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Hypomethylation-mediated overexpression of ITGA2 stimulates cell invasion and migration of thyroid carcinoma

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Summary. Objective. To study the molecular mechanism of DNA methylation-mediated ITGA2 overexpression in thyroid carcinoma (TC).

Methods. First, 450K methylation data and mRNA expression profiles in TCGA-THCA dataset were downloaded from TCGA database. ITGA2 was identified as a methylation-driven gene by using R package "MethylMix". Afterwards, qRT-PCR, western blot and flow cytometry assay were performed to measure ITGA2 expression in TC cells. Methylation-specific PCR was utilized to measure promoter region methylation of ITGA2 in TC cells. Transwell and wound healing assays were carried out to assess cell invasive and migratory properties.

Results. Compared with normal cells, TC cells presented significantly increased ITGA2 expression. In addition, ITGA2 expression was controlled by DNA methylation. Hypomethylation of CpG island resulted in an increased ITGA2 expression. Hence, methylation and expression levels of ITGA2 were inversely associated. Moreover, overexpression of ITGA2 and promoter region hypomethylation facilitated cell invasive and migratory abilities in TC.

Conclusion. These findings authenticated that promoter region hypomethylation of ITGA2 fostered ITGA2 expression as well as TC cell invasion and migration.

Key words: ITGA2, Promoter methylation, Invasion and migration, Thyroid carcinoma

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Introduction

Thyroid carcinoma (TC) is a prevalent endocrine malignant tumor worldwide, occupying 90% of endocrine tumors (Li et al., 2016). According to differentiation levels, TC can be categorized into well-differentiated follicular TC (FTC) and papillary TC (PTC), as well as poorly differentiated TC (PDTC) and anaplastic TC (ATC), which are most invasive and difficult-to-diagnose (Ferrari et al., 2015). Currently, morbidity of TC is still rising. According to the National Cancer Institute of the United States, there were 56870 cases of newly diagnosed TC and deaths related to TC in the United States in 2017 (Kazaure et al., 2012; Siegel et al., 2016; Harlow et al., 2017). Several researchers believed that TC may be associated with epigenetic alteration (Nikiforova and Nikiforov, 2008, 2011; Verdelli et al., 2015). Investigating the molecular mechanism of TC pathogenesis is a continuing concern within diagnosis and treatment of TC due to high recurrence rate of TC among cancer patients,

Epigenetics refers to gene function alteration, which involves alteration of the structure and organization of chromatin instead of DNA sequences (Biswas and Rao, 2017). DNA methylation and histone acetylation are two possible causes of epigenetic alteration, and DNA methylation attracts more attention (Hotchkiss, 1948). DNA methylation transfers a methyl group in 5'-CpG-3' dinucleotide to the fifth carbon of cytosine to form 5-MC (Holcakova, 2018; Morgan et al., 2018). Aberrant methylation of CpG island may change levels of tumor repressors and oncogenes, and thus cancer cells are always accompanied with aberrant DNA methylation (Ehrlich, 2009; Rao et al., 2013; Smith and Meissner, 2013). In transcriptionally active genes, the transcription factors may bind to DNA when CpG islands in the promoter region stay unmethylated (Mersakova et al., 2016). In contrast, methylated CpG islands inhibit such bindings and hence result in gene silencing (Pechalrieu et al., 2017; Dugue et al., 2018). Characterized by methylation of promoter CpG island in tumorigenesis-

associated genes, alterations in DNA methylation affect gene expression and relevant biological functions, which promotes caner progression. For instance, Kim et al. (2019) found that the hypermethylation of TESC leads to the downregulation of TESC mRNA/protein expression. Xia et al. (2019) found that Promoter DNA methylation-silenced miR-204 can serve as a potential diagnostic biomarker of PTC. Besides, miR-204 may be a crucial player in the development of PTC for its involvement in many tumor-related pathways. Promoter region hypermethylation of key genes suppresses gene expression and influences biological functions, thus stimulating cancer progression. Wehbe et al. (2006) revealed that the promoting role interleukin-6 played in cholangiocarcinoma progression is associated with aberrant promoter methylation and gene expression. Another study conducted by Harkaway et al. (1992) further confirmed that increasing DNA methylation in the promoter region of HoxA5 fosters tumor progression via suppressing HoxA5 gene expression. According to the study conducted by Lal et al. (2006), RIZ1 gene expression is epigenetically inactivated by promoter hypermethylation, and RIZ1 is an underlying therapeutic target. Therefore, aberrant promoter methylation may be a therapeutic target for TC.

Recently, it is possible to analyze TC at molecular level with deepening investigations on TC and rapid development of molecular detecting techniques. Through genome-wide microarray gene expression profile analysis, numerous studies have probed the function and mechanism of certain genes in varying cancers. For example, Hou et al. (2011) revealed that BRAF modulates promoter methylation and expression of multiple key genes to stimulate epigenetic mechanism of PTC canceration. Nikolova et al. (2008) identified the essential role of RGS4 in TC pathogenesis and the molecular therapeutic target for TC through genome-wide gene expression profile. This study attempted to investigate the promoter methylation level of ITGA2, and its impact on TC progression. First, bioinformatics analysis was conducted through R program to screen out differentially expressed genes (DEGs) and related CpG sites. Then, methylation-specific PCR (MSP) was utilized to assess DNA methylation level in tumor tissue and normal tissue. ITGA2 was found to be obviously hypomethylated. qRT-PCR and western blot were conducted to measure ITGA2 expression at molecular level. Furthermore, DNMT inhibitor (DNMTI) 5-Aza-Cytidine (5-AzadC) was used to probe its impact on ITGA2 promoter methylation level and cellular function in TC. This research may generate new insights into exploring pathogenesis and novel therapeutic targets of TC.

Materials and methods

Cell resources and cell culture

PTC cell line TPC-1 (BNCC337912), FTC cell line FTC-133 (BNCC337959) and normal thyroid follicular epithelial cell line Nthy-Ori3-1 (BNCC340487) were accessed from BeNa Culture Collection (BNCC, China). Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, USA) with 10% fetal bovine serum (FBS) was recommended for TPC-1 cells culture. FTC-133 and Nthy-Ori3-1 cells were prepared in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher Scientific, USA) with 10% FBS. Cells were cultured in an incubator at 37°C with 5% CO_2.

5-AzadC treatment

First, 5-AzadC (Sigma, USA) was employed to treat TC cell lines to demethylate ITGA2 gene. After 3 d of cell culture in culture medium with 10 μM 5-AzadC, TIANamp Genomic DNA kit (Tiangen, China) was introduced to isolate DNA. Finally, DNA methylation of ITGA2 was analyzed.

MSP

Genome DNA was isolated from TC cells (TPC-1, FTC-133) and normal thyroid follicular epithelial cells (Nthy-Ori3-1). Following the manufacturer's protocol, CpGenome DNA Modification Kit (Chemicon, USA) was used to perform bisulfite treatment of DNA. Unmethylated cytosine residues in DNA were converted to uracil without alteration of methylated cytosine. MSP was utilized to identify DNA methylation level of ITGA2, with ddH_2O as the negative control. The assay was performed in triplicate. Primers are listed in Table 1.

Western blot

Total proteins were isolated from cells with cell lysis buffer (C0481, Sigma, USA) at 4°C for 30 min, and were then subjected to SDS-PAGE, followed by transferring onto PVDF membrane (Millipore, USA).

Table 1. MSP primer sequences.

MSP primer		Sequence (5'-3')
ITGA2 (M)	Sense	GGAGGAATGAATTTTAGTTTACGT
	Anti-sense	CTAAAATACCCATTCTCTACTCGAA
ITGA2 (U)	Sense	TGGAGGAATGAATTTTAGTTTATGT
	Anti-sense	CTAAAATACCCATTCTCTACTCAAA

Table 2. qRT-PCR primer sequences.

Target gene	Primer (5'-3')
ITGA2	F:5'-CTCACCAGGAACATGGGAAC-3'
	R:5'-GTCAGAACACACACCCGTTG-3'
β-actin	F:5'-GATGTACGTTGCTATCCAGGC-3'
	R:5'-CTCCTTAATGTCACGCACGAT-3'

After blocking with 5% skim milk for 1h, the membrane was incubated with primary antibodies anti-ITGA2 (Abcam, UK) and β-actin (Cell Signaling Technology, USA) overnight at 4°C. Later, membrane was cultivated with horseradish peroxidase-conjugated goat anti-rabbit IgG H&L (HRP) (Cell Signaling Technology, USA) for another 1 h at room temperature. Finally, protein blots were visualized with ECL (Baomanbio, China) and quantified using Image J software. Relative protein level was standardized with endogenic control β-actin.

Real-time quantitative polymerase chain reaction (qRT-PCR)

Trizol kit (Invitrogen Inc., USA) was recommended for total RNA isolation. RNA quality and concentration were evaluated via ultraviolet spectrophotometry (ND-1000, NanoDrop Technologies, USA). Next, PrimeScript RT kit (Takara, Japan) was employed for inverse transcription of total RNA (400 ng). qPCR was carried out on Thermal Cycler Dice real-time system amplifier (TP800, Takara, Japan) by SYBR®Premix Ex Taq™II (Tli RNaseH Plus) kit (Takara, Japan). Primers were synthesized by Guangzhou RiboBio Co., Ltd. (China) (Table 2). Relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ method and standardized with β-actin. The assay was carried out 3 times.

Cell transfection

The si1-NC, si1-ITGA2, si2-NC and si2-ITGA2 were obtained from GenePharma (China). TPC-1 and FTC-133 cells (1×10^6 cells/well) were plated to 6-well plates, followed by cell culture in routine conditions until 40-60% confluence. Lipofectamine 2000 (Invitrogen, USA) was implemented for cell transfection per the manufacturer's protocol. Culture medium was replaced by complete medium 6h later.

Transwell invasion assay

Transwell chambers with polyethylene terephthalate (PET) membrane (diameter 6.5 mm, pore size 8 μm) were recommended for cell invasion assay. First, 5×10^4 cells were suspended in 200 μL serum-free DMEM, and then samples were added to the upper chamber pre-coated with BD BioCoat Matrigel. Next, 10% FBS as attractor was added into the lower chamber. After cells

Table 3. The genes whose methylation degrees were inversely correlated with their expression levels.

Gene	Normal Mean	Tumor Mean	logFC	pValue	adjustP	cor	corPavlue
TNFRSF12A	0.188108864	0.077279624	-1.283407863	3.34E-30	1.27E-28	-0.414288498	1.43E-22
CLDN1	0.448980554	0.341718101	-0.39384629	4.55E-28	1.73E-26	-0.634630212	7.72E-59
RAET1E	0.416981129	0.314307291	-0.407806352	6.17E-27	2.35E-25	-0.592939619	9.69E-50
RDH5	0.779144829	0.354048456	-1.137944697	7.31E-25	2.78E-23	-0.638238609	1.08E-59
AGR2	0.77295393	0.64683207	-0.256991219	5.49E-24	2.09E-22	-0.420835795	2.62E-23
ITGA2	0.519382625	0.416732287	-0.317676873	5.64E-24	2.14E-22	-0.427355333	4.66E-24
TNFRSF10B	0.867923393	0.756718923	-0.197810188	9.28E-23	3.52E-21	-0.487776236	7.68E-32
PGA5	0.585955738	0.727896499	0.312941634	1.46E-22	5.56E-21	-0.477975354	1.80E-30
PAX8-AS1	0.484336079	0.699483651	0.530281863	6.79E-22	2.58E-20	-0.376380746	1.31E-18
LGALS1	0.311716227	0.160535692	-0.957339169	8.54E-22	3.25E-20	-0.433974065	7.76E-25
NPC2	0.842681451	0.640674273	-0.39539631	1.16E-21	4.40E-20	-0.503495081	3.92E-34
CSF2	0.628995737	0.495300757	-0.344745413	2.61E-21	9.91E-20	-0.478903927	1.34E-30
DUSP5	0.300580046	0.20400315	-0.559157812	8.84E-21	3.36E-19	-0.561851363	8.99E-44
C15orf62	0.508245741	0.306973072	-0.727414113	1.86E-20	7.08E-19	-0.342151247	1.88E-15
DCSTAMP	0.77621254	0.652167593	-0.251208987	2.64E-20	1.01E-18	-0.733385472	3.44E-87
ODAM	0.522272792	0.635573713	0.283255908	4.08E-20	1.55E-18	-0.48404924	2.58E-31
MIR146B	0.526118443	0.309432914	-0.765760962	1.08E-19	4.12E-18	-0.405314423	1.38E-21
SLPI	0.452314383	0.329916149	-0.455226473	1.24E-18	4.71E-17	-0.320473288	1.21E-13
MYO1G	0.496446333	0.408671776	-0.280695155	2.15E-18	8.17E-17	-0.457596878	9.27E-28
CTXN1	0.614612908	0.492002281	-0.321013063	3.15E-18	1.20E-16	-0.563700421	4.13E-44
ABHD11-AS1	0.461618514	0.332681965	-0.472557428	2.61E-17	9.91E-16	-0.553427204	2.91E-42
CDSN	0.624979186	0.535010202	-0.224241741	3.28E-17	1.25E-15	-0.477326356	2.21E-30
PLAU	0.903226128	0.852501782	-0.08338437	1.67E-16	6.34E-15	-0.47387256	6.55E-30
S100A16	0.75827872	0.622288998	-0.285143497	2.17E-16	8.24E-15	-0.497305187	3.23E-33
RIN1	0.526584564	0.453166463	-0.216624133	7.25E-14	2.76E-12	-0.595563908	2.83E-50
AHR	0.878395959	0.815419538	-0.107328893	5.89E-12	2.24E-10	-0.443979842	4.78E-26
TMEM100	0.534770768	0.452928337	-0.239637804	1.48E-11	5.63E-10	-0.511049288	2.81E-35
SLC1A5	0.550400169	0.455869476	-0.271860102	1.70E-11	6.45E-10	-0.545525363	6.96E-41
B3GALT2	0.641684216	0.677087512	0.077478813	1.88E-08	7.15E-07	-0.637928141	1.28E-59
NFE2L3	0.396863478	0.348679304	-0.186742065	1.03E-06	3.93E-05	-0.698295853	8.73E-76
TACSTD2	0.321126554	0.247318279	-0.376773098	2.00E-06	7.61E-05	-0.719736574	1.51E-82
MIR31HG	0.296974851	0.249177609	-0.253166331	1.37E-05	0.000519828	-0.544066249	1.24E-40
PRR15	0.189365762	0.279475213	0.56154483	0.001059665	0.040267273	-0.447659293	1.68E-26
PHEX	0.641315883	0.587052572	-0.127545432	0.001107441	0.042082775	-0.554571588	1.83E-42

were incubated at 37°C for 24h, a cotton swab was applied to remove the non-invading cells from the upper surface of the membrane, while cells in the bottom chamber were fixed with paraformaldehyde and stained with crystal violet. The stained cells in 5 randomly selected fields were reckoned and quantitated under a microscope (×100).

Wound healing assay

First, 5×10^5 cells were maintained in 6-well plates overnight. The next day, a sterile pipette tip was applied to scratch confluent cell monolayer. PBS was employed to wash off detached cells. Serum-free medium was supplemented for 24h of cell incubation. Afterwards, photographs were taken for observation of the wound healing status.

Flow cytometry assay

Cells were detached using 2 mM EDTA in PBS and ITGA2 expression on cell surface was assessed (Biolegend 359310, USA). Annexin V staining was conducted using FITC Annexin V Apoptosis Detection Kit (BD Biosciences 556547, USA). The experimental method is the same as Adorno-Cruz et al. (2021).

Statistical analysis

All data were analyzed on GraphPad Prism 6.0 (La Jolla, USA). Every experiment was repeated in triplicate. The Student's t-test and the chi-square test were used for difference comparison. $p<0.05$ confirmed statistical significance.

Results

ITGA2 is hypomethylated and highly-expressed in TC

First, 450K methylation data (normal: 56, tumor: 515) of TCGA-THCA were downloaded from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). Subsequently, genes with substantial methylation differences in TC tissues compared with normal tissues were screened. Finally, genes whose methylation degrees were inversely correlated with gene expressions were further screened, and the specific genes are listed in Table 3. According to some reports, ITGA plays an important role in the cell function of TC (Qin et al., 2020; Gao et al., 2021), we, therefore, became interested in ITGA2 and conducted research on ITGA2. As shown in Fig. 1A, compared with normal tissue, ITGA2 promoter was hypomethylated in tumor tissue.

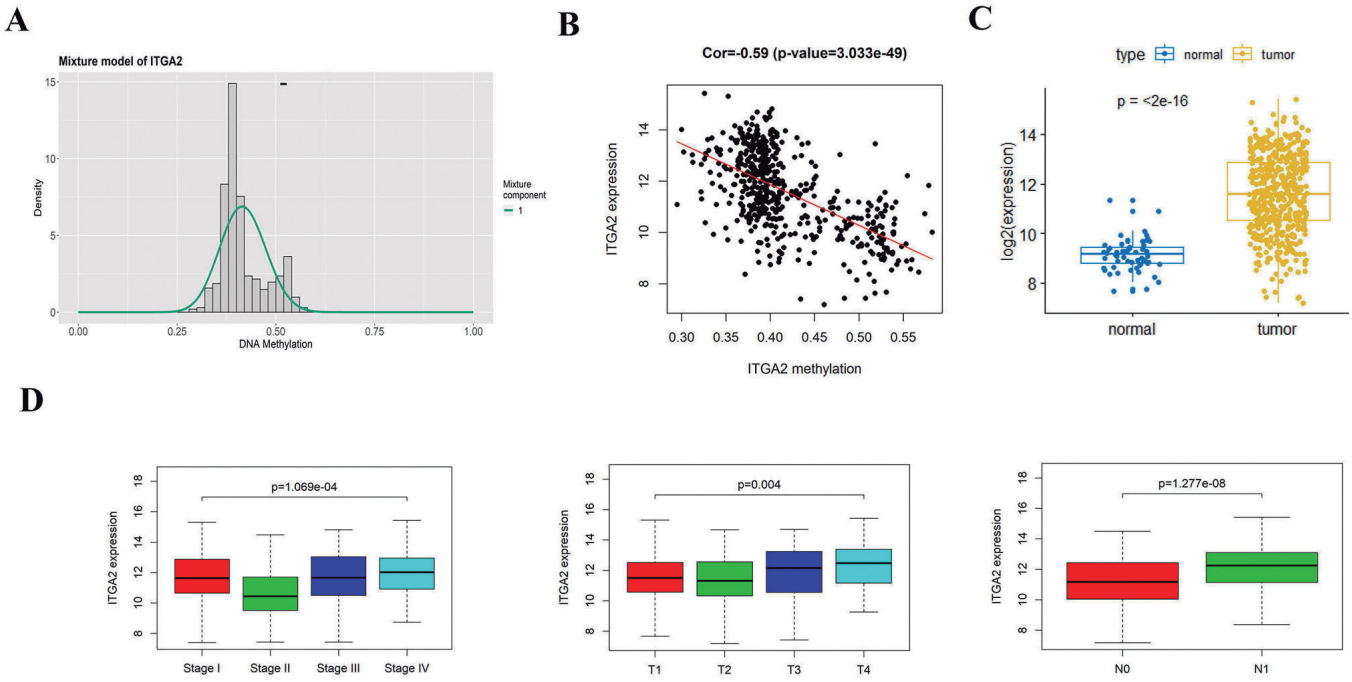

Fig. 1. Analysis of ITGA2 promoter methylation and gene expression in TC. **A.** Methylation mixture model of ITGA2. The distribution map represents the DNA methylation status of ITGA2. The histogram shows the distribution of methylation in tumor samples (n=515), and the horizontal black bar indicates the distribution of methylation in normal samples (n=56). **B.** Pearson correlation analysis of ITGA2 DNA methylation level and gene expression level. **C.** Box plot of ITGA2 expression in the normal group (n=58, blue) and tumor group (n=510, yellow) in TCGA-THCA dataset. **D.** Box plot of ITGA2 expression difference in various tumor stages.

ITGA2 promoter methylation level was also inversely correlated with ITGA2 expression (Fig. 1B). Besides, it could be found from expression profiles of mRNA (normal: 58, tumor: 510) in TCGA-THCA dataset that ITGA2 was conspicuously up-regulated in tumor tissue (Fig. 1C). The Kruskal.Test exhibited a growing trend of ITGA2 expression with tumor progression (Fig. 1D). Existing essays reported that overexpression of ITGA2 notably enhances tumor cells adhering to extracellular matrix (ECM) (Yu, et al., 2019). Co-expression of UCA1 and ITGA2 fosters the malignant progression of pancreatic cancer through the focal adhesion pathway (Gong et al., 2019). Bioinformatics analysis results displayed that ITGA2 was hypomethylated and overexpressed in TC, and its methylation level was inversely correlated with its expression. Moreover, ITGA2 may modulate cancer progression via affecting adhesion of cancer cells to the ECM.

Promoter hypomethylation of ITGA2 up-regulates ITGA2 expression in TC

Through bioinformatics analysis, the high expression of ITGA2 in TC was found to be caused by DNA promoter hypomethylation of ITGA2. A study (Lian et al., 2018) certified that promoter methylation-mediated ITGA2 overexpression leads to dismal outcomes for acute myeloid leukemia patients. Nevertheless, the impact of aberrant promoter methylation of ITGA2 on TC progression was rarely reported, and thus we further investigated the impact of ITGA2 promoter methylation on its gene expression in TC cells through *in vitro* experiments. First, qRT-PCR and western blot were employed to detect ITGA2 level in TC and normal thyroid follicular epithelial cells. As illustrated in Fig. 2A,B, ITGA2 was lowly expressed in normal cell line while it was up-regulated in TC cell lines. CpG islands of ITGA2 were identified on MethPrimer website (Fig. 2C). MSP was used for evaluation of ITGA2 DNA methylation level in normal and TC cell lines. The results in Fig. 2D present no methylation in FTC-133 cells, partial methylation in TPC-1 cells, and complete methylation in Nthy-Ori3-1 cells. These findings denoted that promoter hypomethylation of ITGA2 up-regulated ITGA2 expression in TC.

Promoter hypomethylation of ITGA2 facilitates cell invasion and migration in TC

To investigate the impact of promoter hypomethylation of ITGA2 on TC growth, si-NC and si-ITGA2 were transfected into TPC-1 and FTC-133 cells, respectively. Cells were treated with 10 μM DNMTI 5-

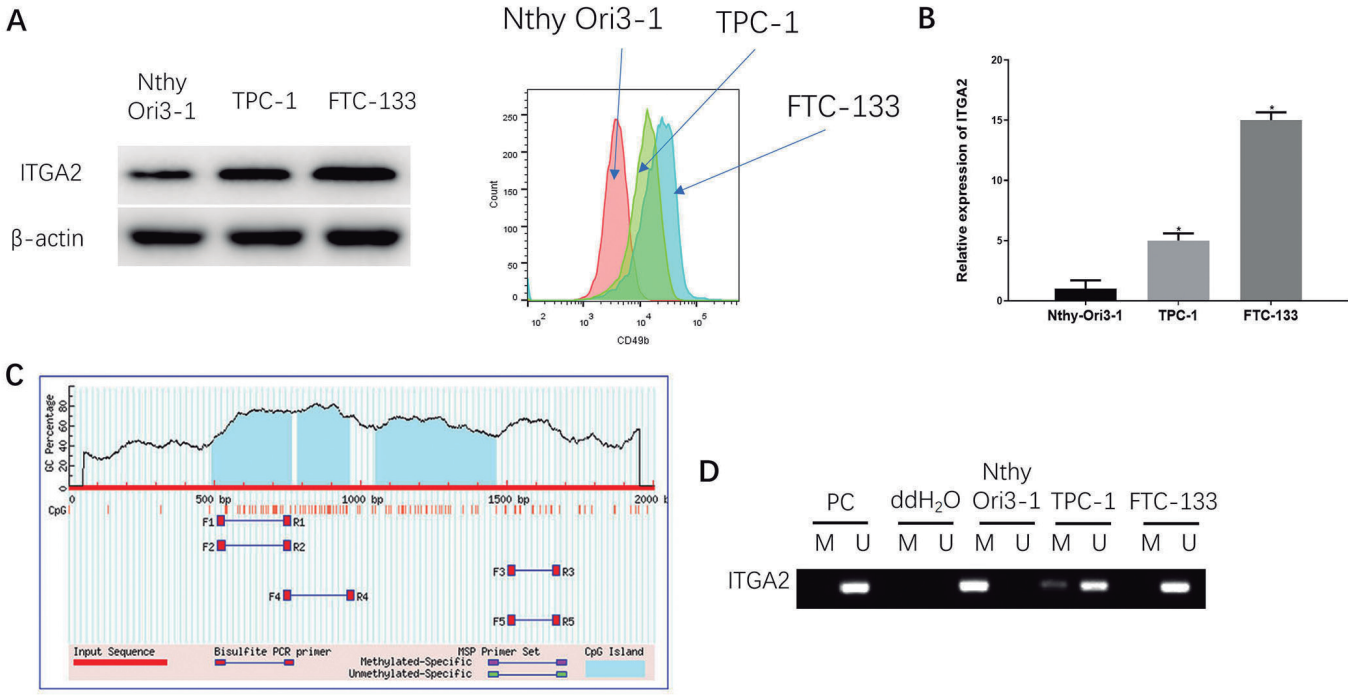

Fig. 2. The methylation level and gene expression of ITGA2 in TC cells and normal cells. **A.** Western blot and flow cytometry assays were used to assess ITGA2 protein expressions in TC cell lines (TPC-1, FTC-133) and normal thyroid follicular epithelial cell line (Nthy-Ori3-1). **B.** qRT-PCR measured ITGA2 mRNA expression in TC cell lines and normal thyroid follicular epithelial cell line. **C.** The CpG islands located in ITGA2 promoter region predicted by MethPrimer website. **D.** The results of MSP of ITGA2 in TC cell lines and normal thyroid follicular epithelial cell line (U: unmethylated alleles; M: methylated alleles); *p<0.05.

AzadC. qRT-PCR was utilized to assess ITGA2 level in TPC-1 and FTC-133 cells after treatment (Figs. 3A, 4A). The result presented that ITGA2 expression decreased with si-ITGA2, whereas its expression significantly increased after adding DNMTI. In Transwell invasion assay, compared with si-NC (si1-NC/si2-NC+-5-AzadC) group, si-ITGA2 (si1-ITGA2/si2-ITGA2+-5-AzadC) group appeared with significantly weak invasive ability of TPC-1 and FTC-133 cells. Besides, the invasive capability was restored in si1-ITGA2/si2-ITGA2+ +5-AzadC group. (Figs. 3B, 4B). As such, wound healing assay enunciated that the migratory property of these two cell lines decreased with si-ITGA2, while it was elevated with the addition of 5-AzadC (Figs. 3C, 4C). Together these results demonstrated that hypomethylated and highly-expressed ITGA2 fostered cell invasion and migration in TC.

Discussion

Through bioinformatics analysis, molecular and cellular experiments, this study investigated the relationship between epigenetic regulation and pathogenesis of TC. A vital role of ITGA2 DNA methylation level and ITGA2 in TC progression was demonstrated here for the first time, which provides a deeper understanding of TC pathogenesis.

TCs are categorized into four presentative histological types, namely PTC, FTC, medullary carcinoma, and ATC (Okada et al., 2014). PTC is the most prevalent one with the lowest malignant degree, which takes up 85% of all TC cases. Okada et al. (2014) disclosed that EpCAM expression in two ATC cell lines is markedly higher than that in TPC-1 and FTC-133 cell lines. D'Agostino et al. (2014) pointed out that the transcriptional levels of TSHIR and NIS in FTC-133 cells are controlled by various forms of epigenetics. Here, we focused on ITGA2 DNA methylation level and its influence on the progression of different subtypes of TC cells.

As an attractive diagnostic and therapeutic target, DNA methylation as the main epigenetic regulatory mechanism is attracting the interest of researchers due to its appearance in precancerous (Moore et al., 2013). Studies on DNA methylation in various cancers manifested that the tumor cells feature global loss of

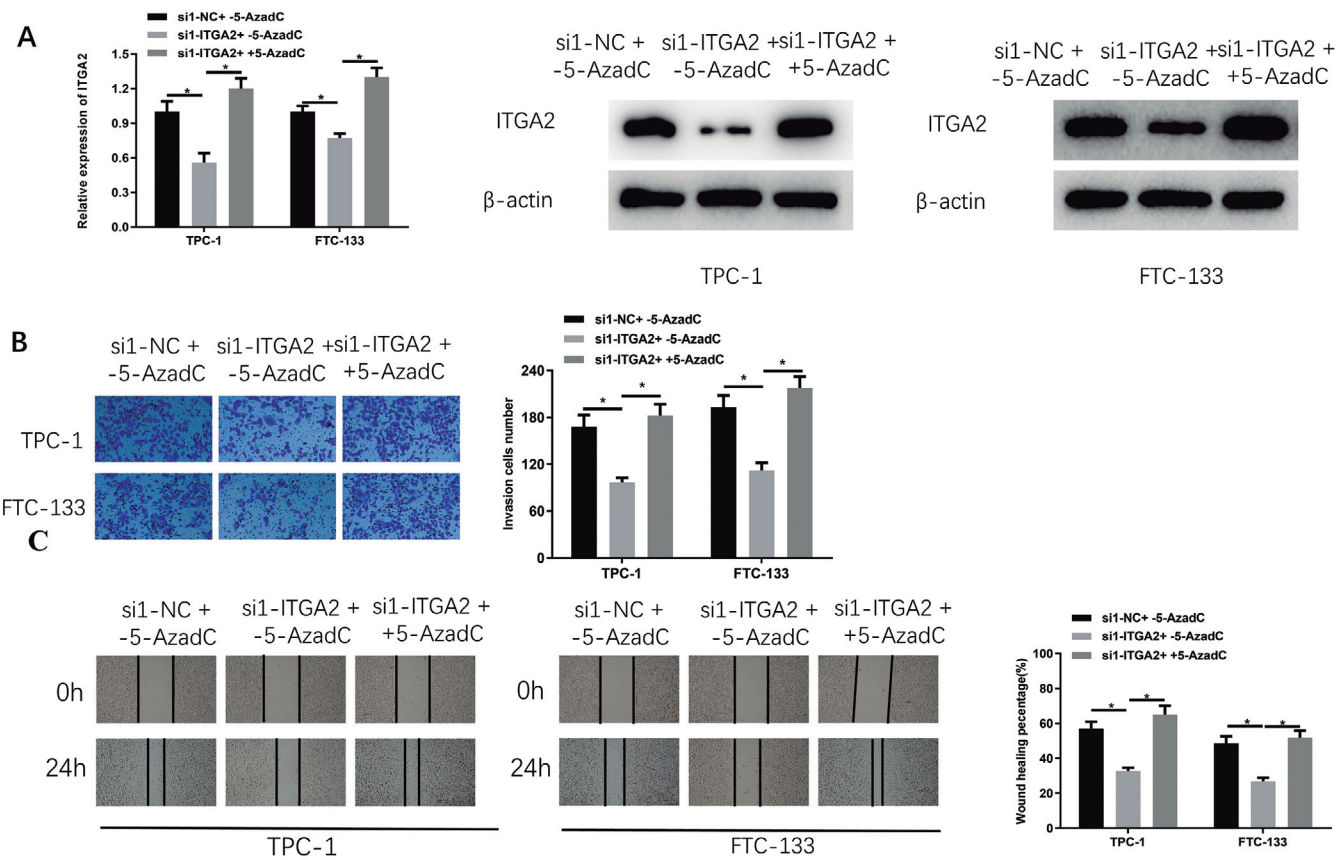

Fig. 3. Hypomethylated and highly-expressed ITGA2 facilitates cell invasion and migration in TC. **A.** qRT-PCR and western blot assays were used to measure ITGA2 mRNA and protein expression respectively among si1-NC+-5-AzadC, si1-ITGA2+-5-AzadC, and si1-ITGA2++5-AzadC groups. **B.** Transwell assay assessed the invasive ability of TPC-1 and FTC-133 cells in different treatment groups. **C.** Wound healing assay detected the migratory ability of TPC-1 and FTC-133 cells in different treatment groups. *p<0.05. B, x 100; C, x 40.

DNA methylation (global DNA hypomethylation), while the promoter region hypermethylation of the specific gene can also be observed (Biswas and Rao, 2017; Bleys et al., 2008). In 2005, Lee et al. determined the presence of GPX3 methylation in esophageal carcinoma while the CPX3 promoter region is unmethylated in normal tissue, and hypermethylation of GPX3 is associated with down-regulated GPX3 expression. Besides, gene methylation level is correlated with the malignant progression of cancer. For instance, Liu et al. (2015) uncovered that GPX3 methylation is related to relatively advanced tumor stages. Epigenetic modification alters gene expression in cancers, thereby changing cancer characteristics such as cell invasion, angiogenesis and proliferation. Through bioinformatics analysis, we found that 34 genes were differentially expressed in TC DNA methylation, as listed in Table 3. We speculated that these genes may affect TC through DNA methylation. Chen et al. (2021) identified key DNA methylation driver genes in PTC development through bioinformatics analysis of TCGA database and this was validated through GEO database to manifest critical roles of TNFRSF1A, CLDN1, and CASP1 in PTC tumorigenesis. Through TC transcriptome analysis and DNA methylation data from TCGA database, Lv et al. (2020) displayed that RDH5, TREM1, BIRC7, and SLC26A7 are highly expressed when hypomethylated and can be used as indicators for poor prognosis of TC patients. Iqbal et al. (2022) found that methylation levels of CHST2, DPP4, DUSP6, ITGA2, SLC1A5, TIAM1, TNIK, and ABTB2 are substantially reduced in TC patients at the gene sequencing level, but no research was done about ITGA2 and TC methylation at the cellular level. Investigations on the effect of DNA methylation on TC are also rare. Additionally, ITGA2 was selected as research object due to our personal interests. However, other genes were not studied at the cellular level, this being one of the shortcomings of this study. Our results showed that Hypomethylation-mediated overexpression of ITGA2 stimulates cell invasion and migration of thyroid carcinoma and the results are the same as other studies. For instance, promoter DNA hypomethylated and highly-expressed S100A15 is correlated with high-metastatic potential and unfavorable prognosis of lung adenocarcinoma (Chen et al., 2017). ITGA2 can be used as a diagnostic marker to

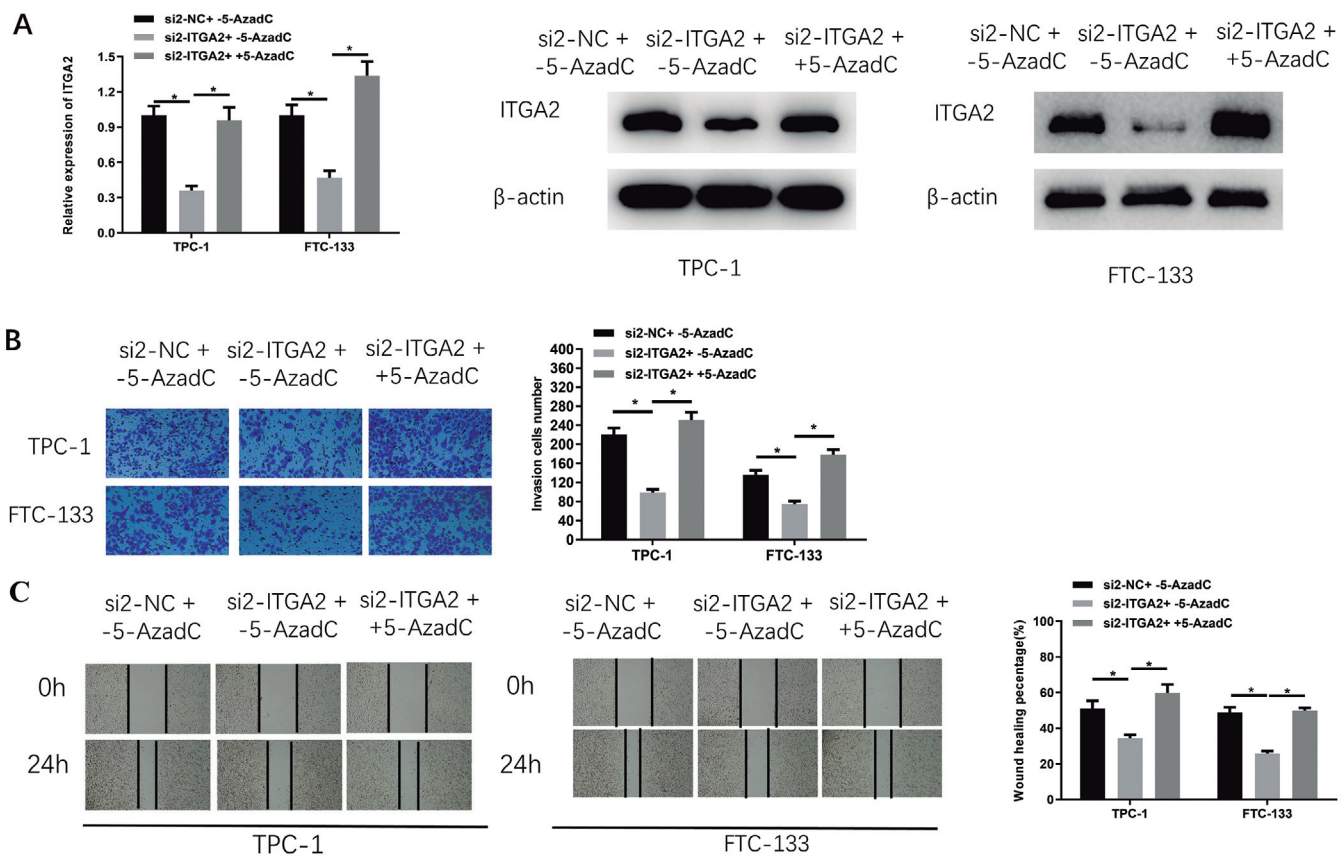

Fig. 4. Hypomethylated and highly-expressed ITGA2 facilitates cell invasion and migration in TC. **A.** qRT-PCR and western blot measured ITGA2 expression in si2-NC+-5-AzadC, si2-ITGA2+-5-AzadC, and si2-ITGA2++5-AzadC groups. **B.** Transwell assay assessed the invasive ability of TPC-1 and FTC-133 cells in different treatment groups. **C.** Wound healing assay detected the migratory ability of TPC-1 and FTC-133 cells in different treatment groups. *p<0.05. B, x 100; C, x 40.

explore the application value of methylation in the clinical diagnosis of TC, and the relationship between the promoter methylation pattern of TC and canceration.

Localized invasion and metastasis of host tissue are markers of cancer progression (Hanahan and Weinberg, 2011). Invasion of tumor cells to host tissue is regulated by the microenvironment of matrix, which is mediated by integrin family members (Fedorov et al., 2001; Desgrosellier and Cheresh, 2010). ITGA2 is aberrantly expressed in cancer pathogenesis (Jost et al., 2014; Adorno-Cruz and Liu, 2019). Regularly, integrins adhere to the ECM to provide traction force for cancer cell invasion. Besides, integrins stimulate tumor cell invasion via modulating the location and activity of matrix degradation proteases (Wu et al., 2008). ITGA2 loss in cancer cells is proven to be associated with metastasis of colon carcinoma and hepatocellular carcinoma (Yu et al., 2019; Wang, et al., 2020; Ruan et al., 2021). Zhang et al. (2008) and San Antonio et al. (2009) disclosed that ITGA2 exerts an essential effect on cell invasion, migration, angiogenesis, and survival. Alonso et al. (2013) and Liu et al. (2016) also found that ITGA2 is an activator of varying cancers *in vivo* and *in vitro*. Gong et al. (2019) manifested that UCA1 and ITGA2 are underlying prognostic factors of pancreatic carcinoma, and they target miR-107 via focal adhesions to modulate cell invasion and migration. To investigate whether promoter hypomethylation of ITGA2 regulates ITGA2 expression and influences TC cell migration and invasion, we conducted MSP and cellular functional experiments with DNMTI. The results clarified that silencing ITGA2 promoter methylation could promote ITGA2 expression. Moreover, promoter hypomethylated and highly-expressed ITGA2 fostered TC cell invasion and migration. These findings further certified the function of ITGA2 in TC and inverse correlation between ITGA2 methylation level and its gene expression. Furthermore, ITGA2 functioned as an oncogene in TC.

All in all, our empirical results denoted that ITGA2 modulated by DNA methylation was an oncogene in TC. Promoter hypomethylation and high expression of ITGA2 facilitated cell migration and invasion in TC. Our findings deepen the understanding of the role of ITGA2 promoter methylation in TC and lay the foundation for novel approaches to targeted therapy for TC.

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References

Adorno-Cruz V. and Liu H. (2019). Regulation and functions of integrin alpha2 in cell adhesion and disease. Genes Dis. 6, 16-24.

Adorno-Cruz V., Hoffmann A.D., Liu X., Dashzeveg N.K., Taftaf R., Wray B., Keri R. A. and Liu H. (2021). ITGA2 promotes expression of ACLY and CCND1 in enhancing breast cancer stemness and metastasis. Genes Dis. 8, 493-508.

Alonso E.N., Orozco M., Eloy Nieto A. and Balogh G.A. (2013). Genes related to suppression of malignant phenotype induced by Maitake D-Fraction in breast cancer cells. J. Med. Food 16, 602-617.

Biswas S. and Rao C.M. (2017).Epigenetics in cancer: Fundamentals and Beyond. Pharmacol. Ther. 173, 118-134.

Bleys J., Navas-Acien A. and Guallar E. (2008). Serum selenium levels and all-cause, cancer, and cardiovascular mortality among US adults. Arch. Intern. Med. 168, 404-410.

Chen Y.C., Lin M.C., Hsiao C.C., Zheng Y.X., Chen K.D., Sung M.T., Chen C.J., Wang T.Y., Lin Y.Y., Chang H.C., Chen Y.M. and Chang J.C. (2017). Increased S100A15 expression and decreased DNA methylation of its gene promoter are involved in high metastasis potential and poor outcome of lung adenocarcinoma. Oncotarget 8, 45710-45724.

Chen Y., Wang K., Shang M., Zhao S., Zhang Z., Yang H., Chen Z., Du R., Wang Q. and Chen B. (2021). Exploration of DNA methylation-driven genes in papillary thyroid carcinoma based on the cancer genome atlas. J. Comput. Biol. 28, 99-114.

D'Agostino M., Sponziello M., Puppin C., Celano M., Maggisano V., Baldan F., Biffoni M., Bulotta S., Durante C., Filetti S., Damante G. and Russo D. (2014). Different expression of TSH receptor and NIS genes in thyroid cancer: role of epigenetics. J. Mol. Endocrinol. 52, 121-131.

Desgrosellier J.S. and Cheresh D.A. (2010). Integrins in cancer: biological implications and therapeutic opportunities. Nat. Rev. Cancer. 10, 9-22.

Dugue P.A., Bassett J. K., Joo J.E., Jung C.H., Ming Wong E., Moreno-Betancur M., Schmidt D., Makalic E., Li S., Severi G., Hodge A.M., Buchanan D.D., English D.R., Hopper J.L., Southey M.C., Giles, G.G. and Milne R.L. (2018). DNA methylation-based biological aging and cancer risk and survival: Pooled analysis of seven prospective studies. Int. J. Cancer 142, 1611-1619.

Ehrlich M. (2009). DNA hypomethylation in cancer cells. Epigenomics 1, 239-259.

Fedorov V.I., Pogodin A.S., Dubatolova T.D., Varlamov A.V., Leont'ev K.V. and Khamoian A.G. (2001). Comparative study of effect of infrared, submillimeter, and millimeter electromagnetic radiation on wing somatic mutations in *Drosophila melanogaster* induced by gamma-irradiation. Biofizika 46, 298-302 (in Russian).

Ferrari S.M., Fallahi P., Politti U., Materazzi G., Baldini E., Ulisse S., Miccoli P. and Antonelli A. (2015). Molecular targeted therapies of aggressive thyroid cancer. Front. Endocrinol. (Lausanne). 6, 176.

Gao Y., Pan Y., Wang T., Yao Y., Yuan W., Zhu X. and Wang K. (2021). MicroRNA-99a-3p/GRP94 axis affects metastatic progression of human papillary thyroid carcinoma by regulating ITGA2 expression and localization. Acta Biochim. Biophys Sin (Shanghai). 53, 1650-1661.

Gong J., Lu X., Xu J., Xiong W., Zhang H. and Yu X. (2019). Coexpression of UCA1 and ITGA2 in pancreatic cancer cells target the expression of miR-107 through focal adhesion pathway. J. Cell Physiol. 234, 12884-12896.

Hanahan D. and Weinberg R.A. (2011). Hallmarks of cancer: the next generation. Cell 144, 646-674.

Harkaway K.S., McGinley K.J., Foglia A.N., Lee W.L., Fried F., Shalita A.R. and Leyden J.J. (1992). Antibiotic resistance patterns in coagulase-negative staphylococci after treatment with topical erythromycin, benzoyl peroxide, and combination therapy. Br. J. Dermatol. 126, 586-590.

Harlow B.E., Flythe M.D. and Aiken G.E. (2017). Effect of biochanin A on corn grain (*Zea mays*) fermentation by bovine rumen amylolytic bacteria. J. Appl. Microbiol. 122, 870-880.

Holcakova J. (2018). Effect of DNA methylation on the development of cancer. Klin. Onkol. 31, 41-45.

Hotchkiss R.D. (1948). The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. J. Biol. Chem. 175, 315-332.

Hou P., Liu D. and Xing M. (2011). Genome-wide alterations in gene methylation by the BRAF V600E mutation in papillary thyroid cancer cells. Endocr. Relat. Cancer 18, 687-697.

Iqbal M.A., Li M., Lin J., Zhang G., Chen M., Moazzam N.F. and Qian W. (2022). Preliminary study on the sequencing of whole genomic methylation and transcriptome-related genes in thyroid carcinoma. Cancers (Basel). 14, 1163.

Jost T., Lacroix C., Braegger C. and Chassard C. (2014). Stability of the maternal gut microbiota during late pregnancy and early lactation. Curr. Microbiol. 68, 419-427.

Kazaure H.S., Roman S.A. and Sosa J.A. (2012). Aggressive variants of papillary thyroid cancer: incidence, characteristics and predictors of survival among 43,738 patients. Ann. Surg. Oncol. 19, 1874-1880.

Kim T.W., Han S.R., Kim J.T., Yoo S.M., Lee M.S., Lee S.H., Kang Y.H. and Lee H.G. (2019). Differential expression of tescalcin by modification of promoter methylation controls cell survival in gastric cancer cells. Oncol. Rep. 41, 3464-3474.

Lal G., Padmanabha L., Smith B.J., Nicholson R.M., Howe J.R., O'Dorisio M.S. and Domann F.E. Jr (2006). RIZ1 is epigenetically inactivated by promoter hypermethylation in thyroid carcinoma. Cancer 107, 2752-2759.

Lee O.J., Schneider-Stock R., McChesney P.A., Kuester D., Roessner A., Vieth M., Moskaluk, C.A. and El-Rifai W. (2005). Hypermethylation and loss of expression of glutathione peroxidase-3 in Barrett's tumorigenesis. Neoplasia 7, 854-861.

Li P., Yang W., Shen B., Li H. and Yan J. (2016). Lentivirus-mediated silencing of MPHOSPH8 inhibits MTC proliferation and enhances apoptosis. Oncol. Lett. 11, 4117-4122.

Lian X.Y., Zhang W., Wu D.H., Ma J.C., Zhou J.D., Zhang Z.H., Wen X.M., Xu Z.J., Lin J. and Qian J. (2018). Methylation-independent ITGA2 overexpression is associated with poor prognosis in de novo acute myeloid leukemia. J. Cell Physiol. 233, 9584-9593.

Liu Q., Jin J., Ying J., Sun M., Cui Y., Zhang L., Xu B., Fan Y. and Zhang Q. (2015). Frequent epigenetic suppression of tumor suppressor gene glutathione peroxidase 3 by promoter hypermethylation and its clinical implication in clear cell renal cell carcinoma. Int. J. Mol. Sci. 16, 10636-10649.

Liu X., Liang Z., Gao K., Li H., Zhao G., Wang S. and Fang J. (2016). MicroRNA-128 inhibits EMT of human osteosarcoma cells by directly targeting integrin alpha2. Tumour Biol. 37, 7951-7957.

Lv L., Cao L., Hu G., Shen Q. and Wu J. (2020). Methylation-driven genes identified as novel prognostic indicators for thyroid carcinoma. Front. Genet. 11, 294.

Mersakova S., Nachajova M., Szepe P., Kasajova P.S. and Halasova E. (2016). DNA methylation and detection of cervical cancer and precancerous lesions using molecular methods. Tumour Biol. 37, 23-27.

Moore L.D., Le T. and Fan G. (2013). DNA methylation and its basic function. Neuropsychopharmacology 38, 23-38.

Morgan A.E., Davies T.J. and Mc Auley M.T. (2018). The role of DNA methylation in ageing and cancer. Proc. Nutr. Soc. 77, 412-422.

Nikiforov Y.E. and Nikiforova M.N. (2011). Molecular genetics and diagnosis of thyroid cancer. Nat. Rev. Endocrinol. 7, 569-580.

Nikiforova M.N. and Nikiforov Y.E. (2008). Molecular genetics of thyroid cancer: implications for diagnosis, treatment and prognosis. Expert Rev. Mol. Diagn. 8, 83-95.

Nikolova D.N., Zembutsu H., Sechanov T., Vidinov K., Kee L.S., Ivanova R., Becheva E., Kocova M., Toncheva D. and Nakamura Y. (2008). Genome-wide gene expression profiles of thyroid carcinoma: Identification of molecular targets for treatment of thyroid carcinoma. Oncol. Rep. 20, 105-121.

Okada T., Nakamura T., Watanabe T., Onoda N., Ashida A., Okuyama R. and Ito K. (2014). Coexpression of EpCAM, CD44 variant isoforms and claudin-7 in anaplastic thyroid carcinoma. PLoS One 9, e94487.

Pechalrieu D., Etievant C. and Arimondo P.B. (2017). DNA methyltransferase inhibitors in cancer: From pharmacology to translational studies. Biochem. Pharmacol. 129, 1-13.

Qin A., Liu Q. and Wang J. (2020). Ropivacaine inhibits proliferation, invasion, migration and promotes apoptosis of papillary thyroid cancer cells via regulating ITGA2 expression. Drug Dev. Res. 81, 700-707.

Rao X., Evans J., Chae H., Pilrose J., Kim S., Yan P., Huang R.L., Lai H.C., Lin H., Liu Y., Miller D., Rhee J.K., Huang Y.W., Gu F., Gray J.W., Huang T.M. and Nephew K.P. (2013). CpG island shore methylation regulates caveolin-1 expression in breast cancer. Oncogene 32, 4519-4528.

Ruan Z., Deng H., Liang M., Xu Z., Lai M., Ren H., Deng X. and Su X. (2021). Overexpression of long non-coding RNA00355 enhances proliferation, chemotaxis, and metastasis in colon cancer via promoting GTF2B-mediated ITGA2. Transl. Oncol. 14, 100947.

San Antonio J.D., Zoeller J.J., Habursky K., Turner K., Pimtong W., Burrows M., Choi S., Basra S., Bennett J.S., DeGrado W.F. and Iozzo R.V. (2009). A key role for the integrin alpha2beta1 in experimental and developmental angiogenesis. Am. J. Pathol. 175, 1338-1347.

Siegel R.L., Miller K.D. and Jemal A. (2016). Cancer statistics, 2016. CA Cancer J. Clin. 66, 7-30.

Smith Z.D. and Meissner A. (2013). DNA methylation: roles in mammalian development. Nat. Rev. Genet. 14, 204-220.

Verdelli C., Forno I., Vaira V. and Corbetta S. (2015). Epigenetic alterations in human parathyroid tumors. Endocrine 49, 324-332.

Wang L., Gao Y., Zhao X., Guo C., Wang X., Yang Y., Han C., Zhao L., Qin Y., Liu L., Huang C. and Wang W. (2020). HOXD3 was negatively regulated by YY1 recruiting HDAC1 to suppress progression of hepatocellular carcinoma cells via ITGA2 pathway. Cell Prolif. 53, e12835.

Wehbe H., Henson R., Meng F., Mize-Berge J. and Patel T. (2006). Interleukin-6 contributes to growth in cholangiocarcinoma cells by aberrant promoter methylation and gene expression. Cancer Res. 66, 10517-10524.

Wu L., Bernard-Trifilo J.A., Lim Y., Lim S.T., Mitra S.K., Uryu S., Chen

M., Pallen C.J., Cheung N.K., Mikolon D., Mielgo A., Stupack D.G. and Schlaepfer D.D. (2008). Distinct FAK-Src activation events promote alpha5beta1 and alpha4beta1 integrin-stimulated neuroblastoma cell motility. Oncogene 27, 1439-1448.

Xia F., Wang W., Jiang B., Chen Y. and Li X. (2019). DNA methylation-mediated silencing of miR-204 is a potential prognostic marker for papillary thyroid carcinoma. Cancer Manag. Res. 11, 1249-1262.

Yu J., Zhang C., Yu Q., Yu H. and Zhang B. (2019). ADAR1 p110 enhances adhesion of tumor cells to extracellular matrix in hepatocellular carcinoma via up-regulating ITGA2 expression. Med. Sci. Monit. 25, 1469-1479.

Zhang Z., Ramirez N.E., Yankeelov T.E., Li Z., Ford L.E., Qi Y., Pozzi A. and Zutter M.M. (2008). alpha2beta1 integrin expression in the tumor microenvironment enhances tumor angiogenesis in a tumor cell-specific manner. Blood 111, 1980-1988.

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