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Overexpression of HMGN3 nucleosome binding protein is associated with tumor invasion and TGF-β expression in cholangiocarcinoma

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ABSTRACT: High mobility group nucleosome binding (HMGN) protein is a non-histone protein that affects the chromatin function, leading to the regulation of gene expression. Several studies reported the aberrant expression of HMGN1–HMGN5 in variety of cancers. This study aimed to investigate a potential HMGN protein and its clinical impact in cholangiocarcinoma (CCA). Analysis of gene expression profiles of HMGNs in liver cancers from public database demonstrated the alteration of HMGN1–HMGN5 in CCA and hepatocellular carcinoma (HCC) tissues. HMGN3 was the only member that overexpressed in CCA, but not HCC, and hence was selected for further study. The immunohistochemistry of HMGN3 revealed that HMGN3 was weakly detected in normal bile ducts and hepatocytes but was gradually expressed in hyperproliferative bile ducts and CCA tissues, indicating the involvement of HMGN3 in development and progression of CCA. CCA exhibited significantly higher expression of HMGN3 than HCC, suggesting HMGN3 as a diagnostic marker distinguishing CCA from HCC. Univariate analysis revealed the association of high HMGN3 expression and tumor invasion of CCA patients. The expression of transforming growth factor β (TGF-β), a major inflammatory cytokine related to liver fluke-related CCA, was positively correlated with the expression of HMGN3 in CCA tissues. Treating CCA cell lines with recombinant TGF-β1 significantly induced the mRNA expression of HMGN3 but not the cholangiocytes. This finding signifies the positive correlation of TGF-β1 and HMGN3 observed in CCA tissues and the influence of TGF-β1 on HMGN3 expression. Further mechanistic studies are necessary to validate these findings.

KEYWORDS: HMGN3, TGF-β, cholangiocarcinoma, tumor invasion

INTRODUCTION

High mobility group nucleosome binding protein (HMGN) is a member of HMGs protein family. Interaction between HMGN and nucleosome promotes chromatin decompaction and facilitates genes expression [1]. HMGN family consists of 5 members: HMGN1–HMGN5. All HMGN proteins share a similar structure consisting of nuclear localized signal, nucleosome binding domain, and c-terminal chromatin regulatory domain [2]. Each HMGN protein regulates a specific set of genes with tissue specific manner [3]. Numerous studies frequently show alteration of HMGN expressions in different types of cancers [4–7]. The significances of HMGNs in cancer aggression have been reported in a wide range of studies. HMGN1 was associated with metastasis of breast cancer cells [8], while HMGN2 was increased in metastatic adenocarcinoma of breast cells and oral squamous cells [5]. Patients with high expression of HMGN4 had high grade tumors, shorter survival in hepatocellular carcinoma [6]. HMGN5 promoted the viability and invasion of human urothelial bladder cancer cells and breast cancer cells [9, 10]. However, the study of HMGN3 in cancers has not yet been reported.

Cholangiocarcinoma (CCA), a cancer of bile duct epithelium, is high prevalence in Northeastern Thailand [11]. Liver fluke (*Opisthorchis viverrini*; *Ov*) infection is a major risk factor that induces chronic inflammation and stimulates inflammatory cytokine secretion, such as transforming growth factor β (TGF-β) and interleukin-6 (IL6) [12, 13], leading to malignant transformation of bile duct cells [14]. TGF-β stimulates Epithelial to Mesenchymal Transition (EMT) process which is a main mechanism for CCA development and metastasis [15, 16].

This study aimed to investigate the HMGN3 and its clinical impact in CCA patients using publicly available dataset. The induction of the expression of candidate HMGN by TGF-β was also shown in CCA cell lines.

MATERIALS AND METHODS

RNA sequencing dataset

Gene expression profiles of HMGN1–HMGN5 in normal bile duct tissues ($n = 9$), CCA ($n = 36$), hepatocel-

lular carcinoma HCC ($n = 369$), and normal adjacent liver tissues ($n = 160$) were retrieved from The Cancer Genome Atlas (TCGA) databases and analyzed using GEPIA (Gene Expression Profiling Interactive Analysis), a web-based tool. The dbGaP accession number is phs000178.

Microarray data

Gene expression profile of liver fluke associated CCA ($n = 47$) were retrieved from GEO database (https://www.ncbi.nlm.nih.gov/geo); the accession number is GSE89749 based on GPL10558 Illumina HumanHT-12 V4.0 expression bead chip platform. The correlation between the expression levels of HMGN3, TGFB1, and IL6 genes and the clinico-pathological data were analyzed using GraphPad Prism® 5.0 software (GraphPad software, Inc., CA, USA).

Patient tissue samples and immunohistochemistry (IHC)

Paraffin-embedded tumor tissues from the 47 CCA patients and 24 HCC patients were obtained from the specimen bank of the Cholangiocarcinoma Research Institute, Khon Kaen University, Thailand. The study protocol was approved by The Human Research Ethics Committee, Khon Kaen University (HE591063). Tissue sections were processed following a standard protocol. In brief, the samples were unmasked with Tris-EDTA buffer pH 9.0 for antigen retrieval, blocked with 1% skim milk, and incubated overnight with HMGN3 antibody (Abcam, Cambridge, UK). The tissue samples were then probed with secondary antibody (EnVision+System-HRP Labelled polymer anti-Rabbit, Dako, CA, USA). Immunoreactivity was detected using 3, 3-diaminobenzidine (DAB) solution and counterstained with Mayer's hematoxylin. The expression levels of HMGN3 were evaluated using H-score, which is ranging from 0–300. The values are based on the intensity and the percentage of positive stained cells. The intensity was rated 0–3: 0, absent; 1+, weak; 2+, moderate; and 3+, strong [17]. H-score = [1 × (% of 1+ cells)] + [2 × (% of 2+ cells)] + [3 × (% of 3+ cells)].

Cell culture

KKU-055, KKU-213A, and KKU-213B cell lines [18] were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% antibiotic-antimycotic. KKU-100 [19] and immortalized cholangiocyte, MMNK-1 [20], were cultured in Ham's F-12 Nutrient mixture supplemented with same concentrations of FBS and antibiotic-antimycotic.

Reverse transcription quantitative PCR (RT-qPCR)

Total RNA was extracted from cell lines using TRIzol (Invitrogen, CA, USA) according to the manufacturer's recommendations. Complementary DNA (cDNA) was synthesized using High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). RT-qPCR was performed on LightCycles® 480 real-time PCR system (Roche Diagnostics, Mannheim, Germany). The expression levels of HMGN3 and β2 microglobulin (B2M) were calculated by $2^{-\Delta Cp}$ value, where ΔCp = Cp of HMGN3 − Cp of B2M. The sequences of primers are as follow: HMGN3 (forward: 5′ AGTCCTGTGCATACTGTGGTG 3′, reverse: 5′ AGCAGGTGGCCACAACTATC 3′); B2M (forward: 5′ AAGATGAGTATGCCTGCCG 3′, reverse: 5′ CGGCATCTTCAAACCTCC 3′).

Statistical analysis

The difference of continuous data between two dependent groups was analyzed by either independent t-test (parametric test) or Mann-Whitney test (non-parametric test). Values are presented as mean ± SD. Student's t-test was used for comparisons between 2 groups. Survival data of patients were performed using Kaplan-Meier analysis. $p < 0.05$ is considered statistically significant. Data were analyzed by GraphPad Prism® 5.0 software (GraphPad software, Inc., CA, USA) and SPSS 17.0 software (SPSS, CA, USA).

RESULTS AND DISCUSSION

HMGN3 was overexpressed in CCA but not HCC

Screening of the mRNA expression of HMGN1–HMGN5 in the primary tissues of CCA and HCC from The Cancer Genome Atlas (TCGA) database unveiled that the expression of HMGN1, HMGN3, and HMGN4 were significantly higher in CCA, whereas HMGN1, HMGN2, and HMGN4 were notably higher in HCC cases when compared with normal adjacent tissues. There were no obvious differential expression levels of HMGN5 in both CCA and HCC (Fig. 1). Comparison between CCA and HCC, HMGN3 was the only HMGN member that significantly overexpressed in CCA but not in HCC. This data implied that aberrant expression of HMGN3 may be specific to CCA, and can be used as a marker to distinguish CCA from HCC. From the literature searching in PubMed, there is a limited report of HMGN3 in cancers [21]. Altogether, we therefore selected HMGN3 for further validation and investigation of the clinical significance of HMGN3 in the liver fluke-associated CCA.

High expression of HMGN3 protein was associated with tumor invasion of CCA patients

To validate the data from TCGA database, IHC staining of HMGN3 was performed in 47 CCA tissues and 24 HCC tissues. HMGN3 was stained weakly in normal bile ducts (NBD), gradually higher in hyperproliferative bile ducts, and the most highly in CCA tissues (Fig. 2a). In addition, HMGN3 was undetected in adjacent normal hepatocytes and slightly increased in

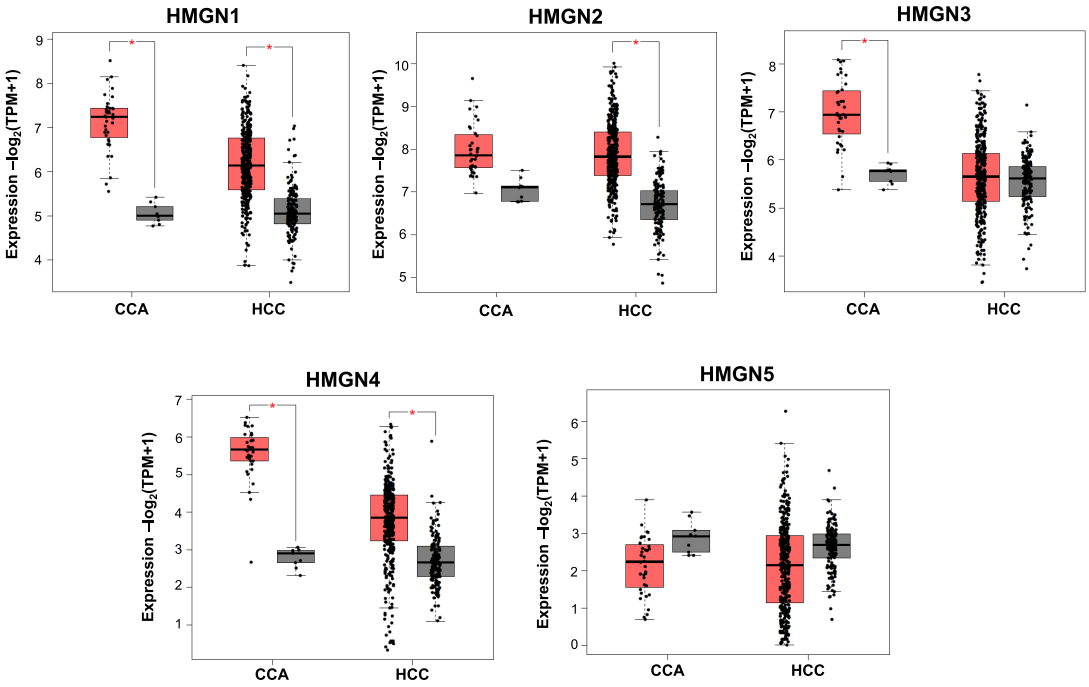

Fig. 1 mRNA expression levels of HMGN1- HMGN5 in CCA and HCC tissues (red) compared with normal adjacent tissues (grey). Data were analyzed from The Cancer Genome Atlas (TCGA) databases using GEPIA (Gene Expression Profiling Interactive Analysis), a web-based tool ($*p < 0.05$).

Table 1 HMGN3 protein expression and clinico-pathological findings of 47 CCA patients.

Clinical characteristic	No. of patients	HMGN3 expression		*p*-value
		Low (H-score <90)	High (H-score ⩾90)	
Age (years)	47			0.028
< 55	23	7	16	
⩾ 55	24	15	9	
Gender	47			0.550
Female	13	7	6	
Male	34	15	19	
Histological type	47			0.753
Papillary	16	8	8	
Non-papillary	31	14	17	
T stage	47			0.011
T1–T2	8	7	1	
T3–T4	39	15	24	
N stage	43			0.287
N0	21	12	9	
N1	22	9	13	
M stage	43			0.089
M0	27	20	17	
M1	6	1	5	
Tumor stage	47			0.201
I–III	11	7	4	
IVA–IVB	36	15	21	

T stages; T1, Solitary tumor without vascular invasion; T2, Solitary tumor with vascular invasion/multiple tumors, with or without vascular invasion; T3, Tumor perforating the visceral peritoneum or involving local hepatic structures by direct invasion; T4, Tumor with periductal invasion. N stage; N0, no regional lymph node metastasis; N1, regional lymph node metastasis present. M stage: M0, no distant metastasis; M1, distant metastasis.

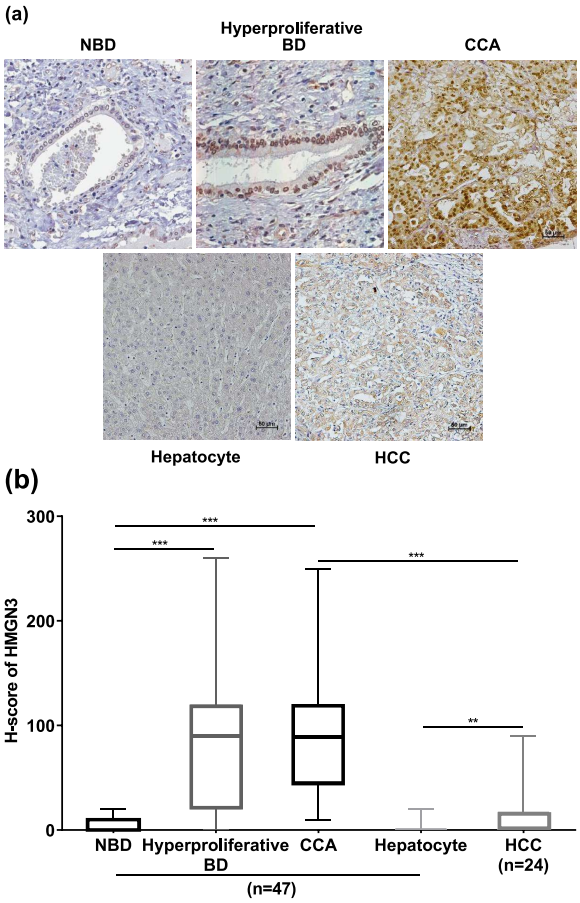

Fig. 2 Elevation of HMGN3 protein expression in CCA and HCC tissues. (a), Representative IHC pictures of HMGN3 staining in normal bile duct (NBD), hyperproliferative BD, CCA, hepatocyte, and HCC; (b), Quantitative analysis of HMGN3 expression (H-score) in all groups. ($** p < 0.01$, $*** p < 0.001$).

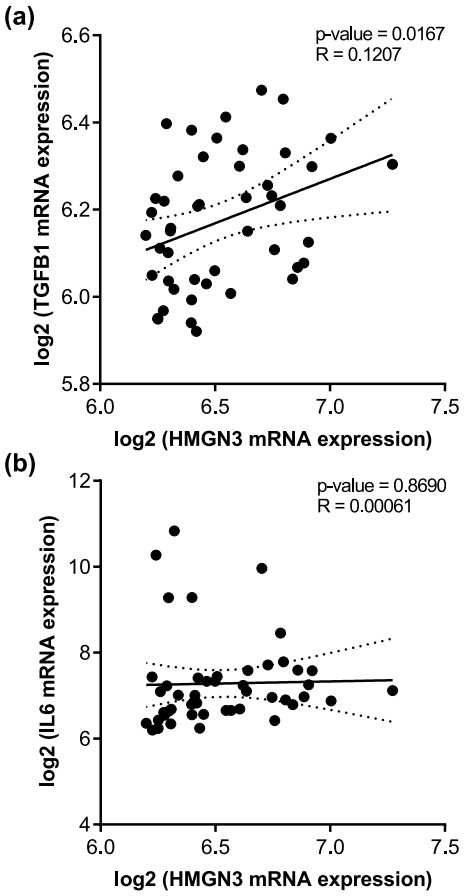

Fig. 3 Correlation between mRNA expression of HMGN3 and two selected inflammatory cytokines: (a), TGFB1; and (b), IL6 using GEO database (GSE89749). Solid line represents mean of mRNA expression, and dotted lines represent standard deviation of mRNA expression.

HCC tissues (Fig. 2a). As displayed in Fig. 2b, the expression of HMGN3 in hyperproliferative bile ducts (median H-score 90, range H-score 0–260) and CCA tissues (median H-score 90, range H-score 10–250) were substantially higher than that in normal bile ducts (median H-score 5, range H-score 0–20). The median H-score value of the HMGN3 in HCC tissues was significantly higher than the hepatocytes (H-score 5 vs. 0). The expression level of HMGN3 in CCA, however, was 18-fold higher than that of HCC tissues ($p < 0.001$). The median value of H-score HMGN3 protein expression of 90 was used as a cut-off value to categorize the patients into low HMGN3 and high HMGN3. Univariate analysis of HMGN3 expression and clinicopathological data in Table 1 demonstrated that high expression of HMGN3 was significantly associated with tumor invasion of CCA patients ($p = 0.011$). Expression level of HMGN3 was not correlated with survival of patients (Fig. S1a). Although no statistical significance, more patients with short survival were observed in the patients with high expression of HMGN3 than those with low expression (Fig. S1b). An increase of sample size may show a significant result. However, our findings reported for the first time the correlation of HMGN3 in the development and the progression of CCA. High expression of HMGN3 may serve as an indicator for invasion of CCA and a differential diagnostic marker between CCA versus HCC. The oncogenic functions of HMGN3 in CCA should be further explored.

Expression of TGFB1 gene was correlated with HMGN3 gene in CCA tissues

Two major CCA-associated inflammatory cytokines, TGF-β and IL6, could induce malignant transformation of bile duct [12, 13]; HMGN3 was upregulated in hyperproliferative bile ducts which is the early

event of cholangiocarcinogenesis. Therefore, we hypothesized that both TGF-β and IL6 may involve in highly expressed HMGN3 CCA cells or may contribute to the development and the progression of HMGN3-dependent CCA cells. To test this hypothesis, the correlations of mRNA expression of TGFB1 gene, or IL6 gene, with HMGN3 gene in 47 CCA cases were analyzed using gene expression profiles dataset of liver fluke-associated CCA (GEO database, GSE89749). Our results in Fig. 3a show a significant positive correlation between TGFB1 and HMGN3 ($p = 0.0167$). In contrast, there was no correlation between IL6 and HMGN3 mRNA expression (Fig. 3b). The association of TGFB1 and HMGN3 expression implied that TGFB1 possibly induced HMGN3 expression.

We continued analyzing the association between TGFB1 and HMGN3 co-expression and clinicopathological features of 26 CCA patients with high level of TGFB1 (median $\geqslant 71$). The median value of HMGN3 mRNA expression of 87 was used as a cut-off value to categorize the patients into low HMGN3 and high HMGN3. Univariate analysis exhibited that there were no significant associations between the co-expression of TGFB1 and HMGN3 and the clinical data of CCA patients (Table S1). Although there was no significant difference between survival of CCA patients expressing high levels of both TGFB1 and HMGN3 versus patients expressing high levels of TGF-β1 and low HMGN3 ($p = 0.315$), the former group tended to have shorter survival than the latter (Fig. S1b).

TGF-β1 induced HMGN3 mRNA expression in CCA cell lines

The endogenous mRNA expression of HMGN3 was determined in MMNK-1, an immortalize cholangiocyte cell line, and four types of CCA cell lines (KKU-055, KKU-100, KKU-213A, and KKU-213B) using q-PCR. The HMGN3 mRNA expressions in all tested CCA cell lines were considerably higher than the MMNK-1 cell line (Fig. 4a). As TGFB1 expression was positively associated with HMGN3 expression (Fig. 3a), the TGFB1 may be the inflammatory cytokine that induces HMGN3 expression. To investigate whether TGF-β1 could activate HMGN3 mRNA expression, MMNK-1 and two CCA cell lines (KKU-100 and KKU-213A) were treated with 10 ng/ml of recombinant human TGF-β1 (R&D Systems, Inc., MN, USA) for 48 h, and HMGN3 mRNA expression was determined using q-PCR. The result demonstrated that HMGN3 mRNA expression was significantly up-regulated 1.2-fold in KKU-100 and 1.6-fold in KKU-213A ($p < 0.05$) (Fig. 4b). TGF-β1 treatment, however, could not induce HMGN3 expression in MMNK-1 cell line. This finding implied that TGF-β1 specifically induced the HMGN3 mRNA expression in CCA cells but not in cholangiocytes.

This was the first time to disclose that HMGN3 expression in CCA cells was TGF-β1 dependent, and the

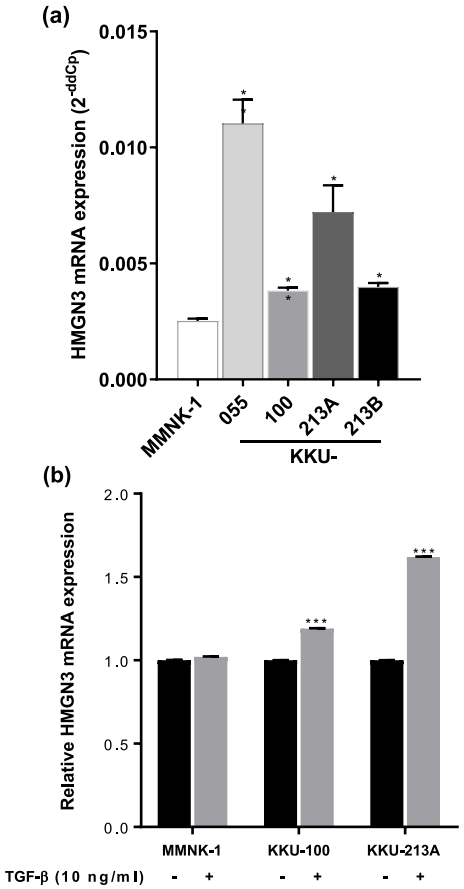

Fig. 4 HMGN3 mRNA expression in MMNK-1 and CCA cell lines: (a), Endogenous HMGN3 expression in four CCA cell lines compared with MMNK-1; (b), TGF-β1 treatment upregulated HMGN3 mRNA expression in CCA cell lines but not in normal cholangiocytes. Data were analyzed from three independent experiments ($*p < 0.05$, $**p < 0.01$ and $***p < 0.001$).

CCA was more sensitive to TGF-β1 treatment than the cholangiocyte. Impacts of TGF-β1 on EMT induction in CCA cells have been reported [15, 16]. TGF-β1 could induce the migration behavior of fluke-associated CCA cell lines via upregulation of Twist transcription factor, N-cadherin and vimentin mesenchymal markers [15]. Up to our study time, the association of HMGN3 expression and EMT has never been studied; it should be further warranted. Moreover, investigating the downstream targets or transcriptional partners of HMGN3 in TGF-β signaling in CCA is required.

CONCLUSION

Overexpression of HMGN3 was detected in the hyperproliferative bile ducts and the CCA tissues but not in the HCC. The overexpression was also associated with tumor invasion in CCA patients, hence, suggesting the role of HMGN3 as: (1) a specific HMGN member of

CCA; (2) a differential diagnostic marker between CCA versus HCC; and (3) an oncogenic protein associated with development and progression of CCA. The mRNA expression of TGFB, a major inflammatory cytokine found in CCA, was positively correlated with HMGN3 mRNA expression in CCA tissues. Additionally, TGF-β1 specifically activated the HMGN3 mRNA expression in CCA cell lines. Our findings showed for the first time the impacts of HMGN3 in CCA and its relationship with TGF-β signaling in CCA.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2022.073.

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Appendix A. Supplementary data

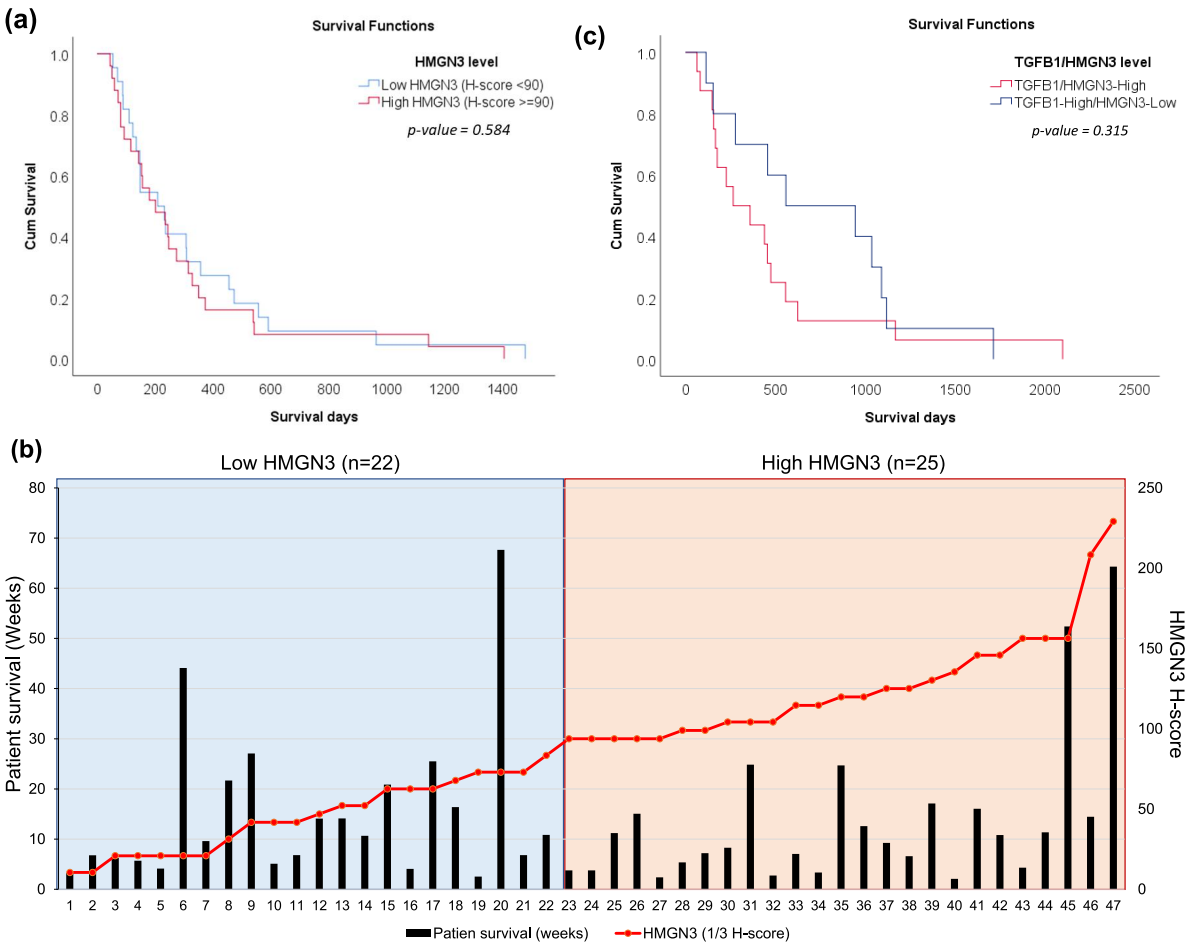

Fig. S1 (a), Kaplan-Meier plot of 47 CCA patients with low and high HMGN3 protein expression; (b), Distribution of H-score of HMGN3 (red line) and survival time (weeks) of CCA patient (black bar); (c), Kaplan-Meier plot of 26 CCA patients who expressed high levels of both TGFB1 and HMGN3 versus patients who expressed high TGFB1 and low HMGN3 levels.

Table S1 Co-expression of TGFB1 and HMGN3 with clinico-pathological findings of 26 CCA patients.

Clinical characteristic	No. of patients	TGFB1/HMGN3 expression		*p*-value
		High/High ($n = 16$)	High/Low ($n = 10$)	
Age (years)	26			0.263
< 56	12	6	6	
$\geqslant 56$	14	10	4	
Gender	26			0.619
Female	12	8	4	
Male	14	8	6	
Histological type	26			0.899
Papillary	16	10	6	
Non-papillary	10	6	4	
Tumor stage	26			0.149
I–III	15	11	4	
IVA–IVB	11	5	6	