# Original Article
# Novel 5-fluorouracil-resistant human esophageal squamous cell carcinoma cells with dihydropyrimidine dehydrogenase overexpression

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**Abstract:** 5-Fluorouracil (5-FU) is a key drug for the treatment of esophageal squamous cell carcinoma (ESCC); however, resistance to it remains a critical limitation to its clinical use. To clarify the mechanisms of 5-FU resistance of ESCC, we originally established 5-FU-resistant ESCC cells, TE-5R, by step-wise treatment with continuously increasing concentrations of 5-FU. The half maximal inhibitory concentration of 5-FU showed that TE-5R cells were 15.6-fold more resistant to 5-FU in comparison with parental TE-5 cells. TE-5R cells showed regional copy number amplification of chromosome 1p including the *DPYD* gene, as well as high mRNA and protein expressions of dihydropyrimidine dehydrogenase (DPD), an enzyme involved in 5-FU degradation. 5-FU treatment resulted in a significant decrease of the intracellular 5-FU concentration and increase of the concentration of α-fluoro-ureidopropionic acid (FUPA), a metabolite of 5-FU, in TE-5R compared with TE-5 cells *in vitro*. Conversely, gimeracil, a DPD inhibitor, markedly increased the intracellular 5-FU concentration, decreased the intracellular FUPA concentration, and attenuated 5-FU resistance of TE-5R cells. These results indicate that 5-FU resistance of TE-5R cells is due to the rapid degradation of 5-FU by DPD overexpression. The investigation of 5-FU-resistant ESCC with *DPYD* gene copy number amplification and consequent DPD overexpression may generate novel biological evidence to explore strategies against ESCC with 5-FU resistance.

**Keywords:** Esophageal squamous cell carcinoma, chemotherapy, 5-fluorouracil, drug resistance, dihydropyrimidine dehydrogenase

## Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the deadliest cancers worldwide [1-3]; the overall 5-year survival rate of patients with ESCC still ranges from 15 to 25% [3, 4]. Chemotherapy is one of the main therapeutic strategies for advanced or metastatic ESCC [5-7]; however, the efficacy of chemotherapy is limited, with only a 6.7-8.9-month median OS [8-10]. To overcome these issues, the mechanism of chemo-resistance of ESCC should be clarified.

Genomic DNA copy number alterations are frequently found in solid tumors, and they are uti-lized as diagnostic markers as well as indicators of the prognosis and drug efficacy [11]. Recently, genome-wide array comparative genomic hybridization (aCGH) has facilitated the analysis of DNA copy number alterations at high resolution [12], and thereby several DNA copy number alterations, such as *EIF2B5* (chromosome 3q), *MYC* (8q), *CCND1* (11q), and *PARP2* (14q), have been detected in patients with ESCC [13, 14]; however, it has not been elucidated whether such alterations are involved in the drug resistance of ESCC.

5-Fluorouracil (5-FU) is a key drug in first-line therapy against ESCC [15]. 5-FU metabolism comprises anabolic and catabolic processes

[16]. To exert its cytotoxicity, 5-FU enters an anabolic process in which it disrupts nucleic acids through various enzymes, such as thymidylate synthase (TS) [16]. On the other hand, 5-FU is degraded by dihydropyrimidine dehydrogenase (DPD), the initial and rate-limiting enzyme encoded by the *DPYD* gene located on the short arm of chromosome 1 (1p21.3) [16, 17], to its metabolites including α-fluoroureidopropionic acid (FUPA). As DPD expression in tumors increases 5-FU resistance [18-22] and exacerbates the prognosis of patients treated with 5-FU [23, 24], DPD is a critical mediator influencing the 5-FU resistance of cancers [25-27].

In this study, we established novel 5-FU-resistant ESCC cells, TE-5R, derived from parental TE-5 cells, and investigated the mechanisms of 5-FU resistance in these cells. We revealed that high DPD expression due to *DPYD* gene copy number amplification is involved in TE-5R cells acquiring 5-FU resistance.

## Materials and methods

### Establishment of 5-FU-resistant ESCC cells

Human ESCC cells, TE-5, were obtained from Riken BioResource Center (Ibaragi, Japan) [28]. The cells were cultured in RPMI1640 medium (Life Technologies Corp., Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Life Technologies Corp.), 100 µg/mL of streptomycin, and 100 units/mL of penicillin (Life Technologies Corp.) at 37°C in a 5% $CO_2$ incubator. TE-5 cells were treated with continuous and step-wise concentrations of 5-FU (1, 2, 5, and 10 µM), based on previous reports [29, 30]. Consequently 5-FU-resistant ESCC cells were established, and named TE-5R cells. Both TE-5 and TE-5R cells have been verified by short tandem repeat analysis and confirmed to be consistent with each other and with the original cell source from Riken BioResource Center.

### WST-1 cell proliferation assays

5-FU resistance of TE-5R cells was assessed by the WST-1 assay. TE-5 and TE-5R cells (5 × $10^3$ cells) were seeded in 96-well plates, and treated with the indicated concentrations of 5-FU for 72 h. Cell viability was measured with Cell Proliferation Reagent WST-1 (Roche Applied Science, Upper Bavaria, Germany) following the

manufacturer's instructions. All data were obtained in sextuplicate. The half maximal inhibitory concentration ($IC_{50}$) of 5-FU in each cell was calculated by probit analysis [31], and the resistance ratio was determined by comparing to the $IC_{50}$ values of parental cells.

### *Genomic DNA preparation, array-comparative genomic hybridization (aCGH) experiments, and copy number assays*

To compare the genomic alterations between TE-5 and TE-5R cells, we performed aCGH analysis. Genomic DNA was extracted from TE-5 and TE-5R cells using AllPrep DNA/RNA/Protein Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. aCGH experiments were performed with SurePrint G3 Human CGH Microarray Kit 2 × 400K (G4448A, Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. Raw aCGH data were analyzed and processed using CytoGenomics 3.0 software (Agilent Technologies). The aCGH data set is available at Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE69494.

To determine the copy number, quantitative real-time PCR was performed with a PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA) using a TaqMan Gene Expression Master Mix (Applied Biosystems) and TaqMan Copy Number Assays: *LPHN2* (Hs00381445_cn), *DPYD* (Hs02103-805_cn), and *PALMD* (Hs01617339_cn). Ribonuclease P RNA component H1 (*RPPH1*), and Human Genomic DNA (Cat No. G3041, Promega Inc.) were used as the endogenous control. The TaqMan copy number assay contained 1 µL of *LPHN2*, *DPYD*, and *PALMD* probes (20 ×, FAM-labeled), 1 µL of *RPPH1* probe mix (20 ×, VIC-labeled), 10 µL of TaqMan Gene Expression Master Mix (2 ×), 4 µL of genomic DNA, and 4 µL of water. PCR cycling conditions were 95°C for 10 minutes, followed by 2-step cycling: 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A manual cycle threshold of 0.2 and an automatic baseline were used to detect the template quantity of target genes and *RPPH1* gene by sequence detection system software (ABI, version 2.4). The target probes and endogenous control were loaded in the same well, and each reaction was performed in triplicate. CopyCaller
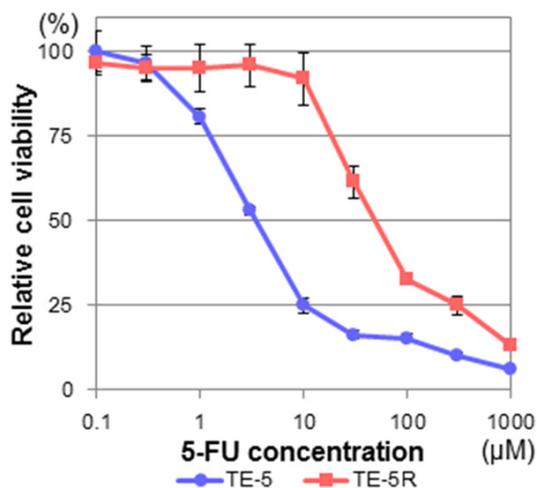
**Figure 1.** 5-FU resistance of TE-5R cells. TE-5 and TE-5R cells were treated with the indicated concentrations of 5-FU for 72 h, and cell viability was assessed using the WST-1 assay. A viability of 100% was defined as the amount of absorption at 450 nm in untreated cells. The mean value ± S.D. of six replicate wells from a representative experiment is shown. Each experiment was repeated at least three times, and consistent results were obtained. $IC_{50}$ values of TE-5 and TE-5R cells were 3.6 ± 1.1 and 55.5 ± 10.1 µM, respectively. Note that TE-5R cells were 15.6-fold more resistant to 5-FU in comparison with parental TE-5 cells.

software (ABI, version 2.0) was used to calculate the copy number of each probe based on real-time PCR data. Copy numbers of each target gene were calculated as the average of those based on two reference genes.

*RNA isolation, cDNA synthesis, and real-time reverse transcriptase-PCR (RT-PCR)*

RNA extraction and cDNA synthesis were conducted as previously described [32, 33]. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed with LightCycler 480 Instrument II Real-Time PCR System (Roche Diagnostics Ltd., Rotkreuz, Switzerland). The relative level of each mRNA was normalized to *ACTB* as an internal control. The primers used in this study were as follows: *DPYD* (Hs_DPYD_1_SG, QuantiTect Primer Assay, QIAGEN GmbH) and *ACTB* (Hs_ACTB_1_SG, QuantiTect Primer Assay).

*Protein extraction and Western blotting*

Whole-cell lysates were collected according to prior reports [34-36], and Western blotting was performed as described previously [37].

Primary antibodies and their titers were as follows: rabbit monoclonal anti-DPD antibody (D35A8, #4654, Cell Signaling Technology, Inc., Danvers, MA, USA; 1:1,000) and rabbit monoclonal anti-β-actin antibody (13E5, #4970, Cell Signaling Technology, Inc.; 1:2,000). β-Actin served as a loading control for whole-cell lysates. The band intensity was quantified using Image Lab 4.1 software (Bio-Rad Laboratories, Hercules, CA, USA).

*Measurement of intracellular concentrations of 5-FU and its metabolite*

The intracellular concentrations of 5-FU and FUPA, a metabolite of 5-FU, were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [38]. TE-5 and TE-5R cells ($5 \times 10^5$ cells) were seeded in 6-well plates with 2 mL of RPMI1640 medium supplemented with 10% fetal bovine serum (Life Technologies Corp.), and treated with 5-FU (10 µM) for 24 h. Cells were harvested and homogenized in methanol (300 µL). The samples were centrifuged and supernatants were filtrated through a filter (SGJVL; 0.45 µm; Millipore, Billerica, MA, USA) and injected into the LC-MS/MS system. The LC-MS/MS assay was performed using a liquid chromatography system consisting of a Prominence series chromatograph (Shimadzu, Kyoto, Japan) coupled to an API 4000 triple-quadrapole tandem mass spectrometer (AB SCIEX, Foster City, CA, USA). Chromatographic separation was carried out on COSMOSIL® $5C_{18}$-MS-II (Nacalai Tesque, Kyoto, Japan). The MS/MS analysis was performed using an elector-spray ionization source in the negative mode. Detection was carried out by monitoring the ion transition of 5-FU from *m/z* 129.0 to 42.0 and FUPA from *m/z* 149.0 to 106.0, respectively [38].

*DPD inhibitor treatment*

A DPD inhibitor, 5-chloro-2,4-pyridinediol (gimeracil), was synthesized by Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan). TE-5 and TE-5R cells ($5 \times 10^3$ cells) were plated in a 96-well plate and cultured for 24 h, and then they were treated with the indicated concentrations of 5-FU in the presence of gimeracil for 72 h at a molar ratio of 1:0.2 (5-FU:gimeracil), referring to a previous report [39]. The effect of gimeracil was assessed by the WST-1 assay and measuring intracellular concentrations of 5-FU and its metabolite, as mentioned above.
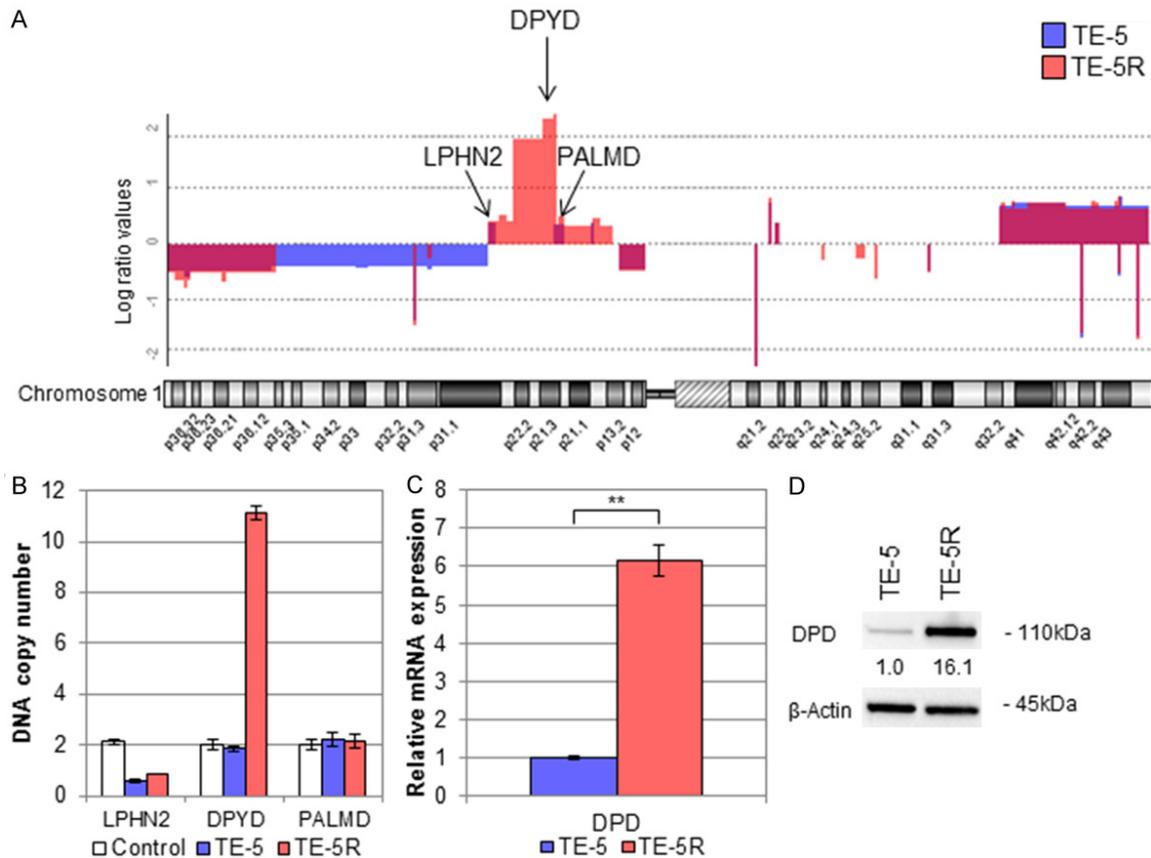
**Figure 2.** Amplification of *DPYD* gene and subsequent DPD expression in TE-5R cells. A. Chromosome 1 copy number alteration in aCGH analysis. The vertical axis indicates the log (base 2) ratio of DNA expression. TE-5R harbored specific regional amplification of the short arm of chromosome 1 around 1p2 including the *DPYD* gene (1p21.3). Note that *LPHN2* and *PALMD* genes are not located in the specific amplified regions. B. qPCR analysis using *LPHN2*, *DPYD* and *PALMD* gene probes. The *DPYD* gene is amplified to produce 10 copies in DNA samples derived from TE-5R cells, but not non-cancerous human genomic DNA or parental TE-5 cells. On the other hand, *LPHN2* and *PALMD* genes are not amplified in any samples. C. DPD mRNA expression levels in TE-5 and TE-5R cells. qPCR revealed significantly increased DPD mRNA expression in TE-5R compared with TE-5 cells. $^{**}P < 0.01$. D. DPD protein expression levels in TE-5 and TE-5R cells. The relative density was calculated by densitometry. β-Actin served as a loading control. The DPD protein expression level in TE-5R cells was higher than in TE-5 cells.

*Statistical analyses*

All data were analyzed by the 2-tailed Student's t-test, and are presented as the mean ± standard deviation (SD). $P < 0.05$ was considered significant. All statistical analyses were performed with SPSS 19 for Windows software (SPSS Inc., Chicago, IL, USA).

**Results**

*Establishment of 5-FU-resistant ESCC TE-5R cells*

TE-5R cells could grow exponentially in the presence of 5-FU (10 μM), although TE-5 cells could not grow under the same condition (data not shown). Dose-response curves indicated that the $IC_{50}$ values of TE-5 and TE-5R cells were 3.6 ± 1.1 and 55.5 ± 10.1 μM, respectively (**Figure 1**), and TE-5R cells had a significantly high $IC_{50}$ value ($P < 0.001$, vs TE-5 cells). Accordingly, TE-5R cells were 15.6-fold more resistant to 5-FU in comparison with parental TE-5 cells. Cross-resistance (e.g., cisplatin and docetaxel) was not noted in TE-5R cells (data not shown). The 5-FU resistance of TE-5R cells was not diminished even though they were cultured in a 5-FU-free medium for eight weeks (data not shown), and so TE-5R cells were incubated with 5-FU-free medium for at least one week prior to use in the subsequent experiments.
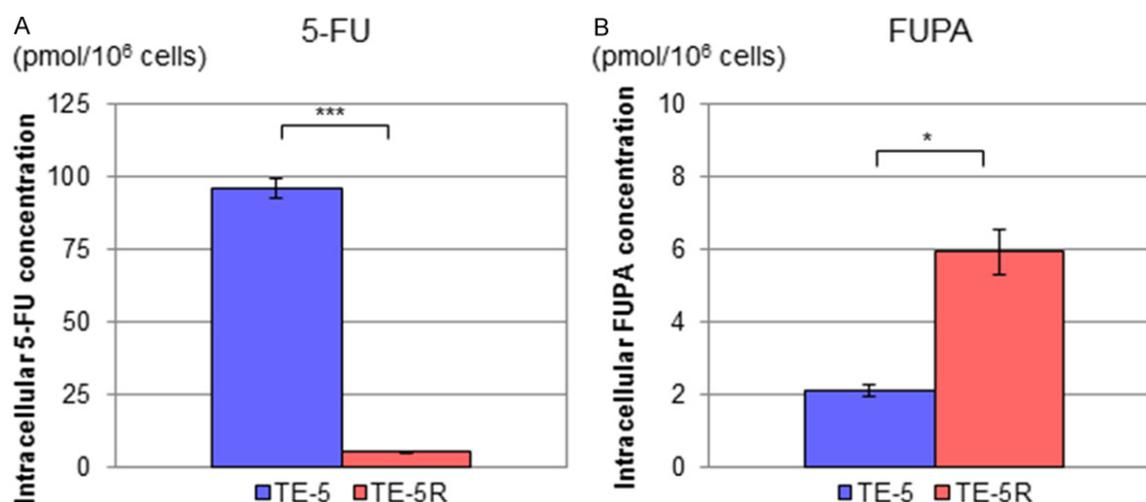
**Figure 3.** Intracellular 5-FU and FUPA concentrations in TE-5 and TE-5R cells treated with 5-FU. TE-5 and TE-5R cells ($5 \times 10^5$ cells) were seeded in 6-well plates, and then treated with 5-FU (10 μM) for 24 h. Cells were harvested, and levels of 5-FU and the FUPA concentration were measured with liquid chromatography-tandem mass spectrometry. A. Intracellular 5-FU concentrations in TE-5 and TE-5R cells treated with 5-FU. Intracellular 5-FU concentrations in TE-5R cells were significantly lower than those in TE-5 cells (n = 3). ***$P < 0.001$. B. Intracellular FUPA concentrations in TE-5 and TE-5R cells treated with 5-FU. Intracellular FUPA concentrations in TE-5R cells were significantly higher than in TE-5 cells (n = 3). *$P < 0.05$.

*Regional chromosome 1p amplification and DPD mRNA/protein overexpression in TE-5R cells*

To compare the fundamental gene alterations between TE-5 and TE-5R cells, we performed aCGH analysis, and found that TE-5R cells acquired regional gene amplification in the short arm of chromosome 1 (1p) including the *DPYD* gene (**Figure 2A**). Quantitative PCR analysis revealed that DNA copies of the *DPYD* gene, which were located within chromosome 1p (**Figure 2A**), were highly amplified in TE-5R cells, but not the *LPHN2* or *PALMD* gene (**Figure 2B**), located outside the specific amplified region (**Figure 2A**). Moreover, DPD mRNA as well as protein expression levels in TE-5R cells were also much higher than those in TE-5 cells (**Figure 2C**, **2D**).

*Intracellular concentrations of 5-FU and its metabolite in TE-5 and TE-5R cells*

To address the functional role of DPD overexpression in TE-5R cells, we measured intracellular concentrations of 5-FU and FUPA in TE-5 and TE-5R cells treated with 5-FU (10 μM) for 24 h. The intracellular 5-FU concentration in TE-5R cells ($5.4 \pm 0.3$ pmol/$10^6$ cells) was markedly lower than in TE-5 cells ($96.2 \pm 3.3$

pmol/$10^6$ cells; $P < 0.001$, **Figure 3A**). Conversely, FUPA concentrations in TE-5R cells ($5.9 \pm 1.6$ pmol/$10^6$ cells) were significantly higher than in TE-5 cells ($2.1 \pm 0.2$ pmol/$10^6$ cells, $P = 0.014$, **Figure 3B**).

*Effects of a DPD inhibitor on 5-FU resistance of TE-5R cells*

Furthermore, we assessed the effects of a DPD inhibitor, gimeracil, on 5-FU degradation in TE-5R cells. As shown in **Figure 4**, gimeracil markedly increased the intracellular 5-FU concentration in TE-5R cells from $5.4 \pm 0.3$ to $62.0 \pm 0.3$ pmol/$10^6$ cells (**Figure 4A**, $P < 0.001$) and decreased the FUPA concentration from $5.9 \pm 1.6$ to $0.6 \pm 0.6$ pmol/$10^6$ cells (**Figure 4B**, $P = 0.006$). Thus, gimeracil significantly reduced 5-FU degradation in TE-5R cells.

Next, we investigated the effects of gimeracil on 5-FU resistance in TE-5R cells. $IC_{50}$ values with and without gimeracil in TE-5 cells were $4.9 \pm 0.9$ and $4.8 \pm 0.9$ μM, respectively (**Figure 4C**, $P = 0.881$), and those in TE-5R cells were $20.8 \pm 9.2$ and $59.0 \pm 14.3$ μM, respectively (**Figure 4D**, $P = 0.023$). Thus, gimeracil significantly reduced the 5-FU resistance of TE-5R cells, but did not affect the 5-FU sensitivity of TE-5 cells.
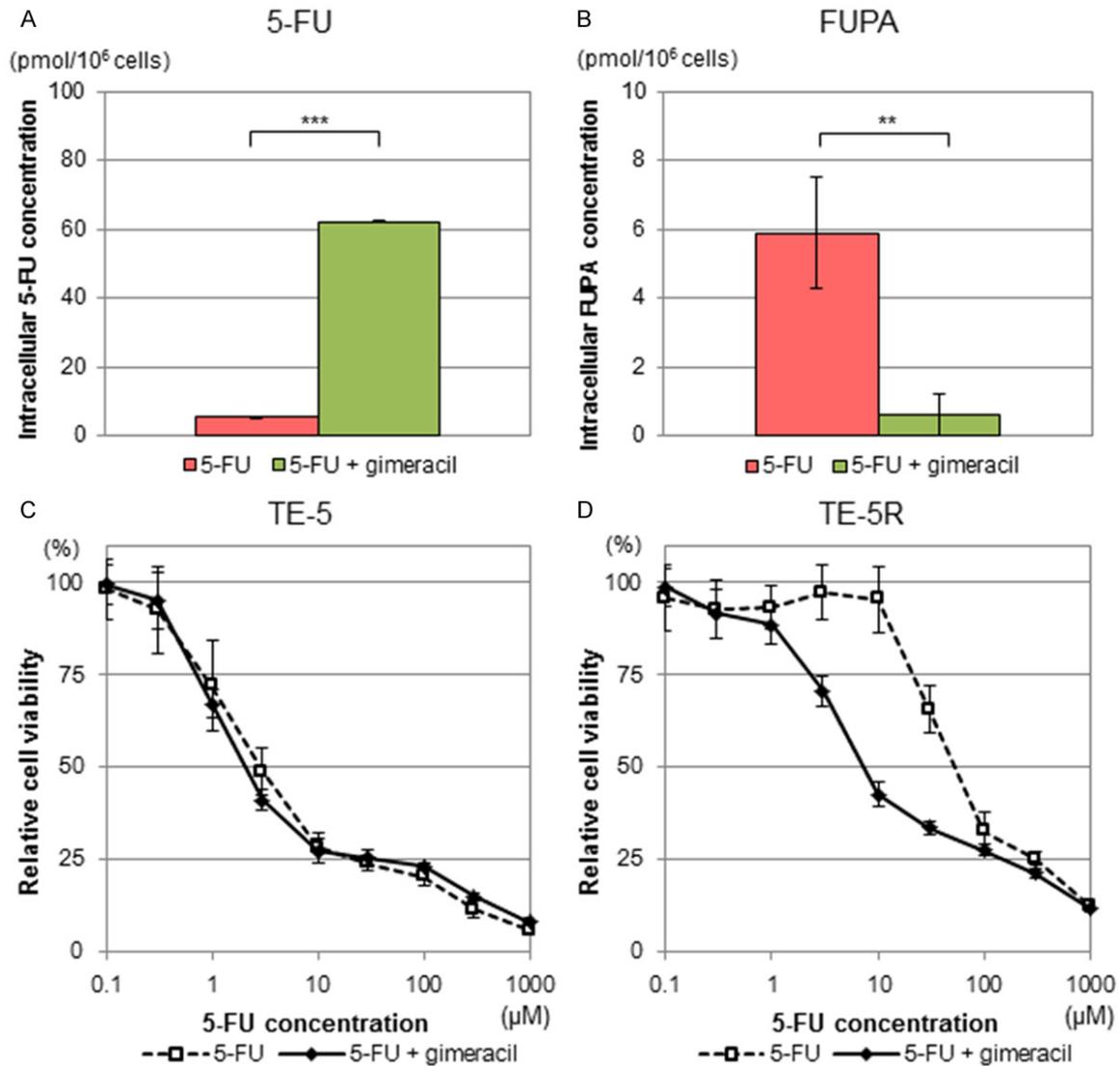
**Figure 4.** Effects of DPD inhibitor on TE-5R cells. A DPD inhibitor, gimeracil, was added to the culture medium with the indicated concentrations of 5-FU at a molar ratio of 1:0.2 (5-FU:gimeracil) for 24 h. (i.e., 2 µM gimeracil to 10 µM 5-FU). (A) Intracellular 5-FU concentrations in TE-5R cells in the presence or absence of gimeracil. Intracellular 5-FU concentrations in TE-5R cells treated with 5-FU and gimeracil were markedly higher than those treated with 5-FU alone. (n = 3). ***$P < 0.001$. (B) Intracellular FUPA concentrations in TE-5R cells in the presence or absence of gimeracil. Intracellular FUPA concentrations in TE-5R cells treated with 5-FU and gimeracil were significantly lower than those treated with 5-FU alone. (n = 3). **$P < 0.01$. (C, D) Effects of gimeracil on 5-FU sensitivity of TE-5 (C) and TE-5R (D) cells. Gimeracil was added to the culture medium with the indicated concentrations of 5-FU at a molar ratio of 1:0.2 (5-FU:gimeracil) for 72 h. Cell viability was assessed by the WST-1 assay. A viability of 100% was defined as the amount of absorption at 450 nm in untreated cells. Each point represents the mean ± S.D. of sextuplicate wells. Note that gimeracil significantly attenuated 5-FU resistance in TE-5R but not TE-5 cells. Representative data from three independent experiments are shown.

## Discussion

In this study, we established novel 5-FU-resistant ESCC cells, TE-5R, by exposing parental TE-5 cells step-wisely to continuously increasing concentrations of 5-FU. TE-5R showed higher DPD expressions and a more rapid degradation of intracellular 5-FU than TE-5 cells. Furthermore, the DPD inhibitor, gimeracil, rapidly elevated the intracellular 5-FU concentration in TE-5R cells treated with 5-FU, and consequently reduced 5-FU resistance. These results indicate that the 5-FU resistance of TE-5R cells is based on DPD-

dependent metabolism. To the best of our knowledge, this is the first report on elucidating the mechanism of 5-FU resistance in ESCC cells.

Our study showed that DPD played a key role in the acquisition of 5-FU resistance by TE-5R cells. DPD is produced in various tumors, including liver [40], breast [24], colorectal [41], and oral [42] cancers; however, the mechanisms of DPD production in cancer cells have not been fully clarified. In the present study, aCGH analysis revealed the regional gene amplification of chromosome 1p including the DPYD gene in TE-5R cells. Consistently, expression levels of DPD mRNA as well as protein were increased comparably to DPD copy number amplification. These findings support the importance of DPD in 5-FU resistance. As DPD expression was not enhanced by short-term (one week) treatment with 5-FU (data not shown), we suggest that long-term 5-FU exposure may lead to the selection of a unique subset of TE-5 cells expressing high levels of DPD.

This was an in vitro study of 5-FU resistance with DPD copy number amplification in ESCC cells. DNA is more stable than mRNA or protein expression [43], and so analysis of copy number alterations, such as of the DPYD gene, might be useful for predicting 5-FU resistance in ESCC patients. We should further examine whether DPD expression is increased by 5-FU treatment in clinical samples.

Consistent with the result that DPD is highly expressed in TE-5R cells, we demonstrated a lower intracellular 5-FU concentration, higher FUPA concentration, and weaker 5-FU cytotoxicity in TE-5R than TE-5 cells. These data suggest that high DPD expression contributes to the degradation of 5-FU in TE-5R cells, which consequently decreases the intracellular 5-FU concentration as well as 5-FU cytotoxicity. Furthermore, we showed that the DPD inhibitor, gimeracil, elevated intracellular 5-FU levels in TE-5R cells treated with 5-FU and attenuated 5-FU resistance. As gimeracil was effective for high DPD-overexpressing ESCC cells such as TE-5R cells, but not low or non-DPD-expressing ESCC cells (data not shown), it is considered to be specifically efficient for the high DPD-expressing ESCC cells. Gimeracil has already been integrated into S-1, a combination drug containing tegafur (5-FU prodrug), potassium oxonate, and gimeracil, and clinically utilized for

several cancers, such as gastric and colon cancers [44]. Therefore, we suggest that the therapeutic utility of S-1 should be verified for ESCC patients with high DPD expression.

In the present study, the growth of TE-5R cells was not accelerated in comparison with that of TE-5 cells, and, moreover, TE-5R cells did not form xenografted tumors in nude mice (data not shown). Thus, high DPD expression did not influence cell growth or tumorigenicity, suggesting that DPD overexpression may not affect the biological characteristics of cancer cells directly. Furthermore, we analyzed other possible underlying mechanisms of 5-FU resistance, such as the modulation of 5-FU metabolism-related proteins [45], MDR1 overexpression [46], epithelial-mesenchymal transition [30], or deregulation of apoptosis [47]; however, none of them were associated with the 5-FU resistance of TE-5R cells (data not shown). As other anti-cancer drugs such as cisplatin and docetaxel were equally cytotoxic to TE-5R cells, the drug resistance of these cells might be limited to 5-FU.

A limitation of this study was that we established only a single 5-FU-resistant cell line; however, further examination of this cell line will contribute to the development of a novel therapeutic strategy against 5-FU-resistant ESCC.

Overall, we established unique 5-FU-resistant ESCC cells with DPD overexpression. Our findings provide not only useful knowledge to explore the mechanism of 5-FU resistance, but also suggest the possibility of combination therapy with gimeracil targeting 5-FU-resistant refractory ESCC.

### Disclosure of conflict of interest

None.

## Abbreviations

ESCC, esophageal squamous cell carcinoma; 5-FU, 5-fluorouracil; TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase; FUPA, α-fluoro-ureidopropionic acid; $IC_{50}$, half maximal inhibitory concentration; RT-PCR, reverse transcriptase-polymerase chain reaction; aC-GH, array comparative genomic hybridization.

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