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MAML3 Contributes to Induction of Malignant Phenotype of Gallbladder Cancer Through Morphogenesis Signalling Under Hypoxia

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Abstract. *Background/Aim: Hedgehog (HH) signalling is a potential therapeutic target for gallbladder cancer (GBC), and Mastermind-like 3 (MAML3) is involved in the transcription of Smoothened (SMO), which is a key protein of HH signalling during hypoxia in the cancer microenvironment. MAML3 is a NOTCH signalling activator, and HH and NOTCH are involved in morphogenesis signalling. However, the association between MAML3-NOTCH and HH signalling and its role in regulating GBC cells remain unknown. This study aimed to determine whether NOTCH signalling affects tumour aggressiveness in GBC under hypoxic conditions and if MAML3 could be a new comprehensive therapeutic target that regulates morphogenesis signalling, HH, and NOTCH in GBC. Materials and Methods: We used three cell lines (NOZ, TYGBK1, and TGBC2TKB) and 58 resected specimens. These samples were subjected to cell proliferation, RNA interference, invasion, western blot, and immunohistochemical analyses. Results: MAML3 expression was higher under hypoxic conditions than under normoxic conditions and was involved in the activation of HH and NOTCH signalling. It contributed to the proliferation, migration, and invasion of GBC cells through the NOTCH signalling pathway and enhanced gemcitabine sensitivity. Immunohistochemical analysis showed that MAML3 expression was related to lymphatic invasion, lymph node metastasis, stage category, and a poor prognosis. Conclusion: MAML3 contributes to the induction of the malignant phenotype of GBC under hypoxia through the HH and NOTCH signalling pathways and may be a comprehensive therapeutic target of morphogenesis signalling in GBC.*

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Key Words: Gallbladder cancer, hypoxia, MAML3, Hedgehog signalling, Notch signalling.

Gallbladder cancer (GBC) is the most common malignant tumour of the biliary system (1). As GBC has no specific clinical manifestations, most cases are detected at the middle or late stages, with low radical resection rates, poor prognosis (2), and poor five-year survival rate, which is estimated at <10% (3). Owing to the thin gallbladder wall and the abundant vascular and lymphatic system surrounding the gallbladder, most patients with GBC have metastatic lesions in the advanced stage at diagnosis. Complete resection may be the only curative treatment for GBC. Gemcitabine has been increasingly used to treat GBC; however, many patients are resistant to gemcitabine (4). Therefore, to improve the prognosis of GBC patients, the exact mechanism of GBC initiation and development needs to be explored.

Hypoxic conditions are part of the cancer microenvironment (5, 6). Morphogenesis signalling, which involves NOTCH and Hedgehog (HH), is activated under hypoxic conditions, especially in cancers (7-10). NOTCH signalling is an evolutionarily conserved pathway that is integral to developmental processes, and defects in this pathway could lead to illnesses (11). According to the classical NOTCH signalling pathway theory, NOTCH cell surface receptors (NOTCH 1, 2, 3, 4) interact with transmembrane ligands on adjacent cells to mediate intercellular communication (12). The binding of ligands to the NOTCH receptor leads to its proteolytic cleavage through processes, such as ADAM-mediated and subsequent γ-secretase-mediated, culminating in the release of the intracellular domain of the NOTCH receptor from the cell membrane into the

Table I. *List of materials and reagents used.*

Materials	Detailed information and supplier
Fetal calf serum	FCS; Life Technologies, Grand Island, NY, USA
Plate reader	Biotrak Visible Plate Reader; Amersham Biosciences; Cytiva, GE Healthcare, Marlborough, MA, USA
DAPT	code: D4257, Tokyo Chemical Industry, Tokyo, Japan
siMAML3	ON-TARGET *plus* SMART pool, L-013813-01-0010, Horizon Discovery Ltd, Cambridge, UK
Negative control siRNA	ON-TARGET *plus* siCONTROL non-targeting pool, D-001810, Horizon Discovery Ltd, Cambridge, UK
The upper surface filter	pore size, 8.0 μm; BD Biosciences, Heidelberg, Germany
Light microscope	Nikon Eclipse TE 300; Nikon Corporation, Tokyo, Japan
MAML3	1:5000; Mouse. no. A300-684A; ThermoFisher Scientific, Waltham, MA, USA
Ki 67	1:200; Cat. no. sc 15402; Santa Cruz Biotechnology, Dallas, TX, USA
E-cadherin	1:200; Cat. no. sc 7870; Santa Cruz Biotechnology, Dallas, TX, USA
Vimentin	1:1,000; Cat. no. ab92547; Abcam, Cambridge, UK
SNAI1	1:200; Cat. no. sc 271977; Santa Cruz Biotechnology, Dallas, TX, USA
Slug	1:200; Cat. no. sc 15391; Santa Cruz Biotechnology, Dallas, TX, USA
PTCH1	1:200; Santa Cruz Biotechnology, Dallas, TX, USA
SMO	1:200; Cat. no. 20787 1 AP; ProteinTech Group, Rosemont, IL, USA
GLI1	1:500; Cat. no. ab151796; Abcam, Cambridge, UK
GLI2	1:500; Cat. no. ab187386; Abcam, Cambridge, UK
GLI3	1:200; Cat.no.sc 6154; Santa Cruz Biotechnology, Dallas, TX, USA
HES1	1:100; goat, sc-13844; Santa Cruz Biotechnology, Dallas, TX, USA
NOTCH1	1:200; Rabbit; c-20, sc-6014-R; Santa Cruz Biotechnology, Dallas, TX, USA
NOTCH2	1:200; Rabbit;25-255, sc-5545; Santa Cruz Biotechnology, Dallas, TX, USA
NOTCH3	1:200; Rabbit; M-134, sc-5593; Santa Cruz Biotechnology, Dallas, TX, USA
NOTCH4	1:200; Rabbit; H-225, sc-5594; Santa Cruz Biotechnology, Dallas, TX, USA
Anti-mouse antibody	1:10,000; Cat. no. NA931; Merck, Cytiva, Marlborough, MA, USA
Anti-rabbit antibody	1:10,000; Cat. no. NA934; Merck, Cytiva, Marlborough, MA, USA
Anti-goat antibody	1:10,000; Cat. no. sc-2020; Santa Cruz Biotechnology, Dallas, TX, USA
α-Tubulin	1:1,000; Cat. no. T6199; Sigma Aldrich; Merck KGaA, Darmstadt, Germany
Microsoft Excel	Microsoft Corp, Redmond, WA, USA
FACS (BD Biosciences)	BD CellQuest™ Pro software 6.0, USA

nucleus. The transcriptional core complex of NOTCH, comprising transcription factor RBPJ and transcriptional Mastermind-like (MAML) coactivator, enables the transcription of NOTCH signalling pathway-related genes (13-16). The three factors constituting the MAML transcriptional coactivator family are MAML1, MAML2, and MAML3 (17, 18). DAPT is a γ-secretase inhibitor that inhibits the NOTCH receptor cleavage and activation, which in turn inhibits cell proliferation and induces apoptosis and epithelial-to-mesenchymal transformation (EMT) (19-21). Our previous study showed that MAML3 causes hyperactivity in pancreatic cancer cells (PDAC) under hypoxia and helps increase transcription of the Smoothened (SMO) gene (22, 23). However, the association between MAML3-NOTCH and HH signalling and its role in regulating GBC cells remain unknown.

In this study, we examined the biological significance of MAML3 in GBC under hypoxic conditions of the tumour microenvironment with regard to activation of the morphogenesis signalling pathway. We aimed to determine whether MAML3 could be a new common therapeutic target of the morphogenesis signalling pathways such as HH and NOTCH, which lead to tumour malignancy in GBC.

Materials and Methods

Cell culture and reagents. A full list of the reagents and suppliers is provided in Table I. Three human GBC cell lines, namely, NOZ [Japanese Collection of Research Bioresource (JCRB) 1033, k-Ras mutation], TGBC2TKB (JCRB1130, k-Ras wild type), and TYGBK-1 [JCRB1499, p53 mutation exon 8 codon 285 (GAG to AGG), k-Ras wild type], were maintained in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with antibiotics and 10% foetal calf serum (FCS; Life Technologies, Grand Island, NY, USA). Under normoxic conditions, the cells were cultured in 95% air and 5% CO_2. Under hypoxic conditions, the cells were cultured in 1% O_2, 5% CO_2, and 94% N2 in a multigas incubator (Sanyo, Tokyo, Japan). All three GBC cell lines were cultured under normoxia or hypoxia for 48 h and used for experimental analyses.

Cell proliferation assay. GBC cells (1×10^5/well) were plated in 6-well plates and cultured under hypoxic conditions for 2 days. The cell count was monitored daily using a light microscope (Nikon Eclipse TE 300; Nikon Corporation, Osaka, Japan). For the DAPT experiment, the number of surviving cells was counted using LUNA-II™ Automated Cell Counter (Cat #L40001, Gyeonggi-do, Republic of Korea). These cells were seeded in 6 well plates (1×10^5 cells/well) and transfected with MAML3 or control siRNA for 48-72 h. For the gemcitabine-sensitivity assays, the siRNA transfected cells were cultured with or

without gemcitabine (0-200 μg/ml) for 48 h at 37˚C. Cell Count Reagent SF (Nacalai Tesque) was then added to the gallbladder cancer cells, which were cultured for 1 h at 37˚C (WST assay). Cell proliferation was assessed by measuring the absorbance at 492 nm using a plate reader (Biotrak visible plate reader; Amersham Biosciences, Cytiva, GE Healthcare, Marlborough, MA, USA) with a 620 nm area. For the DAPT drug experiment, the cells were cultured with different concentrations of DAPT (0-100 μM, D4257, Tokyo Chemical Industry, Tokyo, Japan) for 2 days. The cells in the negative control group were treated with an equal amount of DMSO.

RNA interference. siRNA for MAML3 (L-013813-01-0010) and negative control siRNA (D-001810) were produced by Dharmacon RNA Technologies (Horizon Discovery Ltd, Cambridge, UK). The cells (2×10^5/well) were cultured in 6-well plates and transfected with 100nM siRNA under normoxic conditions using Lipofectamine RNAiMAX Reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's guidelines; the cells were transfected for 2 days.

Matrigel invasion assay. The invasive capacity of GBC cells was assessed using the Matrigel invasion assay (19). The siRNA transfected cells (1×10^5) suspended in 10% FCS RPMI-1640 were added to the upper chamber and incubated for 16h under hypoxia or normoxia. The invading cells on the lower side of the filter were removed and stained using Diff-Quik reagents (Sysmex Corporation, Kobe, Japan); the cells were treated with Diff-Quik Fixative, Diff-Quik Solution I, and Diff-Quik Solution II for 10 min at room temperature (25-28˚C). A light microscope was used to count the stained cells at a 200× magnification.

Western blot analysis. Western blotting was performed according to a previously described protocol (24). The protein-transferred membranes (Whatman GmbH, Dassel, Germany) were incubated with primary antibodies against MAML3 (1:5000; Mouse. no. A300-684A; ThermoFisher Scientific, Waltham, MA, USA), Ki 67 (1:200; cat. no. sc 15402; Santa Cruz Biotechnology, Dallas, TX, USA), E-cadherin (1:200; cat. no. sc 7870; Santa Cruz Biotechnology), vimentin (1:1,000; cat. no. ab92547; Abcam, Cambridge, UK), SNAI-1 (1:200, cat. no. sc 271977; Santa Cruz Biotechnology), Slug (1:200; cat. no. sc 15391; Santa Cruz Biotechnology), α-SMA (1:2000; Rabbit; ab5694; Abcam), PTCH1 (1:200; Santa Cruz Biotechnology, Inc.), SMO (1:200; cat. no. 20787 1 AP; ProteinTech Group, Rosemont, IL, USA), GLI1 (1:500; cat. no. ab151796; Abcam), GLI2 (1:500; cat. no. ab187386; Abcam), GLI3 (1:200; cat.no.sc 6154; Santa Cruz Biotechnology), HES1 (1:100; goat, sc-13844; Santa Cruz Biotechnology), NOTCH1 (1:200; Rabbit; c-20, sc-6014-R; Santa Cruz Biotechnology), NOTCH2 (1:200; Rabbit; 25-255, sc-5545; Santa Cruz Biotechnology), NOTCH3 (1:200; Rabbit; M-134, sc-5593; Santa Cruz Biotechnology), and NOTCH4 (1:200; Rabbit; H-225, sc-5594; Santa Cruz Biotechnology) overnight at 4˚C. The membranes were incubated for 1 h at room temperature (25-28˚C) with horseradish anti-mouse (1:10,000; cat. no. NA931; Merck, Cytiva, Marlborough, MA, USA) peroxidase-conjugated antibody, anti-rabbit (1:10,000; cat. no. NA934; Merck, Cytiva) horseradish peroxidase-linked antibody, or anti-goat (1:10,000; cat. no. sc 2020; Santa Cruz Biotechnology, Dallas, TX, USA) horseradish peroxidase-linked antibody. α-Tubulin (1:1,000; cat. no. T6199; Sigma Aldrich; Merck KGaA, Darmstadt, Germany) was used as the protein loading control.

Immunohistochemistry. Tissue samples were collected between January 2001 and December 2012 from 58 patients with GBC who underwent resection at the Department of Surgery and Oncology at Kyushu University Hospital in Fukuoka, Japan. Patients who had received other anticancer treatments prior to the surgery were excluded from the study. Approval for the use of tissues was granted by the Ethical Committees for Clinical Study at Kyushu University (reference no. 30 230). The study was conducted in accordance with the Helsinki Declaration and the Japanese government's Ethical Guidelines for Human Genome/Gene Research Informed consent was obtained from the patients in accordance with relevant guidelines. A 3% hydrogen peroxide solution was used for 5 min to inhibit endogenous peroxidase activity. The Target Retrieval Solution was used for a 10 min high-pressure antigen retrieval procedure. The slides were incubated with the primary antibodies for an entire day at 4˚C and then with Histofine Simple Stain MAX PO (M) (4 μg/ml, Cat. no. 424131) or Histofine Simple Stain MAX PO (R) (4 μg/ml; Cat. no. 424141; Nichirei Biosciences, Tokyo, Japan) for 40 min at room temperature (25-28˚C). Diaminobenzidine (DAB) was used to visualise the tagged antigens. Haematoxylin was used for counterstaining for 3 min at room temperature (25-28˚C).

Statistical analyses. Data are presented as mean±standard deviation (SD). To compare continuous variables between two groups, Student's *t*-test was performed. Microsoft Excel (Microsoft Corp, Redmond, WA, USA) was used to complete the statistical analysis. Statistical significance was set at p-value <0.05. The relationship between negative and positive expression of MAML3 in human tissue specimens was analysed using Pearson's χ^2 test. The Kaplan–Meier method was used to plot survival curves, and the Wilcoxon test was used to analyse the data.

Results

MAML3 may be hyperactive under hypoxia and induce the proliferation of GBC. To determine MAML3 expression under hypoxic and normoxic conditions in GBC and ascertain the effect of MAML3 on GBC cell proliferation, all three human GBC cell lines were treated with different MAML3 protein concentrations to determine the one exerting optimal developmental effect. Previously, human PDAC cell lines (ASPC-1 and SUIT-2) were used to determine MAML3 expression in pancreatic cancer during hypoxia (19). Fluorescence-activated cell sorting and protein assays were used to observe and confirm the effect of MAML3 knockdown. The results showed that MAML3 protein signal intensity was significantly stronger under hypoxia than under normoxia (Figure 1A-D).

Cell-count results showed that the growth rates were much lower in all three GBC cells lines with MAML3 knockdown (siMAML3, line 2) than in those without MAML3 knockdown (siCONTROL, line 1) ($p<0.01$, Figure 1E). Although apoptosis occurred at 72 h under hypoxia, we found that the growth rates at 48 h were lower in the GBC cells with MAML3 knockdown (siMAML3, line 4) than in those without MAML3 knockdown (siCONTROL, line 3) ($p<0.05$, Figure 1E). We used western blotting to confirm

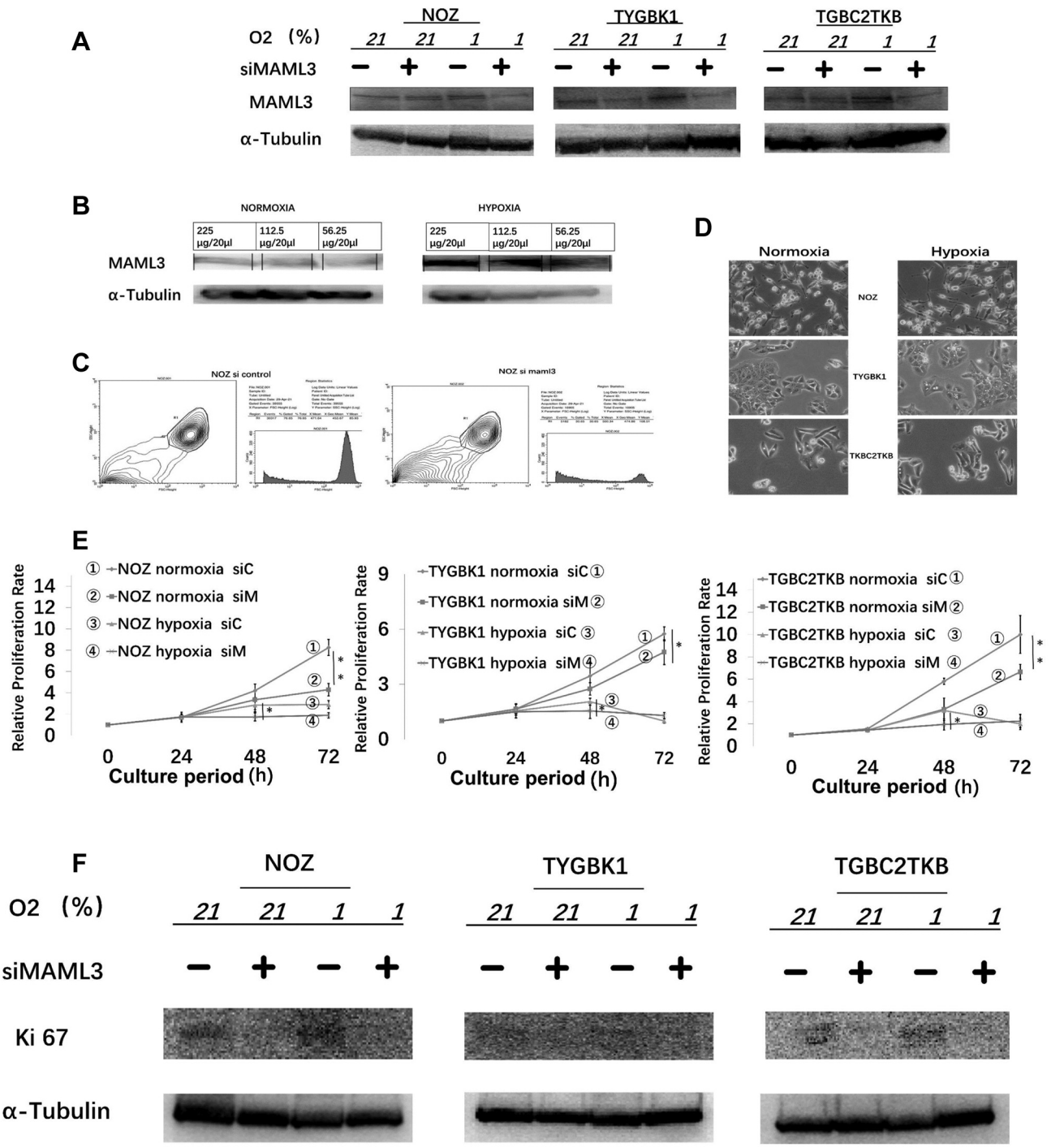


Figure 1. *Master mind like 3 (MAML3) is implicated in gallbladder cancer (GBC) proliferation under hypoxia. (A) Western blot analysis was used to detect the expression of MAML3 in GBC cell lines under normoxia and hypoxia after 48 h of treatment with MAML3 or control siRNA. (B) Proteins were extracted from GBC cells (NOZ) after incubation under normal oxygen and hypoxia for 48 h at three different concentrations of MALM3 protein (225, 112.5, and 56.25 μg/20 μl). Western blot assay showed that MAML3 was significantly up-regulated under hypoxia. (C) GBC cells (NOZ) were transfected with siMAML3 for 48 h in normoxia, and fluorescence activated cell sorting (FACS) was used to represent the successful transfection of siMAML3. (D) Images of GBC cells cultured for 48 h under normoxia and hypoxia. Changes in morphology are observed after incubating the GBC cells (NOZ, TYGBK1, and TGBC2TKB) in a hypoxic environment for 48 h. (E) Proliferation assay of MAML3 or control siRNA-treated GBC cell lines cultured for 24, 48, and 72 h under normoxia and hypoxia. (F) Western blot analysis was used to detect the expression of Ki 67, which indicated proliferation of GBC cells treated with MAML3 or control siRNA under normoxia and hypoxia. *p<0.05, **p<0.01, ***p<0.001, Bar, mean±standard deviation.*

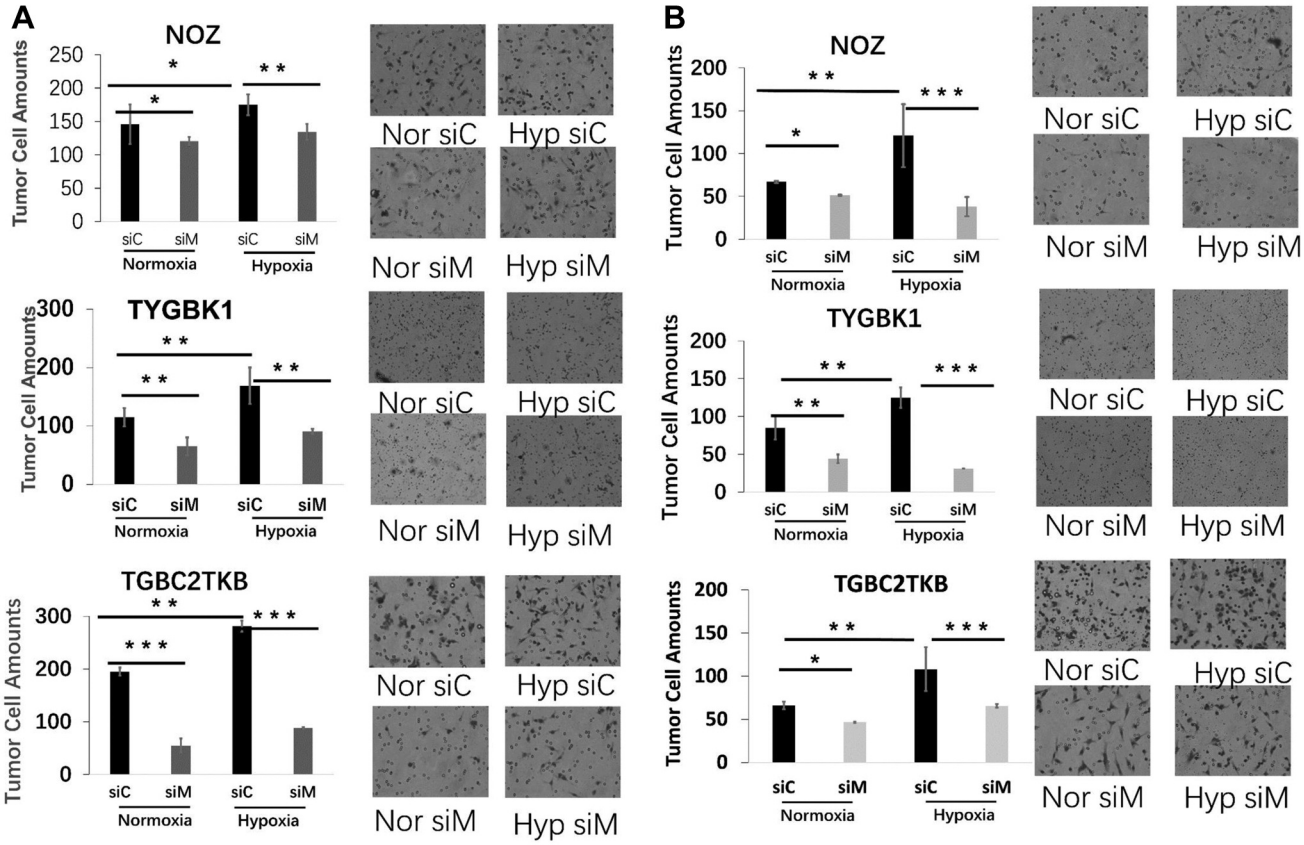
the presence of Ki-67, a nuclear protein that is a well-established marker of cell proliferation and may be required for maintaining cell proliferation (25). The signal intensity of the expressed Ki67 protein was reduced in all three GBC cell lines with MAML3 knockdown (siMAML3) under normoxia and hypoxia (Figure 1F). These findings indicate that MAML3 may play a vital role in the development of GBC by regulating cell proliferation under hypoxia.

MAML3 may enhance in vitro GBC migration and invasiveness under hypoxia. As cancer cells are known to show increased migration and invasion under hypoxia (19, 26, 27), we used transwell chambers to simulate GBC cell migration, and Matrigel invasion assays to simulate GBC cell invasion to determine whether MAML3 expression affected the migratory and invasive behaviour of GBC cells. GBC cells had higher migration and invasion potential under hypoxia than under normoxia, whereas GBC cell lines with MAML3 knockdown had significantly lower migration and invasion potential than that of the controls (Figure 2A and B). We used western blotting to confirm the extent of EMT, as it is a factor that endows cells with migratory and invasive properties (24, 28). Inhibition of MAML3 expression led to an increase in the expression of EMT-related molecules such as E-cadherin (epithelial gene), whereas that of vimentin (mesenchymal gene) decreased under both hypoxia and normoxia (Figure 2C). We also confirmed the protein expression of transcription repressors SLUG and SNAI-1 and the interstitial fibrosis marker α-SMA (29). As shown in Figure 2C, down-regulation of MAML3 resulted in reduced SLUG and α-SMA expression under hypoxia and normoxia, whereas the expression of SNAI-1 did not decrease. These results suggest that GBC cells exhibit migration and invasion hyperactivity in the hypoxic state. Additionally, selective inhibition of MAML3 down-regulates GBC cell migration and invasion in normoxia and hypoxia, which may be because MAML3 mediates the EMT.

MAML3 may affect the sensitivity of GBC cells to gemcitabine. Despite the effectiveness of gemcitabine as a chemotherapeutic agent for treating GBC, some patients do not respond well to this treatment, which is a concern (30). The sensitivity of GBC cells to gemcitabine was previously examined (31), and hypoxia was found to affect the pharmaceutical effectiveness of gemcitabine (32, 33). Thus, to determine whether MAML3 affects the effectiveness of gemcitabine and whether GBC cells develop resistance under hypoxic conditions, we performed the WST assay in all three GBC cell lines in normoxic and hypoxic conditions. The results showed that drug sensitivity of GBC cells decreased at gemcitabine concentrations ranging from 0-200 μg/ml in hypoxic conditions compared with that in normoxic conditions. Additionally, compared with the control group, all three GBC cell lines developed significant drug resistance to gemcitabine when MAML3 expression was reduced (Figure 3A and C). These results suggest that sensitivity of GBC cells to gemcitabine is modulated by MAML3 and the hypoxic state.

MAML3 might participate in the activation of morphogenesis signalling in both the HH and NOTCH signalling pathways. To elucidate the role of MAML3 in inducing proliferation and invasion of GBC under hypoxic conditions, we explored related cancer signal transduction pathways. MAML3 plays a role in hypoxia-induced SMO transcription up-regulation, which activates HH signalling in PDAC (19). Therefore, we evaluated the association between MAML3 (a transcriptional coactivator of NOTCH signalling) and related genes in the HH signalling pathway during hypoxia in GBC cells using western blotting. We observed that the inhibition of MAML3 down-regulated Patched homolog 1 (PTCH1), SMO (a key protein that drives HH signalling), GLI (a target gene for HH signalling), and HES1 (a target gene for NOTCH signalling) (19, 27, 34, 35) to varying degrees. Hyperexpression of GLI1 was observed under hypoxia. GLI2 expression was significantly altered in the TYGBK1 cell line when MAML3 expression was inhibited under hypoxia, but no significant changes were observed under normoxia (Figure 4A). MAML3, as an important co-operative transcription factor in the classic NOTCH signalling pathway, can induce migration in GBC under hypoxia (19, 26, 36, 37), which promotes proliferation and invasion of cancer cells and other pathologic biological phenomena (Figure 4B). In contrast, MAML3 exhibited hyperactivity under hypoxia (Figure 1A and B), inducing the expression of SMO and initiating the activation of the HH signalling pathway (Figure 4C). These findings indicate that MAML3 may mediate both NOTCH and HH signalling pathways, which affect the proliferation and invasion of GBC cells.

γ-secretase inhibitor DAPT can affect the proliferation, migration, and invasion of GBC cells. To investigate the pharmacological effects of the NOTCH inhibitor DAPT (38, 39) on GBC cell lines, we evaluated the pharmacokinetics of DAPT by treating the GBC cells with increasing doses (0-100 μM). The cell-count results revealed that at DAPT concentrations of 0-80 μM the viability of the GBC cells (NOZ) was approximately 90%, whereas the survival rate decreased to approximately 70% at a DAPT concentration of 100 μM (Figure 5A). Thus, DAPT exerted minimal toxicity at 80 μM concentration. The GBC cell lines that were cultured with 40 μM DAPT under normoxic and hypoxic conditions were observed for 72 h and subjected to western blot analysis, which revealed that cleaved NOTCH1 and HES1 (NOZ) levels decreased in a dose-dependent manner. This indicated that DAPT suppressed NOTCH signalling (Figure 5E). Consistent with MAML3-mediated NOTCH inhibition, we found that the proliferation of GBC cells was reduced under



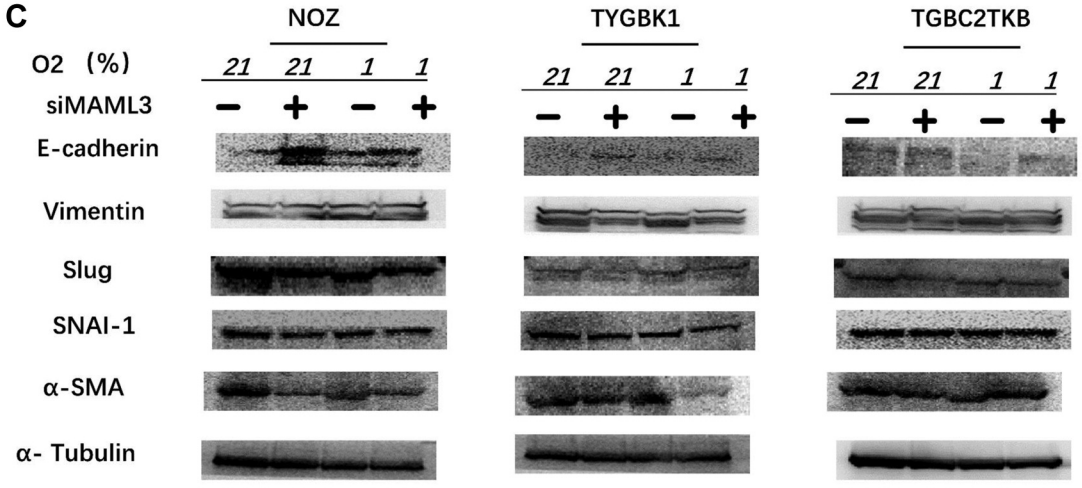


Figure 2. *Master mind like 3 (MAML3) is necessary for cell migration and invasion in gallbladder cancer (GBC) through enhanced epithelial-mesenchymal transition (EMT). (A) MAML3 or control siRNA-transfected GBC cells were added to the upper side of the transwell membrane for the migration assay. The cells that migrated to the bottom side of the membrane were stained with DiffQuik, and the total number of cells was counted after 16 h of incubation under normoxia and hypoxia. Magnification 200×. (B) In a 24-well plate, 100 μl Matrigel-RPMI 5× diluent (200 μg/ml) was added and placed in the upper chamber for 1 h at 4˚C. The upper chamber processed using Matrigel was subjected to the same procedure as in the migration experiment. Magnification 200×. (C) Expression levels of MAML3, EMT-related molecules (E cadherin, vimentin, SLUG, and SNAI-1), and alpha smooth muscle actin (α-SMA) in GBC cell lines treated with MAML3 or control siRNA. Scale bar, 50 μm. * p<0.05, **p<0.01, ***p<0.001, Bar, mean±standard deviation.*

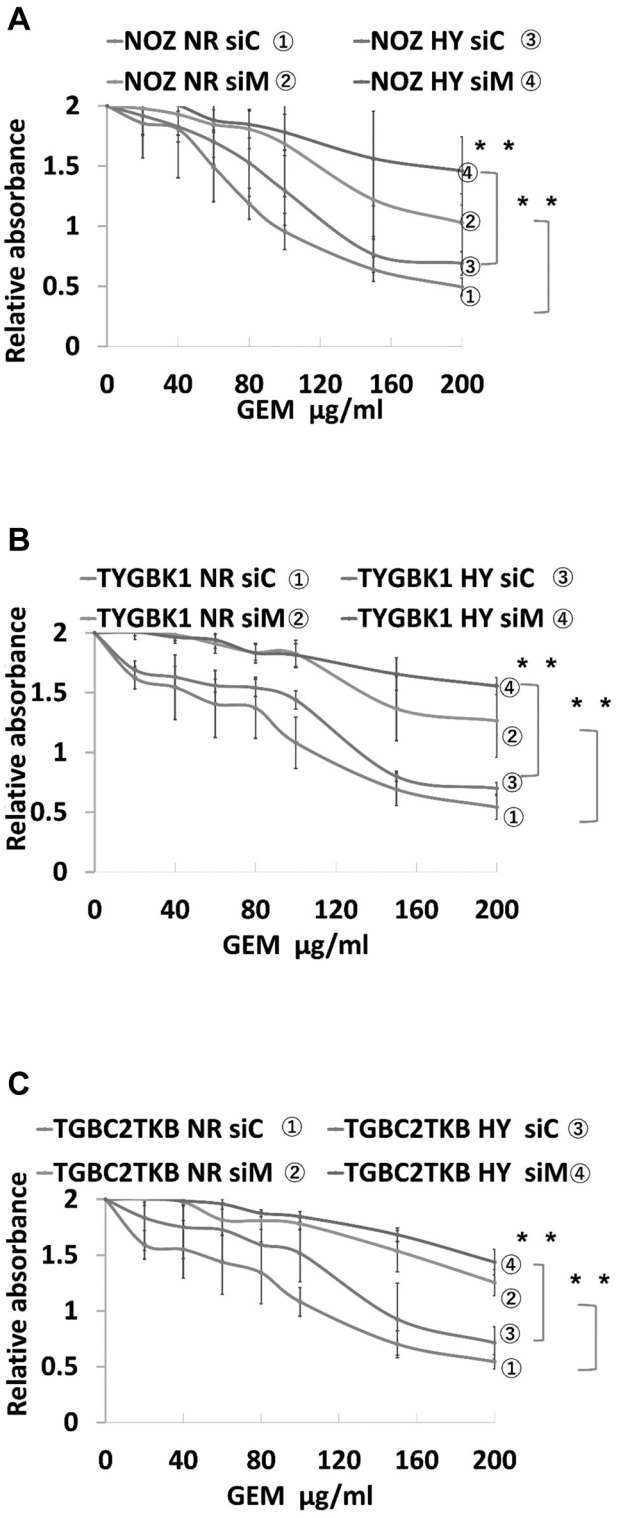


Figure 3. *Inhibition of Master mind like 3 (MAML3) expression reduced the sensitivity of gallbladder cancer (GBC) cells to gemcitabine under normoxia and hypoxia. Relative absorbance ratio of three (A), (B), (C) GBC cell lines transfected with MAML3 or control siRNA and treated with gemcitabine at 0-200 μg/ml for 48 h under normoxia and hypoxia. *p<0.05, **p<0.01, ***p<0.001.*

normoxia and hypoxia, and DAPT treatment had a significant effect on GBC cell proliferation (Figure 5B). Transwell assay results revealed that the number of GBC cells showing migration and invasion was significantly lower in the DAPT treatment groups than in the control group (Figure 5C and D). In normoxic and hypoxic states, GBC cells showed a decrease in vimentin and SNAI-1 expression and an increase in E-cadherin expression (Figure 5F). DAPT did not reduce the expression of other EMT markers such as SLUG (Figure 5F). These *in vitro* results suggest that NOTCH signalling may impact GBC cells and that NOTCH signalling is an essential factor for tumour growth and invasion.

Significant expression of MAML3 component in GBC patients. The tissue sections from 58 surgically resected human GBC specimens were subjected to immunohistochemistry. Based on the observed staining intensity, the cells were divided into two categories–negative and positive expression. MAML3 staining was observed in more than half of the patient tissue sections (n=45, 77.6%), whereas the remaining samples had no visible staining (negative expression, n=13). Additionally, MAML3 expression was detected in the nuclei of gallbladder epithelial cancer cells of patients (Figure 6A, arrow), indicating that MAML3 signalling was activated in GBC and cancer progressed from the epithelial mucosal cells to the external tissues (40, 41). We summarised these GBC cases and the clinicopathological features (sex, age, UICC T category, venous invasion, lymphatic invasion, UICC N category, stage classification) related to MAML3 in Table II. Pearson χ^2 analysis (42) showed that MAML3 expression and the clinicopathological variables, especially T factor, lymphatic invasion, N factor, and tumour stage, were correlated. In addition, Kaplan–Meier survival plot showed that the overall survival between the MAML3-negative and MAML3-positive expression groups was significantly different; the survival was poorer in the MAML3-positive expression group than in the MAML3-negative expression group (Figure 6B).

Discussion

Of the three GBC lines used in this study, NOZ harbours a mutation in k-Ras, which affects the therapeutic effects of various drugs (43); TGBC2TKB and TYGBK-1 are k-Ras wild-type cells. Our analysis focused on cell behaviour under hypoxia. We hypothesised that hypoxia leads to mutations in malignant tissue and acquisition of invasive potential by tumour cells (44-48) in correlation with GBC.

In this study, we demonstrated that MAML3 may be a common therapeutic target for HH signalling and NOTCH signalling. The HH and NOTCH signalling pathways are evolutionarily conserved morphogenic pathways that mediate embryonic development and adult tissue homeostasis (15, 49, 50). Previous studies have provided a foundation for

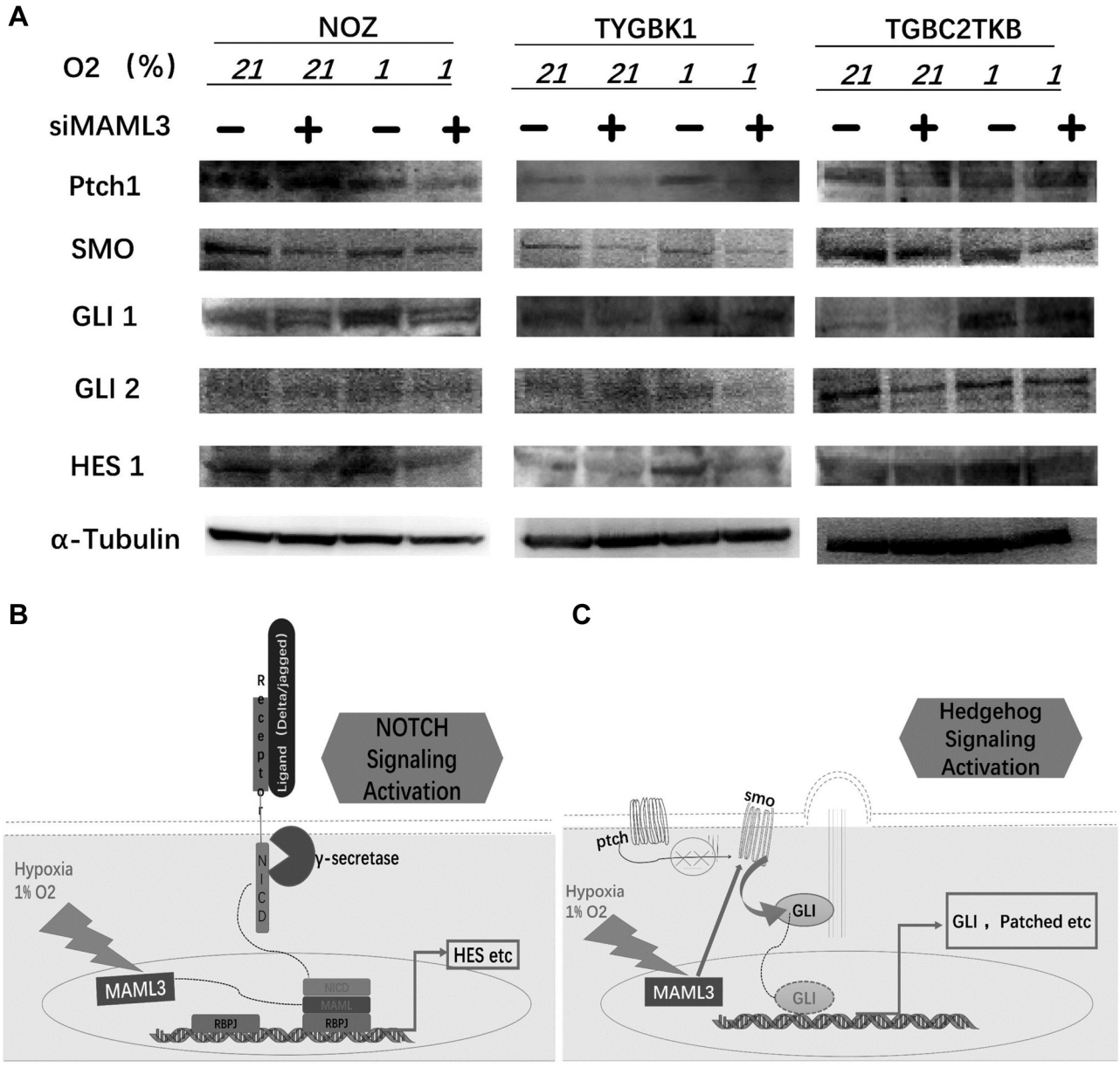


Figure 4. *MAML3 contributes to NOTCH/HH associated signalling in gallbladder cancer (GBC). (A) Western blot analysis was used to examine the expression of HH signal-related molecules (PTCH, SMO, GLI1, and GLI2) and NOTCH signal-related downstream target molecule HES1 in all three MAML3- and control siRNA-transfected GBC cell lines under normoxia and hypoxia for 48h. Schematic diagram of NOTCH signalling (B) and HH signalling (C) activation under hypoxia. In the NOTCH signalling pathway, MAML3 promotes the HES1 hyperexpression (NOTCH target product). In the HH signalling pathway, MAML3 resulting in hyperexpression of SMO and activation of GLI and PTCH. MAML3: Master mind like 3; PTCH1: Patched homolog 1 SMO: Smoothened; GLI1: glioma-associated oncogene homolog 1; GLI2: glioma-associated oncogene homolog 2; HES1: hairy and enhancer of split 1.*

understanding the role of NOTCH–HH signalling in carcinogenesis (14, 36, 51, 52). However, few parallels and variances exist in NOTCH–HH signalling among different cancers. Research has shown that hypoxia increases MAML3 expression, which contributes to increased transcription of *SMO* and *HES1*, promoting proliferation, invasion, and migration in PDAC (19) and small-cell lung cancer (53). These results combined with those of the current study provide an explanation for HH–NOTCH signalling crosstalk with MAML3 under hypoxia and potential cancer treatment targets.

Gemcitabine is a first-line treatment agent for gallbladder cancer in Japan; however, its treatment effect is limited (54).

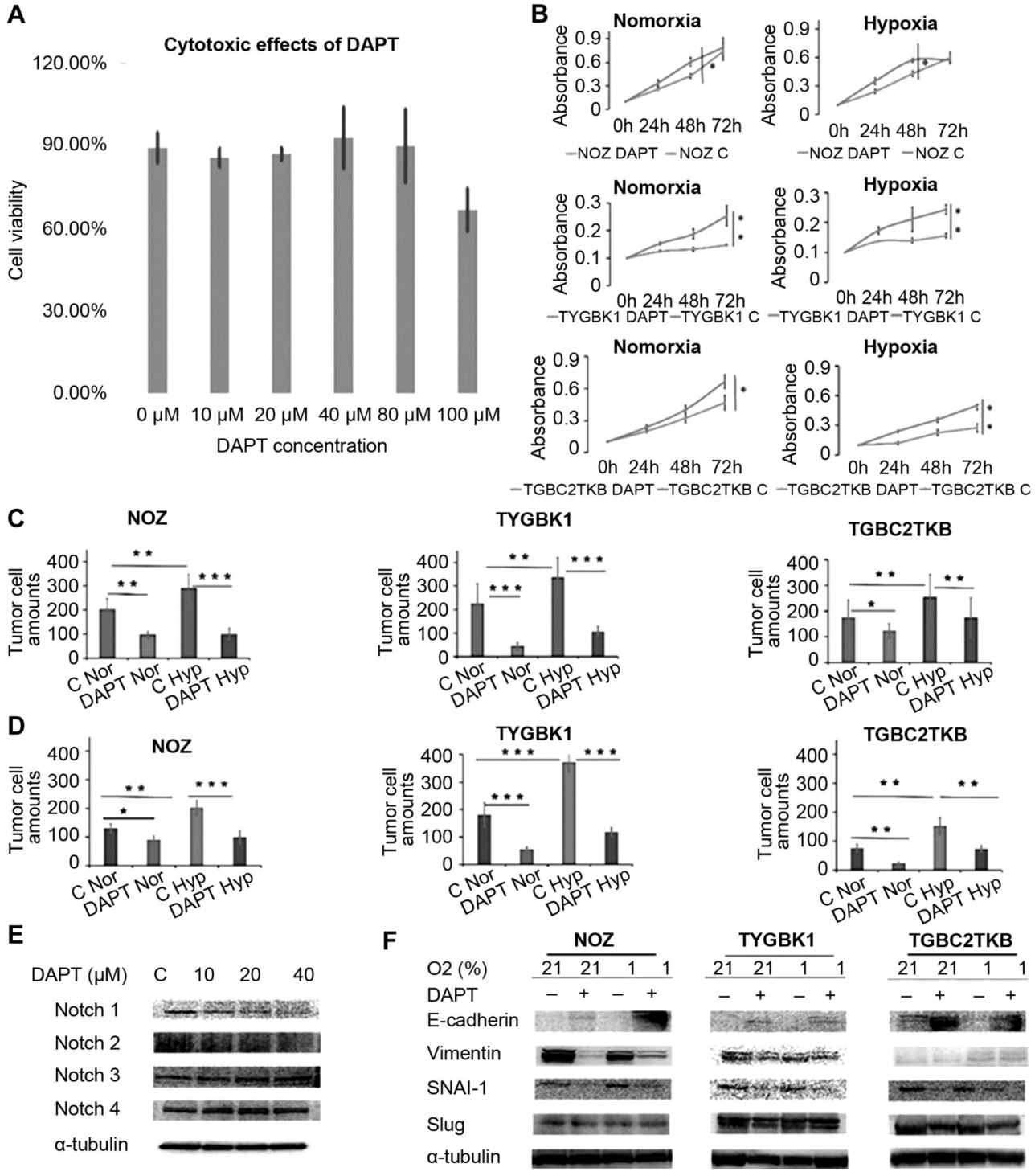


Figure 5. *Treatment with the γ-secretase inhibitor DAPT suppressed growth, migration, and invasion of gallbladder cancer (GBC) cells, and affected epithelial-mesenchymal transition (EMT)-related activity. (A) NOZ cells were treated with different concentrations of DAPT (0-100 μM) for 24 h, and the number of surviving cells was measured using the LUNA-II™ Automated Cell Counter. (B) In the proliferation assay, GBC cell lines were treated with 40 μM DAPT and incubated for 24, 48, and 72 h under normoxia and hypoxia. In the migration (C) and invasion assays (D), GBC cell lines were treated with DAPT (40 μM) and incubated in an upper chamber for 18 h (with DAPI counterstaining) under normoxia and hypoxia. Magnification 200×. Scale bar, 50 μm (E) The expression of NOTCH (1, 2, 3, 4) proteins was determined using western blotting (NOZ cell line) following treatment with 0-40 μM of DAPT. (F) EMT markers were analysed in GBC cell lines using western blotting following treatment with DAPT or DMSO control under normoxia and hypoxia. *p<0.05, **p<0.01, ***p<0.001. Bar, mean±standard deviation.*

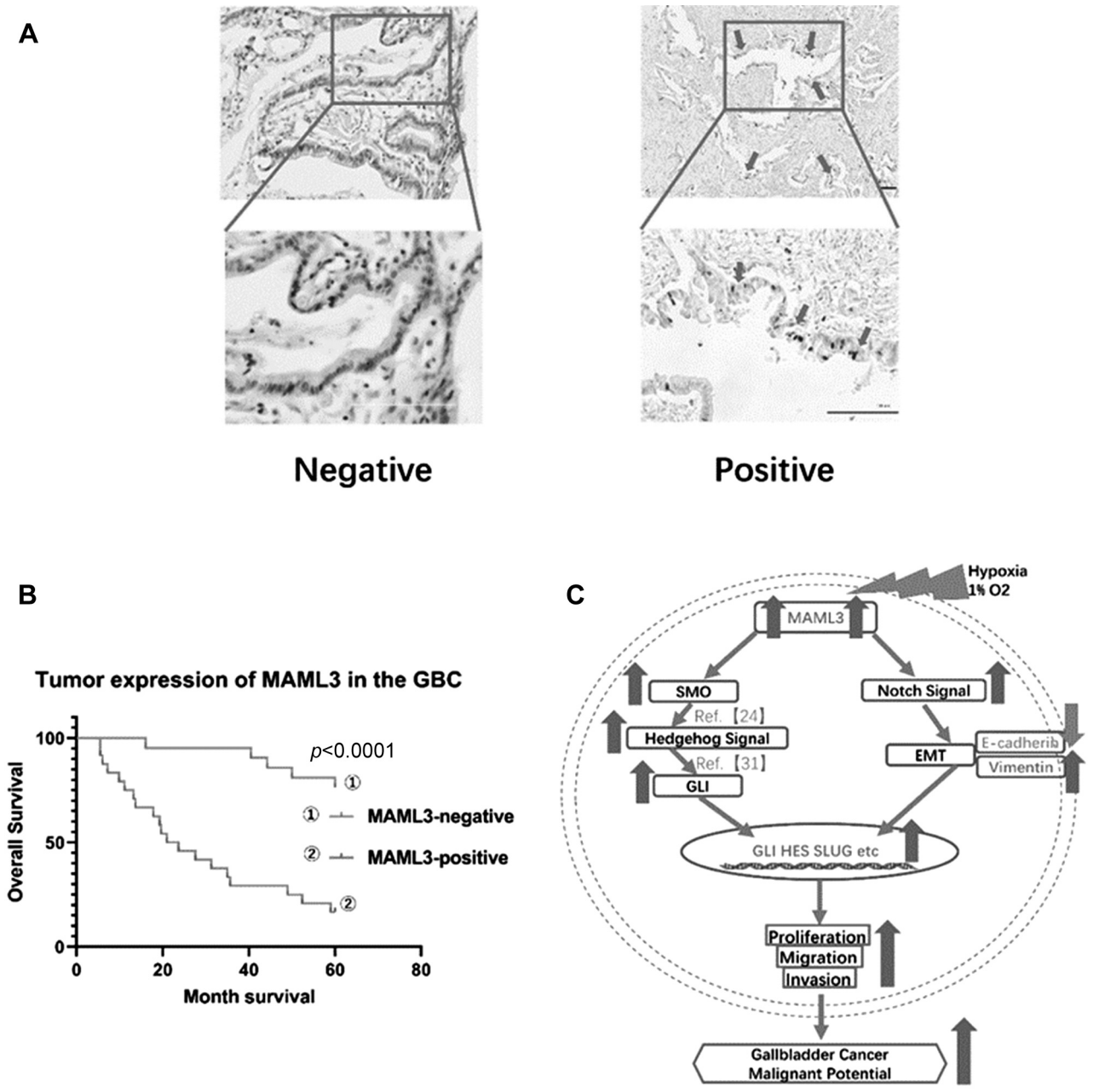


Figure 6. *Immunohistochemical analysis of MAML3 in gallbladder cancer (GBC) specimens and schematic findings of the study. (A) Immunohistochemical staining for MAML3 in GBC cells. Negative reaction, no staining; Positive reaction, brown staining. Red arrows indicate MAML3 expression in the nucleus of cancer cells. (Negative, upper image; original magnification: 200×; lower image original magnification: 400×) (Positive, upper image original magnification: 50×; lower image original magnification: 200×). (B) Kaplan–Meier survival curves of all 58 GBC samples with negative or positive anti-MAML3 expression (p<0.0001). (C) In hypoxic GBC cell microenvironment, MAML3 interacts with the HH and NOTCH signalling pathways [that are associated with epithelial-mesenchymal transition (EMT)], inducing potential for malignancy in GBC.*

Moreover, resistance to gemcitabine has become a major issue in patients with bile duct cancer (26). Therefore, developing a combination therapy with gemcitabine is important. Gemcitabine is a nucleoside analogue that is absorbed by DNA during the S phase and acts directly by blocking DNA synthesis and causing apoptosis (55, 56). Some studies have suggested that hypoxia significantly reduces gemcitabine activity by lowering the production of active gemcitabine deoxynucleotides and potentially down-regulating the rate-limiting enzyme dCK, which decreases the

Table II. *Clinicopathological features of patients with gallbladder cancer.*

Variable	MAML3 Negative expression (–) N=13	MAML3 Positive expression (+) N=45 (Intensity: Low, High)	*p*-Value
Sex			
Male	4	21 (12, 9)	0.30796
Female	9	24 (6, 18)	
Age (median=70.01)			
<70	5	26 (18, 8)	0.218754
≥70	8	19 (11, 8)	
UICC T category			
Tis/1	11	5 (4, 1)	< 0.001
T2/3	2	40 (22, 18)	
Venous invasion			
Absent (VX V0)	11	25 (15, 10)	0.057164
Present (V1a,b,c V2)	2	20 (8, 12)	
Lymphatic invasion			
Absent (LyX Ly0)	11	23 (9, 14)	0.030737
Present (Ly1a,b,c)	2	22 (17, 5)	
UICC N category			
N0	13	23 (12, 11)	0.001375
N1	0	22 (18, 4)	
Stage classification			
Stage 0-I	11	5 (4, 1)	< 0.001
Stage II-IIIB	2	40 (22, 18)	

sensitivity to gemcitabine (33, 57, 58). However, several investigations have demonstrated that the sensitivity of lung cancer cells to gemcitabine does not change considerably under hypoxia (59, 60). Nevertheless, our study found that gemcitabine administered under hypoxic conditions was less effective in GBC cells, and MAML3 inhibition reduced the sensitivity of GBC to gemcitabine. As the mechanisms underlying gemcitabine sensitivity are quite complex, we hypothesised that the hypoxic environment surrounding cancer cells stabilises and activates the hypoxia-inducible factor HIF-1α (61), resulting in activation of the gemcitabine-resistance signalling pathways (62), including HH–NOTCH signalling (63, 64). This hypothesis is consistent with the experimental data obtained in this study.

γ-secretase inhibitors interfere with NOTCH receptor cleavage, preventing NOTCH signalling, reducing cell proliferation, and triggering apoptosis in a variety of cancer models both *in vivo* and *in vitro* (38, 65, 66). DAPT is frequently utilised as a γ-secretase inhibitor to impede NOTCH signalling and cancer cell growth (67, 68). The results of this study also showed that DAPT blocks the NOTCH signalling pathway, resulting in inhibits transcription of the NOTCH1 target gene. In this study, GBC cell lines treated with DAPT had significantly reduced capacity for proliferation, migration, and invasion (29, 69, 70) under both hypoxic and normoxic conditions, with resulting up-regulation of the epithelial marker E-cadherin and down-regulation of the mesenchymal markers vimentin and SNAI-1. This finding suggests that the NOTCH pathway plays an important role in the regulation of EMT in GBC cells. As EMT promotes the migration and invasion of GBC cells, this further shows that the NOTCH signalling pathway mediates tumour metastasis in GBC (Figure 6C).

Although traditional tumour markers such as CEA, CA19-9, and CA125 (71, 72) have been used clinically, their sensitivity and specificity for diagnosis and prognosis are inadequate (73). Patients with GBC are typically diagnosed during the inspection of late tumour lesions owing to the lack of early clinical manifestations or effective biomarkers, resulting in a poor prognosis (74) owing to the aggressiveness of GBC (75). Using immunohistochemical staining, we observed positive and negative MAML3 expression in the surgically resected GBC specimens. In patients with GBC, positive expression of MAML3 was significantly correlated with poor prognosis and overall survival, lymphatic metastasis, and late cancer-stage classification. This supports our *in vitro* experimental MAML3 results, which could be indicators for providing MAML3-positive patients the appropriate aggressive treatment.

Both the HH and NOTCH signalling pathways are involved in morphogenesis. They are major developmental pathways that are frequently deregulated in cancer (36). The HH signalling pathway normally ceases after embryogenesis, however, previous studies have established that HH signalling is re-activated in some cancers and that GLI2, but not GLI1 or GLI3, regulates the proliferative and invasive capacity of GBC (31, 76). Thus, the HH signalling pathway produces signals during GBC, shows hyperactivity, and promotes matrix hyperplasia, myofibroblast differentiation, and the EMT process of cancer cells (77, 78). Our results demonstrated that MAML3 inhibition led to reduced expression of GLI1 and SMO, suggesting that MAML3 contributes to the activation of HH signalling in GBC.

Figure 6C shows a schematic representation of the results of the present study. Our research suggests that MAML3-NOTCH/HH associated signalling is active in GBC and significantly contributes to its proliferative, migratory, and invasive characteristics. We expect that this study will aid in the development of novel treatments for refractory GBC.

Conclusion

MAML3 contributes to the induction of the malignant phenotype of GBC under hypoxia through HH and NOTCH signalling activation, and MAML3 may be a therapeutic target of morphogenesis signalling for GBC.

Conflicts of Interest

The Authors declare that they have no financial or commercial conflicts of interest related to this study.

Authors' Contributions

LN, HO, SM, AI, MN: Conception, Methodology, Formal analysis, Writing, Critical review, and Editing. SI, SM, SN, SK, KN: Critical review, Supervision, and Revision. YY, YO: Funding acquisition and Critical review. All Authors have seen and approved the final draft before submission.

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