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Frequent hypermethylation of *RASSF1A* tumour suppressor gene promoter and presence of Merkel cell polyomavirus in small cell lung cancer

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ABSTRACT

In small cell lung cancer (SCLC), hypermethylation of the tumour suppressor Ras association domain family 1A (*RASSF1A*) is frequent. It is associated with SV40 polyomaviral infection in other tumours. Merkel cell polyomavirus (MCPyV) infection has been reported in Merkel cell carcinoma (MCC), a neuroendocrine carcinoma with biological similarity to SCLC. In our study, we investigated polyomavirus infection (SV40 and MCPyV) and promoter hypermethylation of the tumour suppressors *RASSF1A* and *p16* in 18 SCLCs (14 primaries and 4 regional lymph node metastases) and 18 blood control samples. MCPyV was found in 39% (7 of 18) of the tumour tissues but not observed in controls. SV40 was not observed in the tumour tissue. *RASSF1A* promoter hypermethylation (94%; 17 of 18) was more frequent compared to *p16* methylation (56%, 10 of 18). We found no significant correlation between *RASSF1A* or *p16* promoter hypermethylation and infection with the investigated polyoma viruses. Our results show a high frequency of hypermethylation of the *RASSF1A* promoter and occurrence of MCPyV infection in the tumour tissue of SCLC. These events may contribute to the pathogenesis of SCLC.

1. Introduction

Lung cancer is one of the most frequent malignant neoplasms in humans. This group of pathologically different cancer types caused 7.9 million deaths worldwide in 2007. Small cell lung cancer (SCLC) accounts for approximately 15% of all lung cancer cases.[1] Approximately 40% of those cases are diagnosed in patients older than 70 years. This proportion continues to rise in contrast to the continued decline in the incidence of SCLC among the general population.[2] SCLC originates from neuroendocrine cells of the lung. Distinct genetic and epigenetic aberrations have been reported in SCLC. Loss of the chromosomal region 3p21.3 is the most frequently detected LOH in SCLC.[3] In the chromosomal segment, we isolated a novel tumour suppressor gene termed Ras association domain family 1A (*RASSF1A*). Promoter hypermethylation of *RASSF1A* occurs frequently in lung cancer and is frequently found in SCLC.[4] Hypermethylation of the *RASSF1A* promoter is associated with infection of the rhesus monkey polyomavirus SV40 in malignant mesothelioma, prostate cancer and breast cancer.[5–7]

Another highly aggressive neuroendocrine cancer with high mortality is Merkel cell carcinoma (MCC). Interestingly, MCC shows genetic alterations similar to those in SCLC.[8–10]

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Recently, a novel Merkel cell polyomavirus (MCPyV) was identified in MCC.[11–14] The presence of MCPyV in other neuroendocrine cancers has not been investigated.

To reveal the causality of viral infection and tumour suppressor gene inactivation in SCLC, we investigated the hypermethylation of *RASSF1A* and *p16* and viral infection of SV40 and MCPyV in SCLC and controls. Our study demonstrates frequent hypermethylation of *RASSF1A* tumour suppressor and presence of MCPyV in SCLC.

2. Material and methods

2.1. Material

SCLCs were retrospectively sampled from the tissue bank of the National Centre for Tumour Diseases (NCT) Heidelberg. The protocol was approved by the local Ethics Committee of the University of Heidelberg. All patients gave informed consent for the work. We investigated 18 samples of 18 tumours (14 primary SCLC, 4 regional lymph node metastases from 18 patients (details Table 1). Eighteen blood control samples were obtained from random donors from the outpatient prophylactic skin check programme of the Department of Dermatology (median age 64.9 years, 11 men, 7 women). Exclusion criteria were inflammatory skin disorders, known general inflammatory or auto-immune diseases, known other severe general diseases and any malignancies (current or in history). Diagnosis of SCLC was established histologically according to World Health Organisation WHO criteria.[15] Additionally, expression of tumour cell markers was investigated immunohistologically: CD56 (17 of 18 investigated samples positive), neuron-specific enolase (15/16), synaptophysin (18/18), chromogranin (3/11) and BerEP4 (4/5). A sufficient number of viable tumour cells were guaranteed in each sample (54.9 ± 21.0%, mean ± SD). Ki-67 expressing proliferative tumour cell fraction was 78.8 ± 14.1%. Strictly corresponding successive sections of the paraffin-embedded material were used for haematoxylin and eosin staining, immunostaining and DNA isolation.

2.2. Antibodies

Monoclonal mouse anti-CD56, clone 123C3 (07-5603, Zymed, San Francisco, CA), monoclonal mouse anti-human neuron-specific enolase (M0873, Dako, Glostrup, Denmark), affinity isolated polyclonal rabbit anti-synaptophysin (A0010, Dako), monoclonal mouse anti-human chromogranin (M0869, Dako), monoclonal mouse anti-human epithelial antigen BerEP4 (M0804, Dako), monoclonal mouse-anti Ki-67 and clone MIB1 (M7240, Dako).

2.3. Immunostaining

Five-micrometre-paraffin sections were de-paraffinised (graded ethanol series). Endogenous peroxidase was quenched (0.3% H_2O_2 in methanol). Sections were microwaved (10 mM citrate buffer, pH 6.0, 4 × 5 min, 600 W) and incubated for 30 min at room temperature either with anti-CD56 (1:200), anti-neuron specific enolase (1:500), anti-synaptophysin (1:100), anti-chromogranin (1:100), BerEP4 (1:200) or anti-Ki-67 (1:100). Further steps were performed with the Elite-ABC-Kit (Vector, Burlingame, CA) and AEC substrate system (Dako) following manufacturer's instructions. Sections were counterstained with 25% hemalaun (Merck, Darmstadt, Germany).

2.4. DNA isolation

The tissue specimen from paraffin-embedded tumours was deparaffinised by xylene and ethanol treatment. After a proteinase K restriction, DNA was isolated with a QIAamp DNA

Table 1 – Sample characteristics and results.

Sample characteristics				Results			
Sample	Histological diagnosis	P/LN	TNM	MCPyV	SV40	*RASSF1A*	*p16*
1	SCLC	P	pT1, pN2, pMx	0	0	+	0
2	SCLC	P	pT2, pN0, pMx	0	0	+	+
3	SCLC	P	pT2, pN0,pMx	0	0	+	+
4	SCLC	LN		+	0	+	0
5	SCLC	LN		+	0	+	+
6	SCLC + SCC	P	pT2, pN2, pMx	0	0	0	+
7	SCLC	P	pT1, pN0, pMx	+	0	+	+
8	SCLC	P	pT3 pNx, pMx	+	0	+	0
9	SCLC	P	pT2, pN2, pMx	0	0	+	0
10	SCLC	LN		0	0	+	0
12	SCLC	P	pT2, pN1, pMx	+	0	+	+
13	SCLC + SCC	P	pT2, pN2, pMx	0	0	+	0
14	SCLC	P	pT2, pN0, pMx	0	0	+	+
15	SCLC	LN		+	0	+	0
16	SCLC	P	pT2, pN2, pMx	0	0	+	+
17	SCLC	P	pT1, pN0, pMx	+	0	+	0
18	SCLC	P	pT1, pNx, pMx	0	0	+	+
19	SCLC	P	pT2, pN0, pMx	0	0	+	+

SCLC, small cell lung cancer; SCLC + SCC, combined SCLC and squamous cell carcinoma; P, primary; LN, lymph node filia; +, positive or methylated; 0, negative or unmethylated; MCPyV, Merkel cell polyomavirus; *RASSF1A*, Ras association domain family 1A.

extraction kit (Qiagen, Hilden, Germany). Concentrations of DNA were determined by UV-photospectrometry (Gene Quant Pro, Amersham Biosciences, New York).

2.5. Methylation analysis

Promoter methylation of the tumour suppressor genes *RASSF1A* and *p16* was investigated by methylation-specific PCR (MSP) with primers and conditions listed in Table 2.[16] MSP eliminates the false positive results inherent to previous PCR-based approaches.[17] Briefly, PCR was performed with methylation-specific primers and unmethylation-specific primers using 225 ng of the bisulphite-modified genomic DNA as template for 40 or 38 cycles at 95 °C for 30 s, (T_A see Table 2) for 30 s in 25 μl containing 200 μmol dNTPs, 20 pmol primers and 1 unit of Taq DNA polymerase (Invitek, Berlin, Germany). Ten micro litres of the PCR product was analysed on a 2% Tris–borate EDTA agarose gel. All visible PCR products obtained by methylation-specific PCR amplification were classified as methylated samples.

2.6. Detection of viral DNA

PCR was performed with the Merkel cell polyomavirus and *SV40*-specific primers (Table 2) using 250 ng of the genomic DNA as template for 40 cycles at 95 °C for 30 s, 62 °C for 30 s and 72 °C for 30 s in 25 μl containing 200 μmol dNTPs, 20 pmol primers and 1 unit of Taq DNA polymerase (Invitek, Berlin, Germany). Ten micro litres of PCR product was analysed in a 2% Tris–borate EDTA agarose gel. Visible PCR products obtained by virus-specific PCR amplification were classified as polyomavirus-positive samples. The housekeeping gene *GAPDH* was utilised in parallel as control for DNA integrity (Table 2). For positive control, human Merkel cell carcinoma or a plasmid harboring *SV40* sequence (pTD1-1) was used in MCPyV or SV40 studies, respectively.

3. Results

3.1. Presence of Merkel cell polyomavirus in SCLC

Recently, the presence of novel polyomavirus termed Merkel cell polyoma virus (MCPyV) was reported in neuroendocrine tumours of the skin.[12] The frequency of *MCPyV* occurrence in SCLC has been not determined and, therefore, we investigated its frequency in 18 SCLC (14 primaries, 4 local lymph node metastases) and in 18 blood control samples (Fig. 1 and Table 1). Visible PCR products obtained by *MCPyV*-specific PCR amplification were classified as Merkel cell polyomavirus-positive samples (Fig. 1). The integrity of DNA was controlled by amplification of a genomic region of *GAPDH* (Fig. 1). We found *MCPyV* occurrence in 7 of 18 (39%) of the tumour tissues (Fig. 1, Table 1). Control samples were not positive ($p < 0.001$, Fisher's exact test). *SV40* was not detected in all SCLCs and blood controls (Table 1). Our results show presence of *MCPyV* in primary SCLC, but not in blood controls.

3.2. Frequent hypermethylation of RASSF1A and p16 in SCLC

It has been observed that polyomavirus infection (e.g. SV40) and promoter hypermethylation of *RASSF1A* correlate in different cancer entities.[5–7] Therefore, we investigated the methylation status of CpG island promoter of *RASSF1A* in 14 primary SCLCs, 4 local lymph node metastases and blood controls by methylation-specific PCR (Fig. 2 and Table 1). Interestingly, we observed frequent hypermethylation of the *RASSF1A* promoter in 17 of 18 (94%) of the investigated tumour tissues (controls without hypermethylation; $p < 0.001$, Fisher's exact test). Methylation of *p16* was less frequent and detected in 10 of 18 (56%) of the tumour tissue and in 2 of 18 (11%) blood controls ($p = 0.001$). No correlation was observed between promoter hypermethylation (*RASSF1A* or *p16*) and *MCPyV* presence ($r_s = -0.316$, $p = 0.20$, $n = 18$ or $r_s = -0.149$, $p = 0.55$, $n = 18$, respectively, Spearmańs rank correlation).

4. Discussion

In our study, we investigated the occurrence of *MCPyV* in small cell lung cancer. Recently, Feng and colleagues demonstrated the presence of *MCPyV* in Merkel cell carcinoma (MCC).[12] Similar to SCLC, MCC is a very aggressive neuroendocrine cancer with high mortality. Interestingly, MCC shows genetic alterations comparable to SCLC.[8–10] Our study is the first to report the presence of MCPyV in SCLC. Occurrence of *MCPyV* in SCLC is less frequent compared to that of MCC. It has been reported that an *MCPyV* infection is present in 77–85% of MCCs.[11,12,14] However, it has also been shown that *MCPyV* is less frequently present in Australian MCCs (24%).[13] Our findings might suggest that MCPyV also plays a role in tumourigenesis of SCLC. Nevertheless, further validation is needed before the role of MCPyV in SCLC genesis can be differentiated from an infection without pathogenetical significance.

Table 2 – Primers and conditions for analyses.

	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	T_A in °C	Cycles	Product size (bp)
RASSF1A	M: GTGTTAACGCGTTGCGTATC	M: AACCCCGCGAACTAAAAACGA	60	40	93
	U: TTTGGTTGGAGTGTGTTAATGTG	U: CAAACCCCACAAACTAAAAACAA	60	40	105
p16	M: TTATTAGAGGGTGGGGCGGATCGC	M: GACCCCGAACCGCGACCGTAA	65	40	150
	U: TTATTAGAGGGTGGGGTGGATTGT	U: CAACCCCAAACCACAACCATAA	60	40	151
MCPyV	S: ACTTGGGAAAGTTTTGACTGGTGGCAA	AS: GGGCCTCGTCAACCTAGATGGGAAAG	63	40	195
SV40	S: CAGTTGCATCCCAGAAGCCTCCAA	AS: TCTTGAAAGGAGTGCCTGGGGGAA	63	34	191
GAPDH	S: TGATGCCCCCATGTTCGTCAT	AS: GACCTTGGCCAGGGGTGCTA	59	34	204

M: methylation-specific primer; U: unmethylation-specific primer; S: sense primer; AS: antisense primer; T_A: annealing temperature.

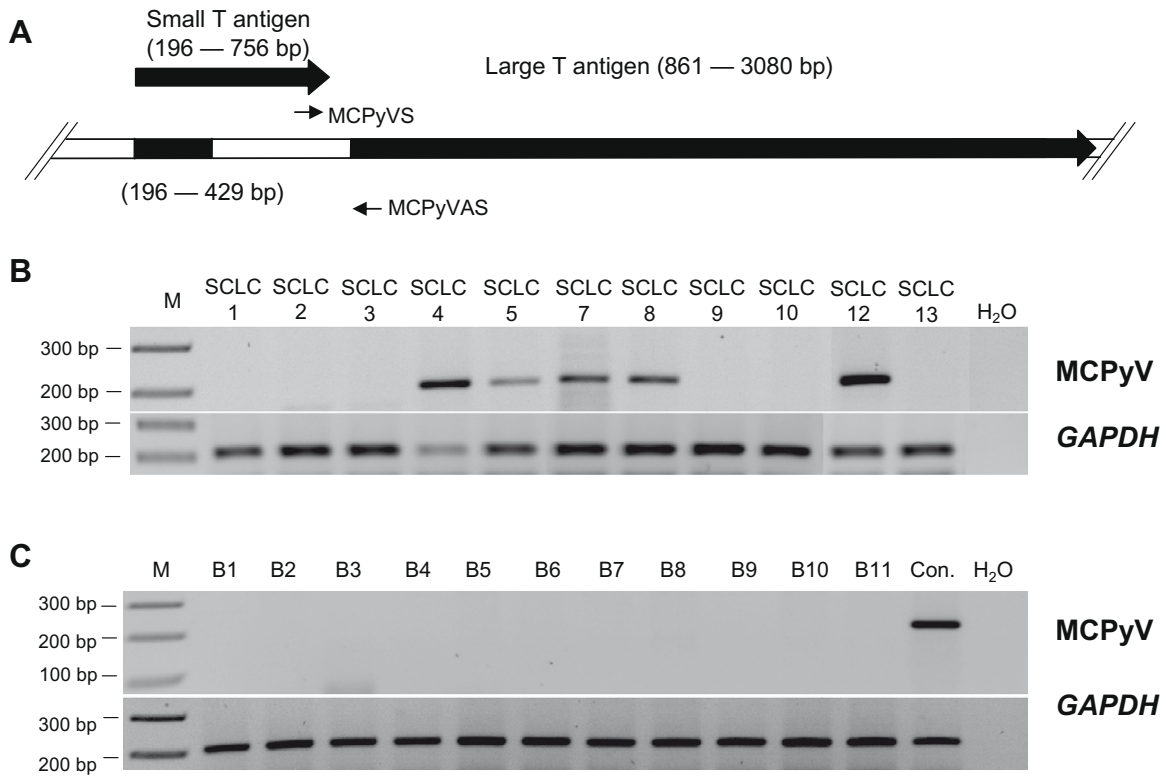

Fig. 1 – Merkel cell polyomavirus (MCPyV) specific PCR. (A) Part of the map of MCPyV genome and location of primers MCPyVS (sense) and MCPyVAS (antisense). (B) DNA extracted from small cell lung cancer (SCLC) was amplified by PCR together with a water control (H_2O). PCR products (195 bp) were analysed in a 2% Tris–borate EDTA agarose gel together with a 100 bp ladder (Marker). Integrity of DNA was confirmed by amplification of *GAPDH* (204 bp). (C) DNA isolated from blood controls (B) and a Merkel cell carcinoma (con.) were analysed by PCR for MCPyV and *GAPDH*.

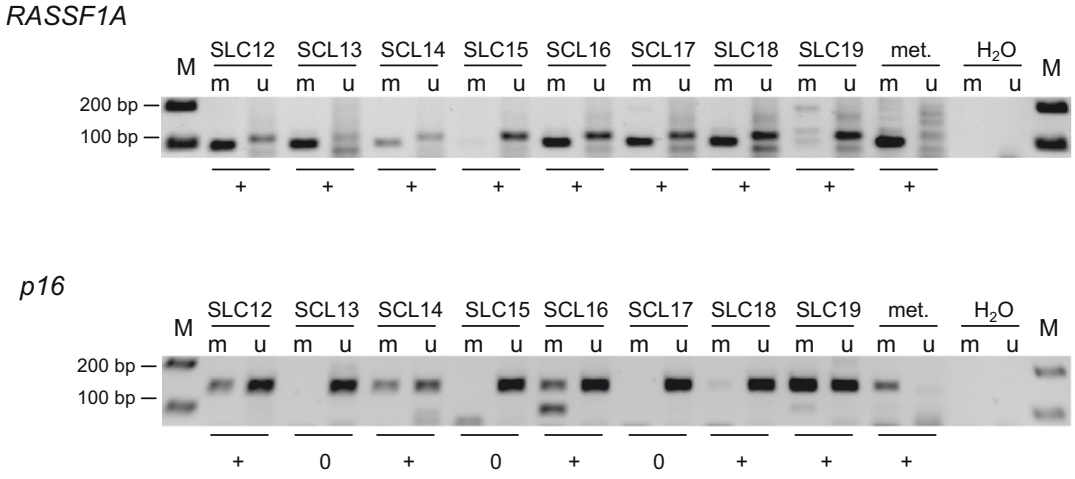

Fig. 2 – Methylation analysis of *RASSF1A* and *p16*. DNA isolated from small cell lung cancer (SCLC) and *in vitro* methylated DNA (met.) were treated with bisulphite and amplified by methylation-specific PCR. Methylation (m)- and unmethylation (u)-specific PCR products of *RASSF1A* (93 bp and 105 bp, respectively) and *p16* (150 bp and 151 bp, respectively) were resolved on a 2% TBE gel together with a negative control (H_2O) and a 100 bp ladder (M).

RASSF1A is a prominent epigenetically tumour suppressor gene and it regulates apoptosis, microtubule stability and cell cycle.[18–20] *RASSF1A* inactivation was previously reported in SCLC.[4,21,22] Here we confirmed this finding and found a methylated *RASSF1A* promoter in 94% of SCLCs. It has been suggested that *RASSF1A* methylation is a promising biomarker

for lung cancer diagnosis.[23,24] It has been reported that infection with SV40 is associated with hypermethylation of *RASSF1A* in malignant mesothelioma, prostate cancer and breast cancer.[5–7] However, no SV40 infection was observed in SCLC and the presence of *MCPyV* and *RASSF1A* hypermethylation did not correlate. This suggests that *RASSF1A* silencing is not necessarily due to MCPyV infection in SCLC. It has been reported that *RASSF1A* hypermethylation is associated with aberrant expression of an isoform of DNMT3B in lung cancer.[25] Another tumour suppressor gene inactivated in SCLC is the cell cycle inhibitor *p16*.[22] We found frequent inactivation of *p16* in 56% of SCLC, but also in 11% of blood controls.

In summary, we report on frequent hypermethylation of *RASSF1A* and detection of *MCPyV* in SCLC. To our knowledge, this is the first study that analysed MCPyV infection in SCLC. Our data indicate that Merkel cell polyomavirus infection may contribute to the pathogenesis of SCLC.

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Conflict of interest statement

None declared.

REFERENCES

1. Herbst RS, Heymach JV, Lippman SM. Lung cancer. *N Engl J Med* 2008;**359**:1367–80.
2. Owonikoko TK, Ramalingam S. Small cell lung cancer in elderly patients: a review. *J Natl Compr Cancer Netw* 2008;**6**:333–44.
3. Hosoe S, Shigedo Y, Ueno K, et al. Detailed deletion mapping of the short arm of chromosome 3 in small cell and non-small cell carcinoma of the lung. *Lung Cancer* 1994;**10**:297–305.
4. Dammann R, Takahashi T, Pfeifer GP. The CpG island of the novel tumor suppressor gene RASSF1A is intensely methylated in primary small cell lung carcinomas. *Oncogene* 2001;**20**:3563–7.
5. Hachana M, Trimeche M, Ziadi S, Amara K, Korbi S. Evidence for a role of the Simian Virus 40 in human breast carcinomas. *Breast Cancer Res Treat* 2008.
6. Liu L, Zhang J, Bates S, et al. A methylation profile of in vitro immortalized human cell lines. *Int J Oncol* 2005;**26**:275–85.
7. Toyooka S, Pass HI, Shivapurkar N, et al. Aberrant methylation and simian virus 40 tag sequences in malignant mesothelioma. *Cancer Res* 2001;**61**:5727–30.
8. Popp S, Waltering S, Herbst C, Moll I, Boukamp P. UV-B-type mutations and chromosomal imbalances indicate common pathways for the development of Merkel and skin squamous cell carcinomas. *Int J Cancer* 2002;**99**:352–60.
9. Van Gele M, Boyle GM, Cook AL, et al. Gene-expression profiling reveals distinct expression patterns for classic versus variant Merkel cell phenotypes and new classifier genes to distinguish Merkel cell from small-cell lung carcinoma. *Oncogene* 2004;**23**:2732–42.
10. Van Gele M, Speleman F, Vandesompele J, Van Roy N, Leonard JH. Characteristic pattern of chromosomal gains and losses in Merkel cell carcinoma detected by comparative genomic hybridization. *Cancer Res* 1998;**58**:1503–8.
11. Becker JC, Houben R, Ugurel S, Trefzer U, Pfohler C, Schrama D. MC polyomavirus is frequently present in Merkel cell carcinoma of European patients. *J Invest Dermatol* 2008.
12. Feng H, Shuda M, Chang Y, Moore PS. Clonal integration of a polyomavirus in human Merkel cell carcinoma. *Science* 2008;**319**:1096–100.
13. Garneski KM, Warcola AH, Feng Q, Kiviat NB, Leonard JH, Nghiem P. Merkel cell polyomavirus is more frequently present in North American than Australian Merkel cell carcinoma tumors. *J Invest Dermatol* 2008.
14. Kassem A, Schopflin A, Diaz C, et al. Frequent detection of Merkel cell polyomavirus in human Merkel cell carcinomas and identification of a unique deletion in the VP1 gene. *Cancer Res* 2008;**68**:5009–13.
15. Travis WD, Colby TV, Corrin B, Shimosato Y, Brambilla E. Histological Typing of lung and pleural tumours. 3rd ed. Berlin: World Health Organization, Springer; 1999.
16. Schagdarsurengin U, Gimm O, Hoang-Vu C, Dralle H, Pfeifer GP, Dammann R. Frequent epigenetic silencing of the CpG island promoter of RASSF1A in thyroid carcinoma. *Cancer Res* 2002;**62**:3698–701.
17. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996;**93**:9821–6.
18. Agathanggelou A, Cooper WN, Latif F. Role of the Ras-association domain family 1 tumor suppressor gene in human cancers. *Cancer Res* 2005;**65**:3497–508.
19. Dammann R, Schagdarsurengin U, Seidel C, et al. The tumor suppressor RASSF1A in human carcinogenesis: an update. *Histol Histopathol* 2005;**20**:645–63.
20. Richter AM, Pfeifer GP, Dammann RH. The RASSF proteins in cancer; from epigenetic silencing to functional characterization. *Biochim Biophys Acta* 2009.
21. Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet* 2000;**25**:315–9.
22. Toyooka S, Toyooka KO, Maruyama R, et al. DNA methylation profiles of lung tumors. *Mol Cancer Ther* 2001;**1**:61–7.
23. Wang Y, Yu Z, Wang T, Zhang J, Hong L, Chen L. Identification of epigenetic aberrant promoter methylation of RASSF1A in serum DNA and its clinicopathological significance in lung cancer. *Lung cancer* 2007;**56**:289–94.
24. Grote HJ, Schmiemann V, Geddert H, et al. Methylation of RAS association domain family protein 1A as a biomarker of lung cancer. *Cancer* 2006;**108**:129–34.
25. Wang J, Bhutani M, Pathak AK, et al. Delta DNMT3B variants regulate DNA methylation in a promoter-specific manner. *Cancer Res* 2007;**67**:10647–52.