Fatty acid beta oxidation enzyme HADHA is a novel potential therapeutic target in malignant lymphoma

Kouhei Yamamoto¹ · Shinya Abe¹ · Ayaka Honda¹ · Jun Hashimoto¹ · Yuuki Aizawa¹ · Sachiko Ishibashi¹ · Taro Takemura² · Nobutaka Hanagata² · Masahide Yamamoto³ · Osamu Miura³ · Morito Kurata¹ · Masanobu Kitagawa¹

Received: 8 February 2019 / Revised: 23 July 2019 / Accepted: 5 August 2019 / Published online: 16 September 2019
© United States & Canadian Academy of Pathology 2019

Abstract

Cancer cells, including malignant lymphoma cells, alter their metabolism, termed “metabolic reprogramming,” on initiation of malignant transformation as well as upon accumulation of genetic abnormalities. Here, to identify a novel therapeutic target involved in the metabolic changes during malignant lymphoma, we performed global analyses combined with shotgun proteomics, in silico database analysis, and clinic-pathologic analysis of nonneoplastic lymphoid tissue and malignant lymphoma tissue and verified the molecular functions in vitro. In total, 2002 proteins were detected from both samples and proteins related to fatty acid beta-oxidation (FAO) were detected more frequently in malignant lymphoma tissue. Consequently, the most frequently detected protein, the mitochondrial trifunctional enzyme subunit-alpha (HADHA), was identified as a potential target. Immunohistochemical analyses revealed that HADHA tended to be overexpressed in a high-grade subtype of malignant lymphoma tissue. Clinicopathologic study revealed that HADHA overexpression was correlated with significantly worse overall survival ($P = 0.013$) and was an independent prognostic predictor in diffuse large B-cell lymphoma ($P = 0.027$). In vitro, downregulation of *HADHA* negatively regulated cell growth by causing G0/G1 arrest ($P = 0.0008$) similar to treatment with etomoxir, an inhibitor of FAO ($P = 0.032$). Moreover, downregulation of *HADHA* increased the susceptibility to doxorubicin ($P = 0.002$) and etoposide ($P = 0.004$). Moreover, these phenotypes were confirmed in an HADHA knockout system. Thus, we provide a basis for a novel therapeutic strategy through the regulation of *HADHA* and FAO in patients with refractory malignant lymphoma.

Supplementary information The online version of this article (<https://doi.org/10.1038/s41374-019-0318-6>) contains supplementary material, which is available to authorized users.

✉ Kouhei Yamamoto
yamamoto.pth2@tmd.ac.jp

¹ Department of Comprehensive Pathology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo 113-8510, Japan

² Nanotechnology Innovation Station for Nanoscale Science and Technology, National Institute for Materials Science, Tsukuba, Ibaraki 305-0047, Japan

³ Department of Hematology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo 113-8510, Japan

Introduction

Malignant lymphoma is a primary malignancy that occurs worldwide [1]. Several long-term efforts, including sophisticated chemotherapeutic strategies or novel drug discovery, have improved clinical outcome in patients with malignant lymphoma [2–4]. The histological grade and molecular profiles vary in these clinical courses, and many cases have been incurable, despite the recent developments in various therapeutic strategies [5, 6]. Therefore, to effectively treat refractory cases and improve the prognosis, novel therapeutic strategies are needed.

Cancer cells possess genetic abnormalities that are responsible for oncogenesis [7]. Many such genetic abnormalities have been reported in malignant lymphoma [8–11]. Certain novel therapeutic reagents against malignant lymphoma have been developed based on these oncogenic mutations [12–14].

However, during malignant transformation, cancer cells undergo metabolic reprogramming to enable abnormal proliferation and prevent cell death signaling [15]. For example, cancer cells strongly depend on aerobic glycolysis for their energy supply, termed “Warburg effect,” which is representative of the metabolic reprogramming occurring in cancer cells [16]. Moreover, recent studies revealed that oncogenic mutations, including mutations in epidermal growth factor and KRAS, could modify glucose metabolism and mitochondrial functions in several cancers [17, 18]. Thus, oncogenic mutation and cancer metabolic reprogramming provide a unique perspective on tumorigenesis and tumor progression. Besides, they are both complex processes involving mechanisms that may work in tandem [15].

Cellular metabolism is controlled by a number of metabolic enzymes, and the amount of enzymes can affect the cellular metabolic rate. However, to date, no reports exist on the quantification of global metabolism-related proteins in lymphoma tissue in comparison with nonneoplastic lymphoid tissue. Therefore, in this study, we aimed to identify a novel therapeutic target protein that is involved in cellular metabolism in malignant lymphoma tissue. We performed global analyses combined with shotgun proteomics, *in silico* database analysis and clinic-pathologic analysis using nonneoplastic lymphoid tissue and malignant lymphoma tissue. In addition, we verified the molecular functions of the identified protein using *in vitro* systems.

Material and methods

Patients and clinicopathologic details

A total of 217 malignant lymphoma, and 13 nonneoplastic tonsil formalin-fixed paraffin-embedded (FFPE) samples were used, obtained from the Tokyo Medical and Dental University Hospital, Tokyo, Japan, between January 2004 and December 2012. The study was approved by the ethical research committee of Tokyo Medical and Dental University Medical Hospital (No. M2000-1818-01). All lymphoma samples were subjected to immunohistochemical and gene translocational analyses for pathologic diagnosis.

For shotgun proteomic analysis, 13 cases with follicular lymphoma (FL) were analyzed. All FL samples were positive for CD20, CD10, and Bcl-2 expression, detected by immunohistochemistry, and were positive for *IgH/BCL2* translocation, detected by fluorescent *in situ* hybridization. Eight of the 13 cases were Grade 3a, and five cases were Grade 2, as diagnosed by more than one hematopathologist. Further, 128 cases with diffuse large B-cell lymphoma (DLBCL) were analyzed for overall survival and clinic-pathologic study. All the 128 patients received R-CHOP

(rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone)-based therapy; special subtypes of DLBCL cases were not included, for example primary central nerve system lymphoma, intravascular large B-cell lymphoma, or primary effusion lymphoma.

Selection of FFPE tissue samples for shotgun proteomics

To reasonably compare the neoplastic lymphoid cells with their normal counterparts, FL and nonneoplastic lymphoid follicles (germinal center; GC) were selected, as the histological structure and protein expression levels of GC markers were similar in both types of cells. Thirteen samples each of the FL and nonneoplastic lymphoid tissue GC were studied by shotgun proteomics. Infiltration of T cells into these follicles was compared by immunohistochemical staining for CD20 (B cells) and CD3 (T cells); it was previously confirmed during diagnosis that the ratios of both cells in the follicles were identical (percentage of infiltrating T cells was 10–30%).

Laser microdissection (LMD) and protein extraction and purification

The experimental flow is indicated in Fig. 1a. For shotgun analysis, 10- μ m-thick sections of the FFPE samples from FL and GC were attached to DIRECTOR™ slides (Expression Pathology, Rockville, MD, USA). These slides were de-paraffinized by treatment with xylene four times (5 min each time), sequentially rehydrated with 100% ethanol, 70% ethanol, and distilled water, and stained with hematoxylin. The stained slides were air-dried, and each follicle was individually dissected using a Leica LMD6000 system (Leica Microsystems GmbH, Wetzlar, Germany) (Fig. 1b, and c). Approximately 30,000 cancerous cells (from a 5 mm² area) were collected into the cap of a 0.2 mL PCR tube. Peptides were extracted and purified using a Liquid Tissue™ MS Protein Prep kit (Expression Pathology, Rockville, MD, USA) [19]. The purified proteins were digested using trypsin (approximately 15–18 U) for 24 h with incubation at 37 °C. Dithiothreitol was then added to the digested samples at a final concentration of 10 mM, and the samples were incubated for 5 min at 95 °C for enzyme inactivation and stored at –20 °C until analysis.

LC-MS/MS shotgun analysis, peptide mass analysis, and protein identification

LC-MS/MS shotgun analysis was performed using reversed-phase (RP)-LC interfaced with an LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, Frankfurt, Germany) with a nanoelectrospray device (AMR,

Fig. 1 “Follicle-specific” global protein identification in nonneoplastic germinal center (GC) and follicular lymphoma samples. **a** Flow chart of the experimental methodology used in this study. **b** Hematoxylin staining of formalin-fixed paraffin-embedded human tonsil specimens (GC). **c** Follicles were individually dissected using the microdissection method. **d** A Venn diagram of the proteins detected from the non-neoplastic germinal center (GC) and follicular lymphoma (FL) cells

Tokyo, Japan), as previously reported [20]. The experimental details are provided in supplemental methods.

Pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database

The protein profiles were analyzed on the KEGG database (<http://www.genome.jp/kegg/>) by inputting data to the KEGG PATHWAY under the section data-oriented entry points [21]. To clarify the differences in each functional profile, we focused on metabolic pathways in this study. We checked glycolysis/gluconeogenesis, citrate cycle (TCA cycle), oxidative phosphorylation, and fatty acid metabolism to compare the ratios and distributions of each protein profile and to extract the specific proteins involved in these pathways.

Immunohistochemistry

The primary antibody used was a rabbit polyclonal antibody against HADHA (clone #HPA015536; Sigma, St. Louis, MO, USA). FFPE sections (4- μ m thick) of lymphoma and tonsil tissues were de-paraffinized in xylene and rehydrated through a graded alcohol series and distilled water. Antigen retrieval was performed for 40 min in an antigen retrieval solution (pH 9.0; Nichirei Bioscience, Tokyo, Japan), using

a microwave (H1850; Energy Beam Science, Toronto, Canada) at 97 °C. Endogenous peroxidase activity was inhibited using 0.3% hydrogen peroxide in methanol for 30 min. Nonspecific protein binding was blocked with normal horse serum (ABC Kit; Vector Laboratories, Burlingame, CA, USA). Sections were incubated overnight with the primary antibody (1:100 dilution) at 4 °C. Detection was performed using the intercalated antibody-enhanced polymer method (Nichirei Bioscience) with the chromogen 3, 3'-diaminobenzidine (Nichirei Bioscience).

Establishment of HADHA-knockdown LCL-K cells and HADHA-knockout(KO) HAP1 cells

HADHA-knockdown LCL-K and *HADHA*-KO HAP1 cells were established by shRNA lentiviral vector and CRISPR-Cas9 system. LCL-K cells were human peripheral blood B cells with Epstein-Barr virus infection [22] and kindly provided by Professor Ayako Arai, St. Marianna University School of Medicine Hospital. HAP1 cells are near-haploid, having a single copy of nearly every chromosome, and purchased from HorizonTM (Cambridge, UK). Details are described in supplemental methods.

Cell growth and cell cycle analysis

Cell growth analyses were performed by treating shHADHA-transduced LCL-K, control LCL-K, and HADHA-KO HAP1 cells and control HAP1 cells compared with etomoxir, a fatty acid beta-oxidation (FAO) inhibitor, and counting the cell number using Countess[®] II FL Automated Cell Counter (Thermo Fisher Scientific). In addition, cell cycle analysis was performed using the above cells by flowcytometry. Details are described in supplemental methods.

Assessment of cell viability after treatment with doxorubicin, etoposide, or etomoxir

Cell viability analysis, after treatment with etoposide and etomoxir, was performed using shHADHA-transduced LCL-K, shCont LCL-K, and HADHA-KO HAP1 cells and control HAP1 cells. Details are provided in supplemental methods.

Statistical analysis

The χ^2 -test and Fisher's exact test were used for the comparison of categorical variables. In the univariate analysis, survival rates were calculated using the Kaplan–Meier method and compared using the log-rank test. Multivariate analysis was performed with the Cox proportional hazard regression model. The comparison of cell viability, cell

cycle progression, and cell viability between the two groups was analyzed by the Student's *t*-test. A value of *P* < 0.05 was defined as statistically significant. StatMate version 5.0 (ATMS, Tokyo, Japan) was used for all the analyses.

Results

Follicle-specific protein identification by shotgun proteomics

From the 13 FL and 13 GC samples, a total of 2002 proteins were detected by shotgun proteomics. Of these, 1519 proteins were from FL samples (Supplemental Table S1), and 1333 proteins were from GC samples (Supplemental Table S2), that is, 669 proteins were FL specific, 483 were GC specific, and 850 occurred in both (Fig. 1d).

Comparative verification by KEGG database analysis

To clarify the differences in characteristics between GC and FL protein profiles, we used information from the KEGG database. At first, the proteins detected by shotgun proteomics were classified based on cellular functions. Metabolic pathway-related proteins were the most frequently detected in both GC and FL (Fig. 2a). Among the metabolic pathway-related proteins, 40 proteins were commonly detected, 18 were GC specific, and 27 were FL specific (Fig. 2b, c). Subsequently, GC and FL specific, metabolic pathway-related proteins were further classified based on the total peptide hit numbers. Thus, fatty acid metabolism-related proteins were the most frequently detected (Supplement Fig. S1a, b). Cellular energy metabolism plays vital roles in cell survival and proliferation, including in cancer cells [15]. Therefore, we focused on several representative

Fig. 2 The details of the metabolic pathway-related proteins in nonneoplastic germinal center (GC) and follicular lymphoma (FL) cells, identified by shotgun proteomics. **a** The number of metabolic pathway-related proteins in GC and FL cells. **b** A Venn diagram of the number of proteins related to metabolic pathway in GC and FL cells. **c** A Venn diagram of the metabolic pathway-related genes in GC and FL cells

energy-related metabolic pathways. Although glycolysis is known to be upregulated in many cancer cells [23], no significant differences were observed in the frequency of glycolysis/gluconeogenesis-related proteins between FL and GC profiles (Supplemental Fig. S2a, b). Regarding TCA cycle-related proteins, the profiles exhibited a similar pattern (Supplemental Fig. S3a, b).

However, oxidative phosphorylation-related proteins occurred more frequently in GC (Supplement Fig. S4a) than in FL (Supplement Fig. S4b) samples. Notably, FAO-related proteins were detected much more frequently in FL (Supplement Fig. S5a and b) samples than in GC samples, even though the profiles of fatty acid synthesis were identical between the two samples (data not shown). Individual proteins involved in oxidative phosphorylation and FAO are summarized in Supplemental Table 3. Among these proteins, ECHA (trifunctional enzyme subunit- α , mitochondrial; Gene name: *HADHA*) was the most frequently detected by shotgun proteomics, not only among FAO-related proteins, but also among FL-specific proteins (Supplemental Table 3); therefore, we focused on the protein encoded by *HADHA*.

Validation by immunohistochemical analysis of FL and other lymphoma subtypes

To evaluate the expression of HADHA protein in situ, we performed immunohistochemical analyses. First, we checked both the FL and GC FFPE samples that were used for shotgun proteomics. In GC follicles, slight HADHA staining was detected only at the perinuclear site, and nonneoplastic T cells exhibited the same staining pattern (Fig. 3a, b). In the 13 FL samples, nine samples positive for HADHA by shotgun proteomics exhibited HADHA staining at the perinuclear and cytoplasmic sites (Fig. 3c). The remaining four cases that were negative for HADHA exhibited the same staining pattern as GC samples (Fig. 3d). We defined the staining patterns as “overexpression” and “normal expression,” in FL and GC samples, respectively.

As shown in Table 1, HADHA was overexpressed in 31% of FL Grades 1 and 2 (4/13 cases) samples, whereas the overexpression pattern was much more frequent in Grades 3a and 3b, including two cases of BCL-2-negative FL (77%, 20/26 cases; $P = 0.005$). These results indicated that HADHA tended to be overexpressed in high-grade lymphoma subtypes.

Next, we performed HADHA immunostaining in lymphoma subtypes other than FL. The percentage of HADHA overexpression tended to be higher in high-grade subtypes, such as DLBCL (69%, 88/128) (Fig. 3e, f), Burkitt lymphoma (100%, 4/4), anaplastic large cell lymphoma (100%, 4/4), and adult T-cell lymphoma/leukemia (100%, 5/5),

whereas the overexpression in low-grade subtypes was <35%, namely in marginal zone B-cell lymphoma (25%, 1/4) and chronic lymphoid leukemia/small lymphocytic lymphoma (13%, 1/8) (Fig. 3g).

Clinical significance of HADHA overexpression in DLBCL

As FL is a generally indolent clinical outcome, we tried to evaluate the relationship between the clinical outcome with various clinical factors and HADHA overexpression in DLBCL which was more aggressive clinical course and worse prognosis than FL. As shown in Supplemental Table 4, the staining patterns did not correlate to age, gender, Ann Arbor status, lactate dehydrogenase (LDH), International Prognostic Index (IPI) score, B symptoms, extranodal site, bone marrow involvement, and Hans algorithm. These data suggested that HADHA overexpression was potentially an independent prognostic factor for DLBCL.

We then investigated the correlation between HADHA overexpression and disease prognosis. Kaplan–Meier analysis revealed that HADHA-overexpressing patients had significantly worse overall survival than did the patients with normal gene expression ($P = 0.013$; Fig. 4). According to the univariate analysis, the advanced stage of Ann Arbor classification ($P = 0.003$), elevated LDH ($P = 0.002$), high performance status ($P = 0.002$), high IPI score ($P = 0.0001$), and HADHA overexpression ($P = 0.013$) were significantly associated with worse overall survival (Table 2). As per the multivariate Cox regression model, HADHA overexpression ($P = 0.027$) remained an independent predictor of poor prognosis (Table 2).

In vitro assessment of HADHA expression in LCL-K cells and HAP1 cells

Based on the findings that HADHA overexpression was related to poor clinical outcome in DLBCL, we further analyzed the function of HADHA in malignant lymphoma cells in vitro. Lentivirus shRNA-mediated HADHA knockdown was performed in LCL-K cells (Supplemental Fig. 6a, b). First, we assessed the relationship between HADHA expression and cell growth. Compared with control cells, lenti-shHADHA-transduced (shHADHA) cells, as well as cells treated with etomoxir exhibited decreased cell growth ($*P = 0.014$, $**P = 0.0008$, $***P = 0.032$) (Supplemental Fig. 6c, d). In cell cycle analysis, both shHADHA and etomoxir-treated LCL-K cells exhibited increased G0/G1 arrest than did the shCont LCL-K cells ($*P = 0.0005$, $**P = 0.0001$) (Supplemental Fig. 6d, e).

We then measured cell viability upon treatment with doxorubicin (a major anti-lymphoma drug), etoposide

Fig. 3 Immunohistochemistry for HADHA in the nonneoplastic germinal center (GC) and follicular lymphoma (FL) cells. **a** “Normal expression” pattern in GC (×20 magnification). **b** High magnification of GC cells (×400 magnification). **c** “Overexpression” pattern in FL (×400 magnification). **d** FL specimen with “normal expression” pattern (×400 magnification). **e** “Overexpression” pattern in diffuse large B-cell lymphoma (DLBCL) (×400 magnification). **f** DLBCL specimen with “normal expression” pattern (×400 magnification). **g** Percentage of HADHA overexpression among the various lymphoma subtypes

(apoptosis-inducing anti-cancer drug), and etomoxir. The cell death ratio was significantly increased in shHADHA-transduced LCL-K cells after treatment with doxorubicin and etoposide, while no significant difference was detected after treatment in cells exposed to etomoxir (Supplemental Fig. 7a, b).

To confirm that these phenotypes were due to down-regulation of *HADHA*, we established *HADHA*-KO HAP1 cells (Supplemental Fig. 8a). As expected, cell

growth analysis showed that etomoxir-treated *HADHA*-KO cells exhibited lower growth and viability than *HPRT*-KO cells for 72 h (* $P = 0.0032$, ** $P = 0.019$) (Supplemental Fig. 8b, c). Moreover, *HADHA*-KO cells were susceptible to treatment with doxorubicin ($P < 0.0001$) and etoposide ($P = 0.0003$), while no significant difference was noted after treatment with etomoxir ($P = 0.521$) (Supplemental Fig. 8d, e). These results were identical to those of knockdown of LCL-K cells.

Table 1 *HADHA* expression and histological grade in follicular lymphoma (FL)

	<i>HADHA</i>		
	Overexpression	Normal expression	
FL Grade 1 and 2	4	9	13
FL Grade 3a and 3b	20	6	26
	24	15	Total 39

 $p = 0.005$ **Fig. 4** Survival analysis in diffuse large B-cell lymphoma (DLBCL) with *HADHA* immunoreactivity by the Kaplan–Meier method. The *HADHA*-overexpression group shows worse prognosis than the *HADHA*-normal expression group ($P = 0.013$)

Discussion

We identified FAO-related proteins involved in malignant lymphoma using combined follicle-specific shotgun proteomics and KEGG in silico analysis. A subset of lymphoma cell lines has been characterized by different metabolic signatures, and this subset expresses mitochondrial and energy metabolism-related proteins, including the FAO-related proteins [24]. Our results are consistent with a previous report that lymphoma cells might require more FAO than do nonneoplastic lymphocytes, with respect to the quantity of the metabolic enzyme utilized. Moreover, the deregulation of pathways in fatty acid synthesis and FAO is related to poor outcomes in several cancer types [25–28].

In malignant lymphoma, *BCL6*, which is dysregulated in 30% of DLBCL and 5–15% of FL cases [1], plays a role in the regulation of lipid metabolism in mice [28]. In addition, another report has shown that the inhibition of FAO positively regulates cell death in malignant lymphoma cells and prevents Myc-induced lymphomagenesis in mice; further, in leukemic cells, the control of FAO is considered a potential therapeutic strategy [29]. Our in vitro analysis revealed that the *HADHA* positively regulated cell growth and negatively regulated anti-cancer reagent-induced cell death signaling. Based on previous reports and our results,

HADHA can be considered not only as a metabolic marker of FAO, but also as a molecular target for therapeutics. Moreover, the inhibition of FAO by etomoxir induced cell death regardless of the expression level of *HADHA*. Etomoxir is an inhibitor of carnitine palmitoyltransferase-1, which is a gateway protein for FAO on the outer layer of the inner mitochondrial membrane [30]. Etomoxir is considered a promising therapeutic reagent for cancer and congestive heart failure; however, etomoxir exerts an adverse effect, causing liver toxicity [31]. To effectively address refractory malignant lymphoma cases, the development of other promising FAO inhibitors is required.

FAO has multiple catalytic steps and four enzymatic reactions that result in the sequential removal of two-carbon and acetyl-coenzyme A units. The *HADHA* gene product ECHA encodes a multifunctional enzyme that plays a role in FAO as hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, collectively termed mitochondrial trifunctional protein subunit-alpha [32]. Mutations in this gene result in the deficiency of the trifunctional protein, resulting in a spectrum of phenotypes consisting of nonketotic hypoglycemia, hepatic dysfunction, and/or skeletal myopathy and cardiomyopathy, caused by energy deficiency from fatty acids [33]. *HADHA* also encodes monolysocardiolipin acyltransferase that remodels monolysocardiolipin to cardiolipin (CL) [34], one of the major phospholipids in mammalian mitochondria and has crucial roles in regulating ATP production [35] and modulation of apoptotic pathway [36, 37]. Disruption of CL remodeling causes cardiomyopathy, cyclic neutropenia, 3-methylglutaconic aciduria, and mild hypocholesterolemia, the characteristic symptoms of Barth Syndrome [38]. Thus, *HADHA* plays a vital role in sustaining the energy required for the maintenance of cellular function and survival.

Interestingly, it has been reported that *HADHA* is involved not only in FAO and CL remodeling but also in miRNA biogenesis [39]. *HADHA* is associated with the Dicer complex, which includes ribonuclease III, and is essential for the cleavage of precursor miRNA to mature miRNA in the cytoplasm. Further, *HADHA* overexpression in vitro has been shown to result in increased mature miRNAs and decreased precursor miRNAs [39]. Here, we found that *HADHA* was overexpressed in the cytoplasm, concurrent with the fact that overexpressed *HADHA* may

Table 2 Univariate and multivariate analyses of clinicopathologic factors influencing overall survival

Variable	Category	No. of patients	<i>P</i> value by log-rank test	HR ^a	95% CI ^b	<i>P</i> value by Cox proportional hazards
Age	≥60 years	90	0.898	0.584	0.230–1.479	0.250
	<60 years	38				
Gender	Male	71	0.294	0.709	0.288–1.744	0.454
	Female	57				
Clinical stage	1.2	53	0.002	1.344	0.423–4.275	0.616
	3.4	71				
LDH ^c	Normal	31	0.070	2.068	0.605–7.069	0.247
	Elevated	88				
Performance status	0–1	90	0.051	0.871	0.300–2.529	0.799
	2–4	29				
IPI ^d score	0–2	83	0.0004	3.078	0.986–9.613	0.053
	3–5	45				
B symptoms	No	86	0.951	0.586	0.225–1.515	0.269
	Yes	39				
Extranodal site	0	32	0.543	0.724	0.228–2.294	0.583
	≥1	95				
Bone marrow involvement	No	81	0.665	0.674	0.149–3.048	0.609
	Yes	12				
Hans algorithm	GCB ^e	45	0.624	1.272	0.486–3.329	0.623
	non-GCB	78				
HADHA	normal expression	40	0.013	3.616	1.155–11.328	0.027
	overexpression	88				

^aHR: hazard ratio^bCI: confidence interval^cLDH: lactate dehydrogenase^dIPI: international prognosis index^eGCB: germinal center B cell

function as a modulator of miRNA biogenesis. As miRNAs play essential roles in the pathology of malignant lymphomas [40], it can be hypothesized that an alteration in miRNA expression by HADHA can cause a dramatic change in the gene expression profile and contribute to lymphomagenesis and lymphoma progression.

In summary, our results provide a basis for the elucidation of the processes involved in lymphomagenesis and lymphoma progression, and for breakthroughs in novel therapeutic strategies. There are several limitations and unaddressed questions in this study, one being the mechanism by which HADHA is overexpressed in malignant lymphoma tissue. HADHA overexpression was not correlated to any clinical factors; therefore, the underlying mechanism is still unknown. Specific genetic mutations or the tumor microenvironment may affect the expression of HADHA. Moreover, measurement of FAO is required for not only in the HADHA-transduced cells, but also in malignant lymphoma tissue samples to acquire solid evidence of FAO upregulation in malignant lymphoma. In

addition, detailed clinicopathologic and molecular analysis of HADHA overexpression in the subtypes of malignant lymphoma, apart from DLBCL, is required. Further studies will address these issues and resolve such questions, leading to clinical application.

Acknowledgements This work was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (No. 21790345). Also, we would like to address the special thanks to Assistant Professor Branden Moriarity, Ph.D., Department of Pediatrics, Masonic Cancer Center, Center for Genome Engineering in University of Minnesota for providence of CRISPR-Cas9 plasmid.

Funding This work was supported by the Ministry of Education, Culture, Sports, Science, and Technology of Japan [No. 21790345, 2010].

Author contributions KY and SA conceived and designed the study. MK(i) guided the project. KY, AH, JH, and YA coordinated and performed most of the experimental work. TT and NH instructed on proteomics analyses. SI and KY performed in vitro analysis. KY wrote the paper, and MY, OM, and MK(u) provided a critical review.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

1. Swerdlow SH, Campo E, Pileri SA, Jaffe ES, Pileri SA, Stein H, et al. WHO classification of tumors of haematopoietic and lymphoid tissues. 4th ed. Lyon: IARC; 2017.
2. Coiffier B, Thieblemont C, Neste VD, Lepeu G, Plantier I, Castaigne S, et al. Long-term outcome of patients in the LNH-98.5 trial, the first randomized study comparing rituximab-CHOP to standard CHOP chemotherapy in DLBCL patients: a study by the Groupe d'Etudes des Lymphomes de l'Adulte. *Blood*. 2010;116:2040–5.
3. Alperovich A, Younes A. Targeting CD30 using brentuximab vedotin in the treatment of hodgkin lymphoma. *Cancer J*. 2016;22:23–6.
4. Zinzani PL, Ribrag V, Moskowitz CH, Michot JM, Kuruvilla J, Balakumaran A, et al. Safety and tolerability of pembrolizumab in patients with relapsed/refractory primary mediastinal large B-cell lymphoma. *Blood*. 2017;130:267–70.
5. Crump M, Neelapu SS, Farooq U, Van Den Neste E, Kuruvilla J, Westin J, et al. Outcomes in refractory diffuse large B-cell lymphoma: results from the international SCHOLAR-1 study. *Blood*. 2017;130:1800–8.
6. Hitz F, Connors JM, Gascoyne RD, Hoskins P, Moccia A, Savage KJ, et al. Outcome of patients with primary refractory diffuse large B cell lymphoma after R-CHOP treatment. *Ann of Hematol*. 2015;94:1839–43.
7. Hanahan D, Weinberg R. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646–74.
8. Morin RD, Johnson NA, Severson TM, Mungall AJ, An J, Goya R, et al. Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. *Nat Genet*. 2010;42:181–5.
9. Lenz G, Davis RE, Ngo VN, Lam L, George TC, Wright GW, et al. Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. *Science*. 2008;319:1676–9.
10. Davis RE, Ngo VN, Lenz G, Tolar P, Young RM, Romesser PB, et al. Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. *Nature*. 2010;463:88–92.
11. Sakata-Yanagimoto M, Enami T, Yoshida K, Shiraishi Y, Ishii R, Miyake Y, et al. Somatic RHOA mutation in angioimmunoblastic T cell lymphoma. *Nat Genet*. 2014;46:171–5.
12. Godwin P, Baird AM, Heavey S, Barr MP, O'Byrne KJ, Gately K. Targeting nuclear factor-kappa B to overcome resistance to chemotherapy. *Front Oncol*. 2013;3:120.
13. McCabe MT, Ott HM, Ganji G, Korenchuk S, Thompson C, Van Aller GS, et al. EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. *Nature*. 2012;492:108–12.
14. Advani R, Buggy J, Sharman J, Smith SM, Boyd TE, Grant B, et al. Bruton tyrosine kinase inhibitor ibrutinib (PCI-32765) has significant activity in patients with relapsed/refractory B-cell malignancies. *J Clin Oncol*. 2013;31:88–94.
15. Zhou Z, Ibekwe E, Chornenkyy Y. Metabolic alterations in cancer cells and the emerging role of oncometabolites as drivers of neoplastic change. *Antioxidants*. 2018;7:16.
16. Warburg O. On the origin of cancer cells. *Science*. 1956;123:309–14.
17. Liou GY, Döppler H, DelGiorno KE, Zhang L, Leitges M, Crawford HC, et al. Mutant Kras-induced mitochondrial oxidative stress in acinar cells upregulates EGFR signalling to drive formation of pancreatic precancerous lesions. *Cell Rep*. 2016;14:2325–36.
18. Makinoshima H, Takita M, Saruwatari K, Umemura S, Obata Y, Ishii G, et al. Signaling through the phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) axis is responsible for aerobic glycolysis mediated by glucose transporter in epidermal growth factor receptor (EGFR)-mutated lung adenocarcinoma. *J Biol Chem*. 2015;290:17495–504.
19. Prieto D, Hood B, Darfler M, Guilel TG, Lucas DA, Conrads TP, et al. Liquid tissue™: Proteomic profiling of formalin-fixed tissues. *BioTechniques*. 2005;38:S35.
20. Kawase H, Fujii K, Miyamoto M, Kubota KC, Hirano S, Kondo S, et al. Differential LC-MS-based proteomics of surgical human cholangiocarcinoma tissues. *J Proteome Res*. 2009;8:4092–103.
21. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res*. 2000;28:27–30.
22. Arai A, Imadome K, Fujiwara S, Miura O. Autoimmune hemolytic anemia accompanied by reactivation of an Epstein-Barr virus infection with suppressed CTL response to EBV-infected cells in an elderly man. *Intern Med*. 2010;49:325–9.
23. Vander H, Cantley T. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*. 2009;324:1029–33.
24. Caro P, Kishan AU, Norberg E, Stanley IA, Chapuy B, Ficarro SB, et al. Metabolic signatures uncover distinct targets in molecular subsets of diffuse large B cell lymphoma. *Cancer Cell*. 2012;22:547–60.
25. Griffiths B, Lewis CA, Bensaad K, Ros S, Zhang Q, Ferber EC, et al. Sterol regulatory element binding protein-dependent regulation of lipid synthesis supports cell survival and tumor growth. *Cancer Metab*. 2013;1:3.
26. Sun Y, He W, Luo M, Zhou Y, Chang G, Ren W, et al. SREBP1 regulates tumorigenesis and prognosis of pancreatic cancer through targeting lipid metabolism. *Tumor Biol*. 2015;36:4133–41.
27. Zaugg K, Yao Y, Reilly PT, Kannan K, Kiarash R, Mason J, et al. Carnitine palmitoyltransferase 1C promotes cell survival and tumor growth under conditions of metabolic stress. *Genes Dev*. 2011;25:1041–51.
28. Samudio I, Harmancey R, Fiegl M, Kantarjian H, Konopleva M, Korchin B, et al. Pharmacologic inhibition of fatty acid oxidation sensitizes human leukemia cells to apoptosis induction. *J Clin Invest*. 2010;120:142–56.
29. Ricciardi MR, Mirabilii S, Allegritti M, Licchetta R, Calarco A, Torrisi MR, et al. Targeting the leukemia cell metabolism by the CPT1a inhibition: functional preclinical effects in leukemias. *Blood*. 2015;126:1925–9.
30. Kruszynska YT, Sherratt HS. Glucose kinetics during acute and chronic treatment of rats with 2[6(4-chloro-phenoxy)hexyl] oxirane-2-carboxylate, etomoxir. *Biochem Pharmacol*. 1987;36:3917–21.
31. Holubarsch CJ, Rohrbach M, Karrasch M, Boehm E, Polonski L, Ponikowski P, et al. A double-blind randomized multicentre clinical trial to evaluate the efficacy and safety of two doses of etomoxir in comparison with placebo in patients with moderate congestive heart failure: the ERGO (etomoxir for the recovery of glucose oxidation) study. *Clin Sci*. 2007;113:205–12.
32. Eaton S, Bursby T, Middleton B, Pourfarzam M, Mills K, Johnson AW, et al. The mitochondrial trifunctional protein: centre of a beta-oxidation metabolon? *Biochem Soc Trans*. 2000;28:177–82.
33. Rector RS, Payne RM, Ibdah JA. Mitochondrial trifunctional protein defects: clinical implications and therapeutic approaches. *Adv Drug Deliv Rev*. 2008;60:1488–96.

34. Taylor WA, Mejia EM, Mitchell RW, Choy PC, Sparagna GC, Hatch GM. Human trifunctional protein alpha links cardiolipin remodeling to beta-oxidation. *PLoS ONE*. 2012;7:e48628.
35. Hatch GM. Cardiolipin: biosynthesis, remodeling and trafficking in the heart and mammalian cells (review). *Int J Mol Med*. 1998;1:33–74.
36. Ascenzi P, Polticelli F, Marino M, Santucci R, Coletta M. Cardiolipin drives cytochrome c proapoptotic and antiapoptotic actions. *IUBMB Life*. 2011;63:160–5.
37. Gonzalez F, Schug ZT, Houtkooper RH, MacKenzie ED, Brooks DG, Wanders RJA, et al. Cardiolipin provides an essential activating platform for caspase-8 on mitochondria. *J Cell Biol*. 2008;183:681–96.
38. Clarke SL, Bowron A, Gonzalez IL, Groves SJ, Newbury-Ecob R, Clayton N, et al. Barth syndrome. *Orphanet J Rare Dis*. 2013;8:23.
39. Kakumani PK, Shanmugam RK, Kaur I, Malhotra P, Mukherjee SK, Bhatnagar RK, et al. Association of HADHA with human RNA silencing machinery. *Biochem Biophys Res Commun*. 2015;466:481–5.
40. Kozloski G, Lossos I. LymphomiRs: microRNAs with regulatory roles in lymphomas. *Curr Opin Hematol*. 2015;22:362–8.