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Original article

Deregulation of *TWIST1* expression by promoter methylation in gastrointestinal cancers

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ABSTRACT

TWIST1, a basic helix-loop-helix transcription factor with versatile roles in cancer, is frequently deregulated in cancers, through established pathway perturbations. However. the significance of *TWIST1* methylation in the deregulation of *TWIST1* in gastrointestinal cancers is not fully clear. This study hypothesized that *TWIST1* promoter methylation deregulates *TWIST1* expression independent of established deregulators such as the *WNT*, *TGFB*, *NOTCH* and miRNA pathways. To prove this hypothesis, colon, gastric and rectal cancer genomic data comprising gene expression, DNA methylation, and miRNA data were retrieved from the Cancer Genome Atlas cohorts which are publicly available in cancer genomic databases, the Genome Data Commons and the cBioportal.org. About 217 variables comprising expression levels of genes of the *WNT*, *TGFB*, *NOTCH* and miRNA signalling pathways, as well as the beta values of 17 *TWIST1* methylation loci were subjected to Principal Component Regression Analysis, and then standard Linear Regression Analysis. The results showed that *TWIST1* methylation is a predictor of *TWIST1* expression in the gastrointestinal cancers, independent of *WNT*, *TGFB*, and *NOTCH* signalling and miRNA deregulation. The results also showed that different *TWIST1* methylation loci may deregulate *TWIST1* expression in different cancer types. The inference that can be drawn from this study is that *TWIST1* DNA methylation is an important *TWIST1* deregulation mechanism in colon, rectal and gastric cancers.

1. Introduction

According to the 2020 GLOBOCAN statistics, the gastrointestinal cancers - colon, rectal and gastric cancers – together comprise the commonest malignancies worldwide ahead of female breast, lung and prostate cancers, accounting for over 3.0 million (15.33 % of all malignancies) new cases (Ferlay et al., 2021; Sung et al., 2021). Together, they constitute the 2nd commonest cause of cancer deaths with 1,703,966 (16.76 % of all malignancies) new cases (Ferlay et al., 2021; Sung et al., 2021). Understanding the molecular genetics of cancers has improved our knowledge of the tumour biology, and enabled the discovery of prognostic and predictive markers for cancer management (Sarhadi et al., 2022; Mehta et al., 2010). However, colon, rectal and gastric cancers remain important public health problems (Ferlay et al., 2021; Sung et al., 2021), and new studies that illuminate its carcinogenesis and progression are warranted.

In this study *TWIST1* methylation was investigated as a molecular basis for altered *TWIST1* expression in colon, gastric and rectal cancers by using the genomic data from the cancer genomics databases. *TWIST1* encodes the twist-related protein 1, a basic helix-loop-helix transcription factor which plays a crucial role in embryonic development and tumour progression (Qin et al., 2012; Cakouros et al., 2010; Fan et al., 2020). *TWIST1* expression is upregulated in CRC, in which it has also been implicated in the enhancement of tumour growth by promoting cell proliferation and survival (Zhao et al., 2017; Zhu et al., 2015). Mechanistically, it regulates cell cycle and apoptosis (Zhao et al., 2017). Moreover, *TWIST1* is regulated by the epithelial-mesenchymal transition (EMT) cellular programme, and through this mechanism it controls invasion and metastasis of CRC (Zhao et al., 2017). Specifically, it downregulates E-cadherin, a cell-to-cell adhesion molecule. E-cadherin loss causes detachment of cells from the body of tumour, forming the basis for invasion and metastasis (Zhao et al., 2017). Clinically, *TWIST1* has shown prognostic relevance in CRC (Zhu et al., 2015, Vu et al., 2017). *TWIST1* is a poor overall survival factor and is implicated in advanced or late-stage disease and lymph node metastases (Zhu et al., 2015, Vu et al., 2017; Yusup et al., 2017).

However, the molecular bases of *TWIST1* expression in gastric, colon and rectal cancers have not been conclusively determined. Preclinical

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and clinical studies have previously established that that *TWIST1* expression in cancers is regulated by complex mechanisms which involve *WNT* (Reinhold et al., 2006; Zanfi et al., 2020), *NOTCH* (Fukusumi et al., 2018; Hsu et al., 2012; Xie et al., 2020; Tian et al., 2015), *TGFB* pathways (Hao et al., 2019; Zhang et al., 2017; Xu et al., 2009; Yang et al., 2020) and microRNA deregulation (Nairismagi et al., 2013; Ghafouri-Fard et al., 2021; Khanbabaei et al., 2016). Adding to this complexity are the findings that both the *WNT* pathway and microRNA signalling regulate gene expression via regulation of DNA and histone methylation (Guo et al., 2018; Liu et al., 2016; Wang et al., 2017). However, the role of *TWIST1* promoter methylation in *TWIST1* regulation has not been extensively studied. The rationale for this study is the absence of clarity about the independent role of *TWIST1* promoter methylation in *TWIST1* expression in gastrointestinal cancers.

This study aims to interrogate the role of epigenetic deregulation of *TWIST1* expression in colon, gastric and rectal cancers by DNA promoter methylation. The study objective is to ascertain whether *TWIST1* methylation predicts *TWIST1* mRNA levels independent of the established regulators of *TWIST1* expression.

2. Materials and methods

2.1. Study cohorts

This study retrospectively analysed the genomic data of 448 colon, 90 rectal and 441 gastric cancers from the cancer genomic atlas (TCGA) PanCancer cases (Liu et al., 2018; Ebili et al., 2021), which are domiciled in the Genome Data Commons (GDC) and cBioPortal database (Liu et al., 2018; Ebili et al., 2021; Gao et al., 2013; Cerami et al., 2012).

2.2. Genomic data

Open-access data, comprising level 3 RNASeq, methylation and microRNA data of the aforementioned cancer cohorts, were retrieved from the GDC and cBioPortal repositories (Gao et al., 2013; Cerami et al., 2012).

2.3. Bioinformatics analyses

Bioinformatic analyses were accomplished using Linux-based codes and scripts that were written in Ubuntu 20.04 environment.

2.4. Study approach

First, expression levels of genes of the *WNT*, *TFGB*, *NOTCH, TWIST1*-related miRNA pathways and for *TWIST1* methylation were subjected to Principal Component Analysis (PCA) in the colon cancer cohort. Then Pearson's Bivariate correlative analysis was used to test the relationships between *TWIST1* expression and the principal components for each pathway. Using the components with significant relationships with *TWIST1* expression, Principal Component Regression (PCR) analysis was used to determine whether the principal components of *TWIST1* methylation loci independently predict *TWIST1* expression. A direct linear regression analysis, which utilized all the variables of the *WNT*, *TFGB*, *NOTCH, TWIST1*-related miRNA pathways and *TWIST1* methylation, was performed to confirm the results obtained with PCR. Finally, validation of the *TWIST1* methylation and expression relationship was sought in the TCGA gastric and rectal cancer cohorts. The list of genes which comprise the *WNT, TFGB, NOTCH* pathways were retrieved from the Gene Set Enrichment Analysis website (Mootha et al, 2003; Subramanian et al, 2005), while the list of miRNAs shown to regulate *TWIST1* expression in cancer was curated from published literature (Nairismagi et al., 2013; Ghafouri-Fard et al., 2021; Khanbabaei et al., 2016) (see Supplementary Materials_TWIST1 Methylation).

2.5. Statistical analyses

The RNASeq, methylation and miRNA data of interest were extracted in Excel spreadsheet from the Ubuntu environment. All the data were then input into SPSS version 24 for statistical analyses. PCA was used to accomplish data reduction in the colon cancer cohort. Bivariate Pearson's correlative analysis was used to seek correlation between continuous variables. Pearson's Chi square (or Fisher) and Linear-by-linear association tests were used to define associations between categorical variables. Linear regression analyses, in standard and PCR forms, were used to test whether *TWIST1* methylation independently predicted *TWIST1* expression. A P value of < 0.05 was utilised as the cut-off level for a two-tailed significant test.

3. Results

3.1. Regulatory correlates of TWIST1 expression

3.1.1. TWIST1 methylation

TWIST1 beta values, which represents the level of *TWIST1* CpG islands methylation, were retrieved from the TCGA methylation data. The beta values of all 17 cg probes were incorporated into a PCA which produced 3 significant components (PCA_TWIST1_Meth1, PCA_TWIST1_Meth2 and PCA_TWIST1_Meth3) using an Eigenvalue of 1.0 and above (see Supplementary Materials_TWIST1 Methylation). Whilst PCA_TWIST1_Meth1 and PCA_TWIST1_Meth2 correlated with *TWIST1* expression at Pearson's coefficients of −0.316 and 0.317, respectively, and P values less than 0.001, PCA_TWIST1_Meth3 did not show any correlation with *TWIST1* expression. These findings showed that *TWIST1* mRNA expression may be regulated by methylation status of *TWIST1* in colon cancer (Supplementary_PCA Correlative Analyses Table 1).

3.1.2. TWIST1 expression and TGFB pathway

Fourteen principal components were obtained by incorporating the mRNA expression of 58 genes of the *TGFB* pathway into a PCA (see Supplementary Materials_TWIST1 Methylation). Seven of the 14 principal components were significantly correlated with *TWIST1* expression at P values of 0.031 or less and at Pearson's coefficient between 0.402 and 0.102 (Supplementary_PCA Correlative Analyses Table 2). The results support the established notion that the TGFB pathway regulates the *TWIST1* activities.

3.1.3. TWIST1 copy number alterations

TWIST1 CNA pattern was retrieved from the TCGA Masked copy

Table 1
Principal Component Regression Analysis Model of Principal Components of *TWIST1* Deregulator pathways in Colon Cancer.

R	**R^2**		**Adjusted R^2**	**S.E. of Estimate**
0.712	0.506		0.494	1.620
Coefficients				
	Unstandardized Coefficients		**t**	***P***
	B	**S.E.**		
(Constant)	1.749	0.108	16.154	< 0.001
PCA_MIR3	0.288	0.119	2.422	0.016
PCA_WNT4	0.793	0.089	8.913	< 0.001
PCA_TGFB3	−0.549	0.112	−4.905	< 0.001
PCA_NOTCH5	−0.310	0.096	−3.234	0.001
PCA_WNT2	0.823	0.102	8.056	< 0.001
PCA_MIR4	0.359	0.113	3.187	0.002
PCA_TWIST1_Meth1	**−0.222**	0.105	−2.111	**0.036**
ANOVA				
	df	**F**	***P***	
Regression	7	41.045	< **0.001**	
Residual	280			
Total	287			

Table 2
Linear Regression Analysis Model of *TWIST1* Deregulators in Colon Cancer.

R	R^2		Adjusted R^2	S.E. of Estimate
0.884	0.782		0.763	1.110
Coefficients				
	Unstandardized Coefficients		**t**	***P***
	B	**S.E.**		
(Constant)	−1.987	0.802	−2.477	0.014
RAB31 Expression	0.139	0.010	14.349	< 0.001
WNT7A Expression	2.070	0.207	9.989	< 0.001
WNT3A Expression	2.141	0.287	7.460	< 0.001
NOTCH2 Expression	−0.172	0.037	−4.586	< 0.001
TWIST1_cg05380019	−3.295	0.561	−5.869	< **0.001**
hsa-mir-337-3p Expression	0.023	0.005	5.125	< 0.001
TGFBR2 Expression	−0.015	0.005	−2.762	0.006
HIPK2 Expression	0.143	0.033	4.368	< 0.001
hsa-mir-106b Expression	−0.001	< 0.001	−3.460	0.001
FNTA Expression	0.093	0.024	3.812	< 0.001
FOSL1 Expression	0.012	0.006	2.183	0.030
TWIST1_cg23244488	3.027	0.717	4.222	< **0.001**
NUMB Expression	−0.183	0.043	−4.292	< 0.001
BMP2 Expression	0.033	0.020	1.691	0.092
KAT2A Expression	0.024	0.007	3.192	0.002
TWIST1_cg24965293	1.558	0.439	3.551	< **0.001**
FZD5 Expression	0.061	0.016	3.945	< 0.001
APH1A Expression	0.013	0.005	2.569	0.011
FZD7 Expression	−0.051	0.018	−2.871	0.004
AXIN2 Expression	0.012	0.003	4.045	< 0.001
TGFB1 Expression	0.023	0.009	2.648	0.009
PPP1R15A Expression	0.009	0.004	2.256	0.025
hsa-mir-181a Expression	< 0.001	< 0.001	−2.153	0.032
ANOVA				
	df	F	*P* value	
Regression	23	41.066	< 0.001	
Residual	264			
Total	287			

number segment data using 0.3 (gain and amplification) and −0.3 (loss and deletion) mean segment as the threshold for copy number change. Based on this threshold a total of 159/443 cases were classed as *TWIST1* gain/amplification, whilst 284/443 cases were wild type. Independent T test showed no difference in *TWIST1* expression between *TWIST1* copy number alterations (Fig. 1).

3.1.4. *TWIST1 expression and WNT signalling pathway*

The mRNA expression levels of 53 members of the *WNT* pathway, comprising upstream-, midstream- and downstream- acting genes were retrieved from the TCGA mRNA expression data and incorporated into a PCA, from which 15 components were obtained (see Supplementary Materials_TWIST1 Methylation). Correlation analysis showed that 6/15 components were correlated with *TWIST1* expression at Pearson's coefficients between 0.482 and 0.123, and at *P* values less than 0.009. Whilst 3/6 of the components had direct correlations with *TWIST1* expression, the remaining showed indirect correlations (Supplementary_PCA Correlative Analyses Table 3).

3.1.5. *TWIST1 expression and NOTCH signalling pathway*

The expression of 50 *NOTCH* signalling genes were included in a PCA which identified 11 components that explained 69.23 % of the variation

Table 3
Principal Component Regression Analysis Model of Principal Components of *TWIST1* methylation loci in Gastric Cancer.

R	R^2		Adjusted R^2	S.E. of Estimate
0.21	0.044		0.034	2.319
	Unstandardized Coefficients		**t**	***P***
	B	**S.E.**		
(Constant)	2.731	0.234	11.685	0.000
PCA_TWIST1_Meth1	−0.507	0.240	−2.116	0.037
ANOVA				
	df	**F**	***P***	
Regression	1	4.478	0.037	
Residual	97			
Total	98			

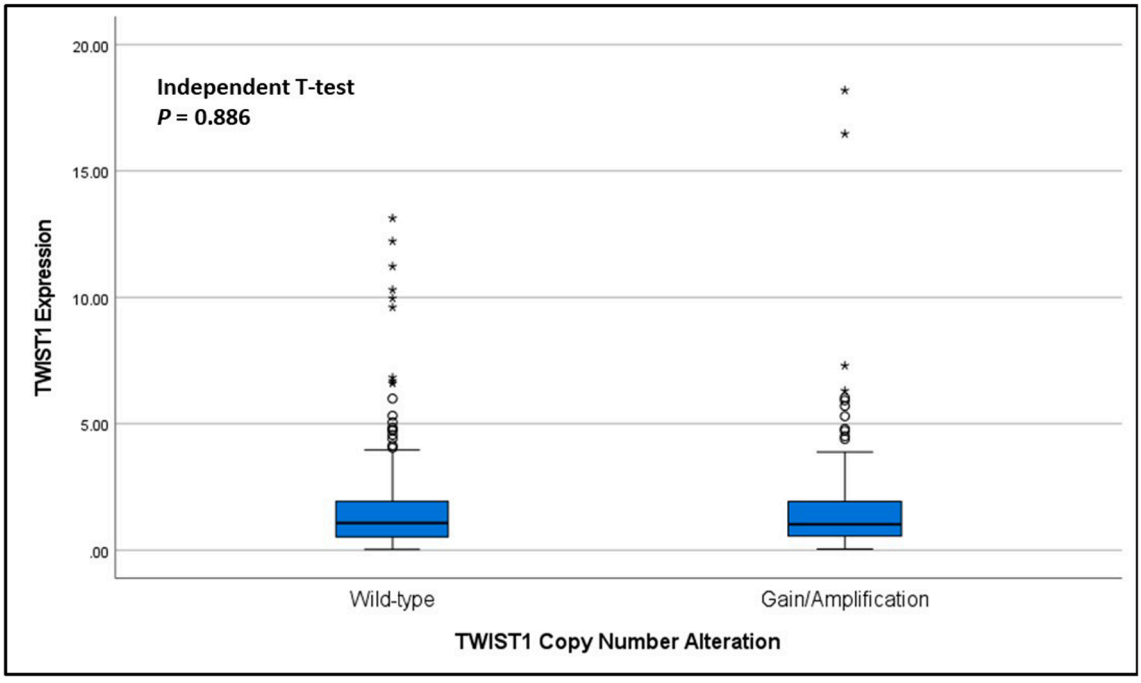

Fig. 1. A Box plot showing the relationship between *TWIST1* copy number alteration and *TWIST1* expression. Independent T test found no difference in the mean *TWIST1*expression levels between wild type and amplified *TWIST1* groups.

inherent in the colon cancer cohort (Supplementary Materials_TWIST1 Methylation). Four of these components showed significant correlations with *TWIST1* expression at correlation coefficient levels between 0.386 and 0.170 and at *P* values less than 0.001. Two of the 13 components were directly correlated with *TWIST1* expression, while the remaining showed inverse correlation (Supplementary_PCA Correlative Analyses Table 4).

3.1.6. *TWIST1 expression and miRNA dysregulation*

The expression levels of 40 *TWST1*-relevant miRNA were incorporated into a PCA from which 11 significant components were obtained (Supplementary Materials_TWIST1 Methylation). Seven of the 11 miRNA PCA components showed significant correlation with *TWIST1* expression at Pearson's correlation levels of 0.339 to 0.102. Two of the components showed indirect correlation with *TWIST1* while the remaining correlated directly, all at *P* values of 0.045 or less (Supplementary_PCA Correlative Analyses Table 5).

3.2. *TWIST1 methylation is an independent predictor of TWIST1 expression*

To determine whether *TWIST1* methylation independently predicts *TWIST1* expression the significantly correlated principal components of the *WNT*, *TFGB*, *NOTCH, TWIST1*-related miRNA gene expression, and *TWIST1* methylation were incorporated into a principal component regression analysis. The best overall regression was statistically significant ($R^2 = 0.506$, F (7, 280) = 41.045, $P < 0.001$). The results indicate that PCA_TWIST1_Meth1 was a significant regressor in a linear regression which included PCA_MIR3, PCA_WNT4, PCA_TGFB3, PCA_NOTCH5, PCA_WNT2, and PCA_MIR4 [PCA_TWIST1_Meth1 (B = -0.222, $P = 0.036$)] (Table 1). *TWIST1* methylation independently predicted *TWIST1* expression. To confirm the results of the principal component regression analysis, all 217 variables of the *WNT, NOTCH, TGFB*, miRNA pathways and *TWIST1* methylation were incorporated into a linear regression analysis with a stepwise introduction of variables. The results showed a best fitted model that was statistically significant ($R^2 = 0.782$, F (1, 264) = 41.066, $P < 0.001$), and identified 22 predictors of *TWIST1* expression, including 3 *TWIST1* methylation loci: TWIST1_cg05380019 (B = -3.295, P < 0.001), TWIST1_cg23244488 (B = 3.027, P < 0.001) and TWIST1_cg24965293 (B = 1.558, P < 0.001) (Table 2). Whilst the TWIST1_cg05380019 and TWIST1_cg23244488 loci showed a canonical inverse correlation with *TWIST1* expression, TWIST1_cg24965293 showed a paradoxical methylation pattern of direct correlation.

3.3. *TWIST1 methylation predicts TWIST1 expression in gastric and rectal cancers*

Having demonstrated that *TWIST1* methylation is an independent predictor of *TWIST1* expression in the colon cancer cohort, we sought to confirm this relationship in the TCGA rectal and gastric cancer cohorts. The principal components derived from reduction of the gastric cancer *TWIST1* methylation data (Supplementary Materials_TWIST1 Methylation) also showed correlations with *TWIST1* expression (Table 3); the rectal cancer TWIST1 methylation data did not pass the test of sampling adequacy (KMO test = 0.487), hence was excluded from PCR. Furthermore, 4 *TWIST1* methylation loci in the gastric cancer cohort – none of which is shared with the colon cancer predictor loci - independently predicted *TWIST1* expression in linear regression analyses that included only the *TWIST1* methylation loci; while 3 distinct *TWIST1* methylation loci independently predicted *TWIST1* expression in the rectal cancer cohort (Table 4 and Table 5).

Table 4
Linear Regression Analysis Model of *TWIST1* methylation loci in Gastric Cancer.

R	R^2		Adjusted R^2	S.E. of Estimate
0.566	0.320		0.291	1.987
Coefficients				
	Unstandardized Coefficients		**t**	***P***
	B	**S.E.**		
(Constant)	3.148	1.587	1.983	0.050
TWIST1_cg23603376	−7.277	1.451	−5.015	< 0.001
TWIST1_cg09864050	8.712	1.908	4.566	< 0.001
TWIST1_cg17447514	−6.184	1.425	−4.341	< 0.001
TWIST1_cg08840152	4.885	2.242	2.178	0.032
ANOVA				
	df	**F**	***P***	
Regression	4	11.056	< 0.001	
Residual	94			
Total	98			

Table 5
Linear Regression Analysis Model of *TWIST1* methylation loci in Rectal Cancer.

R	R^2		Adjusted R^2	S.E. of Estimate
0.766	0.586		0.513	1.141
Coefficients				
	Unstandardized Coefficients		**t**	***P***
	B	**S. E.**		
(Constant)	−3.545	1.246	−2.846	0.011
TWIST1_cg24965293	5.479	1.356	4.040	< 0.001
TWIST1_cg18791205	9.752	2.469	3.950	0.001
TWIST1_cg08560111	−4.228	1.695	−2.494	0.023
ANOVA				
	df	**F**	***P***	
Regression	3	8.026	0.002	
Residual	17			
Total	20			

4. Discussion

This study has demonstrated that *TWIST1* methylation is a predictor of *TWIST1* expression in the TCGA colon, gastric and rectal cancer cohorts. It used a rigorous approach that first tested the relationship between *TWIST1* expression and *TWIST1* methylation within the context of multiple *TWIST1* regulatory pathways using principal component regression, and then confirmed the relationship using standard linear regression. It incorporated 217 variables comprising expression levels of genes of the *WNT, NOTCH*, *TGFB*, T*WIST1*-relevant miRNA pathways and *TWIST1* methylation loci beta values, into regression analyses and determined that *TWIST1* methylation relationship with *TWIST1* expression was independent of other established predictors of *TWIST1* expression or activity. Three *TWIST1* methylation loci were found to predict *TWIST1* expression independent of the other regulatory factors of *TWIST1*.

The co-predictors of *TWIST1* expression that were incorporated into the analyses in this study have been previously shown in many clinical and mechanistic studies to regulate *TWIST1* expression. Hence the necessity to incorporate them into the prediction models to prove *TWIST1* methylation independence. For example, *TWIST1* transcripts were demonstrably regulated by components of the *WNT* signalling pathway in a mechanistic study performed by Reinhold, et al. (2006). Moreover, Zanfi et al. (2020) demonstrated in their study that inhibition of Wnt/CTNNB1 caused changes in expression patterns of *TWIST1* and other EMT regulators in squamous cell carcinoma cell lines. The *TFGB* pathway has long been established as a regulator of the EMT, a program that includes changes in *TWIST1* expression (Hao et al., 2019; Zhang et al., 2017; Xu et al., 2009). In a recent study, Yang et al. (2020) demonstrated that *TWIST1* functions downstream of *TFGB* in *in vitro* breast cancer models, a relationship that sets *TFGB* in control over *TWIST1*. Furthermore, *NOTCH* signalling is an established regulator of *TWIST1* expression (Fukusumi et al., 2018). Tian et al. (2015) showed that *TWIST1* acts downstream of the *NOTCH* signalling to regulate chondrogenesis in mesenchymal progenitor cells. Hsu et al. (2012)

demonstrated a relationship between *NOTCH1* and *TWIST1* levels in SC-M1, HEK293 and K562 cells. Specifically, of the Notch1 receptor over-expression promoted the SC-M1 colony formation, invasion and migration through Twist1 expression, among other factors. Xie et al. (2020) also demonstrated that the *NOTCH* signalling regulated self-renewal and the EMT in adenoid cystic carcinoma cells through upregulation of *TWIST1* and the other EMT-enhancers. Moreover, several studies have demonstrated *TWIST1* expression to be the target of scores of microRNAs. For reviews on the miRNAs that target *TWIST1* see the references Nairismagi et al., 2013; Ghafouri-Fard et al., 2021; and Khanbabaei et al., 2016.

The results of studies investigating the relationship between *TWIST1* methylation and expression have been contradictory. For example, in congruence with the results obtained in this study Galvan et al. (2015) demonstrated a reverse relationship between gene methylation and protein expression in a subset of colorectal cancer cohort with high-grade budding. Furthermore, *TWIST1* methylation was associated with perturbation of *TWIST1* expression in a gastric cancer cell line study (Sakamoto et al., 2015). The aforementioned studies, however, did not clarify whether the *TWIST1* methylation-expression corelations found were independent of the canonical deregulators of *TWIST1* expression. Furthermore, Gort et al. (2008), who evaluated the relationship among *TWIST1* methylation, mRNA and protein expression in breast cancer, found no such *TWIST1* methylation-expression relationship. Moreover, Kwon et al. (2013) analysed *TWIST1* methylation and Twist1 protein expression in a cohort of tonsillar squamous cell carcinoma, but did not show any correlation between gene methylation and protein expression. The reason for this observed discrepancy among the referenced studies may be because the *TWIST1* methylation-expression relationship is tumour-specific, existing only in gastrointestinal cancers. However, it could be due to differences in the methylation loci interrogated in the different studies. In the present study only 3 of the 17 *TWIST1* methylation loci showed independent correlation with *TWIST1* expression in the TCGA colon cancer cohort; and these 3 loci were different than the *TWIST1* expression-relevant methylation loci found in gastric and rectal cancers cohort.

In conclusion, this study has shown that *TWIST1* methylation predicts *TWIST1* expression, and it can thus be inferred that *TWIST1* methylation independently regulates *TWIST1* expression. The study also showed that different *TWIST1* methylation loci may alter *TWIST1* expression in different cancer types.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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