JMJD2A sensitizes gastric cancer to chemotherapy by cooperating with CCDC8

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Received: 22 July 2019 / Accepted: 14 October 2019 / Published online: 1 November 2019
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Abstract

Background Jumonji domain-containing protein 2A (JMJD2A) of the JMJD2 family of histone lysine demethylases has been implicated in tumorigenesis. However, its expression and role in gastric cancer (GC) drug resistance remain unknown. Here, we investigated the role of JMJD2A in GC chemotherapeutic susceptibility and its clinical relevance in GC.

Methods We selected 12 relevant genes from previously identified gene signatures that can predict GC susceptibility to docetaxel, cisplatin, and S-1 (DCS) therapy. Each gene was knocked down using siRNA in GC cell lines, and cell viability assays were performed. JMJD2A expression in GC cell lines and tissues was assessed using qRT-PCR and immunohistochemistry, respectively. A JMJD2A downstream target related to drug susceptibility was examined using whole-gene expression array and immunoprecipitation.

Results Among the 12 candidate genes, down-regulation of JMJD2A showed the maximum effect on GC susceptibility to anti-cancer drugs and increased the IC₅₀ values for 5-FU, cisplatin, and docetaxel 15.3-, 2.7-, and 4.0-fold, respectively. JMJD2A was universally expressed in 12 GC cell lines, and its overexpression in GC tissue was positively correlated with tumor regression in 34 DCS-treated patients. A whole-gene expression array of JMJD2A-knockdown GC cells demonstrated a significant decrease in the expression of pro-apoptotic coiled-coil domain containing 8 (CCDC8), a downstream target of JMJD2A. Direct interaction between CCDC8 and JMJD2A was verified using immunoprecipitation. CCDC8 inhibition restored drug resistance to docetaxel, cisplatin, and S-1.

Conclusions Our results indicate that JMJD2A is a novel epigenetic factor affecting GC chemotherapeutic susceptibility, and JMJD2A/CCDC8 is a potential GC therapeutic target.

Keywords Gastric cancer · Histone lysine demethylases · Jumonji domain-containing protein 2A (JMJD2A) · Coiled-coil domain containing 8 (CCDC8) · Drug resistance

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10120-019-01024-9>) contains supplementary material, which is available to authorized users.

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Introduction

Histone lysine methylation is recognized as an important post-translational modification that regulates chromatin structure and gene expression [1]. Histone demethylases

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remove methyl groups from the lysine residues of histone tails, thereby regulating the transcriptional activity of target genes [2, 3]. Jumonji domain-containing protein 2A (JMJD2A), also known as lysine-specific demethylase 4A (KDM4A), is a member of the histone demethylase JMJD2 (JMJD2A–2D) family that is characterized by the catalytic Jumonji C (JmjC) domain [4]. It catalyzes the demethylation of di- and tri-methylated H3K9, H3K36, and H1.4K26 [5].

Recently, the JMJD2 family has attracted much attention, because JMJD2A, JMJD2B, and/or JMJD2C have been shown to be overexpressed in breast [6], lung [7, 8], colorectal [9], and bladder tumors [10], and required for cancer cell growth [4]. In particular, JMJD2A in human gastric cancer (GC) has been identified as an oncogenic protein based on transcription regulation [11].

GC is the second leading cause of cancer-related death worldwide [12]. Recent advances in diagnostic methods and perioperative management have facilitated the early detection of GC, hence lowering the mortality rate. However, patients with advanced tumor frequently develop recurrence after radical resections, and consequently show poor survival rates [13]. Until now, many chemotherapeutic agents, with promising activity against gastric cancer, have been identified, including S-1, docetaxel, cisplatin; however, most chemotherapy regimens remain insufficient for the improvement of survival rates [14, 15]. Recently, we have shown that triple-drug combination chemotherapy comprising the key therapeutic agents for inoperable metastatic gastric cancer: docetaxel, cisplatin, and S-1 (DCS), has a high response (79.1–87.1%) and conversion surgery (22.6–34.9%) rates in patients with metastatic GC [16–21]. In addition, a phase II trial by Koizumi et al. also indicated a similarly high response rate (81%) [22]. A phase II study of neoadjuvant chemotherapy with DCS showed a sufficiently high R0 resection rate (90.7%) and appreciable pathological response (65.9%) in patients with advanced resectable GC [23]. However, unresponsive cases still occur in DCS therapy. One of the key reasons may be drug resistance, which have prevented successful treatment in these cases [24, 25]. Several research groups are still exploring various mechanisms of drug resistance to overcome this challenge of chemotherapy in advanced GC cases.

We had previously detected 29 functional gene signatures that are associated with significant response to DCS treatment using global gene expression analysis [26]. This finding provided an important clue to uncover the unidentified mechanism of drug resistance in DCS chemotherapy. Among these genes, JMJD2A was found to be one of the functional genes affecting the efficacy of DCS therapy.

Here, to extend the existing findings, we elucidated the role of JMJD2A in drug susceptibility of DCS therapy in patients with GC, and further identified significant positive correlation of tumor reduction rate with JMJD2A expression

in GC. Moreover, using whole-gene expression analysis, we detected coiled-coil domain containing 8 (CCDC8) as a novel functional target of JMJD2A.

Materials and methods

Patients

Before triple-combination chemotherapy using docetaxel, cisplatin, and S-1 (DCS), a total of 34 tissues from 34 patients, with unresectable metastatic gastric cancer in clinical stage IV, were obtained in this study. Patient characteristics are shown in Table S1. Written informed consent was obtained from all patients according to the guidelines approved by the Ethics Committee of Tokushima University Hospital (Approval number; 865). Unresectable distant metastatic disease with an M1 stage was defined according to the Japanese Classification of Gastric Carcinoma (JGCA) version 14 [27]. Metastatic lesions (M1) were evaluated according to the findings of conventional examinations such as contrast computed tomography, ultrasonography, or magnetic resonance imaging. Staging laparoscopy was also performed as needed to exclude occult M1 disease in the peritoneum or other intra-abdominal sites. During DCS therapy, tumor reduction rate was classified based on tumor regression rates (TRR), following the RECIST [28].

Cell lines, culture, and chemical reagents

Mainly, human gastric cancer cell lines, MKN45 and KATO-III, were used in this study. MKN45 cell lines were obtained from Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). KATO-III cells were provided from the department of Medical Oncology and Hematology, Sapporo Medical University School of Medicine (Hokkaido, Japan). Ten other types of human gastric cancer cells, namely MKN1, MKN74, IM95, OCUM-1, GCIY, HGC-27, NUGC-4, AGS, HSC-39, and JRST, were obtained from the JCRB Cell Bank (Osaka, Japan). Cells were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% FBS (Life Technologies, Carlsbad, CA) at 37 °C in an incubator with 5% CO₂. 5-FU, cisplatin, and docetaxel were obtained from Sigma-Aldrich.

siRNA and transfection

Specific double-stranded JMJD2A siRNA (Silencer Select[®] s18635; Ambion, Austin, TX) were transfected into MKN45 and KATO-III cells (1.0×10^4 or 1.0×10^6) using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Silencer Negative Control #1 siRNA (Ambion) was used as negative control.

siRNAs for the other 13 genes and CCDC8 (s38376 and s228331) were also used. The sequences of siRNAs are shown in Table S2.

Quantitative real-time PCR (qPCR)

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany), and reverse transcribed into cDNA using High Capacity RNA-to-cDNA kit (Life Technologies). The probe and primers were from TaqMan gene expression assay reagents (Applied Biosystems, Foster City, CA). The following TaqMan assays were used; *KDM4A* (Hs00206360_m1, Applied Biosystems), *CCDC8* (Hs01003458_s1), *18S* (Hs99999901_s1), and *GAPDH* (Hs02758991). Each mRNA level was normalized with respect to those of *18S* and/or *GAPDH* as internal control.

Plasmid construction and transfection

Expression plasmid-encoding human *JMJD2A* protein was constructed as follows. Human *JMJD2A* was excised from the pOTB7 vector (Riken BioResource Center, Tsukuba, Japan) with *Bam* HI and *Sma* I and ligated into pcDNA3.1/*myc*-His (Invitrogen). In MKN45 cells (1.0×10^4 or 1.0×10^6), *JMJD2A* expression plasmid or an empty vector (pcDNA3.1/*myc*-His) was transiently transfected using Lipofectamine 3000 (Invitrogen).

Cell viability assay

Cell viability was assessed by water-soluble tetrazolium salt (WST) using Cell Counting Kit-8 (Dojindo, Japan). We selected 12 genes (PCGF3, B4GALT5, EGR1, PDGFB, HECA, ANTXR2, ANXA5, CISH, PLK2, ERLIN1, *JMJD2A*, and FAM116A) for silencing. After the incubation of siRNA or negative control siRNA for 24 h, cell growth was assayed in 96-well plates after 48 h with the treatment of 5-FU, cisplatin, and docetaxel. Various concentrations of 5-FU (up to 500 μ M), cisplatin (up to 40 μ M), and docetaxel (up to 1000 nM) were used, and IC_{50} was subsequently calculated.

Western blotting

Cells were washed with PBS and lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate; Sigma-Aldrich) containing protease inhibitors (Sigma-Aldrich). Equal amounts of sample proteins were denatured by boiling for 5 min in SDS-sample buffer containing 1% beta-mercaptoethanol. The proteins were then electrophoresed on an SDS-polyacrylamide gel and transferred onto an Immobilon-P membrane (Millipore).

The membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 for 1 h, and thereafter incubated with a primary antibody overnight. The primary antibodies used are as follows: anti-*JMJD2A* mouse monoclonal antibody (1:100; sc-271210, Santa Cruz Biotechnology, Santa Cruz, CA), anti-*CCDC8* rabbit polyclonal antibody (1:250; HPA041993, Sigma-Aldrich), anti-tri-methyl-histone H3 (Lys9) rabbit monoclonal antibody (1:1000; #13969 Cell Signaling Technology, Beverly, MA), anti-di-methyl-histone H3 (Lys9) rabbit monoclonal antibody (1:1000; #4658, Cell Signaling Technology), and anti-beta-actin mouse monoclonal antibody (1:10,000; A5441, Sigma-Aldrich). Proteins were then visualized with an anti-mouse and rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:15,000; GE Healthcare, UK) using an ECL Western Blotting Detection System (Amersham Pharmacia Biotech, Aylesbury, UK).

Immunohistochemistry

Immunohistochemistry was performed using anti-*JMJD2A* polyclonal antibody (1:50; #5328, Cell Signaling Technology), and biotinylated link antibody LSAB2 System-HRP DAB kit (Dako, Carpinteria, CA) followed by counterstaining with hematoxylin.

Expression levels were visualized and classified by combining an estimate of the percentage of immunoreactive cells (quantity score) and staining intensity (staining intensity score) with minor modification, as in the previous report [8]; this was evaluated independently by two investigators. Staining intensities were divided into three grades: no staining in any tumor cell (score 0), staining of less than 50% tumor cells with weak intensity (score 1), and staining in 50–90% tumor cells with strong intensity (score 2). For instance, 60% labeled cells were scored as staining intensity 2, showing 1.2 immunohistochemistry scores (0.6 multiplied by 2), and average immunohistochemistry scores were described.

Immunoprecipitation

Immunoprecipitation (IP) kit (# K286-25) was purchased from BioVision (Milpitas, CA) and performed following the manufacturer's protocol. Briefly, after the transfection of *JMJD2A* plasmid and an empty vector (pcDNA3.1/*myc*-His) for 48 h in MKN45 cells, cells were lysed with RIPA buffer containing protease inhibitor cocktail provided. Cell suspension were mixed for 30 min at 4 °C and centrifuged for 2 min at 2000 $\times$ g, followed by extraction of the supernatant. To the cell extracts, anti-*JMJD2A* polyclonal antibody (Cell Signaling Technology; #5328) and rabbit IgG (Cell Signaling Technology, #3900) as control, were added and rotated overnight at 4 °C. Protein A/G beads (25 μ L) were added next, and the solution was further incubated for 1 h

at 4 °C, centrifuged, and the pellet washed thrice. Samples were boiled for 5 min at 95 °C and analyzed by western blotting using JMJD2A or CCDC8 antibody. Secondary antibody was used in Clean-Blot IP Detection Reagent (Thermo Scientific, Waltham, MA).

Gene expression microarray

After the transfection of JMJD2A siRNA or negative control #1 into MKN45 cells, total RNA was extracted at 24 h using an RNeasy Mini Kit (Qiagen). Four independent samples were prepared. The concentration of RNA was measured with NanoDrop 1000 spectrophotometer (NanoDrop Technologies; Thermo Scientific), and its integrity was assessed with the Agilent 2100 Bioanalyzer and RNA 6000 LabChip Kit (Agilent Technologies, Palo Alto, CA). Whole-gene expression was determined using a whole human genome microarray (SurePrint G3 Human GE 8 × 60 K; Agilent Technologies). Briefly, total RNA was labeled with Cyamine 3-Cytidine biphosphate by T4 ligase and hybridized to SurePrint G3 human genome microarray. Subsequently, each sample was scanned by a Microarray Scanner (G2505C; Agilent Technologies), and fluorescence signal was extracted using Feature Extraction Software (version 10.7.3.1). Raw intensity of the mRNA data was analyzed by GeneSpring GX 12 (Agilent Technologies). The complete datasets were deposited in the Gene Expression Omnibus database (accession number GSE111590).

Statistical analysis

Statistical significance of the data was analyzed using the unpaired Student's *t* test, using SPSS version 24.0 (SPSS, Inc., Chicago, IL). A probability level of 0.05 was used as the criterion for significance.

Results

Change in drug susceptibility after silencing 12 genes related to DCS therapy

Previously, we had identified 29 candidate predictor genes in GC tissues from both responders and non-responders of DCS therapy [26]. In this study, to extend our observation related to drug susceptibility, we selected 12 genes among the 29 genes by a leave-one-out approach as we described previously [26]. After specific silencing of the 12 genes by siRNA, the IC₅₀ values of 5-FU, cisplatin, and docetaxel were analyzed in MKN45 cells, as shown in Table 1. Among the 12 genes, only JMJD2A showed a significant increment of each of the IC₅₀ values of 5-FU, cisplatin, and docetaxel compared to those in control cells (15.3, 2.7, and 4.0-fold, respectively). Thus, loss of JMJD2A was associated with an increase in IC₅₀, implicating the role of JMJD2A in resistance to the three anti-cancer drugs.

Table 1 Effect of knockdown of 12 genes on IC₅₀ of 5-FU, cisplatin, and docetaxel in MKN45 cells

No.	Gene symbol	Gene name	5-FU		Cisplatin		Docetaxel	
			IC ₅₀ (μM)	<i>p</i> value	IC ₅₀ (μM)	<i>p</i> value	IC ₅₀ (nM)	<i>p</i> value
1	PCGF3	Polycomb group ring finger 3	24.30 ± 3.39	0.0009	62.36 ± 10.02	0.0006	4.27 ± 3.22	NS
2	B4GALT5	UDP-Gal:betaGlcNAc beta 1,4-galactosyl-transferase	3.43 ± 0.12	0.001	6.59 ± 1.16	NS	9.95 ± 2.61	0.01
3	EGR1	Early growth response 1	5.44 ± 1.45	NS	20.43 ± 3.15	0.001	21.05 ± 4.76	0.003
4	PDGFB	Platelet derived growth factor B	3.27 ± 0.07	0.0009	12.18 ± 1.66	0.002	12.72 ± 1.27	0.0002
5	HECA	Headcase homolog	3.63 ± 0.16	0.002	3.92 ± 0.18	NS	7.16 ± 0.43	0.0001
6	ANTXR2	Anthrax toxin receptor 2	3.42 ± 0.02	0.001	13.83 ± 2.38	0.003	12.97 ± 4.56	0.02
7	ANXA5	Annexin A5	6.90 ± 0.81	NS	53.19 ± 7.53	0.001	8.72 ± 1.29	0.002
8	CISH	Cytokine-inducible SH2-containing protein	19.79 ± 3.18	0.002	72.23 ± 8.40	0.0002	0.77 ± 0.42	0.0005
9	PLK2	Polo-like kinase 2	7.72 ± 0.30	NS	3.11 ± 0.24	0.03	12.74 ± 0.69	0.0001 >
10	ERLIN1	ER lipid raft associated 1	23.23 ± 15.13	NS	3.79 ± 0.72	NS	9.78 ± 3.11	0.02
11	JMJD2A	Jumonji domain containing 2A	100.99 ± 21.17	0.002	13.13 ± 2.38	0.005	13.41 ± 1.35	0.0002
12	FAM116A	Family with sequence similarity 116, member A	6.58 ± 1.80	NS	10.73 ± 2.81	0.02	7.64 ± 0.61	0.0003
	Random siRNA		6.62 ± 0.66		4.79 ± 0.84		3.38 ± 0.16	

Experiments were independently performed thrice. *p* values (gene specific siRNA versus random siRNA) were calculated using Student's *t* test

Expression of JMJD2A in 12 types of GC cells

To investigate the gene expression profiles in various histologically typed gastric cancer, we measured the expression level of JMJD2A in 12 GC cell lines (Fig. 1a). Endogenous expression of JMJD2A was universally

observed in all the cell lines measured using TaqMan qPCR, whose values were normalized against both 18S and GAPDH. Among these, we selected MKN45 cells for further experimental analysis, because it showed relatively high expression of JMJD2A and is widely used as a representative cell line derived from poorly differentiated adenocarcinoma.

Fig. 1 Expression of JMJD2A in human gastric cancer tissues and correlation with tumor reduction rate. **a** Expression levels of JMJD2A in 12 human gastric cancer cell lines were measured by TaqMan qPCR. Relative expression levels are shown as the value normalized to 18S (left) and GAPDH (right). Values are presented as means $\pm$ SD from triplicate. **b** Representative image showing histological staining of JMJD2A in pretreated human gastric cancer tissues; hematoxylin and eosin (HE) staining of JMJD2A (at lower (100 $\times$) and higher (200 $\times$) magnifications) (#5328). Upper panels, intestinal type (patient number 8, immunoscore of 1.8); lower panels, diffuse type (patient number 15, immunoscore of 1.7). **c** Correlation plots between immunohistochemistry score and tumor reduction rate (%) after two cycles of DCS therapy. *p* values were calculated using Pearson's test. R^2 indicates the coefficient of determination

Expression of JMJD2A in human GC tissues and association with clinicopathological factors and tumor reduction rate

To examine the expression of JMJD2A and its potential clinical relevance in DCS therapy, we estimated JMJD2A expression levels in pretreatment biopsy specimens from 34 patients with GC using immunohistochemical staining. Representative histological staining of JMJD2A in intestinal type and diffuse type are shown in Fig. 1b. We first evaluated a possible association between the expression levels of JMJD2A and several clinical characteristics in patients treated with DCS. The cases in which the JMJD2A score was 0–1.0 were enrolled in the JMJD2A low group ($n = 11$), whereas cases with JMJD2A scores between 1.1 and 2.0 were enrolled in the JMJD2A high group ($n = 23$). No significant differences were observed in terms of sex, age, histologic type, HER 2 status, T stage, N stage, or distant metastasis sites (peritoneum, liver, lung, and pancreas) between the two groups, whereas significant differences in lymph node distant metastasis and a number of non-curative factors were noted (Table S3). Thereafter, we analyzed the association with reduction rates of gastric tumors. Immunoscores showed positive and significant association with JMJD2A expression after two courses of DCS therapy ($p = 0.012$) (Fig. 1c). Thus, high expression of JMJD2A was correlated with positive reduction rate in gastric tumors, indicating its potential role in increased drug susceptibility to DCS therapy.

Cellular function of JMJD2A

Because JMJD2A was reported to reverse tri-methylated histone H3K9, we checked the enzymatic activity of JMJD2A as a histone demethylase. We examined the loss-of and gain-of functions in JMJD2A in vitro (Fig. 2a). After the knockdown of JMJD2A in MKN45 cells, H3K9 di-methylation status was significantly reduced, and tri-methylation increased, as seen by western blotting. In contrast, with overexpressed JMJD2A, levels of H3K9 di-methylation were remarkably enhanced, and tri-methylation apparently reduced. These results were confirmed in the NUGC-4 cell line (Figure S1). This was opposite to the loss-of function result, indicating genuine methylated activity in overexpressed JMJD2A. H3K9 tri-methylation is well known as a transcriptional repressive factor to form the heterochromatin structure. In our experimental procedure, JMJD2A appeared to regulate transcription through the demethylation of H3K9.

Drug susceptibility after overexpression of JMJD2A

Based on the loss-of-function results in JMJD2A, the IC_{50} values of 5-FU, cisplatin, and docetaxel were significantly

Fig. 2 JMJD2A physically associated with methylated histone H3K9. **a** JMJD2A siRNA was transfected with RNAiMAX into MKN45 (1.0×10^6) cells, and overexpressed with lipofectamine 3000. Cell extracts were measured by western blotting and evaluated by JMJD2A levels. For physiological functions of JMJD2A, cell extracts were evaluated by di- and tri-methylated H3K9 status. β -actin served as a loading control. MW; molecular weight. **b** Effect of JMJD2A overexpression on IC_{50} of 5-FU, cisplatin, and docetaxel in MKN45 cells

increased (Table 1), suggesting the role of JMJD2A in drug resistance. We then analyzed the gain-of-function of JMJD2A to examine whether the drug susceptibility had changed. After the overexpression of JMJD2A in MKN45 cells, IC_{50} values of the three drugs had not changed (Fig. 2b), which was implicated in the lack of induction of drug efficacy after the overexpression of JMJD2A. In addition, using NUGC-4 cells, which showed relatively low JMJD2A expression (Fig. 1a) after overexpression of JMJD2A, we analyzed the changes of IC_{50} values of the three drugs (Table S4). As earlier, there was no significant modification of the IC_{50} values. Against the result of loss-of function in JMJD2A, we found a different status with the gain-of function of JMJD2A.

CCDC8 is a downstream target of JMJD2A

Because the interaction of JMJD2A with the other genes is unknown, we analyzed the downstream genes associated with JMJD2A silencing, using whole-gene expression analysis, to explore the mechanism by which JMJD2A regulates drug susceptibility. After silencing the JMJD2A gene in MKN45 cells, we identified 20 genes that had significantly different expression levels compared to those in control cells (unpaired t test, $p < 0.05$); the changes in expression of these

genes were 4.5-fold greater (Fig. 3a, b). Of note, whole-gene expression analysis showed significant suppression of TXLNB, SPATA21, and CCDC8, ranked from top to third. We selected the CCDC8 gene among these candidate genes, because it is known to be a pro-apoptotic gene related to DNA damage, whereas there have been no reports describing the involvement of other genes in carcinogenesis and drug susceptibility. Moreover, we also considered that the expression of CCDC8 is reported to be relatively high in gastric cancer tissue compared to those of other genes in the TCGA database [29].

To confirm the whole-gene expression results, we used the other cellular samples and analyzed the expression of CCDC8 by qPCR and western blotting. After silencing JMJD2A in MKN45 cells, CCDC8 expression was significantly reduced at both 24 and 48 h, as shown by qPCR (Fig. 4a). In parallel, significant reduction of CCDC8 was also recognized, at 24 and 48 h, by western blot analysis (Fig. 4b).

To further explain the cellular association of CCDC8, we tested the in-vitro interaction of JMJD2A and CCDC8 using immunoprecipitation assay. As shown in Fig. 4c, CCDC8

Fig. 3 Hierarchical clustering of 20 candidate downstream genes related to JMJD2A knockdown.

a Each column represents one gene, and each row represents their cellular origin. Four data from JMJD2A siRNA and four from Random siRNA in MKN45 cells, were considered, using whole human genome microarray (8×60 K; Agilent). A pseudo-color representation of the relative intensities is shown; red color indicates high expression, and green color low expression. **b** After specific gene silencing of JMJD2A, top 20 candidate genes are listed to show significant different expression

b

No.	Gene symbol	Gene name	p value	Fold change	Regulation
1	TXLNB	taxilin beta	0.020	-11.95	down
2	SPATA21	spermatogenesis associated 21	0.038	-9.76	down
3	CCDC8	coiled-coil domain containing 8	0.015	-7.68	down
4	RESP18	regulated endocrine-specific protein 18	0.042	-6.26	down
5	SLC22A9	solute carrier family 22 (organic anion transporter), member 9	0.038	-6.24	down
6	MEGF11	multiple EGF-like-domains 11	0.010	-6.12	down
7	PCDHGB2	protocadherin gamma subfamily B, 2	0.049	-5.96	down
8	JMJD2A	jumonji domain containing 2A	0.0001	-5.75	down
9	WDR17	WD repeat domain 17	0.043	-5.66	down
10	CCT8L2	chaperonin containing TCP1, subunit 8 (theta)-like 2	0.040	-5.29	down
11	DEFB119	defensin, beta 119	0.045	-5.01	down
12	ZAP70	zeta-chain (TCR) associated protein kinase 70kDa	0.020	-4.97	down
13	DDX25	DEAD (Asp-Glu-Ala-Asp) box helicase 25	0.049	-4.90	down
14	TMPRSS11A	transmembrane protease, serine 11A	0.045	-4.84	down
15	KCNJ5	potassium channel, inwardly rectifying subfamily J, member 5	0.034	-4.82	down
16	CKAP5	cytoskeleton associated protein 5	0.000	-4.72	down
17	GABRG1	gamma-aminobutyric acid (GABA) A receptor, gamma 1	0.039	-4.71	down
18	AMHR2	anti-Mullerian hormone receptor, type II	0.043	-4.67	down
19	EFHD1	EF-hand domain family, member D1	0.007	4.63	up
20	SI	sucrase-isomaltase (alpha-glucosidase)	0.030	-4.57	down

Fig. 4 Direct effect of JMJD2A knockdown on CCDC8 level and interaction. **a** After the treatment of MKN45 cells with JMJD2A siRNA, expression of JMJD2A and CCDC8 was measured at 24 h by TaqMan qPCR. Relative expression levels are shown as the value normalized to those of 18S (left) and GAPDH (right). **b** Levels of JMJD2A and CCDC8 were analyzed at 24 and 48 h by western blotting. β -actin was used as a control. MW: molecular weight. **c** MKN45 cells were transfected with expression plasmid of JMJD2A for 48 h. Transfection with empty vector was used as control. Cell lysates were immunoprecipitated with anti-JMJD2A antibody (#5328) or control IgG. The co-immunoprecipitated JMJD2A was detected by western blotting (top). The co-immunoprecipitated CCDC8 was then analyzed using anti-CCDC8 antibody (HPA041993) to evaluate the enrichment of CCDC8 (bottom). MW: molecular weight

Table 2 Effect of CCDC8 knockdown on IC_{50} of 5-FU, cisplatin, and docetaxel in MKN45 cells

	IC_{50} MKN45/random si	IC_{50} MKN45/CCDC8 si	<i>p</i> value
5-FU (μ M)	6.08 ± 1.12	59.47 ± 0.60	<0.0001
Cisplatin (μ M)	4.80 ± 0.25	7.86 ± 0.56	0.001
Docetaxel (nM)	3.81 ± 0.08	13.90 ± 0.39	<0.0001

Experiments were independently performed thrice; *p* values were calculated using Student's *t* test

bound to endogenous JMJD2A, but not to IgG alone, showing that CCDC8 and JMJD2A interact directly.

Effect of CCDC8 knockdown on drug susceptibility

In parallel with the loss of JMJD2A, the IC_{50} values of 5-FU, cisplatin, and docetaxel were found to be significantly increased (Table 1). Thus, we analyzed whether CCDC8 function was involved in drug susceptibility (Table 2). After silencing of CCDC8 (Figure S2), the IC_{50} value of 5-FU, cisplatin, and docetaxel were significantly higher than in control cells, indicating that CCDC8 cooperates with JMJD2A

to affect the modulation of susceptibility to 5-FU, cisplatin, and docetaxel, and induced drug resistance in DCS therapy.

Discussion

In this study, we showed that JMJD2A can potentially regulate drug susceptibility in DCS therapy using siRNA-mediated down-regulation and immunohistochemistry of GC patient tissues. Additionally, using whole-gene expression analysis and *in-vitro* immunoprecipitation, we identified that JMJD2A cooperates with CCDC8 to control the susceptibility of 5-FU, cisplatin, and docetaxel treatment.

Aberrant changes in histone lysine methylation status have been linked to cancer formation [30]. In a recent study, members of the JMJD2 family of histone lysine demethylases (KDM) have been identified as having oncogenic function and are highly expressed in human cancers [31]. In this regard, Hu et al. reported that high JMJD2A levels are associated with disease stage, nodal status, and metastasis in GC patients [11]. Similarly, when we analyzed the relationship between expression of JMJD2A and clinicopathological factors of GC patients, significant differences were observed in distant lymph node metastasis and the number of non-curative metastatic factors (Table S3). Thus, JMJD2A has been

indicated to be involved in tumorigenesis and in enhancing the metastasis of GC. However, its role in anti-cancer drug resistance in GC therapy remains largely unknown.

Histone methylation can positively or negatively impact gene transcription; dysregulation of histone methyl-modifying enzymes has been increasingly recognized as a hallmark of carcinogenesis [32, 33]. JMJD2A is a transcriptional cofactor and enzyme that catalyzes demethylation of H3K9 and H3K36 [4, 34]. Because methylation of H3K9 in promoter regions is associated with transcriptional repression [35], JMJD2A plays an important role in diverse types of cancer, including breast [6], lung [7, 8], colon [9], and bladder cancer [10], possibly activating transcription through demethylation of H3K9 [7]. For instance, overexpression of JMJD2A is sufficient to promote replication and copy gain of specific chromosomal regions, which is associated with the drug-resistant oncogene *CKS1B* in breast cancer [36]. Using a knockdown approach, we observed a significant contribution of JMJD2A to the susceptibility to key anti-cancer drugs (5-FU, cisplatin, and docetaxel) for treatment of GC. Our data raise the possibility that loss of JMJD2A function may result in inactivation of transcription of genes related to drug susceptibility, which leads to increase in the IC₅₀ value of these three drugs.

Given the role of JMJD2A in gene transcription, it was worth investigating a cooperative novel factor in the regulation of drug susceptibility. One advantage of unbiased whole-gene expression approach is the potential to uncover new genes that can be targeted for further investigation. Thus, we analyzed the effects on global gene expression of JMJD2A silencing. We identified CCDC8 as the downstream target of JMJD2A, which was verified using in vitro experiments.

CCDC8 contains two coiled-coil domains [37] involved in diverse biological processes such as the regulation of gene expression, cell division, and membrane fusion [38, 39]. CCDC8 mutation leads to the development of 3 M syndrome in humans, a primordial growth disorder, by interaction with *CUL7* (Cullin 7) and *OBSL1* (Obscurin-Like 1) [37]. In addition, CCDC8 has been previously shown to be a tumor suppressor in several types of cancer, such as breast [40], renal [41], and lung cancers [42], associated with hypermethylation of the CCDC8 gene in some of these tumors.

However, the role and possible mechanisms of CCDC8 in drug susceptibility has not been elucidated to date. Interestingly, CCDC8 is also known as a critical cofactor for p53-mediated apoptosis upon DNA damage and as facilitating activation of proapoptotic targets [43]. In this regard, p53-mediated apoptosis is abrogated in the gastric tumor cells lacking CCDC8 expression, which leads to resistance to chemotherapeutic agents such as CDDP and 5FU that are known to cause p53-dependent apoptosis [44].

Moreover, knockdown of CCDC8 clearly induced drug tolerance against docetaxel in our analysis. This drug, belonging to the taxane family of compounds, exerts anti-tumor activity by promoting the assembly of microtubules and preventing their de-polymerization, resulting in cell apoptosis [45, 46]. The ordered assembly and disassembly of microtubules are tightly regulated by numerous microtubule-associated proteins [47]. In a recent report, deletion of CCDC8 was shown to result in microtubule defects, thus affecting fundamental cellular processes in patients with 3 M syndrome [48]. Interestingly, we found that low levels of CCDC8 expression closely associated with anti-cancer drug resistance, supporting the notion that docetaxel tolerance, mediated by disassembly of microtubules, is caused by loss of CCDC8. However, the detailed mechanism underlying drug susceptibility related to CCDC8 remains unclear. Further research is required to clarify how CCDC8 affects chemotherapeutic drug susceptibility in relation to JMJD2A in GC.

In our experimental settings, overexpression of JMJD2A did not show the same phenotype in chemosensitivity (Fig. 2b, Table S4), although loss of JMJD2A affected drug susceptibility via lack of CCDC8. Genetic studies have shown that deletion of histone demethylases lead to pleiotropic phenotypes. In general, deletion or mutation of histone demethylases has been shown to induce severe phenotypes. For instance, LSD1 (also known as KDM1A) knockout mice display embryonic lethality around E5.5 [49]. In contrast, most studies have revealed the effects of histone demethylases on transcription of a limited number of genes [32]. These studies show that demethylases bind to a large number of genes, but the expression of only a limited number of genes was affected. Thus, the effect of histone demethylases is supposed to be generally modest. In fact, CCDC8 levels were not increased after overexpression of JMJD2A (Fig. 4c), suggesting that JMJD2A may not fully interact with methylation residues of the CCDC8 gene promoter. This may partly explain why overexpression of JMJD2A did not enhance the chemotherapeutic drug susceptibility of GC cells.

On the contrary, a positive correlation of JMJD2A expression and tumor reduction rate was observed in DCS therapy (Fig. 1c), indicating that JMJD2A may sensitize GC to chemotherapeutic drugs. Although the underlying mechanisms of these inconsistent results between in vitro and in vivo functions of JMJD2A were not fully investigated in the present study, a direct interaction between JMJD2A and CCDC8 as evidenced by immunoprecipitation assays (Fig. 4c) suggests that JMJD2A may exert its drug susceptibility function in cooperation with CCDC8. The different mechanisms of this cooperation may depend on the biological environment, such as specific methylation status in vivo and in vitro. To develop therapies targeting JMJD2A/

CCDC8, further research is required to unveil the mechanism by which JMJD2A/CCDC8 interaction modulates anti-cancer drug susceptibility.

In conclusion, our study revealed that JMJD2A is a novel epigenetic factor of chemotherapeutic susceptibility in GC, and high expression of JMJD2A was found to reliably predict tumor regression rates with DCS therapy, thereby showing that JMJD2A could serve as an independent factor in the treatment with triple anti-cancer drugs. Using a whole-gene expression approach, we identified CCDC8 as the direct downstream gene of JMJD2A and demonstrated the cooperation between JMJD2A and CCDC8 in the regulation of anti-cancer drugs, which may be a promising molecular target for the treatment of GC.

Acknowledgements This work was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI [Grant number JP16K19348 and JP18K07942]. We thank Hideaki Horikawa for analysis of microarray data, supported by the Support Center for Advanced Medical Sciences, Tokushima University Graduate School of Biomedical Sciences.

Compliance with ethical standards

Conflict of interest Tetsuji Takayama received a research fund from TAIHO Pharmaceutical Co., Ltd. (Tokyo, Japan).

Human rights statement All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions.

Informed consent Informed consent or a substitute for it was obtained from all patients for being included in the study.

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