


RESEARCH PAPER



## PAX5 gene as a novel methylation marker that predicts both clinical outcome and cisplatin sensitivity in esophageal squamous cell carcinoma

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### ABSTRACT

Therapeutic strategies for esophageal cancer largely depend on histopathological assessment. To select appropriate treatments of individual patients, we examined the background molecular characteristics of tumor malignancy and sensitivity to multidisciplinary therapy. Seventy-eight surgically-resected esophageal squamous cell carcinoma (ESCC) cases during 2001–2013 were examined. *PAX5*, a novel gene methylation marker in ESCC, was evaluated in the specimens, as methylation of this gene was identified as an extremely tumor-specific event in squamous cell carcinogenesis of head and neck. *PAX5* methylation status was evaluated by quantitative MSP (QMSP) assays. Mean QMSP value was 15.7 (0–136.3) in ESCCs and 0.3 (0–8.6) in adjacent normal tissues ( $P < 0.001$ ). The 78 cases were divided into high QMSP value (high QMSP,  $n = 26$ ) and low QMSP value (low QMSP,  $n = 52$ ). High QMSP cases were significantly associated with downregulated *PAX5* expression ( $P = 0.040$ ), and showed significantly poor recurrence-free survival [Hazard Ratio (HR) = 2.84;  $P = 0.005$ ; 95% Confidence Interval (CI): 1.39–5.81] and overall survival (HR = 3.23;  $P = 0.002$ ; 95%CI: 1.52–7.01) in multivariable analyses with histopathological factors. *PAX5*-knockdown cells exhibited significantly increased cell proliferation and cisplatin resistance. *PAX5* gene methylation can predict poor survival outcomes and cisplatin sensitivity in ESCCs and could be a useful diagnostic tool for cancer therapy selection.

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CDDP; esophageal cancer; GLUT1; methylation; *PAX5*

### Introduction

Esophageal cancer is one of the most difficult solid tumors to overcome. Nearly 90% of esophageal cancers in Asian countries are esophageal squamous cell carcinomas (ESCCs).<sup>1</sup> Among Japanese patients diagnosed with ESCC from 2003–2005, the 5-year survival rates after the initial diagnosis were 32.3% in males and 41.3% in females ([http://gdb.ganjocho.jp/graph\\_db/index](http://gdb.ganjocho.jp/graph_db/index)). These rates are much lower than those for other digestive tract cancers (stomach: 64.2%/61.5%; colon: 70.3%/67.9%; rectum: 67.3%/67.8%) and almost equal to those for lung cancer (25.0%/41.0%) and hepatobiliary cancers (liver: 28.7%/26.2%; biliary tract/gall bladder: 22.5%/19.9%). Histopathologically, tumor budding phenomenon could be one of the key findings of poor prognostic ESCCs.<sup>2</sup> To overcome this situation, multidisciplinary treatments are applied to ESCCs, which are generally chemoradiation therapy sensitive.

Diagnostic biomarkers of ESCCs have been widely examined and used clinically. Zhang et al.<sup>3</sup> reviewed 5 serum markers (CEA, Cyfra21–1, p53, SCC, VEGF), and found that all 5 had favorable area under curve (AUC) values for receiver-operating characteristic (ROC) curves: CEA: 0.71; Cyfra21–1: 0.58; P53:

0.73; SCC: 0.69; VEGF: 0.81. However, these biomarkers did not generally have high sensitivity: CEA: 8–70%; Cyfra21–1: 36–63%; p53: 14–60%; SCC: 13–64%; VEGF: 64–85%. Biomarker combinations in panels are necessary for the majority of ESCC cases. Furthermore, gene or protein expressions must be evaluated according to various cutoff values depending on cohort characteristics or experimental assays. Prognostic markers of ESCC have also been extensively examined. Recently, Jia et al.<sup>4</sup> reported TSP1 as an independent poor prognosis factor for ESCC based on high-throughput magnetic bead-based mass spectrometry. Although TSP1 clearly showed stage-dependent overexpression, cancer prognosis depends on not only histopathological findings but also the effectiveness of adjuvant therapy, including cisplatin-based chemotherapy. Focusing on biomarkers of chemoradiation sensitivity is another trend.

One of the authors previously reported that *PAX5* gene methylation could be an excellent marker for head and neck squamous cell carcinoma (HNSCC) detection<sup>5</sup> using methylated DNA-binding domain-based sequencing (MBD-seq) and fluorescence-based quantitative methylation-specific PCR (QMSP). He found that the marker had high sensitivity (80%)

and high specificity (94%) (AUC: 0.86) in 76 tumors and 19 normal tissues. They further revealed that 79% of *TP53*-mutated samples exhibited *PAX5* gene methylation. In another study, we used droplet digital PCR to show that *PAX5* gene methylation can be used as a molecular marker for surgical margin analysis and as a prognostic marker of HNSCCs.<sup>6</sup> *PAX5*, located at chromosome 19p13, was originally identified as a B-cell-specific transcription factor required for B-cell differentiation<sup>7</sup> and neural development.<sup>8</sup> *PAX5* gene methylation has been reported in various neoplasms, including HNSCC,<sup>5</sup> gastric cancer,<sup>9</sup> hepatoma,<sup>10</sup> breast cancer, and lung cancer.<sup>11</sup> *PAX5* is also mutated in human acute B-cell leukemia.<sup>12</sup> To our knowledge, there are no previous reports on *PAX5* in ESCC.

In this study, we applied this versatile marker to ESCCs, which have the same pathological characteristics as HNSCCs, to evaluate its potential as a marker for detection, prognosis, and cisplatin-based chemotherapy sensitivity.

## Results

### *PAX5* gene methylation and expression in 78 clinical ESCC samples

QMSP assays were performed for tumors and adjacent normal tissues in the 78 ESCC samples (Fig. 1a). Median (interquartile range) *PAX5* QMSP of tumors was 5.92 (1.28–23.27), while that of adjacent normal tissues was 0.08 (0.03–0.25). The difference between tumors and normal tissues was significant ( $P < 0.001$ , Mann–Whitney U test). Furthermore, 67 of 78 cases (85.9%) showed more methylation in tumors than in paired adjacent normal tissues.

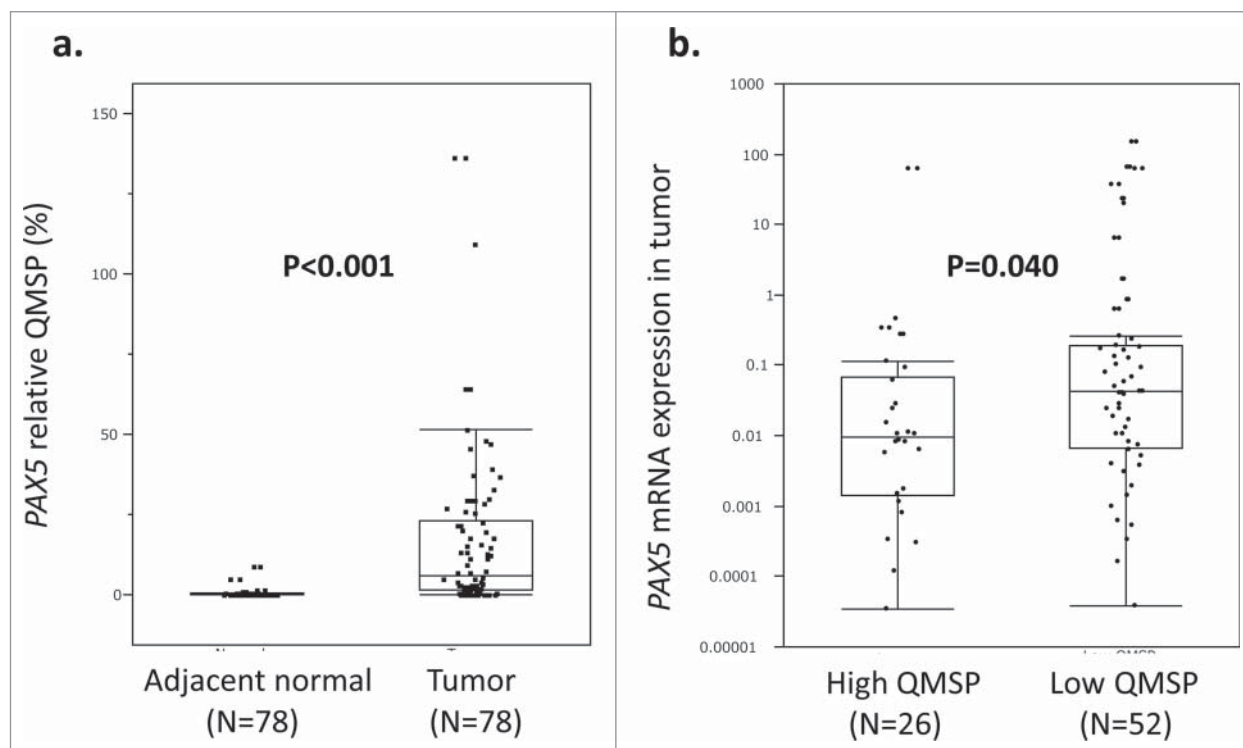
To examine associations between *PAX5* methylation and mRNA expression, all 78 cases were divided into *PAX5* high QMSP group ( $n = 26$ ) and *PAX5* low QMSP group ( $n = 52$ ) tumor groups by the optimal cutoff (QMSP = 17.4), which was calculated by ROC curve analysis of *PAX5* QMSP values and high/low expression in the 78 ESCC tumors. High and low expression groups ( $n = 39$  each) were divided by the median *PAX5* expression value of tumors. qRT-PCR assays showed that *PAX5* mRNA expression was significantly downregulated in the highly methylated tumor group ( $P = 0.040$ , Mann–Whitney U test; Fig. 1b). In the Spearman test, *PAX5* mRNA expression showed marginal correlation with *PAX5* QMSP (correlation coefficient -0.211,  $P = 0.064$ ).

### *PAX5* QMSP and mRNA expression in esophageal cancer cell lines

*PAX5* QMSP values and mRNA expression were also examined in 9 esophageal cancer cell lines (Supplementary Figure S1a). All cell lines except for WSSC had high QMSP values. Although *PAX5* mRNA expression varied, most cell lines showed low expression (Supplementary Figure S1b) with significant upregulation after 5-Aza-dC treatment (Supplementary Figure S1c). Gene hypermethylation was confirmed to be modified after 5-Aza-dC treatment (Supplementary Figure S1d).

### Influence of *PAX5* inhibition on cell proliferation and cell cycle

To observe the effects of *PAX5* knockdown, we transfected *PAX5* siRNA into 2 esophageal cancer cell lines. We chose the



**Figure 1.** a: QMSP assay results of the 78 ESCCs. The tumor tissues showed significantly high relative QMSP values compared with the adjacent normal tissues ( $P < 0.001$ , Mann–Whitney U test). b: The 78 ESCC tumors were divided into *PAX5* high QMSP ( $n = 26$ ) and low QMSP ( $n = 52$ ) groups by the optimal cutoff value (QMSP = 16.0). *PAX5* mRNA expression in the high QMSP group was significantly lower than that in the low QMSP group ( $P = 0.040$ , Mann–Whitney U test). Gene hypermethylation appeared to be one of the major mechanisms for the downregulation of *PAX5* mRNA expression.

NUEC1 and TE3 cell lines based on their relatively high *PAX5* expression. *PAX5* knockdown was confirmed by qRT-PCR (Fig. 2a). Cell proliferation ability and cell cycle activity were evaluated by WST-1 and BrdU assays. The results implied that *PAX5* knockdown induced both cell proliferation and cell cycle promotion (Fig. 2b, c). However, invasion assays did not show a clear difference between control and *PAX5* siRNA transfections (data not shown).

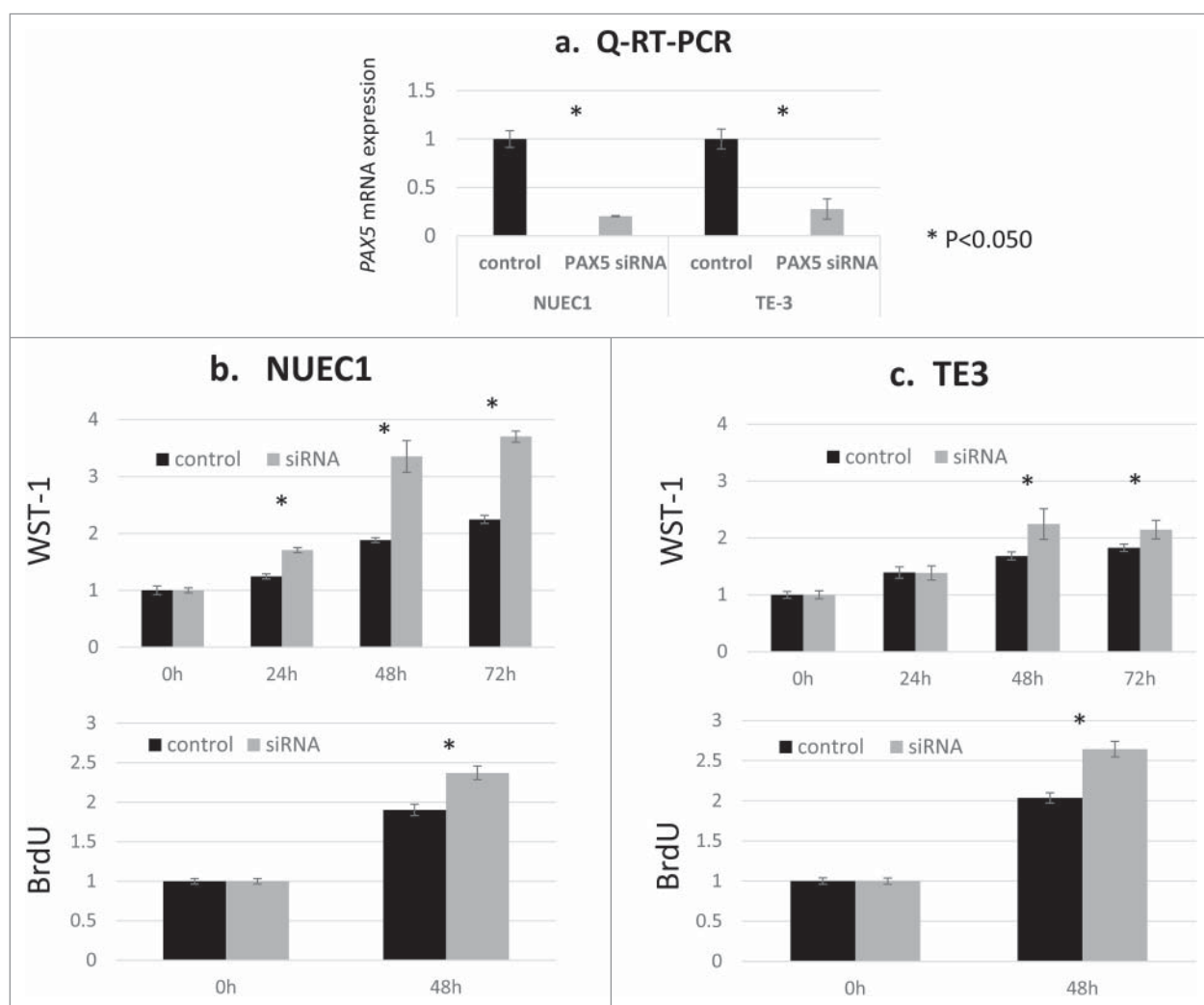
### Detection of *PAX5* knockdown-related cancer pathways

To identify downstream targets in *PAX5*-related cancer pathways, we used the Human Cancer Pathway Finder array to compare well-known carcinogenic gene expressions in control NUEC1 cells and *PAX5*-silenced NUEC1 cells. Using the optimal threshold (fold change >4.0 or <-4.0), *CCL2*, *GSC*, *LPL*, and *SLC2A1* were significantly upregulated, while *CCND2*, *IGFBP5*, and *TEK* were significantly downregulated in *PAX5*-silenced cells (Fig. 3a). We then applied these findings to the 78 ESCC clinical samples. The results for *SLC2A1* gene expression are shown in Fig. 3b as representative data. All cases

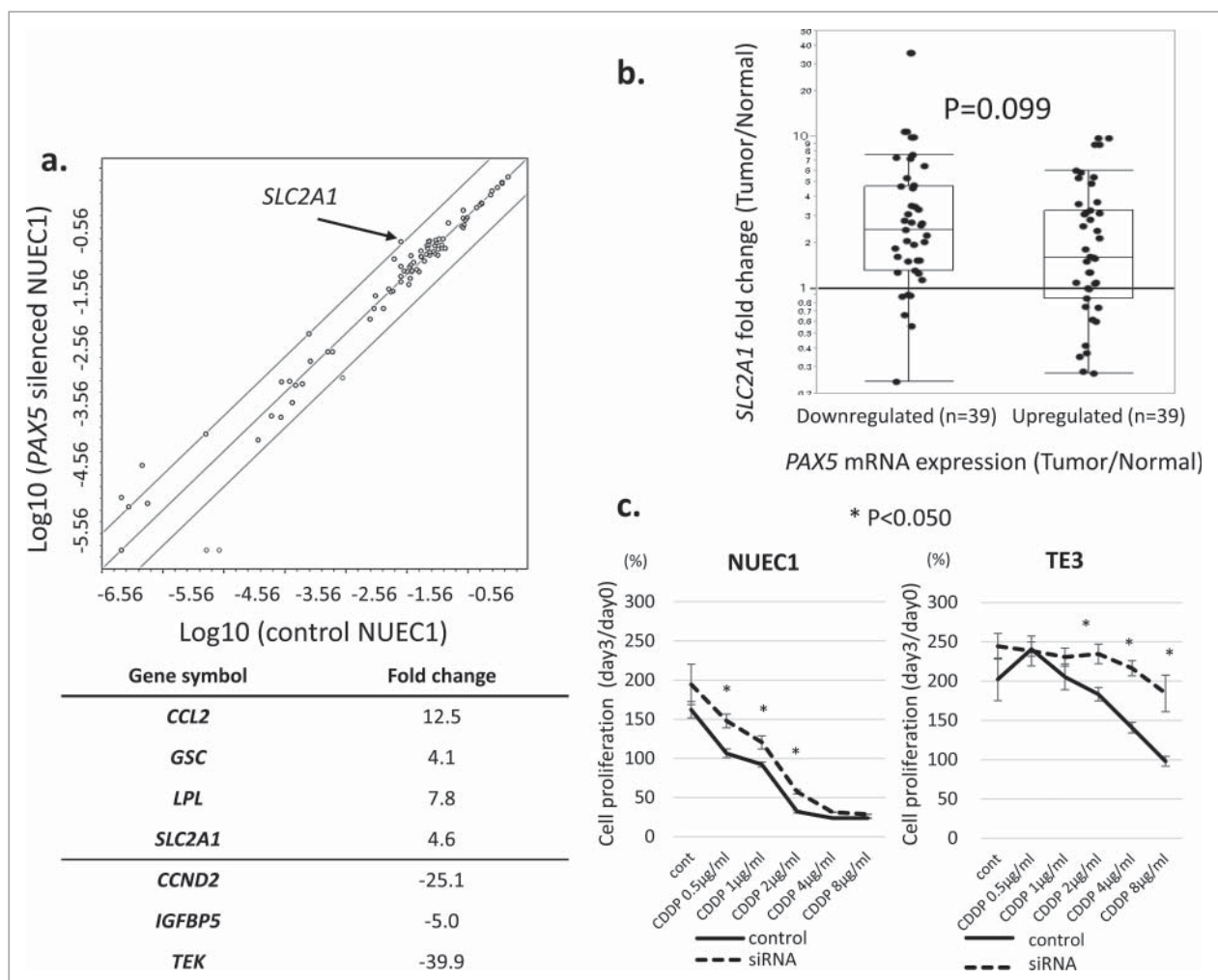
were divided into *PAX5* mRNA downregulated cases ( $n = 39$ ) and upregulated cases ( $n = 39$ ) in the tumor tissues when compared with matched normal tissues. Although not significant ( $P = 0.099$ , Mann-Whitney U test), downregulation of *SLC2A1* expression (fold change <1.0) was mainly seen in *PAX5*-upregulated cases. Meanwhile, western blotting analysis revealed that *SLC2A1* protein was upregulated in the *PAX5*-silenced TE3 cell line (Fig. 2a, Supplementary Figure S2a). Taken together, these data imply that *PAX5* gene expression is inversely associated with *SLC2A1* gene expression.

### RNA-seq and methylation array results from TCGA data

To confirm our findings for *PAX5* and *SLC2A1* more generally, we checked the Cancer Genome Browser website (<https://genome-cancer.ucsc.edu/>),<sup>13</sup> which contains *PAX5* methylation array data in 202 esophageal cancer cases (Illumina HumanMethylation450) (Supplementary Figure S2b) and *PAX5* exon expression data in 198 esophageal cancer cases (IlluminaHiSeq) (Supplementary Figure S2c). We sorted the data by histological cell type (adenocarcinoma or SCC) and sample type (cancer or



**Figure 2.** Functional analyses of the *PAX5* gene using ESCC cell lines. *PAX5* siRNA treatment was performed in NUEC1 and TE3 cells with relatively strong *PAX5* mRNA expression. a: *PAX5* mRNA expression was examined by qRT-PCR in negative control-transfected cells (control) and *PAX5* siRNA-transfected cells (*PAX5* siRNA). Significant downregulation of *PAX5* was confirmed in both cell lines ( $P < 0.050$ , Student's *t*-test, 2-tailed). b, c: Results of WST-1 and BrdU assays. *PAX5*-silenced cells showed significantly increased cell proliferation ability in both NUEC1 and TE3 cell lines ( $P < 0.050$ , Student's *t*-test, 2-tailed).



**Figure 3.** a: Results of the Human Cancer Pathway Finder array. *PAX5*-silenced NUEC1 cells (Y axis) were compared with negative control-transfected NUEC1 cells (X axis). We set the optimal threshold as fold change  $>4.0$  or  $<-4.0$ . Significantly upregulated genes in *PAX5*-silenced cells were *CCL2* (Fold change: 12.5), *GSC* (4.1), *LPL* (7.8), and *SLC2A1* (4.6). Significantly downregulated genes in *PAX5*-silenced cells were *CCND2* (Fold change: -25.1), *IGFBP5* (-5.0), and *TEK* (-39.9). b: *SLC2A1* gene expression in the 78 ESCC clinical samples. *SLC2A1* overexpression in the tumor occurred in the majority of *PAX5*-downregulated tumor cases. c: WST-1 assays comparing *PAX5* siRNA-transfected ESCC cells (NUEC1, TE3) and negative control-transfected cells under a wide range of cisplatin concentrations. In both cell lines, *PAX5*-silenced cells showed significantly higher cell proliferation than control cells.

adjacent normal). As expected, the majority of SCCs (lower part of each heat map) had *PAX5* hypermethylation around the TSS site and low *PAX5* expression. Moreover, SCCs in the same expression array cohort showed high *SLC2A1* expression and low *TEK* expression (Supplementary Figure S2c). Meanwhile, esophageal adenocarcinoma (EAC) mainly showed *PAX5* gene body methylation (red in upper middle area). This different methylation pattern appeared to affect *PAX5* overexpression, *SLC2A1* downregulation, and *TEK* overexpression in the 198 esophageal cancer cases.

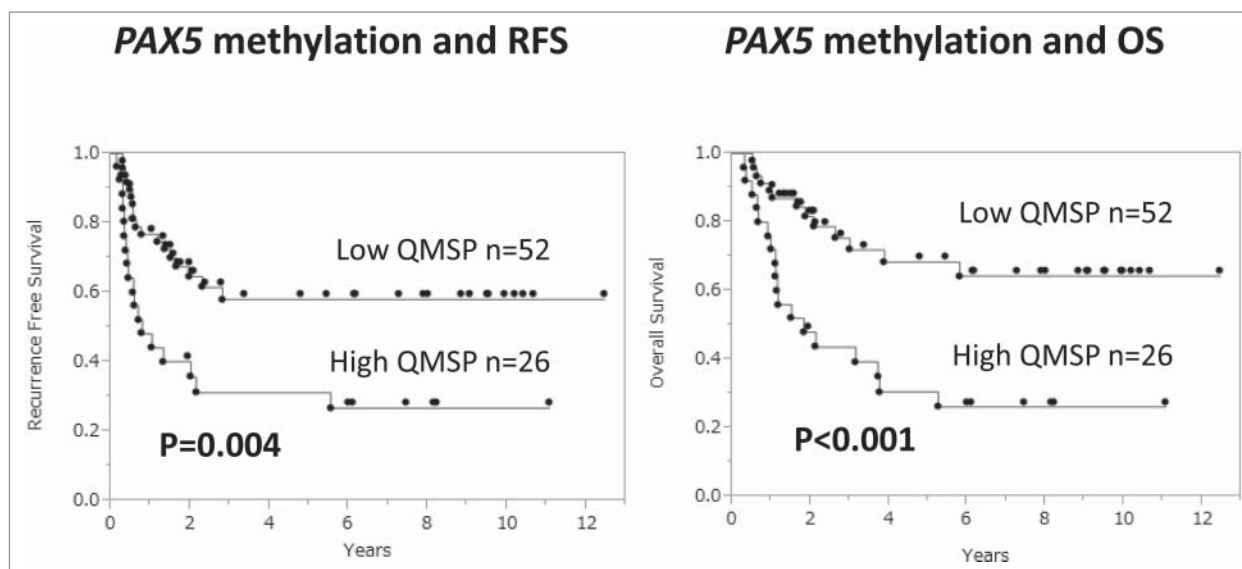
#### Cisplatin resistance induced by *PAX5* knockdown

*SLC2A1*, also known as glucose transporter 1 (*GLUT1*), is an established chemotherapy sensitivity-related gene. Overexpression of *SLC2A1* was reported to cause adriamycin resistance<sup>14</sup> and poor survival after preoperative chemoradiotherapy.<sup>15</sup> Furthermore, inhibition of *SLC2A1* was shown to overcome 5-fluorouracil resistance<sup>16</sup> or cisplatin resistance.<sup>17</sup> Thus, we examined whether *PAX5* inhibition induced cisplatin resistance

through *SLC2A1* overexpression. For this, we performed WST1 assays comparing *PAX5* siRNA-transfected ESCCs (NUEC1, TE3) and control cell lines under a wide range of cisplatin concentrations (Fig. 3c). In both cell lines, *PAX5*-silenced cells showed relatively higher cell proliferation, suggesting acquisition of cisplatin resistance.

#### *PAX5* methylation and clinical outcomes

As *PAX5*-silenced ESCCs showed oncogenic behavior and cisplatin resistance in the *in vitro* experiments, we examined the associations between *PAX5* expression or gene methylation and clinical outcomes of the 78 ESCC cases. The *PAX5* low expression group showed poor RFS and OS ( $P = 0.082$  and  $P = 0.018$ , respectively, log-rank test; Supplementary Figure S3). In particular, the *PAX5* highly methylated group exhibited significantly poorer RFS and OS than the *PAX5* low QMSP group ( $P = 0.004$  and  $P < 0.001$ , respectively, log-rank test, Fig. 4). The Cox proportional hazards model was applied to outcome analyses to determine prognostic histological or



**Figure 4.** The *PAX5* highly methylated group ( $n = 26$ ) showed significantly poorer RFS and OS ( $P = 0.004$  and  $P < 0.001$ , respectively, log-rank test) than the *PAX5* low QMSP group ( $n = 52$ ).

molecular markers (Table 1). In multivariable analyses of RFS and OS, only positive lymphatic invasion and high *PAX5* QMSP were significant poor prognostic factors. The HRs for high *PAX5* QMSP were 2.54 ( $P = 0.006$ ; 95%CI: 1.34–5.29) for RFS and 3.12 ( $P = 0.003$ ; 95%CI: 1.48–6.70) for OS. Although lymph node metastasis could be one of the major prognostic factors, it is sometimes influenced by chemoradiotherapy or sampling error. Zhang et al. reported that the ratio of metastatic lymph node was superior to predict the survival than pN staging.<sup>18</sup> Also, Chen et al. showed that lymphovascular invasion is the more important critical factor of patients' survival.<sup>19</sup> Even in our cohort, positive lymph node metastasis is the significant prognostic factor in univariate analysis, while that is marginally significant in multivariable analysis. We think our results are almost consistent with previous studies.

To evaluate cisplatin-resistant features of *PAX5* methylation, we compared the *PAX5* high QMSP and low QMSP groups in cisplatin-based adjuvant chemotherapy cases ( $n = 26$ ) and no-chemotherapy cases ( $n = 52$ ) (Fig. 5a). Notably, the *PAX5* highly methylated group showed extremely poor RFS in cisplatin-treated cases ( $P < 0.001$ , log-rank test), while no

significant difference was seen in no-chemotherapy cases ( $P = 0.091$ ). We also compared the *PAX5* high QMSP and low QMSP groups in postoperative radiotherapy cases ( $n = 15$ ) and no-radiation cases ( $n = 63$ ) regardless of chemotherapy combination (Fig. 5b). However, *PAX5* methylation status did not appear to affect the prognostic outcome among the irradiated cases ( $P = 0.297$ ). These clinical findings support the notion that *PAX5* highly methylated tumors may have the potential of cisplatin resistance, although the case numbers in these subgroup analyses were very small.

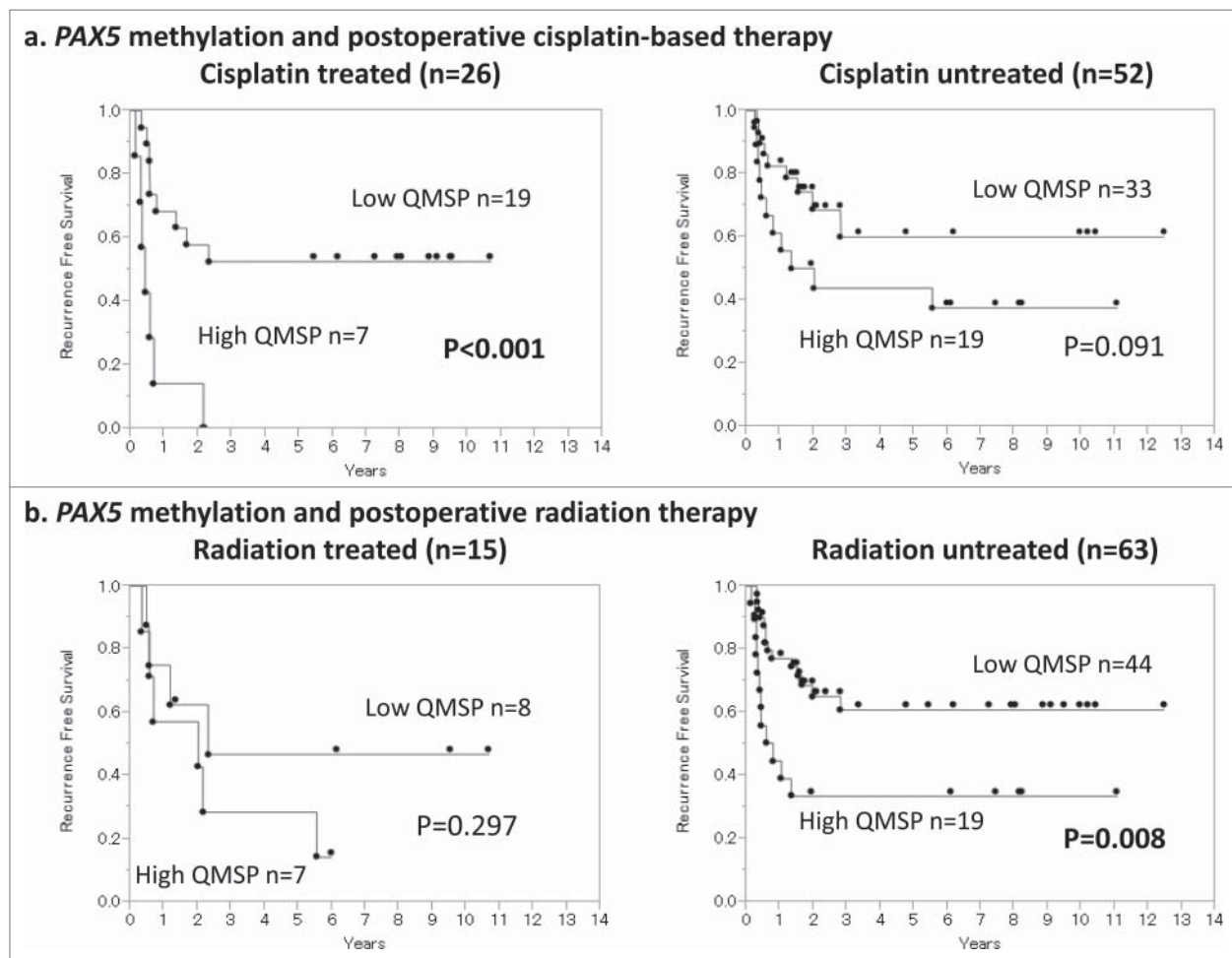
## Discussion

*PAX5* methylation has been reported in several cancer types. The same MSP primer pairs were used for analyses of hepatoma,<sup>10</sup> gastric cancer,<sup>20</sup> and lung cancer,<sup>21</sup> and are located at the promoter region of the *PAX5* gene. A head and neck cancer study<sup>5</sup> from our group defined a new quantitative MSP site around exon 2 containing the most tumor-specific methylated region. This site was also distinctively methylated in ESCCs (Supplementary Figure S2b, yellow rectangle). In addition,

**Table 1.**

Factors	Cases	RFS univariate	RFS multivariable	OS univariate	OS multivariable
pT Stage	T3 / Tis-T2	51 / 27	HR = 1.36 $P = 0.392$ 95%CI:0.68–2.88	HR = 1.72 $P = 0.169$ 95%CI:0.80–4.12	
pN Stage	N1 / N0	46 / 31 (1)	HR = 2.39 $P = 0.017$ 95%CI:1.16–5.39	HR = 1.99 $P = 0.080$ 95%CI: 0.92–4.69	HR = 2.11 $P = 0.089$ 95%CI:0.90–5.58
Differentiation	Poor / Moderate-Well	12 / 66	HR = 1.75 $P = 0.186$ 95%CI: 0.74–3.67	HR = 2.56 $P = 0.036$ 95%CI:0.68–2.88	HR = 1.55 $P = 0.349$ 95%CI:0.59–3.64
Lymphatic invasion	Positive / Negative	58 / 20	HR = 5.18 $P < 0.001$ 95%CI: 1.86–21.55	HR = 3.56 $P = 0.020$ 95%CI: 1.20–15.30	HR = 4.27 $P = 0.019$ 95%CI:1.23–27.04
Vessel invasion	Positive / Negative	29 / 49	HR = 1.89 $P = 0.062$ 95%CI: 0.97–3.66	HR = 1.28 $P = 0.500$ 95%CI:0.61–2.60	HR = 1.12 $P = 0.759$ 95%CI:0.53-2.43
Intraepithelial spread	Positive / Negative	33 / 37 (8)	HR = 1.15 $P = 0.690$ 95%CI: 0.57–2.33	HR = 1.12 $P = 0.759$ 95%CI:0.53-2.43	
<i>PAX5</i> QMSP	High / Low	26 / 52	HR = 2.54 $P = 0.006$ 95%CI:1.31–4.93	HR = 2.67 $P = 0.006$ 95%CI: 1.34–5.29	HR = 3.16 $P = 0.002$ 95%CI:1.55–6.59

(number): unknown



**Figure 5.** a: Influence of *PAX5* methylation status on cisplatin-treated and untreated cases examined by Kaplan–Meier curves. Among the cisplatin-treated cases ( $n = 26$ ), the *PAX5* high QMSP group showed significantly poorer RFS ( $P < 0.001$ , log-rank test), while no significant difference was seen in the non-adjuvant chemotherapy cases ( $n = 52$ ;  $P = 0.091$ , log-rank test). b: Influence of *PAX5* methylation status on radiation-treated and untreated cases examined by Kaplan–Meier curves. The *PAX5* methylation status did not affect the prognostic outcomes among the irradiated cases ( $P = 0.297$ , log-rank test).

*PAX5* highly methylated cases showed poorer overall survival than others in our esophageal SCCs. We also confirmed it using other head and neck SCC cohort from TCGA methylation data (Supplementary Figure S4). Interestingly, EACs showed a completely different methylation profile from ESCCs. Although EACs also exhibited high-level methylation, densely methylated sites were found in the gene body area and appeared to cause *PAX5* overexpression (Supplementary Figure S2c). Cancer-specific *PAX5* overexpression was reported in small-cell lung cancer (SCLC).<sup>22</sup> In their cell line analysis, no *PAX5* expression was clearly demonstrated in head and neck cancers ( $n = 12$ ), esophageal cancers ( $n = 6$ ), non-small cell lung cancers ( $n = 13$ ), and pancreatic cancers ( $n = 6$ ), while almost all SCLCs ( $n = 12$ ) showed positive *PAX5* expression. Thus, *PAX5* expression patterns may depend on cancer pathological cell types, rather than cancer locations. *PAX5* overexpression was also shown in oral squamous cell carcinoma<sup>23</sup> and B-cell lymphomas.<sup>24</sup> The latter paper described that *PAX5* was upregulated through aberrant hypermutation or chromosomal translocation in lymphomas. The authors also thought that alternative mechanisms including gene body hypermethylation could exist.<sup>25</sup> Liang et al. reported that the combination of

histone deacetylase inhibitors and demethylating agent is necessary to induce *PAX5* protein re-expression.<sup>26</sup> It also implies that another inhibition mechanism exists. Anyway, although adenocarcinomas are thought to be less sensitive to chemotherapy,<sup>27</sup> *PAX5*-overexpressing adenocarcinomas might be good candidates for cisplatin-based chemotherapy treatment.

The energy-related process of cancer cell growth is supported by increased glucose uptake through the plasma membrane and metabolism.<sup>28</sup> *SLC2A1*, also known as *GLUT1*, is one of the major glucose transporters. It is frequently overexpressed in various tumors characterized by increased glycolysis rates to meet the requirements of rapidly growing cells<sup>29</sup> and contributes to the Warburg effect, the most characterized metabolic change in tumor cells. Mutant p53 was reported to promote *GLUT1* translocation from the cytoplasm to the cell surface. Meanwhile, *GLUT1* knockdown abolished this stimulatory effect of mutant p53 and reduced glucose uptake.<sup>30</sup> Therefore, *GLUT1* expression is an essential factor to sustain cancer cell metabolism. Our Cancer Pathway Finder array revealed that *PAX5* knockdown was inversely correlated with increasing *GLUT1*. Among the 78 clinical samples, *PAX5* downregulation in tumors might be associated with *GLUT1* upregulation

( $P = 0.099$ ). Furthermore, increased *GLUT1* was reported to be associated with mTOR activation, leading to increased glycolysis and reduced autophagy.<sup>31</sup>

Gene alterations of *p53* have been widely examined in ESCCs. Mutant *p53* proteins are strongly associated with tobacco smoking and alcohol consumption,<sup>32</sup> bind to numerous downstream proteins to irregularly enhance or inhibit their functions and promote cell invasion and metastasis.<sup>33</sup> Immunohistochemical staining showed that accumulation of mutant *p53* proteins could be a poor prognostic marker in ESCCs.<sup>34,35</sup> Zhang et al.<sup>36</sup> performed a meta-analysis of *p53* mutation status and chemotherapy sensitivity, and concluded that wild type *p53* status was correlated with favorable chemotherapy response. Meanwhile, mutant *p53* overexpression could be a key factor in ESCC carcinogenesis. In 2011, Liu and colleagues reported that *PAX5* directly binds to the *p53* promoter and activates *p53* signaling in hepatocarcinogenesis.<sup>10</sup> They demonstrated that *PAX5* induction in a hepatoma cell line caused wild type *p53* overexpression by binding to the *p53* promoter. This may mean that epigenetic silencing of *PAX5* decreases wild type *p53* production, resulting in a mutant *p53*-dominant status that increases the oncogenic potential of tumor cells.

*GLUT1* is also a known chemoresistance factor in cancer cells. Head and neck SCC cell lines with high *GLUT1* levels showed significantly higher resistance to cisplatin.<sup>37</sup> Meanwhile, *GLUT1* inhibition chemosensitized cancer cells toward cisplatin.<sup>17</sup> We did a confirmation experiment about this point in Supplementary Figure S3c. *GLUT1* inhibition increased cis-diamminedichloroplatinum (CDDP) sensitivity, as we expected. Increased *GLUT1* mRNA expression in response to *PAX5* knockdown could be an explanation for our finding of *PAX5* silencing as a biomarker of CDDP resistance (Fig. 3c, Supplementary Figure S2c). Although further mechanistic experiments are required, the association of *PAX5* with *GLUT1* may be mediated by the tumor suppressor *p53*, which is known to be regulated by *PAX5* binding to its promoter site<sup>10</sup> and to repress the expression of key regulators of metabolic genes including *c-Myc* and *GLUT1*.<sup>38</sup> A possible network around *PAX5-p53-SLC2A1* axis in esophageal carcinoma was drawn using cBioPortal,<sup>39,40</sup> and is shown in Supplementary Figure S5.

A methylation marker can be a useful tool for cancer detection if the methylation is a very cancer-specific event.<sup>41</sup> Although preoperative histological examination can distinguish SCC or adenocarcinoma, molecular diagnosis is necessary for evaluation of tumor malignancy, invasive nature, or chemotherapy sensitivity. The *PAX5* QMSP assay might complement this information. Nowadays, molecular-based diagnosis has become less time-consuming. The rapid QMSP procedure requires <3 h from sample collection until data analysis,<sup>42</sup> meaning that intraoperative judgments for additional therapy can be undertaken.

In conclusion, *PAX5* methylation occurred frequently and was a very tumor-specific event in ESCCs. The *PAX5* gene methylation was significantly associated with low expression in tumors. Since *PAX5* silencing was correlated with increased cancer cell proliferation and cisplatin resistance, it might lead to poor RFS (HR = 2.67;  $P = 0.006$ ; 95%CI: 1.34–5.29) and OS (HR = 3.12;  $P = 0.003$ ; 95%CI: 1.48–6.70). *PAX5* gene methylation could be useful as a strong prognostic factor for ESCCs.

## Materials and methods

### Sample collection

Surgical samples of ESCC were collected at Nagoya University Hospital (Nagoya, Japan) between December 2001 and October 2013. All resected specimens were histopathologically proven to be ESCC, and classified by the TNM classification 7th Edition (Union for International Cancer Control). The sample collection protocol was approved by the Review Board of Nagoya University, and written informed consent for surgical sample use was obtained from all patients. A total of 78 ESCC patients without any preoperative chemoradiotherapy were included in the study, and fresh frozen tissues were collected from their surgical samples. Cancers originated from Barrett's esophagus or esophagogastric junction was excluded. The background and clinicopathological features of the patients are summarized in Supplementary Table S1.

### Cell culture

Seven esophageal cancer cell lines (TE1, TE2, TE3, NUEC1, NUEC2, T.Tn, WSSC) and one esophagus epithelial normal cell line (Het-1A) were propagated according to individual cell culture recommendations. TE1, TE2, and TE3 were donated by Tohoku University.<sup>43,44</sup> NUEC1, NUEC2, and WSSC were established in our institute.<sup>45,46</sup> T.Tn were obtained from the Japanese Collection of Research Bioresources. Het-1A was purchased from ATCC (CRL-2692). All cancer cell lines were cultured in RPMI-1640 medium supplemented with penicillin, streptomycin, and 10% fetal bovine serum at 37°C in a 5% CO<sub>2</sub> incubator. Het-1A was cultured in BEBM medium kit without GA-1000 (CC-3170) following the ATCC protocol.

### DNA extraction and bisulfite treatment

DNA was extracted from several 10- $\mu$ m-thick dissected frozen tissues using a DNeasy Blood & Tissue Kit (Qiagen, 69504). Presence of >70% of cancer cells in all tumor samples was histologically confirmed in slides taken before and after sample harvesting. DNA samples (1  $\mu$ g) extracted from the tissues were subjected to bisulfite treatment using an EpiTect Bisulfite Kit (Qiagen, 59104).

### QMSP

The bisulfite-modified DNA was used as a template for fluorescence-based QMSP as described.<sup>5,6,47</sup> The primer and probe sequences are shown in Supplementary Table S2. QMSP assays were performed in triplicate using StepOnePlus (Thermo Fisher Scientific). Thermal cycling was initiated with denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Each plate included patient DNA samples, serially diluted positive standards (Bisulfite Converted Universal Methylated Human DNA Standards; Zymo Research, D5015) for constructing the standard curve, and multiple water blanks as no-template controls. Mean values of triplicate samples were used for analyses. The methylation ratio (QMSP value) is defined as the ratio of the fluorescence emission intensity values for the target gene-specific PCR products to those of the *ACTB* (reference gene) and then multiplied by 100 for easier tabulation.

### RNA extraction, cDNA conversion, and quantitative real-time PCR

Total RNA was isolated from frozen surgical samples or cell lines using an RNeasy Mini Kit (Qiagen, 74104) according to the manufacturer's protocol. cDNA (cDNA) was synthesized with qScript cDNA SuperMix (Quanta BioSciences, 95048) with 1  $\mu$ g of total RNA. PCR amplifications were performed in triplicate with SYBR Premix Ex Taq II (Takara Bio, RR820) as follows: 1 cycle of 95°C for 10 s, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Real-time detection of emission intensity was conducted with a StepOnePlus (Thermo Fisher Scientific). The primer pairs used for RT-PCR are also shown in Supplementary Table S2. *ACTB* was used as an internal control. *PAX5* gene expression levels were calculated by the  $2^{-\Delta\Delta CT}$  method.<sup>48</sup> For esophageal cancer cell lines, we double-checked the expression using TaqMan expression assays (Hs00277134-m1 and Hs02758991-g1; Thermo Fisher Scientific).

### Five-Aza-2'-deoxycytidine (5-Aza-dC) treatment of cell lines

At 24 h before treatment, cells were plated at low density ( $1-3 \times 10^6$  cells depending on growth characteristics of respective cell lines) in T75 75-cm<sup>3</sup> Tissue Culture Flasks. Stock solutions of 100 mM 5-Aza-dC (Sigma, A-3656) and 3 mM Trichostatin A (Sigma, T1952) were prepared with Dimethyl Sulfoxide (Sigma, D2650). Immediately before addition to cell culture medium, appropriate aliquots of stock solutions were dissolved in phosphate-buffered saline (pH 7.5). Cells were treated with 5  $\mu$ M 5-Aza-dC for 5 d, with daily changes of medium. Trichostatin A (300 nmol/L) was mixed into the medium for the final 24 h. The cells were harvested for RNA extraction.

### PAX5 siRNA transfection and cell proliferation assay

Esophageal cancer cell lines with relatively high *PAX5* expression (TE3 and NUGC1) were plated on a 96-well plate at  $5 \times 10^3$  cells/well and incubated overnight at 37°C. The cells were then transfected with *PAX5* small interfering RNA (siRNA) (Silencer Select *PAX5*), *SLC2A1* siRNA (Silencer Select *SLC2A1*) or control siRNA (Silencer Select Negative Control No. 1 siRNA) and RNAiMAX transfection reagent (Thermo Fisher Scientific) (0 h). Cell proliferation was measured at different time points (24 h, 48 h, 72 h) using a Premix WST-1 Cell Proliferation Assay System (Takara Bio Inc., Shiga, Japan), according to the manufacturer's instructions. Briefly, 10  $\mu$ l of Premix WST-1 was added to each well. After incubation in the dark for 4 h at 37°C, the absorbances were measured by spectrophotometric readings (440 nm). All assays were performed in triplicate, and each experiment was repeated at least twice. Cell proliferation values were calculated as percentages of the value at each time point relative to the value at 0 h (baseline).

### BrdU assay

To assess DNA synthesis activity, BrdU cell proliferation enzyme-linked immunosorbent assays (Roche, Tokyo, Japan)

were performed. Briefly, transfected cells in 96-well plates were labeled with BrdU for 2 h at each time point. BrdU incorporated into cellular DNA was quantified using the manufacturer's protocol.

### Cancer pathway finder array

To find *PAX5*-related pathways, we used the RT<sup>2</sup> Profiler PCR Array Cancer Pathway Finder (Qiagen) following the manufacturer's instructions. The NUGC1 and TE3 cell lines were selected as candidate cells with high *PAX5* mRNA expression. *PAX5* siRNA (Applied Biosystems) was transfected into both cell lines and resulted in significantly downregulated mRNA expression in both cell lines rather than negative control (Applied Biosystems) transfected cells. These cells were collected after 48 h. The results were analyzed using RT<sup>2</sup> Profiler PCR Array Data Analysis v3.5 software. *ACTB* was used as a reference gene.

### Western blotting

Cultured cells were washed and lysed with Pierce RIPA buffer (Thermo Fisher Scientific). Protein lysates were homogenized, and supernatants were collected after centrifugation. Protein concentrations were measured with a Pierce BCA Protein Assay Kit (Takara Bio, Otsu, Japan). NuPAGE LDS sample buffer (Thermo Fisher Scientific) was added to adjusted protein samples, and the samples were separated by 10% SDS polyacrylamide gel electrophoresis. The separated samples were electrotransferred onto polyvinylidene fluoride membranes using an iBlot Gel Transfer Device (Thermo Fisher Scientific) and blocked with 5% bovine serum albumin (Sigma-Aldrich). The membranes were immunoblotted overnight at 4°C with a mouse anti-GLUT1 antibody (Abcam, Tokyo, Japan) followed by Anti-mouse IgG, HRP-linked antibody (Cell Signaling Technology, Tokyo, Japan). For  $\beta$ -actin, a mouse monoclonal anti- $\beta$ -actin antibody (Abcam) and the same mouse secondary antibody were used. Signals were detected by an LAS-4010 (GE Healthcare, Tokyo, Japan). Densitometry analysis was performed with ImageJ software.<sup>49</sup>

### Statistical analysis

Continuous variables were analyzed by the Mann-Whitney U test as a non-parametric test and Student's *t*-test (2-tailed) as a parametric test. Categorical variables were analyzed by Fisher's exact test. Recurrence-free survival (RFS) was defined as the time from surgery to first documentation of disease recurrence. Overall survival (OS) was defined as the time from surgery to the date of death from any cause. Associations of gene methylation and other histopathological factors with RFS/OS were evaluated by the Cox proportional hazards model with hazard ratios (HRs) and 95% confidence intervals (95% CIs). Associations with  $P < 0.05$  in univariate analyses were further evaluated in multivariate regression analyses. All tests were 2-sided and considered statistically significant and clinically promising for values of  $P < 0.05$ . Statistical analyses were performed using JMP 9 software (SAS Institute, Cary, NC, USA). Statistical significance was set at  $P < 0.05$ .



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## Conflict of interest

None.

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