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A critical re-assessment of DNA repair gene promoter methylation in non-small cell lung carcinoma

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DNA repair genes that have been inactivated by promoter methylation offer potential therapeutic targets either by targeting the specific repair deficiency, or by synthetic lethal approaches. This study evaluated promoter methylation status for eight selected DNA repair genes (*ATM*, *BRCA1*, *ERCC1*, *MGMT*, *MLH1*, *NEIL1*, *RAD23B* and *XPC*) in 56 non-small cell lung cancer (NSCLC) tumours and 11 lung cell lines using the methylation-sensitive high resolution melting (MS-HRM) methodology. Frequent methylation in *NEIL1* (42%) and infrequent methylation in *ERCC1* (2%) and *RAD23B* (2%) are reported for the first time in NSCLC. *MGMT* methylation was detected in 13% of the NSCLCs. Contrary to previous studies, methylation was not detected in *ATM*, *BRCA1*, *MLH1* and *XPC*. Data from The Cancer Genome Atlas (TCGA) was consistent with these findings. The study emphasises the importance of using appropriate methodology for accurate assessment of promoter methylation.

There is accumulating evidence that tumour response to DNA-damaging agents is associated with the expression levels of DNA repair genes. *BRCA1* mRNA expression has shown to be associated with cisplatin resistance[1–3] and docetaxel sensitivity[4,5] in breast, ovarian, lung and gastric cancers. Tailored chemotherapy based on *BRCA1* mRNA levels has also shown to improve patient survival in lung[2,6], bladder[7] and ovarian cancers[8]. Similarly, high *ERCC1* and *MGMT* mRNA expression has shown to confer resistance to platinum compounds[9] and sensitivity to alkylating agents[10,11]. Expression profiling of selected DNA repair genes thus has potential for the better stratification of cancer patients who are likely to respond to DNA-damaging agents[12].

Formalin-fixed paraffin-embedded (FFPE) tissues are commonly the only available clinical material for diagnostic analysis and for cancer biomarker studies. When FFPE tissue is used, expression profiling of DNA repair genes is often limited by low quantity and degraded RNA due to the detrimental effects of formalin fixation on nucleic acids. Moreover, normal cell contamination that is almost inevitably occurring during RNA extraction, even after macrodissection of tumour-enriched regions, confounds the interpretation of gene expression data.

Promoter methylation is an epigenetic mechanism leading to transcriptional silencing of gene expression and thus has been used for indirect assessment of gene expression. The use of relatively stable DNA for testing of molecular biomarkers provides an important advantage over RNA-based testing with respect to extraction, handling and storage conditions[13]. In human cancers, a number of DNA repair genes have been shown to undergo transcriptional silencing by DNA methylation[14].

Previous studies have reported the occurrence of promoter methylation in several DNA repair genes in non-small cell lung cancer (NSCLC). However, the reported frequency of promoter methylation in NSCLC varies markedly between studies; *ATM* (0–47%), *BRCA1* (4–30%), *MGMT* (8–50%) and *MLH1* (0–68%) (Table 1). This high variation reflects the different methodologies used and emphasises that the DNA methylation status of clinically relevant genes needs to be validated using reliable and reproducible methodology before testing for their methylation has any clinical validity.

Table 1 | Methylation of DNA repair genes in NSCLC reported in the literature

Gene	Sample	Frequency (%)	Method	Reference
ATM	105 tumours	47	MSP	50
	37 tumours	0	MSP	17
	180 tumours	19	MSP	51
BRCA1	28 tumours	18	3-D microarray with linker-PCR	52
	98 tumours	30	MSP	39
	158 tumours	4	MSP	38
MGMT	220 tumours	50	MSP	53
	49 tumours	8	MethyLight	54
	122 tumours	30	MSP	55
	72 tumours	17	MSP	56
	105 tumours	10	MSP	50
MLH1	31 tumours	0	MSP	57
	105 tumours	59	MSP	50
	239 tumours	35	MSP	58
	77 tumours	56	COBRA	59
	116 tumours	68	COBRA	60
	49 tumours	2	MethyLight	54
XPC	158 tumours	33	MSP	23
ERCC1	Not reported	-	-	-
NEIL1	Not reported	-	-	-
RAD23B	Not reported	-	-	-

It is important to note that most of the previous studies used methylation-specific PCR (MSP), a method that has multiple limitations[15,16]. Detection of methylation status by MSP is based on end-point analysis of PCR amplification by gel electrophoresis, which only provides qualitative results. As a consequence, the level of methylation and the pattern of methylation (homogeneous or heterogeneous) cannot be assessed. Furthermore, false positive results can be generated depending on primer design and the stringency of the assay conditions[17].

It is thus important to critically re-evaluate previous methylation reports using methodologies with a low risk of false positives. In this study, we used methylation sensitive–high resolution melting (MS-HRM) methodology for detection of promoter methylation. MS-HRM is a sensitive and closed-tube methodology developed for detection of methylation in a locus-specific manner, utilizing the different melting temperatures (Tm) of methylated and unmethylated DNA after bisulfite modification[18]. Due to the high Tm difference between methylated and unmethylated DNA, methylated samples are readily identifiable by analysing melting profiles. MS-HRM allows semi-quantitative assessment of methylation levels when all the examined CpG sites are methylated, but like other methods cannot quantify when the methylation is heterogeneous[16].

The aim of this study was to assess the promoter region methylation status of DNA repair genes of potential clinical importance. One set of genes had been previously investigated in NSCLC (*ATM*, *BRCA1*, *MLH1*, *MGMT* and *XPC*). We also assessed two DNA repair genes that had been reported as methylated in other tumour types (*NEIL1* and *RAD23B*), and a gene (*ERCC1*) whose gene expression levels had been reported as having predictive significance in NSCLC[19].

Results

Re-assessing the methylation status of DNA repair genes reported to be methylated in NSCLC. DNA methylation of the promoter region CpG islands in the *ATM*, *BRCA1, MGMT, MLH1* and *XPC* genes has been previously reported in lung cancer. We assessed the methylation status of these CpG islands in 11 lung cancer cell lines and 56 NSCLC tumours using methylation sensitive–high resolution melting (MS- HRM). The MS-HRM assay conditions for *ATM*, *BRCA1*, *MGMT* and *MLH1* were robust and published in our previous studies[18,20–22]. We designed our *ERCC1* and *XPC* MS-HRM assay to screen the region where methylation was reported in a previous study[23]. A series of dilutions of methylated DNA in unmethylated DNA (100%, 50%, 25%, 10%, 5%, 1%, 0%) were used in each MS-HRM run as controls.

We did not find promoter methylation in four of these five DNA repair genes (*ATM*, *BRCA1*, *MLH1* and *XPC*) in any of the 11 lung cancer cell lines (Table 2) or in the 56 NSCLC tumours (Figure 1). Although *XPC* methylation was previously reported in the H1355 and Calu-1 cell lines, we did not find any *XPC* methylation in these cell lines (Figure 1). All methylated controls tested in each MS-HRM run were readily interpreted as methylated as their melting profiles differed significantly from that of unmethylated controls.

MGMT methylation was found in three lung cancer cell lines (H69, H1666, and H1755) (Table 2) and seven of the 56 NSCLC tumours (13%) (Figure 2. Panel A). The melting patterns of the methylated lung cancer cell lines and NSCLC tumours were indicative of heterogeneous methylation i.e. the methylation status of individual CpGs varied across the amplicon. The level of heterogeneous methylation cannot be readily estimated by visual examination of the melting curves as the methylation dilution controls are only useful for estimation of methylated alleles of homogeneously methylated samples. However, the melting profiles of the lung cancer cell lines were indicative of low (H69, H1975) and moderate (H1666) numbers of methylated cytosines in the interrogated region. Similarly, only low or moderate levels of heterogeneous *MGMT* methylation were detected in seven NSCLC tumours.

Assessing the methylation status of DNA repair genes not previously tested in NSCLC. We also assessed the promoter methylation of *NEIL1*, *ERCC1* and *RAD23B* as their methylation has been reported in other cancers, but has not previously been examined in NSCLC[24–26].

Frequent *NEIL1* methylation was found in lung cancer cell lines and in NSCLC tumours. In the lung cancer cell lines, high levels of *NEIL1* methylation were detected in H460 (100%) and H1355 (nearly 100%). The other cell lines showed heterogeneous methylation either at moderate levels (H1650, H2087 and H1975) or at low levels (H1395, H1755, H1666, H69, H2228, and Calu-1). Bisulfite Sanger sequencing of selected lung cancer cell lines confirmed the presence of *NEIL1* methylation. *NEIL1* methylation was also detected in 23 of the 56 NSCLC tumours (42%). All methylated

Table 2 | Methylation status of two control genes and eight DNA repair genes in 11 lung cancer cell lines

	Control genes		DNA repair genes							
Cell line	APC	CDKN2A	ATM	BRCA1	ERCC1	MGMT	MLH1	NEIL1	RAD23B	XPC
H1395	UM	UM	UM	UM	UM	UM	UM	Low	UM	UM
H1650	100%	NA	UM	UM	UM	UM	UM	Moderate	UM	UM
H460	50%	NA	UM	UM	UM	UM	UM	100%	UM	UM
H1755	UM	NA	UM	UM	UM	Low	UM	Low	UM	UM
H1666	UM	NA	UM	UM	UM	Moderate	UM	Low	UM	UM
H69	UM	UM	UM	UM	UM	Low	UM	Low	UM	UM
H2087	50%	UM	UM	UM	Low	UM	UM	Moderate	UM	UM
H2228	5%	NA	UM	UM	UM	UM	UM	Low	UM	UM
H1975	UM	UM	UM	UM	UM	UM	UM	Moderate	UM	UM
H1355	100%	100%	UM	UM	UM	UM	UM	~100%	UM	UM
Calu-1	UM	100%	UM	UM	UM	UM	UM	Low	UM	UM

NA; no amplification, UM; unmethylation.

NSCLC tumours showed heterogeneous methylation pattern either at moderate levels (13 samples) or at low levels (10 samples) (Figure 2. Panel B).

Methylation in *ERCC1* and *RAD23B* was rarely detected. *ERCC1* methylation was found in H2087 and one of the NSCLC tumours, the former showing a low-level heterogeneous methylation pattern and the latter showing a moderate-level heterogeneous methylation pattern. *RAD23B* methylation was detected in one NSCLC tumour and was not found in any of the lung cancer cell lines. Bisulfite Sanger sequencing of the tumour samples confirmed the methylation status (Figure 2. Panels C and D).

Methylation status of two control genes using MS-HRM. Given the low frequency of methylation of our target DNA repair gene set, we also assessed the promoter methylation status of the *APC* and *CDKN2A* genes in the cell lines and tumours to confirm that neither the bisulfite modification protocol nor the MS-HRM analysis precluded the detection of promoter methylation. The *APC* and *CDKN2A* genes were chosen as a relatively high methylation frequency for each has been previously found by multiple laboratories[27–29]. A high proportion of samples were methylated for either one or both of the genes, indicating that methylation, where present, could be readily identified by our MS-HRM assays.

APC methylation was found in five lung cancer cell lines (H460, H1355, H1650, H2087, and H2228) (Figure 3. Panel A). The melting patterns of all methylated lung cancer cell lines were suggestive of homogeneous methylation. By comparing with methylation dilution controls, the level of *APC* methylation was estimated to be 100% in

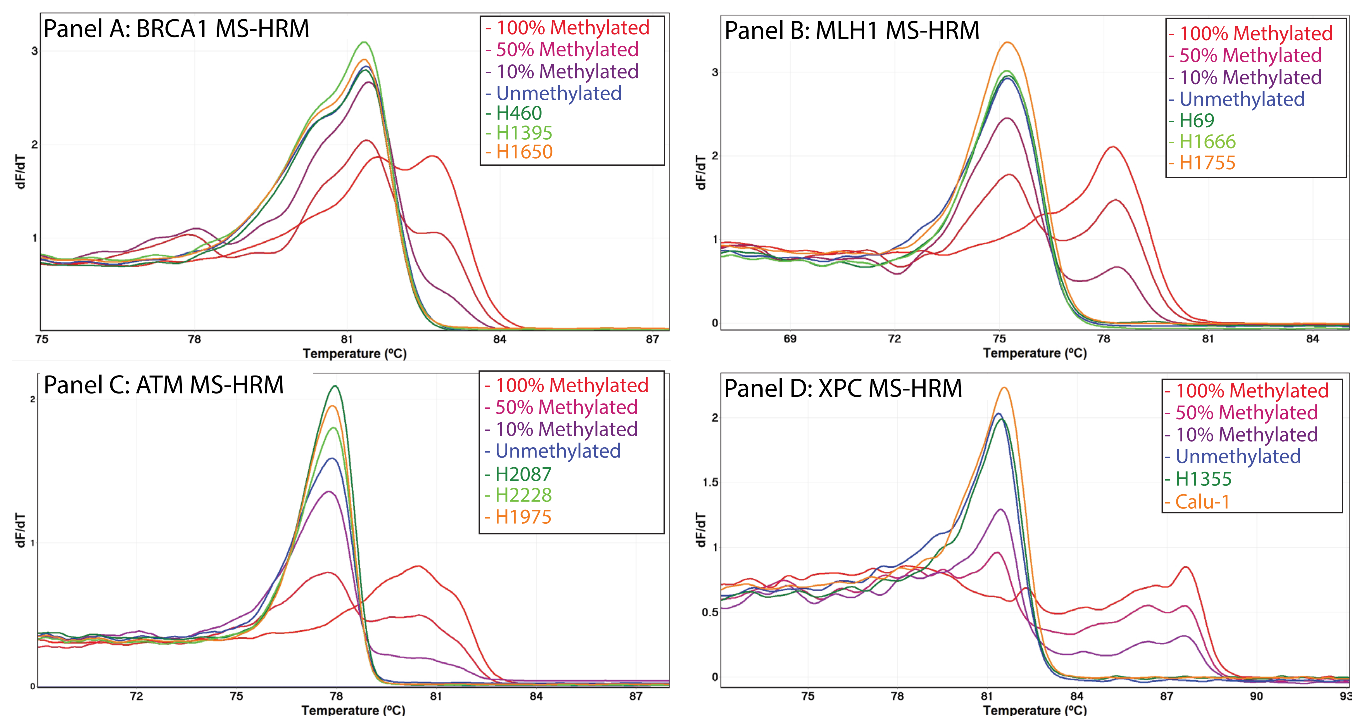

Figure 1 | Absence of *ATM*, *BRCA1*, *MLH1* and *XPC* methylation in lung cancer cell lines and tumours. DNA methylation in the promoter regions of the *ATM*, *BRCA1*, *MLH1* and *XPC* genes was assessed in 11 lung cancer cell lines and 56 NSCLC tumours using MS-HRM. After bisulfite modification, methylated DNA that retains cytosines has a higher melting temperature compared with unmethylated DNA that contains thymine (uracil before PCR). All samples having different melting patterns compared with unmethylated DNA control (in blue) are considered as methylated. Promoter methylation in *ATM*, *BRCA1*, *MLH1* and *XPC* was not detected in any of the lung cancer cell lines and the NSCLC tumours. The negative first derivative plot of three representative lung cancer cell line samples are shown for *BRCA1* (Panel A), *MLH1* (Panel B), *ATM* (Panel C) and *XPC* (Panel D). Absence of *XPC* methylation is seen for the two lung cancer cell lines (H1355 and Calu-1) that were previously reported to be methylated.

Figure 2 | Detection of methylation in *MGMT*, *NEIL1*, *ERCC1* and *RAD23B* in NSCLC tumours. DNA methylation in the promoter regions of the *MGMT*, *NEIL1*, *ERCC1* and *RAD23B* genes was assessed in 56 NSCLC tumours using MS-HRM. Methylation was detected in *MGMT* (25%), *NEIL1* (42%), *ERCC1* (2%) and *RAD23B* (2%). The negative first derivative plots of representative methylated NSCLC tumours for *MGMT* (Panel A) and *NEIL1* (Panel B), *ERCC1* (Panel C) and *RAD23B* (Panel D) are shown. Bisulfite Sanger sequencing traces confirming the *ERCC1* and *RAD23B* methylation status are shown below the negative first derivative plots. Red, green and blue asterisks indicate the positions of methylated cytosines, unmethylated cytosines and bisulfite conversion controls respectively.

H1650 and H1355, and 50% in H460 and H2987. Interestingly, *APC* methylation was estimated to be about 5–10% in H2228, suggestive of differential *APC* methylation status within the H2228 cells.

Homogenous *CDKN2A* methylation was found in two lung cancer cell lines (H1355 and Calu-1) and was estimated to be 100% (Figure 3. Panel B). Five of the lung cancer cell lines (H460, H1650, H1666, H1755, and H2228), that were previously found to have a homozygous *CDKN2A* deletion (CONAN database), were not amplified by the *CDKN2A* MS-HRM assay, confirming the absence of *CDKN2A* template. Four cell lines (H1395, H69, H2987 and H1975) were negative for CDKN2A methylation.

We also tested the 56 NSCLC tumours for methylation in the *APC* and *CDKN2A* promoter regions. *APC* and *CDKN2A* methylation was detected in 14 NSCLC tumours each (25%) (Figure 3. Panels C and D). A total of 23 NSCLC tumours (41%) were methylated for at least one of the genes and methylation in both genes was detected in 5 NSCLC tumours.

External validation using The Cancer Genome Atlas (TCGA) database. As we observed a considerable discrepancy in the methylation frequency of tested DNA repair genes between our MS-HRM results and the literature, we sought to validate our results using a second dataset generated by another methodology with a low false positive rate. We analysed the methylation data from the TCGA NSCLC database that provides genome-wide methylation status assessed by the HumanMethylation 450 k beadchip (Illumina). Methylation data were available from 568 NSCLC tumours, comprising of 341 adenocarcinomas and 227 squamous cell carcinomas.

We searched for HM450k probes overlapping the CpG sites within our eight MS- HRM amplicons. No overlapping CpG sites were detected for *ATM*, *ERCC1* and *MGMT*. Eight probes were identified for the remaining genes; two probes for *BRCA1*, one for *MLH1*, one for *NEIL1*, three for *RAD23B* and one for *XPC* (Figure 4).

Methylation was completely absent in the three CpG sites of *RAD23B* and the CpG site of *XPC* in the 568 NSCLC samples. Very low frequency methylation was seen for *MLH1* (3/567, 0.5%). Three primary squamous cell carcinomas of lung showed methylation at both CpG sites of *BRCA1* (3/568, 0.5%) although one of the sites in the third sample was just below the 20% cut-off. These results were consistent with our MS-HRM results finding either absent or very rare methylation in the *BRCA1*, *MLH1*, *RAD23B* and *XPC* promoters.

The CpG site in the *NEIL1* promoter was frequently methylated (478/568, 84%), although the level of methylation varied among the methylated samples. This TCGA methylation data again confirmed our MS-HRM results demonstrating high frequency *NEIL1* methylation in NSCLC.

Discussion

Epigenetic alterations in cancer are a potential source of therapeutic targets for personalised cancer treatment. *MGMT* methylation in glioma is the best known example where it predicts a durable res-

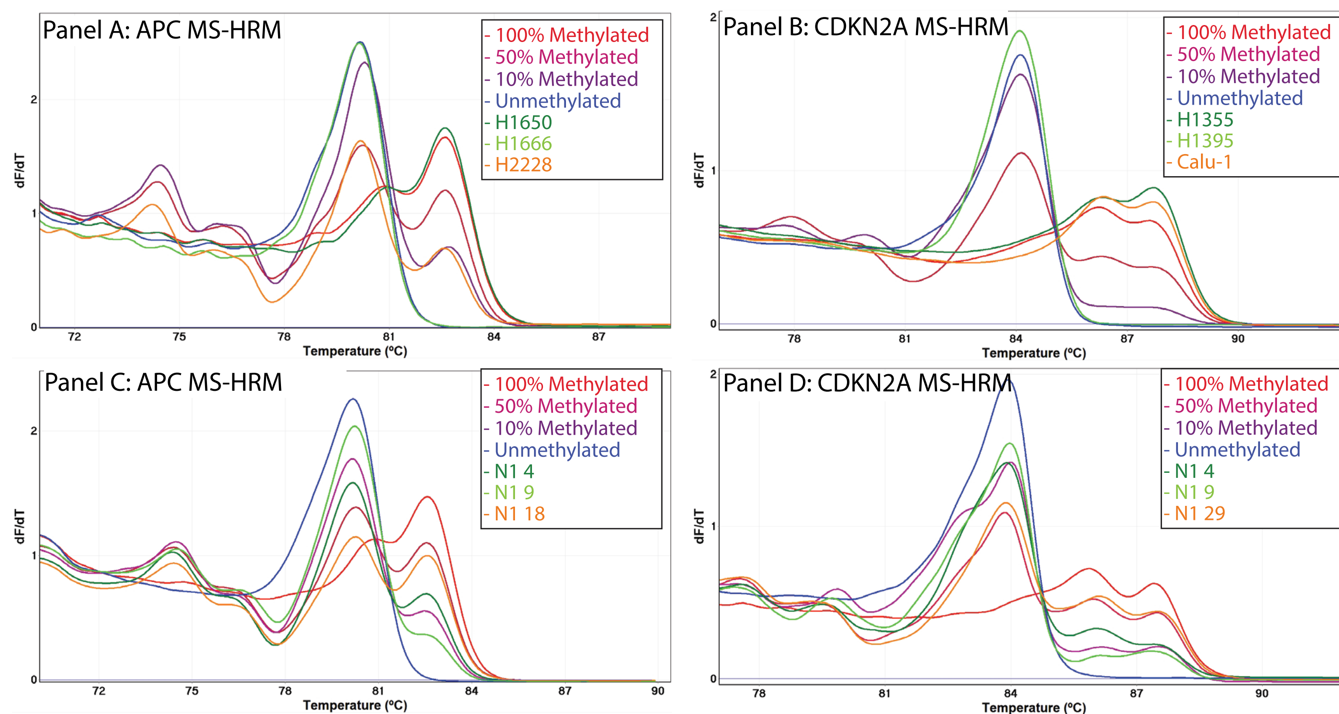

Figure 3 | Assessment of methylation status for *APC* and *CDKN2A* in lung cancer cell lines and tumours using MS-HRM. DNA methylation in the promoter regions of the *APC* and *CDKN2A* genes was assessed in 11 lung cancer cell lines and 56 NSCLC tumours using MS-HRM. *APC* was found in 5 lung cancer cell lines and 14 NSCLC tumours. *CDKN2A* methylation was detected in 2 lung cancer cell lines and 14 NSCLC tumours. The negative first derivative plots of three representative methylated lung cancer cell lines for *APC* (Panel A) and *CDKN2A* (Panel B) and three representative methylated NSCLC tumours for *APC* (Panel C) and *CDKN2A* (Panel D) are shown.

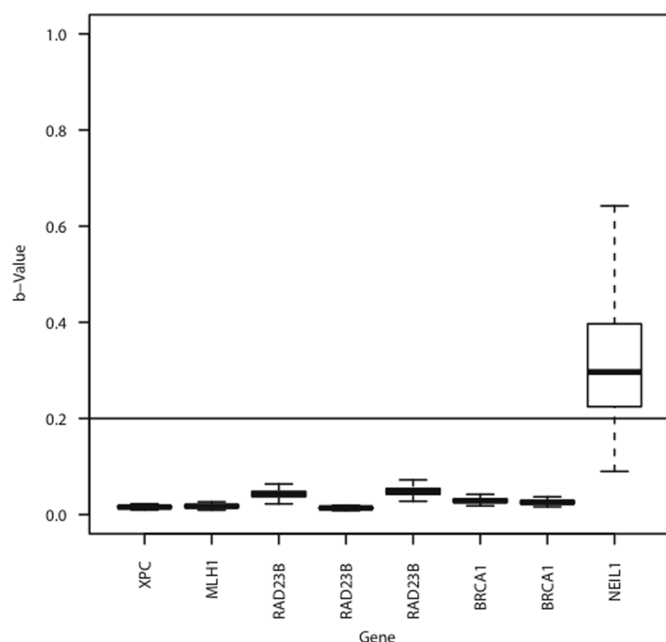

Figure 4 | The TCGA methylation data of five DNA repair genes. TCGA methylation data from 568 non-small cell lung cancers for the eight overlapping CpG sites with our MS-HRM amplicons is presented as boxplots. Two overlapping CpG sites for *BRCA1*, one for *MLH1*, one for *NEIL1*, three for *RAD23B* and one for *XPC* were analysed. A β-value of greater than 0.2 was used to define the presence of DNA methylation as shown by the horizontal line. Consistent with our MS-HRM results, absent or very rare methylation was found for *BRCA1*, *MLH1*, *RAD23B* and *XPC*, and highly frequent methylation was found for *NEIL1*.

ponse to treatment with alkylating agents[30]. Methylation of other DNA repair genes also has been considered for the selection of optimal chemotherapeutic agents for the treatment of cancer, although these have not been clinically implemented up to now[31]. Before clinical implementation, individual methylation markers need to be rigorously validated, ideally by using different methodologies[13]. In this study, we sought to validate a range of previously reported and to examine novel methylated DNA repair markers that could potentially be therapeutically exploited.

In the literature, highly variable estimates of the frequency of methylation for the *ATM* (0–47%), *BRCA1* (4–30%), *MLH1* (0–68%), *MGMT* (8–50%), and *XPC* (33%) genes have been reported in NSCLC tumours (Table 1). As most of the previous studies used the MSP method to determine the methylation status of candidate genes, the previous findings needed to be validated using other methodologies that are less prone to give false results. When we assessed DNA methylation using MS-HRM, we did not find methylation in any of these four DNA repair genes (*ATM*, *BRCA1*, *MLH1*, and *XPC*) in our 11 lung cancer cell lines and 56 NSCLC samples. This was consistent with the TCGA data which showed either absent or very low frequency of methylation for these promoters.

There are several possible explanations for the discrepant results, including differences in ethnicity or clinicopathological features of samples tested in each study. Several studies have reported varying methylation frequencies in cancer between different ethnic groups, including in the promoters of the *IGFBP3*[32], *TMS1*[33] and *GSTP1* genes[34]. However, the real reasons for the discrepant results are likely to be technical such as scoring of low-level methylation, false positives due to the use of inadequately designed primers or amplification of methylated pseudogene sequences.

Low levels of methylation, especially present at ≤1%, can cause discrepant results due to the different analytic sensitivity of detection methods. The lower limit of MSP can be close to 0.1%[35], allowing samples with low level methylation to be interpreted as methylated.

A high frequency of false positive *ATM* methylation calls deriving from the use of inadequate MSP conditions has been previously demonstrated in NSCLC[17]. *ATM* methylation was not detected in NSCLC when strict guidelines for performance of MSP are used[17]. We did not find *ATM* methylation in our 11 lung cancer cell lines and 56 lung tumours, confirming the absence of *ATM* methylation.

The *BRCA1* pseudogene (*BRCA1P1*), a duplicated region of *BRCA1* exons 1A, 1B, and 2, has a strong sequence homology to the *BRCA1* gene. As methylation of *BRCA1P1* has been previously reported in cancers[36,37], there is a risk of false positives due to amplification of the methylated *BRCA1P1* sequence. Two previous studies have assessed the *BRCA1* methylation status in NSCLC tumours using MSP as a detection method[38,39]. The MSP primers designed by Lee *et al*. have 19 (19/21 in the forward) and 17 (17/20 bases in the reverse) matched bases, including the nine consecutive bases from the 3′ end of both primers, to the *BRCA1* pseudogene[39]. The frequency of *BRCA1* methylation (30%) reported by Lee *et al*. was 7-fold higher than that of being reported by Marsit *et al*. (4%) where more stringent MSP primers were used to avoid the amplification of the methylated *BRCA1P1* sequence.

XPC methylation was initially reported in NSCLC tumours and lung cancer cell lines[23]. Wu *et al*. reported that *XPC* methylation was detectable in 34% of NSCLC tumours by *Hpa*II-based PCR and in four lung cancer cell lines harboring *TP53* mutations. Surprisingly, *XPC* methylation was not detected in this cohort of 56 NSCLC tumours and in two of the lung cancer cell lines (H1355 and Calu-1) previously reported as methylated[23]. The absence of *XPC* methylation was confirmed in the TCGA data. None of the 568 NSCLC tumours of the TCGA study had *XPC* methylation at the overlapping CpG site with our MS-HRM amplicon. As our *XPC* MS-HRM assay was designed to amplify those methylated CpG sites in the Wu *et al.* study, the discordant results was thus not likely to be caused by the examination of different CpG sites. To assess the *XPC* methylation status in NSCLC tumours, Wu *et al*. used the *Hpa*II restriction endonuclease for selective cleavage of unmethylated DNA before PCR amplification[23]. However, there is a risk of incomplete enzymatic digestion of unmethylated DNA by *Hpa*II, potentially resulting in false positives[40,41].

This study is the first report showing methylation of *ERCC1*, *RAD23B* and *NEIL1* in NSCLC. The excision repair cross-complementing group 1 (ERCC1) is a rate-limiting protein involved in the recognition and excision of DNA adducts. Recently, Chen *et al.* reported that *ERCC1* methylation was significantly associated with chemosensitivity to cisplatin in glioma cell lines and glioma tumours[25]. In NSCLC, low levels of *ERCC1* expression were correlated with favorable clinical outcomes of prolonged survival and sensitivity to platinum-based chemotherapies[19]. Therefore, *ERCC1* methylation may serve as a predictive biomarker for identification of NSCLC patients who are highly sensitive to platinum-based chemotherapies. These patients potentially would have a more durable response than patients with high levels of *ERCC1* expression that were not methylated.

The RAD23B protein forms a DNA damage recognition complex with the XPC and centrin 2 proteins. The RAD23B/XPC/centrin 2 complex recognises and interacts with the damaged bases or the sugar-phosphate backbone of DNA in the NER pathway[42]. High RAD23B expression has been suggested as a promising biomarker associated with response to histone deacetylase (HDAC) inhibitors in cutaneous T-cell lymphoma patients[43]. As anti-tumour activities of HDAC inhibitors have been demonstrated in NSCLC[44], it can be speculated that silencing of RAD23B expression through promoter methylation makes tumour cells more resistant to HDAC inhibitors. On the other hand, *RAD23B* methylation may make tumour cells more sensitive to DNA-damaging agents.

Nei-like 1 (NEIL1), an ortholog of *E.coli* Nei (endonuclease VIII), is a bifunctional DNA glycosylase that repairs oxidative DNA damage of 8-hydroxyguanine and thymine glycol[45] and protects cells from radiation-mediated cell death[46]. Recently, epigenetic silencing of *NEIL1* through promoter methylation was reported in head and neck squamous cell carcinomas[24]. In this study, we found that the *NEIL1* promoter is methylated in NSCLC at a high frequency (42%). This may identify patients that are sensitive to radiotherapy which deserves further study.

In conclusion, this study showed that methylation frequency of *ATM*, *BRCA1*, *MLH1* and *XPC* in NSCLC is likely to be overestimated in the literature, emphasising the importance of rigorous

Table 3 | Primer sequences and amplicon information for each MS-HRM assay

Name	Primer Sequence	Genomic region^	Number of CpG#	Annealing Tm (°C)	Amplicon (bp)
APC_F	5′-cggggttttgtgttttattg-3′	chr5:112,073,406-112,073,476	4	58.5	71
APC_R	5′-tccaacgaattacacaactac-3′				
ATM_F	¦5′-gtttgcgttawgtttattaatggtt-3′	chr11:108,092,818-108,094,287	12	55	147
ATM_R	5′-acttccgtcctcaaacttaaaa-3′				
BRCA1_F	*5′-ttgttgtttagcggtagtttttggtt-3′	chr17:41,277,396-41,277,474	4	63	79
BRCA1_R	*5′-caatcgcaattttaatttatctataattccc-3′				
CDKN2A_F	5′-cggaggaagaaagaggaggggt-3′	chr9:21,974,843-21,974,935	7	70	93
CDKN2A_R	5′-cgctacctactctcccctct-3′				
ERCC1_F	*†5′-gagtcgtttttttttattIgggtttttttg -3′	chr19:45,932,040-45,932,132	12	65	93
ERCC1_R	*5′-cgcccgcctctaaacttaacc -3′				
MGMT_F	5′-gcgtttcggatatgttgggatagt -3′	chr10:131,265,469-131,265,578	12	61	110
MGMT_R	5′-aacgacccaaacactcaccaaa -3′				
MLH1_F	5′-agttttaaaaacgaattaataggaagag-3′	chr3:37,034,741-37,034,821	5	59	81
MLH1_R	5′-actacccgctacctaaaaaaatatac-3′				
NEIL1_F	*5′-aatttttttttcgttttagggtttttgtattgg-3′	chr15:75,639,328-75,639,472	9	59	145
NEIL1_R	*5′-aaaacgaaaaaataaacccacttaaaataattc-3′				
RAD23B_F	5′-gtcgttaggaggaagttttaggagt-3′	chr9:110,045,332-110,045,451	11	57	124
RAD23B_R	†5′-gaaaactccgccccaaIattaaaaac-3′				
XPC_F	5′-ttttaacgaaggggcgtggttaag-3′	chr3:14,220,061-14,220,178	13	62	118
XPC_R	5′-cgaaccatattacttatctaaacaaattcca-3′				

^UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly was used.
*M13 sequences were attached to these primers.
#CpG dinucleotides flanked by the MS-HRM primers were counted.
†An inosine base (I) was used at the cytosine site of a CpG dinucleotide in the *ERCC1* and *RAD23B* reverse primers to reduce the bias toward methylated templates.
¦The *ATM* forward primer contains W (A/T) at the position of the SNP rs4987880 (A/T) to avoid any amplification bias.

validation of previous data on DNA methylation. In particular, the use of adequate methodology is critical to avoid false positive results. DNA methylation in the *ERCC1, RAD23B* and especially the *NEIL1* DNA repair genes may serve as useful biomarkers for the determination of molecularly tailored therapies in a subset of NSCLC patients.

Methods

Samples. Fifty-six N1 stage NSCLC tumours were collected at the Austin Hospital, Melbourne, Australia with the approval of the Austin Human Research Ethics Committee (project title and approval number "Biomarkers in the Australian Non Small Cell Lung Cancer Population" – H2006-02394). The methylation study was approved by the Ethics of Human Research Committee at the Peter MacCallum Cancer Centre, Melbourne, Australia (project title and approval number "Molecular Pathology of Cancer: Methylation, Mutation & Expression" - 02/26).

DNA extraction from lung cancer cell lines. Lung cancer cell lines were cultured in RPMI 1640 medium with 25 mM HEPES supplemented with 10% fetal bovine serum, and 0.1 units/mL of penicillin and 0.1 μg/mL of streptomycin. Cells were maintained at 37°C in a humidified chamber containing 5% CO2. Cultured cells were harvested and washed twice with Dulbecco's phosphate-buffered saline buffer, followed by centrifugation at the speed of 10,000 rpm for 10 minutes. Cell pellets were then suspended in 200 μL of Dulbecco's phosphate-buffered saline buffer. After addition of 20 μL of proteinase K (20 mg/mL) and 200 μL of buffer AL, the suspended cells were incubating at 56°C for overnight. Genomic DNA was extracted from cultured lung cancer cell lines using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

DNA extraction from NSCLC tumours. The tumour purity of individual NSCLC cases was assessed by pathologists at Peter MacCallum Cancer Centre and was estimated to be 40–95% with a median of 67%. Two to five 5 μm formalin-fixed tissue sections were washed with 1 mL of xylene to remove paraffin and were incubated at 40°C for 10 minutes. Supernatant was removed after a centrifugation at 13,000 rpm for 10 minutes and tissue pellets were sequentially washed with 100% and 70% ethanol. The tissue pallets were then resuspended with 100 μL of ATL buffer of the DNeasy Tissue kit (Qiagen) and incubated at 97°C for 15 mins, followed by proteinase K digestion for 3 days at 56°C. Genomic DNA was then extracted using the DNeasy Tissue kit according to the manufacturer's instructions.

Bisulfite modification. One microgram of genomic DNA was bisulfite modified using the MethylEasy Xceed kit (Human Genetic Signatures, North Ryde, Australia) according to the manufacturer's instructions. Bisulfite modified DNA was eluted twice with 50 μL of elution buffer in an estimated concentration of 10 ng/μL.

Preparation of methylation standards. Commercially available methylated DNA (Millipore, Billerica, MA) was used as a fully methylated control. To prepare unmethylated DNA, peripheral blood DNA obtained from a healthy individual underwent two rounds of whole genome amplification (WGA) using the Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Giles, UK) according to the manufacturer's instructions. The first round of WGA was performed with 1 ng of peripheral blood DNA. One microliter of a 10-fold dilution of the first round WGA product was used for the second round of WGA. After bisulfite modification, a qPCR assay that amplified a region lacking CpG dinucleotides within the *COL2A1* gene was performed to quantify the amount of template[47]. A series of methylation dilution standards of 100%, 50%, 20%, 10%, 5% and 1% were prepared by mixing of the fully methylated DNA with the WGA DNA.

Methylation sensitive-high resolution melting assays. MS-HRM assays were designed either to target the promoter region or to overlap the regions used in previously reported assays. To better work with fragmented FFPE DNA, MS-HRM assays were designed to amplify a short amplicon size of less than 150 bp. Primer sequences, amplicon sizes, $MgCl_2$ concentration, annealing temperatures, and the number of CpG dinucleotides analysed are summarised in Table 3. PCR amplification and MS-HRM were performed on the RotorGene Q (Qiagen). The PCR mixture was prepared in a final volume of 20 μL and contained: 10 ng of bisulfite modified template, 1× PCR buffer, 2.5–4 mM $MgCl_2$, 200–400 nM each primer, 200 μM dNTPs, 5 μM SYTO9 and 0.5 U HotStar Taq (Qiagen). PCR cycling and melting conditions were as follows; one cycle of 95°C for 15 minutes; 50–55 cycles of 95°C for 10 seconds, annealing temperature of each assay for 20 seconds, 72°C for 25 seconds; one cycle of 97°C for one minute and a melt from 70°C to 95°C rising 0.2°C per second. The melting profiles of amplicons were analysed using RotorGene Q Software (v1.7). All samples were tested in duplicate.

Bisulfite Sanger sequencing. To confirm the MS-HRM results for *ERCC1*, *NEIL1* and *RAD23B* methylation, MS- HRM products were sequenced using the Big Dye Terminator v3.1 chemistry (Applied Biosystems). Sequencing products were purified with the Agencourt CleanSEQ reagent (Beckman Coulter) and analysed by capillary electrophoresis on an ABI3730 sequencer (Applied Biosystems). The sequencing data was analysed using Sequencher 4.6 (Gene Codes Corporation).

Analysis of DNA methylation from publically available data. Marmal-aid (http://www.marmal-aid.org) is a data repository of publically available genome-scale DNA methylation analysis using the Illumina HumanMethylation 450 K (HM450K) beadchip. At the time of download, 8,654 data sets were available from marmal-aid and also contained data from The Cancer Genome Atlas (TCGA) study[48]. At the time of download, a total of 568 non-small cell lung cancers (341 adenocarcinomas and 227 squamous carcinomas) were available from marmal-aid from TCGA. We identified 11 HM450K probes interrogating CpG sites within our MS-HRM amplicons. Raw beta values were extracted using marmal-aid in R. Data were extracted according to their annotations in marmal-aid. Samples annotated with "lung" and "cancer" and our probes of interest were used for downstream analysis and visualization in R (http://www.cran.org). Using guidelines previously described[49], a β-value of greater than 0.2 was used to define the presence of DNA methylation.

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Author contributions

Suitable samples were identified by P.M., T.J., B.S. and C.M. The experimental work was carried out by H.D. and the TCGA data was analysed by N.W., H.D. and N.W. prepared figures. H.D. and A.D. co-wrote the manuscript. All authors approved the final manuscript.

Additional information

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