

# Protocadherin $\gamma$ A3 is expressed in follicular lymphoma irrespective of BCL2 status and is associated with tumor cell growth

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**Abstract.** Protocadherin genes (*PCDH*s) have been suggested to act as tumor suppressor genes in various tumor types. Previous studies have demonstrated the upregulation of certain *PCDH*- $\gamma$  subfamily genes in nodal and duodenal follicular lymphoma (FL) using gene expression analyses. However, the mechanisms and associated molecular function of *PCDH*- $\gamma$  subfamily gene upregulation in FL remain to be elucidated. The present study examined the expression of *PCDHGA3*, an upregulated *PCDH*- $\gamma$  gene subfamily member, in B-cell lymphoma 2 (BCL2)-positive and -negative FL, and evaluated its association with tumor cell proliferation in an FL-derived cell line. Immunohistochemical analysis demonstrated that the majority of FL grade 1-2 samples (19/20; 95%) and over half of grade 3A FL samples (5/9; 56%) were *PCDHGA3*-positive, whereas only 1/17 reactive lymphoid hyperplasia samples was positive. Notably, this positivity was widely observed in samples of BCL2-negative FL (13/15; 87%) and FL with diffuse area (10/10; 100%). The FL-derived cell line FL18 exhibited strong *PCDHGA3* expression, similar to the patient samples, and its proliferation was suppressed by *PCDHGA3* gene knockdown. Genes expressed concomitantly with *PCDHGA3* were selected from gene expression data, and *TNFRSF6B*, a member of the tumor necrosis factor receptor superfamily, was among the top five most strongly correlated genes. Coexpression of *TNFRSF6B* and *PCDHGA3* was observed immunohistochemically in FL18 cells, suggesting potential cooperation

in tumor cell maintenance. In conclusion, the results of the present study indicated that *PCDHGA3* was expressed in FL irrespective of BCL2 status and grading and was associated with cell proliferation. Further studies involving molecular genetic analyses are required to elucidate the mechanisms underlying the activity of *PCDHGA3* in FL.

## Introduction

Follicular lymphoma (FL) is the second most common form of non-Hodgkin's B-cell lymphoma (NHL) and the most common subtype of indolent NHL. The majority of FL cases (~85%) harbor the t(14;18)(q32;q21) translocation (1), which juxtaposes the B-cell lymphoma 2 (*BCL2*) gene on chromosome 18 to the immunoglobulin heavy chain gene (*IGH*) locus, resulting in constitutive expression of BCL2. Therefore, BCL2 is an important diagnostic marker of FL. However, 10-20% of cases are BCL2-negative (2), and this loss of BCL2 expression is frequent in high-grade cases (grades 3A and B) (1). Of these BCL2-negative cases, FL is difficult to diagnose even when other immunohistochemical markers are investigated.

It was previously reported that upregulation of certain protocadherin- $\gamma$  (*PCDH*- $\gamma$ ) subfamily genes in nodal and duodenal FL using gene expression analysis (3). *PCDH*- $\gamma$  subfamily genes, which are predominantly expressed in the nervous system, constitute the largest subgroup (~80 members) of the cadherin superfamily of cell-adhesion molecules (4,5). In various tumor types, *PCDH* genes are downregulated or silenced via promoter hypermethylation-mediated gene inactivation, including *PCDH8* (6) in breast carcinoma, *PCDH9* in glioblastoma (7), *PCDH17* in esophageal carcinoma (8) and *PCDH10* in Burkitt and diffuse large B-cell lymphoma (9). However, the mechanisms and associated molecular function of *PCDH*- $\gamma$  subfamily gene upregulation in FL remain to be elucidated. In the present study, *PCDHGA3* was investigated as a potentially useful diagnostic marker, even in BCL2-negative FL cases. Furthermore, a functional analysis of *PCDHGA3* was performed in an FL-derived cell line.

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## Materials and methods

**Patients and cell lines.** A total of 71 formalin-fixed paraffin-embedded tissue (FFPET) samples were selected, including 17 nodal reactive lymphoid hyperplasia (RLH) samples and 54 FL samples, obtained from the routine and consultation files of the Department of Pathology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (Okayama, Japan). Of the 54 FL cases, 20 were grade 1 or 2, 9 were grade 3A, 10 were FL with diffuse area and 15 were BCL2-negative.

Patient samples were diagnosed on the basis of morphological and immunophenotypical observations according to the current World Health Organization classification (1), and all cases were reviewed by three skilled hematopathologists (Dr Katsuyoshi Takata, Dr Yasuharu Sato and Professor Tadashi Yoshino). The study protocol was approved by the Institutional Review Board of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences (Okayama, Japan). All study procedures were conducted in accordance with the guidelines of the Declaration of Helsinki.

The human FL-derived cell lines, FL18 and FL318 were provided by Dr Ohno of Kyoto University (Kyoto, Japan). The cells were cultured at 37°C in 5% CO<sub>2</sub> in RPMI-1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (Biological Industries, Cromwell, CT, USA) and 10,000 U/ml streptomycin and 10,000 µg/ml penicillin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cells were passaged every 3 days and used at 60-70% confluence; 10-20 passages were used for subsequent experiments.

**Microarray data analyses.** Gene expression data of 18 nodal FL and 8 RLH samples from a previous study (3) (GSE48047) were re-analyzed. Expressed genes correlating with *PCDHGA3* expression were identified using GeneSpring software version 11.0.2 (Agilent Technologies, Inc., Santa Clara, CA, USA), as previously described (3).

**Hematoxylin and eosin staining and immunohistochemistry.** Patient samples were fixed with 10% formaldehyde for 24 h at room temperature. The tissues were then embedded in paraffin and cut into 3 µm sections. The slides were soaked in xylene, dipped into a Coplin jar containing Mayer's hematoxylin and agitated for 30 sec. The slides were then rinsed in H<sub>2</sub>O for 1 min, then counterstained with 1% eosin Y solution for 10-30 sec with agitation. The sections were then dehydrated with two changes of 95% alcohol and two changes of 100% alcohol for 30 sec each, then 1-2 drops of mounting medium was added, and covered with a coverslip.

Formalin-fixed paraffin-embedded tissue sections (3 µm thick) were immunohistochemically stained using a BOND-MAX autostainer (Leica Biosystems, Melbourne, Australia), with immune complexes visualized by the polymer method, according to the manufacturer's protocol and as previously described (10). The following primary antibodies were used: Rabbit anti-human *PCDHGA3* (1:50; cat. no. RB33029; Abgent, Inc., San Diego, CA, USA) and mouse anti-human CD21 (1:20; cat. no. IR608; Dako Cytomation, Glostrup, Denmark). For the indirect double immunofluorescence

study, cytospin slides of FL18 cells were subjected to staining with the primary antibodies against *PCDHGA3* and mouse anti-tumor necrosis factor receptor superfamily member 6B (TNFRSF6B; 1:100; cat. no. ab57956; Abcam, Cambridge, UK), as described previously (10).

**Reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was extracted from cells using the miRNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocols. RNA concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA) and reverse-transcribed into cDNA using SuperScript III First-Strand Synthesis System kit (Invitrogen; Thermo Fisher Scientific, Inc.) and a Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's protocol. PCR was performed using GeneAmp Fast (2X) PCR Master Mix (Thermo Fisher Scientific, Inc.), using the following *PCDHGA3*-specific primer sequences: Forward, 5'-CTC ACAAGCTCAGTCCCAGA-3' and reverse, 5'-CATAAGTGA TGCGGGCGTTG-3'.  $\beta$ -actin (*ACTB*) was also amplified as a control, using the following primers: Forward, 5'-CATGTA CGTTGCTATCCAGGC-3' and reverse, 5'-CTCCTTAAT GTCACGCACGAT-3' (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). Reactions were performed using a Bio-Rad T100 Thermal Cycler and thermal cycling conditions consisting of: 94°C for 2 min, 94°C for 30 sec, 55°C annealing temperature for 30 sec, and 72°C for 1 min for 30 cycles, followed by 72°C for 10 min. Following amplification, PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide fluorescence using a Gel Print 2000i VGA, USA (BioImage; Thermo Fisher Scientific, Inc.) The resulting bands were compared to  $\beta$ -actin bands produced using the same cDNA isolated from each cell line.

***PCDHGA3* gene knockdown assay.** Three *PCDHGA3* small interfering (si)RNAs (s31922, s31923 and s31924) and one negative control siRNA (Silencer Select Negative Control #1) were obtained from Thermo Fisher Scientific, Inc. FL18 cells (5x10<sup>5</sup>) were transfected with 200 nM *PCDHGA3* or negative control siRNA using a Neon Transfection System (Invitrogen; Thermo Fisher Scientific, Inc.) under the conditions of 1400 V, 20 ms and 2 pulses. At 24 h post-transfection, the medium was replaced with fresh medium. The cells were grown for a further 48 h and harvested for subsequent analysis.

Total RNA was extracted from cells using a miRNeasy Mini kit (Qiagen, Inc.) according to the manufacturer's protocol. RNA purity and concentration were determined spectrophotometrically. RNA was reverse-transcribed into cDNA using SuperScript III First-Strand Synthesis System kit (Invitrogen; Thermo Fisher Scientific, Inc.) and a Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories, Inc.), according to the manufacturer's protocol. Quantitative PCR (qPCR) was performed using a StepOnePlus Real-Time PCR System and TaqMan Gene Expression assays (Thermo Fisher Scientific, Inc.). Specific probes for *PCDHGA3* (Hs00259230\_s1), *GAPDH* (Hs02758991\_g1) and *ACTB* (Hs99999903\_m1) were obtained from Thermo Fisher Scientific, Inc. The relative degree of change for each gene was calculated using the 2<sup>- $\Delta\Delta C_q$</sup>  method (11), with *GAPDH* as the endogenous control.

**Cell proliferation assay.** At 24 h post-siRNA transfection, cells were seeded in a 24-well culture plate at a density of  $1 \times 10^5$  cells/ml. At 48 and 72 h post-transfection, cell numbers were manually counted via trypan blue staining.

**Western blotting.** Cultured FL18 cells ( $1 \times 10^6$ ) were harvested, washed in cold PBS, and lysed using Ambion RNeasy lysis buffer (Thermo Fisher Scientific, Inc.). Equal amounts of protein (15  $\mu$ g per lane) from each sample were separated by 8% SDS-PAGE (Bolt Bis-Tris Plus Gel, Thermo Fisher Scientific, Inc.), the membranes were blocked with PBS-T (PBS with 0.1% Tween-20) containing 5% skimmed milk powder at room temperature for 1 h, then transferred onto a polyvinylidene difluoride membrane (Invitrogen; Thermo Fisher Scientific, Inc.). Membranes were incubated at 4°C overnight with a rabbit anti-human PCDHGA3 antibody (1:500; cat. no. AP13611C; Abgent, Inc., San Diego, CA, USA); a mouse anti-human  $\beta$ -actin antibody (1:50,000; cat. no. sc47778; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) served as a loading control, as previously described (12). The membrane was washed in PBS-T, and incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The following secondary antibodies were used: Donkey anti-rabbit (1:50,000; cat. no. NA9340V; GE Healthcare Life Sciences, Chalfont, UK) and sheep anti-mouse (1:50,000; cat. no. NA9310V, GE Healthcare Life Sciences). The membrane was washed with PBS-T, and immune complexes were visualized using an enhanced chemiluminescence Prime Detection System (GE Healthcare Life Sciences).

**Statistical analysis.** The Chi-squared test, Fisher's exact test and one-way analysis of variance were conducted using SPSS software, version 14.0 (SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Expression of PCDH- $\gamma$  subfamily genes is upregulated in nodal FL compared with RLH.** Of the differentially expressed genes in terms of gene ontology between nodal FL and RLH, the top five upregulated genes were PCDH- $\gamma$  subfamily members B4, B3, B2, A5 and A3. A heatmap of the PCDH- $\gamma$  subfamily genes is presented in Fig. 1. Therefore, PCDHGA3, as a representative gene, was selected for further analysis.

**PCDHGA3 is strongly expressed in FL, including high-grade and BCL2-negative cases.** Results of the immunohistochemical analysis of RLH and FL samples are presented in Fig. 2 and Table I. Only 1/17 RLH samples were weakly PCDHGA3-positive (Fig. 2A and B), whereas 19/20 (95%) FL grade 1-2 samples were positive (Fig. 2C and D). In addition, approximately half of the FL grade 3A samples were positive (Fig. 2E and F). Notably, all 10 samples of FL with diffuse area were PCDHGA3-positive (Fig. 2G-I) and 13 of 15 (87%) BCL2-negative FL samples were PCDHGA3-positive (Fig. 2J-M). Ki-67 staining (a marker of proliferation) was performed to diagnose BCL2-negative neoplastic follicles. These results revealed that FL cells express PCDHGA3 irrespective of BCL2 status or grade.

Table I. PCDHGA3 expression in RLH and FL samples.

Histological subtype	No. studied	Positive (%)	P-value (vs. RLH)
RLH	17	1 (5.9)	-
FL (Grades 1 and 2)	20	19 (95)	<0.001
FL (Grade 3)	9	5 (55.6)	0.028
FL (Diffuse area)	10	10 (100)	<0.001
FL (BCL2 negative)	15	13 (86.7)	<0.001

RLH, reactive lymphoid hyperplasia; FL, follicular lymphoma; BCL2, B-cell lymphoma 2.

**PCDHGA3 silencing reduces cell proliferation in the FL18 cell line.** As a large proportion of nodal FL cases expressed PCDHGA3, PCDHGA3 expression status was examined in the FL cell lines, FL18 and FL318. The two cell lines expressed PCDHGA3 mRNA, as determined by RT-qPCR (Fig. 3A). PCDHGA3 gene knockdown with PCDHGA3 siRNAs was performed in the FL18 cell line. The silencing effects of three different PCDHGA3-specific siRNAs in FL18 cells were evaluated using RT-qPCR and western blotting. The s31924 siRNA was selected for further analysis as it yielded the greatest PCDHGA3 mRNA inhibition ratio (55.9%) relative to untreated FL18 cells ( $P = 0.005$  Fig. 3B and C). Following PCDHGA3 knockdown, proliferation curves were generated for untreated FL18 cells, and FL18 cells transfected with negative siRNA or PCDHGA3 s31924 siRNA. Proliferation was significantly suppressed in FL18 cells transfected with PCDHGA3 siRNA at 72 h compared with untreated FL18 cells. ( $P < 0.001$ ; Fig. 3D).

**Coexpression of TNFRSF6B and PCDHGA3 in the FL18 cell line.** Genes expressed concomitantly with PCDHGA3 were selected from the gene expression data of nodal FL and RLH (Table II). TNFRSF6B was among the top five correlated genes. Furthermore, the protein encoded by this gene may be involved in regulating the suppression of Fas ligand-mediated cell death (13). Coexpression of TNFRSF6B and PCDHGA3 was observed in the FL18 cell line by double immunostaining (Fig. 3E).

## Discussion

PCDHs, which are downregulated via epigenetic mechanisms in various tumor types, have been suggested as candidate tumor suppressor genes (6-9,14). It has been previously demonstrated that expression of PCDH- $\gamma$  subfamily genes is upregulated in low-grade FL (3). In the present study, PCDHGA3 expression was investigated in various grades of FL using immunohistochemistry, and it was examined whether this gene product may contribute to cell proliferation in an FL-derived cell line. As an antibody against PCDHGA3 suitable for use in immunohistochemical studies was available, this gene and protein was selected for further analysis.

For the pathological diagnosis of FL, detection of aberrant BCL2 expression in neoplastic follicles is important

Table II. Top 10 genes correlated with PCDHGA3.

Gene name	Gene symbol	Median value
Opiate receptor-like 1	OPRL1	0.6731
Tumor necrosis factor receptor superfamily, member 6b, decoy	TNFRSF6B	0.6721
ADAMTS-like 5	ADAMTSL5	0.6635
1-Aminocyclopropane-1-carboxylate synthetase homolog	ACCSL	0.6401
Carbohydrate sulfotransferase 4	CHST4	0.6375
Transmembrane channel-like 6	TMC6	0.6331
Solute carrier family 16, member 12	SLC16A12	0.6299
MAM domain containing 4	MAMDC4	0.6281
PRKC, apoptosis, WT1, regulator	PAWR	0.6271
Cullin-associated and neddylation-dissociated 1	CAND1	0.6288

Expressed genes correlating with *PCDHGA3* expression were identified using GeneSpring software. Median value represents the correlation index between the gene and PCDHGA3. PCDHG, protocadherin  $\gamma$ .

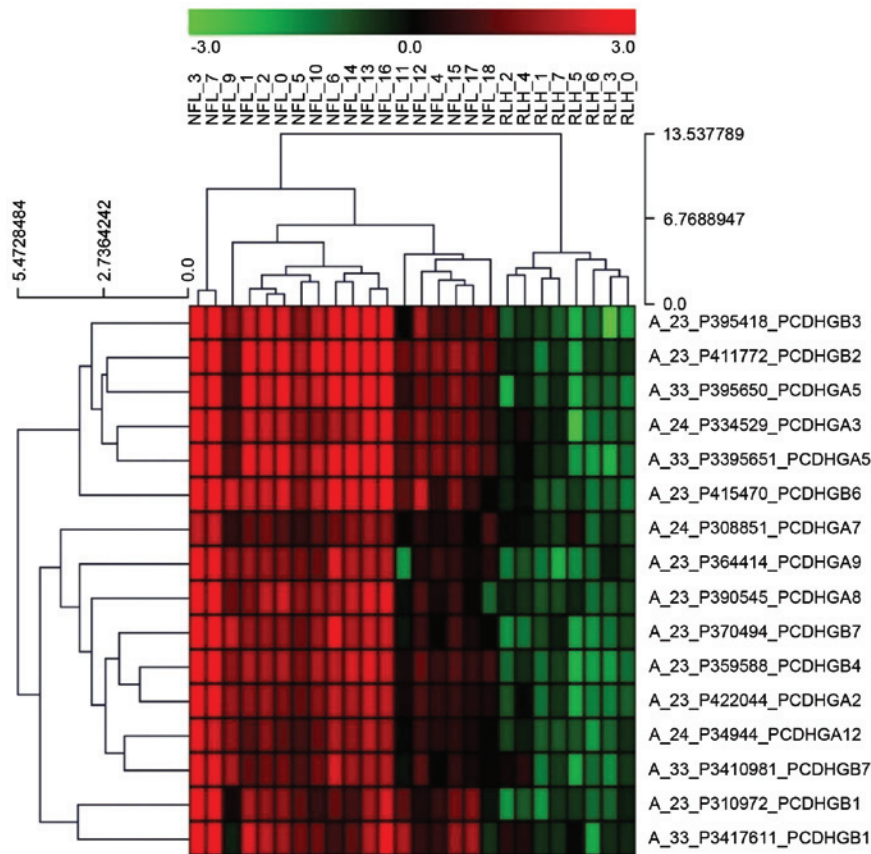


Figure 1. Heatmap of PCDHG gene subfamilies in FL and RLH. The top five upregulated genes were PCDH- $\gamma$  subfamily members B4, B3, B2, A5 and A3. Red indicates gene upregulation and green indicates gene downregulation. PCDHG, protocadherin  $\gamma$ ; FL, follicular lymphoma; RLH, reactive lymphoid hyperplasia.

and useful, as 80-90% of cases of low-grade FL harbor the IGH-BCL2 translocation. Although only 10-20% cases of low-grade FL lack BCL2 expression, cases of high-grade FL less commonly harbor this translocation and therefore less frequently express BCL2 (15). As it may be difficult to diagnose BCL2-negative FL, other diagnostic markers have been investigated. For example, Masir *et al* (16) reported that a rabbit monoclonal BCL2 antibody (E17) facilitated the

diagnosis of BCL2-negative FLs, whereas Zamo *et al* (17) reported the lack of TUBB3 expression in BCL2-negative FLs. The present study hypothesized that PCDHGA3 is a useful diagnostic marker for all cases of FL, as PCDHGA3 expression was observed irrespective of BCL2 status. The positivity of PCDHGA3 in FL Grade 3 (55.6%) was lower than the rest of the listed FL group (86.7-100%) although it was still high enough to use as a diagnostic tool. This may be associated

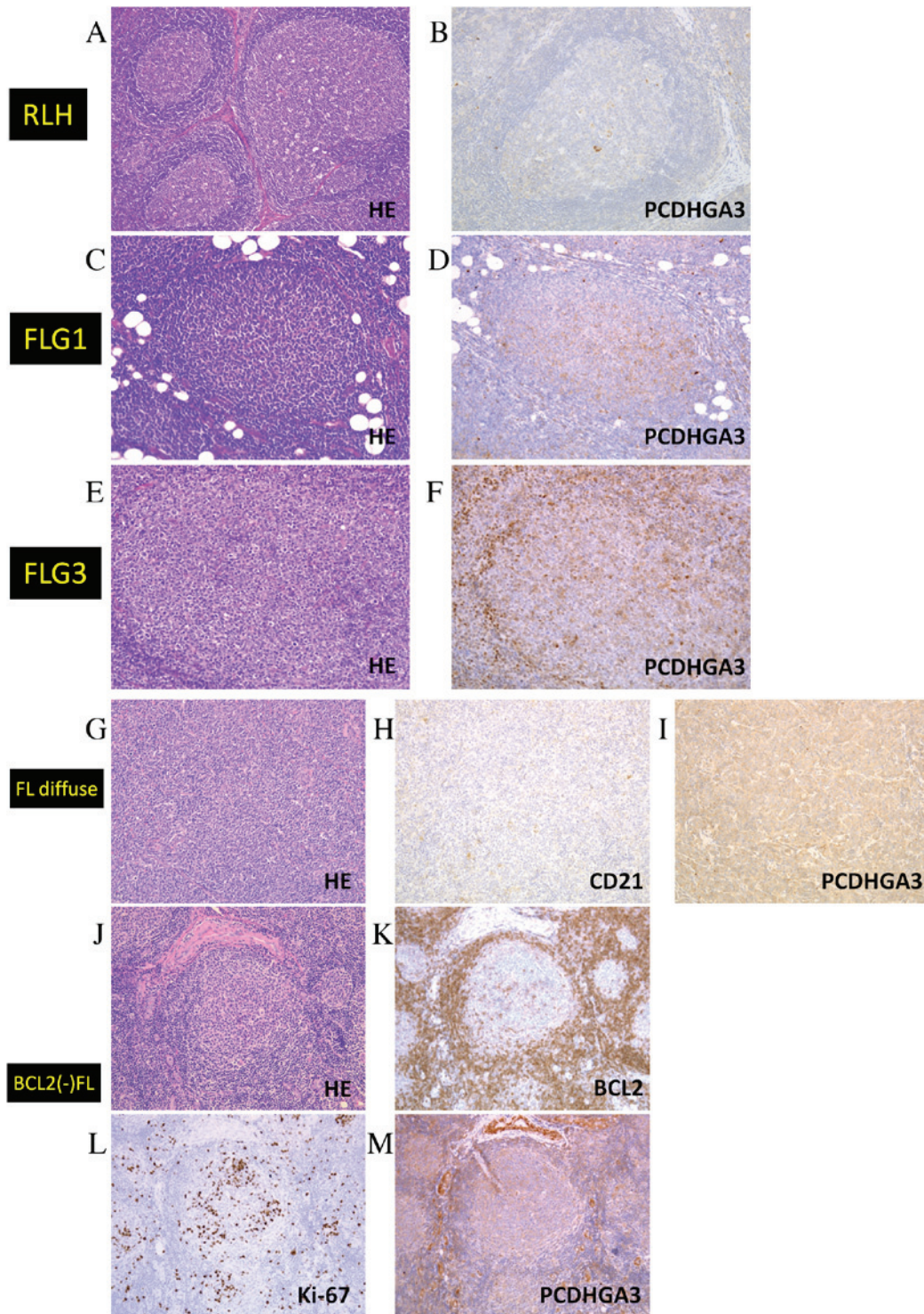


Figure 2. Immunohistochemical analysis of PCDHGA3 in FL samples. (A) HE staining of RLH. (B) PCDHGA3 was negative in RLH. (C) HE staining of an FL grade 1 sample, which was (D) positive for PCDHGA3. (E) HE staining of an FL grade 3 sample, which was (F) positive for PCDHGA3. (G) HE staining of an FL with diffuse area sample, which was (H) negative for CD21-expressing follicular dendritic cells and (I) positive for PCDHGA3. (J) HE staining of a BCL2-negative FL sample, which was (K) negative for BCL2, (L) positive for Ki-67 and (M) positive for PCDHGA3. All these images were representative of their groups. Original magnification A and G-M, x100. Original magnification B-F, x200. PCDHG, protocadherin  $\gamma$ ; FL, follicular lymphoma; HE, hematoxylin and eosin; RLH, reactive lymphoid hyperplasia; CD, cluster of differentiation; BCL2, B-cell lymphoma 2.

with the high-grade feature of the tumor cells, and should be investigated further in future studies.

Similarly high levels of *PCDHGA3* expression were detected in FL18 cells and samples from FL cases, and FL18 cell proliferation was suppressed following *PCDHGA3* gene

knockdown, suggesting that *PCDHGA3* is associated with FL tumor cell growth.

To investigate the mechanism underlying the effect of *PCDHGA3* on tumor cell proliferation, gene expression profiles were reanalyzed to identify genes that were

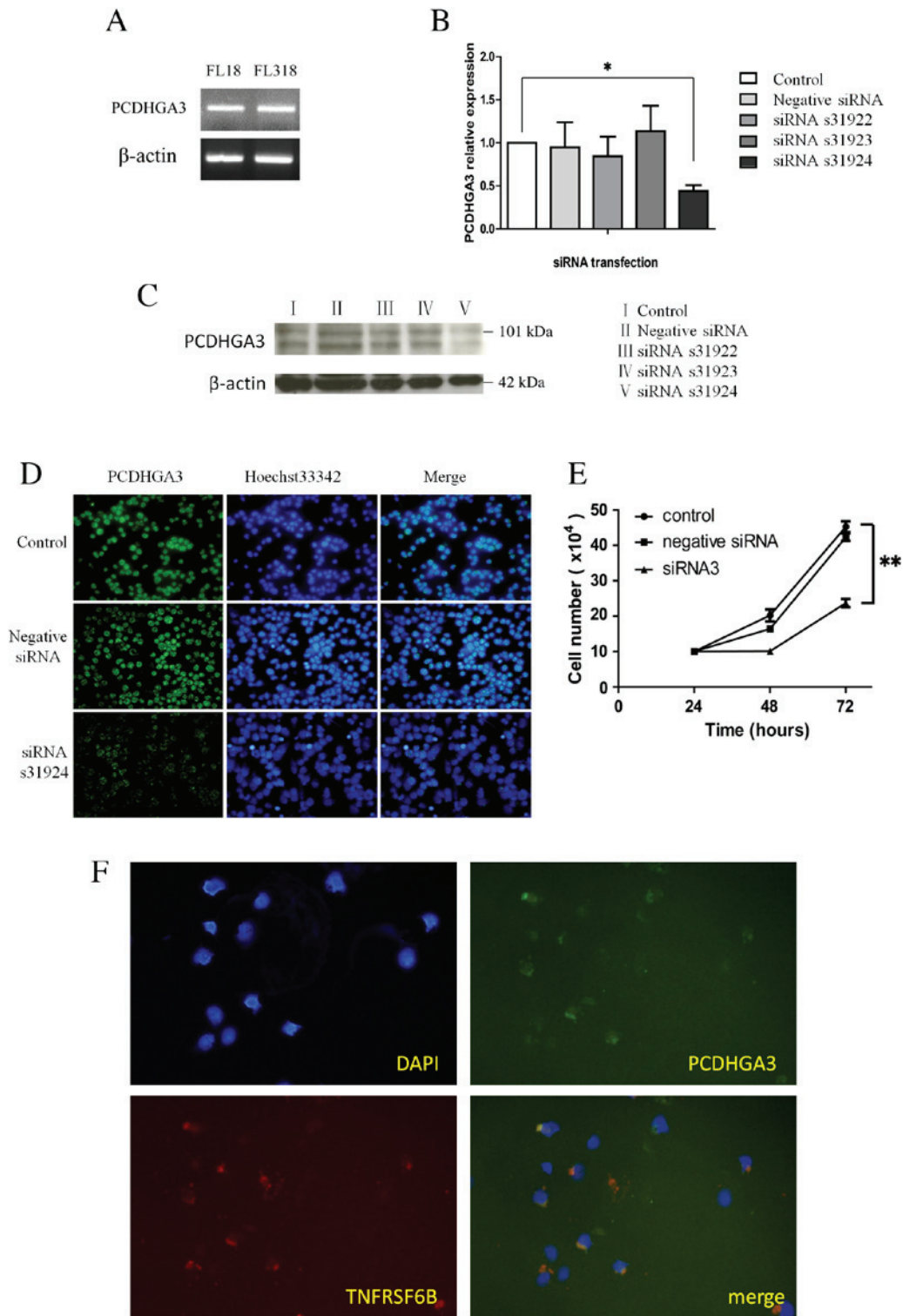


Figure 3. *PCDHGA3* gene knockdown. (A) *PCDHGA3* mRNA was detected in the FL-derived cell lines, FL18 and FL318. Knockdown of the *PCDHGA3* gene was confirmed by (B) reverse transcription-quantitative polymerase chain reaction, (C) western blotting and (D) immunofluorescence. The siRNA, s31924, was selected for further analysis because it yielded the greatest *PCDHGA3* mRNA inhibition ratio (55.9%) relative to untreated FL18 cells, original magnification  $\times 100$ . (E) Cell proliferation was significantly suppressed in *PCDHGA3* siRNA-treated FL18 cells at 72 h. (F) Coexpression of *PCDHGA3* and *TNFRSF6B* was observed in the FL18 cell line by double immunofluorescence staining, original magnification  $\times 200$ . \* $P < 0.01$ ; \*\* $P < 0.001$ . PCDHG, protocadherin  $\gamma$ ; FL, follicular lymphoma; siRNA, small interfering RNA; *TNFRSF6B*, tumor necrosis factor receptor superfamily member 6B; DAPI, 4,6-diamidino-2-phenylindole.

differentially expressed concomitantly with *PCDHGA3*. *TNFRSF6B* was among the top five coexpressed genes. *TNFRSF6B* overexpression has been reported in various types

of malignant tumors (18-20) and its protein product inhibits Fas ligand-mediated cell death. With regards to lymphoma, a study by Bedewy *et al* (21) revealed that *TNFRSF6B*

expression was associated with shorter event-free survival in an aggressive subtype of B-cell lymphoma. In the present study, coexpression of TNFRSF6B and PCDHGA3 proteins was observed in the FL18 cell line. The association between these genes remains to be elucidated, although the proteins may interact with each other. Further investigation is required to clarify this.

In conclusion, the results of the present study revealed that PCDHGA3 was expressed in FL, irrespective of the BCL2 status and tumor grade, and was demonstrated to be associated with cell proliferation. Further studies involving molecular genetic analysis are required to clarify the mechanisms underlying the effect of *PCDHGA3* in FL.

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