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The Prognostic Impact of Jumonji Domain-containing 2B in Patients with Resected Lung Adenocarcinoma

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Abstract. *Background: Jumonji domain-containing 2B, JMJD2B, has been shown to play an important role in the pathogenesis of lung cancer cells. However, the significance of JMJD2B in patients with lung cancer remains to be elucidated. Patients and Methods: Seventy-eight patients with resected adenocarcinoma, whose data regarding oncogenic drivers in lung cancer were available, were included in the study. Immunohistochemical analysis was performed with a specific antibody for JMJD2B to investigate JMJD2B expression and the significance of JMJD2B expression in survival after surgery was evaluated. Results: Among the 78 patients, 50 (64%) exhibited JMJD2B immunopositivity. The overall survival (OS) of the 50 JMJD2B-positive patients after surgery was significantly inferior to that of the JMJD2B-negative patients (five-year survival=56.7% vs. 92.6%; log-rank: p=0.01). Multivariate analysis showed that JMJD2B positivity was an independent prognostic factor. Conclusion: JMJD2B may be a novel prognostic factor for resected lung adenocarcinoma.*

Lung cancer is a devastating neoplasm and the leading cause of cancer-related deaths in many countries; its poor prognosis is a concern that urgently needs to be addressed for the management of lung cancer (1). Intriguingly, the genetic pathogenesis of lung cancer has gradually been elucidated. For instance, mutations in the epidermal growth factor receptor (*EGFR*) gene and rearrangement in the anaplastic lymphoma kinase (*ALK*) gene are known to be deeply involved in tumorigenesis, cancer cell proliferation and survival in non-small cell lung cancer (NSCLC), specifically adenocarcinoma (2, 3). Importantly, specific inhibitors against lung cancer with such oncogenes exhibit remarkable antitumor efficacy, which results in improved survival (4, 5). With regard to the role of the dysregulation of epigenetics in human cancer, there is growing evidence demonstrating its possible involvement in cancer pathogenesis (6); however, the true significance of dysregulation of epigenetics in oncogenesis and cancer progression has not been fully elucidated.

Epigenetic processes include DNA methylation, histone modifications and non-coding RNA. Among these processes, histone modifications, such as acetylation, methylation and phosphorylation, modulate chromatin dynamics, which result in the alteration of multiple cellular functions (7). Methylation marks on lysine residues of histones, such as H3K4, H3K9, H3K27, H3K36 and H4K20, may actively or repressively affect the transcriptional process. These methylated lysine residues are located in the N-terminal tails of histones (7). Although these methylation modifications had been thought irreversible, they were identified to be reversed by some enzymes, such as lysine-specific demethylase 1 (LSD1) and the Jumonji C (JmjC) domain-containing protein family (8). The demethylation of histone lysine residues is also involved in the regulation of transcription, as documented by several reports that have clarified the physiological functions of the enzymes, as well as their possible association with human cancer (9); however, their significance in lung cancer remains unclear.

JMJD2B (KDM4B), a member of the JmjC domain-containing protein family, was identified *in silico* (10). It is

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composed of the JmjN, JmjC, PHD and TUDOR domains. The JmjC domain catalyzes demethylation and JMJD2B promotes transcription by demethylating trimethylated H3K9 *via* the JmjC domain (11). Recent reports indicate that hypoxic conditions can induce the expression of some JmjC family members, including JMJD2B (12). In fact, *JMJD2B* has been shown to harbor hypoxia-inducible factor (HIF) binding sites in its promoter sequences and HIF-1α-induced JMJD2B expression contributes to enhanced hypoxic gene expression and tumor growth (12). With regard to the significance of JMJD2B in cancer, we have previously reported that it is indispensable for the survival of bladder and lung cancer cell lines and that it is overexpressed in clinical bladder and lung cancer tissues in comparison to corresponding normal tissues (13). Furthermore, an association of JMJD2B with other types of human cancers, such as breast, gastric, colorectal and hepatocellular cancer, has been reported (14-17). However, the significance of JMJD2B in resected lung adenocarcinoma and on the prognosis of patients with resected adenocarcinoma remains unclear.

In this report, we demonstrate that up-regulation of JMJD2B in patients with resected adenocarcinoma is associated with a poorer prognosis in comparison to patients without JMJD2B up-regulation, thus suggesting that JMJD2B may be a novel prognostic factor in surgically resected adenocarcinoma.

Patients and Methods

Patients. The present retrospective study investigated 78 patients with surgically resected adenocarcinoma in which oncogenes, such as *EGFR, K-ras, ALK, BRAF, ROS1* and *RET*, were analyzed in the Department of Thoracic Oncology, National Kyushu Cancer Center, Japan. Pathological staging was performed using the 7th edition of the TNM Classification of Malignant Tumors. In addition to the pathological stage and the status of the oncogenic drivers, the patients' gender, age, smoking history, pathological tumor and pathological lymph nodal factors, were analyzed. The present study was approved by our institutional review board.

Genetic analysis. Genomic DNA and RNA samples were extracted and purified from tumors that were snap-frozen in liquid nitrogen at surgery. *EGFR* and *K-ras* mutations were identified using a polymerase chain reaction (PCR)-based fragment analysis and direct sequencing. *B-RAF* mutations were identified using direct sequencing, whereas the *ALK, ROS1* and *RET* fusion genes were assayed using a multiplex RT-PCR and direct sequencing methods. Detailed information on the primer sets and the experimental procedure has been described previously (18, 19).

Immunohistochemistry. A specific antibody against JMJD2B (A301-478A; Bethyl Laboratories, Montgomery, TX, USA) was used for the immunohistochemical analysis, as described previously (13). EnVision+ kit/horseradish peroxidase (DAKO, Carpinteria, CA, USA) was applied. After deparaffinization, specimens were autoclaved under high pressure (121˚C, 15 min) in target retrieval solution at pH 6 (S1699; DAKO) and, then, treated with peroxidase blocking reagent and protein blocking regent (080-01186 and 137-01823; Wako). Thereafter, the tissue sections were incubated with a rabbit anti-JMJD2B polyclonal antibody at a dilution of 1:100, followed by DAKO ENVISION+ polymer/HRP (anti-rabbit; Dako Cytomation). Liquid DAB+ chromogen (K4007; DAKO) was used for antigen visualization. Finally, tissue specimens were stained with Hematoxylin 3G (8656; SAKURA, Tokyo, Japan) to discriminate the nucleus from the cytoplasm. Allred scores were applied to discriminate between the positive (≥4) and negative (<4) cases (20). Representative positive and negative cases are shown in Figure 1A and B.

Statistical analysis. The associations between JMJD2B expression in NSCLC and patients' characteristics were analysed using Fisher's exact test. Overall survival (OS) was defined as the time from the initial surgery until death from any cause, while disease-free survival (DFS) was defined as the time from the initial surgery until recurrence. The Kaplan-Meier method was used to estimate the survival probabilities. The curves of the two groups were statistically compared using the log-rank test. For the relationships between clinical factors and survival, univariate and multivariate analyses were performed using a Cox proportional hazard model, respectively. All statistical analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC, USA) or StatView 5.0 for Windows (SAS Institute). All p-values of <0.05 were considered to indicate statistically significant differences.

Results

Association between the JMJD2B expression and the clinicopathological characteristics. From January 2002 to November 2006, a total of 78 patients with surgically resected adenocarcinoma in which driver mutations, such as *EGFR, K-ras, ALK, BRAF, ROS1* and *RET*, were analyzed; Table I shows the characteristics of the patients. The 78 patients included 48 males and 30 females; the median age was 67 years (range=51-82). Fifty-one and 27 patients were diagnosed with pathological stage I and stage ≥II, respectively. Mutations in the *EGFR, K-ras* and *BRAF* genes were detected in 29 (37%), seven (9%) and four (5%) patients, respectively. *ALK* rearrangement was observed in six patients (8%), whereas no *RET* or *ROS1* fusion genes were identified. Among the 78 patients, 50 cases (64%) exhibited JMJD2B immunopositivity, while 28 cases (36%) were JMJD2B-negative. No significant associations between JMJD2B expression and the various clinicopathological factors were identified, while tumors with a more advanced pathological stage tended to express JMJD2B more frequently than stage I tumors (p=0.0857).

The difference in OS between JMJD2B-positive and -negative lung adenocarcinoma. We performed an analysis to determine the associations between the various clinicopathological factors and DFS and OS; the findings are summarized in Table II. The median follow-up time was 49.5 months. Univariate analysis using a Cox proportional hazard model

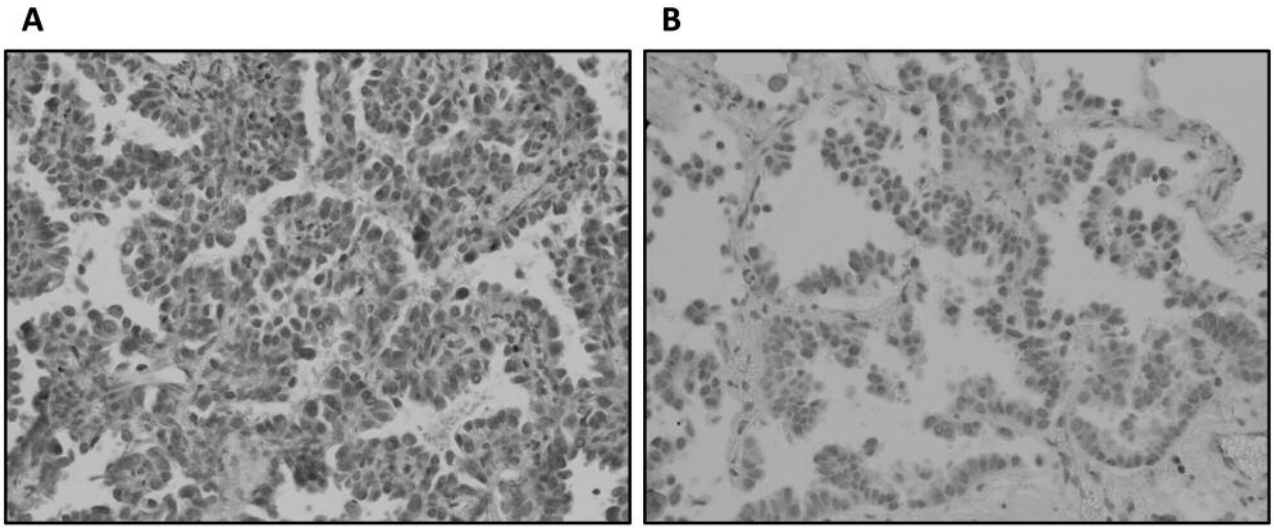

Figure 1. *Representative images of JMJD2B-positive (A) and -negative (B) cases. Original magnification: ×200.*

revealed that gender (male, 39.9 months; female, 68.2 months), age (<70 years, 42.5 months; ≥70 years, 63.4 months) and pathological stage (stage I, 61.8 months; stage ≥II, 30.7 months) were significant prognostic factors for five-year DFS. With regard to JMJD2B, a non-significant trend toward worse five-year DFS was observed in JMJD2B-positive patients (47.4%) in comparison to JMJD2B-negative patients (53.6%) (p=0.76). With regard to five-year OS, a more advanced pathological stage and JMJD2B positivity were associated with worse survival (p=0.0078 and 0.0101, respectively). Figure 2 demonstrates the Kaplan-Meier curves for OS according to JMJD2B expression. The five-year OS of JMJD2B-positive patients was 56.7%, while that of the JMJD2B-negative patients was 92.6% (log-rank: p=0.01). In addition, smoking status and genetic alterations in *EGFR, K-ras* and *ALK* were not significant prognostic factors for either DFS or OS. In multivariate analysis, age ≥70 and pathological stage I were found to be independent predictive factors for improved DFS, while gender was not observed to significantly affect the prognosis (Table III). Furthermore, pathological stage ≥II and JMJD2B positivity were identified as significant predictive factors for worse OS.

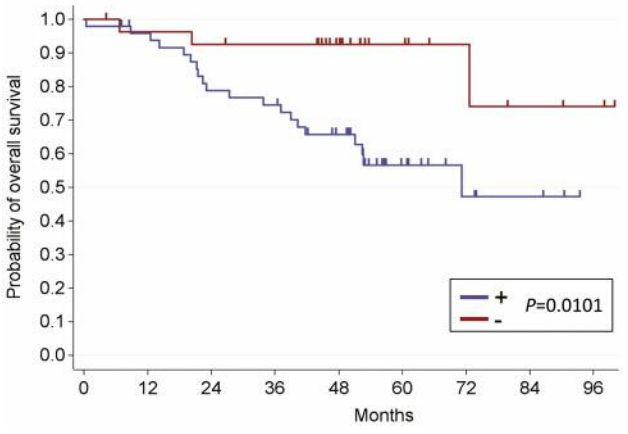

Figure 2. *The Kaplan-Meier curves of overall survival according to JMJD2B expression.*

Discussion

JMJD2B is a member of the JMJD2 family, which demethylates methylation marks on H3K9 and H3K36; JMJD2B specifically removes dimethylated and trimethylated H3K9, resulting in the activation of transcription (11). JMJD2B is involved in the self-renewal of embryonic stem cells and generation of induced pluripotent stem cells (21). Furthermore, several studies have demonstrated the transcriptional regulation of *JMJD2B* by hypoxia-inducible factor due to the presence of hypoxia response elements in its promoter region (12, 22). Furthermore, JMJD2B has been shown to be expressed in several types of cancer, including breast, gastric, colorectal and hepatocellular cancer, and involved in cell proliferation (14-17). In addition, our previous study indicated that JMJD2B promotes cell growth through regulation of cyclin-dependent kinase 6, which is one of the key modulators of the cell cycle in bladder and lung cancer cell lines (13). Thus, the involvement and expression of JMJD2B in several types of cancer has gradually been clarified. With regard to its clinical significance, a previous

Table I. *Clinicopathological characteristics and their association with JMJD2B expression.*

Factor		Total (78)	JMJD2B-positive (50)	JMJD2B-negative (28)	*p*-Value
Gender	Male	48	28	20	0.2282
	Female	30	22	8	
Age	<70	46	26	20	0.1493
	≥70	32	24	8	
Smoking status	+	43	26	17	0.3076
	–	35	24	11	
pT factor	T1, T2	72	47	25	0.6611
	T3, T4	6	3	3	
pN factor	N0, 1	68	41	27	0.0857
	N2	10	9	1	
pStage	I	51	29	22	0.0849
	≥II	27	21		6
EGFR mutation	+	29	18	11	0.8105
	–	49	32	17	
K-ras mutation	+	7	6	1	0.4113
	–	71	44	27	
EML4-ALK fusion	+	6	2	4	0.1801
	–	72	48	24	
BRAF mutation	+	4	2	2	0.6089
(unknown: n=1)	–	73	48	25	

JMJD2B, Jumonji domain-containing 2B; EGFR, epidermal growth factor receptor; K-ras, Kirsten rat sarcoma viral oncogene homolog; EML4, echinoderm microtubule associated protein like 4; ALK, anaplastic lymphoma kinase; BRAF, B-Raf proto-oncogene, serine/threonine kinase.

Table II. *Univariate analysis of the relationships between clinical factors and survival.*

Factor	n	5-year DFS (%)	*p*-Value	5-year OS (%)	*p*-Value
Gender	Male (48)	39.9	0.0411	59.9	0.086
	Female (30)	68.2			85.2
Age	<70 (46)	42.5	0.0111	66.6	0.5135
	≥70 (32)	63.4			71.7
Smoking status	+ (43)	56	0.2112	73.2	0.3079
	– (35)	46.3			65.6
pStage	I (51)	61.8	0.0004	79.7	0.0078
	≥II (27)	30.7			51.7
EGFR mutation	+ (29)	67	0.3071	91.3	0.1208
	– (49)	43.2			60.2
K-ras mutation	+ (7)	50.5	0.6487	66.7	0.3877
	– (71)	50.5			69.3
EML4-ALK fusion	+ (6)	50	0.6738	100	Not evaluable
	– (72)	60.6			65.9
JMJD2B	+ (50)	47.4	0.7602	56.7	0.0101
	– (28)	53.6			92.6

DFS, Disease-free survival; OS, overall survival; EGFR, epidermal growth factor receptor; K-ras, Kirsten rat sarcoma viral oncogene homolog; EML4, echinoderm microtubule associated protein like 4; ALK, anaplastic lymphoma kinase; JMJD2B, jumonji domain-containing 2B.

report showed that JMJD2B expression is associated with significantly worse OS in breast cancer (16); however, the clinical significance of JMJD2B in resected lung cancer has yet to be elucidated. In the present study, OS of the JMJD2B-positive patients after surgery was shown to be significantly inferior to that of the JMJD2B-negative patients (five-year survival=56.7% *vs.* 92.6%; log-rank: $p=0.01$), while multivariate analysis showed that JMJD2B positivity, as well as pathological stage, were independent prognostic factors. Therefore, this is the first report to show the significance of

Table III. *Multivariate analysis of the relationships between clinical factors and survival.*

Factor	HR (95% CI) of DFS	*p*-Value	HR (95%CI) of OS	*p*-Value
Gender (Female/Male)	0.620 (0.298-1.288)	0.1996	-	-
Age (<70/≥70)	2.269 (1.094-4.541)	0.0273	-	-
pStage (I/≥II)	0.37 (0.197-0.697)	0.0021	0.426 (0.184-0.986)	0.0462
JMJD2B (–/+)	-	-	0.282 (0.082-0.970)	0.0446

DFS, Disease-free survival; HR, hazard ratio; CI, confidence interval; OS, overall survival; JMJD2B, jumonji domain-containing 2B.

JMJD2B in the survival of patients with resected lung adenocarcinoma, suggesting that JMJD2B can be a useful biomarker to predict survival of patients with surgically resected lung adenocarcinoma.

The targeting of enzymes associated with epigenetics has attracted much attention in the treatment of cancer and some drugs, inhibiting DNA methyltransferase and histone deacetylases, have already been approved for hematological malignancies (23). In addition, several inhibitors against histone methylating and demethylating enzymes are under development and some are under clinical scrutiny in a number of trials (24). With regard to the treatment of lung cancer by inhibitors targeting epigenetics, a specific inhibitor against LSD1 is currently under evaluation in a phase I trial for patients with small-cell lung cancer. The JMJD family is also expected to be a promising therapeutic target as supported by our previous study that showed JMJD2B might be a feasible molecular-therapeutic target for the treatment of lung cancer (13). In lung cancer, although specific tyrosine kinase inhibitors targeting EGFR and ALK exhibit excellent efficacy in patients with such oncogenes, resistance inevitably occurs in almost all patients, suggesting the urgent need for the development of novel therapeutic inhibitors (25). JMJD2B is expected to be such an inhibitor and it is hoped that it will prolong the survival of lung cancer patients.

The present retrospective study is associated with some limitations. First, the analysis of JMJD2B expression was confined only to adenocarcinoma histology. Although this is due to the need to examine the association of JMJD2B with oncogenic drivers, further studies should focus on both squamous cell carcinoma and small-cell lung cancer. Furthermore, preclinical experiments should be performed to elucidate whether JMJD2B is a promising therapeutic target in the treatment of both histological types. Another limitation is that we only investigated the expression and significance of JMJD2B in patients with lung adenocarcinoma. Since JMJD2B is just one member of the JMJD family, we should comprehensively examine the other JMJD constituents in future studies.

In conclusion, our results suggest that JMJD2B may be a novel prognostic factor for patients with resected lung adenocarcinoma, which should be investigated by further studies with a larger cohort and other histological types of lung cancer.

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Conflicts of Interest

All Authors declare no conflicts of interest in association with this study.

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