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Original Article

Expression of EZH2 in renal cell carcinoma as a novel prognostic marker

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Enhancer of zeste homolog 2 (EZH2) is a member of the Polycomb group proteins and a part of Polycomb repressive complex 2. EZH2 is important for transcriptional regulation through nucleosome modification and interaction with other transcription factors. Particularly, aberration of EZH2 has been implicated in oncogenesis and progression of various neoplasms. The objective of this study was to evaluate EZH2 expression in renal cell carcinoma (RCC), especially clear cell RCC (CRCC) and correlate the expression with prognostic factors. EZH2 expression was determined by immunohistochemical staining with additional Western blotting. High expression of EZH2 was significantly correlated with higher pT stage or more frequent distant metastases (P = 0.001 and 0.024, respectively). Survival analyses displayed that patients with high EZH2 expression had a significantly shorter disease-free survival than those with low expression (P = 0.019). High expression of EZH2 tended to reduce the overall survival, however, differences did not reach statistical significance (P = 0.066). From our results, we propose that EZH2 is a useful prognostic marker for aggressive behavior of CRCC and may be applicable as a therapeutic target molecule.

Key words: enhancer of zeste homolog 2, immunohistochemistry, kidney, prognosis, renal cell carcinoma

Renal cell carcinoma (RCC) originates from the renal parenchyma, and comprises 90% of primary malignant renal tumors in adults.[1] Although worldwide temporal trends by kidney cancer type have not been elucidated, the incidence of RCC in the United States has continued to rise, with 58 240 new diagnoses per year and 13 040 deaths from the disease in 2010.[2] RCC remains a major cause of morbidity and mortality. Approximately 40% of patients eventually die of cancer progression and RCC has the highest fatality rate of the common urologic malignancies.[3]

The enhancer of zeste homolog 2 (*EZH2*), located on chromosome 7q35,[4] is a member of the Polycomb group (PcG) genes. The PcG genes play a central role in determination and maintenance of cell identity, cell cycle regulation and oncogenesis.[5] Moreover, PcG genes have recently featured because of involvement in the cellular aging process.[6] EZH2 constitutes the polycomb repressive complex (PRC) 2 along with embryonic ectoderm development (EED), Yin-Yang-1 (YY1) and suppressor of zeste 12 (SUZ12), and serves as a histone methyltransferase. As a result of direct methylation of lysine-27 of histone H3, various genes are silenced, including tumor suppressor genes.[7] This proves that dysregulated expression of EZH2 is associated with diverse human hematologic malignancies, such as malignant myeloid disorders[4] and lymphomas[8] as well as epithelial malignancies including prostate,[9] breast,[10] bladder,[11] and skin[12] cancers. To date, however, little is known about the role of EZH2 in RCC. The objective of this study was to determine EZH2 expression in RCC, especially clear cell RCC (CRCC) and correlate the expression with various clinicopathologic factors.

MATERIALS AND METHODS

Patients

A total of 210 cases were obtained by retrieval of the pathology reports of patients who underwent curative surgery for RCC at the Keimyung University Dongsan Hospital from January 1997 to December 2008. The clinicopathological parameters of the patients were re-evaluated by a review of the patients' medical records and slides. The data of patients lost to follow-up were obtained from family members via telephone survey. The TNM stage was determined according to the seventh edition of the American Joint Committee on

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Cancer (AJCC) staging system.[13] Patients dying of causes other than RCC were excluded from this study. The study was approved by institutional review board (IRB).

Tissue microarrays (TMAs) and immunohistochemical staining

Immunohistochemical staining was done at primary as well as metastatic tumors. All tissue samples were formalin fixed and paraffin embedded. Paraffin blocks containing representative tumor lesions were selected after review of the corresponding hematoxylin and eosin slides. Two to three representative lesions from each case were marked on the source blocks and cored with a 3.0-mm diameter cylindrical device, and then, the cores were re-embedded into the recipient block. Tissue microarray slices of 5 μm thickness were cut by microtome and placed on slides and the sections on the slides were deparaffinized and rehydrated in the graded series of alcohol solutions. Immunohistochemical staining for the EZH2 antigen was performed using an autostainer ((LV360-2D), LabVision Corporation, Fremont, CA, USA) and UltraVision LP Kit ((TL-060-HD), LabVision Corporation), according to the manufacturer's protocol. The rabbit polyclonal EZH2 antibody (1:300, Zymed (36–6300), San Francisco, CA, USA) was used for primary antibody. The slides were counterstained with hematoxylin. After the autostainer process, the slides were dehydrated through 100% alcohol, cleared and mounted with permanent mounting media. Tissue from B-cell lymphomas and high grade prostate adenocarcinoma known to have high EZH2 expression were used as external positive control. The slides incubated without primary antibody were used as negative control.

Immunohistochemical staining evaluation

Nuclear pattern of immunostaining was considered positive. EZH2 were stained in more than 70% of tumor cells in most of the cases. Intensities of EZH2 staining were diverse. EZH2 expression was assessed according to staining intensity of positive tumor cells, and was scored from 0 to 3 as follows: 0, absence of staining; 1, weak staining; 2, moderate staining; and 3, strong staining. Representative examples of immunostainings are shown in Fig. 1. All sections were evaluated by two pathologists blinded to clinicopathological features and clinical outcome. In the adjacent normal tissues, EZH2 was sparsely expressed in the nuclei of vascular and lymphatic endothelial cells, smooth muscle cells, schwann cells and adipocytes. The histiocytes, proximal and distal tubules showed a diffuse nuclear staining for EZH2. EZH2 were not detected in the mesangial cells in glomeruli.

Extraction of nuclear protein and western blot analysis

Western blot analysis was performed to validate the EZH2 expression by immunohistochemical staining. Ten fresh frozen CRCC tissues were separated into cytoplasmic and nuclear fractions for Western blot analysis. Briefly, the tissues were pulverized by homogenizer and lysated in the hypotonic lysis buffer (10 mM HEPES, 10 mM KCl, 3 mM $MgCl_2$, 0.5% NP-40, 2 mM PMSF, 200 nM aprotinin) for 10 min. After centrifugation in 12 000 rpm for 10 min, the precipitate was lysated again by buffer for nuclear protein extraction (10 mM Tris-Cl (pH 7.5), 0.5 M NaCl, 2.5% glycerol, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 2 mM PMSF, 200 nM aprotinin) for 10 min. The obtained supernatant was used in the experiment as a nuclear protein from the process of centrifugation in 12 000 rpm for 10 min. Nuclear proteins (50 μg/lane) were resolved by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gels) and transferred onto nitrocellulose membrane (Millipore Co., Bedford, MA, USA). The membrane was lightly washed with distilled water and then separated by size followed by blocking with TBST (Tris-buffered saline, 10 mM NaCl supplemented with 0.05% Tween 20) containing 5% non-fat dried milk. The membranes were further incubated with primary antibodies such as EZH2 (Zymed (36–6300)) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with TBST, the membranes were incubated with anti-rabbit and anti-mouse secondary antibodies coupled to horseradish peroxidsase, respectively. The membranes were subsequently developed in the ECL Western detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical analyses

For statistical analyses, the immunohistochemical staining scores were grouped into low (scores 0 to 2) and high (score 3) expression. Cross tabulations with the χ^2-statistic and Fisher's exact test were constructed to determine the association between EZH2 expression and clinicopathological parameters, i.e., gender, age, histologic type, histologic grade (Fuhrman's), T stage, N stage, total TNM stage and distant metastases. For survival analysis, two end points were considered, i.e., disease relapse (defined as either a local recurrence or metastasis) and death with disease. Kaplan–Meier curves were used to estimate the distributions of disease-free survival (DFS) and overall survival (OS), and the differences in survival between the groups were compared using the log-rank test. DFS was measured as the duration from surgical resection to clinical evidence of disease relapse, or the last follow-up in patients with no evidence of recurrence or metastasis. The period of OS was measured from the date of surgical resection to the

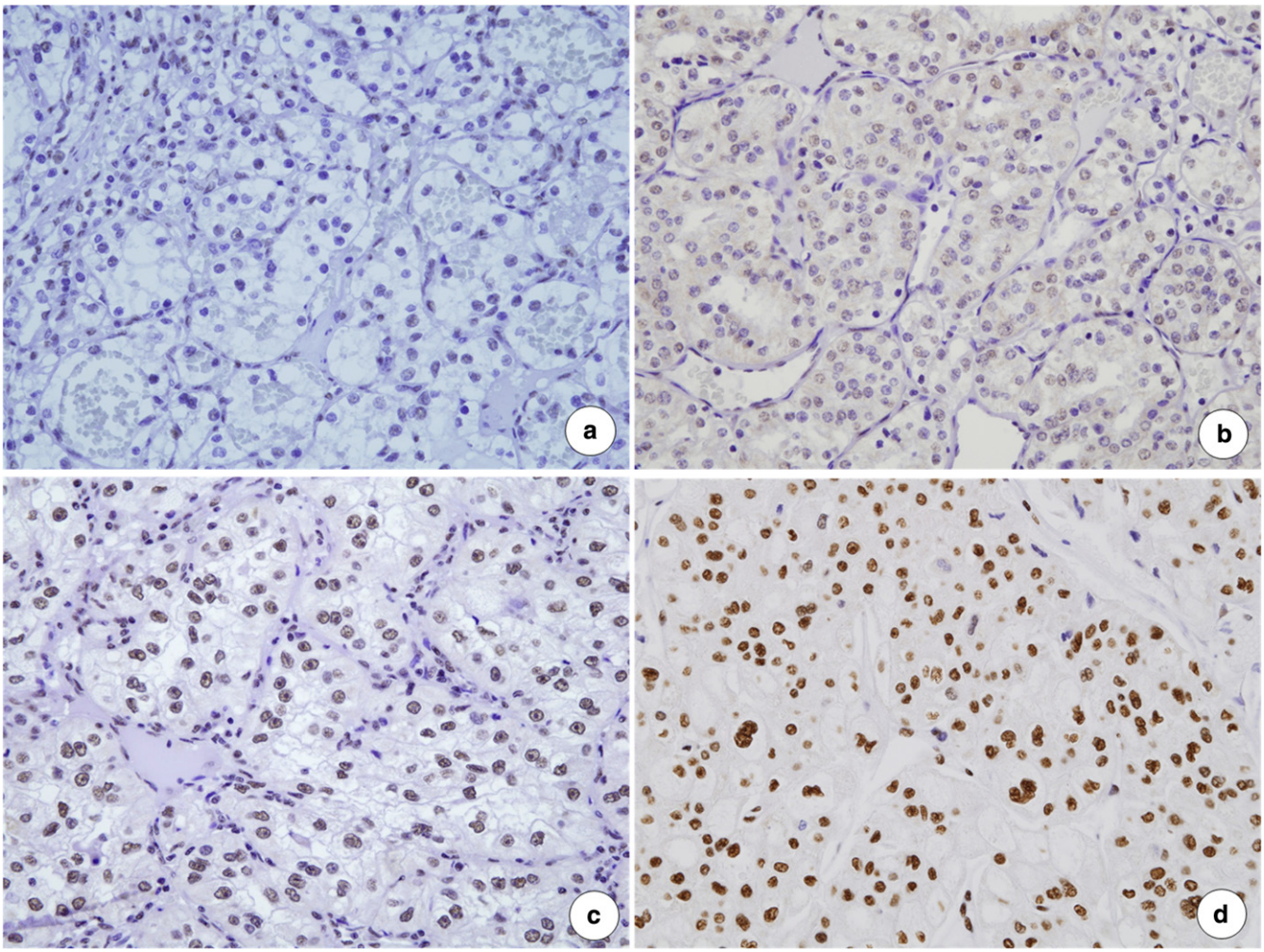

Figure 1 Immunohistochemical analysis of enhancer of zeste homolog 2 (EZH2) in clear cell renal cell carcinoma tissues. (**a**) EZH2 is not expressed in tumor cells (scored as 0). (**b**) EZH2 is weakly expressed in nuclei of tumor cells (scored as 1). (**c**) EZH2 is moderately expressed in nuclei of tumor cells (scored as 2). (**d**) Strong nuclear expression of EZH2 is observed in tumor cells (scored as 3).

date of death or the last follow-up. The median duration of follow-up was 1395 days (range 8 to 5235). With TNM staging, stages I to II was considered as early, and stages III to IV as late. All statistical analyses were carried out using the SPSS version 19 software package (SPSS-IBM Inc, Chicago, IL, USA), and $P < 0.05$ was considered statistically significant.

RESULTS

Clinicopathological features

In total there were 210 RCC cases, which included the following: 171 (81.4%) CRCCs; 10 (4.8%) papillary RCCs; 17 (8.1%) chromophobe RCCs; 5 (2.4%) Xp11.2 translocation RCCs; 1 (0.5%) multilocular cystic RCC; and 6 (2.8%) unclassified. The types of RCC have no relationship with the EZH2 expression. Each RCC type other than CRCC cannot be statistically analyzed due to small sample size, and thus this study was confined to the CRCC group. The CRCC group's characteristics, incidence of disease progression and status of the patients are illustrated in Table 1. The study subjects comprised 123 (65.3%) men and 48 (34.7%) women, aged 19–82 years (mean 56 years). According to the seventh edition of the AJCC TNM staging system, the cases consisted of 101 (59.1%) T1 stage, 31 (18.1%) T2 stage, 37 (21.6%) T3 stage and 2 (1.2%) T4 stage tumors; 163 (95.3%) N0 stage and 8 (4.7%) N1 stage tumors; and 166 (97.8%) M0 stage and 5 (2.9%) M1 stage (defined as distant metastasis at the time of diagnosis) tumors. Ninety six patients (56.1%) had stage I disease; 30 patients (17.5%), stage II; 38 patients (22.2%), stage III; and 7 patients (4.1%), stage IV. Evidence of lymphovascular invasion was present in 10 cases (5.8%). The histologic grades of tumor were as follows: 5 (2.9%) grade 1, 45 (26.3%) grade 2, 84 (49.1%) grade 3 and 37

Table 1 Associations between clinicopathological parameters and EZH2 expression in clear cell renal cell carcinoma patients

	EZH2		
	Low N (%)	High N (%)	*P*-value
Age (yr)			
≤56	43 (25.1)	39 (22.8)	0.312
>56	57 (33.3)	32 (18.7)	
Gender			
Male	69 (40.4)	54 (31.6)	0.124
Female	31 (18.1)	17 (9.9)	
Histologic grade			
Grade 1–2	34 (19.9)	16 (9.4)	0.126
Grade 3–4	66 (38.6)	55 (32.2)	
pT stage			
T1–2	86 (50.3)	46 (26.9)	**0.001**
T3–4	14 (8.2)	25 (14.6)	
TNM stage			
I-II	82 (48.0)	44 (25.7)	**0.003**
III-IV	18 (10.5)	27 (15.8)	
Distant metastases			
Absent	90 (52.6)	55 (32.2)	**0.024**
Present	10 (5.8)	16 (9.4)	
Status of the patients			
Alive	91 (53.2)	58 (33.9)	0.073
Disease related-death	9 (5.3)	13 (7.6)	
Total	100 (58.5)	71 (41.5)	

P-values of χ^2-tests are indicated; bold, statistically significant ($P < 0.05$).

N, number; pT, pathologic tumor; TNM, tumor node metastasis; yr, years old.

(21.6%) grade 4. One hundred and forty-five patients (84.8%) showed no evidence of disease relapse, and 26 patients (15.2%) had distant metastases. A total of 22 patients (12.9%) died of the disease during the follow-up period.

EZH2 expression by western blotting

To confirm the specificity of the immunohistochemical results, Western blot analysis was performed in nuclear extracts of 10 CRCC tissues, in which freshly frozen materials were available. Western blotting revealed double or triple bands, which were positioned between 75 kDa and 150 kDa, for isoforms of the EZH2 protein. The protein levels of EZH2 which were quantified by Western blotting were in accord with immunohistochemical results (Fig. 2).

Correlation of EZH2 expression with clinicopathological variables

The associations between EZH2 expression levels and clinicopathological parameters are shown in Table 1. Low expression of EZH2 revealed lower pT stage and total TNM stage. These are statistically significant results (P = 0.001 and 0.003, respectively). Increased risk for distant metastases was significantly related to high EZH2 expression (P = 0.024). On the other hand, EZH2 had no significant association with age, gender, histologic grade, N stage or lymphovascular invasion (data not shown). High EZH2 expression tended to increase the possibility of disease related death, but it did not reach statistical significance (P = 0.073). Table 2 represents distant metastases correlated with EZH2 expression (P = 0.024), age (P = 0.02) and other well known prognostic features, histologic grade (P = 0.002), pT stage ($P < 0.0001$) and pN stage ($P < 0.0001$).

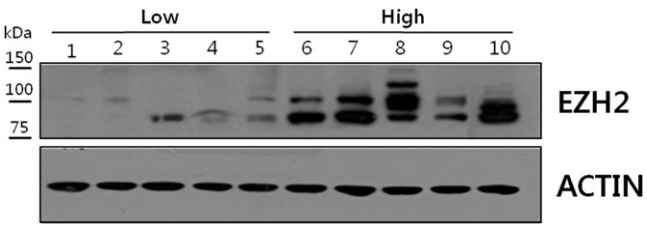

Figure 2 Western blot analysis of enhancer of zeste homolog 2 (EZH2) levels in primary clear cell renal cell carcinoma tissues. Immunohistochemically, the left five cases were graded as low EZH2 expression and the right five cases were graded as high. Western blot results correspond to those of immunohistochemistry.

Table 2 Associations between clinicopathological parameters and patients' outcome

	N (%)		
	Distant metastases Absent	Distant metastases Present	*P*-value
EZH2 expression			
Low	90 (52.6)	10 (5.8)	**0.024**
High	55 (32.2)	16 (9.4)	
Age (yr)			
≤56	75 (43.9)	7 (4.1)	**0.02**
>56	70 (40.9)	19 (11.1)	
Gender			
Male	103 (60.2)	20 (11.7)	0.538
Female	42 (24.6)	6 (3.5)	
Histologic grade			
Grade 1–2	49 (28.7)	1 (0.6)	**0.002**
Grade 3–4	96 (56.1)	25 (14.6)	
pT stage			
T1–2	123 (71.9)	9 (5.3)	**<0.0001**
T3–4	22 (12.9)	17 (9.9)	
pN stage			
N0	142 (83.0)	21 (12.3)	**<0.0001**
N1	3 (1.8)	5 (2.9)	

P-values of χ^2-tests are indicated; bold, statistically significant ($P < 0.05$).

N, number; pN, pathologic node; pT, pathologic tumor; yr, years old.

Survival analyses

Kaplan–Meier curves revealed statistical correlation between high expression of EZH2 and poor DFS (P = 0.019) (Fig. 3a).

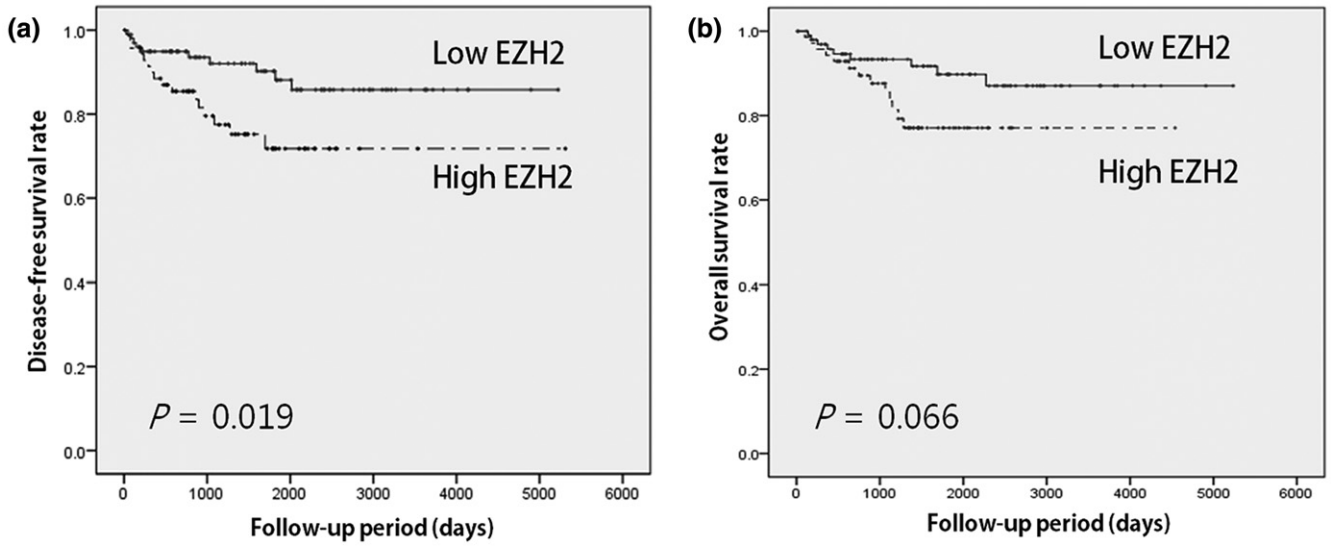

Figure 3 (**a**) Disease-free survival and (**b**) overall survival according to enhancer of zeste homolog 2 (EZH2) groups in patients with clear cell renal cell carcinoma.

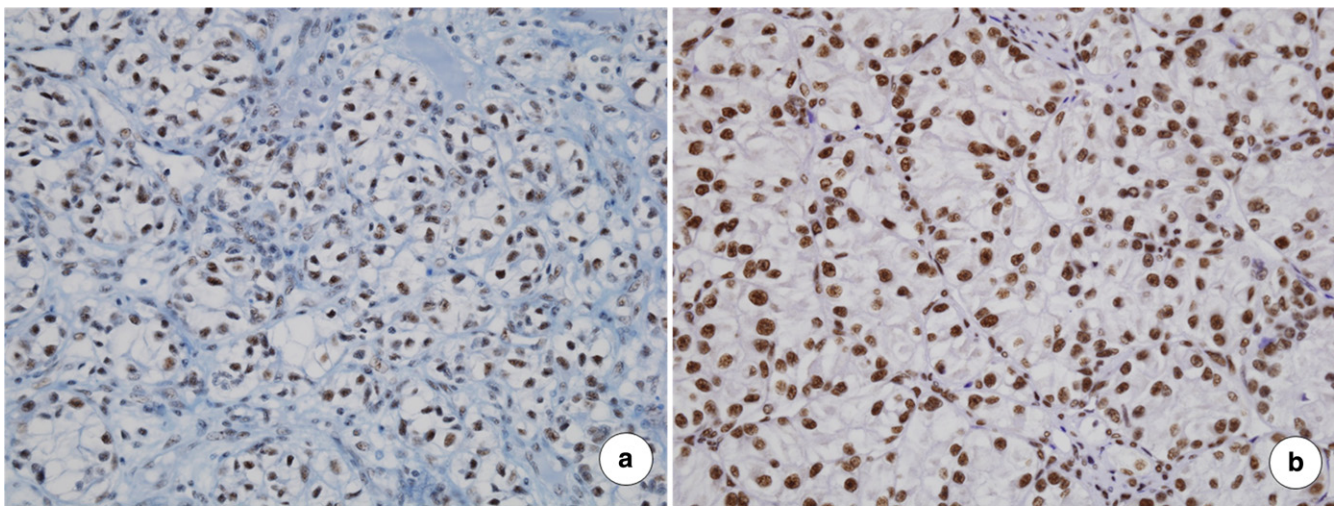

Figure 4 Immunohistochemical staining for enhancer zeste homolog 2 (EZH2) in (**a**) primary clear cell renal cell carcinoma (CRCC) tissues along with (**b**) matched metastatic tumor. Both primary and metastatic CRCC reveal diffuse and strong immunoreactivity of EZH2.

The high expression of EZH2 was associated with worse OS; however, the difference did not reach statistical significance ($P = 0.066$) (Fig. 3b). The estimated five-year survival rate of low expression group (90.0%) is comparatively different from that of high expression group (76.0%).

EZH2 expression in metastatic lesions

The most common metastatic organ is the lung (13 cases) followed by bone (6 cases), liver (4 cases), brain (2 cases) and carcinomatosis peritonei (1 case). Eight distant metastatic lesions were available to immunohistochemistry. With only one exception, all metastatic tumors revealed similar EZH2 expression to primary tumors (6 high expression and 1 low expression) (Fig. 4). One case revealed high expression in the primary lesion, but low immunoreactivity in its bony metastasis.

DISCUSSION

Following advances in genetic engineering and biotechnology, cancer-specific biomarkers have become critical for tar-

geted therapy and predicting prognosis. Although various RCC biomarkers have been proposed previously, the available biomarkers and clinicopathologic parameters are not adequate. Non-surgical treatment for advanced RCC has not been as well developed as those available for other malignancies, and resistance to chemotherapy is a common occurrence in case of RCC. Currently, eight drugs have been approved for systemic treatment of advanced RCC, among which only interferon-α (INFα) and temsirolimus have provided statistically significant improvement in the overall survival.[14,15] Therefore, a biomarker that can predict prognosis or cancer progression and aid in the development of a new drug is required.

EZH2 represses the transcription of various genes via trimethylation of lysine-27 at histone H3. EZH2 demonstrates different effects depending on the target genes. It is generally involved in cell proliferation, anti-differentiation, apoptosis resistance and cell invasion, and can eventually lead to oncogenesis. Notably, it is implicated in aggressive tumor features and unfavorable outcome. For example, prostate cancer, a relatively well-researched cancer, shows aberrant expression of EZH2. As EZH2 expression increases, *adrenergic receptor beta 2* (*ADRB2*) is repressed, consequently enabling cell invasion and transformation.[16] Further, *INK4B–ARF–INK4A* (*INK/ARF*), *BMP receptor 1B* (*BMPR1B*), *NOTCH1, Dapper antagonist of β-catenin 3* (*DACT3*), *Dickkopf 1* (*DKK1*) and *Disabled homolog2-interacting protein* (*DAB2IP*) are targeted by EZH2. EZH2 also suppresses the expression of *Vasohibin1* (*VASH1*), *E-cadherin* (*CDH1*), SNAIL1, *RAD51* and *Aldehyde dehydrogenase 1 family, member A1* (*ALDH1A1*).[17–19]

In this study, we investigated EZH2 expression and its importance in CRCC. Significant correlations were observed between EZH2 expression and distant metastases, pT, TNM stage and DFS rate. High-EZH2 expressing tumors tended to metastasize more and metastasized tumor cells also showed high EZH2 expression.

Only a few studies have been performed on EZH2 expression in RCC[20–24] and show conflicting results. Wagener *et al*.[21] performed immunohistochemical staining of RCC tissues obtained from 520 patients and demonstrated that high-EZH2 tumors had poorer outcomes than low-EZH2 tumors. In addition, several highly unfavorable clinicopathologic features were correlated with high EZH2 expression. They scored the tumors according to the percentage of nuclear staining of EZH2. Contradictory to these findings, Hinz *et al*.[22] evaluated mRNA in 119 CRCC patients by using quantitative real-time PCR and reported that, compared to the adjacent benign renal parenchyma, RCC tissues showed EZH2 mRNA overexpression and patients with high EZH2 levels independently showed longer DFS than those with low EZH2 levels. Our results are similar to those of Wagener *et al*., suggesting that high EZH2 expression in RCC is a predictor of aggressive tumor characteristics. These findings correspond to the generally accepted function of EZH2 in most other malignancies. Only a few studies suggest an anti-tumor function of EZH2.[25,26] An RCC cell line study[20] showed that EZH2 contributes to proliferation and apoptotic resistance, which supports our results. Such conflicting results may occur because of differences in the methods for estimating EZH2 expression, that is, immunohistochemistry and quantitative real-time PCR. We used immunohistochemistry and immunoblotting to assess the EZH2 protein level, which directly performs various functions. Another reason for the conflicting results may be the use of a limited number of samples in these studies. Further larger-scale studies are required to clarify the significance of EZH2 in RCC. In our study, the RCC group was divided into histologic types, that were analyzed separately in order to improve the accuracy of analysis. Moreover, our study included an Asian population only.

We found that when CRCC cells were strongly stained for EZH2, they also tended to be expressed in the endothelial cells (data not shown). Therefore, EZH2 may be overexpressed in RCC-associated endothelial cells. EZH2 has been reported to be upregulated in tumor-associated endothelial cells related to VEGF-mediated downregulation of miR-101 in glioblastoma cells.[27] EZH2 overexpression has been reported in both tumor cells and tumor vasculature of ovarian carcinoma. It increases tumor angiogenesis, leading to poor clinical outcomes.[28]

A number of possible mechanisms for EZH2 upregulation have been revealed in various malignant tumors. The pRB-E2F pathway regulates EZH2 expression by transcriptional activation and leads to tumor cell proliferation.[29] The loss of miRNAs such as miR-26a, miR-214, and miR-101 is also known to lead to EZH2 accumulation.[30–32] The upstream pathways of aberrant EZH2 expression are not clearly defined in RCC and need to be investigated for controlling RCCs with EZH2 overexpression. Treatment with medications such as 3-deazaneplanocin A[33], an EZH2 inhibitor, or by blocking the upstream mechanism of aberrant EZH2 expression may provide a major breakthrough in the treatment of advanced RCCs that are refractory to conventional chemotherapeutic agents. Recently, several cell line studies have shown that 3-deazaneplanocin A induces tumor cell death.[33]

In summary, EZH2, which is a member of the PcG protein family, acts as a transcriptional repressor and is implicated in various malignancies. Our results showed that high EZH2 expression in CRCC correlates with unfavorable prognosis and shorter DFS. Aberrant EZH2 expression may be involved in renal cell carcinogenesis and predict unfavorable clinical behavior. EZH2 can be used potentially as a therapeutic target, which would represent a major breakthrough in the treatment of advanced RCC.

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