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From Cell Biology to Tissue Engineering

Retrospective analysis of 25 immunohistochemical tissue markers for differentiating multilocular cystic renal neoplasm of low malignant potential and multicystic renal cell carcinoma

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Summary. Multilocular cystic renal neoplasm of low malignant potential (MCRNLMP) and multicystic renal cell carcinoma (MCRCC) are morphologically indistinguishable. MCRNLMP is a tumor composed entirely of numerous cysts, the septa of which contain individual or groups of clear cells without expansile growth. However, unlike MCRCC, neither recurrence nor metastasis has been reported in MCRNLMP. The aim of this study was to identify significant differential pathological characteristics in resected specimens from patients diagnosed with MCRNLMP (n=13) and MCRCC (n=17) using immunohistochemistry of 25 tissue markers. Staining interpretation was performed semi-quantitatively using the H-score (0-300) or intensity score (0-3), and differences between groups were evaluated using the Fisher exact and Wilcoxon rank-sum tests. During a median follow-up of 66.2 months (1-141.9 months), there was only one case of MCRCC recurrence among all 30 patients, including 19 (63.3%) at stage pT1a, 8 (26.7%) at stage pT1b, and 3 (10.0%) patients at stage pT2. Tumor necrosis rate (0% vs. 52.9%) and median tumor size (3.2 cm vs. 4.1 cm) significantly differed between MCRNLMP and MCRCC samples. Among the 25 tissue markers, only HIF1a, PDGFRα, SMA, VEGFR1, VEGFR2, VEGFR3, CD10, CD31, CD34, CK7-tubule, TGAse-2, and Ki-67 showed significantly different expression between the groups. These tissue markers with differential expression between MCRNLMP and MCRCC can provide a clue to understanding their distinct pathophysiology.

Key words: Renal cell carcinoma, Comparison, Cyst, Immunohistochemistry

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Introduction

Multilocular cystic renal cell carcinoma (RCC) became an established disease entity under the 2004 World Health Organization (WHO) classification of kidney tumors. RCC is characterized by clear tumor cells composed of numerous cystic linings of cystic tumors that are indistinguishable from grade-1 clear cell RCC but without formation of a malignant and expansive nodule (Eble et al., 2004). Multilocular cystic RCC has been reported to show indolent behavior without any recurrence and metastasis at more than five years of follow-up, with an overall prevalence of less than 2% (Montironi et al., 2013).

A new term was adopted for multilocular cystic RCC at the 2012 International Society of Urological

Pathology (ISUP) consensus conference on renal neoplasia (Srigley et al., 2013): multilocular cystic renal neoplasm of low malignant potential (hereafter MCRNLMP) (Srigley et al., 2013; Kristiansen et al., 2015). MCRNLMP is diagnosed based on its pathological characteristics, representing a tumor composed entirely of numerous cysts, the septa of which contain individual or groups of clear cells without expansile growth (Moch et al., 2016). Histologic diagnosis of MCRNLMP is dependent on available clinical and radiologic information as well as standard hematoxylin and eosin (H&E)-based histologic evaluation, but not on immunohistochemistry (IHC) (Kristiansen et al., 2015). Its epidemiology is still unclear because of its rarity with a prevalence of less than 2% among all cases of cystic RCC, and only a few case reports and descriptive prognostic studies with small sample sizes have been published to date (Li et al., 2016).

The most controversial and relevant issue related to MCRNLMP at present is the ability to differentiate this subtype from multicystic RCC (hereafter MCRCC). This distinction is important because of the favorable prognostic outcomes for MCRNLMP, including no recurrence and metastases and no other visible objective key imaging findings until the cystic renal mass is resected and pathologically diagnosed (Siegel, 2005; You et al., 2011). In addition, patients with MCRNLMP are currently managed by strictly following the guidelines for MCRCC with either radical or partial nephrectomy and excessively regular follow-ups involving frequent radiologic and laboratory examinations. Proper discrimination between the subtypes would therefore avoid such invasive and unnecessary procedures.

Therefore, the aim of this study was to identify any significant differences in the IHC expression of 25 tissue markers known to be involved in the pathogenesis and progression of RCC between nephrectomized tissue specimens of patients with MCRNLMP and morphologically similar MCRCC to evaluate their potential for differentiating between the two subtypes (Kim et al., 2017a,b).

Materials and methods

Ethics statement

Following approval of this retrospective study by the Institutional Review Board (IRB) of the National Cancer Center (No. NCC2017-0096), the IRB approved exemption from the written consent procedure. This study was conducted according to the principles expressed in the Declaration of Helsinki.

Inclusion criteria and tissue samples

Between 2009 and 2016, 20 cases of MCRNLMP and 60 cases of MCRCC were selected for retrospective review of the patients' medical records and tissue samples. After excluding 7 (25.0%) MCRNLMPs and 43 (71.7%) MCRCCs because of follow-up loss, old tissue samples inadequate for tissue microarray (TMA) and IHC staining, and less than 10 grossly cystic components within the sample, as reviewed by a senior uropathologist with 15 years of experience, a total of 30 (37.5%) patients, including 13 (43.3%) MCRNLMP and 17 (56.7%) MCRCC patients, were enrolled in the study. The patients' RCC specimens staged at T1a, T1b, or T2 were evaluated with IHC using the 25 tissue markers described below.

IHC and TMA

The TMA and IHC analyses were performed according to previous studies using our established procedure of manufacturing TMAs (Kim et al., 2015, 2017). In brief, TMA blocks were constructed using representative tumor areas and paired normal control tissue samples from formalin-fixed paraffin-embedded tumor material. Suitable areas for tissue retrieval from duplicate 2-mm-diameter cores taken from the tumor block were marked on standard H&E-stained sections, punched out from the paraffin block, and inserted into a recipient block. The deparaffinization, heat-induced antigen retrieval, and reactivity were performed using a standard protocol for each tissue biomarker with the Ventana Ultra-View detection kit and automatic immunostainer (Ventana, Tucson, AZ, USA).

The following 25 tissue biomarkers were selected and IHC-stained on all 30 TMA blocks of primary nephrectomized specimens according to suitable environmental conditions indicated by the manufacturer: AMACR, BAP1, CD10, CD31, CD34, CK7-tubule, CK-PAN, HIF1α, HIF2α, Ki-67, pS6, PAX8, PBRM1, PDGFRα, PDGFRβ, PDL1, PSMA, PTEN, RCC, SMA, TGase-2, VEGFR1, VEGFR2, VEGFR3, and Vimentin.

Quantification of tissue expression

The expression of each tissue marker was semi-quantitatively assessed by a senior uropathologist (WSP) using a continuous H-score ranging from 0 to 300 (Kim et al., 2017a,b). The H-score was calculated with an intensity score (0, none; 1, low; 2, intermediate; 3, severe intensity) multiplied by the area of expression (0-100% of cells stained). The percentage of cells stained and the intensity of that staining were assessed within both the nuclei and the cytoplasm of malignant cells and compared to those of the paired benign cells by a single pathologist (WSP) blinded to the clinical outcome who was assisted by one urologist (SHK). Among the 25 tissue markers, CD31, CD34, CK7-tubule, CK-PAN, PD-L1, and Ki-67 were quantified only based on the intensity score (0-3) and the other 19 markers were quantified based on the H-score. In addition, Ki-67

expression was categorized according to different degrees of intensity with respect to the percentage of positively stained cells as follows: none, 0%; low intensity, 1-10%; intermediate intensity, 11-30%; and severe intensity, >30%.

Statistical analysis

The semi-quantitative H-scored and intensity-scored immunostaining results were statistically analyzed to compare the expression scores for all 25 tissue biomarkers between the MCRNLMP and MCRCC groups. The highest H-score for the expression level was selected for all markers, except for the case of BAP1 loss, PBRM1 loss, and PTEN loss, in which the lowest H-score for negative values was chosen.

To investigate the distribution of clinicopathological characteristics and the associations with tissue biomarkers between the two groups, different statistical analyses were conducted according to the characteristics of each variable. All continuous variables are summarized as the median (range; min-max) and all categorical variables are presented as frequency and percentages. The differences in distribution between the two groups were compared using Fisher's exact test for categorical variables, and using Wilcoxon rank-sum test for continuous variables. The intensity score and semi-quantitative H-score for tissue biomarkers were visually examined using bar graphs and box plots.

Results

The clinicopathological characteristics of the 30 patients and results of IHC staining are summarized in Table 1. During a median follow-up period of 62.9 months (1-141.9 months), there was only one case of recurrence among the 17 MCRCC patients. A tumor was resected in 19 (63.3%) radical and 11 (36.7%) partial nephrectomies with a median tumor size of 3.8 (0.5-8) cm. The majority of patients were diagnosed at pathologic stage pT1a (63.3%).

Among all clinicopathological variables, only tumor necrosis rate (0% vs. 52.9%) and tumor size (3.2 cm vs. 4.1 cm) differed significantly between the MCRNLMP and MCRCC groups ($p<0.05$, Table 1). Among the 25 tissue markers, only the expression of HIF1α, PDGFRα, SMA, VEGFR1, VEGFR2, VEGFR3, CD10, CD31, CD34, TGase-2, CK7-tubule, and Ki-67 significantly differed between the two groups ($p<0.05$; Table 2, Figs. 1, 2). The expression of CK-PAN, PDL1, AMACR, HIF2a, and PDGFRβ was similar between the two RCC subtypes ($p>0.05$, Table 2, Figs. 1, 2). The expression of RCC, pS6, PBRM1 loss, PAX8, PSMA, Vimentin, BAP1 loss, and PTEN loss was also indistinguishable

Table 1. Baseline characteristics (N=30).

		Total (N=30)	MCRNLMP (N=13)	MCRCC (N=17)	p-value
Age	median (range)	48.5 (27-82)	50 (35-73)	47 (27-82)	0.586
Gender	male	27 (90.0)	12 (92.3)	15 (88.2)	>0.999
	female	3 (10.0)	1 (7.7)	2 (11.8)	
HTN	yes	14 (46.7)	6 (46.2)	8 (47.1)	>0.999
DM	yes	5 (16.7)	2 (15.4)	3 (17.7)	>0.999
ASA	1	14 (46.7)	7 (53.9)	7 (41.2)	0.839
	2	15 (50.0)	6 (46.2)	9 (52.9)	
	3	1 (3.3)	0 (0.0)	1 (5.9)	
ECOG	0	28 (93.3)	12 (92.3)	16 (94.1)	>0.999
	1	2 (6.7)	1 (7.7)	1 (5.9)	
Operation method	radical Nx	19 (63.3)	7 (53.9)	12 (70.6)	0.454
	partial Nx	11 (36.7)	6 (46.2)	5 (29.4)	
T stage	T1a	19 (63.3)	10 (76.9)	9 (52.9)	0.289
	T1b	8 (26.7)	3 (23.1)	5 (29.4)	
	T2	3 (10.0)	0 (0.0)	3 (17.7)	
Sinus fat involvement	positive	1 (3.3)	0 (0.0)	1 (6.3)	>0.999
Tumor necrosis	positive	9 (30.0)	0 (0.0)	9 (52.9)	0 003
Lymphovascular invasion	positive	2 (6.7)	0 (0.0)	2 (12.5)	0.492
Tumor size (cm)	median (range)	3.8 (0.5-8.0)	3.2 (0.5-6.0)	4.1 (2.0-8.0)	0.030
Operative time(min)	median (range)	225 (115-390)	225 (115-390)	220 (120-340)	0.539
Recurrence	yes	1 (3.3)	0 (0.0)	1 (5.9)	>0.999

HTN, hypertension; DM, diabetes mellitus; ASA, American Society of Anesthesiologists score; ECOG, Eastern Cooperative Oncology Group performance status.

between the two groups (Table 3).

Discussion

In spite of its rarity, favorable prognosis, and lack of distinguishable objective clues with preoperative clinicopathological findings, MCRNLMP represents an important disease entity as it could provide pathological insight into the oncogenesis of RCC given that it represents the earliest stage of the disease; therefore, there might be a trigger point that induces the malignant potential from the MCRNLMP form (Li et al., 2016). Li et al. (2016) reported 76 cases of MCRNLMP without any recurrences and metastases during a median follow-up of 52 months after surgery, and one patient diagnosed with Von Hippel-Lindau (VHL) disease had a unilateral renal tumor identified with both MCRNLMP and clear cell RCC. Based on these results, the authors suggested that both MCRNLMP and MCRCC could arise from the same event owing to a mutation of the *VHL* gene. Concurrent tumors might arise from similar embryologic processes due to abnormal factors such as carcinogens and hormones (Ailles and Weissman, 2007). Different renal tumors could arise from cancer stem cells that follow dissimilar differentiation pathways regulated by tissue microenvironmental interactions. In this study, one (7.7%) of the 13 cases of MCRNLMP had both clear cell RCC and MCRNLMP.

However, the staging criteria used in Li et al. (2016) might not be suitable for MCRNLMP, which are based

Fig. 1. Intensity score tissue 6 biomarkers.

Table 2. Comparison of 17 tissue biomarkers between two diseases.

		Total (N=30)	MCRNLMP (N=13)	MCRCC (N=17)	p-value
CD31	1: mild	8	8 (61.5)	0 (0.0)	<0.001
	2: intermediate	11	4 (30.8)	7 (41.2)	
	3: severe	11	1 (7.7)	10 (58.8)	
CD34	1: mild	1	1 (7.7)	0 (0.0)	0.026
	2: intermediate	3	3 (23.1)	0 (0.0)	
	3: severe	26	9 (69.2)	17 (100)	
CK7-tubule	0: none	6	0 (0.0)	6 (35.3)	0.010
	1: mild	6	2 (15.4)	4 (23.5)	
	2: intermediate	2	0 (0.0)	2 (11.8)	
	3: severe	16	11 (84.6)	5 (29.4)	
CK-PAN	2: intermediate	3	0 (0.0)	3 (17.7)	0.238
	3: severe	27	13 (100.0)	14 (82.4)	
Ki-67	1: mild	24	13 (100.0)	11 (64.7)	0.024
	2: intermediate	6	0 (0.0)	6 (35.3)	
PDL1	0: none	3	0 (0.0)	3 (17.7)	0.358
	1: mild	17	8 (61.5)	9 (52.9)	
	2: intermediate	10	5 (38.5)	5 (29.4)	
AMACR	median (range)	30 (10-290)	10 (10-290)	30 (10-280)	0.194
CD10	median (range)	30 (10-280)	10 (10-280)	150 (10-250)	0.002
HIF1α	median (range)	115 (10-290)	30 (10-80)	250 (30-290)	<0.001
HIF2α	median (range)	150 (20-290)	150 (20-180)	230 (20-290)	0.256
PDGFRα	median (range)	270 (230-290)	250 (230-270)	280 (260-290)	<0.001
PDGFRβ	median (range)	255 (20-290)	260 (240-280)	250 (20-290)	0.656
SMA	median (range)	255 (140-280)	150 (140-170)	270 (240-280)	<0.001
TGase-2	median (range)	280 (30-290)	270 (60-290)	280 (30-290)	0.027
VEGFR1	median (range)	280 (240-290)	260 (240-290)	280 (270-290)	0.001
VEGFR2	median (range)	260 (220-290)	250 (220-280)	270 (250-290)	0.009
VEGFR3	median (range)	15 (10-70)	10 (10-20)	20 (10-70)	0.008

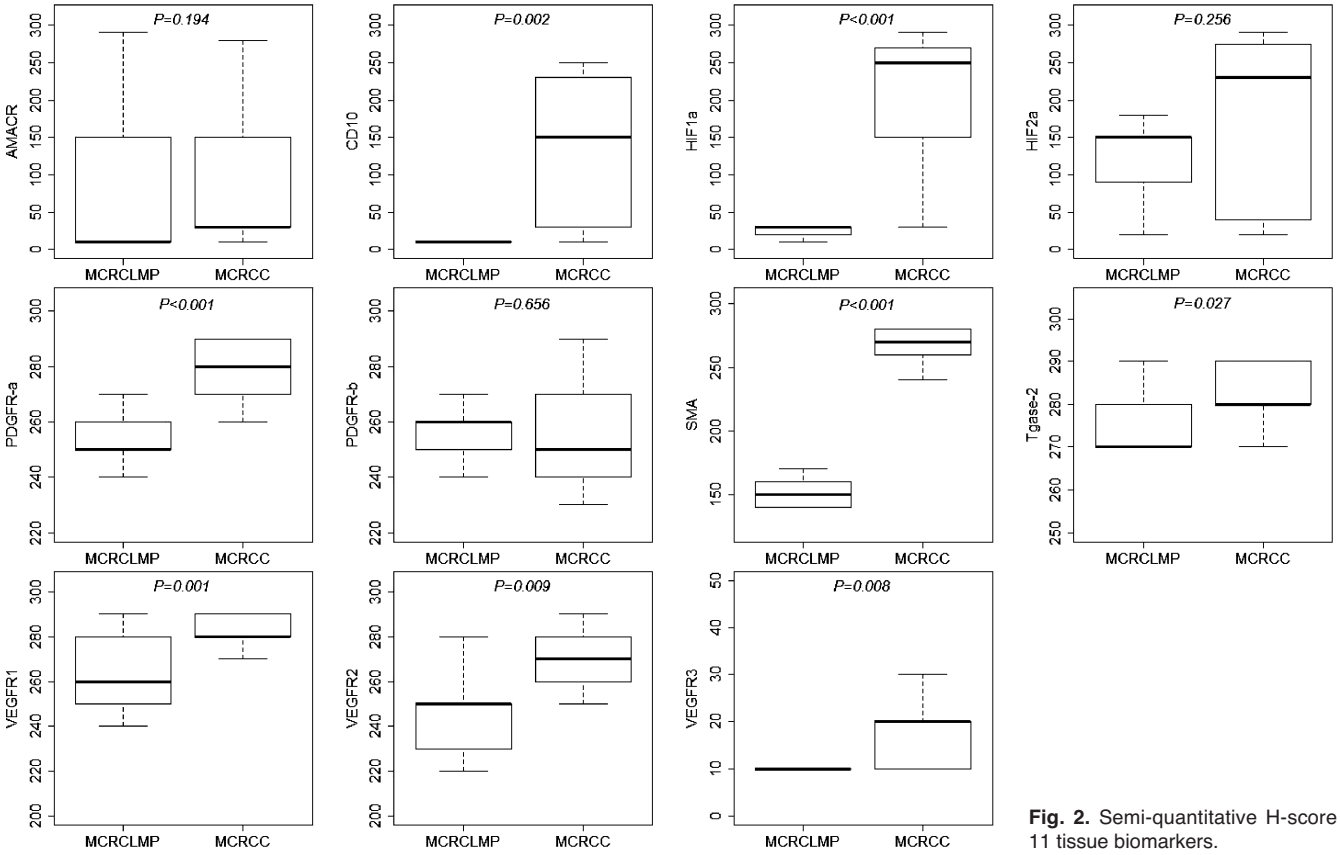

Fig. 2. Semi-quantitative H-score 11 tissue biomarkers.

only on the stage, nuclear grade, and TNM stages. No unique symptomatologic or anthropometric characteristics have been observed in MCRNLMP compared to other cystic RCCs, because the majority of patients with cystic RCC show no symptoms and no abnormal physical examinations. Only a few patients presented kidney-related symptoms such as flank discomfort, a palpable mass, pain, hematuria, or hypertension (Gong et al., 2008; Kuroda et al., 2012; Udager and Mehra, 2016), whereas all the patients with MCRNLMP in this study were asymptomatic and the mass was only found during annual healthcare screening.

The 17 MCRCC samples analyzed in this study were selected under more stringent and concrete criteria by a senior pathologist, with a requirement of 10 cysts in the gross specimen, than applied in previous studies (Gong et al., 2008; Kuroda et al., 2012; Udager and Mehra, 2016). The 25 markers selected were those shown to be related to the oncogenesis of RCC and other differential tissue markers from similar cystic RCCs such as cystic clear cell papillary RCC and tubulocystic carcinoma (von Teichman et al., 2011; Li et al., 2012a,b; Alexiev and Drachenberg, 2013; Chowdhury et al., 2013; Kuroda et al., 2013; Kristiansen et al., 2015). Some of the markers showed similar expression levels, whereas others showed slightly but insignificantly different expression levels (AMACR, RCC, BAP1, CK-PAN, HIF2α, pS6, PAX8, PBRM1, PDGFRβ, PDL1, PTEN, and Vimentin). Some final markers, including HIF1α, PDGFRα, SMA, VEGFR1, VEGFR2, VEGFR3, CD10, CD31, CD34, CK7-tubule, TGase-2, and Ki-67, were significantly different between the two subtypes ($p<0.05$). No other baseline characteristics differed between the two groups ($p>0.05$, Table 1), except for necrosis and tumor size ($p<0.05$).

Among these 12 significant tissue markers, several overexpressed markers related to VHL-hypoxia-inducible factor (HIF) pathways such as HIF1a, PDGFRα, VEGFR1, VEGFR2, and VEGFR3 are known to be involved in oncogenic pathways in clear cell RCC (Chittiboina and Lonser, 2015; Su et al., 2015; Foshat and Eyzaguirre, 2017). Inactivation of the VHL gene leads to a dysfunctional *VHL* protein and increased HIF-α levels, resulting in enhanced angiogenesis of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). The proliferating cell index based on Ki-67 expression indicates enhancement of cell proliferation and apoptosis (Scholzen and Gerdes, 2000; Foix et al., 2016; Haddad et al., 2017), while enhanced SMA, CD31, and CD34 expression promotes neovascularization in angiogenesis and contributes to oncogenetic progression in clear cell RCC (Pouessel and Culine, 2007). Furthermore, CD31 and CD34 were reported to be pan-endothelial cell parameters associated not only with newly formed microvessels but also with existing vessels in RCC TMAs (Phucoc et al., 2008). In a study of metastatic clear cell RCC, Kuroda et al. (2015) conducted a TMA analysis and found that HIF-1a, CA-9, CD31, VEGFR1, VEGFR2, PDGFRα, and PDGFRβ were significantly associated with the therapeutic responses of targeted therapy. However, in the present study, since MCRCC was associated with significantly different baseline characteristics of greater tumor size and a higher necrotic rate within the tumor relative to those in MCRNLMP, these nine significantly overexpressed markers might not be distinctive and differential markers of MCRNLMP on their own, because the higher expression of angiogenic markers might be induced by a faster growing rate in the larger tumors in the MCRCC group compared to those in the MCRNLMP group.

The other three significant markers, including significantly overexpressed CD10 and TGase-2 (transglutaminase-2) and the non-expressed CK7-tubular area, demonstrated some distinctive patterns between the two disease groups ($p<0.05$). With respect to CD10 and CK7 expression, Raspollini et al. (2016) conducted an IHC analysis of clear cell papillary RCC, and found that negative CD10 and AMACR expression and strong CK7 expression were associated with indolent behavior and low malignant potential, similar to the results for the MCRNLMP group in the present study. TGase-2 has been associated with multiple cancers such as melanoma, pancreatic cancer, lung cancer, and glioblastoma, playing a role in disease invasion/progression, drug resistance, metastasis, and autophagy,

Table 3. Comparison of 8 tissue biomarkers between two diseases.

		Total (N=30)	MCRNLMP (N=13)	MCRCC (N=17)
pS6	10	24	11 (84.6)	13 (76.5)
	20	1	0 (0.0)	1 (5.9)
	30	1	1 (7.7)	0 (0.0)
	150	3	1 (7.7)	2 (11.8)
	280	1	0 (0.0)	1 (5.9)
PBRM1	250	1	0 (0.0)	1 (5.9)
	270	4	2 (15.4)	2 (11.8)
	280	15	9 (69.2)	6 (35.3)
	290	10	2 (15.4)	8 (47.1)
PAX8	230	1	0 (0.0)	1 (5.9)
	280	8	2 (15.4)	6 (35.3)
	290	21	11 (84.6)	10 (58.8)
PSMA	10	23	13 (100.0)	10 (58.8)
	20	6	0 (0.0)	6 (35.3)
	50	1	0 (0.0)	1 (5.9)
VIMENTIN	210	1	0 (0.0)	1 (5.9)
	250	1	0 (0.0)	1 (5.9)
	290	28	13 (100.0)	15 (88.2)
BAP1	280	10	6 (46.2)	4 (23.5)
	290	20	7 (53.9)	13 (76.5)
PTEN	280	5	1 (7.7)	4 (23.5)
	290	25	12 (92.3)	13 (76.5)
RCC	10	30	13 (100.0)	17 (100.0)

resulting in a poor prognosis (Gentile and Cooper, 2004). The immunoreactivity of TGase-2 in MCRCC has already been demonstrated by our research group. We previously showed that TGase-2 plays an important role in clear cell RCC using both RCC cell lines and human samples (Ku et al., 2013, 2014; Kim et al., 2017a,b), which reflects its higher inflammatory response and tumor progression than MCRNLMP (Kim, 2004). This study also showed that a higher expression rate of TGase-2 in MCRCC significantly distinguished the two groups (p=0.027). Accordingly, the markers CD10, CK7-tubular, and TGase-2 appear to have clinical value with respect to their effects on disease progression and other angiogenic activities within the renal tumor environment and peri-tumoral environment, which can contribute to the enhanced angiogenic pathogenesis in RCC to transform a tumor of low malignant potential to one of aggressive malignant potential.

The concept of focusing on the early pathogenesis of cystic RCC might also be applied in further genetic analyses of microenvironmental neovascularities to identify multiple target genes related to the angiogenesis of RCC. Genetic analyses on MCRNLMP have shown that it is a distinct subtype of clear cell RCC based on pathological features and genetic features similar to other clear cell RCCs (Moch, 2010; Williamson et al., 2012; Wahal and Mardi, 2014). The chromosome 3p deletion and mutation of the *VHL* gene were observed in approximately 74% and 25% of cases, respectively (Halat et al., 2010; von Teichman et al., 2011). Although no significant genetic differences have been detected between clear cell RCC and MCRNLMP, Raspollini et al. (2015) found that a *KRAS* gene mutation could distinguish between the two subtypes in spite of their histologic similarities.

This study had several inborn limitations, including its retrospective design with small sample numbers and some possibilities of technical errors in the TMA preparations and IHC staining. The interpretation of the expression of the 25 tissue markers in IHC TMA could have also been influenced by the specific experimental conditions as well as subjectivity in the interpretation of outcomes. Moreover, further multivariate analysis adjusted by other different clinicopathological parameters would be needed because the results from this study were derived from univariate analysis. Nevertheless, this study is of clinical significance because it is the first to evaluate all 25 tissue markers related to the pathophysiology of MCRCC in comparison with the pathologically and histologically similar MCRNLMP form using TMA-IHC analysis, and suggested three significant potential markers related to the life-threatening conversion of MCRNLMP to aggressive MCRCC. Several agents targeting the tumoral vascularity in RCC have been developed to date, including TGase-2 in systemic therapy for metastatic RCC (Gentile and Cooper, 2004; Kim, 2004; Pouessel and Culine, 2007). Further studies with larger samples will be required to understand the differential pathophysiology of MCRNLMP and other MCRCCs using genetic analyses.

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