Diagnostic and prognostic impact of desmocollins in human lung cancer

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ABSTRACT

Objective Desmosomes are intercellular junctions that confer strong cell–cell adhesion. Two main members of desmosomal cadherins, desmogleins (DSGs) and desmocollins (DSCs), are involved in carcinogenesis. However, their role in human lung cancer remained elusive. The aims of this study were to analyse the expression of DSCs and to evaluate their clinical application in lung cancer.

Methods The expression of DSC1-3 mRNAs was analysed by RT-PCR. The methylation status of DSCs was analysed by demethylation tests and bisulphite sequencing. Protein expression of DSCs in primary lung cancer was evaluated by immunohistochemistry on tissue microarrays.

Results DSC1-3 mRNAs were downregulated in lung cancer cells, and the expression was restored in four out of seven cell lines, respectively, after 5-aza-2′-deoxycytidine treatment. A heterogeneous methylation pattern was detected by bisulphite sequencing in exon 1 of DSC2 and DSC3. In 199 patients with primary lung cancer, we found that lower protein expression of DSC1 was significantly linked to worse tumour differentiation ($p=0.017$), DSC3 proteins were more expressed in squamous cell carcinoma (SCC) compared with adenocarcinoma (ADC) ($p<0.001$), and reduced expression of DSC1 and DSC3 was significantly correlated with poor clinical outcome ($p=0.045$ and $p=0.007$, respectively).

Conclusions Our data suggest that downregulation of DSC1-3 may be explained by DNA methylation, DSC1 may be a marker for tumour differentiation, DSC3 has a potential diagnostic value in subclassification of non-small cell lung carcinoma into SCC and ADC, and furthermore, DSC1 and DSC3 may be prognostic markers for lung cancer.

INTRODUCTION

The desmosomes are intercellular junctions that act as anchors for intermediate filaments, and they are crucial to tissues that experience mechanical stress.[1] Desmosomes are made of proteins from three families: the desmosomal cadherins, the armadillo family and the plakin family of cytolinkers. The cell adhesion proteins of the desmosomes, DSGs (DSG1-4) and DSCs (DSC1-3), are members of the cadherin family.[2] DSGs and DSCs are membrane-spanning glycoproteins that mediate adhesion by interacting with other family members on adjacent cells.[3][4]

The role of desmosomal proteins in cancer development and progression is not well understood, though a body of evidence indicates that they may influence epithelial cell invasion and metastasis since an important function of desmosomes related to cancer is their ability to inhibit cell motility.[5] For example, loss of expression of desmoplakin, a component of desmosomal plaque proteins, promotes increased local tumour invasion in a mouse model of pancreatic neuroendocrine carcinogenesis;[6] loss of DSC2 contributes to the growth of colorectal cancer cells by regulating Akt/β-catenin signalling.[7]

Both tumour-promoting and tumour-suppressive functions have been assigned to desmosomal proteins.[2] For instance, DSG2 was found to be overexpressed in skin cancer,[8] DSG3 was upregulated in head and neck cancer as well as lung cancer.[9][10][11] By contrast, DSC3 ablation increased the incidence of Ras-induced skin tumour in mice,[12] and furthermore, DSC3 was found to be downregulated in breast and colorectal cancer due to promoter hypermethylation.[13][14]

In a previous study, we performed suppression-subtractive hybridisation to compare gene expression between normal human bronchial epithelial cells (HBEC) and a lung squamous carcinoma cell line H2170.[15] Two cDNA libraries were cloned. The clone, HBEC-80 with high similarity to desmocollin 3, was found in the library enriched for the genes which were downregulated in tumour cells. Further investigations revealed that DSC3 plays an important role in lung carcinogenesis (Cui et al. 2012, in press).

In this study we analysed the expression of DSC1-3 in human lung cancer and investigated their clinical relevance.

MATERIALS AND METHODS
Cell lines and cell culture

Human lung carcinoma cell lines, including non-small cell lung carcinomas (SCC: H157, H226, H2170; adenocarcinoma (ADC): H23, H2030, H2228 and H322), and small cell lung carcinomas (SCLCs: SHP77, CPC-N, H82, COLO677, COLO668 and H526) were purchased from the American Type Culture Collection (ATCC, Rockville, Maryland, USA), and from the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany). Cells were grown in RPMI 1640 medium (BIOCHROM AG, Germany) supplemented with 10% (v/v) foetal bovine serum (FBS), and maintained in a humidified atmosphere with 5% CO_2 at 37°C. In addition, three ADC cell lines including D51, D54 and D117 were established in our laboratory. They were derived from primary tumours of patients who were operated at the University Hospital Charité. These cells were grown in Leibovitz 15 media supplemented with 10% (v/v) FBS and 1% (w/v) glutamine.

RNA extraction and RT-PCR

Total RNA was extracted from the cells using the Trizol reagent (Invitrogen, Karlsruhe, Germany). One microgram of total RNA was reverse-transcribed into cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany).

PCR amplification of DSC1-3 was performed under the following conditions: 94°C 1 min, 53°C 30 s, 72°C 30 s, for 35 cycles, with initial denaturation at 95°C 15 min, and final elongation at 72°C 7 min. Glyceraldehyde-3-phosphate dehydrogenase was used as control. Primer sequences are shown in supplementary data 1.

Demethylation tests

For demethylation tests, seven lung cancer cell lines were plated and cultured in 10 cm dishes. At 50% confluence, 10, 20 and 25 μM of 5-aza-2′-deoxycytidine (DAC) (Sigma Chemical Co, St Louis, MO USA) were added to the medium on days 0 and 2. Cells were harvested on day 4 for total RNA isolation.

Bisulphite sequencing

Bisulphite-treated DNA was subjected to PCR amplification. Two pairs of primers (supplementary data 1) were designed for amplification of both unmethylated and methylated sequences. Hot-start PCR (Peqlab, Germany) was carried out to amplify the CpG sites of DSC2 and DSC3 with the following conditions: 95°C 15 min, 40 cycles of 95°C 1 min, 60°C 45 s and 72°C 45 s, with a final extension at 72°C 7 min. PCR products were applied for direct sequencing by capillary electrophoresis (LGC, Berlin, Germany).

Immunohistochemistry

Three hundred and ninety-eight tumour specimens from 199 patients with primary lung cancer were used for the construction of tissue microarrays (TMAs). All the patients were undergoing surgical operation at the department of surgery of the Central Hospital Bad Berka GmbH. No adjuvant radiotherapy or chemotherapy was administered before surgery. The clinico-pathologic features of the samples are listed in table 1 according to the WHO/TNM classification of lung cancer.[16]

Table 1 Study cohort

Clinical parameters	No. of patients Total n=199 (%)
Tumour differentiation (191)	
G1	79 (41.4)
G2	67 (35.1)
G3	45 (23.5)
T stage (194)	
T1	46 (23.7)
T2	106 (54.6)
T3	27 (13.9)
T4	15 (7.8)
N stage (173)	
N0	92 (53.2)
N1	52 (30.1)
N2	27 (15.6)
N3	2 (1.1)
Histology (199)	
SCC	128 (64.3)
LCLC	3 (1.5)
ADC	63 (31.7)
SCLC	5 (2.5)

ADC, adenocarcinoma; LCLC, large-cell lung carcinoma; SCLC, small-cell lung carcinoma; SCC, squamous cell carcinoma.

TMAs were constructed as previously described.[17] All cases were reviewed on whole sections stained by standard H&E and AB-PAS alcian blue-periodic acid schiff by two pathologists (M Mireskandari and T Knösel). Two tissue cylinders per tumour with a diameter of 0.6 mm were present in one TMA.

A section from the TMA was dewaxed with xylene and gradually hydrated. Antigen retrieval was performed by treatment in a pressure cooker for 6 min. The primary antibodies used were as follows: polyclonal antidesmocollin 1 antibody (Sigma, USA; 1:100), polyclonal antidesmocollin 2 antibody (Acris, Germany; 1:800), and monoclonal antidesmocollin 3 antibody (Acris, Germany; 1:100). Detection took place according to the manufacturer's instructions (LSAB 2-kits, DAKO, Denmark). All slides were read by two pathologists (M Mireskandari and T Knösel). Immunohistochemistry was scored semiquantitatively as negative (less than 5% cells were stained) and positive (more than 5% cells were stained).

Statistical analysis

To compare the protein expression of DSC1-3 with clinicopathologic parameters, χ^2 or Fisher's exact test was applied. Kaplan–Meier survival curves were constructed for statistical significance with the logrank tests. Cox proportional hazard analysis was used to quantify the HR of each risk factor, and forward stepwise multivariable analysis was used to identify the prognostic factors. All p values were calculated two-sided. Difference was considered statistically significant when p value was 0.05 or less. The statistical analysis was performed by using the software package SPSS 19.0 (SPSS, Chicago, Illinois, USA).

RESULTS

mRNA expression of DSCs in lung cancer cell lines

RT-PCR showed that mRNA of DSC1-3 was expressed in human bronchial epithelial cells. DSC1 was expressed in H23, H226, H2170 and H322, but in other cell lines, there was no signal detectable. DSC2 was expressed in most of the cell lines tested, except for two SCLC cell lines CPC-N and COLO677. DSC3 was only expressed in H226, while silenced in other cell lines (figure 1A).

### Restoration of DSCs mRNA expression by 5-aza-2′-deoxycytidine (DAC)

After treatment with different concentrations of the demethylation agent DAC for 96 h, DSCs mRNA expression was restored in four out of seven cell lines for DSC1-3, respectively (figure 1B). In some of the cell lines, the reexpression of DSCs was in a concentration-dependent manner (eg, DSC1 re-expression in SHP77, DSC2 re-expression in H157 and COLO677, as well as DSC3 re-expression in COLO677).

Analysis of DSCs methylation status in lung cancer cell lines

We evaluated the methylation status of DSC2 DNA in 31 CpG sites and DSC3 DNA in 20 CpG sites (table 2). There was no CpG island in the promoter region/exon1 of DSC1, therefore, we could not analyse the methylation status of DSC1 in lung cancer cells. We found that DSC2 and DSC3 were highly methylated in four cell lines where restoration of mRNA expression was detected by demethylation tests. As expected, in normal control HBEC, neither DSC2 nor DSC3 DNA was methylated.

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Figure 1 A: DSCs expression analysis in lung cancer cell lines and human bronchial epithelial cells (HBEC). DSCs mRNA expression was analysed by RT-PCR, showing that DSC1-3 was expressed in HBEC. In lung cancer cell lines, DSC1 was found to be expressed in H23, H2170, H322, as well as H226, and DSC3 was merely expressed in H226. Unlike DSC1 and DSC3, DSC2 was expressed in the most of the cell lines tested except for CPC-N and COLO677. Glyceraldehyde-3-phosphate dehydrogenase was used as loading control. B: Demethylation tests in lung cancer cell lines. RT-PCR showed that after treatment with different concentrations of deoxycytidine for 96 h, DSCs mRNA expression was heterogeneously upregulated in four out of seven cell lines. * indicates non-small cell lung carcinoma cell lines.
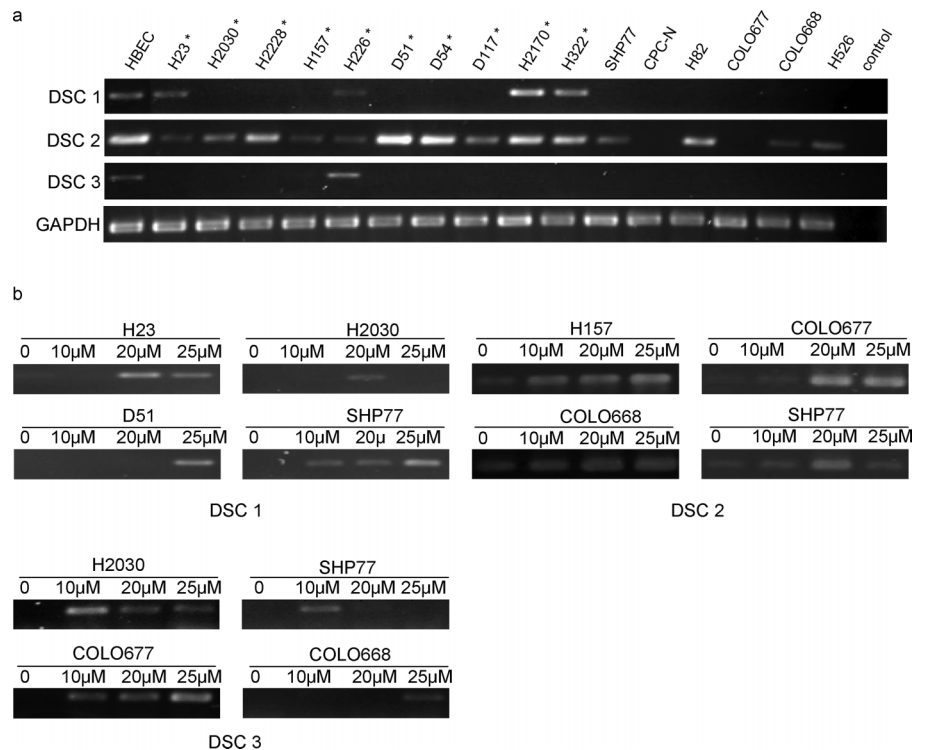
Protein expression of DSCs in primary lung tumours

Immunohistochemistry showed that DSC1-3 proteins were mainly localised in membrane.

As shown in table 3, 47 cases (23.6%) exhibited expression of DSC1. A reverse association between DSC1 expression and tumour differentiation was found: high-grade tumours showed significantly decreased expression of DSC1 (p=0.017, table 3). However, the expression of DSC1 did not differ significantly by other clinicopathological parameters. DSC2 and DSC3 were expressed in 68.3% and 31.7% of primary lung tumours, respectively. Additionally, DSC3 seemed to be a marker to distinguish SCC from ADC, since SCC showed significantly more positive staining of DSC3 compared with ADC (p<0.001, table 3). Examples of DSC1-3 protein expression in primary SCC of lung are shown in figure 2. In bronchial epithelial cells of normal bronchus, DSC1-3 staining was not visible (figure 2E–G).

From follow-up data we found that the median survival time for DSC1, DSC2, DSC3 positive and negative patients were 1158 and 787 days, 948 and 835 days, as well as 1218 and 744 days, respectively. Kaplan–Meier survival analysis revealed that downregulation of DSC1 and DSC3 was significantly linked to shorter overall survival (p=0.045 for DSC1; p=0.007 for DSC3, figure 3A,C). However, the expression of DSC2 was not correlated with clinical outcome (p=0.544, figure 3B).

Cox hazard analyses of lung cancer patient prognosis

The correlation between DSC1-3 expression and clinical outcome was analysed in a multivariable Cox proportional hazard regression model to discover optimal independent prognostic factors. The results are shown in table 4. DSC1 (HR=1.682, p=0.037) and DSC3 (HR=2.004, p=0.004) were the optimal independent prognostic factors in the final regression model, but not DSC2 (HR=0.759, p=0.229).

DISCUSSION

Desmosomes, together with adherens junctions, represent the major adhesive cell junctions of epithelial cells.[18] Desmosomal components are downregulated in certain types of carcinoma, suggesting their possible role in tumour suppression.[19–22]

In this study, we found that the expression of DSC1-3 was significantly downregulated compared with normal control cells. The molecular mechanism responsible for the altered DSCs expression in lung cancer has not yet been well elucidated. So far, no genetic alterations of DSCs have been found. Except for the detailed description of methylation patterns of DSC3 in breast and colon cancer,[13 14] no epigenetic regulation of DSCs has been reported in any other tumour entities. In our study, demethylation tests of DSCs by using the pharmaceutical agent DAC restored the expression of DSCs in four out of seven lung cancer cell lines. In line with this, methylation was detected in 31 CpG sites and 20 CpG sites of exon 1 in DSC2 and DSC3 respectively. These results suggested that DNA hypermethylation is at least partially responsible for the gene silencing of DSC2 and DSC3 in lung cancer cells.

It has been suggested that desmosomal components may contribute to the differentiation of human endometrial carcinoma.[23] Besides, Monica et al[24] and Tsuta et al[25] reported that DSC3 was a marker for poorly differentiated SCC and also for squamous differentiation in undifferentiated large cell lung carcinoma (LCLC). Consistent with these results, we observed that lose of DSC1 expression was associated with poor differentiation in primary lung tumours.

Despite extensive study, there are few clinically useful immunohistochemical markers for lung cancer diagnosis and prognosis.[26] Late diagnosis is a fundamental obstacle to improving lung cancer outcomes.[27–29] In our study, we could only divide the primary tumours into two major subgroups of SCC and ADC for statistical analysis, since the sample size of small cell lung carcinoma (SCLC) and LCLC was too small to be further evaluated. In line with the report from Boelens et al,[30] we found that DSC1-3 expression was not visible in normal bronchial epithelial cells, indicating that DSC1-3 may be upregulated in primary SCC of lung, and additionally, DSC3 seemed to be a marker to distinguish SCC from ADC, since 45.3% of

Table 2 Methylation status of DSC2 and DSC3 in lung cancer cell lines

DSC2 exon 1

cell lines	244	258	263	265	281	294	299	303	305	312	317	319	335	340	343	347	352	354	364	388	396	408	421	441	443	456	458	465	470	482	486
HBEC																															
H23																															
H2030																															
H2228																															
H226																															
H2170																															
H157																															
SHP77																															
COLO677																															
COLO668																															

DSC3 exon1

Cell lines	60	63	79	81	93	103	109	120	123	127	129	147	155	162	165	175	178	183	187	189
HBEC																				
H23																				
H2030																				
H2228																				
H226																				
H2170																				
SHP77																				
H82																				
COLO677																				
COLO668																				

black: methylated CpG site; gray: partially methylated CpG site; white: unmethylated CpG site

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Table 3 Correlation between DSCs expression and clinicopathologic parameters

Clinicopathological parameters	DSC1 (−)	DSC1 (+)	p Value	DSC2 (−)	DSC2 (+)	p Value	DSC3 (−)	DSC3 (+)	p Value
T stage									
T1	31 (15.6)	15 (7.5)	0.895*	10 (5.0)	36 (18.1)	0.570*	35 (17.6)	11 (5.5)	0.157
T2	86 (43.2)	20 (10.1)		36 (18.1)	70 (35.2)		72 (36.2)	34 (17.1)	
T3	23 (11.6)	4 (2.0)		10 (5.0)	17 (8.5)		15 (7.5)	12 (6.0)	
T4	9 (4.5)	6 (3.0)		3 (1.5)	12 (6.0)		9 (4.5)	6 (3.0)	
N stage									
N0	72 (36.2)	20 (10.1)	0.993*	31 (15.6)	61 (30.7)	0.613*	64 (32.2)	28 (14.1)	0.640*
N1	44 (22.1)	8 (4.0)		13 (6.5)	39 (19.6)		31 (15.6)	21 (10.6)	
N2	18 (9.0)	9 (4.5)		9 (4.5)	18 (9.0)		20 (10.1)	7 (3.5)	
N3	2 (1.0)	0 (0)		2 (1.0)	0 (0)		2 (1.0)	0 (0)	
Tumor differentiation									
G1	66 (34.6)	13 (6.8)	0.017	27 (14.1)	52 (27.2)	0.182	47 (24.6)	32 (16.8)	0.086
G2	51 (26.7)	16 (8.4)		21 (11.0)	46 (24.1)		48 (25.1)	19 (9.9)	
G3	29 (15.2)	16 (8.4)		10 (5.2)	35 (18.3)		33 (17.3)	12 (6.3)	
Histology									
SCC	105 (52.8)	23 (11.6)	0.072	38 (19.1)	90 (45.2)	0.771	70 (35.2)	58 (29.1)	<0.001*
ADC	39 (20.4)	24 (12.6)		20 (10.1)	43 (21.6)		58 (29.1)	5 (2.5)	

Note: the values in parenthesis are present in percentage.
*Fisher' exact test.
ADC, adenocarcinoma; SCC, squamous cell carcinoma.

SCCs exhibited expression of DSC3, while less than 8% of ADCs were DSC3 positive (table 3). This result was also in good agreement with the expression pattern of DSC3 in non-small cell lung carcinoma (NSCLC) cell lines, since DSC3 mRNA was only expressed in a SCC cell line H226, while lost in all the ADC cell lines (figure 1A). Compared with the study by Tsuta et al which showed that 100% of SCC samples were positive for DSC3 staining, our positive rate was lower. The discrepancy is probably due to different antibodies, technologies and the staining evaluation. So far, only p63 and CK5/6 are widely used as diagnostic markers for SCC of lung, while thyroid transcription factor-1 (TTF-1) together with CK7 is considered as a marker for lung ADC.[31–34] Based on the expression of DSC3 alone, it might be not sufficient to make a diagnosis for SCC of lung, however, we hope that in combination with the classic markers, DSC3 may contribute to subclassification of NSCLC.

Desmosomal proteins have also been considered as prognostic markers in various cancer types. For example, downregulation of desmoplakin expression provides prognostic information in human oropharyngeal cancer.[21] Decreased DSG3 expression was associated with poor prognosis in lung cancer.[11] Recently,
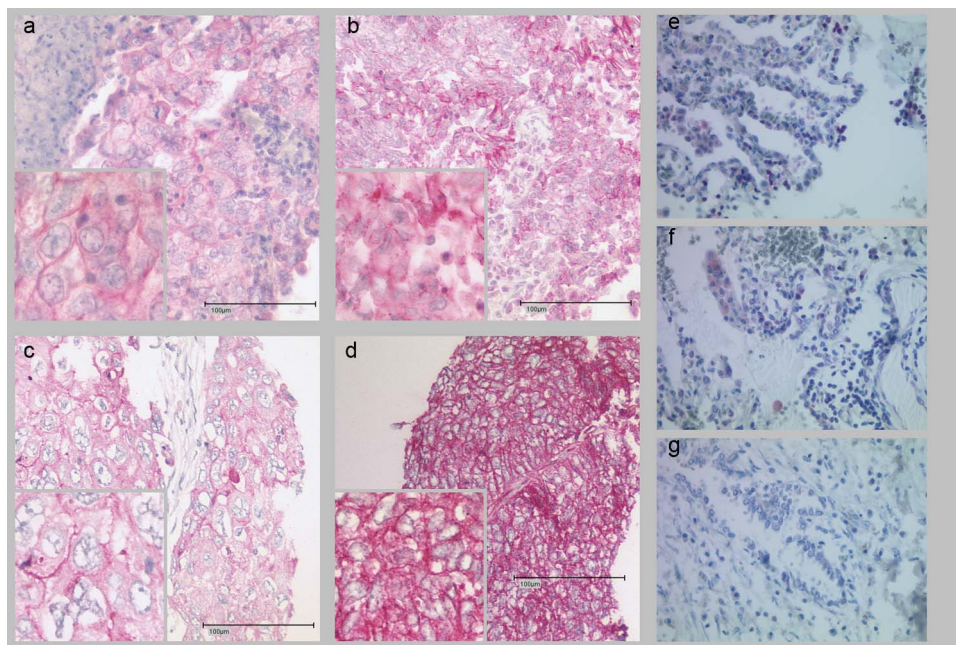

Figure 2 Examples of DSC1-3 protein expression in primary squamous cell carcinoma of lung and bronchial epithelial cells of normal bronchus. DSC1-3 proteins were mainly localised in membrane. A: DSC1 positive staining; B: DSC2 positive staining; C: DSC3 moderately positive; D: DSC3 strongly positive. E-G: DSC1-3 negative staining in normal bronchial epithelial cells. Image with 20×magnification of the corresponding case is showed in the inset. This figure is only reproduced in colour in the online version.
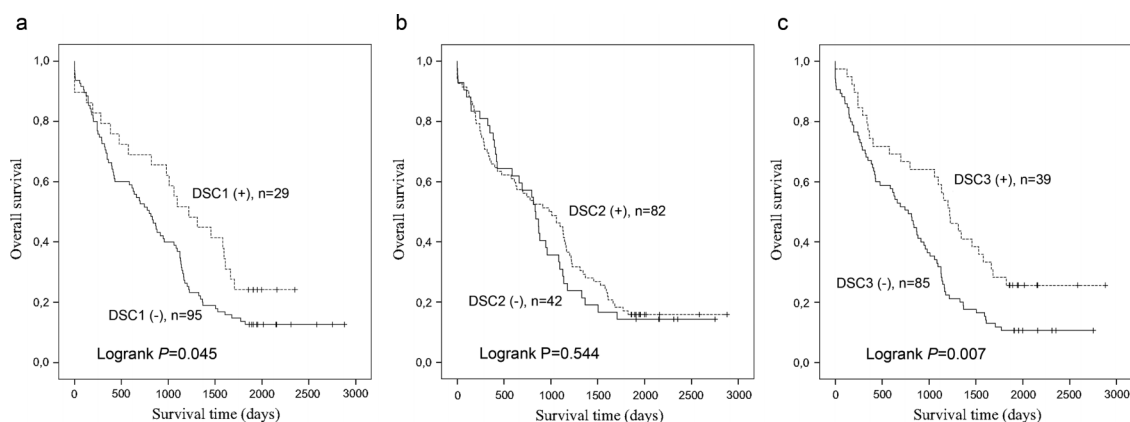
Figure 3 Expression of DSC1 and DSC3 predicted clinical outcome in primary lung cancer. Kaplan Meier curves showed that patients with lower expression of DSC1 or DSC3 had shorter overall survival time compared to patients with higher expression of DSC1 or DSC3 (A, C). In contract, DSC2 expression was not significantly linked to patients' survival (B).

Table 4 Cox hazard analyses of overall survival of lung cancer patients

Variables	df	HR	95% CI Lower	95% CI Upper	p Value
DSC1					
Negative/Positive (95 : 29)	1	1.682	1.032	2.741	0.037
DSC2					
Negative/Positive (42 : 82)	1	0.759	0.485	1.190	0.229
DSC3					
Negative/Positive (85 : 39)	1	2.004	1.254	3.202	0.004

we reported that methylation of DSC3 DNA is a prognostic marker in human colorectal cancer.[13] In this study, we found that reduced expression of DSC1 and DSC3 was associated with an unfavourable prognosis in lung cancer. Like DSG3, DSC1 and 3 seem to be a potential prognostic marker in lung cancer.

In summary, our data showed that DSC1 may be a marker for tumour differentiation, DSC3 protein expression may have diagnostic value for subclassification of NSCLC into SCC and ADC; moreover, patients with reduced DSC1 and DSC3 expression had poor prognosis. Further studies are needed to explore the functional role of DSCs in lung carcinogenesis.

Take home messages

▶ Epigenetic regulation is involved in the downregulation of DSC1-3 in lung cancer cells.
▶ We propose novel diagnostic marker DSC3 in distinguishing lung SCC from adenocarcinoma.
▶ Additionally, DSC1 and DSC3 were thought to be novel prognostic markers for patients with lung cancer.

Contributors All authors have participated in the planning, execution, or analysis of the study. I Petersen und Y Chen conceived the experiment; TC and LY performed the experiments; TK, MM, and QZ analysed the data; LK and AK collected the samples.

TC and YC interpreted data and wrote the manuscript. All authors have approved this manuscript and believe that the manuscript represents honest work.

Competing interests None.

Ethics approval The study was performed according to common ethical standards.

Provenance and peer review Not commissioned; externally peer reviewed.

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