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ARTICLE

Molecular Diagnostics

Schlafen 11 predicts response to platinum-based chemotherapy in gastric cancers

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BACKGROUND: Although unresectable or recurrent gastric cancers (GC) are frequently treated with platinum-based chemotherapy, response to treatment remains unpredictable. Because Schlafen 11 (SLFN11) is recently identified as a critical determinant of platinum sensitivity, we investigated the potential clinical utility of SLFN11 in the treatment of GC.

METHODS: We analysed the correlation between SLFN11 expression and overall survival in 169 GC patients by our established immunohistochemical approach. The impact of SLFN11 expression on the response to platinum and transition of SLFN11 expression upon long-term treatment with platinum were examined using GC cell lines and organoids.

RESULTS: GC patients with high-SLFN11 expression exhibited significantly better survival than those with low-SLFN11 expression, and the significance increased when we selected patients treated with platinum-based chemotherapy. Knockout of SLFN11 and reactivation of SLFN11 in GC cells conferred resistance and sensitivity to platinum, respectively. In GC cells and organoids, long-term treatment with oxaliplatin suppressed SLFN11 expression while imparting drug resistance. The acquired resistance to oxaliplatin was reversed by reactivation of SLFN11 with epigenetic modifying drugs.

CONCLUSIONS: This is the first report revealing definitive clinical implications of SLFN11 in the treatment of GC patients and providing novel strategies for the drug selection based on SLFN11 expression.

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BACKGROUND

Gastric cancer (GC) is the fourth leading cause of cancer-related deaths, accounting for approximately 750,000 deaths worldwide each year.[1] According to the Japanese GC Association guideline, chemotherapies are strongly recommended for GC patients with progressive unresectable or recurrent tumours, or who undergo non-curative resection as an additional therapy. The typical primary chemotherapy regimen for HER2 (human epidermal growth factor receptor type 2)-negative GC includes a combination of cisplatin or oxaliplatin with capecitabine or S1 prodrugs of 5-fluorouracil (5-FU).[2] For HER2-positive GC, trastuzumab, an anti-HER2 antibody, can be added to the combination of cisplatin or oxaliplatin with capecitabine or S1.[2–4] Cisplatin and oxaliplatin (platinum derivatives) are classified as DNA-damaging agents (DDAs) that exert antitumour effects by inducing DNA damage that renders lethality in a replication-dependent manner.[5] Although these drugs have been widely used since the 1960s in various cancers, like many chemotherapy agents, the major challenges include unpredictable initial response, development of resistance following initial benefit and recurrence. This is, in part, because clinically meaningful biomarkers that can predict responders/non-responders to the platinum are not available. Hence, major goals for improving treatment for GC patients are to identify novel predictive biomarkers and to develop treatments that overcome resistance to platinum.

Schlafen 11 (SLFN11), a nuclear protein belonging to the Schlafen family, has a putative DNA/RNA helicase domain at its C terminus and a conserved nuclease motif in its N terminus.[6,7] In 2012, two groups analysing the cancer cell line database NCI-60 or the Cancer Cell Line Encyclopedia (CCLE) independently identified that *SLFN11* mRNA expression level was the most highly correlated with the sensitivity to DDAs, including platinum derivatives, topoisomerase 1 and 2 inhibitors, alkylating agents and DNA synthesis inhibitors.[8,9] Since then, multiple preclinical studies have confirmed SLFN11 expression as a causal and dominant determinant of sensitivity to DDAs, and moreover to poly (ADP-ribose) polymerase (PARP) inhibitors.[10–18] Several clinical studies in small-cell lung cancers, oesophageal cancers and breast cancers have correspondingly shown that high-SLFN11 expression significantly

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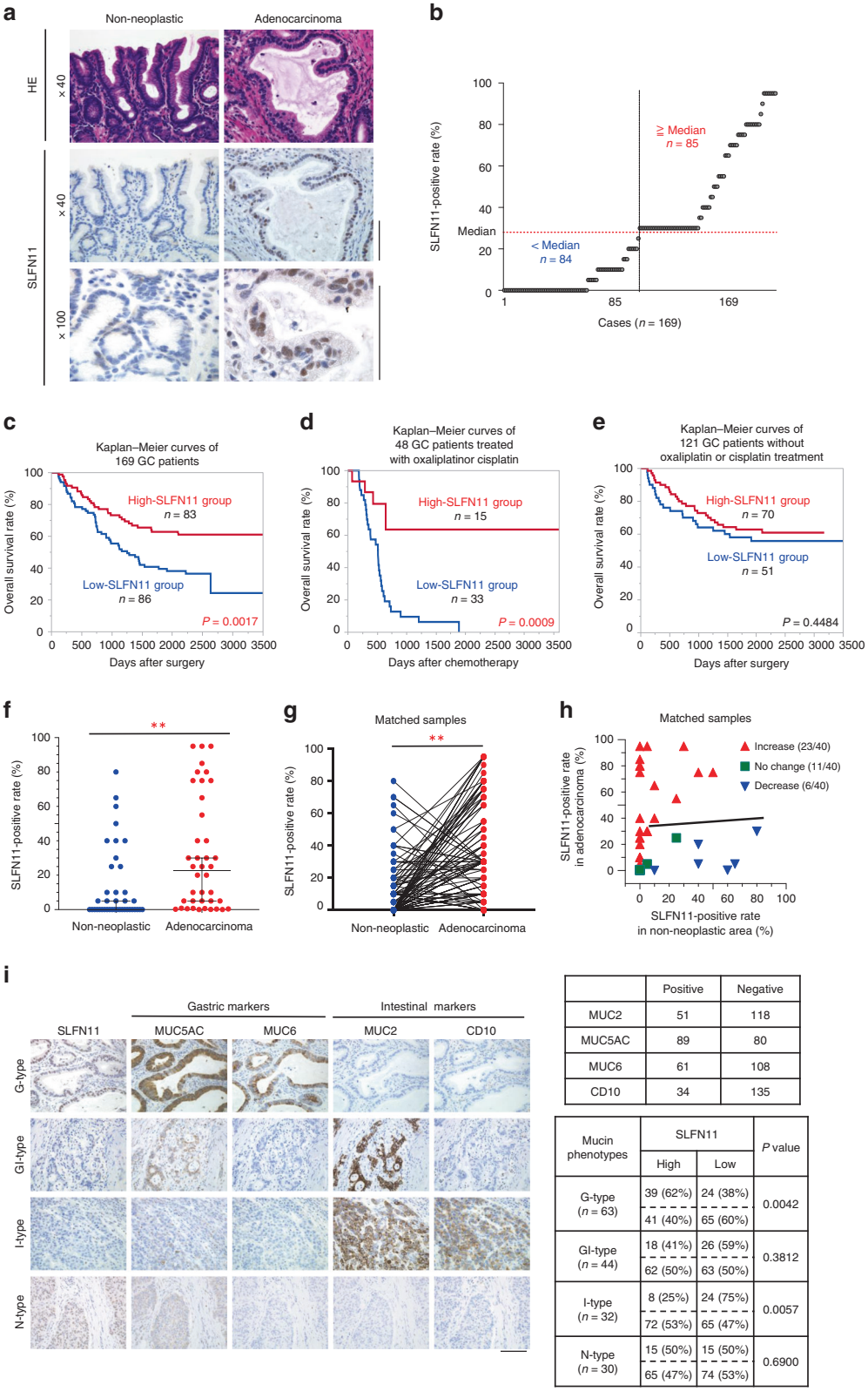


	Positive	Negative
MUC2	51	118
MUC5AC	89	80
MUC6	61	108
CD10	34	135

Mucin phenotypes	SLFN11		*P* value
	High	Low	
G-type (*n* = 63)	39 (62%)	24 (38%)	0.0042
	41 (40%)	65 (60%)	
GI-type (*n* = 44)	18 (41%)	26 (59%)	0.3812
	62 (50%)	63 (50%)	
I-type (*n* = 32)	8 (25%)	24 (75%)	0.0057
	72 (53%)	65 (47%)	
N-type (*n* = 30)	15 (50%)	15 (50%)	0.6900
	65 (47%)	74 (53%)	

correlates with enhanced responses to DDAs, indicating the therapeutic potential of using SLFN11 levels as a predictive biomarker of response to DDAs.[17,19–21] Mechanistically, SLFN11 binds stressed replication forks on chromatin and induces cell death, in part, through enforcing prolonged replication arrest under the treatment of DDAs.[22,23]

Deleterious mutations of SLFN11 are rarely reported in GC public databases such as bioportal.org (http://bioportal.org). Rather, aberrant SLFN11 expression appears to be regulated by DNA methylation levels within the SLFN11 promoter and gene body and/or histone modifications.[10,16,24] Hence, the expression of SLFN11 is subject to change by epigenetic modifier drugs such as

Fig. 1 High expression of SLFN11 is a favourable prognostic marker for gastric cancer (GC) treated with platinum-based chemotherapy. a Representative images of HE and immunohistochemical (IHC) staining for SLFN11 in non-neoplastic epithelium and adenocarcinoma. Scale bars are 100 μm. **b** Plots of SLFN11-positive rate (%) in 169 GC patients. The median (30%) was used as a cut-off value. **c** Kaplan–Meier curves for overall survival rate (%) in 169 GC patients divided into high-SLFN11 group ($n = 83$) and low-SLFN11 group ($n = 86$). Hazard ratio (HR), 0.5; 95% confidence interval (CI), 0.32–0.77; $P = 0.0017$ (log-rank test). **d** Kaplan–Meier curves for overall survival rate (%) in 48 GC patients (15 in high-SLFN11 group and 33 in low-SLFN11 group) previously treated with oxaliplatin or cisplatin. HR, 0.2; 95% CI, 0.06–0.51; $P = 0.0009$ (log-rank test). **e** Kaplan–Meier curves for overall survival rate (%) in 121 GC patients (70 in high-SLFN11 group and 51 in low-SLFN11 group) without oxaliplatin or cisplatin treatment. HR, 0.8; 95% CI, 0.46–1.42; $P = 0.4484$ (log-rank test). **f–h** Comparison of SLFN11 expression in non-neoplastic epithelium and the adjacent adenocarcinoma in the same samples ($n = 40$). **f** Plots of SLFN11-positive rate (%) in the indicated regions. Bars represent the median and 95% CI. $^{**}P < 0.01$ by unpaired *t*-test. **g** Plots of SLFN11-positive rate (%) of matched samples in the indicated regions were connected $^{**}P < 0.01$ by paired *t*-test. **h** SLFN11-positive rate (%) of matched samples in the indicated regions is represented on a scatter plot. $r = 0.0022$. **i** Relationship between SLFN11 expression and GC mucin phenotypes in 169 GC patients. Representative IHC images for the indicated markers. Accordingly, samples were classified into Gastric type (G-type), Gastric and Intestinal mixed type (GI-type), Intestinal type (I-type) and Null type (N-type). Scale bars are 100 μm in enlarged images. Positive and negative numbers of samples for each marker are summarised (right upper). Fisher's exact test was used for SLFN11 expression and GC mucin phenotype (each type and other types) (right lower).

inhibitors for DNA methyltransferases, EZH2, a histone methyltransferase and histone deacetylases.[10,16,24] The methylation at SLFN11 promoter is also reported in GC across 201 patient samples.[25]

We are recently aware that tissue RNA-seq data being the mixture of stroma and tumour cells can disturb the accurate evaluation of SLFN11 expression level in tumour cells because tumour- infiltrating lymphocytes in stroma usually exhibit high-SLFN11 expression.[26] Therefore, we encourage to utilise an optimised immunohistochemistry (IHC) with a proper anti-SLFN11 antibody rather than tissue RNA-seq to score the SLFN11 expression level in tumour tissues.[26] To determine the utility of our validated SLFN11 IHC assay in the context of emerging data of the importance of SLFN11 to understanding platinum treatment for GC, in the present study, we assessed the correlation between SLFN11 expression measured by IHC and platinum-based therapeutic outcomes. In addition, we used GC cell lines and patient-derived GC organoids to determine the impact of manipulating SLFN11 expression on platinum sensitivity.

METHODS

Tissue samples

Formalin-fixed paraffin-embedded blocks from 169 GC patients were obtained from the archives of the National Hospital Organization Kure Medical Center and Chugoku Cancer Center. In the 169 GC patients, 121 were untreated, and 48 were treated with regimens containing oxaliplatin or cisplatin. The 48 patients were negative for HER2 and did not receive treatment for anti-HER2 antibody.

Immunohistochemistry (IHC) and scoring

The SLFN11 immunostaining protocol was described previously.[26] Briefly, IHC was performed using a Dako Envision+ Mouse Peroxidase Detection System (Dako Cytomation, Carpinteria, CA, USA). The sections were incubated with the first antibody for 1 h at room temperature, followed by incubation with Envision+ anti-mouse or rabbit peroxidase for 1 h. Three surgical pathologists (N. S., D.T. and K.K.) independently counted the SLFN11 IHC scores without knowledge of clinical and pathological parameters or patient outcome. When >30% of tumour cells were stained, immunostaining was considered positive for SLFN11. Inter-observer differences were resolved by consensus review at a double-headed microscope.

Cell lines and generation of SLFN11-knockout cells

GC cell lines MKN-1, MKN-7, MKN-45 and MKN-74 were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). All cell lines were maintained in RPMI-1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 10% foetal bovine serum (BioWhittaker, Walkersville, MD), 2 mM L-glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37 °C. SLFN11-knockout (SLFN11-KO) cells were generated in MKN-1 and MKN-45 cell lines by CRISPR/Cas9 methods, with details as described previously.[26]

Phenotypic analysis of GC based on mucin expression pattern

GC was classified into four phenotypes: gastric (G), intestinal (I), gastric and intestinal mixed (GI) and null (N) types according to the mucin expression pattern. To analyse the phenotypic expression of GC, we performed IHC with antibodies against MUC5AC, MUC6, MUC2 and CD10. The criteria for the classification of GC subtypes were described previously.[27]

Viability assay

The viability assay of cell lines and organoids was performed with a 3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay. Five-thousand cells were seeded in each well of 96-well culture plates. After 24-h, the cells were continuously treated with various concentrations of drugs. After 2 days, the culture medium was removed, and 50 μL of a 0.5 mg/mL solution of MTT (Sigma-Aldrich) was added to each well. The plates were then incubated for 1 h at 37 °C. The MTT solution was then removed and replaced with 50 μL of dimethyl sulfoxide (Wako) per well, and the absorbance at 540 nm was measured using an Envision 2104 Multilabel Reader (Perkin Elmer, Waltham, MA). For the organoids, Matrigel was dissolved in 100 μL of 2% SDS (Wako) before adding DMSO.

Cell growth assay

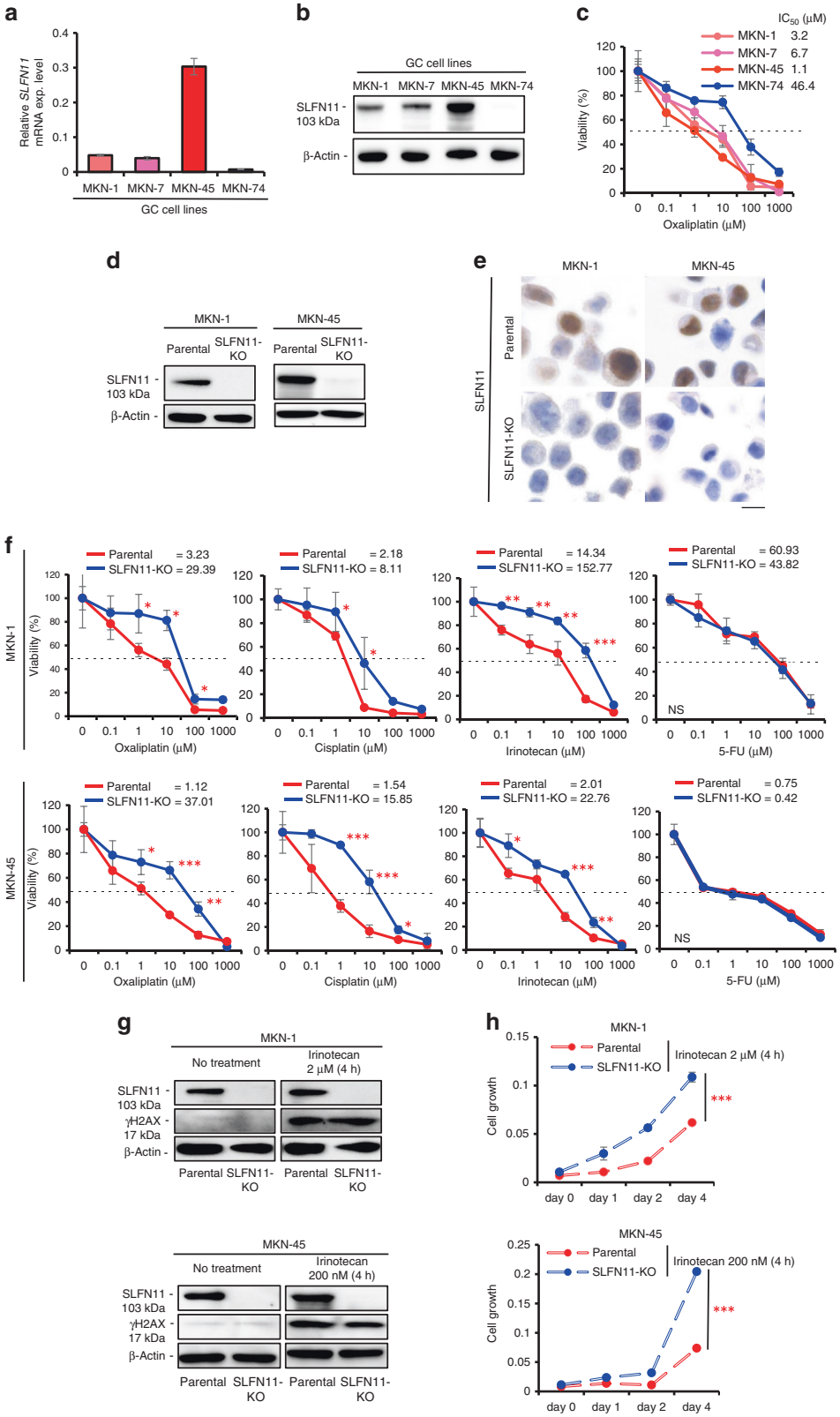
MKN-1 and MKN-45 cell lines were treated for 4 h with irinotecan at 2 μM and 200 nM, respectively. Then, the cells were washed and released into a drug-free medium. Cell number was monitored by MTT assay at 1, 2 and 4 days after the drug treatment (irinotecan).

siRNA transfection

Short-interfering RNA (siRNA) transfection was performed using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions. A mixture of *SLFN11*-targeting siRNAs (#L-016764-01-0005, SMARTpool: ON-TARGETplus *SLFN11* siRNA, Dharmacon) or a mixture of control siRNAs (D-001810-10-05, ON-TARGETplus Non-targeting Pool, Dharmacon) was transfected at a final concentration of 10 nM. Cells were seeded at 50% confluence one day before transfection and transfected with siRNAs.

Establishment and culture of human GC organoids

Human GC organoids were obtained from patients who underwent surgery at the Department of Gastroenterological and



Transplant Surgery at Hiroshima University Hospital and the Kure Medical Center and Chugoku Cancer Center. Human GC organoids were established and cultured as described previously.[28] Clinical data of patient-derived organoids are summarised in Supplemental Table S1.

Establishment of oxaliplatin-resistant cells and organoids

First, the parent cells or organoids were continuously treated with oxaliplatin at a concentration lower than each IC_{50} (50% inhibitory concentration). Then, the concentration of oxaliplatin was gradually increased at the timing of passages when the cells

Fig. 2 SLFN11 expression is a major determinant of sensitivity to DNA-damaging agents in GC cell lines. a Results of quantitative real-time PCR (qRT-PCR) showing *SLFN11* mRNA expression in GC cell lines (MKN-1, MKN-7, MKN-45 and MKN-74). **b** Western blotting showing SLFN11 protein expression in the indicated GC cell lines. Actin was used as a loading control. **c** Viability curves of the indicated GC cell lines to various concentrations of oxaliplatin. Viability was examined by MTT 2 days after the drug treatments. **d** Western blotting showing SLFN11 expression in the MKN-1 parental and SLFN11-knockout (SLFN11-KO) cells (left) and in the MKN-45 parental and SLFN11-KO cells (right). **d** Immunohistochemical staining for SLFN11 expression in the MKN-1 parental and SLFN11-KO cells (left), and the MKN-45 parental and SLFN11-KO cells (right). Original magnification: ×100. Scale bars are 50 μm in the enlarged images. **f** Viability curves of the indicated cell lines to oxaliplatin, cisplatin, irinotecan and 5-FU. Viability was determined by MTT assays 2 days after the drug treatments. NS, not significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by *t*-test. **g** Western blotting for the indicated proteins in the indicated cell lines. Each cell line was treated as indicated for 4 h. **h** Cell growth curves of the indicated cell lines. The cells were treated with the indicated concentration of irinotecan for 4 h and released into drug-free medium. Cell growth was measured by MTT assays at the indicated timings. $^{***}P < 0.001$ by *t*-test. **a**, **c**, **f** and **h** Representative results in triplicate from three independent experiments are shown as mean ± SD.

became confluent. The concentration of oxaliplatin was increased until it reached 2-fold higher the original IC_{50} of the parent cells or organoids. At this point, oxaliplatin-resistant (oxa-resistant) cells or organoids were considered established.

Statistical analysis

Statistical differences were evaluated by Student *t*-test. The overall survival rate was analysed using a Kaplan–Meier curve, log-rank test and multivariate analysis based on the Cox proportional hazards method. The correlation between expression levels of SLFN11 and clinicopathological characteristics was analysed with Fisher's exact test. The results are expressed as the mean ± standard deviation of triplicate measurements. We considered $P < 0.05$ to be statistically significant.

RESULTS

SLFN11 is a prognostic marker for GC patients treated with platinum-based chemotherapy

We first assessed the clinical implications of SLFN11 in GC patients. We performed IHC on 169 samples from GC patients using a pre-established protocol.[26] Gastric epithelial cells showed nuclear-specific staining patterns of SLFN11 as reported (Fig. 1a).[26] We then scored the SLFN11-positive rate within tumour areas of the 169 samples and found two major populations at 0% and 30% (Fig. 1b). We used the median value (30%) to divide patients into the high-SLFN11 group ($n = 85$) and low-SLFN11 group ($n = 84$). High-SLFN11 expression was correlated significantly with the clinicopathological characteristics of lower tumour classification and lower stage (Supplemental Table S2). The 5-year overall survival rates of the 169 GC patients by Kaplan–Meier analysis were 63% and 40% for the high-SLFN11 and low-SLFN11 groups, respectively. Moreover, the high-SLFN11 group had significantly higher overall survival rate than that of the low-SLFN11 group (hazard ratio (HR), 0.5; 95% confidence interval (CI), 0.32–0.77; $P = 0.0017$, Fig. 1c). Univariate analyses revealed that age, T, N and M classifications, stage, lymphatic invasion, vascular invasion and SLFN11 expression were significantly associated with survival. Multivariate analyses identified high-SLFN11 expression as an independent marker of better prognosis (Supplemental Table S3).

Because SLFN11 augments the antitumour effect of DDAs,[9] we further assessed if SLFN11 expression is associated with clinical outcomes of the platinum-based chemotherapy. For this purpose, we selected 48 GC patients who had undergone platinum-based chemotherapy and re-analysed their data. There was no significant relationship between SLFN11 expression and any clinicopathological characteristics (Supplemental Table S4). The 5-year overall survival rates were 64% and 0% for the high-SLFN11 and low-SLFN11 groups, respectively (Fig. 1d). The high-SLFN11 group had significantly higher overall survival rate than the low-SLFN11 group (HR, 0.2; 95% CI, 0.06–0.51; $P = 0.0009$, Fig. 1d). Univariate and multivariate analyses across clinical characteristics and SLFN11 expression uniquely identified high-SLFN11 expression as an independent marker of better prognosis (Supplemental Table S5).

Re-analyses of the data of the 48 patients using different cut-off scores of 20% or 10% also showed that the high-SLFN11 group had significantly better overall survival (HR, 0.46; 95% CI, 0.21–0.93; $P = 0.0341$ for 20%, and HR, 0.49; 95% CI, 0.24–0.95; $P = 0.0348$ for 10%) (Supplemental Fig. S1a, b). Conversely, re-analyses of the 121 GC patients not treated with platinum-based chemotherapy revealed that SLFN11 expression was not associated with overall survival for any analyses tested (Fig. 1e, Supplemental Table S6). High-SLFN11 expression significantly correlated with clinicopathological characteristics of lower tumour classification and negative vascular invasion (Supplemental Table S7). Collectively, these results indicate that high-SLFN11 expression can be a predictive biomarker of better prognosis in GC patients, especially when the patients are treated with platinum-based chemotherapy.

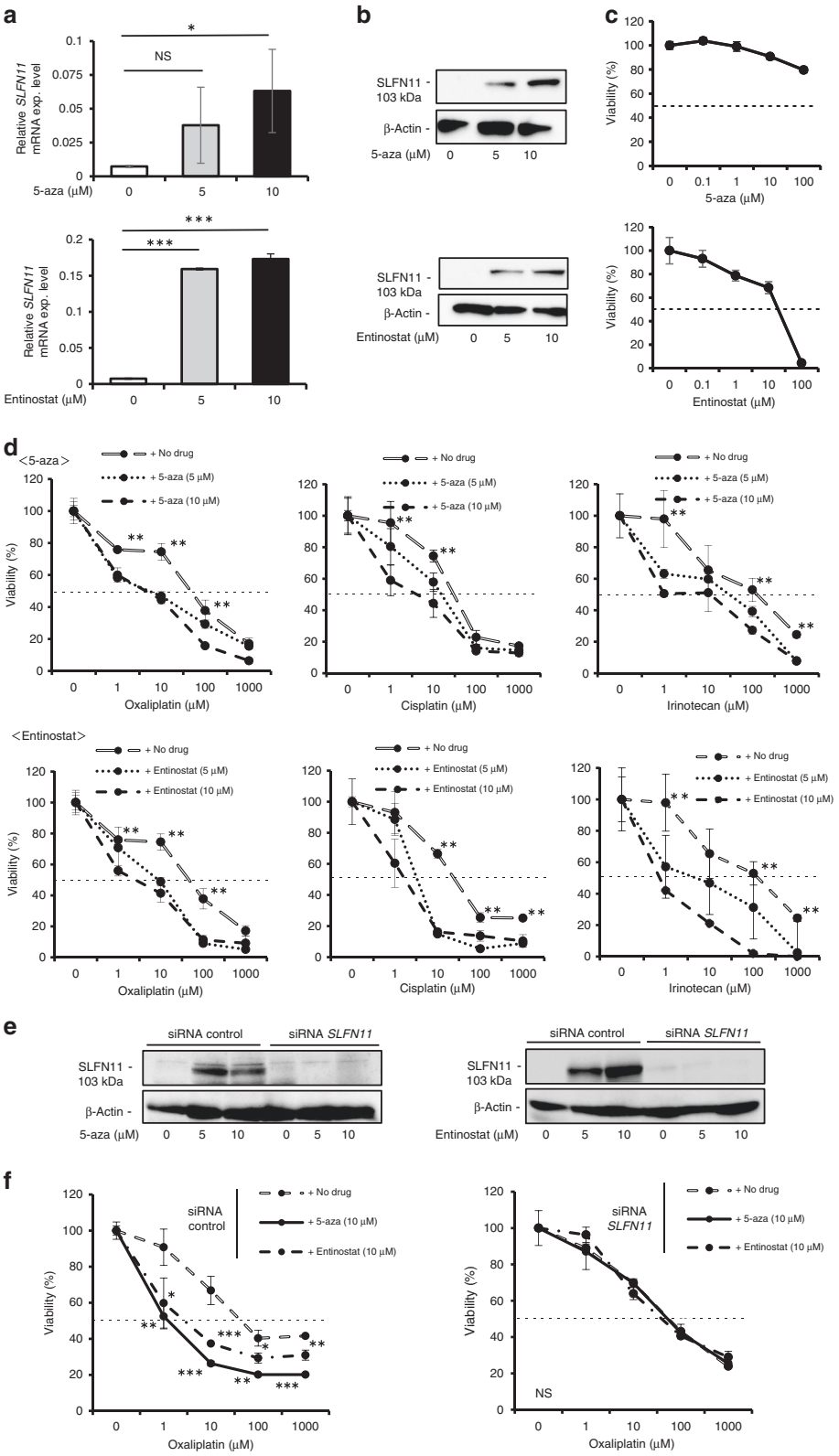
SLFN11 expression varies during tumorigenesis of GC

To examine possible altered SLFN11 expression during tumorigenesis, we randomly chose 40 samples and also scored SLFN11-positive rates in non-neoplastic epithelium next to tumour areas (Fig. 1a, f).[26] The rate of SLFN11 expression was significantly lower within non-neoplastic areas than tumour areas (Fig. 1f). A paired test revealed that the SLFN11 expression rate was significantly increased during tumorigenesis (Fig. 1g). The SLFN11 expression rate increased in 23 of 40 (57%) samples, did not change in 10/40 (25%), and decreased in 7/40 (17%) during tumorigenesis. There was no significant correlation of SLFN11-positive rate between non-neoplastic and tumour areas (Fig. 1h). These results indicate that SLFN11 expression is flexible and can either increase or decrease during tumorigenesis in GC.

Gastric-type GC expresses SLFN11 at higher rates than intestinal-type GC

We next examined an association between SLFN11 expression and a mucin phenotype of GC. GC can be classified into four phenotypes, gastric (G), intestinal (I), gastric and intestinal mixed (GI) and null (N) types, according to mucin expression patterns.[27] We performed IHC with antibodies for gastric marker mucins (MUC5AC and MUC6) and intestinal marker molecules (MUC2 and CD10) and classified our 169 GC samples into 63 G-type, 44 GI-type, 32 I-type and 30 N-type phenotypes (Fig. 1i). We found that the G-type GC has significantly higher SLFN11 expression than the others, while the I-type GC has significantly lower SLFN11 expression than the others (Fig. 1i). Considering our previous study showing fewer SLFN11-positive cells in colon cancers,[26] intestinal-type of GC might share characterisation with colon cancer in SLFN11 regulation.

According to the previous study of GC in The Cancer Genome Atlas (TCGA), GC is classified into four molecular subtypes: Epstein–Barr virus-infected tumours (EBV), microsatellite-instability tumours (MSI), genomically stable tumours (GS) and chromosomally instability tumours (CIN).[29] We attempted to find any correlation between SLFN11 expression and the subtypes, yet no obvious correlation was found (Supplemental Fig. S1c and Supplemental Table S8).



SLFN11 is a key determinant of sensitivity to DDAs in GC cell lines

We used four GC cell lines (MKN-1, MKN-7, MKN-45 and MKN-74) to examine the functions of SLFN11 in GC. MKN-45 cell line showed high-SLFN11 expression for both mRNA and protein levels, whereas MKN-1 and MKN-7 cell lines showed relatively low mRNA but detectable protein levels of SLFN11 (Fig. 2a, b). MKN-74 cell line showed little mRNA and undetectable protein levels of SLFN11 (Fig. 2a, b). Viability assay revealed that the SLFN11-negative MKN-74 cell line was highly resistant to oxaliplatin compared to the other SLFN11-positive GC cell lines (Fig. 2c). To

Fig. 3 Epigenetic activation of SLFN11 further sensitises a GC cell line MKN-74 to DNA-damaging agents. a Results of qRT-PCR showing *SLFN11* mRNA expression in the MKN-74 cells that were treated/untreated with 5-aza (top) or entinostat (bottom) for 2 days. NS, not significant; $^{*}P < 0.05$, $^{***}P < 0.001$ by *t*-test. **b** Western blotting showing SLFN11 protein expression under the same condition as (**a**). Actin was used as a loading control. **c** Viability curves for 5-aza (top) and entinostat (bottom) as single agents in MKN-74 cell line. Viability was determined by MTT assays 2 days after the drug treatments. **d** Viability curves of the MKN-74 cell line. MKN-74 cells were pretreated with 5-aza (top) or entinostat (bottom) for 2 days, washed and then treated with the indicated concentrations of oxaliplatin, cisplatin or irinotecan for 2 additional days. Viability was determined by MTT assays 2 days after the drug treatments. $^{**}P < 0.01$ by *t*-test. **e** Western blotting for SLFN11 expression in MKN-74 cells treated with siRNA control (left) or siRNA *SLFN11* (right). Two days after the siRNA transfection, cells were treated by 5-aza or entinostat for 2 additional days and whole-cell lysate was collected. Actin was used as a loading control. **f** Viability curves of the MKN-74 cell line transfected with siRNA control or siRNA *SLFN11*. The transfected MKN-74 cells were further treated with 5-aza or entinostat and with or without oxaliplatin. Viability curves were determined by MTT assays 2 days after the drug treatments. NS, not significant $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by *t*-test. **a**, **c**, **d** and **f** Representative results in triplicate from three independent experiments are shown as mean ± SD.

investigate the functional significance of SLFN11 in GC chemosensitivity, we employed previously established SLFN11-knockout (SLFN11-KO) MKN-45 cells[26] and newly established SLFN11-KO MKN-1 cells. We confirmed successful SLFN11-KO by Western blot and IHC in both cell lines (Fig. 2d, e). Both SLFN11-KO cell lines showed indistinguishable morphology and cell proliferation rate under normal conditions, compared to their parental cells (Fig. 2e and Supplemental Fig. S2a).

We then examined drug sensitivity to several anticancer agents in the parental and SLFN11-KO cells of the MKN-1 and MKN-45 cell lines. After continuous treatments for 48-h, the parental MKN-1 and MKN-45 cell lines were highly sensitised to DDAs of oxaliplatin, cisplatin and irinotecan compared to the SLFN11-KO counterparts (Fig. 2f). However, a non-DDA 5-FU sensitised the parental and SLFN11-KO cells of the MKN-1 and MKN-45 cell lines to similar degrees (Fig. 2f). Next, we examined whether SLFN11 can determine the cell fate after brief exposures. After 4 h of irinotecan treatment, the amount of DNA damage measured by Western blot and immunofluorescence of γH2AX was comparable between the parental and SLFN11-KO cells (Fig. 2g and Supplemental Fig. S2b, c), indicating that SLFN11 did not alter the amount of initial DNA damage. Yet, after the transient exposure with irinotecan for 4 h, SLFN11-positive parental cells proliferated significantly slower than SLFN11-KO cells in the MKN-1 and MKN-45 cell lines (Fig. 2h). Hence, SLFN11 already changes the future cell fate within 4 h after drug treatment. Overall, these results demonstrate that SLFN11 expression determines the sensitivity to representative DDAs in MKN-1 and MKN-45 cell lines.

Epigenetic modification reactivates SLFN11 expression and restores sensitivity to DDAs in SLFN11-negative chemoresistant GC cell line

As overcoming resistance to DDAs is an unmet need in GC, we tested whether SLFN11 reactivation can restore the sensitivity to DDAs in chemoresistant GC. The GC cell line MKN-74 with very low SLFN11 was highly resistant to DDAs (Fig. 2a–c), and we treated the cell line with DNA-methyltransferase inhibitor 5-aza or an HDAC inhibitor entinostat. Both mRNA and protein levels of SLFN11 increased after continuous treatment with 5-aza or entinostat (Fig. 3a, b). Both 5-aza and entinostat were less cytotoxic at concentrations of 5–10 μM where SLFN11 was well reactivated (Fig. 3c). Using the drugs in that range, we performed viability assays using MKN-74 cell line in combination with oxaliplatin, cisplatin or irinotecan. Any combination significantly decreased the viability of the MKN-74 cell line compared to single treatments with the DDAs (Fig. 3d), implying that the combination worked synergistically possibly because the reactivation of SLFN11 enhanced the drug sensitivity.

To examine whether the observed synergistic effect is directly connected to SLFN11 re-expression, we knocked down *SLFN11* using siRNA targeting *SLFN11* in the MKN-74 cell line. We confirmed that SLFN11 protein was not induced after continuous treatment with 5-aza or entinostat in the *SLFN11*-knockdown MKN-74 cells yet increased in the siRNA control-treated cells (Fig. 3e). We then examined the synergistic effect of 5-aza or entinostat with oxaliplatin in the siRNA control and siRNA *SLFN11*-treated MKN-74 cells. The synergistic effect by either combination observed in the control cells mostly disappeared in the *SLFN11*-knockdown cells (Fig. 3f). Therefore, despite genome-wide transcriptional activation by the epigenetic modifying drugs, we conclude that SLFN11 is the primary factor that causes the synergism by the epigenetic modifying drugs with oxaliplatin.

In contrast, the MKN-45 cell line with a high basal SLFN11 expression did not respond to the treatments of 5-aza or entinostat in terms of mRNA and protein expression of SLFN11 (Supplemental Fig. S3a, b). The combination of non-toxic doses of 5-aza or entinostat with DDAs did not further sensitise the MKN-45 cell line compared to each DDA alone (Supplemental Fig. S3c, d). These results suggest that reactivation of SLFN11 can be a key to enhancing the antitumour effects of DDAs and that combination of DDAs with epigenetic modifiers may be a promising strategy for overcoming the resistance to DDAs in GC.

A GC cell line acquires resistance to oxaliplatin by losing SLFN11 while restores sensitivity by epigenetic activation of SLFN11

GC is often refractory to the 2nd chemotherapy.[30,31] To investigate the mechanism of refractory post chemotherapy, we attempted to mimic in vivo clinical conditions by using the MKN-45 cell line having high-SLFN11 and exhibiting hypersensitivity to DDAs (Fig. 2). First, we continuously treated the MKN-45 cell line with a gradually increasing concentration of oxaliplatin (see 'Methods' and Fig. 4a). After 3 months, we obtained an oxaliplatin-resistant (oxa-resistant) population that can survive at 5 μM of oxaliplatin (about 5-fold higher than the IC_{50} of the parental cells). Surprisingly, the oxa-resistant cell line drastically decreased *SLFN11* mRNA expression compared to the parental cells (Fig. 4b). We confirmed the loss of both nuclear SLFN11 expression by IHC (Fig. 4c) and SLFN11 protein in whole lysates (Fig. 4d). As expected, the oxa-resistant MKN-45 cell line was significantly resistant to oxaliplatin compared to the parental cells in the 48-h viability and colony-formation assays (Fig. 4e, f). Acquired resistance was also observed in cisplatin and irinotecan (Supplemental Fig. S4a). Second, we examined the effect of SLFN11 reactivation in the oxa-resistant MKN-45 cell line by epigenetic modification as summarised in Fig. 3. Treatment with non-toxic doses of 5-aza or entinostat reactivated SLFN11 expression in mRNA and protein levels (Fig. 4g–i). The reactivated SLFN11 protein also located exclusively in the nucleus as in the parental cells (Fig. 4c, h) and was expressed as highly as that in the parental cells (Fig. 4i). Lastly, we examined drug sensitivity to oxaliplatin in combination with 5-aza or entinostat in the oxa-resistant cell line and verified re-sensitisation by the combination compared to the single treatment with oxaliplatin (Fig. 4j). Comparable results were obtained with cisplatin and irinotecan instead of oxaliplatin (Supplemental Fig. S4b). Collectively, our results reveal that SLFN11 expression can decrease during the process of the acquisition of resistance to DDA,s but is reversible through epigenetic modification, suggesting a novel strategy to overcome the acquired drug resistance in GC.

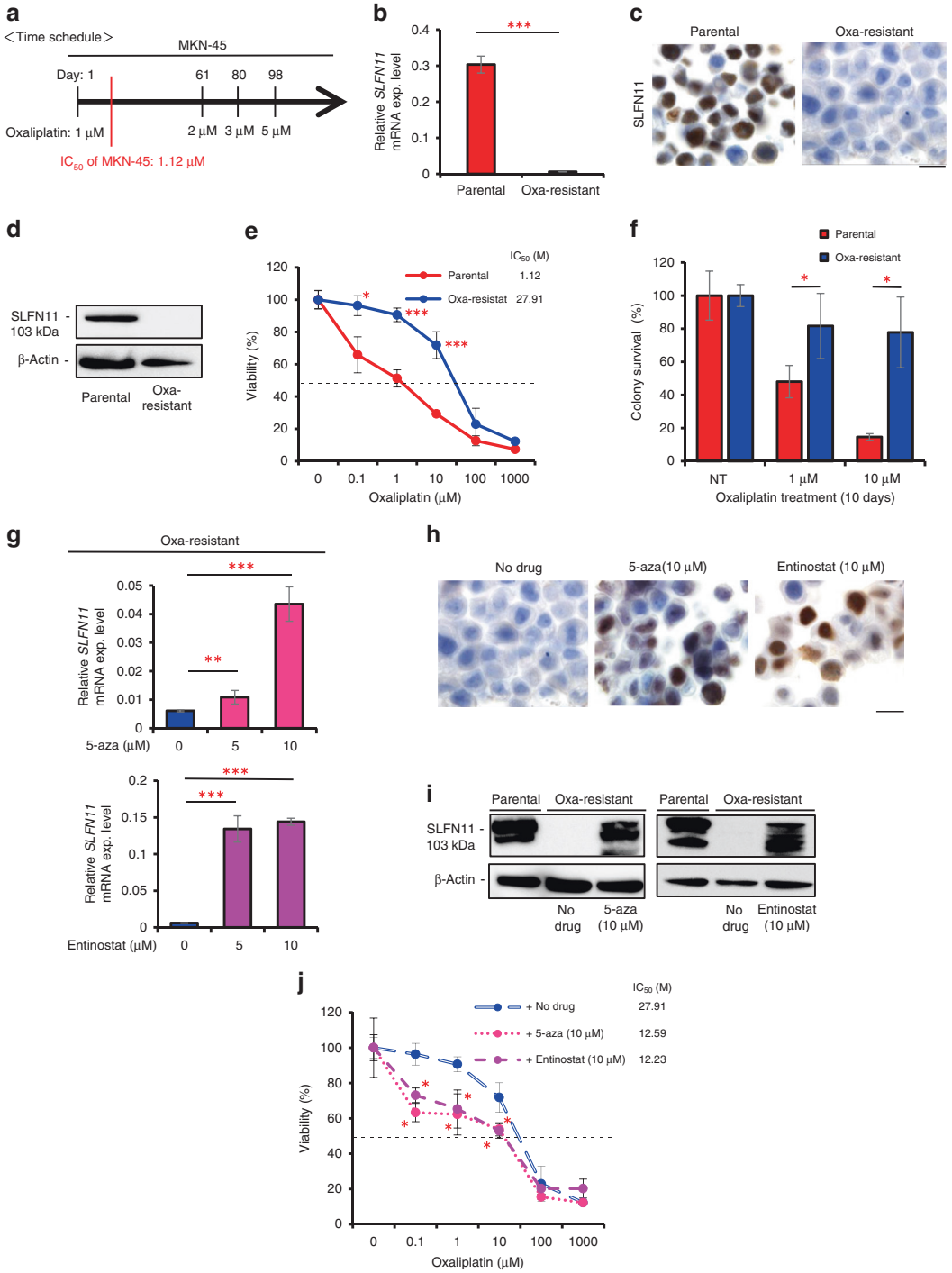
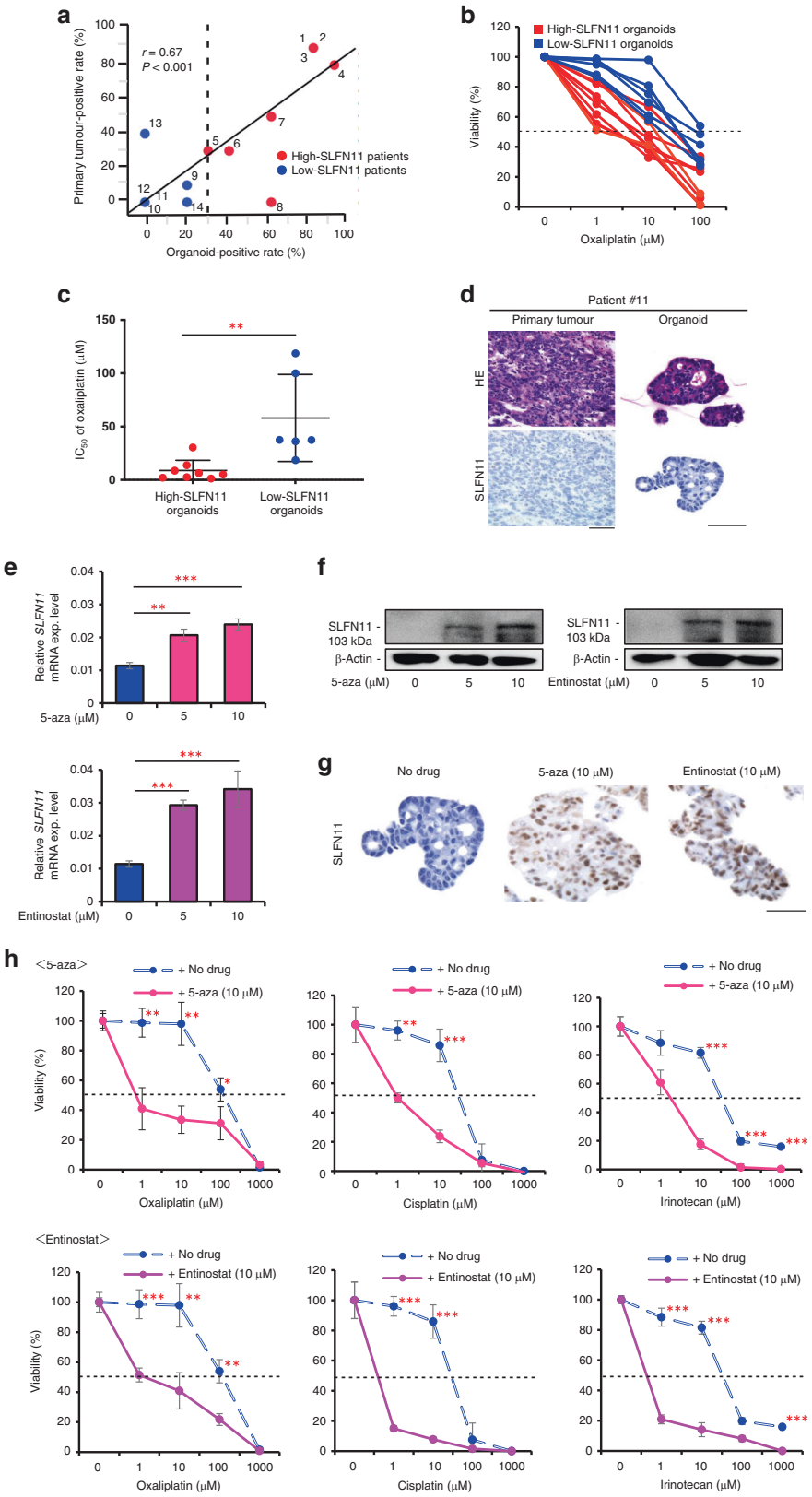


Fig. 4 GC cells acquire resistance to oxaliplatin while inactivating SLFN11. a A scheme of oxaliplatin treatment to establish the oxaliplatin-resistant (oxa-resistant) MKN-45 cell line. **b** qRT-PCR showing *SLFN11* mRNA expression in MKN-45 parental and oxa-resistant cells. ***$P < 0.001$ by *t*-test. **c** IHC for SLFN11 in MKN-45 parental and oxa-resistant cells. Original magnification: ×100. Scale bars are 10 μm in the enlarged images. **d** Western blotting examining SLFN11 expression in MKN-45 parental and oxa-resistant cells. Actin was used as a loading control. **e** Viability curves of MKN-45 parental and oxa-resistant cells for oxaliplatin. Viability was determined by MTT assays 2 days after the drug treatments. *$P < 0.05$, ***$P < 0.001$ by *t*-test. **f** Colony-formation assays performed in MKN-45 parental and oxa-resistant cells. Colony numbers were counted 10 days after the drug treatments and normalised by the number of untreated cells. *$P < 0.05$ by *t*-test. **g** qRT-PCR showing *SLFN11* mRNA expression in the oxa-resistant MKN-45 cell line treated/untreated with 5-aza (top) or entinostat (bottom) for 2 days. *$P < 0.05$, ***$P < 0.001$ by *t*-test. **h** IHC for SLFN11 in the oxa-resistant MKN-45 cell line treated/untreated with 5-aza or entinostat for 2 days. Original magnification: ×100. Scale bars are 10 μm in the enlarged images. **i** Western blotting under the same conditions as (**h**). Actin was used as a loading control. **j** Viability curves in the oxa-resistant MKN-45 cell line in combination with 5-aza or entinostat for 2 days. Viability was determined by MTT assay 2 days after the drug treatments. *$P < 0.05$ by *t*-test. **b**, **e**, **f**, **g** and **j** Representative results in triplicate from three independent experiments are shown as mean ± SD.



Endogenous or epigenetically reactivated SLFN11 confers oxaliplatin hypersensitivity to GC organoids

Organoids are three-dimensional cell cultures that retain various features of original organs. Patient-derived organoids recapitulate drug sensitivities of primary tumours well.[32–34] In this study, we newly established organoids from 14 GC patients and compared SLFN11 expression between the organoids and tumour tissues in individual patients (Supplemental Fig. S5a and Supplemental Table S1). SLFN11-positive rates were highly conserved among the 14 samples, confirming that GC organoids recapitulated the

Fig. 5 Low-SLFN11 organoids acquire sensitivity to DNA-damaging agents in combination with epigenetic modifying drugs. a Correlation analysis of SLFN11-positive rate between primary tumours vs. patient-derived organoids across 14 GC patients. Patients were classified as indicated by the threshold (30%) of organoid SLFN11-positive rate. **b** Viability curves of 14 GC organoids to oxaliplatin. Viability was determined by MTT assays 2 days after the drug treatments. Samples with colours corresponded to those of (**a**). **c** Comparison of IC_{50} of oxaliplatin in 8 high-SLFN11 and 6 low-SLFN11 GC organoids. $^{**}P < 0.01$ by *t*-test. Bars represent the median and 95% CI. **d** Representative images of HE staining and IHC for SLFN11 in the primary tumour and organoids from patient #11. Original magnification: ×40 (primary tumour) and ×100 (organoids). Scale bars are 100 μm in the enlarged images. **e** qRT-PCR showing *SLFN11* mRNA expression in the organoids from patient #11 treated with 5-aza (top) or entinostat (bottom) for 2 days. $^{**}P < 0.01$, $^{***}P < 0.001$ by *t*-test. **f** Western blotting for the indicated proteins with the organoids from patient #11 treated/untreated with 5-aza (left) or entinostat (right) for 2 days. Actin was used as a loading control. **g** IHC for SLFN11 in the organoids from patient #11 treated/untreated with 5-aza or entinostat for 2 days. Original magnification: ×100. Scale bars are 100 μm in enlarged images. **h** Viability curves of the organoids from patient #11. The organoids were pretreated with 5-aza (top) or entinostat (bottom) for 2 days and then treated with the indicated concentrations of oxaliplatin, cisplatin or irinotecan for another 2 days before MTT assays were performed. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by *t*-test. **b**, **e** and **h** Representative results in triplicate from three independent experiments are shown as mean ± SD.

phenotype of their parental tumours as for SLFN11 expression (Fig. 5a and Supplemental Table S1).

We applied the threshold of a 30% positive rate that was determined from tissue samples to the organoids (Fig. 1). Six GC organoids were assigned to the low-SLFN11 group and eight GC organoids were assigned to the high-SLFN11 group (Supplemental Fig. S5a and Supplemental Table S1). We next performed viability assays with the 14 GC organoids and obtained an IC_{50} of oxaliplatin for each organoid (Fig. 5b and Supplemental Table S1). The IC_{50}s of the SLFN11-positive group were significantly lower than those of the SLFN11-negative group (Fig. 5c), indicating that SLFN11 acts as a determinant of oxaliplatin sensitivity in GC organoids.

We then attempted to reactivate SLFN11 in a SLFN11-negative and oxaliplatin-resistant organoid. For the aim, we chose an oxaliplatin-resistant GC patient # 11 organoid (Fig. 5d). Treatment with 5-aza or entinostat clearly reactivated SLFN11 expression in mRNA and protein levels (Fig. 5e, f). Nuclear-specific localisation of SLFN11 was observed by IHC in the 5-aza- or entinostat-treated GC organoids (Fig. 5g). The organoid in GC patient #11 treated with a less-toxic dose of 5-aza or entinostat (Supplemental Fig. S5b) was significantly highly sensitised to oxaliplatin, cisplatin or irinotecan compared to the untreated isogenic organoid in 48-h viability assays (Fig. 5h). We found that, in another GC organoid from patient #14 with low-SLFN11 expression, reactivation of SLFN11 was partly detected by entinostat but not by 5-aza (data not shown). These results imply that not all but some cases of SLFN11-negative GC that are refractory to platinum can acquire the sensitivity by combining epigenetic modifiers such as HDAC inhibitors and DNA-methyltransferase inhibitors possibly through reactivation of SLFN11.

GC organoids acquire platinum resistance while reducing SLFN11 expression

Finally, we intended to mimic the clinical situation of acquired platinum resistance after primary chemotherapy with GC organoid. After challenging with several different organoids, we succeeded in establishing an oxa-resistant organoid from GC patient #7. We speculate that the other high-SLFN11 organoids were too sensitive to acquire the resistance (Fig. 6a). As we realised in a cell line model (Fig. 4), the resistance-acquired GC organoid of patient #7 drastically decreased SLFN11 in mRNA and protein levels, which was also confirmed by IHC (Fig. 6b–d). As expected, the oxa-resistant GC organoid was significantly resistant to oxaliplatin compared to the parental organoid in 48-h viability assays (Fig. 6e). The suppressed mRNA and protein levels in the oxa-resistant GC organoid were recovered by treatment with 5-aza or entinostat (Fig. 6f, g). The oxa-resistant GC organoid treated with 5-aza or entinostat gained significantly higher sensitivity to oxaliplatin compared to the untreated oxa-resistant GC organoid in 48-h viability assays (Fig. 6h). In summary, our results reveal that GC organoids can acquire platinum resistance by inactivating SLFN11 through epigenetic conversion, but can regain platinum sensitivity with a combination of epigenetic modifying drugs, a scenario that is potentially applicable clinically to GC patients.

DISCUSSION

DNA-damaging platinum derivatives are standard of care for GC, but the response is unpredictable, and resistance often develops. This study first showed that SLFN11 expression examined by our established IHC method can predict overall survival of GC patients. This is particularly operative for GC patients treated with platinum-based chemotherapy. Studies in GC cell lines and GC organoids reveal that intrinsic or acquired resistance to oxaliplatin appears to be secondary to epigenetic suppression of SLFN11. Moreover, epigenetic modifiers reverse the suppression and restore high sensitivity to oxaliplatin. Considering that 5-aza and entinostat or other HDACi are already in clinical use,[35–37] this study provides practical strategies to improve treatment outcomes for GC patients. However, tolerability of the combination of epigenetic modifiers with DDAs was not assessed in vivo in this study and should be examined in further study.

SLFN11, a breakthrough gene after a decade-long lack of a biomarker for platinum

Although numerous genes and pathways are involved in the sensitivity or resistance to platinum,[5,38–41] and some of them have proven usefulness in personalised precision medicine, there remain no practical biomarkers for platinum in clinical use. This is probably because individual prereported factors, such as homologous recombination (HR)-related genes, are not frequently mutated or inactivated in general tumours including GC. A recent study analysing HR defect in GC by genome sequencing reported that mutations of HR genes are present in 10% of GC.[42]

In this study, we revealed that SLFN11 expression in GC is divided into two discrete populations of high and low levels by approximately half-and-half. Such a polar distribution gene expression suggests the possibility of a useful biomarker for clinical decision-making. Because IHC methods are commonly available in hospitals and institutions in most countries, we propose that careful IHC quantification for SLFN11 expression should be considered for GC patients who are under consideration for platinum-based chemotherapy. This situation is not always applicable for all organs because in the case of colon cancers, there are few SLFN11-positive adenocarcinomas, as noted in our previous report.[26] In the present study, we showed that I-type GC showed low-SLFN11 expression levels and that G-type GC showed high-SLFN11 expression levels. Hence, we speculated the presence of common transcription or epigenetic factors that can determine the I-type or G-type as well as SLFN11 expression. As SLFN11 is a druggable protein, such switching factors are worthy of investigation in future studies.

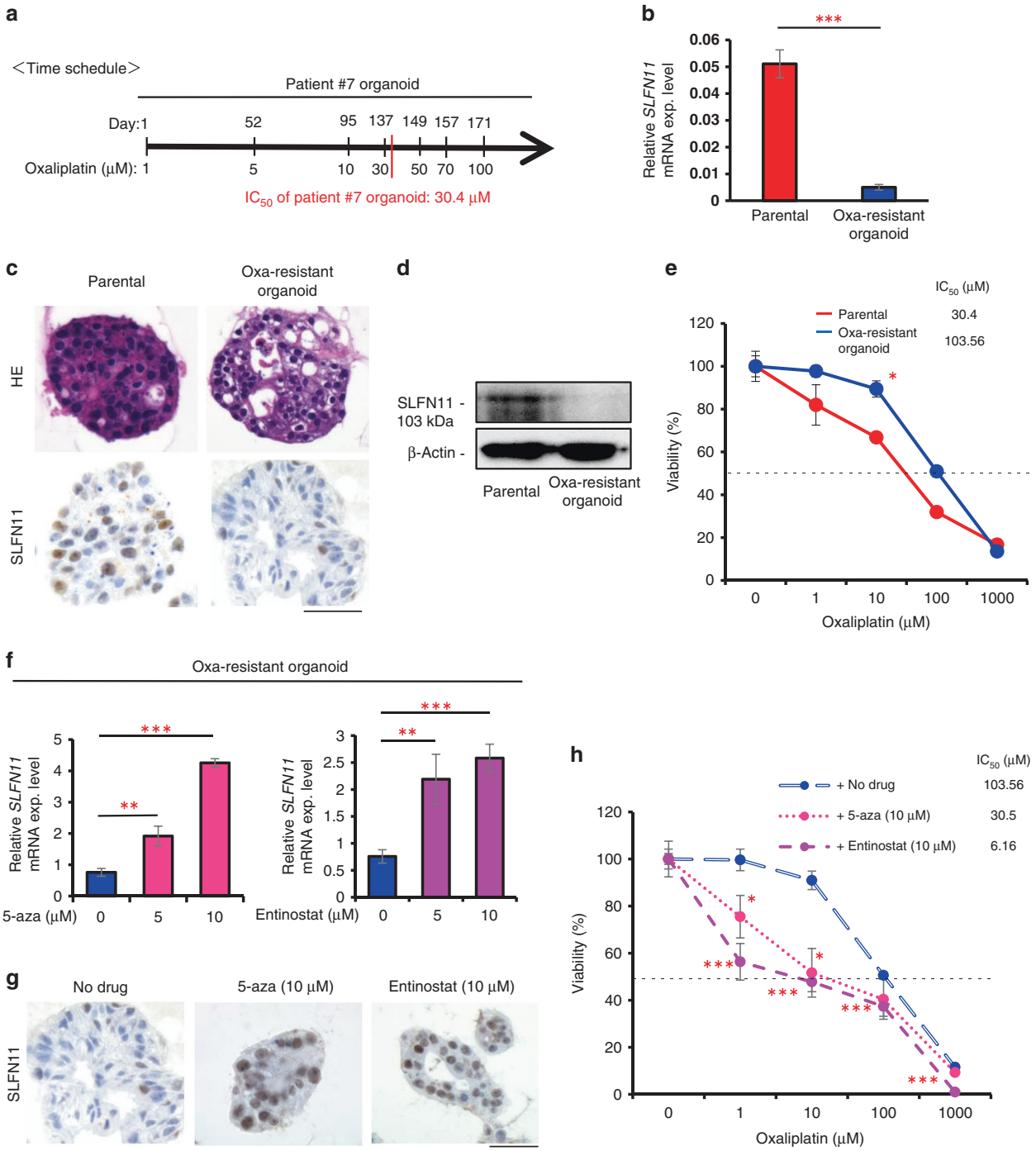


Fig. 6 GC organoids acquire resistance to oxaliplatin while suppressing SLFN11 expression. **a** A scheme of oxaliplatin treatment to establish the oxaliplatin-resistant (oxa-resistant) GC organoids from patient #7. **b** qRT-PCR showing *SLFN11* mRNA expression in parental and oxa-resistant organoids from patient #7. ***$P < 0.001$. **c** Representative images of parental and oxa-resistant organoids from patient #7 (HE, top; IHC for SLFN11, bottom). Original magnification: ×40. Scale bars are 100 μm in the enlarged images. **d** Western blotting showing SLFN11 expression in parental and oxa-resistant organoids from patient #7. Actin was used as a loading control. **e** Viability curves of parental and oxa-resistant organoids from patient #7 for oxaliplatin. Viability was determined by MTT assay 2 days after drug treatments. *$P < 0.05$ by *t*-test. **f** qRT-PCR showing *SLFN11* mRNA expression in oxa-resistant organoids from patient #7 treated/untreated with 5-aza or entinostat for 2 days. *$P < 0.05$ by *t*-test. **g** Representative images of oxa-resistant organoids from patient #7 treated/untreated with 5-aza or entinostat for 2 days. Original magnification: ×40. Scale bars are 100 μm in the enlarged images. **h** Viability curves of oxa-resistant organoids from patient #7 for oxaliplatin concomitantly treated with 5-aza or entinostat. Viability was determined by MTT assay after 2 days of drug treatment. *$P < 0.05$, ***$P < 0.001$ by *t*-test. **b**, **e**, **f** and **h** Representative results in triplicate from three independent experiments are shown as mean ± SD.

Importance of evaluating SLFN11 expression precisely

SLFN11 was first identified only in 2012 as a candidate predictive biomarker for DDAs.[6,8,9] Although several papers have reported about SLFN11 and the relevance of clinical outcomes, standard methods to evaluate SLFN11 from patient samples remain only in development. For example, SLFN11 IHC staining of GC reported by the Human Protein Atlas shows a cytoplasmic staining pattern, while our IHC method indicates an exclusive nuclear location in both non-

neoplastic epithelial cells and GC. In IHC, we strongly recommend confirming nuclear staining of SLFN11 in internal controls of vascular endothelial cells and tumour-infiltrating lymphocytes for any organs.

Interpreting SLFN11 expression from biopsy samples is challenging because they do not always contain such positive control cells. Unfortunately, in case of unresectable GC that is regularly treated by platinum-based chemotherapy, only biopsy samples are available. A recent paper on prostate cancers showed that SLFN11 expression in circulating tumour cells measured by immunofluorescence was consistent and reflected the response of platinum-based chemotherapy.[18] Although further research is needed, accurate detection of SLFN11 expression with minimal invasiveness by biopsy samples or circulating tumour cells in GC would bring additional clinical advantages.

Utility of SLFN11 in the treatment of GC

Chemotherapy for GC includes platinum derivatives (cisplatin and oxaliplatin), thymidylate synthase (TS) inhibitors (capecitabine and S1, prodrugs of 5-fluorouracil [5-FU]) and an anti-HER2 antibody (trastuzumab). Platinum derivatives induce inter-cross-link lesions that block replication forks, whereas TS inhibitors reduce the production of thymidine, a nucleoside required for DNA replication, leading to inhibition of DNA synthesis. In this study, we revealed that SLFN11 promotes a better response to platinum derivatives but not to TS inhibitors in cell-based assays. Although we have not clarified the mechanisms of the differential responses between these drugs, SLFN11 is recruited to abnormal DNA structures carrying RPA (replication protein A) on single-strand DNA, whose structures are generated by acute replication blocks.[6,22,43] Hence, it is likely that platinum derivatives induce such abnormal structures acutely, whereas TS inhibitors, by slowly reducing replication with keeping normal replication structure, would not in comparison. As SLFN11-relevant drugs include not only platinum derivatives but also topoisomerase and PARP inhibitors,[44] our study could expand the drug choices for GC based on the expression levels of SLFN11.

In summary, we propose that SLFN11 expression assessment in GC, when performed accurately, should be considered as a biomarker predictive of response to platinum treatments, a marker of drug selection beyond platinum derivatives, a cause of refractory and recurrence to platinum derivatives and a druggable target for DDAs with epigenetic modification drugs.

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AUTHOR CONTRIBUTIONS

T.T., N.S. and J.M. designed the study. T.T., D.T., N.S., R.A., R.H., U.K., K.K., K.T. and H.O. collected and analysed the patient clinical data. T.T., D.T, M.Y., R.A., T.H., R.H., P.Q.T., S.U., R.M. and K.H. performed the experiments and collected and analysed the data. K.K., H.O., AT.S., E.M. and W.Y. interpreted and analysed the results. T.T., D.T., N.S., AT.S. and J.M. drafted and edited the paper. All of the authors read and approved the final paper.

ADDITIONAL INFORMATION

Ethics approval and consent to participate This study was approved by the Ethics Committee of Kure Medical Center and Chugoku Cancer Center, Kure, Japan (No. 2019-36), Human Genome Research of Hiroshima University, Hiroshima (E 597 01) and conformed to the ethical guidelines of the Declaration of Helsinki. All patients provided written informed consent to participate in this study.

Consent to publish Not applicable.

Data availability All the data supporting the findings of this study are available within the Supplementary Information files and from the corresponding authors on reasonable request.

Competing interests The authors declare no competing interests.

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