Tumor Biol. (2012) 33:287–296
DOI 10.1007/s13277-011-0282-2

RESEARCH ARTICLE

DNA methylation biomarkers for lung cancer

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Received: 30 September 2011 / Accepted: 21 November 2011 / Published online: 6 December 2011

Abstract Changes in DNA methylation patterns are an important characteristic of human cancer including lung cancer. In particular, hypermethylation of CpG islands is a signature of malignant progression. Methylated CpG islands are promising diagnostic markers for the early detection of cancer. However, the full extent and sequence context of DNA hypermethylation in lung cancer has remained unknown. We have used the methylated CpG island recovery assay and high-resolution microarray analysis to find hypermethylated CpG islands in squamous cell carcinomas (SCC) and adenocarcinomas of the lung. Each tumor contained several hundred hypermethylated CpG islands. In an initial microarray screen, 36 CpG islands were methylated in five of five (=100%) of the SCC tumors tested and 52 CpG islands were methylated in at least 75% of the adenocarcinomas tested (n=8). Using sodium-bisulfite-based approaches, 12 CpG islands (associated with the *BARHL2, EVX2, IRX2, MEIS1, MSX1, NR2E1, OC2, OSR1, OTX1, PAX6, TFAP2A,* and *ZNF577* genes) were confirmed to be methylated in 85% to 100% of the squamous cell carcinomas and 11 CpG islands (associated with the *CHAD, DLX4, GRIK2, KCNG3, NR2E1, OSR1, OTX1, OTX2, PROX1, RUNX1,* and *VAX1* genes) were methylated in >80% of the adenocarcinomas. From the list of genes that were methylated in lung adenocarcinomas, we identified the gene *FAT4* and found that this gene was methylated in 39% of the tumors. FAT4 is the closest mammalian homologue of the *Drosophila* tumor suppressor Fat which is an important component of the Hippo growth control pathway. Many of these newly discovered methylated CpG islands hold promise for becoming biomarkers for the early detection of lung cancer.

Keywords DNA methylation · CpG islands · Methylation marker · Lung cancer · FAT4

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Introduction

5-Methylcytosine, present at 70–80% of all CpG dinucleotides, is the major modified base found in mammalian DNA. It has been known for some time that the level of 5-methylcytosine is globally reduced in tumor tissues relative to corresponding normal tissues [14, 16, 36], mostly due to hypomethylation of different classes of repetitive DNA sequences [34]. It was also observed that gene-specific hypermethylation at CpG-rich, so-called CpG-island sequences occurs specifically in cancer tissues [2]. CpG island hypermethylation is a phenomenon commonly observed during the development and progression of human tumors [21]. In the 1990s, researchers reported hypermethylation of CpG

islands of several known tumor suppressor genes and other genes involved in important growth control or genome defense pathways, for example DNA repair genes [8, 12, 17, 20, 21, 23, 24, 27]. Today, there are many reports documenting methylation of CpG islands associated with a large number of different genes, including almost every type of human malignancy. In lung cancer, many specific CpG islands are methylated, for example those associated with the genes *CDKN2A*, *RASSF1A*, *RARbeta*, *MGMT*, *GSTP1*, *CDH13*, *APC*, *DAPK*, *TIMP3*, and a number of others [1, 9, 11, 42, 47, 49]. The methylation frequency of a particular CpG island (defined as the percentage of tumors analyzed in a study that carry methylated alleles) generally ranges from less than 10% to over 80% of the tumors depending on the histological subtype of tumor, the study population, and/or the methodology used to assess and quantitate DNA methylation.

Detection of methylated CpG islands in accessible biological materials such as serum or sputum has the recognized potential to be useful for the early detection and diagnosis of cancer, including lung cancer [3, 25, 43]. Highly sensitive techniques that can clearly detect the methylated tumor-associated DNA fragments among a large excess of unmethylated molecules are necessary to accomplish this goal. Most useful as DNA methylation markers would only be those genes that show close to background levels of methylation in normal human tissues and those that are methylated in a large fraction of the tumors.

Current research approaches in cancer epigenetics are focused on the characterization of the complete set of DNA methylation changes in cancer. Several techniques have been introduced and have been summarized comprehensively [13]. We recently developed a useful method, the methylated CpG island recovery assay (MIRA) that—unlike most other approaches—does not depend on the use of sodium bisulfite, restriction enzymes, or antibodies for identifying the methylated regions [31]. The MIRA method is based on the high affinity of the MBD2b/MBD3L1 protein complex for methylated CpG dinucleotides. For efficient pull-down of methylated DNA by this method, two or more methylated CpG sites in a DNA fragment of 50 base pairs or less are required [32]. MIRA is compatible with microarray analysis [32] or high-throughput DNA sequencing [35]. We have used the MIRA method in combination with high-resolution CpG island microarrays to characterize the full extent of DNA methylation changes that occur at CpG islands in non-small cell lung cancer tissues.

DNA methylation analysis of lung cancer

To analyze tumor-associated DNA methylation changes, we compared stage I lung squamous cell carcinomas (SCCs) or adenocarcinomas (AC) to normal matched lung tissues [34]. We used the MIRA-assisted microarray method for DNA methylation analysis [32, 33]. MIRA-enriched methylated DNA fractions obtained from tumor tissue and from matching normal lung tissue removed with surgery were analyzed on Agilent CpG island arrays covering a total of 27,800 CpG islands.

A complete set of hypermethylated CpG islands and discovery of new DNA methylation biomarkers

Five stage I squamous cell carcinomas and eight stage I adenocarcinomas of the lung were analyzed on these arrays along with matched normal lung. Using the criteria and cutoffs defined previously [34], the number of methylated CpG islands ranged from 216 to 744 in the five individual squamous cell tumors (Table 1). For adenocarcinomas, between 219 and 908 CpG islands were methylated per tumor (Table 1).

Squamous cell carcinomas

Using MIRA-assisted microarray analysis, we identified 36 CpG islands that were methylated in all of the five SCC tumors analyzed (Fig. 1 and Table 2). A large fraction of the methylated CpG islands were mapped to homeobox genes in the genome. Since these 36 loci had excellent potential to be specific and sensitive methylation biomarkers for SCC, we analyzed 12 randomly chosen markers (*BARHL2, EVX2, IRX2, MEIS1, MSX1, NR2E1, OC2, OSR1, OTX1, PAX6, TFAP2A,* and *ZNF577*) in a larger series of 20 SCCs [34] by bisulfite-based COBRA assays [46]. This assay is semi-quantitative, technically robust and is commonly used for

Table 1 Number of methylated CpG islands in stage I lung adenocarcinoma (AC) and squamous cell carcinomas (SCC)

Sample	Methylated CpG islands[a]
AC1	408
AC2	219
AC3	315
AC4	319
AC5	260
AC6	355
AC7	447
AC8	908
SCC1	245
SCC2	633
SCC3	744
SCC4	216
SCC5	608

[a] See Rauch et al. [34]

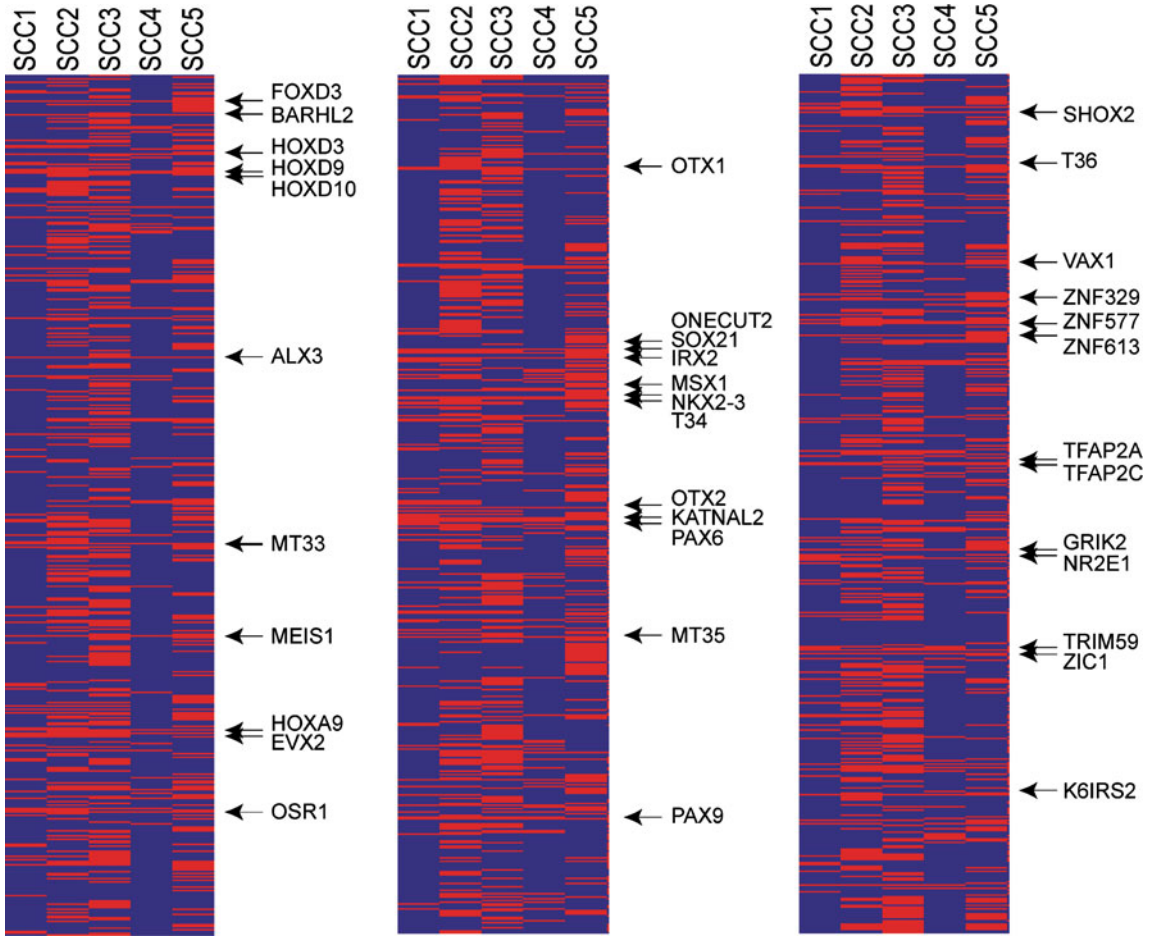

Fig. 1 Methylation of CpG islands in lung squamous cell carcinomas. The *red bars* indicate methylation of individual CpG islands across a series of five stage I lung squamous cell carcinomas. The CpG islands methylated in all five tumors are marked by *arrows*

assessing the methylation status of CpG islands in smaller to medium size sample series; this method has a very low rate of false positive results. The methylation frequency of the individual markers ranged from 17/20 (=85%) to 20/20 (=100%) of the tumors. The *OTX1* and *NR2E1* associated CpG islands were methylated in all SCC tumors tested (=100%). Several of these SCC markers were highly specific for tumor-associated methylation, i.e., little or no detectable methylation was observed in tumor-adjacent normal lung tissue or in the lungs of non-tumor patients. These included the CpG islands of the *OTX1*, *BARHL2*, *MEIS1*, *OC2*, *TFAP2A*, and *EVX2* genes [34]. None of these CpG islands was methylated substantially in white blood cell DNA from healthy individuals.

Adenocarcinomas

Using MIRA-assisted microarray analysis, we identified 52 CpG islands that were methylated in at least six out of eight adenocarcinomas (Table 3). Several of these adenocarcinoma methylation markers (*CHAD, DLX4, GRIK2, KCNG3, NR2E1, OSR1, OTX1, OTX2, PROX1, RUNX1,* and *VAX1*) were chosen at random for verification by bisulfite-based COBRA assays. These selected adenocarcinoma markers were methylated in more than 80% of the tumors (Fig. 2). The *CHAD* gene was methylated in 8 of 11 tumors tested (data not shown). None of these CpG islands was substantially methylated in blood DNA from healthy individuals or in non-cancerous lung DNA. The tumor specificity and high methylation frequency of these genes makes them excellent candidates for use in future diagnostic applications for early detection of lung cancer.

Methylation of *FAT4* encoding a component of the Hippo tumor suppressor pathway in lung adenocarcinomas

One of the methylated targets in adenocarcinomas and SCCs was the gene *FAT4*. To confirm the microarray results, we

Table 2 List of hypermethylated CpG islands as potential markers for stage 1 lung squamous cell carcinoma (SCC)

Location in hg18			Position relative to known genes			
Chr	Start	End	Upstream	Promoter	Intragenic	Downstream
chr1	47682300	47683607				FOXD2
chr1	63554983	63563059			FOXD3	
chr1[a]	90955098	90955445		BARHL2		
chr1	90963078	90965392	BARHL2			
chr1	110411789	110414826			ALX3	
chr1	119350668	119352843				
chr2[a]	19419271	19421884		OSR1		
chr2	20733488	20734761			GDF7	
chr2	24251150	24251698		FLJ30851		
chr2[a]	63134539	63134851			OTX1	EHBP1
chr2[a]	66525936	66527140			MEIS1	
chr2	66662073	66662908				MEIS1
chr2	80383189	80384357			LRRTM1	
chr2[a]	176652334	176656692	HOXD13		EVX2	
chr2	176672309	176673755	HOXD11		HOXD12	HOXD13
chr2	176689012	176689669	HOXD10			HOXD11
chr2	176690354	176690648	HOXD9		HOXD10	HOXD11
chr2	176694671	176696537	HOXD8	HOXD9		HOXD10
chr2	176737660	176738187			HOXD3	
chr3	148591199	148594390			ZIC4	
chr4	1386292	1391730				FLJ34443
chr4[a]	4910534	4911092	MSX1			
chr4	174686622	174688044		HAND2		
chr5[a]	2791954	2794237				IRX2
chr5	3647468	3656054			IRX1	
chr5	54554812	54555385				UNG2
chr5	72629904	72631564				
chr5	140790679	140792801		PCDHGA12		
chr6	10489545	10490340				
chr6[a]	10498025	10498551				TFAP2A
chr6	10530308	10530634	TFAP2A			MGC40222
chr6	101953488	101953856		GRIK2		
chr6[a]	108592365	108597232		NR2E1		
chr7[a]	27170441	27172987	HOXA7	HOXA9		HOXA10
chr7	27219207	27220360				
chr7	121743780	121744577				CADPS2
chr7	153214251	153216599		DPP6		
chr7	154857319	154860615				
chr8	100054910	100056159				
chr9	959530	963276	DMRT3			DMRT1
chr9	125813068	125820774		LHX2		
chr10	94170296	94170734				
chr10	118882152	118882629			VAX1	
chr11[a]	31783382	31783583			PAX6	
chr12	52726910	52727810	HOXC4		HOXC4	

Table 2 (continued)

Location in hg18			Position relative to known genes			
Chr	Start	End	Upstream	Promoter	Intragenic	Downstream
chr12	60871036	60872535			FAM19A2	
chr13	94152191	94153185				SOX21
chr14	36205265	36206099			PAX9	
chr14	56344361	56346593			OTX2	
chr14	60045486	60047933		SIX6		
chr15	77511155	77512698		KIAA1024		
chr15	87750378	87752134				
chr15	87753276	87754065				
chr18	53170706	53172603		ST8SIA3		
chr18[a]	53254153	53259851			ONECUT2	
chr20	54012011	54014085		CBLN4		
chr21	36990064	36995761		SIM2		
chrX	136459743	136460985				

Chromosome coordinates are according to the UC Santa Cruz Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway) March 2006 assembly (hg18). These CpG islands were methylated in five of five stage I SCCs according to the Agilent CpG island microarray data [34]

[a] Methylation status of these CpG islands was verified by COBRA assays

analyzed methylation of the *FAT4*-associated promoter CpG island in 18 stage I adenocarcinomas and matched normal lung tissue (Fig. 3a). *FAT4* was methylated in 7/18 tumors (=39%). We also confirmed that *FAT4* is silenced in a series of non-small cell lung tumors compared to corresponding normal tissue when a cDNA panel from Origene, which contains various types of non-small cell lung tumors of different stages (stage I and II), was used. According to this RT-PCR analysis, expression of *FAT4* was reduced in 18 of 23 stages I and II lung tumors relative to matched normal lung tissue (Fig. 3b) suggesting that methylation of this gene is perhaps only one mechanism leading to reduced expression in tumors. *FAT4* encodes the closest mammalian homologue of the *Drosophila* Fat protocadherin (37% identical at the amino acid level). Fat is an upstream component of the Hippo pathway in flies [6]. The Hippo pathway is a signaling cascade involved in organ size control, tumor suppression, and apoptosis [29, 39]. Mutations of this pathway in mice has been linked to tumorigenesis [26, 40, 48]. However, it has been difficult to clearly demonstrate functional inactivation of this tumor suppressor pathway in human tumors. Mutations in Hippo pathway components, such as the MST and LATS kinases, are very infrequent. Epigenetic inactivation is seen most commonly for RASSF1A, a regulator of MST kinases [18, 19, 30] and sometimes for *MST* genes themselves [38]. In lung tumors, *RASSF1A* is most frequently inactivated by promoter methylation in small cell lung cancers [10]. In non-small cell lung cancers, *RASSF1A* methylation occurs at a frequency of 30–40% in adenocarcinomas and squamous cell carcinomas [5, 9]. We have not yet tested if methylation of *RASSF1A* and *FAT4*, both being upstream regulators of the Hippo pathway, are mutually exclusive or cooperate in lung tumors. Interestingly, *Fat4* has recently been identified as a susceptibility gene for pulmonary adenomas in mice [4]. Thus, methylation of the promoter of *FAT4* may have functional consequences for tumor initiation or progression in the lung. In this sense, methylation of *FAT4* can be considered a potential epigenetic "driver" event for tumorigenesis rather than just a "passenger" event that would be without functional consequences [22].

DNA methylation markers for lung cancer

The novel aspect of our work has been the comprehensive methylation analysis of all CpG islands in human lung cancer using microarrays [32–34]. We were able to directly measure the methylation levels at over 27,000 CpG islands and found that between approximately 200 and 900 of these islands were methylated in individual lung SCC and AC samples (Table 1). These numbers are compatible with earlier estimates derived from analysis of only a subset of CpG islands methylated in cancer [7]. It is clear, of

Table 3 Methylation markers for lung adenocarcinoma

Methylated CpG island	Positive tumors (total of 8)[a]	Gene	Region
chr1:110411789-110414826	8	ALX3	Promoter CGI
chr2:131513833-131514558	6	ARHGEF4	Exon CGI
chr1:90963078-90965392	6	BARHL2	Upstream CGI
chr17:45900570-45901899	7	CHAD[b]	Promoter CGI
chr17:45403953-45405615	6	DLX4	Alternative promoter CGI
chr2:72996564-73001768	6	EMX1	Promoter CGI
chr19:60283718-60285792	6	EPS8L1	Alternative promoter CGI
chr3:129688190-129694969	6	GATA2	Promoter CGI
chr6:101953487-101953856	6	GRIK2[b]	Promoter CGI
chr3:142998750-142999334	6	GRK7	Intron CGI
chr2:176672309-176673755	6	HOXD12	Coding region
chr2:176854920-176855448	6	HOXD3	1st intron
chr5:3647467-3656054	6	IRX1	Promoter and gene
chr3:42702088-42702920	6	KBTBD5	Promoter CGI
chr2:42573289-42575670	6	KCNG3[b]	Promoter CGI
chr1:196157102-196157447	7	LHX9	Exon 4
chr13:34947570-34948159	7	MAB21L1	Coding region
chr14:36122288-36122589	7	NKX2-8	Upstream CGI
chr6:108592364-108597232	6	NR2E1[b]	Promoter CGI
chr2:19419271-19421884	8	OSR1[b]	Promoter CGI
chr2:63134539-63134851	7	OTX1[b]	Exon 4
chr2:63127980-63132934	6	OTX1	Promoter CGI
chr2:63136019-63136626	6	OTX1	Last exon
chr14:56344360-56346593	8	OTX2[b]	1st intron
chr14:36205264-36206099	7	PAX9	Exon 3
chr4:30330303-30333940	7	PCDH17	Promoter CGI
chr5:134390991-134393045	8	PITX1	Last exon
chr4:111758678-111758932	6	PITX2	Last exon
chr1:212225350-212225703	6	PROX1[b]	Upstream CGI
chr5:40715259-40717838	6	PTGER4	Promoter CGI
chr21:35320830-35321129	7	RUNX1[b]	1st intron
chr14:60178707-60179539	6	SIX1	Downstream CGI
chr2:45085286-45086054	6	SIX2	Promoter CGI
chr4:48180120-48181230	6	SLC10A4	Promoter CGI
chr18:53170705-53172603	6	ST8SIA3	Promoter CGI
chr17:56827842-56838048	6	TBX2	Promoter CGI
chr6:10518095-10518676	6	TFAP2A	Exon CGI
chr20:54633686-54640196	7	TFAP2C	Promoter CGI
chr10:118885953-118888027	6	VAX1[b]	Promoter CGI
chr19:63407032-63407845	6	ZNF274	intron CGI
chr19:63559209-63560680	6	ZNF497	Last exon
chr19:57082653-57083180	7	ZNF577	Promoter CGI
CpG islands with no known gene association			
chr10:22804714-22807056	6	chr10 CGI	
chr10:119484483-119484981	6	chr10 CGI	
chr13:49599000-49600287	7	chr13 CGI	
chr13:94152190-94153185	7	chr13 CGI	
chr2:45,013,398-45,013,616	6	chr2 CGI	
chr4:24,699,205-24,699,608	7	chr4 CGI	
chr5:54554811-54555385	7	chr5 CGI	
chr6:10498024-10498551	7	chr6 CGI	
chr7:35267676-35268256	7	chr6 CGI	
chr6:30203152-30203589	6	chr6 CGI	

[a]All genome locations are for the human genome build 18 (March 2006) of the UC Santa Cruz Genome Browser

[b]Genes with COBRA verification data

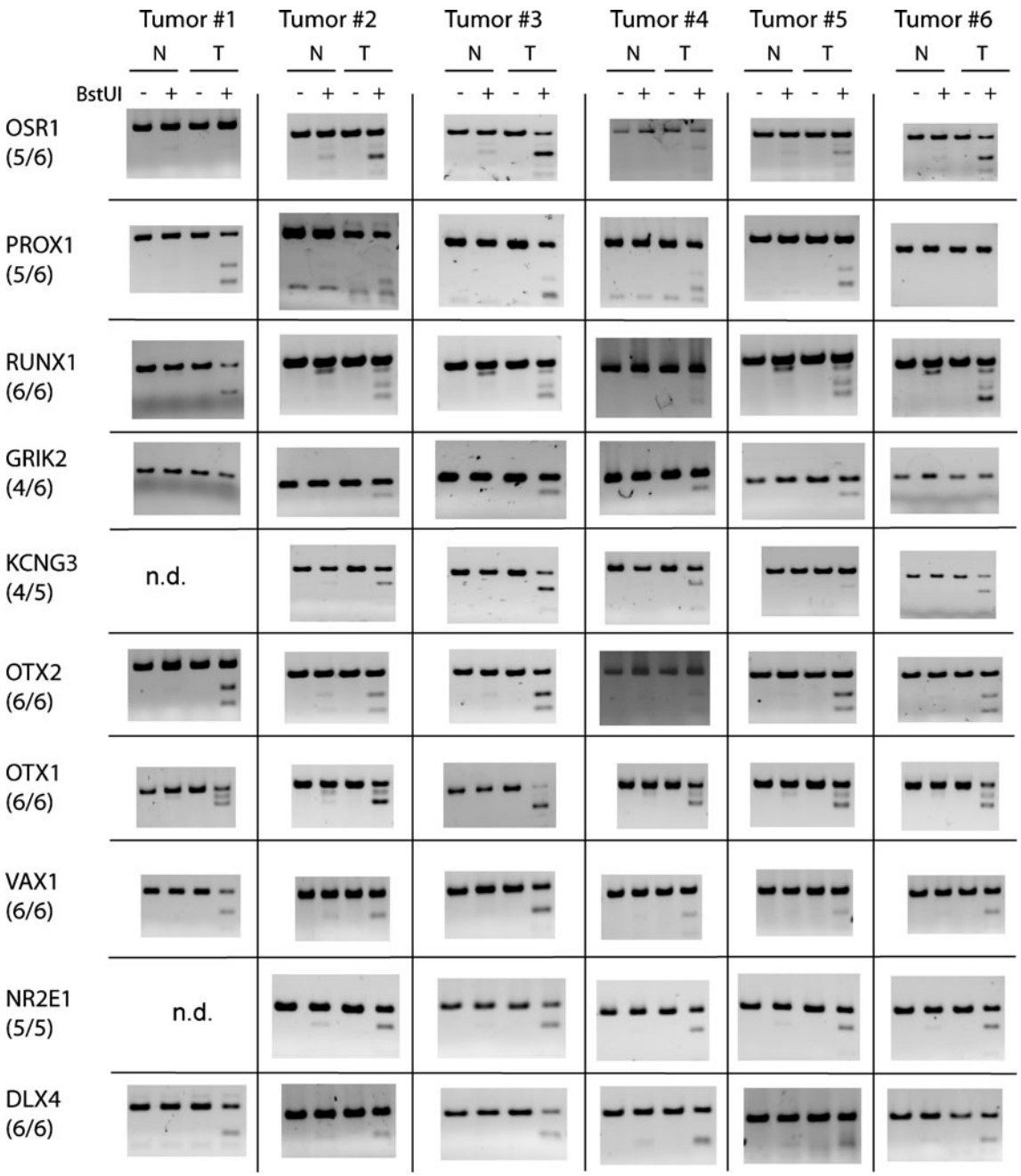

Fig. 2 Verification of DNA methylation markers in normal lung tissue and in matching adenocarcinoma samples. Methylation differences between adenocarcinomas (*T*) and matching normal pairs (*N*) were detected by COBRA assays for the indicated gene targets. "–" refers to control digestion with no BstUI, "+" refers to BstUI-digested samples. Digestion by BstUI indicates methylation of the sequence tested (5′CGCG). The indicated CpG islands were analyzed (see Table 3 for chromosomal location of the CpG islands)

course, that not all of these genes can be tumor suppressor genes or in other ways can be driver events for tumorigenesis. For example, our earlier observations indicated that a substantial subset of the methylated genes (20–40% depending on the individual tumor) were homeobox genes [32, 33]. Homeobox genes are regulated by the Polycomb complex, and it is a common observation that Polycomb-associated genes are frequently methylated in cancer [28, 33, 37, 41, 44, 45]. Homeobox gene-associated CpG islands were among the most useful DNA methylation markers identified in lung cancer. The CpG islands of the *OTX1*, *BARHL2*, *MEIS1*, *OC2*, *TFAP2A*, and *EVX2* genes were tumor-specifically methylated in SCC with little or no detectable methylation seen in normal lung tissue or in blood DNA

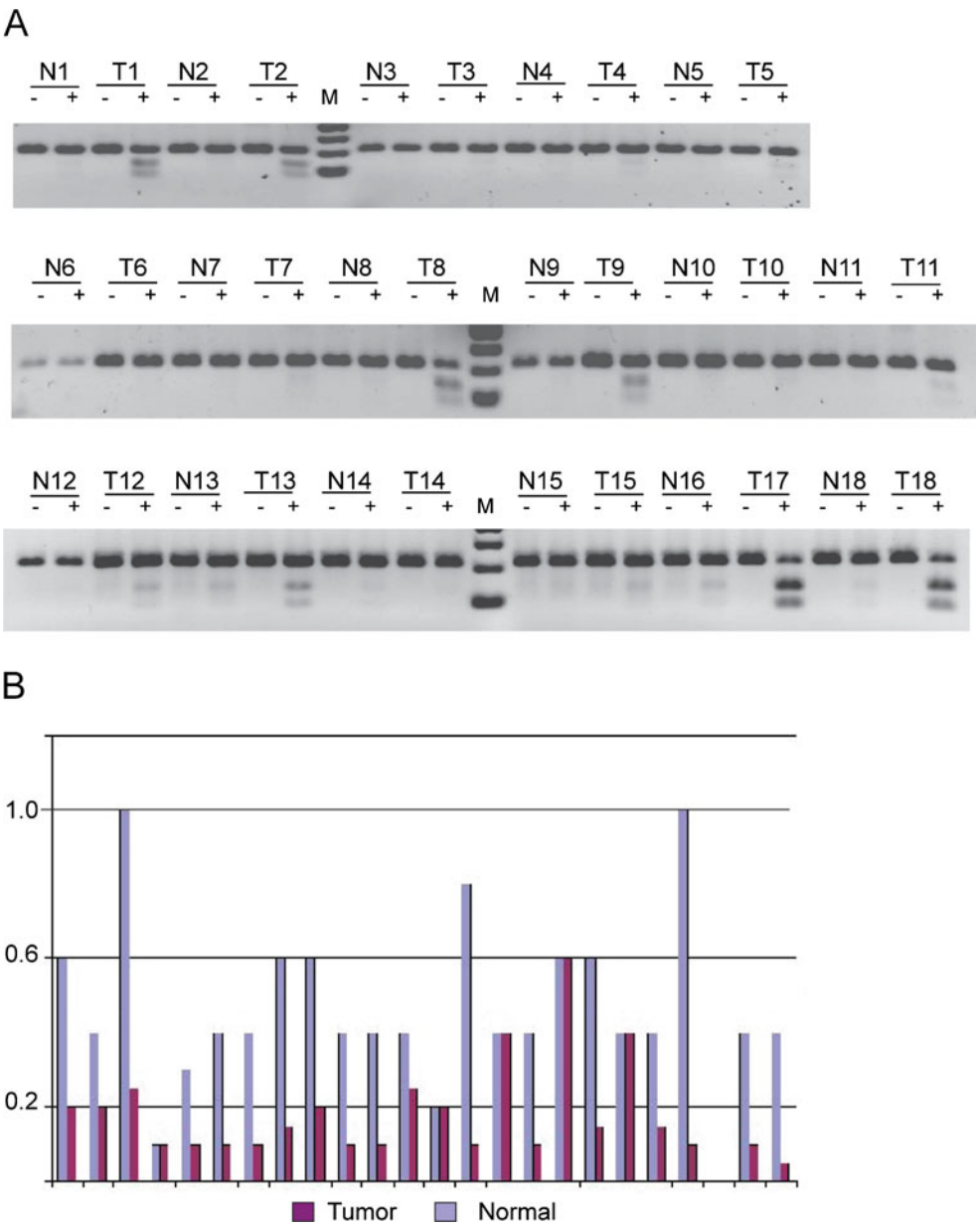

Fig. 3 Methylation of the *FAT4* gene in lung adenocarcinomas. **a** Methylation of the *FAT4* promoter-associated CpG island was tested in 18 stage I lung adenocarcinomas (*T*) and matched normal lung tissue (*N*). Methylation differences were detected by COBRA assays. "–" refers to control digestion with no BstUI, "+" refers to BstUI-digested samples. Digestion by BstUI indicates methylation of the sequence tested (5′CGCG), which was found in seven of the tumors. **b** *FAT4* silencing in a series of non-small cell lung tumors. Normalized cDNA from 23 paired lung tumor/normal samples were tested with TaqMan qRT-PCR and the expression level of *FAT4* was derived from comparing with β-actin internal control with two highly expressed samples set as 1

[34]. Importantly, the methylation frequency of these markers (85% to 100% of the tumors were methylated) is much higher than methylation frequencies of most other lung cancer DNA methylation markers reported previously. For example, *OTX1* was tumor specifically methylated in 20/20 (=100%) of the SCC tumors. For adenocarcinomas, several promising markers have been identified including *CHAD, DLX4, GRIK2, KCNG3, NR2E1, OSR1, OTX1, OTX2, PROX1, RUNX1,* and *VAX1*. Methylation of these genes in lung cancer has not yet been reported, except for RUNX1 [15]. Very commonly, frequently methylated genes are methylated in both subtypes of non-small cell lung cancers. For example, the CpG islands associated with the *NR2E1, OSR1,* and *OTX1* genes were methylated in both adenocarcinomas and squamous cell carcinomas at a frequency of over 95%. These markers are excellent candidates for future clinical or diagnostic applications aimed at either detection of early disease in body fluids such as blood or sputum, or at disease management and follow-up using molecular diagnostic testing.

Acknowledgments This work was supported by NIH grant CA084469 and a grant from the University of California Tobacco Related Disease Research Program (TRDRP) (to G.P.P.)

Conflicts of interest Under a licensing agreement between City of Hope and Active Motif (Carlsbad, CA), the methylated CpG island recovery assay (MIRA) technique was licensed to Active Motif, and the authors T.A.R. and G.P.P. are entitled to a share of the royalties received by City of Hope from sales of the licensed technology.

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