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 resistance2,3 and docetaxel sensitivity4,5 in breast, ovarian, lung and gastric cancers. Tailored chemotherapy based on BRCA1 mRNA levels has also shown to improve patient survival in lung6,7, bladder8 and ovarian cancers9. Similarly, high ERCC1 and MGMT mRNA expression has shown to confer resistance to platinum compounds9 and sensitivity to alkylating agents10,11. 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 agents12.

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 conditions13. In human cancers, a number of DNA repair genes have been shown to undergo transcriptional silencing by DNA methylation14.

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 emphasizes 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.
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 endpoint 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 differences 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.

| Gene | Sample | Frequency (%) | Method | Reference |
|------|--------|---------------|--------|-----------|
| ATM  | 105 tumours | 47 | MSP | 50 |
| BRCA1| 37 tumours | 0 | MSP | 17 |
| 180 tumours | 19 | MSP | 51 |
| MGMT | 28 tumours | 18 | 3-D microarray with linker-PCR | 52 |
| 98 tumours | 30 | MSP | 39 |
| 158 tumours | 4 | MSP | 38 |
| 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 |
| 31 tumours | 0 | MSP | 57 |
| MLH1 | 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 |
| 105 tumours | 35 | MSP | 58 |
| 122 tumours | 30 | MSP | 55 |
| 72 tumours | 17 | MSP | 56 |
| 105 tumours | 10 | MSP | 50 |
| 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 |

### 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\(^14–20\).

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
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. 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 the tumour samples (Figure 3. Panel A).

| Control genes | DNA repair genes |
|---------------|-----------------|
| **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 | NA | 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 |

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

NA: no amplification, UM: unmethylation.

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.
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 samples, 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).

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-
response to treatment with alkylating agents. 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. Before clinical implementation, individual methylation markers need to be rigorously validated, ideally by using different methodologies. 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, TMS1 and GSTP1 genes. 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%²⁵, allowing samples with low level methylation to be interpreted as methylated.

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 3 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.
A high frequency of false positive ATM methylation calls deriving from the use of inadequate MSP conditions has been previously demonstrated in NSCLC\(^{37}\). ATM methylation was not detected in NSCLC when strict guidelines for performance of MSP are used\(^{38,39}\). 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\(^{38}\). 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\(^{25}\). Wu et al. reported that XPC methylation was detectable in 34% of NSCLC tumours by HpaII-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 HpaII restriction endonuclease for selective cleavage of unmethylated DNA before PCR amplification\(^{28}\). However, there is a risk of incomplete enzymatic digestion of unmethylated DNA by HpaII, 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\(^{42}\). In NSCLC, low levels of ERCC1 expression were correlated with favorable clinical outcomes of prolonged survival and sensitivity to platinum-based chemotherapies\(^{37}\). 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\(^{38}\). 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.


equiv-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\(^{28}\). 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’-cgggttgttttgactcttag-3’ | chr5:112,073,406-112,073,476 | 4 | 58.5 | 71 |
| APC_R | 5’-cattacctatgctattttaaa-3’ | chr5:112,073,308-112,073,387 | 12 | 55 | 147 |
| ATM_F | 5’-ggtggtgtttagtatcaggt-3’ | chr1:108,092,818-108,094,328 | 12 | 55 | 147 |
| ATM_R | 5’-cattacctatgctattttaaa-3’ | chr5:112,073,308-112,073,387 | 12 | 55 | 147 |
| BRCA1_F | 5’-cgggttgttttgactcttag-3’ | chr5:112,073,406-112,073,476 | 4 | 58.5 | 71 |
| BRCA1_R | 5’-cattacctatgctattttaaa-3’ | chr5:112,073,308-112,073,387 | 12 | 55 | 147 |
| CDK2A1_F | 5’-cgggttgttttgactcttag-3’ | chr5:112,073,406-112,073,476 | 4 | 58.5 | 71 |
| CDK2A1_R | 5’-cattacctatgctattttaaa-3’ | chr5:112,073,308-112,073,387 | 12 | 55 | 147 |
| ERCC1_F | 5’-cgggttgttttgactcttag-3’ | chr5:112,073,406-112,073,476 | 4 | 58.5 | 71 |
| ERCC1_R | 5’-cattacctatgctattttaaa-3’ | chr5:112,073,308-112,073,387 | 12 | 55 | 147 |
| MGMT_F | 5’-cgggttgttttgactcttag-3’ | chr5:112,073,406-112,073,476 | 4 | 58.5 | 71 |
| MGMT_R | 5’-cattacctatgctattttaaa-3’ | chr5:112,073,308-112,073,387 | 12 | 55 | 147 |
| MLH1_F | 5’-cgggttgttttgactcttag-3’ | chr5:112,073,406-112,073,476 | 4 | 58.5 | 71 |
| MLH1_R | 5’-cattacctatgctattttaaa-3’ | chr5:112,073,308-112,073,387 | 12 | 55 | 147 |
| NEIL1_F | 5’-cgggttgttttgactcttag-3’ | chr5:112,073,406-112,073,476 | 4 | 58.5 | 71 |
| NEIL1_R | 5’-cattacctatgctattttaaa-3’ | chr5:112,073,308-112,073,387 | 12 | 55 | 147 |
| RAD23B_F | 5’-cgggttgttttgactcttag-3’ | chr5:112,073,406-112,073,476 | 4 | 58.5 | 71 |
| RAD23B_R | 5’-cattacctatgctattttaaa-3’ | chr5:112,073,308-112,073,387 | 12 | 55 | 147 |
| XPC_F | 5’-cgggttgttttgactcttag-3’ | chr5:112,073,406-112,073,476 | 4 | 58.5 | 71 |
| XPC_R | 5’-cattacctatgctattttaaa-3’ | chr5:112,073,308-112,073,387 | 12 | 55 | 147 |

1) UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly was used.
2) M13 sequences were attached to these primers.
3) CpG dinucleotides flanked by the MS-HRM primers were counted.
4) An inosine base (I) was used at the cytosine site of a CpG dinucleotide in the M13 sequences were attached to these primers.
5) #CpG dinucleotides flanked by the MS-HRM primers were counted.
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 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 tissues 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. Cell pellets were then suspended with 100 µL of ATL buffer of the DNAeasy Tissue kit (Qiagen) and incubated at 97 °C for 15 minutes, followed by proteinase K digestion for 3 days at 56 °C. Genomic DNA was then extracted using the DNAeasy 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 undergoing a routine 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 product was used for the second round of WGA. After bisulfite modification, a qPCR assay that amplified a region lacking CpG dinucleotides within the product was used for the second round of WGA.

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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 TGCA 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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