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High expression of Polycomb group protein EZH2 predicts poor survival in salivary gland adenoid cystic carcinoma

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ABSTRACT

Background: The prognosis of adenoid cystic carcinoma (ACC), a malignant salivary gland tumour, depends on clinicopathological parameters. To decipher the biological behaviour of ACC, and to identify patients at risk of developing metastases, additional markers are needed.

Methods: Expression of the cell cycle proteins p53, cyclin D1, p16^{INK4a}, E2F1 and Ki-67, together with the Polycomb group (PcG) proteins BMI-1, MEL-18, EZH2 and EED was investigated immunohistochemically 21 formalin-fixed, paraffin-embedded primary ACCs in relation to tumour characteristics.

Results: ACC revealed significantly increased expression of the cell cycle proteins compared to normal salivary tissue (n = 17). Members of the two PcG complexes displayed mutually exclusive expression in normal salivary gland tissue, with BMI-1 and MEL-18 being abundantly present. In ACC, this expression pattern was disturbed, with EZH2 and EED showing significantly increased expression levels. In univariate analysis, presence of recurrence, poor differentiation and high EZH2 levels (>25% immunopositivity) significantly correlated with unfavourable outcome. ACCs with high proliferative rate (>25% Ki-67 immunopositivity) significantly correlated with high levels of EZH2 and p16. Only the development of recurrence was an independent prognostic factor of survival in multivariate analysis.

Conclusions: Expression of PcG complexes and of essential cell cycle proteins is highly deregulated in ACC. Also, EZH2 expression has prognostic relevance in this malignancy.

Adenoid cystic carcinoma (ACC) is a relatively common malignant salivary gland tumour, representing about 10–15% of all head and neck neoplasms.¹ This tumour shows bidirectional differentiation towards luminal (ductal) and abluminal (myoepithelial and basal) cells, in which the myoepithelial cells predominate.² Histologically, three distinct patterns are recognised, namely the cribriform, tubular and solid subtype.³

Given that this neoplasm is characterised by slow progression, about 65% of patients are still alive after 5 years. However, local recurrences and late distant metastases result in a 20 year survival rate of only about 20%.⁴ To identify patient groups with unfavourable outcome, several studies have attempted to uncover clinical and pathological parameters with prognostic relevance in ACC.^{5–7} However, these clinicopathological parameters are far from satisfactory as prognostic predictors and

several studies have been undertaken to search for additional markers based on protein levels.^{8–13} Only p53 proved to be a consistent marker of aggressiveness, being highly expressed in the solid pattern,¹⁰ correlating with unfavourable clinical outcome¹⁴ and having high loss of heterozygosity rates.¹⁵

Polycomb group (PcG) proteins control the transcriptional memory of a cell by maintaining the stable silencing of specific sets of genes through chromatin modifications.¹⁶ They form two distinct complexes and their members exhibit a mutually exclusive expression pattern in differentiated, healthy tissues.¹⁷ A growing body of work has linked deregulated expression of human PcG genes to malignant transformation, loss of differentiation in tumour cells, metastatic behaviour and poor prognosis.^{18,19} At the molecular level, the link between aberrant PcG gene expression and cancer development remains largely unclear. However, recent *in vitro* studies have demonstrated that PcG proteins interact with several negative (p16^{INK4a}, p53, pRb, p14)^{20–22} and positive regulators (E2F1, cyclin D1)²³ of the cell cycle. Disturbances in PcG protein interaction with these cell cycle controllers have been suggested to be an important step in malignant transformation.

The present study was undertaken to evaluate immunohistochemically the expression patterns of various cell cycle-associated proteins (p16^{INK4a}, cyclin D1, p53, E2F1 and Ki-67) and PcG proteins (BMI-1, MEL-18, EZH2 and EED), and to assess their potential prognostic role in predicting tumour characteristics and patient survival in the (myo)epithelial tumour ACC.

MATERIALS AND METHODS

Patient data

Twenty-one patients, from whom formalin-fixed, paraffin-embedded (FFPE) primary ACCs were available, were included in this study. In 17 cases, matched normal salivary gland tissue could be obtained as a control. All tumour samples were derived from the archives of the Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands. Patients were diagnosed with ACC between 1993 and 1999. The design of this study adheres to the code for proper secondary use of human tissue of the Dutch Federation of Biomedical Scientific Societies (<http://www.federa.org>).²⁴ Patient follow-up data were available for all 21 cases. The male to female ratio was 1:1.3 and patients ranged in age from 25

Table 1 Antibodies and staining conditions for immunohistochemistry

Antibody	Clone	Source	Species	Dilution	Detection method
P53	D07	Dako	Mouse	1:500 o/n	sABC-BT
P16	16P04	Neomarkers	Mouse	1:200 1 h	Envision
E2F1	KH95	Neomarkers	Mouse	1:250 1 h	Powervision Plus
Ki-67	MIB-1	Dako	Mouse	1:40 o/n	sABC-BT
Cyclin D1	DCS-6	Neomarkers	Mouse	1:400 o/n	Envision
BMI-1		*	Mouse	1:1000 o/n	sABC-BT
EZH2	M18	*	Mouse	1:5 o/n	sABC-BT
MEL-18	C-20	Santa Cruz	Goat	1:400 o/n	sABC-BT
EED	M26	*	Mouse	1:25 o/n	sABC-BT

*Non-commercial.

o/n, overnight at 4°C; 1 h, 1 hour at room temperature; sABC-BT: horseradish-peroxidase conjugated streptavidin/biotin complex and biotinylated tyramine.

to 81 years (median 54.9). Nineteen tumours were located in the major salivary glands (9 parotid, 8 submandibular, 2 sublingual) and two were located elsewhere in the oral cavity. Tumours were classified by a pathologist (EB) according to the predominant histological pattern (6 solid, 15 cribriform/tubular). Except for two patients who received surgery alone, all patients were treated by surgery and postoperative radiotherapy. Five patients (24%) developed a recurrence, eight patients (38%) developed a metastasis, and two patients (9%) developed both during the time of follow-up (range 7–128 months; median 73.6). Most ACCs spread to the lungs ($n = 5$), one tumour metastasised to the liver, one to the liver and lungs, and one spread to the liver, lungs and bones. Development of either recurrence or metastasis was independent of the radicality of the surgical resection margins. Disease-free survival ranged from 2 to 121 months (median 27). Six (29%) patients died during the time of follow-up, all due to the disease.

Immunohistochemistry

All tumour samples were fixed in 4% buffered formalin, processed, and embedded in paraffin according to routine procedures. Endogenous peroxidase was inhibited with 0.3% H_2O_2 in methanol at room temperature; antigens were retrieved by heating in either a microwave (antibodies BMI-1 and p16^{INK4a} in Tris/EDTA buffer pH 9; Ki-67 and p53 antibodies in 0.01 M sodium citrate buffer pH 6.0) or an autoclave (EZH2 in Tris/EDTA buffer pH 8; MEL-18 and cyclin D1 in 0.01 M sodium citrate buffer pH 6.0). In case of PcG antibodies, the slides were rinsed in phosphate-buffered saline (PBS) containing 0.5% Triton-X (5 min), followed by PBS only (3×1 min). The slides were washed in 0.1 M glycine (10 min) and rinsed in PBS. After preincubation with normal rabbit serum (NRS), primary and biotinylated secondary antibodies (rabbit anti-mouse F(ab')₂ and rabbit anti-goat together with 5% human pool serum (HPS)) were applied; immunostaining was performed using the

Figure 1 Significantly increased expression of the cell cycle proteins Ki-67, cyclin D1, p16^{INK4a}, p53 and E2F1 in adenoid cystic carcinoma (ACC) compared to normal salivary gland tissue (all $p < 0.001$). (A) Area of boxplot displays 50% of middle values; thick black line represents median number of immunopositive cells. Representative staining patterns of (B) Ki-67, (C) p16 and (D) p53 in normal salivary gland tissue (right side of picture) and in ACC (left side). Magnification, ×200.

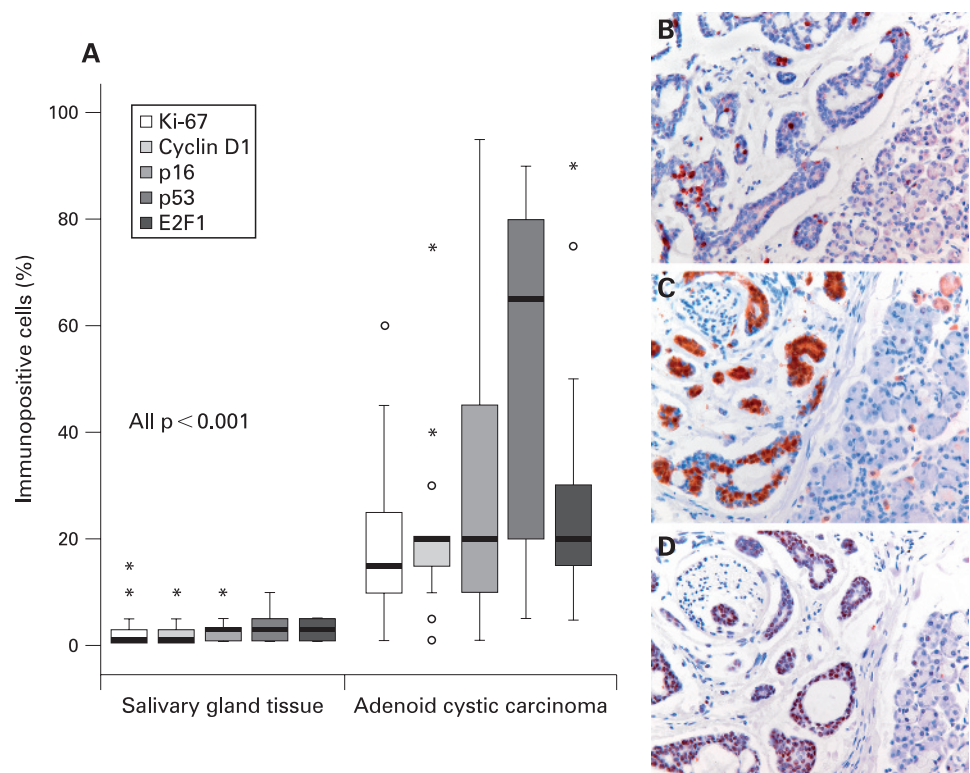
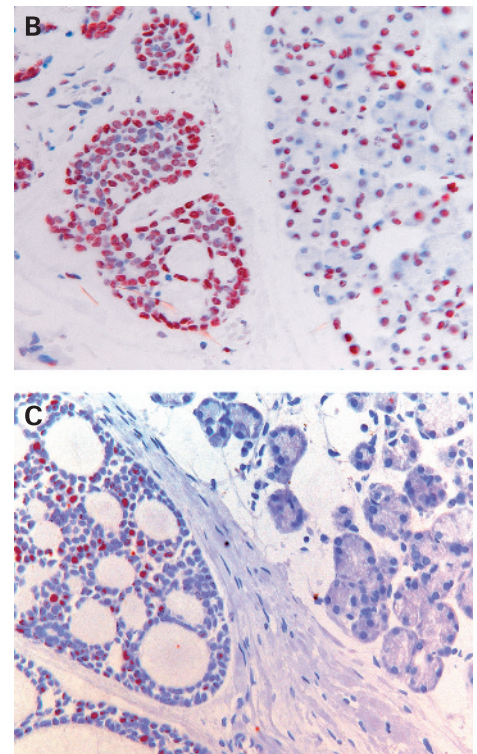
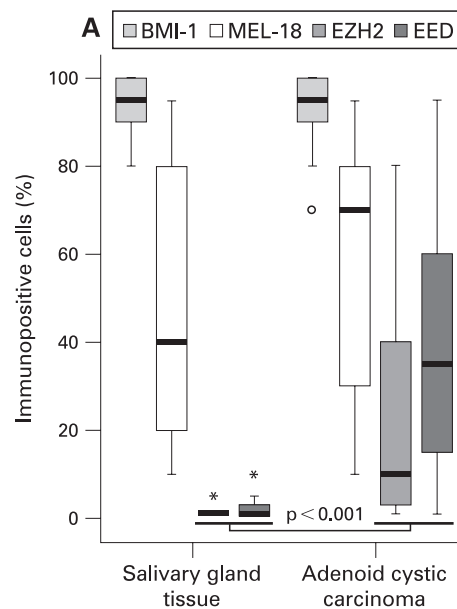


Figure 2 Significantly increased expression of members of the PRC2 complex in adenoid cystic carcinoma (ACC) compared to normal salivary gland tissue ($p < 0.001$). (A) Area of boxplot displays 50% of middle values; thick black line represents median number of immunopositive cells. Representative staining patterns of (B) BMI-1 and (C) EZH2 in normal salivary gland tissue (right side of picture) and in ACC (left side). Magnification, $\times 200$.



appropriate detection method (table 1). Negative controls were included by substitution of the primary antibody with 1% bovine serum albumin (BSA) in PBS. The streptavidin and biotinylated horseradish peroxidase complex (sABC) method was performed with a biotinylated tyramine (BT) intensification step (1:1000 with 0.01% H_2O_2 in sterile PBS). Envision horseradish peroxidase system (Dako, Glostrup, Denmark) and Powervision Plus (Immunologics, Duiven, The Netherlands) were carried out following the protocol supplied by the manufacturer. 3-Amino-9-ethylcarbazole (AEC) (Zymed, San Francisco, California, USA) was used as substrate for the sABC-BT method, and diaminobenzidine (DAB) for the Envision and Powervision Plus method. Sections were counterstained with haematoxylin, dehydrated and mounted. Interpretation of the staining was done by semi-quantitative scoring by at least two investigators (HV and EB). Percentage of tumour cells with positive staining was grouped as low (expression in $\leq 25\%$ of cells) or high (expression in $> 25\%$ of cells).

Statistical analysis

Because of the non-normal distribution of the protein expression levels and the relatively small sample size, statistical

evaluation was performed using non-parametric tests. Comparison between the protein expression levels in tumour and normal tissue was done using the Mann-Whitney U test. Associations between protein expression and clinicopathological parameters were performed using Fischer's exact probability test. Disease-free and disease-specific survival rates were determined using the Kaplan-Meier method; differences in survival were evaluated with the log-rank test. Univariate analysis with the Cox proportional hazards regression model was used to determine each identified prognostic factor. The multivariate Cox proportional hazards regression model was employed in a forward stepwise manner to analyse the relevance of independent prognostic factors. A two-sided p value < 0.05 was considered to be statistically significant. Statistical analyses were performed using SPSS, V.12.0.1 (SPSS, Chicago, Illinois, USA).

RESULTS

Cell cycle protein expression in ACC is disturbed compared to normal salivary gland tissue

Seventeen cases of normal salivary gland tissue were investigated immunohistochemically for the expression of the cell

Table 2 Significant prognostic factors by univariate analysis

Clinical parameter	n	Mean DSS (95% CI)	p Value	HR (95% CI)	χ^2	p Value
Histological subtype				0.2 (0.04 to 1.12)	3.63	0.05
Cribriform/tubular	14	115 (97 to 132)	0.04			
Solid	7	70 (49 to 91)	(LR = 4.11)			
Recurrence				18.4 (2.03 to 167.4)	9.03	0.003
Absent	16	114 (97 to 132)	0.001			
Present	5	58 (29 to 86)	(LR = 12.64)			
EZH2				5.04 (0.91 to 27.82)	3.72	0.05
Low ($\leq 25\%$)	15	115 (98 to 132)	0.04			
High ($> 25\%$)	6	68 (45 to 91)	(LR = 4.23)			

DSS, disease-specific survival in months; LR, log rank.

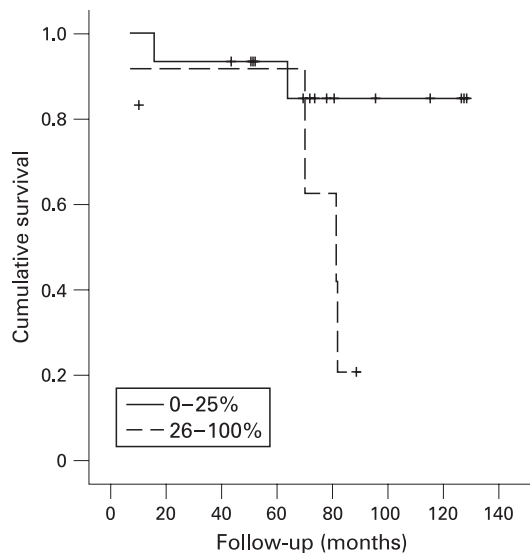


Figure 3 Overall survival curves of patients with adenoid cystic carcinoma showing low ($\leq 25\%$; $n = 15$) and high ($> 26\%$; $n = 6$) EZH2 levels. A significant difference was observed between the two groups (log rank = 4.23; $p = 0.04$).

cycle proteins E2F1, Ki-67, p16^{INK4a}, p53 and cyclin D1. All proteins showed low median expression levels (fig 1A): E2F1, 3% (range 1–5); Ki-67, 3% (range 1–15); p16^{INK4a}, 3% (range 1–10); p53, 3% (range 1–10); and cyclin D1, 1% (range 1–10). In ACC, expression of all the cell cycle proteins was significantly increased compared to normal salivary gland tissue (fig 1A–D). E2F1 showed a median expression of 20% (range 5–90), Ki-67 of 15% (range 1–60), p16^{INK4a} of 20% (range 1–95), p53 of 65% (range 5–90), and cyclin D1 of 20% (range 1–75).

Mutually exclusive expression pattern of PcG complexes in normal salivary gland tissue is deregulated in ACC

Members of the PRC1 complex, BMI-1 and MEL-18, showed abundant nuclear staining in normal salivary gland tissue (median expression 95% (range 80–100) and 40% (range 10–95), respectively; fig 2A). In contrast, members of the PRC2

complex showed almost no immunoreactivity (both EED and EZH2 1% median expression; range 1–10 and range 1–5, respectively). Expression patterns of the PcG proteins were disturbed in ACC when compared to normal salivary gland tissue (fig 2A–C). Members of the PRC1 complex were still abundantly expressed; BMI-1 exhibited a median expression of 95% (range 70–100) and median MEL-18 immunoreactivity increased to 70% (range 10–95). Interestingly, EED and EZH2 displayed significantly raised expression levels in the tumour (both $p < 0.001$). Median expression of EZH2 increased to 10% (range 1–80%) and in the case of EED to 35% (range 1–95).

Presence of locoregional recurrence and poor differentiation significantly correlate with poor patient outcome in ACC

To examine the influence of clinicopathological characteristics on patient outcome, we performed univariate survival analysis with sex, age (< 55 vs ≥ 55 years), perineural spread, histological differentiation (solid vs cribriform/tubular), presence of recurrence, and presence of metastasis as prognostic factors. The presence of recurrence and the solid subtype significantly correlated with poor disease-specific survival rate (log rank = 12.64, $p = 0.001$ and log rank = 4.11, $p = 0.04$, respectively; table 2). No parameters showed a significant association with disease-free survival.

Correlation of protein expression with clinical data

To correlate the expression of cell cycle and PcG proteins with categorical clinical data, we divided the expression rates into low ($\leq 25\%$) and high ($> 25\%$) expression groups. All the proteins analysed were correlated with sex and age of the patient, presence of recurrence or metastasis, patient outcome, histology (solid vs cribriform/tubular), and presence of perineural growth. Table 3 summarises significantly correlating variables. Additionally, high p16^{INK4a} and high Ki-67 expression showed a tendency towards more aggressive tumour behaviour ($p = 0.08$ and $p = 0.09$, respectively), defined as development of either a recurrence or a metastasis. Correlation between individual proteins revealed that ACCs with high proliferative rate ($> 25\%$ Ki-67 immunopositive cells) also displayed significantly higher EZH2 ($p = 0.05$) and p16 levels ($p = 0.02$). Additionally, tumours with low E2F1 status tended to express

Table 3 Significant correlations between various cell cycle and Polycomb group (PcG) proteins and clinicopathological parameters as determined by Fischer's exact test

Clinical parameter	Total patients	Patients with low staining*	Patients with high staining†	p Value
		Patients with low cyclin D1 staining (n = 16)	Patients with high cyclin D1 staining (n = 5)	
Metastasis				
Absent	10	5 (23.8%)	5 (23.8%)	0.01
Present	11	11 (52.4%)	0 (0%)	
		Patients with low Ki-6 staining (n = 16)	Patients with high Ki-67 staining (n = 5)	
Histology				
Cribriform/tubular	14	14 (66.7%)	0 (0%)	0.006
Solid	7	3 (14.3%)	4 (19%)	
		Patients with low EZH2 staining (n = 15)	Patients with high EZH2 staining (n = 6)	
Patient outcome				
Alive	15	13 (61.9%)	2 (9.5%)	0.03
Dead	6	2 (9.5%)	4 (19%)	

* $\leq 25\%$ of the cells immunopositive; † $> 25\%$ of the cells immunopositive.

low amounts of EZH2 ($p = 0.07$). Univariate survival analysis classified EZH2 expression as a significant prognostic factor (log rank = 4.23; $p = 0.04$; fig 3). However, multivariate survival analysis determined the presence of recurrence to be the only independent prognostic indicator ($p = 0.003$).

DISCUSSION

Adenoid cystic carcinoma (ACC) is a malignant tumour of the salivary glands with an unpredictable clinical course. It has an unusually slow biological growth, resulting in a relatively favourable 5-year survival. About 30% of patients are alive after 15 years.⁶ Since current prognostic indicators are not as reliable as desired, there is a need for novel molecular predictors of tumour behaviour at the time of diagnosis. We assessed immunohistochemically the expression patterns of various cell cycle and Polycomb group (PcG) proteins in ACC and correlated our findings with patient follow-up data.

Univariate analysis of clinicopathological characteristics in relation to patient survival revealed recurrence of the primary tumour and the solid histological subtype as significant prognostic factors. Previous retrospective studies have also acknowledged the importance of histology concerning survival of patients with ACC.^{25–27} The cribriform and tubular subtypes are considered to be more differentiated than the solid subtype; loss of differentiation is related to aggressive behaviour in several cancer types.

Varambally *et al* were the first to correlate increased EZH2 expression with aggressive tumour behaviour in hormone-refractory, metastatic prostate cancer and to show that clinically localised tumours with higher EZH2 expression have a poorer prognosis.²⁸ Since then, raised EZH2 levels have been found in several epithelial tumours,^{29–34} and in various haematological malignancies.^{35–37} Studies which investigated the prognostic relevance of EZH2 revealed that high protein status predicted recurrence,²⁹ invasiveness and metastatic potential,³⁸ and poor survival.²⁹ In corroboration with this, high EZH2 (>25%) expression significantly correlated in our group of ACCs with poor patient outcome in univariate analysis, showing that in malignant tumours with myoepithelial differentiation PcG expression is disturbed and EZH2 is related to more aggressive tumour behaviour. In multivariate analysis however, only recurrence remained as an independent prognostic factor.

It has been suggested that specific amplification of the *EZH2* gene at 7q35 is responsible for its overexpression in primary human tumours.²³ In our previous paper,³⁹ we investigated this same group of ACCs for frequent DNA aberrations by microarray-based comparative genomic hybridisation. Although no amplifications were detected at 7q35, two tumours with high EZH2 protein expression displayed a chromosomal gain at its locus. Our findings imply that copy number elevations are not the main mechanisms of EZH2 overexpression; disturbances at other levels, such as chromatin modification, or most likely a deregulation in the pRb-E2F pathway, are probably more important.

Recently, the finding that EZH2 expression is critical for S-phase entry and G₂-M transition has implicated this PcG protein in cell cycle regulation.²³ *EZH2* transfection in lymphoma cells increased their proliferation,³⁷ and in cancers of the breast, endometrium and prostate, and in cutaneous melanoma, EZH2 expression levels were strongly associated with increased tumour cell proliferation.⁴⁰ Similarly, Ki-67 expression in our group of ACCs showed a significant correlation with EZH2 expression ($p = 0.05$).

Take-home messages

- ▶ This is the first study describing Polycomb group (PcG) gene expression in a salivary gland tumour.
- ▶ Mutually exclusive expression pattern of the two PcG complexes is highly deregulated in adenoid cystic carcinoma (ACC).
- ▶ High EZH2 expression correlates with poor survival in ACC.
- ▶ Cyclin D1, p16^{INK4a}, Ki-67, E2F1 and p53 show significantly increased expression in ACC compared to normal salivary gland tissue.

After the discovery of the *INK4a* locus as a downstream target of Bmi-1 in murine models,²² several in vitro studies have demonstrated the same negative regulation in human primary cells.^{20 41 42} More specifically, EZH2 trimethylation of histone H3 on lysine 27 at the *INK4a* promoter and coding region serves as a mark to recruit the BMI-1-containing PRC1 complex. Binding of this complex to the locus prevents transcription by blocking the association of RNA polymerase II.²⁰ In our tumours, the observed high p16^{INK4a} expression, even in the presence of BMI-1, suggests a deregulation of normal protein interactions. Two different explanations for this might be the impairment of BMI-1 function or the disruption of the pRb pathway downstream of p16. It has been discovered that in cases of overexpression/amplification of cyclin D1 or mutation/deletion of pRb, p16 levels can accumulate as a result of oncogenic stress without causing proliferative arrest.^{43 44}

In conclusion, derailments in these essential cell cycle pathways on chromosomal and protein levels are very common in a wide range of human cancers,⁴⁴ and we speculate that similar alterations might be involved in ACC tumourigenesis.

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