Measuring the METH-2 promoter hypermethylation and transcript downregulation in non-small cell lung carcinomas with the Agilent 2100 bioanalyzer

Application Note

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Abstract

The antiangiogenetic factor METH-2 (ADAMTS-8) was identified by a previous dual-channel cDNA microarray analysis to be at least two-fold under-represented in 85 % of primary non-small cell lung carcinomas (NSCLCs). The Agilent 2100 bioanalyzer was used to validate this observation in an independent series of NSCLCs and adjacent normal tissues by comparative multiplex RT-PCR. This Application Note shows that METH-2 mRNA was dramatically reduced in all 23 tumour samples analysed. DNA methylation analysis of the proximal promoter region of this gene revealed abnormal hypermethylation in 67 % of the adenocarcinomas and 50 % of the squamous cell carcinomas, indicating that epigenetic mechanisms are involved in silencing this gene in NSCLC. In conclusion, the downregulation of METH-2 in NSCLC, often associated with promoter hypermethylation, is a frequent event, which may be related to the development of the disease.

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Introduction

The ability of a tumour to grow more than 2-3 mm^3 relies on the process of angiogenesis, creation of a new blood supply, to facilitate growth by allowing the delivery of nutrients and oxygen to the new tissue (Folkman and Coltran, 1976; Folkman et al 1989). Antiangiogenetic approaches are considered to be possible adjuncts to conventional therapy in the treatment of lung cancer patients.

METH-2 (ADAMTS-8) is a member of the ADAMTS family and was, along with its counterpart METH-1 (ADAMTS-1), fully characterised by Vazquez et al 1999. METH-2 is expressed in various human tissues, exhibiting high levels in the adult and fetal normal lung. The METH-2 protein has a powerful antiangiogenetic effect and can specifically suppress endothelial cell proliferation (Vazquez et al, 1999). To our knowledge, there was no specific report on the involvement of this gene in lung cancer. However, METH-2 was highlighted in a previous study (Heighway et al, 2002) in which a panel of cDNA microarrays comprising 47 650 transcript elements was used to analyse gene expression patterns in 39 ressected primary human non-small-cell lung tumours that were compared to normal lung tissues. The gene was found to be significantly underrepresented (two-fold or lower) in 85 % of the analysed non-small cell tumours. This suggested the possibility that the loss of METH-2 function might be an important factor in tumour angiogenesis.

In this study, the Agilent 2100 bioanalyzer and the DNA 1000 LabChip kit was used to validate our previous observations of METH-2 in an independent series of non-small cell lung carcinomas (NSCLCs). In addition we developed a DNA 1000 LabChip based competitive methylation-specific PCR (cMSP) in order to investigate possible epigenetic abnormalities responsible for this inactivation.

Experimental

Tissues

In all, 12 adenocarcinomas (ADCs) and 11 squamous cell carcinomas (SCCs) were selected from our tumour bank, along with their paired normal tissues. All tumours were classified as T stage = 2. The mean age of the patients was 64. All 23 tissue pairs were used for METH-2 expression analysis. DNA from 27 additional NSCLCs (12 ADC, 15 SCC) was used in the methylation analysis. The following nine lung cancer cell lines obtained from American Type Culture Collection (Rockville, USA) were used in the homozygous deletion screening: adenocarcinoma: A549, SKLU1; squamous: CRL5802, HTB182, SKMES, LUDLU1; small cell: DMS53; large cell: CORL23.

DNA extraction, RNA extraction and cDNA synthesis

Genomic DNA from the set of 27 unpaired tumours was extracted as previously described using standard phenol/chloroform extraction protocol (Liloglou et al. 2004). Total RNA and genomic DNA from the 23 paired samples was extracted from 20 x 40 mm frozen tissue sections using the Qiagen RNA/DNA extraction kit, following the manufacturer's protocol. RNA was divided into 1-2 mg aliquots for subsequent cDNA synthesis in 20-µL reactions using the Promega Reverse Transcription system, using oligo-dT primers. RNA aliquots were stored at –80 °C; cDNA and genomic DNA samples were stored at –20 °C.

Comparative multiplex PCR

To compare the relative expression levels of METH-2 in paired normal and tumour lung cancer tissues, multiplex PCR reactions were set up as described. Two primer pairs were used in the same PCR reaction: METH-2 primers spanning exons 3 and 4 (primer set METH-2-A, for all primers see table 1) and a control gene primer pair (control gene, KIAA0228: amyloid beta precursor protein-binding protein, ABPPBP-2, acc. no. D86981) that had previously been found to be expressed at a constant level in normal and lung cancer tissues (Heighway et al 2002, primer set ABPPBP-2). PCR reactions (50 µL) were set up, containing 1 µL of cDNA, 5 µL of 10 x buffer, 0.5 U of Roche Taq polymerase, 1 µL 1 of dNTPs (5 mM), 1 µL of ABPPBP-2 primer set (10 pMol/µL l), 10 µL of METH-2 primer set (10 pMol/µL), and 32.75 µL ddH20. cDNA was amplified under the following conditions: 95 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 59 °C for 30 s, 72 °C for 30 s and a final extension step of 72 °C for 10 min PCR was optimized (data not shown) in terms of cDNA input and cycles in order to discriminate meaningfull between tissues with high (normal) and low (tumour) expression of METH-2.

Homozygous deletion screening in lung cancer cell lines

To determine whether the chromosomal region containing the METH-2 gene was deleted in lung cancer, 100 ng of genomic DNA was amplified by PCR using METH-2 primers located within exon 2 and intron 2 (primer set METH-2-B). PCR mix was composed of 1 µL of gDNA, 5 µL of 10 x buffer, 0.5 U of Roche Taq polymerase, 1 µL of dNTPs (5 mM), 1 µL primers (10 pMol/mL) and 32.75 mL ddH20. The reaction profile was 95 ºC for 5 min, followed by 30 cycles of 94 ºC for 1 min, 58 ºC for 30 s, 72 ºC for 30 s and a final extension of 72 ºC for 10 min.

Allelic imbalance at the METH-2 locus

Allelic imbalance (AI) in METH-2 was assessed in 23 normal/tumour pairs using the intragenic, intronic single nucleotide polymorphism (SNP) rs1552330. SNP templates were generated by PCR using the same PCR mix and reaction profile as for homozygous deletion screening. PCR primer set was METH-2-C. A 10-µL volume of PCR template was then digested with 5 U BstUI in a total volume of 30 µL for 3 h at 60 ºC. Allelic imbalance was visually assessed as an imbalance of the digested-undigested product ratio in comparison to the normal counterpart.

Methylation analyis

Sodium bisulphite treatment of DNA

A 2-mg portion of genomic DNA was digested with 20 U of HindIII. During a subsequent bisulphite treatment cytosines were stepwise sulfonated and deaminated which converts them to uracil sulphonates. A desulphonation at a basic pH completes the transition from cytosines to uracils. C5-methylated cytosines are not modified under these conditions. A detailed protocol is given at Dunn et al., 2004. DNA was precipitated with ammonium acetate/ethanol, recovered by centrifugation and eluted in 50 mL of 1 mM Tris-HCl and 0.1 mM EDTA (pH 8.0). DNA samples were stored at –20 ºC until further use.

	Name	Gene, Primer-Sequence (5'– 3')	Size
		Comparative multiplex PCR	
1	METH-2-A	Spanning exons 3 and 4	175 bp
	fw	AAC AAA AGC TGC TCC GTG AT	
	rev	TCT GGT TCA GGT GGA CGA AC	
2	ABPPBP-2	Amyloid beta precursor protein-binding protein, control gene	201 bp
	fw	GAA CTG TGT GCA CTC CTA TTT G	
	rev	CCG TGC CAA ATA CAC TGC ATG GT	
		Homozygous deletion screening	
3	METH-2-B	Within exon 2 and intron 2	151 bp
	fw	TGC GTA ACT TCT GCA ACT GG	
	rev	CTT TGA TCT GCC CAT CCT GT	
		Allelic imbalance	
4	METH-2-C	Intron, intragenetic SNP, rs152330	181 bp
	fw	ATG GAG TCT TCC CAG GTG GT	
	rev	TGC CAA AGC TGG TCT CAC TA	
		Competitive methylation specific PCR	
5	METH-2-D	Exon1 methylation specific	169 bp
	fw	CGC GGT ATA GGT TGA TCG TC	
	rev	TGC CAA AGC TGG TCT CAC TA	
6	METH-2-E	Intron3 methylation independent	299 bp
	fw	TTG ATT GGG GTT TGA GAG GAT T	
	rev	CCC AAC TAA CCA CAC TCC AAA CT	

Table 1
Primers used in this study.

Competitive methylation-specific PCR

A competitive methylation-specific PCR (cMSP) was designed by combining methylation-specific primers (primer set METH-2-D) annealing with the CpG island located in exon 1 of METH-2. For bisulphite treated DNA with C5-methylation in the CpG island an amplification is possible. For non-methylated cytosines from bisulphite treated DNA insufficient hybridization of the respective primer prevents amplification completely. Uracil would

hybridize with adenine but not with the given guanine. The principle is depicted in figure 1. The methylation specific analysis was carried out as multiplexed PCR together with methylation-independend primers (primer set METH-2-E) annealing in intron 3 of the gene. The PCR mix was composed of 5 µL 2 x Qiagen Multiplex PCR Master Mix (Qiagen, UK), 0.35 pmol control primers, 5 pmol methylation-specific primers and 2 mL bisulphite-treated DNA. The reaction profile was 95 °C for 15 min, followed by 36 cycles consisting of 94 °C for 30 s, 60 °C for 40 s, 72 °C for 70 s and a final extension of 72 °C for 20 min. PCR products (control: 299 bp, methylation-specific: 169 bp) were analysed using the Agilent 2100 bioanalyzer and the DNA 1000 LabChip kit.

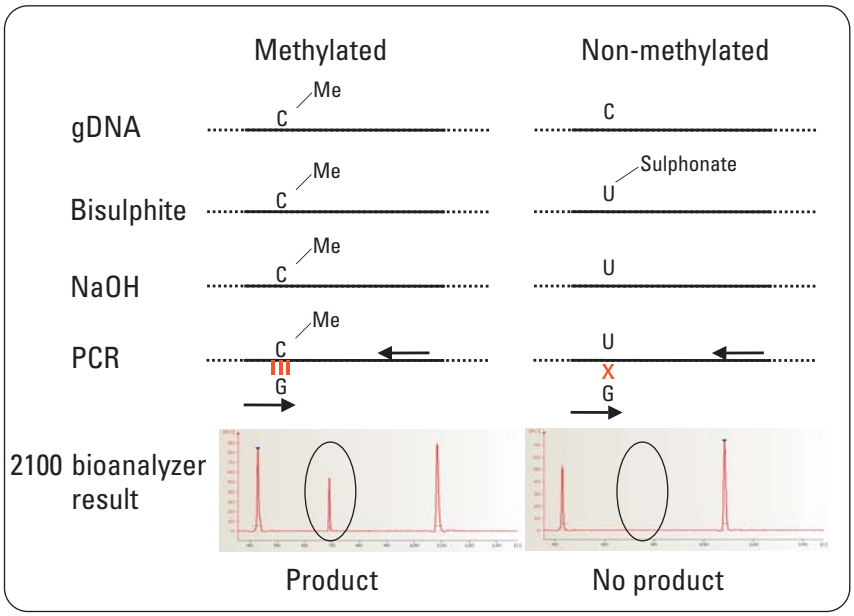

Figure 1
Principle of methylation detection by bisulphite treatment of DNA and subsequent PCR. Non-methylated cytosines are sulfonated and deaminated, converted to uracil sulphonates and subsequently transformed to uracils. C5-methylated cytosines are not modified under these conditions. Finally, a PCR with specific primers with insufficient (x) or optimal hybridization (III) allows to distinguish between methylated and non-methylated DNA. This analysis is carried out as competitive, multiplex PCR (cmRT-PCR) with an internal control PCR for a methylation independend gene.

Results and discussion

Angiogenesis is regulated through a balance of angiogenetic and antiangiogenetic factors, and the angiogenetic switch, which permits the expansion of a small neoplastic lesion, is a key event in tumour progression (Cox et al, 2000). In this study, we have used the Agilent 2100 bioanalyzer to validate the expression pattern of the antiangiogenetic factor METH-2 in NSCLC. Furthermore, we have investigated possible genetic and epigenetic phenomena such as allelic loss and promoter hypermethylation, which may be responsible for this downregulation.

Comparative multiplex RT-PCR (cmRT-PCR) analysis of METH-2 in 23 paired and normal lung cancer tissue cDNAs demonstrated a dramatic reduction of METH-2 transcript in 23 out of 23 (100 %) lung tumour samples (figure 2). Given that the tumours were randomly selected from our database, and were independent of those analysed in the initial microarray study, this downregulation is likely to represent a common state for neoplastic lung tissue. The global reduction of METH-2 transcript in the lung tumour tissues could suggest abnormal downregulation in tumours, providing a positive selective advantage.

Hypermethylation of the METH-2 promoter, indicated by the presence of methylation-specific PCR product, was demonstrated in 58 % of the 50 NSLC samples analysed (figure 3). METH-2 hypermethylation was more frequent in adenocarcinomas (16 out of 24, 67 %) than in squamous cell carcinomas (13 out of 26, 50 %), although this difference is not statistically significant (Fisher's test, P=0.18). Methylation in normal tissue was found only in one case while the rest were negative. Concerning the possible causes of METH-2 silencing, interestingly, over 50 % of tumours demonstrated the presence of hypermethylat-

ed alleles in the gene's promoter region, with the majority (49 out of 50) of the normal samples showing no hypermethylation. These results suggest that DNA methylation may be an important mechanism of transcriptional inactivation of METH-2 in NSCLC. The MSP approach used is not quantitative; therefore, no conclusions can be currently drawn regarding the percentage of cells carrying methylated alleles.

Homozygous deletion and allelic imbalance analysis of METH-2

No homozygous deletions of METH-2 were identified in any lung cancer cell lines tested (data not shown). This suggests that this phenomenon may be infrequent in NSCLC. Allelic imbalance analysis of METH-2 using an RFLP assay for the rs152330 intronic SNP demonstrated nine heterozygous and 14 homozygous (non-informative) samples. Allelic imbalance was observed in 44 % of the informative samples (data not shown). The use of additional SNPs and/or microsatellites in the region providing higher percentage of heterozygotes is required in future studies to confirm this initial observation.

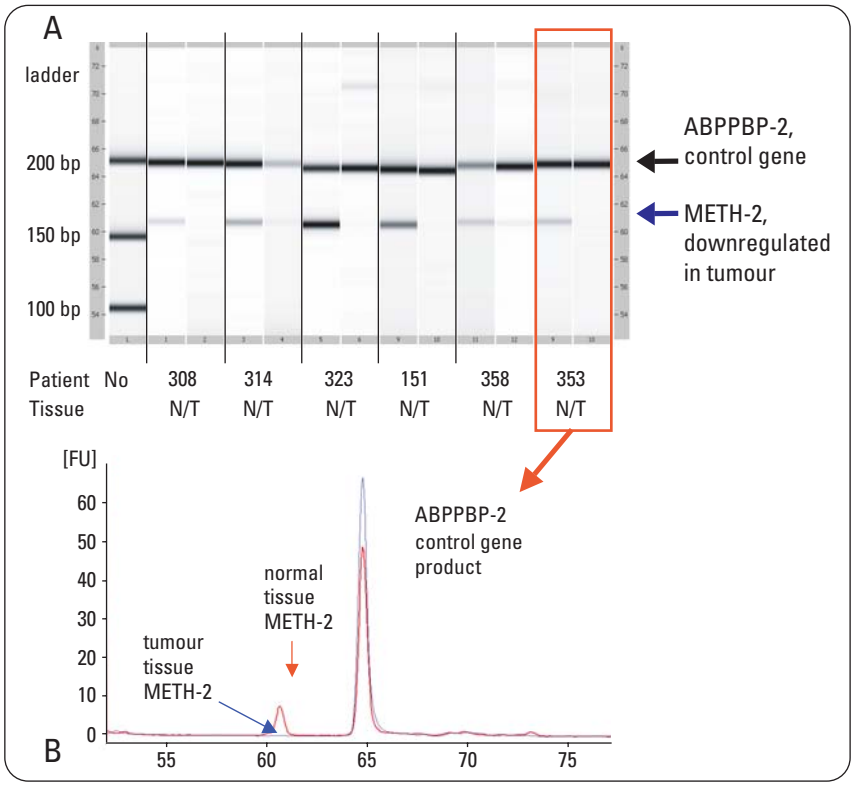

Figure 2
Relative expression of METH-2 in paired normal and tumour tissue. Patient numbers are shown. N=Normal; T=Tumour. The heavier band represents the control gene (ABPPBP-2) and the lower band represents METH-2. (A) Gel-like image (zoomed in). A remarkable reduction of METH-2 transcript is observed in all tumour samples. (B) Electropherogram overview of normal and tumour tissue samples from patient number 353.

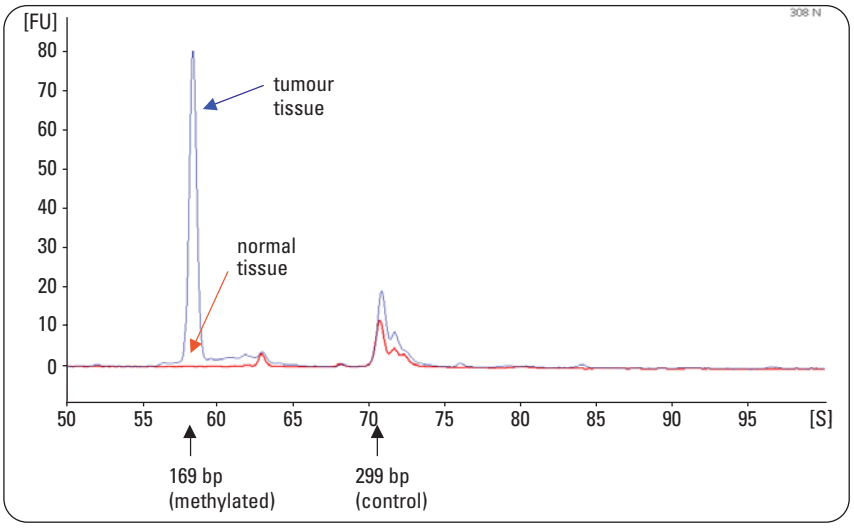

Figure 3
METH-2 cMSP analysis on lung tumour DNAs. Electropherogram overview of normal and tumour tissue samples from patient number 308. The presence of the methylation-independent control peak (299 bp) indicates sufficient amount of converted DNA, while the presence of the methylation-specific PCR product peak (169 bp) demonstrates the existence of methylated copies in tumour tissue.

Conclusion

The Agilent 2100 bioanalyzer is a quick, reliable and easy-to-use technique to detect and quantitate PCR fragments, allowing analysis of 12 DNA samples in 30 minutes. It is highly versatile allowing numerous applications on PCR-based assays. In this study we have used the bioanalyzer and the DNA 1000 LabChip kit to validate the expression pattern of METH-2 in NSCLC, and also to examine the methylation status of the gene's promoter. METH-2 transcript was dramatically reduced in 100 % of the lung tumour samples analysed, confirming our previous results. More than 50 % of the tumours showed hypermethylation of the METH-2 promoter region, indicating that promoter hypermethylation may be an important mechanism in transcriptional inactivation of the gene. Finally, we report that, homozygous deletion of the METH-2 gene seems to be infrequent in NSCLC, whereas allelic imbalance will need to be studied further before any conclusions can be made as to its frequency.

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