INTRODUCTION

Metabolic reprogramming is a major hallmark of cancer and is characterized by the alteration of glycolysis, glutaminolysis, lipid metabolism, amino acid metabolism, mitochondrial metabolism, nucleic acid biogenesis, pentose phosphate pathway, autophagy-lysosomal degradation pathway, and other biosynthetic and bioenergetic pathways. These cancer metabolisms provide tumor cells not only with essential energy, but also with the materials required to support large-scale biosynthesis for their rapid proliferation, survival, stemness, metastasis, and resistance to anticancer drugs. As cancer is a genetic disease caused primarily by genetic and epigenetic alterations, metabolic reprogramming is often triggered by gene aberrations, including copy number aberrations (gene amplification...
and deletion) and somatic mutations of metabolism-related genes. Thus, the metabolism is often reprogrammed to meet the energetic and substrate demands of the tumor; however, this reprogramming often generates metabolic vulnerabilities in the tumor, providing new opportunities for cancer therapy. Therefore, the identification of metabolically essential genes for cancer cells leads the development of novel cancer therapies targeting the specific metabolic vulnerability.

Esophageal cancer is the eighth most common cancer and the sixth most common cause of cancer-related death worldwide. There are two major histological types of esophageal cancer: squamous cell carcinoma and adenocarcinoma. In Asia, the most common type is ESCC, which is associated with environmental factors such as chronic smoking and alcohol consumption. Although our understanding of the pathogenesis has improved and there have been advances in therapeutic strategies, the 5-year relative survival rate of patients with ESCC with distant metastasis remains low at only 4.3% due to rapid progression, local recurrence, and distant metastasis. Recent studies demonstrated that immunotherapy can improve the survival of patients with locally advanced and metastatic ESCC. However, novel therapeutic strategies are needed for advanced ESCC and the molecular pathogenesis of this disease remains unclear. We previously identified many genes related to the pathogenesis of ESCC by high-throughput screening of copy number aberrations and epigenomic alterations, including DNA methylation. Among them, genes expected to be druggable targets were found. In particular, small molecule compounds for the inhibitor of cIAP1/2, which we identified as a target of 11q22 amplification in ESCC, have been developed. However, metabolic vulnerability as a therapeutic target in ESCC has not been adequately understood.

Cancer cells utilize aerobic glycolysis, known as the Warburg effect, to generate energy for their survival and proliferation due to mitochondrial dysfunction resulting from the mutations of genes coding enzymes associated with the TCA cycle. Although there is interest in the glycolytic phenotype of many cancers, recent studies have demonstrated that cancer cells require fully functional mitochondria to support their proliferation and survival. The connecting link between glycolysis and the TCA cycle is the PDH complex, which catalyzes pyruvate, a metabolite from glucose, to acetyl-CoA in the mitochondria. Thus, the PDH complex is a rate-limiting enzyme that connects glycolysis to the TCA cycle, and consists of E1 (PDH composed of catalytic α [PDHA] and regulatory β [PDHB] subunits), E2 (dihydrolipoyl transacetylase [DLAT]), E3 (dihydrolipoyl dehydrogenase [DLD]), and PDHX as an E3-binding protein. The inhibition of PDH activity induces aerobic glycolysis as the Warburg effect by catalyzing pyruvate to lactate in cancer cells, promoting the tumor suppressor function of the PDH complex. In contrast, inhibition of PDH activity by depleting PDHB was reported to resemble the Warburg effect, inhibiting breast cancer growth. Therefore, the role of the PDH complex in cancer is controversial and may be dependent on the cellular context, including the pathological condition of cancer cells. In this study, using siRNA-based screening for genes related to several metabolic pathways, we identified PDHX as a metabolically essential gene for the growth of ESCC cells. Our study provides new insights related to the development of novel therapeutic strategies against ESCC by targeting PDHX as a key molecule in its metabolic vulnerability.

2 | MATERIALS AND METHODS

2.1 | Antibodies and reagents

The antibody against PDHX (10951-1-AP) was purchased from Proteintech, the antibody against β-actin (A5441) was from Sigma-Aldrich, the antibody against CD44v9 (RV3) was from Cosmo Bio, and the antibody against Ki-67 was from DAKO (#M7240). CPI-613 was purchased from Cayman Chemical.

2.2 | Construction of the siRNA library and cell growth assay

The Cherry-Pick siRNA library containing four pooled siRNAs for 224 metabolism-related genes was synthesized by Horizon Discovery. Cells (1 × 10⁴ cells/well) were plated in 96-well plates and siRNA was transfected the next day at 10 nmol/L using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturer’s instructions. After 3 days, cells were stained with 0.1% crystal violet (CV) as described in previous papers. The stained cells were lysed with a 2% SDS solution and the optical density (OD) was measured at 560 nm using a microplate reader. The OD values of cells relative to the control cells transfected with pooled siRNA for the negative control (siNC) were arbitrarily set to 100% to determine the percentage of viable cells.

2.3 | In vivo tumor growth assay

All animal experiments were carried out according to the guidelines and approval by the Tokyo Medical and Dental University Animal Care and Use Committee as described in previous papers. Six-week-old female BALB/c nude mice were purchased from Charles River Laboratories. Cells (1 × 10⁷ cells in 100 µL of PBS with 50% Matrigel) were injected subcutaneously into the flank of nude mice. The tumor volume was calculated using the following formula: V = 4/3 × π × (1/2 × smaller diameter)² × (larger diameter)/2. shVector cells and PDHX-inhibited HSC-40A cells (1 × 10⁷ cells in 200 µL of PBS) were injected into the intraperitoneal cavity of nude mice. For treatment with CPI-613, when the tumor volume reached 100-150 mm³, CPI-613 was administered by intraperitoneal (IP) injection at 25 mg/kg two times per week. D5W (5% dextrose in water) was used as a control vehicle.
2.4 | Tumor sphere formation assay

Cells were seeded at a density of $10^3$ cells/ml in 6-well Ultra-Low Attachment Plates in DMEM-F12 1:1 media (Wako) containing 1x B-27 supplement (Thermo Fisher Scientific), 4 μg/mL of insulin (L6634, Sigma-Aldrich), 20 ng/mL of FGF2 (F0291, Sigma-Aldrich), 10 ng/mL of EGF (E9644, Sigma-Aldrich), and 5% Pen/Strep (Thermo Fisher Scientific). At 10 days, three random fields per well were digitally imaged using an EVOS cell imaging system (Thermo Fisher Scientific). The diameters of each sphere were measured on the long axis using Adobe Photoshop Elements 14 (Adobe). The average size of spheres from three wells was calculated and tumor spheres with a diameter $>70$ μm were counted.

2.5 | Publicly available data for the gene expression and the copy number in human cancer

An expression dataset (GSE44021) for tumor and nontumor tissue in 113 paired samples of primary ESCC was downloaded from the Gene Expression Omnibus (GEO). The frequencies of genes highly expressed in tumor tissues relative to the corresponding nontumor tissue were calculated. The Cancer Genome Atlas (TCGA) data for the expression and copy number were downloaded from the “cBioportal” website (https://www.cbioportal.org). For correlation analysis in 92 ESCC cases, a log2 transformation was applied to the expression values and copy number values, and the Pearson scores for the correlation were computed. The top 20% (18 of 92 cases) of the cohort was considered “PDHX-high” and the remaining was considered “PDHX-low”.

2.6 | Statistical analysis

Significance was assessed by the two-tailed Student’s t-test using GraphPad Prism Version 7.0 (GraphPad Software, Inc.). The correlation between PDHX expression in primary ESCC samples and the clinicopathological variables was analyzed using the chi-squared test. Differences with $P$ values lower than .05 were considered to be significant.

Additional materials and methods are described in the Supporting Information.

3 | RESULTS

3.1 | Identification of metabolically essential genes for cell growth in ESCC

To identify the metabolic vulnerability in the cell growth of ESCC, we constructed an siRNA library containing each of four pooled siRNAs for a total of 224 genes related to various metabolic pathways, including the TCA cycle and glycolysis, referring to the KEGG pathway (https://www.genome.jp/kegg/pathway.html) (Tables S1 and Table S2). We then transfected each of the four pooled siRNAs for 224 genes into two ESCC cell lines, KYSE850 and KYSE170 cells, and identified 43 genes causing a greater than 30% reduction in cell survival rate in both cell lines (Figure 1A and Table S3). Furthermore, the in silico analysis of the expression dataset (GSE44021), which was obtained by NCBI Gene Expression Omnibus (GEO), revealed that the expression of 13 of 43 genes was frequently upregulated in ESCC tumor tissues relative to the corresponding nontumor tissues in $>$30% of 113 ESCC cases (Figure 1B). They included genes related to the TCA cycle (PC, PDK1, PDHX), glycolysis (LDHB, HK2, HIF1A), amino acid metabolism (ASNS, SHMT2, PSAT1, DHFR, GLDC), nucleotide metabolism (PRPS1), and autophagy (RB1CC1), suggesting possible metabolic vulnerability in ESCC (Table S3). Among these 13 genes, we focused on the PDHX (PDHX) gene because its oncogenic significance is largely unknown. When the pooled siRNAs (named siPDHX-1) and two individual siRNAs (siPDHX-2 and -3) were transfected into KYSE850 and KYSE170 cells, respectively, we confirmed the inhibition of cell growth following effective knockdown of PDHX expression (Figure 1C). This growth inhibition was also demonstrated by shRNA-mediated knockdown of PDHX expression (Figure 1D). Furthermore, tumor growth in xenograft mice was markedly suppressed in PDHX-inhibited KYSE850 cells compared with the control cells (shVector) (Figure 1E). Tumors composed of PDHX-inhibited KYSE850 cells had a significant reduction in the number of Ki67-positive cells, a marker of cell proliferation, compared with tumors composed of control cells (shVector) (Figure 1F). Therefore, PDHX expression was suggested to be required for the growth of ESCC cells in vitro and in vivo.

PDHX is a component of the PDH complex that catalyzes the conversion of pyruvate to acetyl-CoA, thereby connecting glycolysis to the TCA cycle, where it can generate ATP as a form of energy in the mitochondria (Figure 2A). Inhibition of PDH activity suppresses this conversion, resulting in glycolysis via the conversion of pyruvate to lactate by lactate dehydrogenase (18,19,24). When PDHX expression was inhibited in KYSE850 and KYSE170 cells, PDH activity was reduced, followed by an increase in intracellular lactate and pyruvate (Figures 2B,C and Figure S1). Furthermore, the production of intracellular ATP markedly decreased in PDHX-inhibited cells compared with the control cells (shVector) (Figure 2D). A decrease in ATP production was also observed after siRNA-mediated knockdown of PDHX expression (Figure 2E). Taken together, PDHX plays a metabolically essential role in the growth of ESCC cells through the maintenance of PDH activity followed by ATP production via the TCA cycle.

3.2 | Expression status of PDHX in primary ESCC samples

We next examined the expression levels of PDHX protein by immunohistochemical analysis in 70 primary ESCC samples. PDHX expression was assessed using the Histoscore (H-score) based on the staining intensity and percentage of positive cells. In normal
esophagus tissue, PDHX is moderately expressed in differentiating cells, but not in the basal layer, as the main location of stem cells ($H$-score = 100) (Figure 3A). When cases with an $H$-score of >150 were defined as having "up-regulation", as demonstrated by the immunostaining of the ESCC tumor in case 1 (Figure 3A), PDHX expression was up-regulated in 24 of 70 ESCC cases (34.3%) (Figure 3B). However, there was no significant association between clinical factors and PDHX expression status (Table S4). Of note, the distribution of PDHX staining was heterogeneous within the tumor and its expression was lower in the keratin pearl, a structure representing terminal differentiation of cancer cells, than that around undifferentiating cells (Figure 3C). A similar distribution of PDHX expression was observed in xenograft tumors of KYSE850 control cells (shVector) (Figure 3D). These observations suggested an association
between cancer stemness and PDHX expression. CD44, especially the variant form (CD44v9), is a well-known marker of CSCs in human cancers, including ESCC, and is also known as an adhesion molecule localized on the cell surface of CSCs. Immunofluorescence analysis revealed that PDHX was co-expressed in CD44v9-positive stem cells within the ESCC tumor tissue; however, this was not the case in CD44v9-positive stem cells within the basal layer of adjacent noncancerous tissue (Figure 3E). Thus, PDHX expression may be closely associated with CSC properties in ESCC cells.

3.3 PDHX is involved in the proliferation of CSCs in ESCC by regulating CD44 expression

To evaluate the effects of PDHX knockdown on the proliferation of CSCs in ESCC cells, we performed a sphere formation assay using control cells (shVector) and PDHX-inhibited cells (shPDHX). The number and size of the spheroids were markedly reduced in PDHX-inhibited cells compared with control cells in KYSE850 and KYSE170 cells (Figure 4A). This inhibitory effect on sphere formation was confirmed by PDHX knockdown via another target sequence (Figure S2). Of note, the expression of both PDHX and CD44 genes was transcriptionally upregulated under the sphere condition compared with the two-dimensional (2D) condition. Moreover, the level of CD44 expression markedly decreased in PDHX-inhibited cells compared with that in control cells (shVector) under both culture conditions (Figure 4B). This decrease of CD44 expression was also observed after siRNA-mediated knockdown of PDHX expression in ESCC cells (Figure S3). Furthermore, fluorescence-activated cell sorting (FACS) and immunofluorescence analyses using specific antibodies against CD44v9 protein revealed that the population of CD44v9-positive CSCs markedly decreased within the spheroids from PDHX-inhibited cells compared with those from control cells (Figure 4C,D). Thus, PDHX expression is closely associated with the proliferation of CSCs in ESCC by positively regulating CD44 expression.

3.4 Co-amplification of PDHX and CD44 genes in human cancers, including ESCC tumors

Both PDHX and CD44 genes are located within 200 kb in the 11p13 region of the chromosome, which is well known for gene amplification in many types of human cancers, including gastric (stomach) cancer (Figure S3). Indeed, these two genes were involved in co-amplification in 114 of 10,967 cases (1.04%) of 32 cancer types in the TCGA PanCancer Atlas study, and their mutation frequencies were higher in gastric cancer (stomach adenocarcinoma) (17 of 440 cases, 3.9%), ESCC (3 of 92 cases, 3.3%), and head and neck squamous cell carcinoma (14 of 523 cases, 2.7%) (Figure 5A,B). Analysis of the copy number ratio of the genomic region within 15 Mb of 11p12-p13 revealed that the PDHX and CD44 genes were localized within the smallest region of overlapping (SRO) in three ESCC cases.
with the 11p13 amplification, strongly suggesting that these genes are targets of this amplicon (Figure 5C). In a correlation analysis of 92 ESCC cases, mRNA expression levels of PDHX and CD44 were positively correlated with their respective copy numbers, and the mRNA levels of both genes were significantly correlated (Figure 5D). We found that PDHX was co-amplified with CD44 in YES3 cells, an ESCC cell line, using genomic-PCR and FISH analyses, and its knockdown inhibited cell growth under both 2D and sphere culture conditions (Figure S4). In addition, our previous study demonstrated that the 11p13 region is frequently amplified in gastric cancer cell lines, including HSC-40A cells, which were established from the ascites of a patient with gastric cancer, and the CD44 gene is a potential target of this 11p13 amplification (28). Moreover, the PDHX and CD44 genes were co-amplified in HSC-40A cells (Figure S5A,B), and PDHX knockdown inhibited in vitro cell growth and in vivo tumor formation in the peritoneal cavity after intraperitoneal injection into nude mice (Figure S5C,D). Thus, PDHX and CD44 are co-activated by gene amplification and may coordinately function in cancer stemness in human cancers, including ESCC and gastric cancer.

3.5 | Antitumor effects of CPI-613, a PDH inhibitor, in ESCC cells

The PDH complex consists of five major subunits, PDHA1, PDHB, DLAT, DLD, and PDHX.20,21 Based on the mRNA expression in 92 ESCC cases, PDHA1, PDHB, DLAT, and DLD expression was significantly upregulated in the highest 20% of 92 ESCC tumors with high PDHX expression (PDHXhigh, cases, n = 18) compared with the lowest 20% with low PDHX expression (PDHXlow, cases, n = 18), suggesting that activation of PDH complex is associated with the expression of PDH-related genes in ESCC tumors (Figure 6A). Furthermore, the expression of PDHA1, PDHB, DLAT, and DLD was transcriptionally upregulated and partially decreased in PDHX-inhibited cells.
compared with that in control cells (shVector) under the sphere condition, not the 2D condition (Figure S6). As lipoic acid (lipoate) is covalently joined as a catalytic co-factor for the inactivation of the PDH complex, a lipoate derivative, CPI-613, can potently induce mitochondrial dysfunction via the inhibition of PDH activity and exhibits strong antitumor activity in numerous cancers.33,34 We examined the antitumor effects of CPI-613 on tumor growth of KYSE850 and KYSE170 cells highly expressing PDHX in vitro and in vivo. As shown in Figure 6B, CPI-613 markedly reduced the number and size of the spheroids formed by both KYSE850 and KYSE170 cells. Furthermore, in vivo tumor growth was markedly inhibited by intraperitoneal administration of CPI-613 compared with a vehicle in the KYSE850 xenograft model (Figure 6C). Thus, high expression of PDHX may be closely associated with the upregulation of other PDH-related genes, and treatment with CPI-613 is therapeutically effective for ESCC tumors highly expressing PDHX, especially ESCC stem cells.

4 | DISCUSSION

The identification of genes that are metabolically essential for cancer cells is a useful approach for the development of cancer therapies targeting metabolic vulnerability. In this study, siRNA-based screening of genes related to metabolic pathways identified PDHX as a metabolically essential gene in the cell growth of ESCC cells. PDHX expression is necessary for the maintenance of PDH activity and the production of ATP energy via the TCA cycle, and functions in the proliferation of CSCs along with in vivo tumor growth in ESCC. The PDHX gene was co-upregulated with the CD44 gene, which is
involved in cancer stemness, by amplification at the 11p13 region in ESCC tumors. CD44v9, a variant isoform of CD44, interacts with and stabilizes xCT, a subunit of the cystine-glutamate transporter, which positively regulates the intracellular level of glutathione (GSH) for ROS scavenging, thereby affecting antioxidant levels in CSCs. As increased PDH activity generates ROS in the mitochondria, CD44v9-mediated antioxidants may protect CSCs from cytotoxicity via the accumulation of ROS. Therefore, the PDHX/CD44 axis coordinate functions in cancer stemness in ESCC and the co-amplification of these two genes may promote the addiction to energy production via mitochondrial metabolism in CSCs. Furthermore, high expression of PDHX is closely associated with the upregulation of other PDH-related genes, and pharmacological targeting of the PDH complex using CPI-613 was therapeutically effective for ESCC tumors highly expressing PDHX. Thus, our study provides new insights related to the development of novel therapeutic strategies targeting the metabolic vulnerability of ESCC (Figure 6D).

A central connection between glycolysis and the mitochondrial TCA cycle is the PDH complex. PDH activity is negatively regulated by the phosphorylation of PDHA, a component of the PDH complex.
complex, via pyruvate dehydrogenase kinase (PDK). The PDK family of enzymes comprises four members (PDK1-4) and is frequently overexpressed in cancers, and inhibition of PDKs induces cancer cell death through increased PDH activity in the mitochondria and subsequent excessive ROS production. As PDK inhibitors have been reported as potential therapeutic agents for cancer, PDKs have been implicated in oncogenesis by promoting glycolysis as the Warburg effect via the reduction of PDH activity. Consistent with these studies, our siRNA-based screening demonstrated that knockdown of PDKs (PDK1-4) inhibited the growth of KYSE850 and KYSE170 cells. However, PDHX knockdown, followed by the reduction of PDH activity, also inhibited the growth of ESCC cells. Thus, biased metabolic conditions, such as excess or reduced PDH activity, are toxic to cancer cells, suggesting the importance of the metabolic balance between glycolysis and the TCA cycle for cancer cell growth. The expression level of PDHX was increased in the spheroids of ESCC cells and in CD44v9-positive cells within primary ESCC tumors. Therefore, activation of the mitochondrial metabolism via PDH activity is indispensable for CSC properties, suggesting the PDH complex to be a metabolically reasonable target for CSCs in the pathological context of ESCC.

The molecular mechanism underlying the transcriptional regulation of PDHX expression in the CD44v9-positive cell population of ESCC remains unknown. Recently, Kumazoe et al. reported that PDHA1 expression was upregulated in CD44v9-positive pancreatic CSCs via transcriptional factors, such as forkhead box O3 (FOXO3) and peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α), and suggested that the upregulation of PDHA1 is essential for CSC properties.
for CSC properties.43 Our study suggests that PDHX expression is closely associated with the upregulation of other PDH-related genes, including PDHA1. Thus, FOXO3 and PGC-1α may be common transcriptional regulators of PDH-related genes; however, this requires further investigation. In our present study, we were unable to define the mechanism by which PDHX positively regulates CD44 expression. The components of the PDH complex, including PDHX and PDHA1, were reported to localize in the nucleus in addition to the mitochondria, and the nuclear PDH complex controls gene expression by mediating histone acetylation.44,45 Thus, the nuclear PDH complex may be associated with epigenetic regulation controlling the expression of CSC-essential genes, including the CD44 gene.

Cancer cells require both aerobic glycolysis and mitochondria metabolism to generate energy. Indeed, combined treatment with 2-deoxyglucose (2DG), which acts as an inhibitor of glucose metabolism, and metformin, a commonly prescribed drug for type 2 diabetes, which prevent oxidative phosphorylation in mitochondria, could synergistically induce cell death by reducing intracellular ATP levels in ESCC cells.66 Furthermore, treatment with sulfasalazine, an inhibitor of xCT that functions as an antioxidant, effectively suppresses tumor growth, cancer stemness, and metastasis of ESCC cells.57-49 Thus, the potential applications of metabolic inhibitors have been experimentally demonstrated in ESCC. For future clinical application, it will be necessary to identify specific metabolic vulnerability in ESCC tumors of individual patients.

The inhibition of mitochondrial metabolism as a new cancer therapeutic treatment has been evaluated in clinical trials.50 Recent evidence suggests that defining the genetic status of enzymes related to the mitochondrial metabolism and mitochondrial activity using PET imaging can help stratify cancer patients who will benefit from treatment with inhibitors targeting the mitochondrial metabolism.50 The lipoate derivative CPI-613 is a first-in-class agent that targets the PDH complex in mitochondrial metabolism and is currently used in clinical trials for acute myeloid leukemia (AML), pancreatic cancer, myelodysplastic syndromes (MDS), and lymphoma.51-53 PDHX was amplified in human cancers, including in ESCC and gastric cancer, and high expression of PDHX is closely associated with the upregulation of other PDH-related genes. Thus, gene amplification and/or high expression of PDHX may be a biomarker to predict high PDH activity in the tumor and to identify tumors susceptible to CPI-613. Further validation of this idea using a large cohort of tumor samples and cancer cell lines is required for the development of precision cancer medicine based on PDH activity in human cancers, including ESCC and gastric cancer.

ACKNOWLEDGEMENTS

The authors thank Ayako Takahashi and Rumi Mori (Tokyo Medical and Dental University, Japan) for their technical assistance. This study was supported in part by Grants-in-Aid for Scientific Research (18K06954 to Jun Inoue; 16K14630 to Johji Inazawa), a Grant-in-Aid for Scientific Research on Innovative Areas “Conquering cancer through NEO-dimensional systems understandings” (15H05908 to Johji Inazawa) from JSPS and MEXT, a research programme of the Project for Cancer Research and Therapeutic Evolution (P-CREATE) and the Tailor-Made Medical Treatment with the BioBank Japan Project (BBJ) from the Japan Agency for Medical Research and Development (AMED). This study was supported by Nanken-Kyoten, TMDU.

DISCLOSURE

The authors declare no competing interests.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Inoue J, Kishikawa M, Tsuda H, Nakajima Y, Asakage T, Inazawa J. Identification of PDHX as a metabolic target for esophageal squamous cell carcinoma. Cancer Sci. 2021;112:2792–2802. https://doi.org/10.1111/cas.14938