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Induction of kallikrein-related peptidase 13 and TET2/3 by anticancer drugs and poor prognosis of patients with esophageal squamous cell carcinoma after preoperative treatment

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Running head: NAC/NACRT/dCRT, KLK13, and prognosis of ESCC

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Synopsis

Anticancer drugs induce KLK13 status conversion and TET2/3 induction in esophageal squamous cell carcinoma (ESCC). The positive conversion of KLK13 after preoperative treatment is a predictor of poor prognosis in patients with ESCC.

Abstract

Background: Preoperative chemotherapy/chemoradiotherapy has been generally considered for the treatment of ESCC (ESCC) to improve prognosis. We examined the effects of anticancer drugs on the expression of kallikrein-related peptidase 13 (KLK13), a potential ESCC prognostic marker, and its clinical relevance in patients who received chemotherapy/chemoradiotherapy for ESCC.

Methods: Overall, 105 patients with ESCC who received chemotherapy or chemoradiotherapy before esophagectomy were enrolled. The expression of KLK13 in biopsy samples obtained before chemotherapy/chemoradiotherapy and resected ESCC tumors was assessed by immunohistochemical staining. The effects of 5-fluorouracil (5-FU) and/or cisplatin (CDDP) exposure on the expressions of KLK13 and ten-eleven translocation dioxygenases (TET) in ESCC cells were examined by RT-PCR.

Results: Immunohistochemical staining of paired ESCC specimens before (biopsy samples) and after (resected specimens) chemotherapy/chemoradiotherapy demonstrated a change in KLK13 expression. KLK13 and TET2/3 transcriptions were induced when human ESCC cell lines were treated with 5-FU and/or CDDP. Among patients with KLK13-negative status before chemotherapy/chemoradiotherapy, those with KLK13-positive resected tumors had a significantly poorer prognosis than those with KLK13-negative resected tumors ($P = 0.0477$). By using tumor cells isolated from ESCC biopsy tissues obtained before chemotherapy/chemoradiotherapy, we established a primary culture system and detected the induction of KLK13 expression by anticancer drugs.

Conclusions: Preoperative treatments alter KLK13 expression in ESCC. The conversion of KLK13 expression from a negative status in biopsy samples to a positive one in resected tumor samples is a predictor of poor prognosis. KLK13 status is a potential

marker for decision-making to avoid harmful chemotherapy/chemoradiotherapy in patients with ESCC.

Introduction

Esophageal squamous cell carcinoma (ESCC) is a major histological type of esophageal cancer. The incidence of ESCC has been increasing in some Asian countries (e.g., Taiwan), probably because of the increase in tobacco and alcohol consumption [1]. In Japan, ESCC accounts for >90% of esophageal malignancies [2]. Patients with ESCC have a markedly poor prognosis, with an overall 5-year survival rate of 18.9% [3]. One of the causes of poor prognosis is the high propensity of ESCC to invade the adjacent organs, probably due to the absence of serosa in the esophagus, and its close proximity to vital structures such as the trachea, vertebral body, and aorta [4]. Although endoscopic resection is diagnostic and curative for intramucosal tumors (T0–T1a), surgery is the first-choice treatment for ESCC, except for advanced ESCCs, such as T4b tumors and tumors with distant recurrence. The high incidence of recurrence after primary resection (35.7%) is also a cause of poor prognosis [5, 6].

To improve the prognosis of patients with ESCC, preoperative chemotherapy with cisplatin (CDDP) plus 5-fluorouracil (5-FU) is generally considered for cStage II and III cases in Japan [7, 8]. In addition, studies have demonstrated the efficacy of synchronous chemoradiotherapy before surgery for ESCC [9]. Surgery is not indicated for patients with advanced ESCC; instead, definitive chemoradiotherapy (dCRT), such as chemotherapy, chemoradiotherapy, and palliative radiotherapy, is selected. In addition, some patients with advanced ESCC undergo salvage surgery for localized residual tumors or recurrent lesions after dCRT. These preoperative treatments have helped improve the prognosis of ESCC [8-10]; however, these are highly invasive and extremely burdensome treatments for the patients. Moreover, clinical markers that can help avoid unnecessary treatment are limited; therefore, the identification of novel clinical markers that can

facilitate treatment decision-making for patients with ESCC is imperative and essential.

Previously, we performed comprehensive transcriptome analysis using serial analysis of gene expression to identify genes with altered expression levels in ESCC [11]. Among the candidate genes whose products were identified as possible prognostic markers and therapeutic targets, we further analyzed kallikrein-related peptidase 13 (*KLK13)* and found an association between KLK13 expression in ESCC with tumor progression and poor prognosis [12]. However, KLK13 expression may be altered by preoperative ESCC treatment. Maeda et al. reported an increase in the mRNA expression of *KLK13* in two patients with advanced gastric cancer after three and four courses of chemotherapy, with fluoropyrimidine S-1 plus CDDP [13]. Furthermore, exposure to CDDP, 5-FU, epirubicin, or methotrexate induced *KLK13* upregulation at the mRNA level in gastric cancer cell lines *in vitro* [14]. Therefore, preoperative treatment, such as neoadjuvant chemotherapy (NAC), neoadjuvant chemoradiotherapy (NACRT), and dCRT, may alter KLK13 expression in ESCC; however, it has not been elucidated.

In the present study, we aimed to clarify the effect of anticancer agents on KLK13 expression in ESCC. We compared the KLK13 expression in paired ESCC specimens between before (biopsy samples) and after (resected specimens) chemotherapy/chemoradiotherapy. In addition, we examined the effect of anticancer drugs using ESCC cell lines and found upregulation of KLK13 and ten-eleven translocation dioxygenases (TET2/3), enzymes oxidizing 5-methylcytosine (5-mC) and involved in DNA demethylation[15], after the administration of anticancer agents. We further retrospectively analyzed the relationship between KLK13 expression, clinicopathological parameters, and ESCC prognosis and found that the conversion of KLK13 from a negative status in biopsy samples obtained before preoperative treatment

to a positive status in resected tumor samples is a predictor of poor prognosis in patients with ESCC.

Materials and Methods

Patients

This study included 130 patients with ESCC who received chemotherapy/chemoradiotherapy at the National Center for Global Medical Research (NCGM) between October 2010 and December 2020 for immunohistochemical analysis using formalin-fixed, paraffin-embedded sections of biopsy and surgical specimens. In all cases, endoscopy was performed at the time of diagnosis and biopsy samples were collected to confirm the pathological diagnosis of ESCC. Of the 130 patients, 26 did not undergo surgery, of which 20 had deteriorating general conditions and 6 did not require surgery because local control was achieved by preoperative treatment. Of the remaining 104 patients who underwent esophagectomy, 11 did not have tumor cells in the resected specimens; therefore, these patients were excluded from the immunohistochemical analysis of resected specimens. Furthermore, 110 patients with ESCC who received chemotherapy/chemoradiotherapy at NCGM between October 2010 and December 2021 and whose biopsy as well as resected specimens were suitable for immunohistochemical analysis were included for prognostic analysis; however, 5 patients whose observation period was <1 year were excluded because of the extremely short follow-up duration. Patient data were collected retrospectively from medical records. Findings of endoscopic examination, blood examination, and imaging studies such as esophagography, computed tomography (CT), and fluorodeoxyglucose positron emission tomography (FDG-PET) were used to determine the tumor stage according to the Eastern Cooperative Oncology Group performance status. Clinical and pathological tumor stages were assessed using the Union for International Cancer Control TNM Classification of Malignant Tumors, 8th edition [16]. Consent was retrospectively obtained from these patients in accordance with

the dictates of the Research Ethics Committee of the NCGM (2417). Biopsy samples were endoscopically obtained from other ten patients with ESCC before preoperative chemotherapy/chemoradiotherapy between October 2022 and March 2023 and were used for primary culture. This study was approved by the Research Ethics Committee of the NCGM (2464). Consent was obtained from these ten patients before sample collection.

Surgical procedure and chemotherapy/chemoradiotherapy

Our standard surgical procedure was esophagectomy with three-field lymph node dissection, reconstruction with a gastric tube via the posterior mediastinum, cervical esophagogastric anastomosis, and jejunostomy. NAC was considered for patients with tumors staged as cT2 Nx M0 or cTx N + M0 (excluding cT4b) and who were eligible for surgical resection. Three types of NAC treatment were used: FP regime comprising 5-FU (800 mg/m^2/day) and CDDP (80 mg/m^2/day), DCF regime comprising docetaxel (70 mg/m^2/day), CDDP (70 mg/m^2/day), and 5-FU (750 mg/m^2/day), or NED + 5FU regime comprising nedaplatin (90 mg/m^2/day) and 5-FU (800 mg/m^2/day). These treatments were followed by radical esophagectomy. NACRT was considered for patients with cT4b Nx Mx or cTx N + Mx tumors with metastatic lymph nodes invading other organs. NACRT comprised FP treatment plus radiation therapy (40 Gy). dCRT comprised radiation (>50 Gy) and FP treatment or DCF treatment. Eight of 30 patients, who received dCRT for unresectable advanced tumors, received salvage surgery.

Evaluation of clinical and histopathological responses to preoperative treatment

The clinical response to preoperative treatment was assessed using upper endoscopy, esophagography, CT, and FDG-PET according to the revised RECIST

guidelines (version 1.1) [17]. The histopathological response to preoperative treatment was evaluated using the Japanese Classification of Esophageal Cancer [18] [grade 0 (ineffective): no significant response to preoperative treatment in cancer tissue or cancer cells; grade 1 (slightly effective): several significant responses to preoperative treatment in cancer tissues or cancer cells and more than one-third residual tumor cells in the lesion; grade 2 (moderately effective): less than one-third residual tumor cells and almost necrotic cancer tissues; and grade 3 (markedly effective): no viable residual tumor cells or only cancer scar tissues].

Cell lines and culture

Human ESCC cell lines KYSE70 and KYSE140 were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). KYSE70 cells were cultured in RPMI1640 medium supplemented with 10% fetal calf serum (FCS). KYSE140 cells were maintained in Ham's F12/RPMI1640 medium containing 2% FCS. To evaluate the mRNA levels for each cell line, ESCC cells were cultured in a 24-well plate at a density of 4×10^4 cells/well for 18 h and treated with 5-FU, CDDP, or 5-FU + CDDP (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 48 h. For primary culture, endoscopically obtained ESCC samples were washed twice in calcium- and magnesium-free HBSS (CMF-HBSS) supplemented with penicillin (10 U/mL), streptomycin (10 mg/mL), gentamicin (100 mg/mL), and amphotericin B (1 mg/mL). Then, tissues were incubated in TrypLE Express (Thermo Fisher Scientific, Rockford, IL, USA) for 20 min at 37 °C, and the supernatant was retained. Incubation and supernatant harvesting were repeated three more times. The pooled supernatants were then centrifuged and filtered with 40-μM nylon mesh. The harvested cells were washed

twice and subjected to primary cell culture.

Quantitative reverse transcription–PCR

Total RNA was isolated from cultured cells using the RNA easy Mini Kit (QIAGEN, Hilden, Germany). After treating RNA with DNase I, double-stranded cDNA was synthesized using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed using ABI TaqMan. Threshold cycle numbers were determined using the Sequence Detector software and transformed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the calibrator gene according to the manufacturer's instructions. The TaqMan Gene expression assay IDs for the genes used in this study were: KLK13, Hs00210264_m1, TET1, Hs00286756_m1, TET2, Hs00325999_m1, TET3, Hs00896441_m1, and GAPDH, Hs00266705_g1 (Applied Biosystems).

Immunohistochemical analysis

Formalin-fixed, paraffin-embedded sections of biopsy and surgical specimens from patients with ESCC were deparaffinized and rehydrated. Antigens were retrieved using a Target Retrieval Solution (Dako, Glostrup, Denmark) in an autoclave for 10 min at 121 °C. The sections were stained using an anti-KLK13 antibody (HPA019487, Sigma-Aldrich, Inc., St. Louis, MO, USA). To confirm the specificity of KLK13 staining signals, PrEST Antigen KLK13 (Sigma-Aldrich) was used as blocking peptides. Diaminobenzidine staining was performed using an ImmPACT™ DAB Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA, USA). Hematoxylin was used for counterstaining. All slides were reviewed by two or more observers who were blinded to

clinical or pathological data. Based on the proportion of KLK13-positive areas in the malignant tissue, ESCC cases were classified into KLK13-negative (percentage of KLK13-positive tumor cells ≤5% on immunostaining) and KLK13-positive groups (percentage of KLK13-positive tumor cells >5%). Owing to the distinct difference, distinguishing KLK13-positive/negative status was not difficult. The intraobserver reliability of the KLK13 staining results showed no significant differences, and a high interobserver reliability was noted between two observers.

Combined bisulfite restriction analysis

We used EpiTect Bisulfite Kit (Qiagen) for bisulfite modification and assessed the *KLK13* gene methylation using combined bisulfite restriction analysis (COBRA). PCR primers used for the analysis are mentioned as follows: 5'-GGCGGGAGGTTCGAAGTCGTTA-3' and 5'-GCTACAATTCGCCTCGCAAA-3', yielding a PCR product of 172 bp. The products were digested using restriction endonuclease Hha*I*, which cleaves only methylated CpG sites.

Statistical analysis

Each tumor was classified based on its location, size, pathology, condition of lymph nodes, and the degree of metastasis (pTNM, 8th edition, 2017). KLK13 staining results were compared using *t* test for age and Chi-squared test or Fisher's exact test for sex, cT status, cN status, cM status, pT status, pN status, pM status, clinical and pathological cancer stage (cStage and pStage), preoperative treatment, and histopathological and clinical response. The overall survival rate was estimated using the Kaplan–Meier method, and between-group differences were assessed using the log-rank

test. The observation period for prognostic analysis was from the data of initiation of preoperative treatment; 5 cases in which observation period was <1 year were excluded from the prognostic analysis because of the extremely short follow-up duration. All statistical analyses were performed using the Prism 7 statistical program (GraphPad Software, Inc., La Jolla, CA, USA). All tests were two-tailed and p-values of <0.05 were considered indicative of statistical significance.

Results

Participants

This study evaluated 130 patients with ESCC whose biopsy samples were collected at the time of diagnosis and who subsequently received NAC, NACRT, or dCRT at our institution between October 2010 and December 2020. Of these patients, 77, 23, and 30 received NAC, NACRT, and dCRT, respectively. Moreover, 80% of the patients in our cohort were male, and the mean age of the patients was 66.8 (range 38–83) years. Of the 130 patients, 26 did not undergo surgery because 20 had deteriorating general condition and 6 did not require surgery as local control was achieved by preoperative treatment. Of the remaining 104 patients who underwent esophagectomy, 11 had no tumor cells in resected specimens; therefore, we performed immunostaining using 93 paired ESCC specimens before (biopsy samples) and after (resected specimens) chemotherapy/chemoradiotherapy. KLK13 was expressed in the extracellular region of the stratum granulosum and the spinus cell layer in normal esophageal mucosa (Fig. 1a). In tumors, KLK13 expression was not detected in 53 (57%) of 93 biopsy samples (Fig. 1b and Table 1). Contrastingly, ectopic cytoplasmic expression was observed in 40 (43%) biopsy specimens but not in the nuclei of tumor cells. In particular, KLK13 expression was upregulated in the peripheral region of keratinized cancer pearls (Fig. 1c). The specificity of the staining signals in tumors was confirmed by their disappearance in the presence of blocking peptides (Fig. 1d). The 93 ESCC cases were classified into two groups according to KLK13 expression in biopsy samples collected before

chemotherapy/chemoradiotherapy, and the relationship between KLK13-negative/positive status and clinical features were analyzed. Accordingly, no significant between-group differences were found with respect to age, sex, type of chemotherapy/chemoradiotherapy, clinical/pathological response, classification, or stage (Table 1).

Effects of anticancer drugs on KLK13 expression in ESCC tumors and cell lines

Because the present results, i.e., the use of biopsy samples collected before preoperative treatment revealed no relationships between KLK13 status and clinicopathological features (Table 1), were contrary to our previous report using surgical specimens[12], we suspected that preoperative treatment affected KLK13 expression in ESCC. Therefore, we performed additional immunostaining and compared KLK13 status in tumor specimens obtained from the same patients between before (biopsy samples) and after (resected specimens) chemotherapy/chemoradiotherapy. Of the 53 cases with KLK13-negative biopsy samples before preoperative treatment, 11 had KLK13-positive surgical specimens after chemotherapy/chemoradiotherapy. By contrast, of the 40 cases with KLK13-positive biopsy samples before preoperative treatment, 28 had KLK13-negative surgical specimens after chemotherapy/chemoradiotherapy. These results suggest that preoperative treatment may have resulted in the alteration of KLK13 status. To confirm the effect of anticancer drugs on KLK13 expression in ESCC, we treated the ESCC cell

lines KYSE70 and KYSE140 with 5-FU and/or CDDP and compared the mRNA induction of KLK13 before and after treatment. In the two cell lines, CDDP treatment resulted in a significant concentration-dependent increase in KLK13 mRNA expression, whereas 5-FU alone did not. The combination of 5-FU plus CDDP showed a synergistic effect on KLK13 transcription (Fig. 2).

Association between the positive conversion of KLK13 expression after preoperative treatment and poor prognosis

To investigate the prognostic relevance of anticancer drugs–mediated KLK13 alteration, we analyzed 105 patients with ESCC who received chemotherapy/chemoradiotherapy at NCGM between October 2010 and December 2021 and whose observation period was >1 year. Of the 105 patients, 45 and 60 were positive and negative for KLK13 ESCC in biopsy samples obtained before initiating preoperative treatment, respectively. Among patients with KLK13-positive ESCC in biopsy samples, we found no difference in the prognosis of the negative converted (whose KLK13 status were converted to negative in resected samples) and unchanged (whose KLK13 status were positive in both biopsy and resected samples) groups (P = 0.6489, Fig 3a). In contrast, when differences between the positive converted (negative KLK13 status in biopsy samples became positive in surgical specimens) and unchanged (negative KLK13 status in biopsy samples and surgical specimens) groups were examined among patients with KLK13-negative ESCC in biopsy samples, we found that the prognosis of the converted group was significantly worse than that of the unchanged group (P = 0.0477, Fig 3b).

Induction of KLK13 in primary cultured tumor cells and demethylation enzymes TET2/3 in ESCC cell lines by anticancer drugs

For clinical application, the influence of anticancer drugs on KLK13 expression must be predicted before preoperative treatment. By using ESCC biopsy samples, we enzymatically isolated and cultured tumor cells *in vitro*. With this primary culture system, we assessed the effect of anticancer drugs on KLK13 expression. Of the 10 cases studied, a marked elevation of KLK13 mRNA expression was observed in two CDDP-treated cases and two cases 5-FU-treated cases (Fig.4). Finally, the molecular mechanisms involved in KLK13 induction by anticancer drugs were investigated. Previously, we reported that KLK13 expression was suppressed by DNA hypermethylation in the promoter region of KLK13 in ESCC[11]. TET1-3 mediates the conversion of 5-mC to 5-hydroxymethylcytosine during the demethylation process and therefore contributes to the recovery of the expression of silenced genes by DNA hypermethylation. In addition, a recent study demonstrated that 5-FU-resistant colorectal cancer cell lines expressed higher levels of TET1 than non-resistant cells[19]. To examine whether anticancer drugs affect TET expression in ESCC, we treated the ESCC cell lines with 5-FU and/or CDDP and compared the mRNA induction of TETs before and after treatment. In the two cell lines used, the combination treatment with 5-FU and CDDP significantly increased the mRNA expressions of TET2 and TET3 (Fig. 5). In KYSE70 cells, 5-FU or CDDP alone induced the concentration-dependent expressions of TET2 and TET3, whereas 5-FU alone did not increase the TET expression in KYSE140 cells. Additionally, we examined methylation status of the upstream region of the *KLK13* gene using COBRA to clarify whether it was demethylated through anticancer drugs. DNA methylation in the *KLK13*

gene was detected in both KYSE70 and KYSE140 cells. However, when these cells were treated with 5-FU and CDDP, DNA methylation in the *KLK13* was not detected (Fig. 5d). These results may suggest that anticancer drugs promote KLK13 demethylation by TET2/3 upregulation, resulting in the positive conversion of KLK13 status after preoperative treatment.

Discussion

In our previous study, KLK13 expression in surgically resected tumor specimens predicted shorter survival of patients with ESCC [12]. However, in the present study, no relationships were found between KLK13 status in tumor biopsy samples collected before chemotherapy/chemoradiotherapy, clinicopathological parameters, and ESCC prognosis. These contrasting results were caused by the preoperative treatment, such as NAC, NACRT, and dCRT, which altered KLK13 expression. Anticancer agents used for preoperative treatment induced DNA demethylation enzymes TET2/3 and subsequently decreased the frequency of DNA methylation in the *KLK13* gene. The conversion of KLK13 from negative in biopsy samples obtained before preoperative treatment to positive in resected tumor samples was found to be a predictor of poor prognosis in patients with ESCC. Furthermore, we established a primary culture system to possibly predict the induction of KLK13 expression and eventually poor prognosis.

The most important finding in this study is that the positive conversion of KLK13 expression after preoperative treatment is a poor prognostic factor for ESCC. High KLK13 expression levels after preoperative treatment may eventually promote ESCC progression and metastasis. Recent studies, including our previous report [12], have demonstrated the profound functions of KLK13 in many cancer-related processes. In lung adenocarcinoma cells, *KLK13* overexpression enhanced the capability to degrade extracellular laminin, which subsequently facilitated cell metastatic potential in SCID mouse xenograft model. Moreover, *KLK13* knockdown caused a significant decrease in cell migratory and invasive properties [20]. In ESCC, ectopic KLK13 expression was often observed in Ki67-positive proliferating tumor cells [12].

In the present study, we also examined the causes of the positive conversion of KLK13 and found that anticancer drugs used as preoperative treatment directly induced KLK13 expression in ESCC cell lines (Fig. 2). Previous reports have demonstrated that the addition of anticancer drugs such as CDDP, 5-FU, epirubicin, and methotrexate to cultured cell lines and chemotherapy induced *KLK13* mRNA expression in gastric cancers[13,14]; to our knowledge, this is the first report to demonstrate the promoting effects of anticancer drugs on KLK13 expression in ESCC. In addition, the upregulation of TET2/3 was observed after the treatment with anticancer drugs. Besides ESCC, the expression of TET1 is also increased in 5-FU-resistant colorectal cancer cell lines, compared with 5-FU-sensitive ones[19]. In gastric cancer cell lines, the relationship between TET2 upregulation and CDDP resistance was found[21]. Thus, our findings of the induction of TET2/3 by 5-FU and/or CDDP may explain the induction of KLK13 in ESCC after preoperative treatment because KLK13 expression was suppressed by DNA hypermethylation in the promoter region of KLK13 in ESCC[11]. In accordance with TET2/3 induction, the frequency of DNA methylation in the *KLK13* gene was decreased when ESCC cell lines were treated with 5-FU and CDDP (Fig. 5d), suggesting that anticancer drugs induce KLK13 expression probably owing to DNA demethylation via TET2/3 induction. The present results encourage us to develop methods to predict KLK13 conversion by preoperative treatment for clinical application.

Since preoperative treatment is considered the standard of care for advanced esophageal cancer [8], identification of biomarkers that facilitate the selection of appropriate therapy must be identified. For the treatment of colorectal cancers, the mutation states of *RAS* and *RAF* are useful factors that facilitate the selection of anti-EGFR agents, i.e., cetuximab and panitumumab [22]. In addition to these biomarkers,

which can potentially help identify patients with effective or ineffective response to preoperative treatment, molecular markers that can identify patients whose prognosis may worsen after preoperative treatment are clinically valuable. The results of this study indicate that positive conversion of KLK13 status after preoperative treatment is a potential biomarker in predicting poor prognosis in patients with ESCC. By using tumor cells isolated from biopsy tissues obtained from patients with ESCC, we established a primary culture system that could detect the promoting effect of anticancer drugs on KLK13 expression. Additionally, the assessment of KLK13 status may become easier using this primary culture system compared with using immunohistological staining, because quantitative PCR method is used to examine KLK13 expression. Since chemotherapy is considered effective as a preoperative treatment, alternative anticancer drugs are necessary to treat patients with ESCC in whom KLK13 induction via 5-FU and/or CDDP is predicted using this primary culture system. We have confirmed that docetaxel and nedaplatin do not induce KLK13 expression in ESCC cell lines (data not shown). Despite the need for further prospective studies to confirm whether the response to anticancer drugs of primary cultured tumor cells isolated from patients before chemotherapy/chemoradiotherapy will be consistent with the KLK13 status after preoperative treatment, this method to predict the KLK13 status after preoperative treatment may promote the quality of life of the patients by avoiding ineffective and harmful treatment based on scientific evidence.

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Figure legends

Fig. 1 Kallikrein-related peptidase 13 (KLK13) expression in esophageal squamous cell carcinoma (ESCC) biopsy samples. (a–c) Typical images of formalin-fixed, paraffin-embedded biopsy samples stained with hematoxylin and eosin (upper panels) or anti-KLK13 antibody (lower panels). (a) KLK13 expression in noncancerous mucosa (normal) included in biopsy samples. (b) Representative staining of KLK13-negative ESCC samples. (c) Representative images of KLK13-positive ESCC samples. (d) Representative images of formalin-fixed, paraffin-embedded biopsy samples of ESCC stained with anti-KLK13 with (right) or without (left) blocking peptide.

Fig. 2 Induction of *KLK13* mRNA in ESCC cell lines after treatment with 5-fluorouracil (5-FU) and/or cisplatin (CDDP). KYSE70 (a) and KYSE140 (b) cells were treated with the indicated concentrations of 5-FU and/or CDDP. Data are reported as the fold increases in induction relative to untreated cells and are shown as the mean ± SD of assays performed in triplicate. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

Fig. 3 Positive conversion of KLK13 status is a predictor of poor prognosis in patients with ESCC. (a) Kaplan–Meier survival curves of 45 patients with ESCC with KLK13-positive biopsy samples. (b) Kaplan–Meier survival curves of 60 patients with ESCC with KLK13-negative biopsy samples. Significant differences in the survival were found between the two groups.

Fig. 4 Induction of *KLK13* mRNA in primary cultured ESCC cells isolated from biopsy samples after treatment with 5-FU and/or CDDP. Tumor cells were isolated

from biopsy samples obtained from 10 patients with ESCC before preoperative treatment and cultured with the indicated concentrations of 5-FU and/or CDDP. The fold increase in induction in the treated cells relative to that in the untreated cells is shown for each case.

Fig. 5 mRNA induction of TET2/3 and demethylation of KLK13 in ESCC cell lines after treatment with 5-FU and/or CDDP. Transcript levels of TET1 (a), TET2 (b), or TET3 (c) were determined by RT-PCR in KYSE70 (left panels) and KYSE140 (right panels) cells treated with the indicated concentrations of 5-FU and/or CDDP. Data are reported as fold increases in induction relative to that in untreated cells and are shown as the mean ± SD of assays performed in triplicate. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. (d) Representative results obtained from combined bisulfite restriction analysis to assess methylation of *KLK13* gene using KYSE70 and KYSE140 cells treated with or without 5-FU and CDDP. M, methylated alleles; U, unmethylated alleles.

Tables

Table 1. KLK13 staining status in prechemotherapy/chemoradiotherapy biopsy specimens and clinical features of ESCC

Characteristics	Number of patients (%)			P value
	Total	KLK13-positive	KLK13-negative	
Number of patients	93 (100)	40 (43)	53 (57)	
Mean age ± SD (yrs)	66.4 ± 9.85	65.9 ± 10.18	66.9 ± 9.67	0.618
Sex				
Male	77 (83)	33 (82.5)	44 (83.0)	0.948
Female	16 (17.2)	7 (17.5)	9 (17.0)	
cT classification				
T1	4 (4.3)	1(2.5)	3 (5.7)	0.8232
T2	18 (19.4)	9 (22.5)	9 (17.0)	
T3	50 (53.8)	21 (52.5)	29 (54.7)	
T4	21 (22.3)	9 (22.5)	12 (22.6)	
cN classification				
N0	18 (19.4)	9 (22.5)	9 (17.0)	0.7373
N1	42 (45.2)	19 (47.5)	23 (43.4)	
N2	29 (31.2)	11 (27.5)	18 (34.0)	
N3	4 (4.3)	1 (2.5)	3 (5.7)	
cM classification				
M0	85 (91.4)	37 (92.5)	48 (90.6)	0.7419

M1	8 (8.6)	3 (7.5)	5 (9.4)	
Clinical Cancer stage†				
I	5 (5.4)	3 (7.5)	2 (3.8)	0.3162
II	23 (24.7)	13 (32.5)	10 (18.9)	
III	52 (55.9)	20 (50.0)	32 (60.4)	
IV	13 (14.9)	4 (10.0)	9 (17.0)	
pT classification				
T1	29 (31.2)	15 (37.5)	14 (26.4)	0.0695
T2	18 (19.4)	10 (25.0)	8 (15.1)	
T3	40 (43.0)	15 (37.5)	25 (47.2)	
T4	6 (6.5)	0 (0)	6 (11.3)	
pN classification				
N0	33 (35.5)	16 (40.0)	17 (32.1)	0.2954
N1	22 (23.7)	12 (30.0)	10 (18.9)	
N2	34 (36.6)	11 (27.5)	23 (43.4)	
N3	4 (4.3)	1 (2.5)	3 (5.7)	
pM classification				
M0	83 (89.3)	36 (90)	47 (88.7)	0.8387
M1	10 (10.7)	4 (10)	6 (11.3)	
Pathological Cancer stage†				
I	21 (22.6)	11 (27.5)	10 (18.9)	0.2475

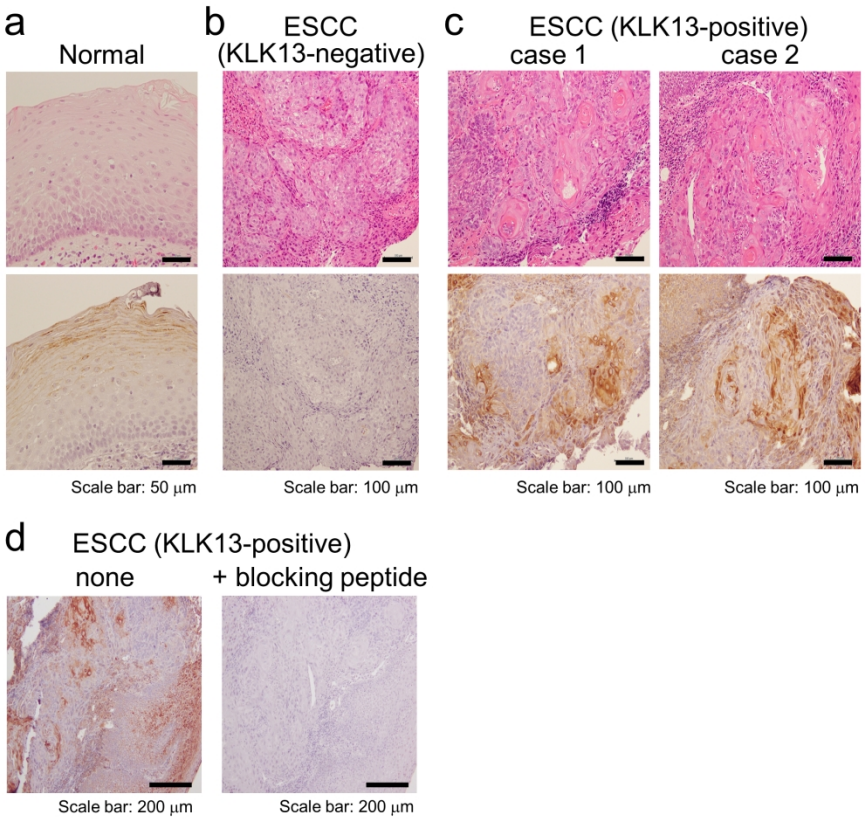
II	22 (23.7)	12 (30)	10 (18.9)	
III	39 (41.9)	13 (32.5)	26 (49.1)	
IV	11 (11.8)	4 (10)	7 (13.2)	
Preoperative treatment				
NAC	71 (76.3)	32 (80)	39 (73.6)	0.5640
NACRT	16 (17.2)	5 (12.5)	11 (20.7)	
dCRT	6 (6.5)	3 (7.5)	3 (5.7)	
Clinical response				
CR	3 (3.2)	2 (5)	1 (1.9)	0.5442
PR	47 (50.5)	19 (47.5)	28 (52.8)	
SD	42 (45.2)	18 (45)	24 (45.3)	
PD	1 (1.1)	1 (2.5)	0 (0)	
Pathological response				
Grade 0	5 (5.4)	4 (10)	1 (1.9)	0.1055
Grade 1	62 (66.7)	28 (70)	34 (64.2)	
Grade 2	26 (28)	8 (20)	18 (34)	
Grade 3	0 (0)	0 (0)	0 (0)	

†Based on the Union for International Cancer Control, 8th edition.

ESCC: esophageal squamous cell carcinoma; SD: standard deviation; NAC: neoadjuvant chemotherapy; NACRT: neoadjuvant chemoradiotherapy; dCRT: definitive chemoradiotherapy; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease

1

FIG 1



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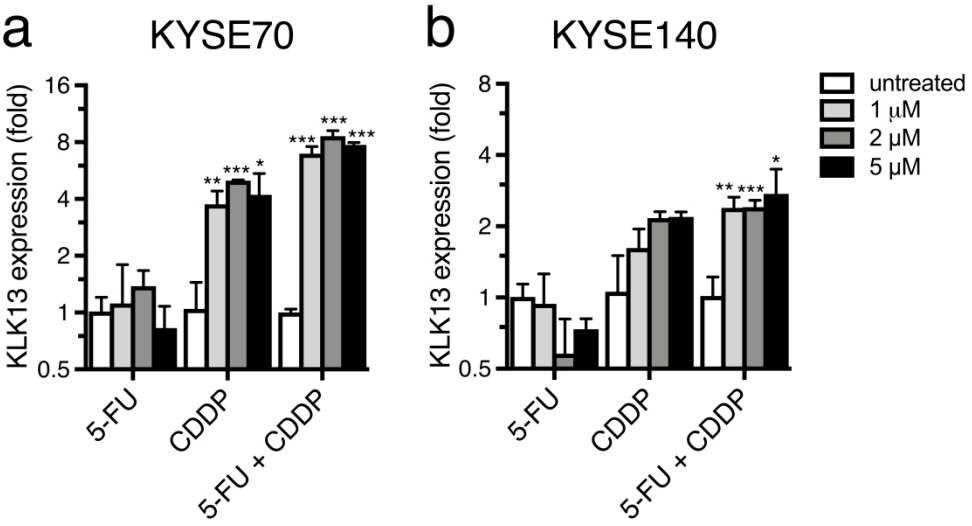
190x254mm (300 x 300 DPI)

FIG 2



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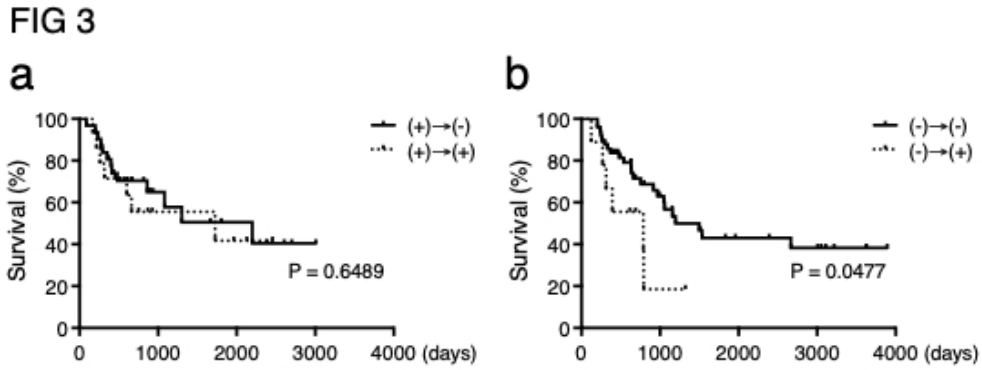

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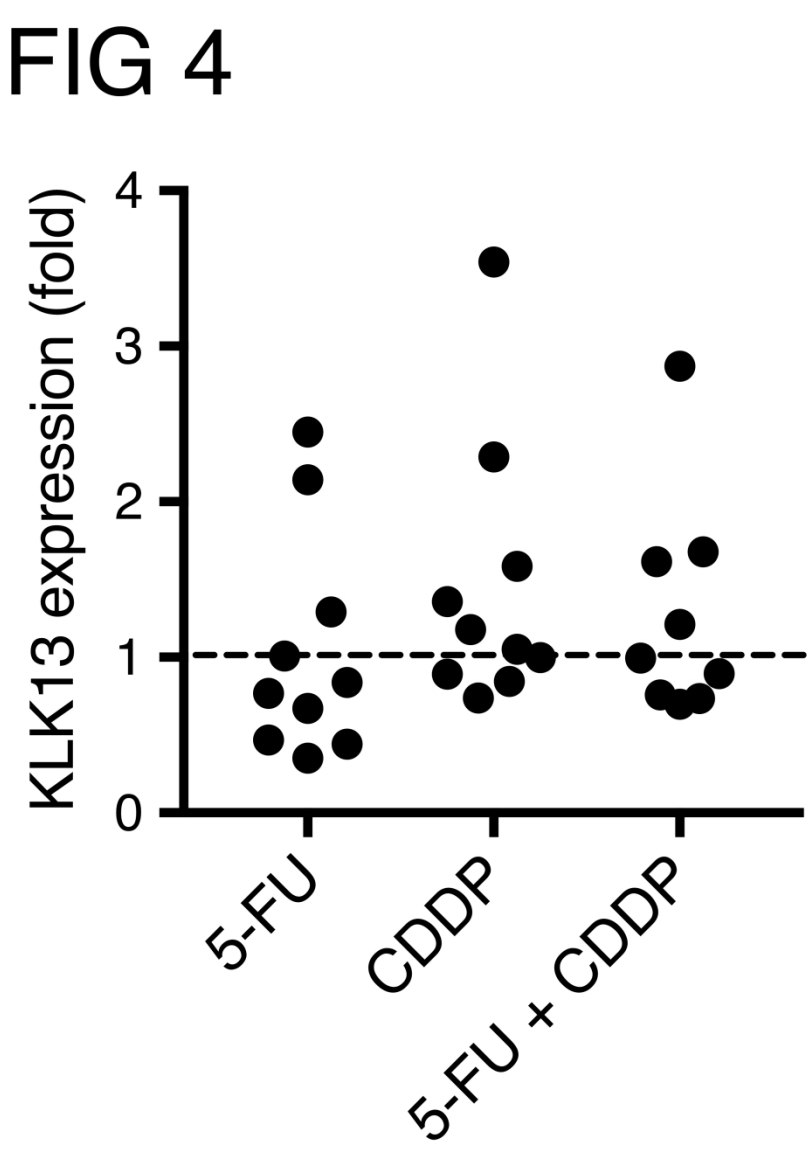

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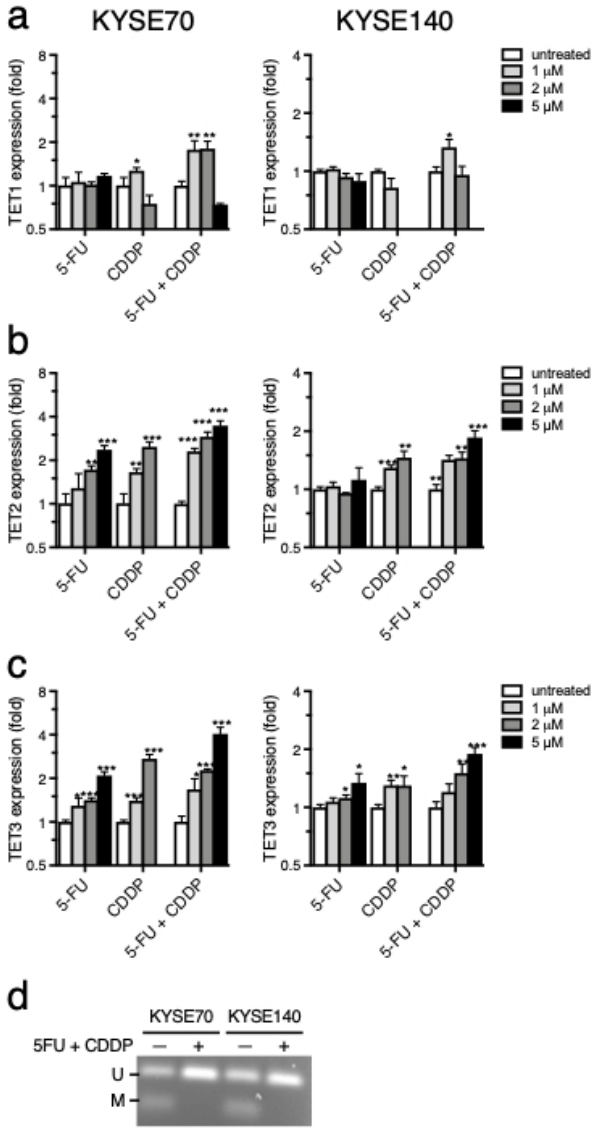
64x86mm (1200 x 1200 DPI)

FIG 5



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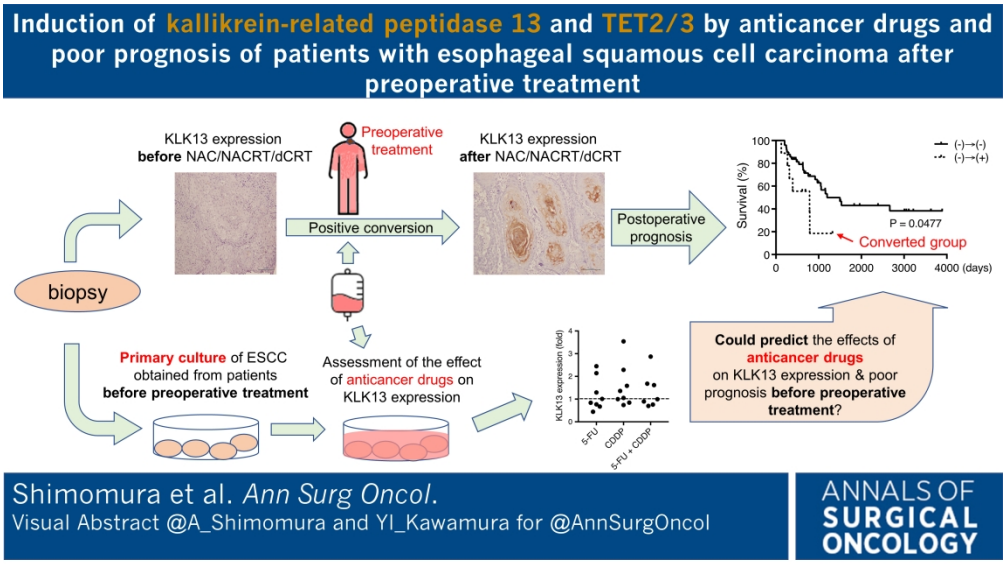

brief summaryThis retrospective study demonstrates that the preoperative treatment, such as NAC, NACRT, and dCRT, altered KLK13 expression. In addition, Anticancer drugs using for preoperative treatment upregulated of KLK13 and DNA demethylation enzymes in ESCC cell lines. The conversion of KLK13 from negative in biopsy samples obtained before preoperative treatment to positive in resected tumor samples was found to be a predictor of poor prognosis in patients with ESCC. Furthermore, we established a primary culture system to possibly predict the induction of KLK13 expression and eventually poor prognosis.

338x190mm (300 x 300 DPI)