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LMP2A induces DNA methylation and expression repression of AQP3 in EBV-associated gastric carcinoma

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ABSTRACT

Epstein-Barr virus (EBV)-associated gastric carcinoma (EBVaGC) is a unique type of gastric carcinomas that promoter hypermethylation of tumor-related genes is extremely frequent to be found. Aquaporin 3 (AQP3) is a small membrane transport protein that plays a crucial role in cancer progression and metastasis. However, there is no experimental study on the expression of AQP3 in EBVaGC and the regulation mechanism of EBV on AQP3. In this study, the loss of AQP3 was contributed by the hypermethylation status of AQP3 promoter in EBVaGC which was caused by elevated expression of DNMT3a. In addition, stable and transient transfection system in SGC7901 showed that viral latent membrane protein 2A (LMP2A) activated phosphorylated ERK and up-regulated DNMT3a. Taken together, LMP2A induced the phosphorylation of ERK, which activated DNMT3a transcription and caused AQP3 expression loss through CpG island methylation of AQP3 promoter in EBVaGC.

1. Introduction

Epstein-Barr virus (EBV), also called human herpes virus 4, was discovered more than 50 years ago from a Burkitt's lymphoma biopsy. It is the first virus to be known to have direct association with human cancer and to be considered as an important DNA tumor virus (Pattle SB, Farrell PJ., 2006; Vereide DT, Sugden B., 2011). The exposure to EBV is high, more than 95% of the adult population are infected with EBV and carry the virus lifelong (Chang CM et al., 2009; Tempera I., 2014). EBV genomes and gene products are consistently detected in a variety number of human cancers, including endemic Burkitt's lymphoma, nasopharyngeal carcinoma, about 50% of Hodgkin's disease, and approximately 10% of gastric carcinomas. Besides, they are detected in most lymphoproliferative disorders of immuno-suppressed individuals (Young LS, Rickinson AB., 2004; Howlader NNA et al., 2011). More than 80% of EBV-associated carcinomas are epithelial malignancies, and the vast majority of them are nasopharyngeal carcinoma and EBV-associated gastric carcinoma (EBVaGC) (Tsao SW et al., 2015). EBVaGC belongs to latency type I or II, in which EBERs, EBNA1, BARTs, LMP2A and BART miRNAs are expressed (Shinozaki-Ushiku A et al., 2015). As a viral factor, LMP2A has a unique NH_2-terminal intracellular domain which is critical for the interaction with cellular proteins, such as tyrosine kinases (Syk and Lyn) and E3 protein-ubiquitin ligases (Fukuda M, Longnecker R., 2005; Winberg G et al., 2000). Therefore, LMP2A could probably participate in eliciting the signaling pathway to mediate CpG island methylation of the host gene promoter.

Aquaporins (AQPs) acting as permeable tetramers are a family of small membrane transport proteins (approx. 30 kDa/monomer) located on the hydrophobic cell membrane (Carbrey JM, Agre P., 2009; Agre P et al., 2002). AQPs are responsible for the transport of small solutes such as glycerol, gas and ions. The expression of AQPs has been found in more than 20 types of human cancer and is significantly correlated with the degree of tumors histological malignancy and the prognosis of patients (Wang J et al., 2015). Recently, it has been reported that, several aquaporins have great implications in cancer and increasing evidence strongly suggests that aquaporin 3 (AQP3) plays a crucial part in cancer progression and metastasis (Satooka H, Hara-Chikuma M., 2016; Wang L et al., 2016). AQP3 was found to express in the basolateral plasma membranes of multiple human epithelia including gastrointestinal tract, upper and lower airways, brain, breast, pancreas, ovary, liver, prostate, bladder, and many other epithelia (Mobasheri, A et al., 2005; Gregoire, F. et al., 2015; Wang, J. et al., 2007; Rubenwolf, P.C. et al., 2009). In addition to facilitating transepithelial water transport, AQP3 is also permeable to glycerol, urea and other solutes (Ishibashi, K. et al., 1994). Serving as a water- and glycerol-transporter, AQP3 facilitates skin hydration (Tonghui, M. et al., 2002) and may also be involved in cell migration during wound healing (Hara-Chikuma, M.

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et al., 2008). Some studies have found that AQP3 was overexpressed in hepatocellular carcinoma, pancreatic ductal adenocarcinoma, lung cancer, colon cancer, esophageal and oral squamous cell carcinoma (Guo X et al., 2013; Kusayama, M., 2011). AQP3 participates in the development of tumors through a variety of signaling pathways. A research reported that aquaporin 3 promoted epithelial-mesenchymal transition in gastric cancer through PI3K/AKT/SNAIL signaling pathway (Chen J et al., 2014). In non-small cell lung cancer (NSCLC), AQP3 was involved in angiogenesis through the HIF-2α-VEGF signaling pathway and was involved in tumor cell invasion through AKT-MMPs signaling (Xia H et al., 2014). However, to our knowledge, the relationship between AQP3 and EBV in gastric carcinoma has not been investigated. Numerous studies revealed that the carcinogenesis of EBVaGC was attribute to genomic features of host DNA, mRNA, microRNA, and CpG methylation profiles (Esteller, M. et al., 2005; Hanahan, D. & Weinberg, R. A., 2000). EBVaGC displays global and non-random DNA methylation of promoter regions of various cancer-associated genes (Shinozaki-Ushiku A. et al., 2015; Chang MS. et al., 2006a, b, c). Some studies reported that promoter methylation of numerous tumor-related genes such as p16INK4A, E-cadherin and p73 were found in EBVaGC, which resulted in gene silencing and the characteristic CpG island methylator phenotype (CIMP) (Sakuma K. et al., 2004; Sudo M. et al., 2004a). DNA methylation has crucial roles in the control of gene activity, it inactivates chromatin by affecting nuclear architecture, leading to transcriptional repression and inhibition of related gene expression (Jaenisch R, Bird A., 2003; Robertson KD., 2002; Suzuki MM, Bird A., 2008).

Herein, in order to investigate the underlying mechanism of the expression loss of AQP3, we detected the methylation status of AQP3 promoter region and the possible signal pathways that EBV may regulate AQP3.

2. Materials and methods

2.1. Cell lines and tumor samples

Three EBV-positive gastric carcinoma cell lines GT38, GT39 and SNU719 were used. GT38 and GT39 cell lines were gifted from Sairenji T. (Division of Biosignaling, Department of Biomedical Science, Tottori University). SNU719 was kindly provided by Prof. Qian Tao (Cancer Epigenetics Laboratory, The Chinese University of Hong Kong). EBVnGC cell line BGC823 was gifted from Chunkui Shao (Sun Yat-sen University). The other EBVnGC cell lines SGC7901 and HGC27 were purchased from the Cell Bank of the Chinese Academy of Sciences. All cell lines were routinely cultured in D-MEM/F-12 (Life Technologies, Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Biological Industries), and 100 U/ml penicillin/streptomycin (Initrogen, USA) at 37 °C in a humidified atmosphere of 5% CO_2.

Paraffin embedded and fresh tumor tissues were collected from the Department of Pathology of the Affiliated Hospital of Qingdao University and Qingdao Municipal Hospital. The positivity of EBV in gastric carcinoma tissues was determined by in situ hybridization of EBV-encoded small RNA1, as described previously (Y. Wang et al., 2007). This study was approved by the Medical Ethics Committee at the Medical College of Qingdao University, China. All the methods mentioned in this manuscript were performed according to the approved guidelines.

2.2. Treatment of cell lines with demethylation agent

When cells were cultivated to a 70%–80% confluency, GT39 and SNU719 cells were treated daily with 10 μmol/L or 15 μmol/L 5-aza-2′-deoxycytidine (5-Aza-CdR) (Aza, Sigma-Aldrich, USA) for three or five days. The untreated cells were used as controls. The cells were harvested for DNA, RNA and protein extraction after the treatment.

2.3. DNA extraction

The standard method with proteinase K digestion and phenol-chloroform purification was used to extract the DNA from cell lines and fresh tumor tissues (Xia Liu et al., 2012). The DNA from paraffin-embedded tumor tissues was extracted by the QIAamp DNA FFPE Tissue kit (QIAGEN GmbH, Germany).

2.4. RNA extraction and reverse transcription

Total RNA was extracted from the cells using the Trizol reagent (Life Technologies, NY, USA) under RNase-free condition. Subsequently, 1 μg of total RNA was reverse transcribed to cDNA using PrimeScript RT Reagent Kit (TAKARA, Dalian, China). The cDNA was stored at −20 °C for later use.

2.5. DNA bisulfite treatment and methylation analysis

Bisulfite modification of DNA was carried out as described previously (Xia Liu et al., 2012). The methylation status of AQP3 CpG locus in cell lines were determined using bisulfite sequencing PCR (BSP) before and after the treatment of 5-Aza-CdR. Meanwhile, methylation-specific PCR (MSP) was used to determine the methylation status of AQP3 promoter in gastric carcinoma tissues.

The MSP product was analyzed on a 2% agarose gel. The sequences of the primers for MSP and BSP are listed in Table 1.

Table 1
Sequences of primers for MSP, BSP, and real-time PCR in this study.

	Primers	Sequence (5′-3′)	Annealing temp (°C)	Product size (bp)
MSP	AQP3-MF	GCGTTTTTATAAAGGGAGTTATTAGC	56.02	118
	AQP3-MR	CCTTCTATCGACCCATAACGA	55.58	
	AQP3-UF	TGTTTTTATAAAGGGAGTTATTAGTGT	53.31	120
	AQP3-UR	ACTCCTTCTATCAACCCATAACAAA	55.66	
BSP	AQP3BSP-F	TTTATTATTGGTTTTTAGATTTGTTTAAGT	51.73	447
	AQP3BSP-R	CAAAATAAAAATCCCCAAACACTC	53.57	
qRT-PCR	AQP3-F	CATCCTGGTGATGTTTGGCTG	57.8	105
	AQP3-R	GTGACAGCAAAGCCAAAGGC	57.45	
	GAPDH-F	CAAATTCCATGGCACCGTCA	59.11	106
	GAPDH-R	ATCGCCCCACTTGATTTTGG	58.81	
	DNMT3a-F	CTCCATCGTCAACCCTGCTC	60.46	200
	DNMT3a-R	TCATCACAGGGTTGGACTCG	59.39	
	DNMT3b-F	ACCCGGGATGAACAGGATCT	60.33	141
	DNMT3b-R	TAGTCCTTCAGAGGGGCGAA	59.96	
	DNMT1-F	TAACCAGGTTGAGCTCGGGT	60.83	167
	DNMT1-R	GAGGATGGGCTGGTACTGTG	59.82	

MSP: methylation-specific PCR; BSP: bisulfite sequencing PCR; qRT-PCR: quantitative Real-Time PCR; F: forward primer; R: reverse primer; Bp: base pair.

2.6. Real-time quantitative PCR (RT-qPCR)

Total RNA of all cell lines mentioned above and the RNA of GT39, SNU719 after 5-Aza-CdR treatment were extracted. The primer pairs used in RT-qPCR experiments were listed in Table 1.

All reagents used for RT-qPCR were obtained from Faststart Essential DNA Green Master (Roche, Germany). The total volume for PCR was 20 μl, consisting of 10 μl of 2 × Faststart Essential DNA Green Master Mix, 0.5 μl of forward and reverse primers each, and 1 μl of cDNA template (equivalent to 50 ng RNA) and supplemetary RNase-free water. The RT-qPCR was carried out by LightCycler® 96 System (Roche, Switzerland).

Experiments were performed in triplicates, and the average values of the 3 test results were analyzed. The relative mRNA expression of AQP3 was calculated by the formula $2^{-\Delta\Delta Ct}$.

2.7. Protein extraction and western blot assay

Cells were washed with ice-cold phosphate-buffered saline (PBS) and total protein from cell lines were extracted using RIPA Buffer (GBCBIO Technologies Inc.) with 1% PMSF (Biosharp). The protein extracts were loaded, size-fractionated by SDS-polyacryl-amide gel electrophoresis and transferred to PVDF membranes (Merck KGaA, Darmstadt, Germany). After blocking with 5% skim milk, the members were incubated with specific first antibodies in dilution buffer at 4 °C overnight. The HRP-conjugated anti-rabbit IgG (1:2000) or HRP-conjugated anti-mouse IgG (1:1000) were incubated at room temperature for 2 h after wash. Then the targeting protein expression level was detected by exposure to autoradiographic film (Fusion Fx, VILBER LOURMAT). Antibodies against AQP3 (Abcam, Cambridge, MA, USA, ab125219, 1:1000 dilution), β-actin (Abcam, ab8227, 1:1000 dilution), β-tubulin (BOSTER Biological Technology, BM1453, 1:1000 dilution), DNMT3a (Abcam, ab188470, 1:2000 dilution), DNMT3b (Abcam, ab79822, 1:1000 dilution), DNMT1 (Abcam, ab188453, 1:1000 dilution) were used.

2.8. Immunohistochemistry (IHC)

Paraffin sections were deparaffinized and hydrated as per routine. Rabbit anti-human polyclonal antibody AQP3 was 1: 1000 diluted. The reagents (PV9000 and DAB) were obtained from ZSGBBio and staining was performed according to the manufacturer's protocol. PBS was used in replacement of primary antibody as a blank control. Positive IHC expression was defined as > 25% staining with an intensity of 2–3 + following the methods described previously (Chen J et al., 2014).

2.9. Plasmids and transfection

Each EBV latent gene LMP1, LMP2A and EBER was cloned into pcDNA3.1 containing FLAG tag. Stable transfection of reporter plasmids was performed using Lipofectamine 2000 Reagent (Bio-Rad Laboratories, Hercules, CA) according to the manufacture's protocol. SGC7901 cells were transfected with LMP1, LMP2A, EBER, and control vector, respectively. The expression of each gene was confirmed by RT-PCR, and the expression of LMP1 and LMP2A were also confirmed by Western blot.

2.10. Small interfering RNAs

Small interfering RNA (siRNA) sequences directed against ERK1 (MAPK3-homo-933) or ERK2 (MAPK1-homo-355) and control siRNA were purchased from Genepharma. LMP2A-transfected SGC7901 cells were transfected with MAPK3-homo-933 siRNA, MAPK1-homo-355 siRNA and control siRNA at 50 nmol/L by Lipofectamine (2000) Reagent (Bio-Rad Laboratories, Hercules, CA) according to the manufacture's protocol. Protein was collected at 48 h and 72 h after transfection.

2.11. DNMT activity assays

DNMT activity was measured using DNMT Activity Quantification Kit (Colorimetric) according to the manufacturer's instructions (Abcam, ab113467). DNMT activity was calculated by the following formula: DNMT activity (OD/h/mg) = (Sample OD - Blank OD)/(Protein Amount (μg) × hour). The amount of nuclear extracts were 5 μg and the incubation time was 2 h.

2.12. Statistical analysis

Statistical significance of two groups was determined by Student's t-test. The correlation between the DNA methylation level or clinical characteristics and EBV expression was determined by Chi-square test. The $2^{-\Delta\Delta CT}$ method was adopted to quantify the mRNA expression level between EBV-positive group and EBV-negative group, where ΔΔCT was defined by the following equation: ΔΔCT = [(CT of AQP3 - CT of GAPDH) $_{Treatment}$ - (CT of AQP3 - CT of GAPDH) $_{Control}$].

3. Results

3.1. AQP3 expression is down-regulated in EBV-positive gastric carcinoma cell lines and EBV-associated gastric carcinoma tissues

The amount of AQP3 mRNA was quantified relatively in EBV-positive and EBV-negative cells by RT-qPCR. The relative quantitative ratio of AQP3 was normalized to the house keeping gene GAPDH. In gastric

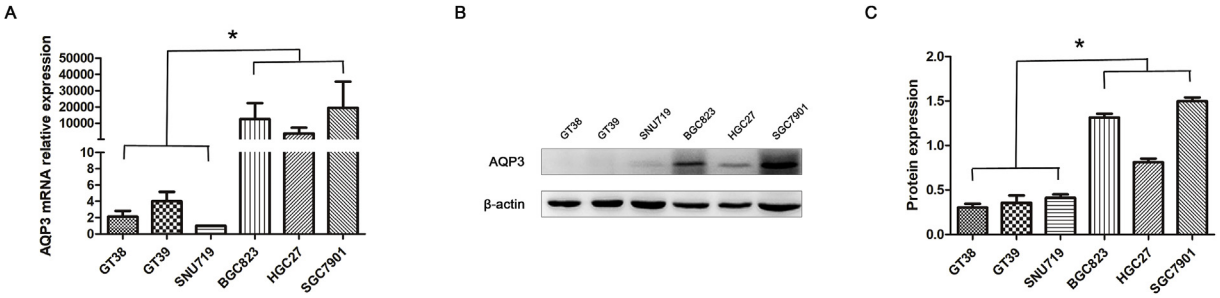

Fig. 1. AQP3 expression is down-regulated in EBV-positive gastric carcinoma cell lines. (A) AQP3 mRNA expression level in three EBV-positive gastric carcinoma cell lines (GT38, GT39, SNU719) and three EBV-negative gastric carcinoma cell lines (BGC823, HGC27, SGC7901). Expression levels were calculated by quantitative real-time PCR. GAPDH gene was used as internal reference and the $2^{-\triangle\triangle Ct}$ method was used for relative quantification. Error bars represent the standard deviations. (B) Western blot analysis of AQP3 protein in EBV-positive and EBV-negative gastric carcinoma cell lines. (C) The densitometry of AQP3 Western blot analysis in EBV-positive and EBV-negative cell lines. * The difference was significant between two groups ($P < 0.05$).

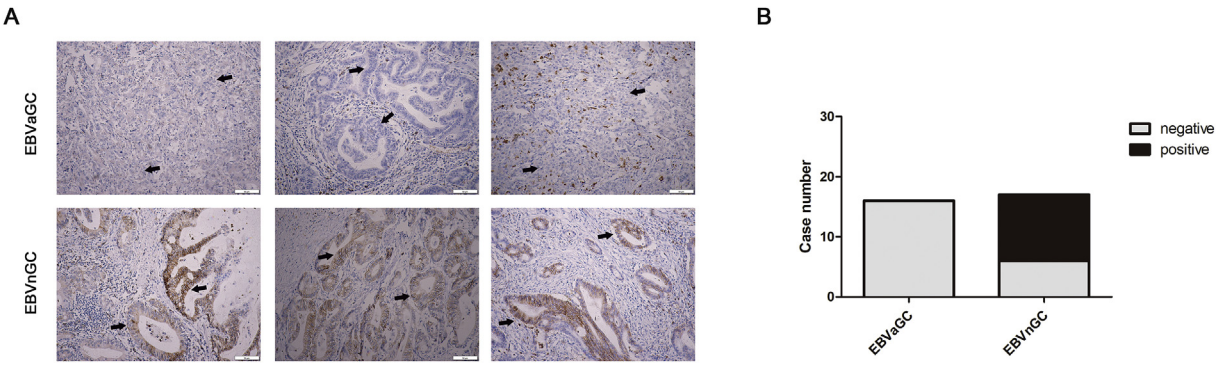

Fig. 2. AQP3 expression is down-regulated in EBV-associated gastric carcinoma tissues. (A) Immunohistochemical staining pattern of AQP3 in three cases of EBV-associated gastric carcinoma tissues and three cases of EBV-negative gastric carcinoma tissues. Original magnification × 200 for all panels. (B) AQP3 immunohistochemical positive and negative case numbers of EBV-associated and EBV-negative gastric carcinoma specimens. Differences on immunohistochemical positive rate between the two groups were statistically significant, $\chi^2 = 15.529$, $P < 0.01$.

carcinoma cell lines, the mRNA level of AQP3 in EBV-positive GC cell lines (GT38, GT39, SNU719) was significantly down-regulated compared to EBV-negative GC cell lines (HGC27, SGC7901, BGC823) (Fig. 1A). Consistently, the expression of AQP3 protein in EBV-positive cell lines was significantly lower than that in EBV-negative cell lines (Fig. 1B and C). AQP3 was reported to be overexpressed in gastric carcinoma (Shen L. et al., 2010; Wang J. et al., 2012), but our data showed its low expression in EBV infected GC cells. It is suggested that EBV may regulate the transcription expression of AQP3 in gastric carcinoma in an unknown manner.

AQP3 protein expression was detected by IHC in gastric carcinoma tissues. Results were shown in Fig. 2. The positive rate of AQP3 expression in EBVnGC was 64.71% (11/17), meanwhile, all the EBVaGC samples were detected AQP3 negative. The difference was statistically significant ($\chi^2 = 15.529$, $P < 0.01$) and this result was consistent with the expression of AQP3 at the cell line level. The clinical characteristics (gender, age, histological type, location and invasion) between the two types of gastric carcinoma patients were similar, as shown in Table 2.

3.2. Methylation of the AQP3 promoter in EBV-positive tumor cell lines

Methprimer was used to predict the CpG island of AQP3. It was predicted that there is a CpG island in the AQP3 promoter region and the first exon region (Fig. 3A), therefore, we investigated the DNA methylation status of AQP3 in this region. Bisulfite sequencing PCR (BSP) was applied to test the methylation status of AQP3 first exon and promoter region in different cell lines. We observed that the methylation rate in EBV-positive gastric carcinoma cell lines was much higher than that in EBV-negative gastric carcinoma cell lines (Fig. 3B). The methylation rates of AQP3 first exon and promoter in EBV-positive cell lines GT38, GT39 and SNU719 were 71.95%, 72.76% and 79.27% respectively, while in EBV-negative cell lines HGC27, BGC823 and SGC7901, the methylation rates were 58.94%, 1.22% and 2.85% respectively. The difference in methylation rate between EBV-positive GC cell lines and EBV-negative GC cell lines was statistically significant ($P < 0.05$).

Table 2
Clinicopathological data for EBVaGC and EBVnGC patients.

	EBVaGC (n = 16)	EBVnGC (n = 17)	*P*
Age (years)			
< 50	6	3	0.259
≧50	10	14	
Gender			
Male	11	12	1
Female	5	5	
Pathologic types			
Poorly differenced adenocarcinoma and signet ring cell carcinoma	15	12	0.175
Well-differentiated and moderately differentiated adenocarcinoma	1	5	
Location			
Gastric cardia	4	1	0.169
Gastric body	8	8	
Antrum	4	8	
Depth of invasion			
Invasion to serosa and invasion through serosa	14	16	0.601
Not invading serosa	2	1	

EBVaGC: EBV-associated gastric carcinoma; EBVnGC: EBV-negative gastric carcinoma.

3.3. Methylation of the AQP3 promoter in EBVaGC tissues

In order to find out whether EBV influence DNA methylation patterns of the promoter of AQP3, methylation specific PCR (MSP) was used to test the methylation status of the AQP3 promoter in DNA extracted from 24 randomly selected EBVaGC tissues and 35 EBVnGC tissues. The results were summarized in Fig. 3C and D. GC tissues that gave a band with methylation-specific primers were scored as methylated (M). GC tissues that only gave a band with primers of unmethylated DNA were scored as unmethylated (U). Sample were repeatedly tested for at least three times. The methylation rate of EBVaGC was 23/24 and EBVnGC was 7/35. The difference between EBVaGC and EBVnGC was statistically significant ($P < 0.01$).

3.4. EBV infection induces DNMT3a up-regulation and subsequent CpG methylation in EBVaGC cell lines

In human carcinoma, constitutive or transient activation of DNA methyltransferase is considered as a mechanism for CpG island methylation. The expression of three DNA methyltransferases (DNMT), DNMT1, DNMT3a and DNMT3b was then evaluated in three EBV-positive GC cell lines (GT38, GT39, SNU719) and three EBV-negative GC cell lines (BGC823, HGC27, SGC7901). The mRNA expression of DNMT3a in EBV-positive GC cell lines was higher than that in EBV-negative cell lines, while the mRNA expression of DNMT1 and DNMT3b was almost the same in EBV-positive/negative GC cell lines (Fig. 4A).

We also detect the protein expression of three DNMTs in EBV-positive and EBV-negative gastric carcinoma cell lines. The protein expression of DNMT3a was higher in three EBV-positive cell lines than that in EBV-negative cell lines while the expression of DNMT1 showed the opposite trend. Moreover, DNMT3b protein expression had no difference between EBV-positive and EBV-negative gastric carcinoma cell lines (Fig. 4B and D).

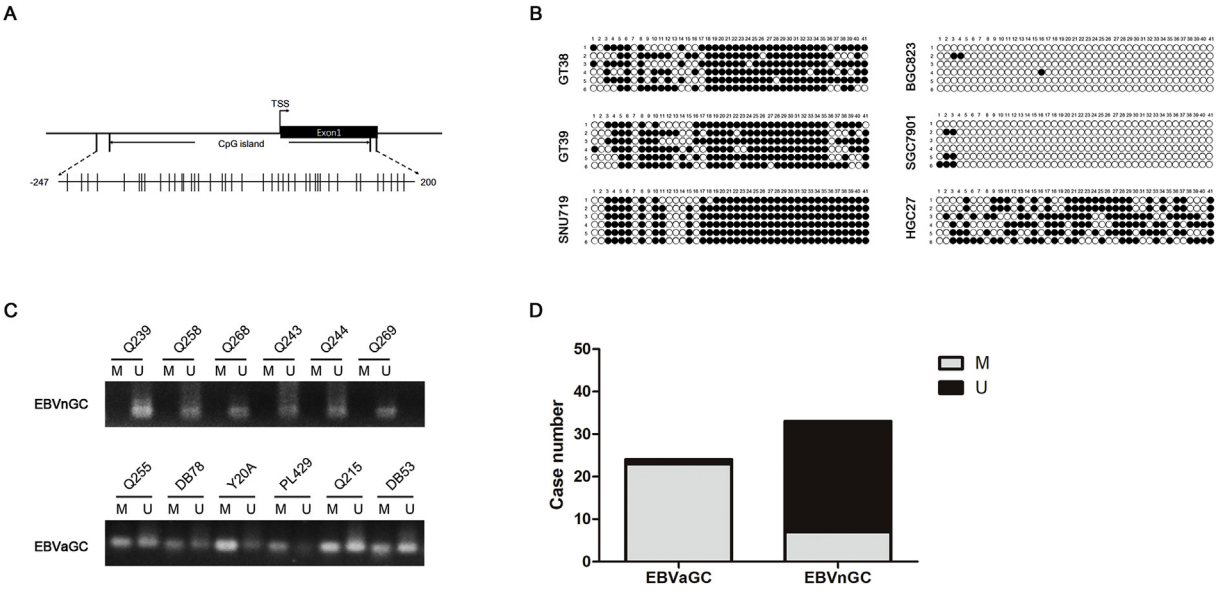

Fig. 3. Methylation of the AQP3 promoter in EBV-positive tumor cell lines and EBVaGC tissues. (A) The CpG-island of AQP3 promoter region. The predicted CpG-island contains 41 CpG sites. (B) The methylation status of CpG sites in AQP3 gene promoter in three EBV-positive and three EBV-negative gastric carcinoma cell lines. The methylation rates of AQP3 in GT38, GT39, SNU719, BGC823, SGC7901 and HGC27 were 71.95%, 72.76%, 79.27%, 1.22%, 2.85% and 58.94% respectively. ● represent methylated CG site; ○ represent unmethylated CG site. (C) MSP analysis of promoter hypermethylation of AQP3 gene in EBV-associated and EBV-negative gastric carcinoma tissues. Six cases representative results of EBVaGC (Q255, DB78, Y20A, PL429, Q215, DB53) and EBVnGC (Q239, Q258, Q268, Q243, Q244, Q269). All 6 EBVaGC cases were considered methylation-positive and all 6 EBVnGC cases were considered methylation-negative. U, unmethylated primer set; M, methylated primer set. (D) Methylation rate of 24 EBVaGC and 35 EBVnGC cases. The difference between the two groups were statistically significant, $\chi^2 = 31.034$, $P < 0.01$.

To demonstrate that EBV infection can influence DNMT activity in gastric carcinoma, we detected DNMT activity in EBV-positive and EBV-negative cell lines. We found that DNMT activity were higher in EBV-positive cell lines than that in EBV-negative cell lines ($P < 0.01$). This suggested that EBV infection can lead to the increase of DNMTs activity (Fig. 4C). Therefore we speculated that the hypermethylation status of AQP3 was caused by the high activity of DNMTs in EBV-positive cell lines.

3.5. 5′-aza-2′-deoxycytidine inhibits DNMT3a protein expression and restores AQP3 expression in a time and dose dependent manner

GT39 and SNU719 cell lines were treated daily with 10 μmol/L or 15 μmol/L 5-aza-2′-deoxycytidine (5-Aza-CdR) for three or five days. The untreated cells were used as controls. BSP was used to detect the methylation status of AQP3 in promoter and the first exon region after the treatment of 5-Aza-CdR. In GT39 cell line, with the treatment of 5-Aza-CdR in 10 μM 3 days, 10 μM 5 days, 15 μM 3 days, 15 μM 5 days, the methylation rate of AQP3 reduced to 55.29%, 41.87%, 40.06% and 32.52%, respectively. In SNU719 cell line, with the treatment of 5-Aza-CdR in 10 μM 3 days, 10 μM 5 days, 15 μM 3 days, 15 μM 5 days, the methylation rate of AQP3 reduced to 39.84%, 29.67%, 0.81% and 0%, respectively (Fig. 5A). Expression of AQP3 gene was evaluated in EBV-positive gastric carcinoma cell lines (GT39 and SNU719) by RT-qPCR and Western blot. The protein expression of DNMT3a and the mRNA expression of AQP3 displayed a dose and time dependent manner with 5-Aza-CdR treatment. With increased dose or time, DNMT3a decreased while AQP3 increased in both mRNA and protein level (Fig. 5B and C).

3.6. LMP2A induces DNMT3a up-regulation and subsequent down-expression of AQP3 in EBV-infected GC cell lines

EBV-negative GC cell line SGC-7901 was stably transfected with LMP1, LMP2A or EBER gene. Cells that successfully transfected express green fluorescent protein (Fig. 6A). The corresponding mRNA and protein expression was detected by RT-PCR and Western blot, three EBV-positive cell lines were used as positive control (Fig. 6B and C). The expression of DNMT3a was greatly elevated in LMP2A-transfected SGC7901 cells (Fig. 6C). We then transiently transfected the LMP2A gene into SGC7901 cell line for 24 h and 48 h. LMP2A-mediated DNMT3a overexpression and AQP3 hypoexpression were also observed in the transient expression system in SGC7901 cells (Fig. 6E). In the transiently transfected cell line, the expression of the target gene is regulated by a strong promoter, whereas, in stable transfected cell line, the promoter may lost or the physiological characteristics of the cells may change and the target gene may not express, therefore the expression of the gene should be verified in both transient and stable transfected cell lines. It was found that the expression of DNMT3a in cells transfected with LMP2A was significantly higher than that in NC cells, while AQP3 exhibited a lower expression level in LMP2A transfected cells than that in NC cell (Fig. 6C). By RT-qPCR, the mRNA expression level of AQP3 was shown to decrease more than 3 fold at both 24 and 48 h and this level was comparable to stable LMP2A-transfected SGC7901 cells (Fig. 6F). The DNMT activity was also detected in LMP2A transient transfected cell line. We found that the activity of DNMTs in SGC7901 that transfected LMP2A for 24 and 48 h were higher than that of NC (Fig. 6G).

3.7. LMP2A-induced ERK phosphorylation leads to DNMT3a overexpression

In the stable transfection system as presented in Fig. 6C, the level of p-ERK1/2 was significantly increased in LMP2A-transfected SGC7901 cells. To confirm the correlation between the phosphorylation of ERK1/2 and expression of DNMT3a and AQP3 in EBV-infected GC cells, ERK1 and ERK2 were knocked down respectively with siRNA in LMP2A-transfected SGC7901 cells. The results showed that the level of DNMT3a protein expression was decreased and the level of AQP3 protein expression was restored in parallel with the decrease of p-ERK1/2 (Fig. 6H).

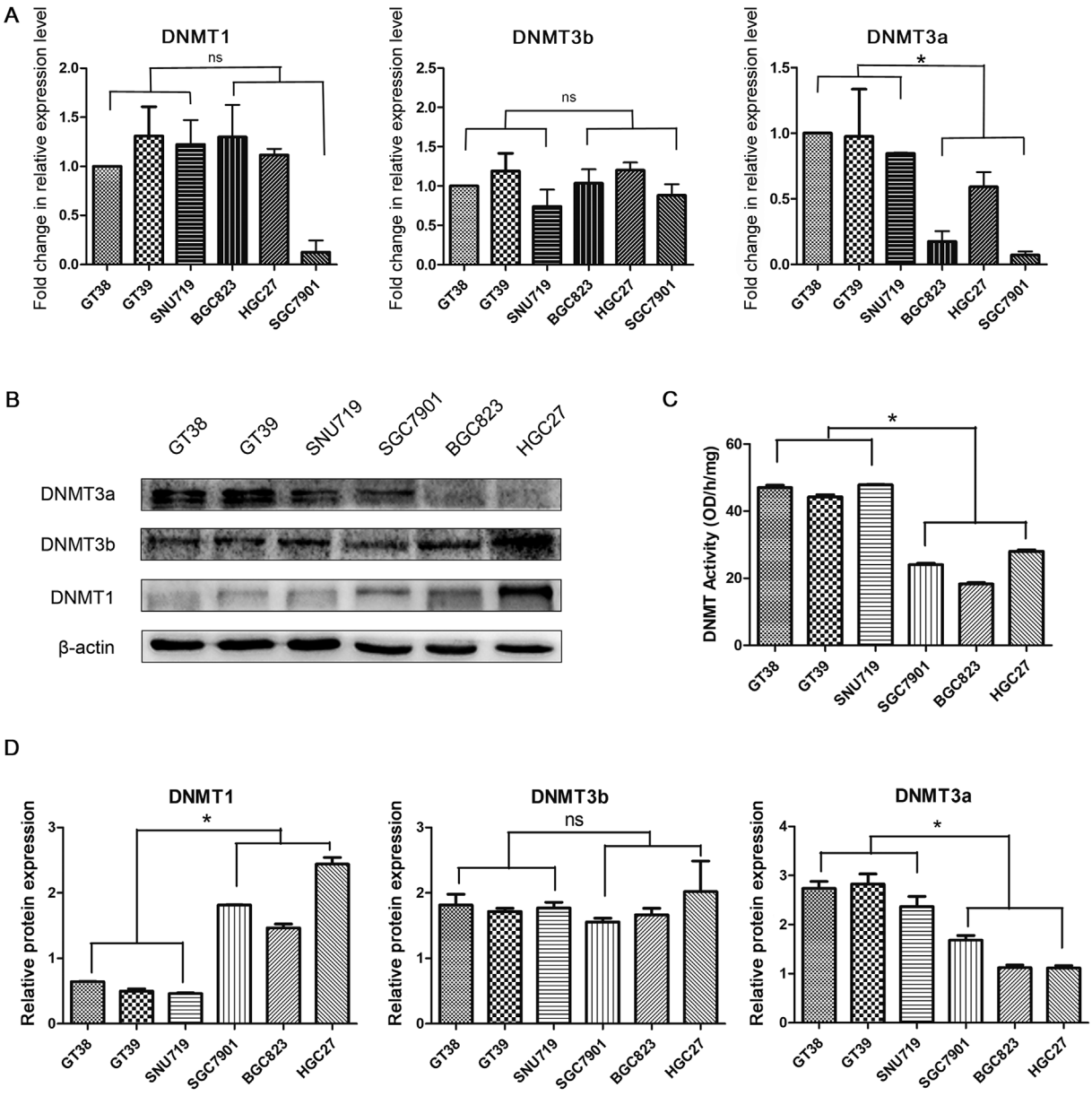

Fig. 4. The expression level of DNMT1, DNMT3a and DNMT3b in EBV-positive GC cell lines (GT38, GT39, SNU719) and EBV-negative GC cell lines (BGC823, HGC27, SGC7901). (A) The mRNA expression level of DNMT1, DNMT3b and DNMT3a in EBV-positive/negative GC cell lines. (B) The protein expression level of DNMT1, DNMT3b and DNMT3a in EBV-positive/negative GC cell lines. (C) The DNMT activity in EBV-positive/negative GC cell lines. (D) The densitometry of DNMT1, DNMT3b and DNMT3a Western blot analysis in EBV-positive/negative GC cell lines. * The difference was significant between two groups ($P < 0.05$). ns There was no significant difference between two groups.

4. Discussion

EBV-associated gastric carcinomas show global CpG island methylation of the promoter region of various cancer-related genes, which is the most characteristic abnormality in genetic and epigenetic anomalies studied in EBV-associated GCs (Chang MS. et al., 2006a, b, c). CpG islands were typically common near transcription start sites and might be associated with promoter regions (Chang MS. et al., 2006a, b, c), the fact was compatible with our results. In the present study, it was demonstrated that AQP3 represented as a typical example. The hypermethylated AQP3 promoter and the first exon region and the repression of AQP3 expression were observed concurrently in EBV-associated GCs, whereas, in EBV-negative GCs, the expression of AQP3 was not inhibited and the CpG island of AQP3 gene was hypomethylated. However, the HGC27 cell line was different from other EBV-negative cell lines. In the HGC27 cell line, the AQP3 promoter region was more methylated while AQP3 expression was lower than other negative cell lines. This might be related to the degree of differentiation of the cell lines. HGC27 is an undifferentiated human gastric cancer cell line, while BGC823 is a poorly-differentiated human gastric cancer cell line and SGC-7901 is a lymph node metastasis gastric cancer cell line (Yuanyi Xu. Et al., 2018). Recently, a manuscript reported that the features of DNA methylation are not the same for all cells. Major differences have been found between differentiated cells and stem cells (Sallustio F. et al., 2019).

To clarify the mechanism of CpG island methylation in AQP3, three enzymatically active DNA methyltransferase (DNMTs), including DNMT1, DNMT3a and DNMT3b, were detected. A study reported that LMP2A induced the phosphorylation of STAT3, which activated DNMT1 transcription and caused PTEN expression loss in EBVaGC (Hino R. et al., 2019). In our study, it was shown that the expression of DNMT3a was markedly increased with EBV infection while the

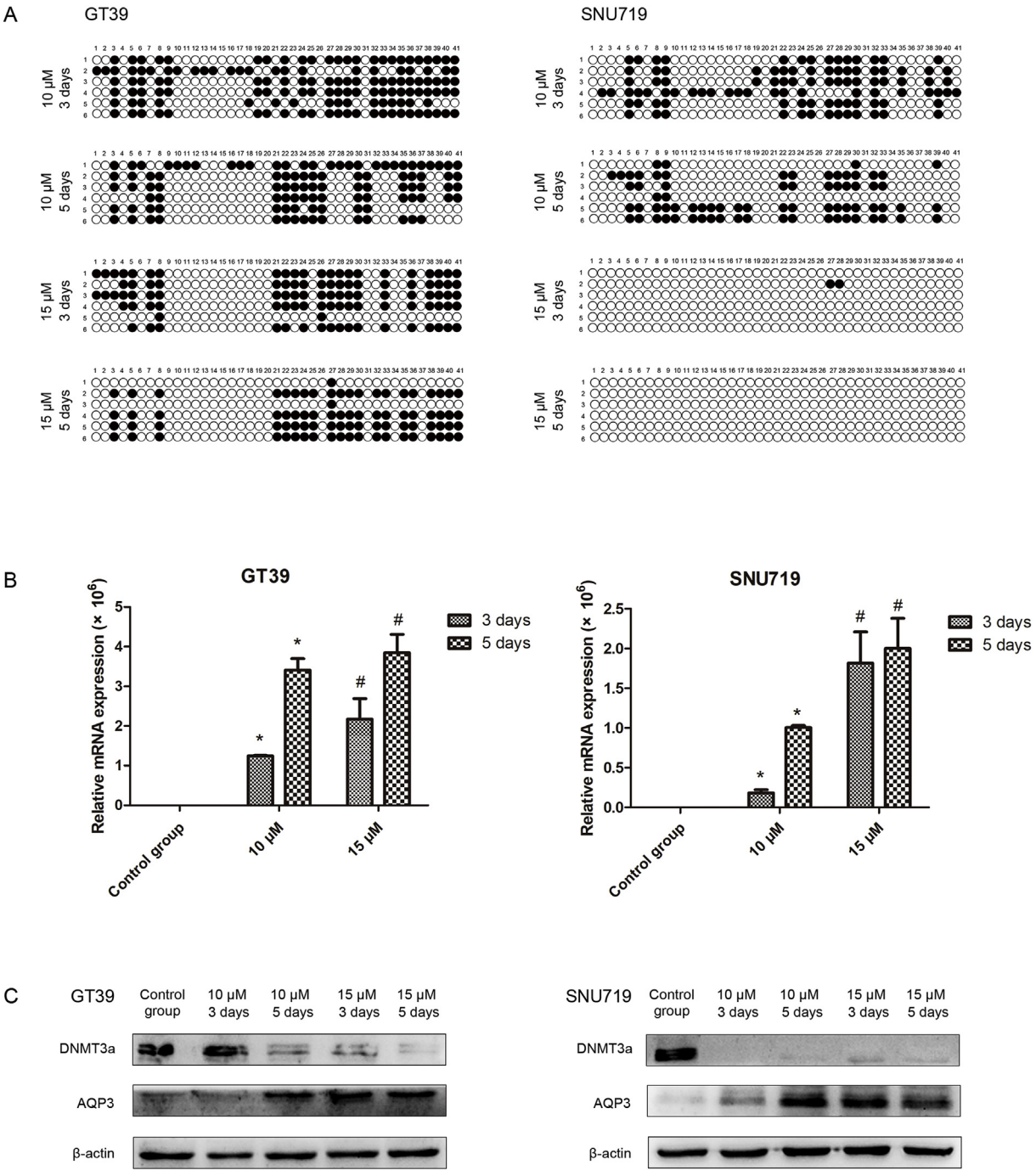

Fig. 5. 5-Aza-CdR inhibits DNMT3a expression and restores AQP3 expression based on different time and concentration patterns. (A) The methylation status of CpG sites in AQP3 gene promoter in GT39 and SNU719 cell line after the treatment of 5-Aza-CdR. (B) AQP3 mRNA expression level in GT39 and SNU719 cell lines before and after the treatment of 5-Aza-CdR. Expression levels were calculated by quantitative real-time PCR. GAPDH gene was used as internal reference and the $2^{-\triangle Ct}$ method was used for relative quantification. *, # The differences were significant as compared with the control group ($P < 0.05$). (C) AQP3 and DNMT3a protein levels were assessed by Western blot in GT39 and SNU719 cell lines before and after the treatment of 5-Aza-CdR.

expression of DNMT1 and DNMT3b had no significant difference between EBVaGC and EBVnGC. However, to our knowledge, there was no report on the regulation mechanism of EBV on DNMT3a.

Oncogenic viruses were reported to modulate the expression of DNMTs. The major EBV oncogene, latent member protein 1 (LMP1) gene, was reported to up-regulate DNMT1, DNMT3a and DNMT3b in nasopharyngeal carcinoma cell lines, which in turn led to the methylation of the tumor suppressor genes *RARB* and *CDH13* (Seo, S. Y. et al., 2008; Tsai, C. N. et al., 2002). Another study showed that EBV infection of germinal center B cells was followed by up-regulation of DNMT3a (bound to the viral promoter Wp) and down-regulation of DNMT3b and DNMT1 (Sarah Leonard. et al., 2011). EBV-encoded RNA (EBER) might not affect the expression of DNMT3a and AQP3. LMP2A, another EBV latent member protein, was reported to suppress the expression of PTEN by up-regulate DNMT1 in gastric carcinoma cell lines MKN-1 and MKN-7 (Hino R. et al., 2019; Chang MS. et al., 2006a, b, c). A study reported that EBV infection in AGS cell line induced significantly specific hypermethylation patterns by up-regulation of DNMT3b through LMP2A (Zhao J et al. Cancer, 2013, 119: 304–12). However, our result showed that the expression of DNMT3b had no difference between EBV-positive

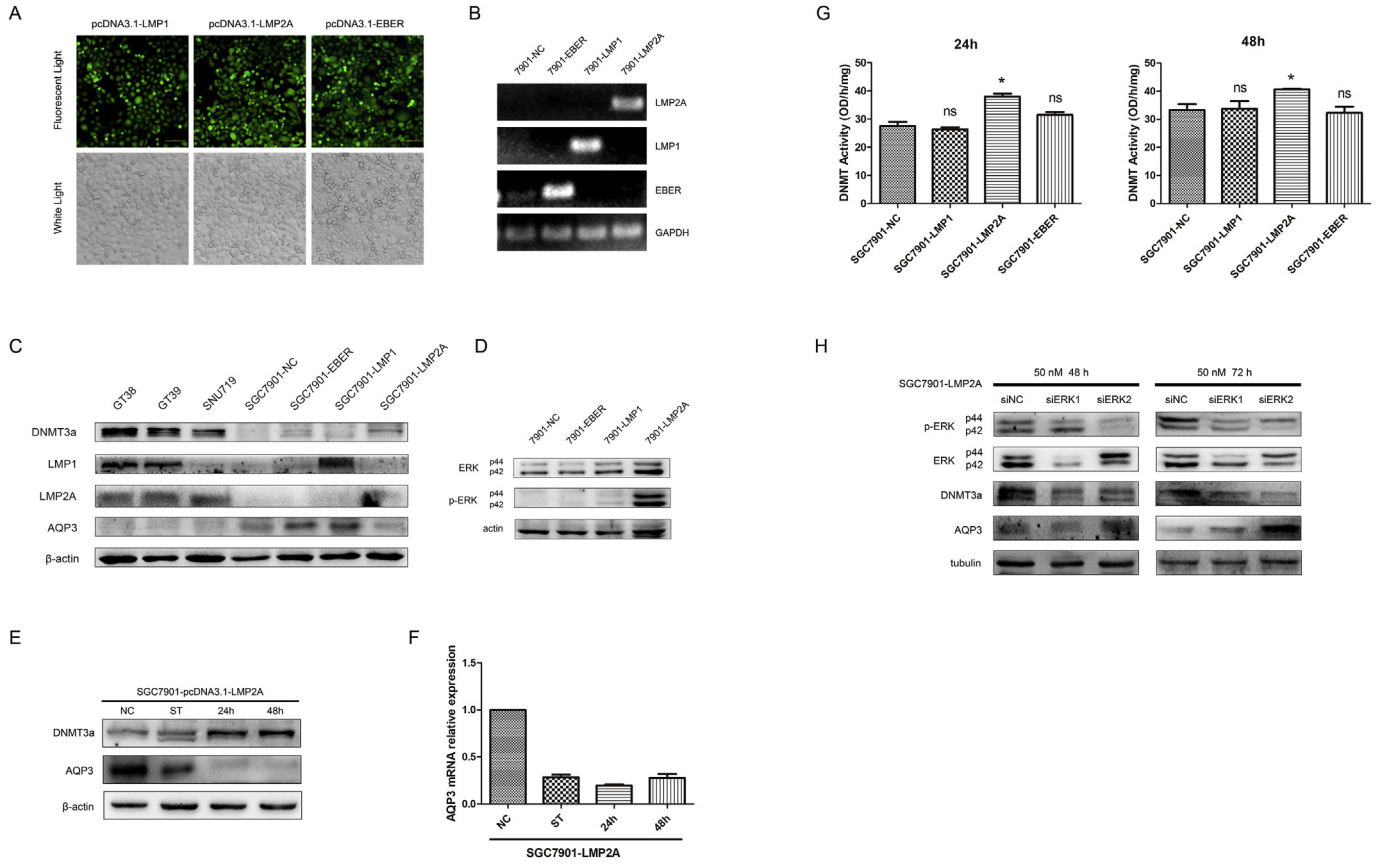

Fig. 6. LMP2A induces DNMT3a up-regulation and subsequent down-expression of AQP3 in EBV-infected GC cell lines. (A) Fluorescence efficiency of SGC7901 cells transfected with plasmids. Original magnification × 200 for all panels. (B) RT-PCR analysis to detect the expression of LMP2A, LMP1 and EBER in stable transfected SGC7901 cells. (C) Western blot analysis of protein extracts from EBV-positive cell lines and SGC7901 that stably transfected with LMP1, LMP2A and EBER. (D) Western blot analysis of protein extracts from SGC7901 that stably transfected with LMP1, LMP2A and EBER. (E) Western blot analysis of protein extracts from SGC7901 that transfected with LMP2A for 24 h, 48 h and from stable transfected cells. ST: stable transfection. (F) mRNA expression of AQP3 in transcient or stable transfected SGC7901 cells. (G) DNMT activity in SGC7901 that transfected with LMP2A for 24 h and 48 h. (H) Western blot analysis of protein extracts from SGC7901-LMP2A with or without siRNA of ERK1 or ERK2.

cell line (GT38, GT39, SNU719) and EBV-negative cell line (SGC7901, BGC823, HGC27). In the present study, we found that the expression of DNMT3a was elevated after transient or stable transfection of LMP2A into EBV-negative GC cell line SGC7901. Based on this phenomenon, we investigated the potential mechanism of EBV LMP2A in regulating of DNMT3a.

A study demonstrated that LMP2A provides a surrogate pre-BCR signal through activation of the extracellular signal-regulated kinase (ERK) pathway (Anderson LJ, Longnecker R., 2008). Another study illustrated that LMP2A activates the ERK pathway and promotes cell mobility (Chen SY. et al., 2002). Furthermore, a recent study reported that N-terminal region of LMP2A containing tyrosines 74, 85, 101, and 112 are required for ERK activation (Dai Iwakiri. et al., 2013). These articles demonstrated that LMP2A can activate the ERK/MAPK signaling pathway. Moreover, ERK/MAPK pathway has been shown to be involved in the regulation of expression of DNMTs in mammalian cells, and inhibition of ras-MAPK pathway has been found to decrease the expression of DNMTs (Deng, C. et al., 2003). Consistently, we found that LMP2A induced the phosphorylation of ERK and thus up-regulated DNMT3a in SGC7901-LMP2A cell line.

EBVaGC is a unique type of GC that accounts for 5–18% of GCs reported around the world (Sudo M. et al., 2004b). EBVaGC has some characteristic clinicopathologic features. For example, EBV positive was negatively correlated with tumor stage and positively correlated with low mortality and low heterogeneity (Sudo M. et al., 2004a). Besides, EBVaGC patients have a relatively longer survival time because EBVaGC may be more susceptible to appear genetic changes associated with better prognosis (Hanahan, D. & Weinberg, R. A. et al., 2000). Given these many distinctions, the carcinogenic process of EBVaGC is thought to be quite different from that of EBVnGC. Numerous studies have found that AQP3 is over-expressed in tumors and low-expressed in normal tissues (Kusayama, M. et al., 2011; Chen J. et al., 2014). Furthermore, AQP3 is involved in tumor metastasis and invasion (Satooka H, Hara-Chikuma M., 2016; Wang L et al., 2016). In our study, we found that the expression of AQP3 was significantly reduced in EBVaGC, either in cell lines or in tissues, suggested that low-level of AQP3 expression caused by EBV infection might be associated with the well prognosis of EBVaGC.

5. Conclusions

Our study has shown that a latent viral protein, LMP2A, constitutively or transiently inducing the phosphorylation of ERK, might lead to the overexpression of DNMT3a. Highly expressed DNMT3a may further cause the hypermethylation of the AQP3 gene at its promoter and the first exon region, which limited the expression of AQP3 in EBV-positive gastric carcinomas.

Author contributions

B.L. conceived and designed the experiments; J.-Y.W., X.-Y.Z., H.X. performed the experiments; J.-Y.W., W.L., Y.Z., H.X. analyzed the experimental data; J.-Y.W. drafted and revised the manuscript; All authors reviewed and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Ethics approval and consent to participate

All procedures involving human participants in this study met the ethical standards of the Medical Ethics Committee at the Medical College of Qingdao University, as well as the 1964 Helsinki declaration and its subsequent revisions or comparable ethical standards.

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