

Monitoring of lung malignant epithelial cells by gene methylation analysis in the conditionally reprogrammed cell cultures

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Conditionally reprogrammed cell (CRC) technology is an effective method for culturing primary malignant cells and non-malignant epithelial cells *in vitro*. This can be useful for precision medicine applications, such as drug sensitivity assays. However, this approach is commonly hindered by the non-specific growth of non-malignant epithelial cells in CRC cultures and the lack of effective biomarkers/assays to distinguish them from primary tumor cells. In this study, we developed a DNA methylation-based, real-time PCR assay to investigate *SHOX2* and *PTGER4* gene promoters as sensitive markers for human lung cancer. We first found that in formalin-fixed, paraffin-embedded (FFPE) malignant lung samples, 90% (28/31) had increased *SHOX2* and/or *PTGER4* promoter methylation as compared with their adjacent non-malignant samples. We then applied this assay to fresh surgical tumors and found increased *SHOX2* and/or *PTGER4* promoter methylation in 80% (20/25) of tumor samples as compared with their corresponding adjacent non-malignant tissues. Increased methylation of *SHOX2* or *PTGER4* promoter regions was also detected in 52% (13/25) of CRC cultures. The presence of malignant cells was confirmed by growth in soft agar cultures, a hallmark of malignant transformation, as well by *EGFR* mutation analysis. These results demonstrate that *SHOX2* and *PTGER4* promoter methylation levels can be used to detect malignant lung epithelial cells in CRC cultures.

Key words: SHOX2, PTGER4, gene methylation, CRCs, soft agar, EGFR

Lung cancer is the leading cause of cancer mortality worldwide [1]. Although the overall prognosis for lung cancer has improved since the application of targeted therapies and checkpoint immune therapies, there is still a strong clinical need for systemic chemotherapy due to evolving tumor heterogeneity [2]. Because ineffective therapies can lead to patient harm, loss of treatment opportunities and a waste of medical resources, the approaches to predict and improve clinical efficacy are urgently needed.

One of these approaches is *in vitro* cell culturing of primary tumors that can be applied as drug sensitivity assays to guide chemotherapy selection. Despite many efforts to optimize this approach, very little success has been achieved in the clinical setting for a number of reasons [3]. First, the success rate for the conversion of a primary tumor to the cell culture is very low, ranging from 10% to 40% [3]. In addition, successful *in vitro* inoculations can take 6–8 weeks, or even longer, to obtain sufficient cell numbers for drug sensitivity testing [4]. This is much longer than the therapeutic window (generally 2–3 weeks) between surgery and initiation of adjuvant treatment. The concordance between traditional

2D *in vitro* drug sensitivity assays and actual clinical efficacy is also low, ranging from 46–80%, possibly due to different cellular structures and microenvironments between *in vivo* tumors and *in vitro* cultures [4].

The recent development of conditionally reprogrammed cells (CRCs), has significantly improved success rates of cell cultures of primary tumors [5, 6]. Tumor cells in CRC cultures are “immortalized” and maintain the characteristics of the original tumors, which better facilitate *in vitro* drug sensitivity assays [5]. Indeed, several groups have reported using CRC cultures to guide personalized therapy, including lung cancer [5, 7–11]. However, the preferential proliferation of non-malignant epithelial cells over the malignant cells from primary tumors in CRC cultures hinders the establishment of reliable malignant cell models [12]. The lack of effective biomarkers and assays to distinguish malignant from non-malignant epithelial cells in CRC cultures also impedes the selective cultivation of malignant cells.

One promising solution relies on DNA methylation patterns, which play an important role in regulating cellular differentiation and development [13]. Aberrant methyla-

tion comprises a major epigenetic feature of human cancers, including lung cancer [14, 15]. Previous studies showed that increased *SHOX2* and *PTGER4* promoter methylation was detected in lung cancer tissues when compared with matched, non-malignant tissues [16, 17]. In this study, we established a sensitive and specific, PCR-based, DNA methylation assay examining *SHOX2* and *PTGER4* gene promoters to detect malignant epithelial cells in CRC cultures.

Patients and methods

Tissue collection and processing. Malignant and non-malignant FFPE lung samples were collected from pathology laboratories of Xiaolan People's Hospital of Zhongshan under the regulations of its ethics board and routing diagnostic. 2–3 pieces of paraffin slice prepared from FFPE blocks were used for DNA extraction.

All fresh cancer tissue specimens were collected from curative intent surgeries at Shenzhen Second People's Hospital under the regulations of its ethics board. Both malignant and adjacent non-malignant lung tissue samples were collected from patients to allow for matching. Resident pathologists inspected and classified all resected lung carcinomas by histopathology – tumor tissues were defined as less than 15% normal cells. The tumor and non-cancerous tissues were transported to the laboratory in ice-cold sterile transport medium. Tissues were washed extensively in Hank's balanced salt solution containing penicillin, streptomycin, and amphotericin B (Gibco, Thermo Fisher Scientific), then minced into 1–2 mm small fragments. Tissues were digested with dispase II and collagenase I (Sigma, St. Louis, MO, USA) at 37°C for 2 h to obtain cell suspensions.

Cell culture. Swiss-3T3-J2 mouse fibroblast and A549 cells were obtained from the American Type Culture Collection (ATCC, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, Australia) and 1% (v/v) penicillin/streptomycin. All cells used in the culture were tested for and found to be mycoplasma-free. Swiss-3T3-J2 mouse fibroblasts (J2 feeder cells) were irradiated at 30 Gy as described previously [7]. For CRC cultures, epithelial cells from tumor samples and their adjacent tissues were co-cultivated with irradiated (30 Gy) J2 feeder cells culture in F medium consisting of 3:1 (v/v) F-12 Nutrient Mixture (Ham) – DMEM, 5% (v/v) FBS, 1% (v/v) penicillin/streptomycin, 0.4 µg/ml hydrocortisone, 5 µg/ml insulin, 8.4 ng/ml cholera toxin, 10 ng/ml epidermal growth factor, and 24 µg/ml adenine (all Sigma-Aldrich) with addition of 5 µM Y-27632 (ROCK inhibitor, Enzo Life Sciences) [9].

Methylation specific PCR assay. DNA samples were extracted from FFPE slides, fresh tumor tissues, matched non-malignant tissues and cell cultures by utilizing the QIAamp DNA Mini Kit (50) (Qiagen, Hilden, Germany) according to the recommended protocol [18]. The DNA

samples were then subjected to bisulfite modification using the EpiTect Fast DNA Bisulfite Kit (200) (Qiagen, Hilden, Germany) according to the protocol. The methylation PCR assay for detecting methylated *SHOX2* and *PTGER4* genes has been described previously [19, 20]. Real-time PCR (Taqman probe assay) was performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA) using the following temperature profile: 2 min/95°C and 50 cycles with 15 s/95°C and 30 s/59°C. The probes and primers for *SHOX2*, *PTGER4*, and *ACTB* (Table 1) were optimized according to the previous publication [21]. 1 ng bisulfite transformed DNA template and 0.2 µM probe were added to 50 µl reaction. Bisulfite transformed DNA purified from A549 and human lung fibroblast cell lines were used as positive and negative controls, respectively. For each sample, a relative methylation value was determined using the $\Delta\Delta CT$ method adapted for DNA methylation analyses as previously described [21]. The percentage of methylated sites was calculated using the following formula: Methylation Sample = 100% * $2^{\Delta\Delta CT}$ Sample [22].

EGFR mutation analysis. *EGFR* mutations were analyzed with the amplification refractory mutation system (ARMS)-based *EGFR* mutation detection technology as described previously [23–27]. Primers and probes for mutation analysis were synthesized by Invitrogen (Thermo Fisher Scientific, China). Genomic DNA was isolated from non-cancerous tissues, tumor tissues and CRCs using the Qiagen Genomic DNA Extraction Kit (Qiagen, Hilden, Germany) [10]. After DNA extraction, samples were subjected to the ARMS-based PCR assay, which detects 31 specific *EGFR* gene mutations by real-time polymerase chain reaction (RT-PCR). This includes 19 distinguished deletions in exon 19, L858R, L861Q, G719X (X = C, S, A), S768I, T790M, C797S, T790M-C797S, and three insertions in exon 20. RT-PCR assays were carried out on an ABI 7500 PCR machine according to the manufactur-

Table 1. Oligonucleotide specifications.

Oligonucleotide	Sequence 5' → 3'
SHOX2	
Forward Primer	GTTTTTTGGATAGTTAGGTAAT
Reverse Primer	TAACCGACTTAAACGACGA
Forward Blocker	TAATTTTGTGTTGTTGTTGATTGGGGTTG-TATGA-SpacerC3
Hydrolysis Probe	FAM-CTCGTACGACCCCGATCG-BHQ1
PTGER4	
Forward Primer	GGTAAGGTTGGGGAGGTAG
Reverse Primer	AAACACCGAACAACACCAC
Reverse Blocker	ACCTCAACAACCTTCAACACCACCA-Spacer C3
Hydrolysis Probe	CY5-ACCGCGACCGCCTCGATTA-BHQ1
ACTB	
Forward Primer	GTGATGGAGGAGGTTTAGTAAGTT
Reverse Primer	CCAATAAAACCTACTCCTCCCTTAA
Hydrolysis Probe	JOE-ACCACCACCCAACACACAATAACAAACA-CA-BHQ1

er's protocol. Data analyses were performed using the 7500 software v2.3.

Soft agarose assay. Soft agar cultures of cells for anchorage-independent growth in 96-well plates were performed as previously described [28]. A mixture of 25 μ l pre-warmed (37°C) F-medium containing 10% FBS, 4 mM Glu, 100 U/ml penicillin/streptomycin, and 25 μ l pre-warmed (56°C) 1.2% soft agar was plated onto each well of a 96-well microplate to serve as a pre-layer for the assay. 50 μ l of 0.4% agarose containing 1×10^3 cells was layered over the solidified pre-layer. Finally, 50 μ l of F-medium was added on top of the solidified cell layers to prevent desiccation. The cells were cultured in a humidified 37°C incubator with 5% CO₂ for 1–2 weeks, and then cell proliferation and viability were scored using the Alamar Blue assay. Cell growth was measured using a Fluoroskan FL Reader, with excitation at 530 nm and emission at 590 nm.

Statistics. Statistical analysis and data normalization were conducted in repeated experiments. The receiver operating characteristic (ROC) and the area under the ROC curve (AUC) were constructed using IBM SPSS statistics 19. The test variable was the percentage of methylation and state variable were 0 and 1, in which 1 represents tumor tissues and 0 represents non-malignant tissues in ROC curve analysis. The cut-off was chosen to reduce the false positive rate to less

than 5% for non-malignant samples. Data were analyzed and plotted in GraphPad Prism 5.

Results

Increased *SHOX2* and *PTGER4* promoter methylation in malignant lung tumors. Previous studies showed that increased levels of *SHOX2* and *PTGER4* promoter methylation were observed in non-small cell lung cancer (NSCLC). Using our DNA methylation RT-PCR assay to analyze 31 matched FFPE samples, we found increased *SHOX2* and *PTGER4* promoter methylation in tumor tissues as compared to their non-cancerous adjacent tissues (Figure 1). Using a cut-off of 8.9% *SHOX2* methylation, we obtained a sensitivity of 78% in malignant samples with an area under the curve (AUC) of 0.87. Using the same cut-off of 8.9% for *PTGER4* methylation, we obtained a sensitivity of 84% and a specificity of 97%. *PTGER4* methylation thus demonstrated significant discriminatory power (AUC=0.91). Combined the *SHOX2* and *PTGER4* methylation can increase the sensitivity to 90% with a specificity of 90% (AUC=0.96, Supplementary Figure S1). Our results confirmed that the DNA methylation levels of *SHOX2* and *PTGER4* were selectively upregulated in lung cancer tissues, suggesting its potential for distinguishing tumor cells and non-cancerous cells.

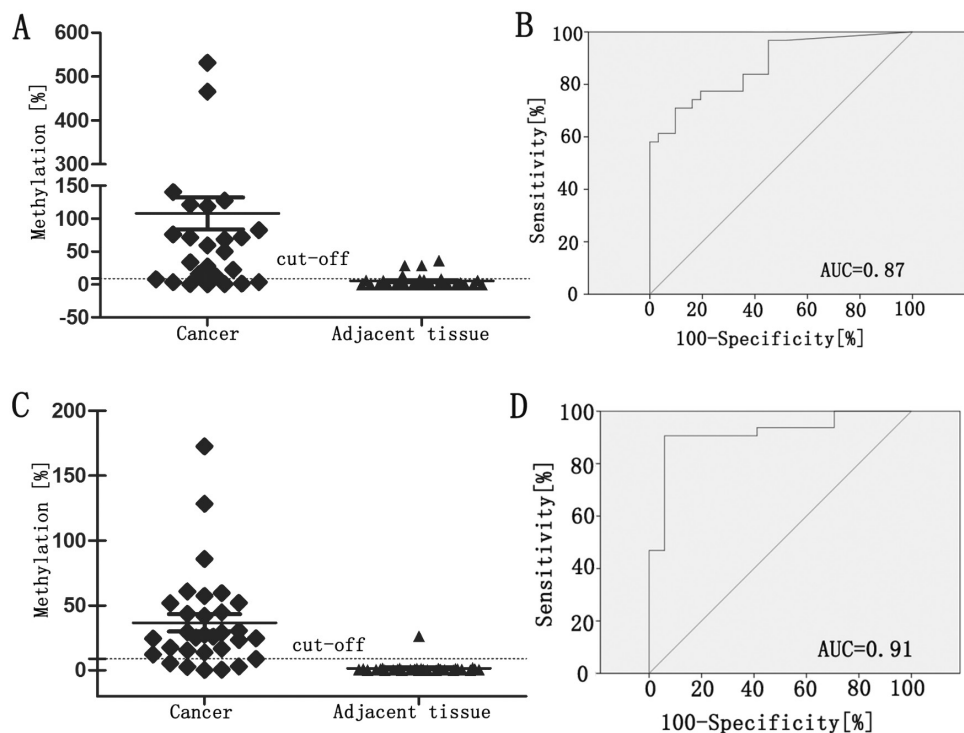


Figure 1. Validation of the *SHOX2* and *PTGER4* DNA methylation expression in lung tumor tissues. DNA was isolated from 31 matched FFPE patients' samples (cancer and non-cancerous) and subjected to methylation specific PCR analysis. A) *SHOX2* methylation level measured in cancer and adjacent tissues when using optimal cut-off 8.9% for samples analysis. B) ROC and the resulting AUC analysis of *SHOX2* study (AUC=0.87). C) *PTGER4* methylation level measured in tumors and adjacent samples. D) ROC and AUC analysis of *PTGER4* study (AUC=0.91).

Next, we measured *SHOX2* and *PTGER4* promoter methylation levels in 25 fresh surgical lung tumors and their adjacent non-malignant samples, as well as in their corresponding *in vitro* CRC cultures. The characteristics of these patients who contributed fresh tissues are described in Table 2, which includes information on histological type and disease stage. The mean age of our patients was approximately 60 years. The majority of these specimens were adenocarcinomas and early stage tumors.

When the same 8.9% methylation was used as a cut-off, increased methylation of *SHOX2* and *PTGER4* promoters was detected in 80% of tumor samples (20/25) (Figure 2A). Using the same cut-off value, increased *SHOX2* and *PTGER4* promoter methylation were detected in 52% (13/25) of CRC cultures, while it was undetectable in all CRC cultures from the matched non-malignant tissues (0/25) (Figures 2C, 2D). These results demonstrate a discriminatory utility in assaying *SHOX2* and *PTGER4* promoter methylation to detect malignant cells in CRC cultures.

Correlation between *SHOX2* and *PTGER4* promoter methylation and anchorage-independent CRC growth.

Our previous results demonstrate that the detection of increased *SHOX2* and *PTGER4* promoter methylation correlates with tumor presence in CRC cultures. Because only transformed cells are able to grow in soft agar cultures, we further tested the growth property of these CRC cultures in soft agar cultures. Among 13 CRC cultures with high levels of *SHOX2* and *PTGER4* promoter methylation, 8 of them could proliferate in soft agar and form colonies (Figure 3, Table 3). Most of the CRC cells with low levels of *SHOX2* and *PTGER4* promoter methylation, including those from non-malignant tissues, were not able to proliferate or form colonies in agarose. Together, our results demonstrate that *SHOX2* and *PTGER4* promoter methylation can be used to discriminate tumor cells in CRC cultures and guide *in vitro* functional assays.

Maintenance of *EGFR* mutations in the CRC cultures.
To verify that our CRC cultures continued to grow tumor

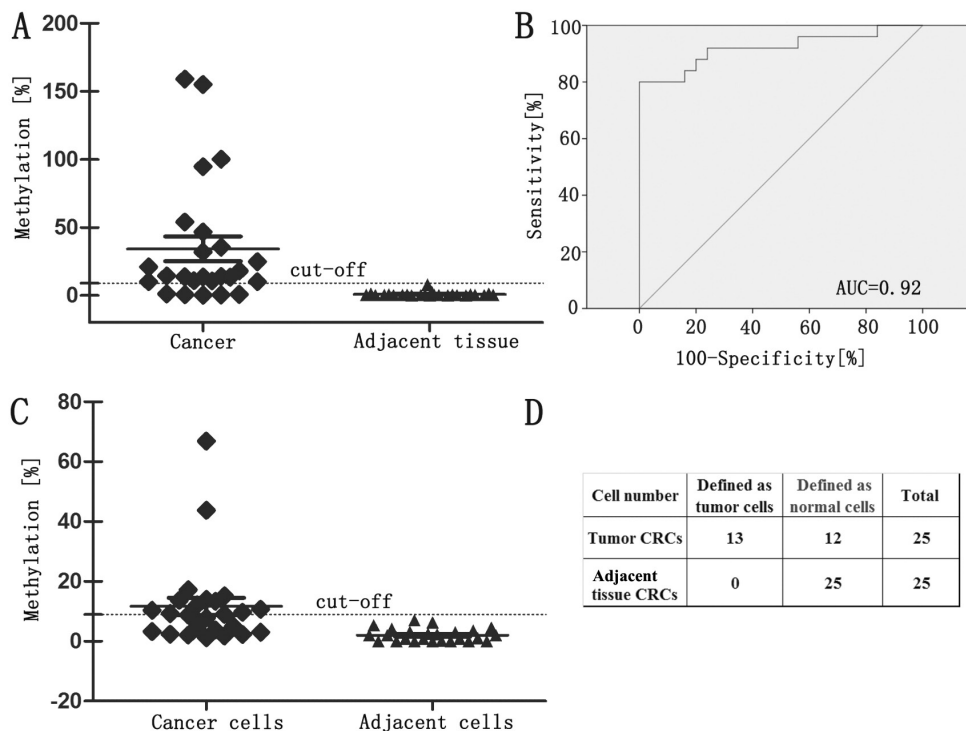


Figure 2. Maintenance of DNA methylation of *SHOX2* and *PTGER4* genes in lung cancer CRC cultures. DNA was isolated from 25 paired fresh tissue samples (cancer and non-cancerous) and their CRC cultures, and subjected to methylation specific PCR analysis. A) The methylation levels of *SHOX2* and *PTGER4* measured in fresh cancer and adjacent tissues. B) ROC and the resulting AUC analysis of *SHOX2* and *PTGER4*. C) *SHOX2* and *PTGER4* methylation levels measured in CRCs from tumors and adjacent samples. D) Summary of the methylation status of *SHOX2* and *PTGER4* gene in CRC cultures.

Table 2. Clinical information of the patients with lung cancer.

Number of patients	Average age (range)	Female/Male	Histological Subtype			Tumor Stage			
			Adenocarcinoma	Squamous cell carcinoma	Other type	I	II	III	IV
25	60.36 (36-80)	13/12	22	2	1	12	7	6	0

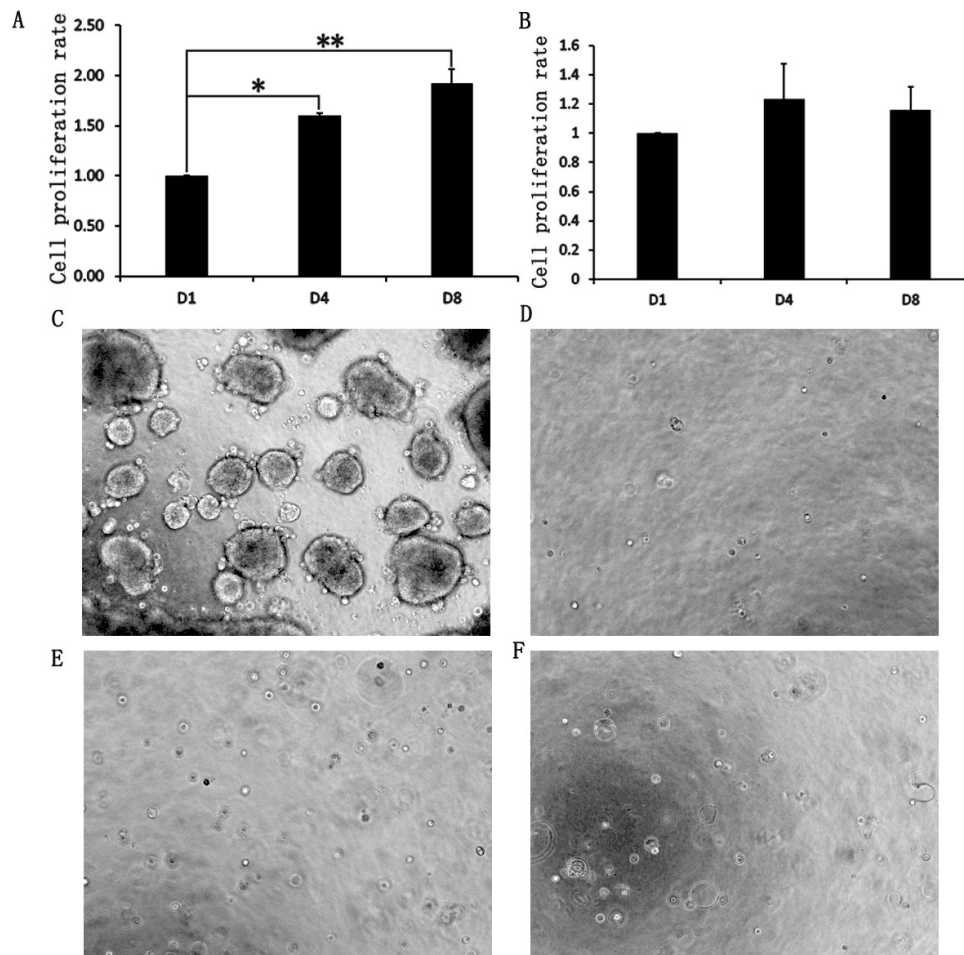


Figure 3. Growth properties of CRCs in soft agar cultures. The average proliferation rate of 13 tumor cell cultures with high methylation levels on day 1, day 4, and day 8 (A) and 12 tumor cell cultures with low methylation levels on day 1, day 4, and day 8 (B) in agarose culture was determined. Data were generated from three independent experiments * $p < 0.05$; ** $p < 0.01$, Student's t test, $n = 3$. Error bars depict mean \pm S.E. Tumor cells with high methylation of *SHOX2* and *PTGER4* (C) and the corresponding adjacent tissues (D) and tumors with low methylation of *SHOX2* and *PTGER4* (E) and the corresponding adjacent tissues (F) were plated in 0.4% agarose with conditioned F-media and overlaid on 0.6% agar.

cells from the primary lung cancers, we used an ARMS-based assay to test for mutations in *EGFR*, the most frequently mutated driver gene in lung cancer. This was performed on our 25 fresh tumor tissues and their corresponding 2nd generation of CRC cultures. *EGFR* mutations were detected in 56% (14/25) of the tissue samples, of these, *EGFR* mutations were sustained in 50% (7/14) CRC cultures, indicating the maintenance of lung cancer cells in a portion of the CRC cultures (Table 4).

Discussion

Since being initially reported in 2014, the success rate of CRC-based *in vitro* cultures for numerous types of primary malignant and non-malignant cells has increased significantly [7]. Recently, however, Gao et al. reported that non-malignant

lung epithelial cells over-proliferated in CRC cultures from resected NSCLC specimens [12]. Due to the lack of feasible biomarkers, they had to identify malignant epithelial cells in CRC cultures by more intensive approaches. These included next generation sequencing (NGS) for mutation and copy number variation (CNV) analysis, and microarray assays of the whole transcriptome mRNA expression. These approaches, however, are not practical in clinical or routine laboratory settings.

In the present study, we aimed to develop a more feasible assay for distinguishing tumor from normal epithelial cells to assist further optimization of CRC culture conditions. Our results demonstrated that *SHOX2* and *PTGER4* promoter methylations have an average sensitivity of 85% and an average specificity of 95% for detecting malignant lung epithelial cells, which is consistent with previous studies

Table 3. Correlation between hypermethylation and cell growth in soft agar.

Cell number	Soft agar grow	Hypermethylated cells
Sample 1	+	+
Sample 2	+	+
Sample 3	-	-
Sample 4	+	+
Sample 5	+	+
Sample 6	-	+
Sample 7	-	+
Sample 8	-	-
Sample 9	+	+
Sample 10	+	+
Sample 11	-	+
Sample 12	-	-
Sample 13	-	-
Sample 14	-	-
Sample 15	-	+
Sample 16	+	+
Sample 17	-	-
Sample 18	-	+
Sample 19	-	-
Sample 20	-	-
Sample 21	-	-
Sample 22	-	-
Sample 23	+	-
Sample 24	+	+
Sample 25	+	-
Total numbers	10	13

Table 4. EGFR mutations detected in tumor tissues and CRC cultures.

Sample	Mutation site	Tumor tissues	CRCs
Sample 1	-	-	-
Sample 2	-	-	-
Sample 3	L861Q	+	-
Sample 4	19del	+	-
Sample 5	L858R	+	+
Sample 6	-	-	-
Sample 7	-	-	-
Sample 8	-	-	-
Sample 9	-	-	-
Sample 10	L858R	+	+
Sample 11	-	-	-
Sample 12	-	-	-
Sample 13	-	-	-
Sample 14	19del	+	-
Sample 15	L861Q	+	-
Sample 16	L858R	+	+
Sample 17	719X	+	+
Sample 18	-	-	-
Sample 19	-	-	-
Sample 20	719X	+	-
Sample 21	L858R/L861Q/719X	+	-
Sample 22	719X	+	+
Sample 23	L858R	+	+
Sample 24	L858R	+	+
Sample 25	L861Q	+	-
Total		14	7

[20]. Using this DNA methylation assay, we also observed cells with detectable methylation of *SHOX2* and *PTGER4* promoters in CRC cultures from lung cancer tissues, which were further confirmed as malignant by anchorage-independent soft agar growth (a hallmark of transformed cells) and *EGFR* mutation analysis [29].

Although a positive correlation between cells with methylation status and proliferation in soft agar culture was observed, we found that 5 out of 13 CRC cultures, testing positive for increased *SHOX2* and *PTGER4* promoter methylation, could not proliferate in soft agar culture. These phenomena were consistent with previous observations that not all cancer cell lines can grow in soft agar [29–31]. On the other hand, CRC cultures negative for *SHOX2* and *PTGER4* methylation could grow in soft agar, indicating that *SHOX2* and *PTGER4* methylation is not an exclusive biomarker for all lung cancer cases.

Compared with Gao et al. [12], we observed more malignant cells in CRC cultures. This could be due to the differences in sample processing, such as tissue storage and digestion conditions. Overall, we also observed a reduced population of malignant lung cancer cells in CRC cultures,

measured by either loss of or reduced *SHOX2* and *PTGER4* promoter methylation, as compared with their precedent tissues. This suggests that non-malignant lung cancer cells over-grow malignant cells in CRC cultures, which is consistent with Gao's observation.

The application of *SHOX2* and *PTGER4* promoter methylation assays to monitor malignant lung cancer cells in CRC cultures can facilitate the optimization of culture conditions for primary cancer cells. This could broaden the utility of CRC cultures to finally be applied in a clinically meaningful way, such as for *in vitro* drug sensitivity assays and new drug development [4]. Moving forward, we hope that the validity of this approach can be expanded to other types of cancers and their respective biomarkers to improve the treatment and management of potentially devastating cancers.

Supplementary information is available in the online version of the paper.

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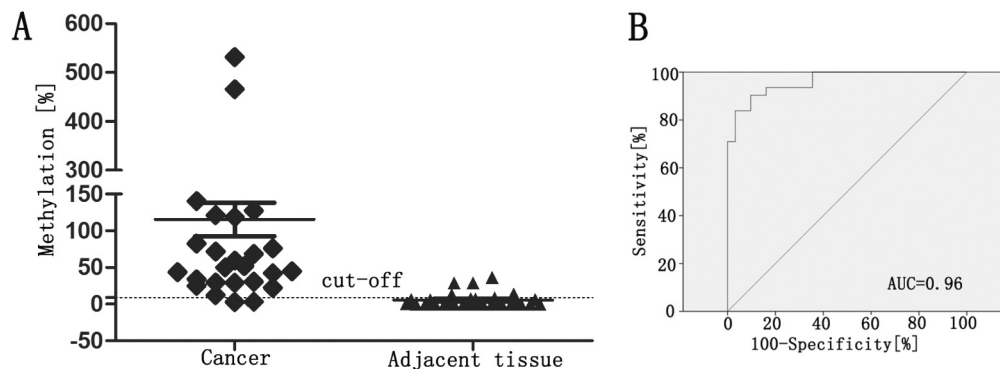
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Monitoring of lung malignant epithelial cells by gene methylation analysis in the conditionally reprogrammed cell cultures

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Supplementary Information



Suppl. Figure S1. Combined *SHOX2* and *PTGER4* DNA methylation expression in lung tissues. DNA was isolated from 31 matched patients' FFPE samples (cancer and adjacent tissue) and subjected to methylation specific PCR analysis. A) The higher methylation level of *SHOX2* and *PTGER4* measured in tumor and adjacent samples when using optimal cut-off 8.9% for sample analysis. B) ROC and the resulting AUC analysis of combined *SHOX2* and *PTGER4* study (AUC=0.96).