The epigenetic landscape of oral squamous cell carcinoma

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Background: There is relatively little methylation array data available specifically for oral squamous cell carcinoma (OSCC). This study aims to compare the DNA methylome across a large cohort of tumour/normal pairs.

Methods: DNA was extracted from 44 OSCCs and paired normal mucosa. DNA methylation analysis employed the Illumina GoldenGate high-throughput array comprising 1505 CpG loci selected from 807 epigenetically regulated genes. This data was correlated with extracapsular spread (ECS), human papilloma virus (HPV) status, recurrence and 5-year survival.

Results: Differential methylation levels of a number of genes distinguished the tumour tissue sample from the matched normal. Putative methylation signatures for ECS and recurrence were identified. The concept of concordant methylation or CpG island methylator phenotype (CIMP) in OSCC is supported by our data, with an association between 'CIMP-high' and worse prognosis. Epigenetic deregulation of NOTCH4 signalling in OSCC was also observed, as part of a possible methylation signature for recurrence, with parallels to recently discovered NOTCH mutations in HNSCC. Differences in methylation in HPV-driven cases were seen, but are less significant than that has been recently proposed in other series.

Conclusion: Although OSCC seems as much an ‘epigenetic’ as a genetic disease, the translational potential of cancer epigenetics has yet to be fully exploited. This data points to the application of epigenetic biomarkers and targets available to further the development of therapy in OSCC.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy with a worldwide incidence of 615,000 cases per annum, of which around 300,000 are oral squamous cell carcinomas (OSCC) (Kademani, 2007). The UK incidence of OSCC has recently increased, reaching over 6000 cases per annum (CR-UK press release, 2012). It is now apparent that cancer is as much a disease of misdirected epigenetics as genetic mutations, losses and amplifications, and recent published evidence suggests that as many as 5% of known gene promoters (i.e. 1500–2000 genes) are abnormally methylated in a typical solid tumour (Schuelbel et al, 2007) compared with, typically, 11 gene mutations (Sjoblom et al, 2006). Next generation sequencing in HNSCC has shown that, although common in TP53 and NOTCH pathways, somatic mutations in specific genes are relatively heterogeneous, individually occurring in <10% of cases (Agrawal et al, 2011). Detection of methylation at known target CpG sequences can be made highly sensitive, opening up the possibility of the development of minimally invasive biomarkers in body fluid surrogates (Laird, 2003; Shaw et al, 2006a). However, genome-wide methylation platforms have lagged behind other genomic methods because bisulphite conversion necessitates an inherent loss of sequence complexity resulting in lack of hybridisation specificity. Consequently, the literature has concentrated on a rather narrow cohort (Ha and Califano, 2006; Shaw, 2006) of methylated gene promoters, and the translational potential of the broader DNA methylome in HNSCC remains relatively unexplored. The concept of a CpG island methylator phenotype (CIMP) (Toyota et al, 1999), although well explored in several tumour types, has yet to be
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MATERIALS AND METHODS

Clinical. Forty four OSCC patients were selected for inclusion in this study, all of whom provided informed consent under a specific institutional ethical approval (REC EC.47.01), between May 2006 and March 2007. Inclusion criteria included histologically confirmed OSCC with a treatment decision for primary surgery. At the time of surgery, 5 mm² tumour samples were excised from resected specimens within the tumour mass, but not involving the margin. Normal samples of similar dimension were taken from the resection margin, ~10 mm from the macroscopic tumour edge and subsequently confirmed as benign by routine histopathology. Tumour and matched normal samples were snap-frozen and stored at ~ 80 °C until DNA preparation. Detailed histopathological analysis was recorded for each surgical resection and the clinical outcome for each patient was recorded at 5th year follow-up. The Office of National Statistics supplied death certification details comprising the immediate cause of death plus the associated factors.

Laboratory. DNA was extracted from 2 mm³ tissue samples using DNeasy tissue kit (Qiagen, Crawley, UK). DNA concentration and quality was measured by spectrophotometry (Nanodrop; Thermo Fisher Scientific Inc., Wilmington, DE, USA). Bisulphite treatment of each sample was undertaken using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA). An aliquot of the converted DNA (corresponding to 500 ng starting gDNA) was subjected to microarray analysis (GoldenGate array-Illumina Ltd, San Diego, CA, USA). The array examination was conducted using microarray analysis (GoldenGate array-Illumina Ltd, San Diego, CA, USA). An aliquot of the converted DNA (corresponding to 500 ng starting gDNA) was subjected to microarray analysis (GoldenGate array-Illumina Ltd, San Diego, CA, USA), examining 1505 CpG sites on 807 genes. Raw methylation data and associated clinicopathological data have been deposited in ArrayExpress (accession: E-MTAB-1298).

HPV testing. As some of the tumours overlapped the junction between oral cavity and oropharynx sites (in particular tonsillar pillar and soft palate), it was felt appropriate to include HPV status to clarify the underlying molecular aetiology. Tumour HPV status was determined using quantitative PCR (qPCR) analysis for HPV16 DNA presence as previously described (Schache et al., 2011). Briefly, duplicate real-time PCR reactions were conducted using primers and a FAM-MGB-labelled Taqman probe designed and optimised to amplify the E6 region of HPV16. Commercially available primers and a VIC-TAMRA-labelled probe for the housekeeping gene Rnase P were included in the multiplex reaction to standardise for DNA input. The HPV16-positive cervical cancer cell line, CaSkI (U.K. Health Protection Agency Culture Collections – 87020501) was used as a positive control and calibrator. The detection threshold for HPV-positive status was set at one or more copies of HPV16 E6 gene per diploid genome. A sample was only considered positive if it met the threshold in both of the duplicate runs.

Bioinformatics analysis. The array data were initially assessed for quality. One tumour sample found as an outlier in principal components analysis (PCA), together with the matching normal sample were excluded from further analysis. Initial processing of the data was conducted using the bioconductor package methylumi (Davis et al., 2012). All 84 CpG sites on the X-chromosome were excluded from data analysis to avoid gender-specific bias.

R statistical package version 2.14.1 (R, 2012) was employed to perform Wilcoxon signed-rank and Wilcoxon rank sum tests. Hierarchical clustering; PCA and survival analysis were performed using Partek Genomic Suite (Partek Inc., St Louis, MO, USA). Hypergeometric analysis of pathways enriched by the differential genes was conducted using MetaCore (GeneGo Inc, Part of Thomson Reuters, Carlsbad, CA, USA). Genes represented on the Illumina GoldenGate Cancer Panel I methylation array were used as reference in the pathway analysis. In statistical analyses, a 5% FDR cutoff was employed after multiple testing correction of P-values.

Methylation in tumour/normal paired tissue. When more than half of the samples (at least 22/43) from a group had average beta values >0.2, the CpG locus was considered methylated in that group. The Wilcoxon signed-rank test was employed to identify statistically significant differential methylation between tumour and matching normal samples using R statistical package. M-value, that is, the log2 ratio of intensities of methylated probe versus unmethylated probe, was employed for Wilcoxon rank sum or signed-rank tests, as it was recently reported that beta values have severe heteroscedasticity for highly methylated or unmethylated CpG sites and M-value is a better measure for statistical analyses (Du et al., 2010). However, as beta values provide more intuitive biological interpretation, differences in methylation levels were derived using average beta values, representing the ratio of methylated probe intensity and overall intensity, that is, the sum of methylated and unmethylated probe intensities. An offset of 100 was added to regularise beta when both probe intensities were low. Thus, for each CpG locus, differential methylation values (Δ β) were calculated by subtracting the average beta values of tumour samples from the average beta values of the normal samples.
Comparison was made with previously published HNSCC methylation data (Poage et al, 2010; Guerrero-Preston et al, 2011) as well as against a metanalysis of 63 previous publications reporting mRNA expression microarray data in HNSCC (Yu et al, 2008). Limiting inclusion criteria to ≥20 tumour/normal pairs, this analysis was restricted to four datasets published by Ye (Ye et al, 2008), Dysvik (Dysvik et al, 2006), Kuriakose (Kuriakose et al, 2004) and Ibrahim (Ibrahim et al, 2003).

Hierarchical agglomerative clustering of tumour samples was performed with Euclidean distance measure and average linkage using average β values of probes selected for differential methylation between tumour and normal samples. Tumours identified by this method as having the CpG island methylator phenotype (CIMP) were validated using Rand Index.

**Tumour methylation.** Methylation data from tumour samples were normalised for sequence length and GC content, and important probes were selected using LumiWCluster package, thus eliminating arbitrary detection P-value cutoffs in removing unreliable methylation values (Kuan et al, 2010). Wilcoxon rank sum test was employed in identifying statistically significant differences in methylation between samples with and without extracapsular spread (ECS); human papilloma virus (HPV)-induced tumour and HPV negative tumours; with and without lymph node metastasis.

For the generation of a methylation signature associated with recurrence, univariate Cox regression analysis was performed with methylation as predictor and freedom from recurrence (FFR) as response. Freedom from recurrence after resection was calculated from the date of surgery to the date of recurrence, with censoring of all other patients. Significant methylation markers associated with recurrence were selected by filtering on P-value < 0.01. Multivariate Cox regression was also performed with additional covariates including age, ECS and HPV status. A composite marker risk score was calculated for each patient from the linear combination of the methylation values weighted by the Cox regression coefficient for each selected marker. To quantify the predictive accuracy of the composite marker, time-dependent ROC curve at 5th year was generated. Estimation of time-dependent sensitivity and specificity based on censored outcome was performed using nearest neighbours algorithm. The area under the time-dependent ROC curve (AUC) at 5th year was calculated to quantify the predictive accuracy of the methylation signature. To assess the uncertainty in the predictive accuracy of the marker, using 10 000 bootstrap replicates and the bootstrap percentile method, 95% confidence intervals (CI) of the AUC were calculated. R packages survivalROC (Heagerty and Saha, 2012) and boot method, 95% confidence intervals (CI) of the AUC were calculated using 10 000 bootstrap replicates and the bootstrap percentile to quantify the predictive accuracy of the marker.

### RESULTS

**Summary of cohort characteristics.** The cohort comprised 29 males (66%) and 15 females (34%) with an age range of 29–86 years (median 60 years) at diagnosis. Clinicopathological characteristics of the cohort were similar to our larger OSCC series, 1992–2001 (Table 1), although it is clear that this cohort has higher pT and pN stage with more involved margins and involved nodes (Rogers et al, 2009). Although this bias is regrettable, it is principally related to the inclusion of fewer very small tumours (14% pT1 here, vs 25% in larger series). This reflects the understandable reticence and ethical dilemma in sacrificing the majority of very small tumour for research purposes rather than for pathological staging. Treatment of tumours was primary surgery in all cases and post-operative radiotherapy/chemoradiotherapy was given in 31 (74%) reflecting, again the rather advanced clinical stages related to the exclusion of the smallest tumours (Table 1). At the end of study, 19 (43%) of the patients had died, 11 (25%) of OSCC, 6 (14%) of other causes and 2 (5%) unknown. Amongst the 25 (57%) survivors, median follow-up data was 52 months and in all but two cases, the follow-up data was ≥43 months. Twelve (27%) cases had histologically proven recurrence of OSCC.

**Promoter methylation analysis in tumour vs normal.** Tumour and matched normal tissues from 43 OSCC patients were analysed in this study. Unsupervised principal components analysis identified two clusters along the first principal component (accounting for 10.7% of variability in the data) that separated...
tumour from normal samples, with a few samples misplaced (Figure 1). There was a tighter clustering of normal samples compared with tumour, which may reflect the expected greater biological heterogeneity in the tumour samples compared with the paired oral mucosal samples. A number of markers demonstrated significantly different methylation levels between tumour and matched normal samples in Wilcoxon signed-rank test. Forty-eight probes were identified as differentially methylated when a corrected P-value (FDR) smaller than 0.05 and difference in average β values (Δβ) > +0.2 or < −0.2 were used as filtration criteria. Of these, 32 probes were hypermethylated in tumour compared with normal tissue, whereas 16 were hypomethylated in tumour (Supplementary Tables 1 and 2).

**Comparison with published HNSCC methylation and mRNA expression microarray data.** A comparison with two previously published HNSCC methylation array data demonstrated a number of genes differentially methylated between tumour and normal in common with our study, all showing concordant methylation status (Table 2). When the top 25 methylation markers from Poage et al (2011) were compared with our data, there were 11 common in the list. A total of 7 out of 10 hypermethylated and 4 out of 15 hypomethylated in that study were common with our list. In comparison with Guerrero-Preston et al (2011), 8 genes were common in the differentially hypermethylated lists. A comparison of gene promoter methylation with gene expression presented in the selected microarray datasets (Ibrahim et al, 2003; Kuriakose et al, 2004; Dysvik et al, 2006; Ye et al, 2008) did not identify any genes in common with our list demonstrating greater promoter methylation in tumour. However, 5 differentially hypomethylated genes were found to be differentially expressed between tumour and normal in either all or some of the four datasets analysed, with 4 of the hypomethylated genes (SPP1, AIM2, PTHLH and IFNG) upregulated in tumour (Table 2).

**Pathway analysis.** Hypergeometric analysis of pathways and processes altered due to aberrant promoter methylation in tumour identified the role of VDR in transcriptional regulation of genes involved in osteoporosis (P = 0.03) as a significant canonical pathway along with glutathione metabolism (P = 0.04). On a process network level, a network representing response to hypoxia and oxidative stress (P = 0.03) was significantly enriched. However, since the pathway and network analysis was conducted on a limited set of genes selected, interpretation of the results needs caution.

**HPV status.** HPV-associated HNSCC has distinct demographic, biology and prognosis (Schache et al, 2011; Shaw and Robinson, 2011), and there is growing evidence that the DNA methylation regulatory pathways are targets for E6 and E7 oncoproteins (Burgers et al, 2007). Accordingly, differences in promoter methylation between HPV(+) and HPV(−) tumours were sought. Genome-wide methylation differences between these two classes were previously noted in squamous cell carcinoma cell lines (Sartor et al, 2011). In our study, PCA and unsupervised hierarchical clustering were capable of distinguishing the two classes based on methylation data in tumour samples, though to a lesser extent (Figure 2), although this may be slightly underpowered as only six samples were HPV-16 DNA positive (13%). However, Wilcoxon rank sum

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**Table 2. Genes showing differential methylation between tumour and normal in the present study, which are in common with other methylation or gene expression studies compared**

| Gene | Methylation status in present study | Methylation status in Poage et al | Methylation status in Guerrero-Preston et al | Expression status in Ye, Dysvik, Kuriakose or Ibrahim |
|------|-----------------------------------|----------------------------------|-----------------------------------------------|-----------------------------------------------------|
| FOXP4 | Hyper | Hyper | Hyper | — |
| HS3ST2 | Hyper | Hyper | Hyper | — |
| NPY | Hyper | Hyper | Hyper | — |
| P1R1B1 | Hyper | Hyper | Hyper | — |
| SOX17T | Hyper | Hyper | Hyper | — |
| MM1 | Hyper | Hyper | Hyper | — |
| EPHRA5 | Hyper | Hyper | Hyper | — |
| CALC4 | Hyper | — | Hyper | — |
| ETA4 | Hyper | — | Hyper | — |
| FOXAT1 | Hyper | — | Hyper | — |
| TAL1 | Hyper | — | Hyper | — |
| W1T1 | Hyper | — | Hyper | — |
| EMR3 | Hypo | Hypo | Hypo | — |
| PI3 | Hypo | Hypo | Hypo | — |
| SPP1 | Hypo | Hypo | Hypo | High (All) |
| AIM2 | Hypo | Hypo | Hypo | High (Ye) |
| PTHLIH | Hypo | — | High (Ye, Kuriakose) |
| IFNG | Hypo | — | High (Ibrahim) |
| CEACAM1 | Hypo | — | Low (Ye, Kuriakose) |

Methylation status from an Illumina GoldenGate study of 19 HNSCC tumours vs 11 non-malignant samples (Poage et al, 2011) and an Illumina HumanMethylation27 array study of 4 OSCC tissue versus 4 normal tissue (Guerrero-Preston et al, 2011) are presented. Comparison with gene expression differences in four HNSCC studies with ≥20 tumour/normal pairs (Ye et al, 2008; Dysvik et al, 2006, Kuriakose et al, 2004 and Ibrahim et al, 2003) is also presented. Complete list of methylation markers significantly differential between tumour and normal in our study are presented in Supplementary Tables 1 and 2.
test identified nine probes with higher methylation levels in the HPV(+) and five probes with higher methylation levels in HPV(−) tumours (P<0.05, Δβ > 0.2). Genes methylated at a higher level in HPV(+) were ALOX12, CCNA1, CTSL1, DAPK1, F2R, FLT1, CADM1, PDX1 and SEZ6L whereas FLJ20712, HIC1, MT1A, OSM and PDGFB were methylated more in HPV(−) samples. It is of note that CCNA1 and CTSL1 were also identified in the previous cell line study as highly methylated and with lower expression in HPV(+) compared with HPV(−) (Sartor et al, 2011).

CpG island methylator phenotype (CIMP). CIMP denotes the concordant tumour-specific DNA methylation observed in subgroup of tumours, and is associated with distinct clinicopathological characteristics. When the 48 differentially methylated promoters in this cohort were clustered based on average β values using hierarchical agglomerative clustering, two visibly distinct clusters were identified. One cluster comprising 19 tumour samples had mostly low β values (median = 0.3) and was designated as ‘CIMP-low’. The other cluster with 24 tumour samples had mostly high β values (median = 0.56) and was designated as ‘CIMP-high’ (Figure 3). A PCA plot of average β values from the 48 markers demonstrated a visible distinction between CIMP-high and CIMP-low groups of tumour samples along the first principal component (Supplementary Figure 1). External cluster validation also showed good agreement between clustering and the CIMP classes (Rand Index: 0.75). A Kaplan–Meier curve using freedom from recurrence (FFR) as endpoint demonstrated a statistically non-significant difference between the CIMP groups (Figure 4), with a trend towards worse prognosis in patients belonging to the CIMP-high category (Log-rank P = 0.06). This is in contrast to our previous observation based on only 10 selected gene promoters.

**Figure 2.** Principal components analysis showing HPV(+) (hexagon) and HPV(−) (sphere) tumour samples. Average β values from 43 tumour samples were employed in the analysis. One sample with unknown HPV status is also displayed.

**Figure 3.** Hierarchical clustering heatmap and dendrogram of tumour samples based on methylation markers differential between tumour and normal tissues. Average β values from the selected 48 probes were used for hierarchical agglomerative clustering using Euclidean distance and average linkage. CpG island methylator phenotype (CIMP) groups are shown by the two clusters on the dendrogram left of the heatmap, the bottom cluster designated as ‘CIMP-high’ and the top as ‘CIMP-low’.
(Shaw et al., 2007). However, CIMP was not an independent factor determining survival ($P = 0.3$) when a multivariate Cox proportional hazards regression was conducted with other covariates such as ECS and HPV status.

**Methylation signature for extracapsular spread (ECS).** Wilcoxon rank sum test identified 15 probes differentially methylated in patients with extracapsular spread when compared with patients without ECS ($P < 0.05$, Delta-beta $> 0.2$); Table 3. Two distinct clusters were observed in hierarchical clustering of the samples based on the markers selected (Figure 5), one containing samples from patients without ECS exclusively and the other a mix of both ECS and non-ECS samples. Genes differentially methylated between ECS and non-ECS patient samples included DCC, CALCA and WT1.

**Methylation signature of recurrence.** Cox proportional hazards regression with freedom from recurrence (FFR) identified a 17-gene signature associated with recurrence ($P < 0.01$; Table 4). A multivariate Cox regression including covariates age, ECS and HPV status also found the signature genes to be statistically significant ($P < 0.05$) except MT1A (0.068) and ATP10A (0.057). When a risk score generated from linear combination of the marker values weighted with regression coefficients was assessed using time-dependent ROC analysis with nearest neighbours method for censored outcome data, a good concordance of the marker values with freedom from recurrence (FFR) was observed with recurrence ($AUC = 0.897$, 95%CI: 0.847–0.951). With a risk grouping was observed with recurrence ($AUC = 0.916$, 95%CI: 0.870–0.954). With a risk grouping was observed with recurrence ($AUC = 0.916$, 95%CI: 0.870–0.954).

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In this study, we explored the epigenetic landscape of series of oral squamous cell carcinomas (OSCC) using an array approach. Significant difference in methylation levels of a number of genes distinguished tumour tissue samples from their matched normal, demonstrating epigenetic modifications acquired in the development of tumours. It was observed that a number of these differentially methylated genes in tumours were also identified in other recent investigations in HNSCC (Poage et al., 2010; Guerrero-Preston et al., 2011), with three genes in common with both these studies: HOXA9, HS3ST2 and NPY. In addition, EYA4 and WT1 were also among the list of genes selected for methylation in oral cancer tissue and pre-operative saliva in a study using similar array as in the present study (Viet and Schmidt, 2008). It is notable that all these common genes had consistently concordant methylation status, even though the studies differed in terms of heterogeneity of tumours, experimental design or methylation array platform employed. Methylation of one such gene, HOXA9 that codes for a homeobox protein, has been suggested as a biomarker for early detection of OSCC (Guerrero-Preston et al., 2011). In addition to HNSCC, methylation-associated silencing of the HS3ST2 gene has been observed in human breast, colon, lung and pancreatic cancers (Miyamoto et al., 2003) as well as in haematological neoplasms (Martin-Subero et al., 2009). Interestingly, there was no correlation between those gene promoter methylation targets and commonly downregulated genes as noted in the publicly available datasets. This may be explained by the multiplicity of molecular mechanisms regulating gene expression, only one of which is DNA methylation. Similarly, a metaanalysis of gene expression data from OSCC samples with matched normal samples, identified commonality with our list of 16 genes differentially hypomethylated between tumour and normal tissues. Among the genes showing lower levels of methylation and higher levels of expression in tumour was SPP1, which codes for osteopontin. This secreted glycoprotein has an important role in determining the oncogenic potential of tumour cells.
The identified putative methylation signatures for ECS and recurrence in this study require meticulous confirmation in an independent cohort, however, they may offer the promise of...
translation into diagnostic and prognostic biomarkers in tumour and surrogate tissue. In particular, epigenetic deregulation of NOTCH signalling in OSCC has been demonstrated as part of a methylation signature for recurrence. This complements the recent discovery of common NOTCH1, NOTCH2 and NOTCH3 mutations in HNSCC (Agrawal et al, 2011) using next generation sequencing. The NOTCH family of genes code for evolutionarily conserved intracellular signalling pathways which regulating cell–cell interactions. Further functional exploration of this pathway in HNSCC and identification of druggable targets are self-evidently a priority in translation of these findings to the clinic. Genes differentially methylated between ECS and non-ECS patient samples included DCC, CALCA and WT1. Interestingly the role of DCC as an epigenetically regulated tumour suppressor gene in HNSCC has already been documented in detail (Carvalho et al, 2006; Guerrero-preston et al, 2011). WT1 promoter methylation has subsequently been proven in our own group to be a clinically valuable biomarker in lung cancer screening (Nikolaidis et al, 2012) and has also been identified similarly as a biomarker for cervical cancer (Lai et al, 2008). CALCA promoter methylation has recently been established as an independent adverse prognostic factor in leukaemia (Ismail et al, 2011).

Tissue processing with macromolecule extraction was performed on whole biopsy tissue rather than on microdissected tissue and the composition of both the epithelial and the stromal compartments was unknown. Epithelial or stromal specific methylation patterns have been observed previously in both breast and prostate and so, a more compartment-specific approach may provide additional insights than a whole-tissue approach (Rodriguez-Canales et al, 2007; Qiu et al, 2008). The findings in particular of NOTCH involvement in this mixed cell population of undefined neoplastic cellularity could have greater significance if the epithelial component alone was utilised. The relevance of the epigenome from mixed whole tissue may however be more likely to reflect in vivo tumour phenotypes as these are reliant upon epithelial and mesenchymal interactions.

One of the aims of this study was to explore the occurrence of tumour subgroups with concordant methylation levels at multiple loci, known as CpG island methylator phenotype (CIMP) (Toyota et al, 1999). A wide range of genetic and environmental factors may lead to CIMP, including the increased expression and abnormal targeting of DNA methyltransferases (DNMTs) (Etoh et al, 2004; Kim et al, 2006; Teodoridis et al, 2008). We observed distinct clustering of tumour samples into CIMP-high and CIMP-low groups with genes differentially methylated between tumour and normal tissues. We have previously investigated the association of CIMP with allelic imbalance, pathology and clinical behaviour of OSCC (Shaw et al, 2007). In that study, CIMP was shown to correlate with a marked inflammatory response and less aggressive tumour biology (Shaw et al, 2007). However, in the present study, we observed a trend for CIMP-high tumours to be associated with poor prognosis, though CIMP was not an independent factor in predicting prognosis. This difference in observation may stem from the difference in technology and markers used, as in the previous study we had used only 10 genes involved in a variety of cellular processes. Although the current observation of CIMP-high tumours presenting poor survival, requires external validation with other HNSCC or OSCC series, it is in line with many other studies in various cancers (Maruyama et al, 2001; Wei et al, 2002; Brock et al, 2003; Roman-Gomez et al, 2004; Abe et al, 2005).

Although the epigenetic signature associated with HPV-driven HNSCC has been recently highlighted (Richards et al, 2009; Sartor et al, 2011), our data offers a somewhat less convincing distinction in the DNA methylation between HPV (+) and HPV (−) cases than these reports may imply. However, it has to be noted that we had only very few samples from HPV (+) patients. We have recently documented that many HPV (+) cases in our clinical population are also smokers (Schache et al, 2011), and although this seems to have little impact on prognostic benefit, this may add genotoxic stress and blur the lines drawn between smoking-related and HPV (+) cases. Notably, the CCNA1 promoter methylation seen in HPV (+) cases reported by Sartor (Sartor et al, 2011), is confirmed in our data, whereas epigenetic downregulation of this gene has been shown to be previously common in OSCC by pyrosequencing methylation assay (Shaw et al, 2006b, 2008). The differences in DNA methylation and underlying perturbations of epigenetic regulatory cellular machinery await further confirmation, elucidation and possible translational application.

We present methylation array data for a significant cohort of OSCC cases, incorporating detailed pathology and long-term clinical outcomes in keeping with our previous approach (Rogers et al, 2009). The benefits of using carefully curated snap-frozen tissue from paired tumour and normal tissues are self-evident in undertaking this work. Although comprehensive validation has not been attempted using single gene methylation assays, the reliability of this array in similar settings has been previously demonstrated (Bibikova et al, 2006; Bibikova and Fan, 2009). Additionally, comparison with expression array datasets (Yu et al, 2008) and the limited methylation array data available in OSCC (Viet and Schmidt, 2008; Poague et al, 2010; Guerrero-Preston et al, 2011) has been carried out, with confirmation of a number of targets. Although the spectrum of OSCC presented here is skewed towards larger tumours where tissue procurement is safely achieved, in other respects, this cohort represents a consecutive series treated uniformly according to written and audited protocols. The tissue procurement is associated with an academic tertiary centre responsible for treatment of all OSCC cases from a discrete geographical population of 2.5 million.

It is evident that epigenetic regulation features as a major mechanism in the carcinogenesis of OSCC and that several pathways appear to be specifically epigenetically deregulated in the cases showing greatest biological aggression that often prove refractory to aggressive conventional therapies. The potential to target epigenetics with novel agents seems a rational approach. Additionally, the translational capacity of epigenetic biomarker panels may be clinically applicable in screening, diagnosis, prediction of response and prognostication.

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