterns of target molecule expression in tumor tissue, in tumor tissue relative to normal kidney tissue, or in the absolute expression level of specific molecule efficacy.

Furthermore, the target molecular expression profile may predict the safety of targeted therapy as well as clinical efficacy. In our current study, severe adverse events ($\geq$ grade 3) were developed in three out of 10 patients, and we observed the expression of some molecules related to the RTK pathway in normal kidney tissue was significantly different according to the severity of adverse events. Although our study was conducted in a small number of patients, it has shown a correlation between VEGFR and PDGFR expression in normal kidney tissue and severe adverse events ($\geq$ grade 3). This suggests that target molecule expression profiles serve as a biomarker to predict the severity of adverse events, and aid in the development of personalized therapy.

We believe that the gene expression pattern of molecules related to RTK pathways in RCC and normal kidney tissue could contribute to the realization of personalized targeted therapy. It is important to recognize that there is a relationship between target gene expression and drug sensitivity, in addition to interpersonal variations in the expression level. Therefore, increased targeted therapy efficacy and safety could be achieved by analysis of clinical specimens for mRNA expression before prescribing medication. Our results could have important implications for the selection of individual targeted therapies for patients, particularly if it is found that different molecular expression profiles confer sensitivity to a specific drug, even if that drug acts on the same pathway as another drug. All patients who showed a poor response after first-line RTK targeted therapy received the mTOR inhibitor everolimus as a second-line targeted therapy; however, their response to this drug was also poor. Using real-time PCR, we were able to show that the expression of mTOR in RCC tissue was significantly lower than that in the corresponding normal kidney tissue. This second-line treatment result reconfirmed the importance of personalized drug selection based on variation in target molecule expression.

On the basis of our initial findings, we proceeded to investigate whether there were other valid prognostic pathways in patients who showed a poor response to the first-line targeted therapy. Target molecule expression in the mTOR, MAPK, HIF, and AMPK pathways were analyzed. We identified PIK3 in the mTOR pathway (cases 6 and 10), HIF1A and CAIX in the HIF pathway (cases 6, 7, 8 and 9), and FINK and PDK4 in the AMPK pathway (cases 6 and 9) as potential therapeutic target molecules (Fig. 4). This suggests that RCC is not equally ame-

nable to treatment strategies targeting the RTK and mTOR pathways. With this in mind, physicians should consider which is the most efficacious and safe pathway to target in metastatic RCC based on an individual's gene expression patterns. Similarly, gene expression profiling could be used to identify the most effective treatment for metastatic RCC since many studies have identified new target molecules in RCC [32-36], and a variety of targeted agents have been developed to suppress these [37-40].

Currently, the mainstream of management for RCC is composed of primary lesion resection and adjuvant target therapy. Resection of diseased kidney can result in renal insufficiency that regeneration is needed because the kidney is far beyond the potential of self-healing. Therefore, regeneration of the kidney is one of the most demanding procedures in nephrologic/urologic area, especially in the patients with kidney cancer. A major concern of regenerative therapies during cancer remission/repression is the possibility of cancer recurrence. Our study analyzes the level of target molecules expression according to the each pathway using real-time PCR and immunohistochemistry, and the results approve these genes' effectiveness and safety for personalized target therapy. Especially, target molecule profile can provide the information about the molecular expression of cancer cells and microenvironment under reproducible conditions, as well as the meaning of the tumor suppression.

Several limitations of our study should be considered. This was a nonrandomized retrospective study that included only ten patients, treated at a single institution and with a short follow-up period. Furthermore, there was selection bias regarding the inclusion criteria, such as the presence of a resectable tumor and the patient's race. In addition, the expression of molecules was evaluated in RCC specimens after radical nephrectomy. Unfortunately, considering the situation at our clinical fields, it is not easy to acquire the sufficient tumor and donor matched normal kidney tissues in RCC patients who have not undergone the neo-adjuvant targeted therapy. In order to overcome these limitations, it would be necessary to perform a prospective study using target molecule expression profiles in a larger number of metastatic RCC patients treated with different targeted therapies. Nevertheless, this study has identified that quantification of target molecule expression in tumor and normal tissue could be used to predict a patient's clinical response to targeted therapy. Previous studies have used IHC analysis to quantify a predictive biomarker; however, this approach is subject to inter-observer variability and cannot be performed for all molecular markers. In addition, in the not too distant future, if the gene expression profile of the targeted molecule can be measured in the blood, urine, or a small amount of tissue sample using needle biopsy, personalized medicine before

neoadjuvant targeted therapy also should be available because it can predict the efficacy and safety.

In this study, we found a correlation between target molecule expression and clinical outcomes, including efficacy and safety, in metastatic RCC patients who received targeted therapy. Based on target gene expression patterns, we identified other valuable pathways for therapeutic targeting in patients who showed a poor response to RTK and mTOR inhibitors. Prediction of efficacious and safe therapeutic options, according to targeted gene expression profiles, could prevent serious adverse events and the burden of unnecessary treatment regimens for metastatic RCC patients. The analysis of interpersonal variability in molecular expression profiles could help to optimize therapy and increase targeted therapy efficacy and safety.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

This study was approved by the Ethics Committee of the Kyungpook National University School of Medicine (IRB no. KNUMC BIO_13-1005). This study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

REFERENCES

1. Di Lorenzo G, Autorino R, Sternberg CN. Metastatic renal cell carcinoma: recent advances in the targeted therapy era. *Eur Urol* 2009;56:959-971.
2. Hutson TE. Targeted therapies for the treatment of metastatic renal cell carcinoma: clinical evidence. *Oncologist* 2011;16 Suppl 2:14-22.
3. Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, Oudard S, et al. Overall survival and updated results for sunitinib compared with interferon alfa in patients with metastatic renal cell carcinoma. *J Clin Oncol* 2009;27:3584-3590.
4. Park JW, Kerbel RS, Kelloff GJ, Barrett JC, Chabner BA, Parkinson DR, et al. Rationale for biomarkers and surrogate end points in mechanism-driven oncology drug development. *Clin Cancer Res* 2004;10:3885-3896.
5. Taneja SS. Re: molecular stratification of clear cell renal cell carcinoma by consensus clustering reveals distinct subtypes and survival patterns. *J Urol* 2011;185:1224.
6. Brannon AR, Reddy A, Seiler M, Arreola A, Moore DT, Pruthi RS, et al.

- Molecular stratification of clear cell renal cell carcinoma by consensus clustering reveals distinct subtypes and survival patterns. *Genes Cancer* 2010;1:152-163.
7. Garcia-Donas J, Leandro-García LJ, Del Alba AG, Morente M, Alemany I, Esteban E, et al. Prospective study assessing hypoxia-related proteins as markers for the outcome of treatment with sunitinib in advanced clear-cell renal cell carcinoma. *Ann Oncol* 2013;24:2409-2414.
 8. Kusuda Y, Miyake H, Behnsawy HM, Fukuhara T, Inoue TA, Fujisawa M. Prognostic prediction in patients with metastatic renal cell carcinoma treated with sorafenib based on expression levels of potential molecular markers in radical nephrectomy specimens. *Urol Oncol* 2013;31:42-50.
 9. Terakawa T, Miyake H, Kusuda Y, Fujisawa M. Expression level of vascular endothelial growth factor receptor-2 in radical nephrectomy specimens as a prognostic predictor in patients with metastatic renal cell carcinoma treated with sunitinib. *Urol Oncol* 2013;31:493-498.
 10. Siraj MA, Pichon C, Radu A, Ghinea N. Endothelial follicle stimulating hormone receptor in primary kidney cancer correlates with subsequent response to sunitinib. *J Cell Mol Med* 2012;16:2010-2016.
 11. Heng DY, Xie W, Regan MM, Warren MA, Golshayan AR, Sahi C, et al. Prognostic factors for overall survival in patients with metastatic renal cell carcinoma treated with vascular endothelial growth factor-targeted agents: results from a large, multicenter study. *J Clin Oncol* 2009;27:5794-5799.
 12. Liou LS, Shi T, Duan ZH, Sadhukhan P, Der SD, Novick AA, et al. Microarray gene expression profiling and analysis in renal cell carcinoma. *BMC Urol* 2004 22;4:9.
 13. Cowey CL, Hutson TE. Molecularly targeted agents for renal cell carcinoma: the next generation. *Clin Adv Hematol Oncol*. 2010;8:357-360, 361-364.
 14. Potti A, George DJ. Tyrosine kinase inhibitors in renal cell carcinoma. *Clin Cancer Res* 2004;10(18 Pt 2):6371S-6376S.
 15. Motzer RJ, Bukowski RM. Targeted therapy for metastatic renal cell carcinoma. *J Clin Oncol* 2006;24:5601-5608.
 16. Levy AR, Levy NS, Wegner S, Goldberg MA. Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. *J Biol Chem* 1995;270:13333-13340.
 17. Berger DB, Herbstritt L, Dengler WA, Marmé D, Mertelsmann R, Fiebig HH. Vascular endothelial growth factor (VEGF) mRNA expression in human tumor models of different histologies. *Ann Oncol* 1995;6:817-825.
 18. Ferrara N. Role of vascular endothelial growth factor in the regulation of angiogenesis. *Kidney Int* 1999;56:794-814.
 19. Tomisawa M, Tokunaga T, Oshika Y, Tsuchida T, Fukushima Y, Sato H, et al. Expression pattern of vascular endothelial growth factor isoform is closely correlated with tumour stage and vascularisation in renal cell carcinoma. *Eur J Cancer* 1999;35:133-137.
 20. Takahashi A, Sasaki H, Kim SJ, Tobisu K, Kakizoe T, Tsukamoto T, et al. Markedly increased amounts of messenger RNAs for vascular endothelial growth factor and placenta growth factor in renal cell carcinoma associated with angiogenesis. *Cancer Res* 1994;54:4233-4237.
 21. Farooqi AA, Waseem S, Riaz AM, Dilawar BA, Mukhtar S, Minhaj S, et al. PDGF: the nuts and bolts of signalling toolbox. *Tumour Biol* 2011;32:1057-1070.
 22. Song SH, Jeong IG, You D, Hong JH, Hong B, Song C, et al. VEGF/VEGFR2 and PDGF-B/PDGFR- β expression in non-metastatic renal cell carcinoma: a retrospective study in 1,091 consecutive patients. *Int J Clin Exp Pathol* 2014;7:7681-7689.
 23. Schutz FA, Je Y, Richards CJ, Choueiri TK. Meta-analysis of randomized controlled trials for the incidence and risk of treatment-related mortality in patients with cancer treated with vascular endothelial growth factor tyrosine kinase inhibitors. *J Clin Oncol* 2012;30:871-817.
 24. Sivendran S, Liu Z, Portas LJ Jr, Yu M, Hahn N, Sonpavde G, et al. Treatment-related mortality with vascular endothelial growth factor receptor tyrosine kinase inhibitor therapy in patients with advanced solid tumors: a meta-analysis. *Cancer Treat Rev* 2012;38:919-925.
 25. Schmidinger M, Zielinski CC. Novel agents for renal cell carcinoma require novel selection paradigms to optimise first-line therapy. *Cancer Treat Rev* 2009;35:289-296.
 26. Simon RM, Paik S, Hayes DF. Use of archived specimens in evaluation of prognostic and predictive biomarkers. *J Natl Cancer Inst* 2009;101:1446-1452.
 27. Gadgil SM, Cote ML, Schwartz AG, Matherly LH, Wozniak A, Bepko G. Parameters for individualizing systemic therapy in non-small cell lung cancer. *Drug Resist Updat* 2010;13:196-204.
 28. Peña C, Lathia C, Shan M, Escudier B, Bukowski RM. Biomarkers predicting outcome in patients with advanced renal cell carcinoma: results from sorafenib phase III Treatment Approaches in Renal Cancer Global Evaluation Trial. *Clin Cancer Res* 2010;16:4853-4863.
 29. Choueiri TK, Regan MM, Rosenberg JE, Oh WK, Clement J, Amato AM, et al. Carbonic anhydrase IX and pathological features as predictors of outcome in patients with metastatic clear-cell renal cell carcinoma receiving vascular endothelial growth factor-targeted therapy. *BJU Int* 2010;106:772-778.
 30. Perez-Gracia JL, Prior C, Guillén-Grima F, Segura V, Gonzalez A, Panizo A, et al. Identification of TNF-alpha and MMP-9 as potential baseline predictive serum markers of sunitinib activity in patients with renal cell carcinoma using a human cytokine array. *Br J Cancer* 2009;101:1876-1883.
 31. Barrios CH, Hernandez-Barajas D, Brown MP, Lee SH, Fein L, Liu JH, et al. Phase II trial of continuous once-daily dosing of sunitinib as first-line treatment in patients with metastatic renal cell carcinoma. *Cancer* 2012;118:1252-1259.
 32. Koyama S, Yu H, Dalgarno DC, Shin TB, Zydowsky LD, Schreiber SL. Structure of the PI3K SH3 domain and analysis of the SH3 family. *Cell* 1993;72:945-952.
 33. Wei H, Ke HL, Lin J, Shete S, Wood CG, Hildebrandt MA. MicroRNA target site polymorphisms in the VHL-HIF1 α pathway predict renal cell carcinoma risk. *Mol Carcinog* 2014;53:1-7.
 34. Stillebroer AB, Mulders PF, Boerman OC, Oyen WJ, Oosterwijk E. Carbonic anhydrase IX in renal cell carcinoma: implications for prognosis, diagnosis, and therapy. *Eur Urol* 2010;58:75-83.
 35. Razorenova OV, Finger EC, Colavitti R, Chernikova SB, Boiko AD, Chan CK, et al. VHL loss in renal cell carcinoma leads to up-regulation of CUB domain-containing protein 1 to stimulate PKC[delta]-driven migration. *Proc Natl Acad Sci U S A* 2011;108:1931-1936.
 36. Lee JH, Kim EJ, Kim DK, Lee JM, Park SB, Lee IK, et al. Hypoxia induces PDK4 gene expression through induction of the orphan nuclear receptor ERR γ . *PLoS One* 2012;7:e46324.
 37. Kim J, Jonasch E, Alexander A, Short JD, Cai S, Wen S, et al. Cytoplasmic sequestration of p27 via AKT phosphorylation in renal cell carcinoma. *Clin Cancer Res* 2009;15:81-90.
 38. LoPiccolo J, Blumenthal GM, Bernstein WB, Dennis PA. Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations. *Drug Resist Updat* 2008;11:32-50.
 39. Podar K, Anderson KC. The pathophysiologic role of VEGF in hematologic malignancies: therapeutic implications. *Blood* 2005;105:1383-1395.
 40. Keith B, Johnson RS, Simon MC. HIF1 α and HIF2 α : sibling rivalry in hypoxic tumour growth and progression. *Nat Rev Cancer* 2011;12:9-22.