CpG island methylation phenotype (CIMP) in oral squamous cell carcinoma: Associated with a marked inflammatory response and less aggressive tumour biology

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Abstract

Studies in several tumour sites highlight the significance of the CpG island methylation phenotype (CIMP), with distinct features of histology, biological aggression and outcome. We utilise pyrosequencing techniques of quantitative methylation analysis to investigate the presence of CIMP in oral squamous cell carcinoma (OSCC) for the first time, and evaluate its correlation with allelic imbalance, pathology and clinical behaviour. Tumour tissue, control tissue and PBLs were obtained from 74 patients with oral squamous cell carcinoma. Pyrosequencing was used to analyse methylation patterns in 75–200 bp regions of the CpG rich gene promoters of 10 genes with a broad range of cellular functions. Allelic imbalance was investigated using a multiplexed panel of 11 microsatellite markers. Corresponding variables, histopathological staging and grading were correlated with these genetic and epigenetic aberrations. A cluster of tumours with a greater degree of promoter methylation than would be predicted by chance alone ($P = 0.001$) were designated CIMP+ve. This group had less aggressive tumour biology in terms of tumour thickness ($p = 0.015$) and nodal metastasis ($P = 0.012$), this being apparently independent of tumour diameter. Further, it seems that these CIMP+ve tumours excited a greater host inflammatory response ($P = 0.019$). The exact mechanisms underlying CIMP remain obscure but the association with a greater inflammatory host response supports existing theories relating these features in other tumour sites. As CIMP has significant associations with other well documented prognostic indicators, it may prove beneficial to include methylation analyses in molecular risk modelling of tumours.

Introduction

Increasing interest in cancer epigenetics has been reflected in the head and neck cancer literature[1-3]. It is now beyond dispute that aberrant cytosine methylation within CpG islands of tumour suppressor gene promoter regions[4] is critical to cancer development, and in particular, the earlier stages. This is not to suggest a diminished role of genetic changes such as deletion or mutation. Indeed it is suggested, in a modification of Knudson's double hit hypothesis[4;5], that in some circumstances one allele of a tumour suppressor gene may be inactivated by methylation and the other by genetic means. As with other fields of molecular biology, increasing knowledge is precipitated by technological advances. In epigenetics, the roles of bisulphite modification[6], methylation dependent restriction enzymes[7], real-time PCR[8], pyrosequencing[9;10] and various array techniques[11] have meant that DNA methylation is now one of the better characterised of molecular abnormalities in cancer.

Evidence suggests that the distribution of methylation at various gene promoters across a tumour series follows identifiable patterns. Tumours that have methylation at one gene are more likely to have other sites of methylation. This has been described as concordant methylation and the CpG island methylation phenotype (CIMP) and was first described 1999 in colorectal cancer[12], and shortly after in gastric cancer[13]. There was also a distinct tumour morphology and behaviour associated with highly methylated tumours ("CIMP high"). This concept mirrored in the epigenetic field what had been a long accepted genetic concept in colorectal cancer, i.e. microsatellite instability (MSI). In MSI mediated tumours, a defect in DNA repair leads to high rates of mutation and the selection of clones with growth advantage leading to the development of a malignant tumours[14]. The concept of these selective processes existing in the epigenetic field, however, has not been universally accepted by the scientific community[15]. The issue was further clouded by the linking of these two phenomena after the discovery that promoter methylation of the mismatch repair gene MLH1 was implicated in MSI[14]. A search of the literature fails to throw any light on the initiating factor in CIMP, analogous to MLH1 down-regulation in MSI, although work has understandably focussed on the DNA methyl-transferase mechanism[16].

Subsequent work in various tumour sites has confirmed the presence of CIMP in T cell leukaemia[17], hepatocellular carcinoma[18], neuroblastoma[19] as well as gastric and colorectal adenocarcinoma. In a recent large study, Samowitz[20] evaluated the CIMP and MSI concepts in colorectal adenocarcinoma. They found that CIMP was associated with mutations of *BRAF* and *KRAS2*, older age, increased stage and poorer prognosis and showed a trend with mucinous histology. In addition, they also found that MSI has a major effect on phenotype, in effect describing four categories of tumour defined by CIMP-high/low and MSI-high/low. Recent work in colorectal cancer even suggests a different pathogenesis in right and left sided tumours that correlates with the presence of CIMP[21], while in gastric cancers, CIMP has been associated with EBV DNA

positivity[22;23]. There has also been some speculation that CIMP represents a molecular signature consistent with inflammation mediated neoplasia across a variety of tumour sites[24] and it would also seem logical that sensitivity to epigenetic therapy may be profoundly linked to CIMP.

There are, as yet, neither published accounts of CIMP, nor any phenotypic or prognostic associations in head and neck squamous cell cancer (HNSCC). Various accounts have classified the pattern of methylation over several genes[25;26] or the global methylated cytosine "load" found in the whole genome[27] in HNSCC, but the investigation of the CIMP phenomenon in HNSCC is overdue, particularly as the most conclusive evidence stems from other regions of the aerodigestive tract. Whilst MSI appears to be rather rare in HNSCC, it might be informative to instead examine any relationship between allelic imbalance at key loci with CIMP.

We are convinced by the argument that quantitative methylation analysis offers convincing benefits in the accurate identification of CIMP[28] and aimed to use pyrosequencing methods to investigate a consecutive HNSCC series. The pattern of allelic imbalance at chromosomal regions implicated in HNSCC was also defined using a panel of microsatellite markers in order to ascertain and correlate the genetic contribution in the same tumour series. These epigenetic and genetic aberrations were then compared with histopathological parameters and clinical outcome data in order to clarify whether the CIMP concept has validity in HNSCC.

Methods

Patient cohort and tissue procurement

74 patients presenting to a head and neck cancer regional referral unit were selected for this study. The selection criteria included biopsy proven oral or oro-pharyngeal squamous cell carcinoma (ICD-O C02 –C09), an intention to treat by primary surgery and the absence of previous similar malignancy or treatment. $5mm^3$ tumour and "normal" margin samples were taken during surgery and snap frozen in liquid N_2. 5ml venous blood was collected into EDTA and centrifuged at 2000 x g for 5 minutes. Plasma was drawn off and the cell pellet was snap frozen. All samples were subsequently stored at–85^oC until DNA preparation. Detailed tumour and patient characteristics were documented, including clinical and pathological TNM grading. The commonest tumour sub-sites included anterior floor of mouth (C04) 20 (27%), tongue (C02.0,C02.2) 17 (23%) and tonsil (C09.0,C09.1,C09.9) 7 (10%) . pTNM pathological grading subsequent to resection was: pT1: 14 (19%); pT2: 30 (41%); pT3: 7 (9%); pT4: 23 (31%). 29 cases (39%) were pN positive and 18 (24%) had extra-capsular spread.

Sample preparation

DNA was extracted from the $2mm^3$ frozen tissue and white cell samples using a DNeasy™ tissue kit (Qiagen Ltd). Both tumour and "normal" margin tissues were prepared in parallel for methylation analysis. Bisulphite treatment of 2µg of each sample (for use in methylation assays) was undertaken using the EZ DNA Methylation Kit™ (Zymo Research) and the converted DNA eluted in 50µl of 0.1 X TE buffer.

Selection of genes for methylation assay

By combining review of the existing literature with our recently generated experimental data, a panel of 10 genes was formulated and appropriate primers were designed to amplify CpG islands within their promoter regions. *p16* is the most commonly studied gene down regulated by promoter methylation[2] and is involved in cell cycle checkpoint control. Another cell cycle control gene, cyclin A1, was recently identified by a pharmacological unmasking array approach[29] and has since been validated by pyrosequencing in OSCC[9]. The DNA repair mechanisms are represented by *ATM*[30], *hMLH1*[31] and *MGMT*[32], all of which have been suggested to be controlled by epigenetic means. There is a relatively extensive literature describing promoter methylation of cell differentiation regulation gene *RARβ*[33;34] and the cell adhesion molecule E-cadherin[35]. In contrast, only recently has this mechanism been suggested in the cell signaling molecule *STAT1*[36] and the matrix metalloproteinase inhibitor *TIMP3*[37]. Cytoglobin is a gene of unknown function that has been identified as a candidate tumour suppressor gene from our research group's recent work on tylosis, an inherited cancer syndrome[38-41]. Its downregulation by promoter hypermethylation has

been recently demonstrated in upper aerodigestive tract squamous carcinoma including HNSCC[39].

Quantitative pyrosequencing methylation analysis (PMA)

PMA was carried out as previously described[9]. Briefly, hot start PCR was carried out using 3µl of bisulphite treated DNA template in each reaction. Primer sequences, PCR conditions and pyrosequencing primer sequences are available on request. Confirmation of PCR product quality and freedom from contamination was established on 2% agarose gels with ethidium bromide staining. Pyrosequencing was carried out using the PSQ96MA System (Biotage™) according to manufacturer's protocol, including single strand binding protein (PyroGold™ reagents). An average methylation index (MtI) was calculated from the mean of the CpG sites evaluated (between 4 and 20 CpG dinucleotides per promoter per sample) as previously described[39].

Multiplex microsatellite analysis of allelic imbalance

A multiplexed panel of microsatellite markers was used as described before[41-43]. Briefly, PCR primers for the microsatellite loci D3S1263, D3S1289, D3S1300, D3S1566, D5S644, D9S157, D9S161, D13S153, D13S171, D13S263, and D17S938 were selected from Applied Biosystems linkage sets. These markers have previously been shown to show consistent allelic imbalance in aerodigestive tract squamous carcinoma samples[42]. Forward primers had 5' fluorescent modification and the reverse primers had a 5' biotin modification. Multiplex PCR reactions (10 μl) contained 2 μl (0.2 μg) of genomic (non-bisulphite treated) DNA from tumour or blood cells, 1µl relevant primers, 5 μl of Qiagen Multiplex PCR Kit Master Mix and 2 μl dH_2O. Cycling conditions were 95°C for 15 min, 30 cycles (94°C for 30 s, 56°C for 90 s, 72°C for 60 s) followed by 30 min at 60°C. PCR products were purified using 2 μl Dynabeads M-280 Streptavidin and resuspended in 4 μl of loading buffer (10:2:1 formamide, dextran blue/EDTA, ROX 400HD size standard). Samples were denatured at 95°C for 5 min, chilled on ice and 1 μl was loaded on a 5% denaturing polyacrylamide gel on a 377 ABI™ sequencer. The gel image was analysed using the ABI Genescan™ and Genotyper™ software.

Allelic imbalance was scored according to previously established performance criteria[41] and was present if the allelic ratio (A1/A2 tumour)/(A1/A2 leukocyte) was outside a range of 1.25-0.75. Assays were carried out in duplicate and AI scored only if both results for an individual sample were outside the normal range. A chromosomal arm was recorded as showing allelic imbalance if any one of the corresponding informative (heterogeneous) microsatellite markers showed a loss. Fractional allelic loss (FAL) was also calculated for each tumour, representing the proportion of informative chromosomal arms that demonstrated loss.

Detailed characterisation of histopathological features

The 13 tumours with highest or lowest overall methylation status (as defined below) were selected. H&E stained histopathological slides were submitted for further detailed analysis by two experienced oral pathologists (GH & JAW). In total 22 tumours were available for analysis (12/13 high and 10/13 low). The 4 other cases were rejected because either their tumours were principally intra-osseous, missing, or otherwise unsuited for comparison. The pathologists were blinded to the methylation status. The morphology and architecture of each tumour was classified according to Anneroth & Hansen[44] including assessment of the invasive front, nuclear polymorphism mitotic rate, degree of keratinisation and inflammatory response. Thus a range of scores for each component and total score of biological aggression was obtained for this subset of 22 tumours.

Statistical Analysis

The methylation index (MtI) for the tumour samples were normalised and subdivided into three groups (unmethylated, weakly and strongly methylated) in order to allow meaningful comparison between individual gene promoters. This was undertaken because the source tissue was not microdissected and consequently the MtI values for each sample were considered to more precise than accurate. The lower threshold, separating the unmethylated from the weakly methylated group, was derived as previously described[9] by identifying the MtI that best separated the tumours from normal controls.. For the genes not displaying tumour specific promoter methylation, an arbitrary MtI value of 5% was used, which is consistent with our previous approach in considering the upper level of background noise generated during pyrosequencing[9].

The tumour samples with MtI below the lower threshold MtI were considered to be essentially unmethylated. The methylated samples were then divided using a median split into a “weakly methylated” group (MtI>lower threshold but <median value) and a “strongly methylated” group (MtI>median value). Each tumour sample was classified separately for each of the ten gene promoters considered. The threshold between strongly and weakly methylated samples was quite consistent at around MtI 0.25 ± 0.05 for each gene. Further corroboration for this approach arises from the (unpublished) observation that an MtIP 0.20–0.25 in these samples was necessary to silence mRNA expression for the corresponding gene. Additionally an aggregate methylation score was calculated for each tumour by adding the number of gene promoters with strong methylation.

Correlations were made between the methylation status, allelic imbalance, pathological and clinical data available using SPSS software. For statistical calculations using aggregated measures of methylation scores or fractional allelic loss, a level of significance of $p<0.05$ was used. In considering the much larger number of correlations made between individual gene promoters and microsatellite markers, a more stringent level of $P<0.01$ was used.

Results

The percentages of unmethylated, weakly and strongly methylated tumours are illustrated for each gene in Table 1. The promoters of genes *ATM*, *STAT1* and *hMLH1* were methylated at less than 5% in all samples analysed and excluded from all further calculations.

Concordant methylation

In order to discover whether high methylation levels were occurring in an interrelated way ("concordant") or merely as random events, a goodness of fit calculation of observed versus expected methylation was performed (Table 2). As an illustration, if methylation had occurred as a random, non-interrelated process, it would be expected that the number of tumours with 3 or more of the 7 genes showing high methylation would be 5.7 however the actual count was 14. It was seen that the observed degree of methylation was greater than expected at both ends of the distribution. The overall goodness of fit indicated that promoter methylation occurred in a highly significantly interdependent or concordant manner ($P<0.001$). The fourteen tumours with high MtI at several of the gene promoters seemed to form a distinct cluster and will be referred to as CIMP+ve tumours below.

Further correlation between individual gene promoters was made (Table 3). It was seen that significant correlations existed between cytoglobin and RARb (Spearman correlation = 0.46, $P < 0.01$), cyclin A1 and MGMT (Spearman correlation = 0.35, $P < 0.01$).

Correlation of methylation phenotype with allelic imbalance

High levels of fractional allele loss (FAL), calculated from data at all 11 microsatellites, were observed in strongly methylated tumours (Spearman correlation 0.25, $P = 0.031$). Further exploration of correlations between individual chromosomal arms and individual gene promoters highlighted two noteworthy associations. Loss at 17p (D17S938) correlated with strong methylation at p16 ($P=0.001$ Pearson chi squared) and at cyclin A1 ($P=0.010$). Eight of 25 (32%) cases with allelic imbalance at D17S938 were strongly methylated at the p16 promoter and 6/24 (25%) at cyclinA1. By comparison, of those cases informative for D17S938 but without allelic imbalance, strong methylation was seen in 0/29 (0%) and 2/29 (7%) at p16 and cyclin A1, respectively. No other significant correlations were apparent between promoter methylation and allelic imbalance (at either $P < 0.05$ or $P < 0.01$ level).

Correlation of methylation phenotype with clinical features and outcome

When considering the association of promoter methylation with clinico-pathological parameters, there were significant associations between CIMP+ve cases and shallower tumour depth (P=0.015) and lower pN stage (P=0.012). Weak trends showing a lower incidence of extra-capsular nodal involvement (P=0.100 chi squared) and improved disease specific survival at 24 months (P=0.156) were also observed with CIMP+ve tumours. It also seemed that there was less likely to be a premalignant lesion co-existent with the index tumour in CIMP+ve cases (P=0.084). There were however, no significant correlations of CIMP status with gender, age, smoking, alcohol consumption, tumour sub-site, pT stage, mandibular invasion, or tumour involvement of surgical margins.

Taken overall, a case builds that the CIMP+ve cases had less aggressive tumour biology. The significant correlations seem perhaps more notable as they were in keeping with the Anneroth score data presented below, and further, were independent of any relationship with tumour stage.

Correlation of methylation phenotype with histopathological features

The individual components within, and total sum of, Anneroth scores were compared between 12/14 CIMP+ve and 10 CIMP-ve cases as shown in Table 4. Whilst there was a trend (P=0.134 Mann-Whitney) between the CIMP-ve tumours and a higher overall Anneroth score (more aggressive), this was largely explained by an association between methylation and the inflammatory response. The CIMP+ve group had much greater inflammatory response in tissue at the deep margin adjacent to the tumour than the CIMP-ve group (P=0.019). This is further illustrated by figure 1.

Discussion

We have brought together genetic, epigenetic, histopathological and clinical data in a series of 74 oral squamous cell carcinoma cases to investigate the importance of promoter methylation on tumour behaviour. We have found that there is a cluster of 14 tumours with a greater degree of promoter methylation than would be predicted by chance (P=0.001) and we adopt the term CIMP+ve to identify this group. The CIMP+ve group have less aggressive tumour biology in terms of tumour thickness (p=0.015) and pathological nodal invasion (p=0.012), this being apparently being independent of tumour diameter. Further, it seems that these CIMP+ve tumours excite a greater host inflammatory response (P=0.019) which might conceivably be related to this difference in clinical behaviour.

This study has the advantage of combining data from several disciplines relevant to cancer biology. We have previously published a subgroup of the pyrosequencing derived methylation data analysed in this paper[9] but the value of this quantitative approach is further highlighted in our current analysis of the methylation phenotype. We have categorised the status of these tumour samples as unmethylated (or within the threshold for normal tissue or experimental error), weakly, or strongly methylated and have observed a number of possible clinically relevant associations. It seems clear that a tumour with 1% promoter methylation of a given gene might be expected to show a biologically significant difference of expression of the gene compared with another tumour with 50% methylation and that this difference would not be apparent using a non-quantitative approach for detecting gene promoter methylation. We remain convinced that the pyrosequencing methylation assays described, whilst not the only quantitative method available, represent progress over simpler methods such as methylation specific PCR. We also have the benefit of a multiplexed panel of microsatellite markers which has previously been rigorously validated and optimised in similar studies in aerodigestive tract malignancy[41]. The inclusion of data from both routine post-operative pathological staging and from further focused examination of specimens has been essential, as has the clinical data derived from the Regional Maxillofacial Unit's head and neck database[45].

The number of gene promoters & microsatellites markers used in this study might be considered limited and could perhaps be usefully extended. In particular, there are several other genes[2] thought to be epigenetically silenced in OSCC such as DAPkinase, *RASSF1*, and *DCC*[2]. However, in this study we have attempted to incorporate a wide range of cellular functions within our panel and clearly these techniques are expensive in terms of logistics and in demand for high quality bisulphite treated DNA. A number of differing approaches are currently being considered in the development of a promoter methylation micro-array[11]. This has obvious attractions in the context of a study of CIMP, however they are not yet commercially available and it remains to be proven if they are sufficiently quantitative for this purpose. We also recognise that if the samples

had been microdissected, there might have been differences in the results obtained. In particular we have evidence that much higher levels of methylation might be detected in pure tumour tissue compared with non-microdissected specimens (unpublished). There are, however, some drawbacks to this technique. Firstly the total quantity of DNA obtained is much smaller and, as has been commented above, these techniques demand relatively high quantity and quality of DNA, particularly as the bisulphite treatment essential to the assay causes a degree of fragmentation. Secondly, the use of microdissection adds significant logistical burden that might result in fewer cases being included in any study.

The clear association that we observed between allelic imbalance at a locus close to *p53* (D17S938) and methylation of the two cell cycle control genes studied (*p16* and cyclin A1) is previously unreported. We speculate that aberrations in cell cycle control will lead to higher cell turnover and consequently predispose to further aberrations. It is difficult to know, assuming causation exists, which is the primary and which is the secondary event, although a recent paper by Maley et al[46] propose that *p16* inactivation (including by methylation) is the first event in the development of premalignant Barrett's oesophagus with *p53* inactivation developing later. It would certainly be of interest to investigate other head and neck tumour series, and other sites, to see if this relationship is duplicated.

A striking difference between the CIMP+ve and -ve cases was the host inflammatory response to the tumours seen on routine histopathology adjacent to the leading edge of the tumour. Whilst this response is usually taken as a favourable prognostic feature and may explain the correlation between CIMP+ve tumours and less aggressive tumour characteristics, there is also growing evidence that inflammation may have a role in carcinogenesis[47]. Clearly a distinction must be made between the inflammatory response to the tumour that we report here and any relationship to an inflammatory mechanism of pathogenesis. However, an association between CIMP+ve tumours and inflammation is also described in research on several other tumour sites. It is thought that in some circumstances, chronic inflammation or hyperplasia becomes complicated by an abnormal epigenetic programme. This then becomes 'locked in', pre-disposing that clone to ultimate malignant transformation[48]. In gallbladder[49] and colon cancer, the development of CIMP+ve tumours secondary to chronic inflammation have been described[50] and a similar association between chronic inflammation and viral aetiology has been discovered in liver cancer[51]. The parallel finding that CIMP+ve gastric tumours had greater evidence of previous EBV infection[52] allows speculation as to the viral aetiology of the methylation phenotype, which might also be pertinent to head and neck cancer. The work of Kang et al clarify the role of chronic inflammation and viral aetiology in both malignant and non-malignant gastric lesions which display CIMP[53;54] while Issa comments that the genes responsible for the creation of CIMP+ve tumours may not be in direct control of methylation,

but perhaps be involved in the predisposition to exaggerated and chronic inflammation to certain stimuli[24]. Further work is required in oral squamous carcinoma to determine if HPV, EBV or other inflammatory triggers might predispose to CIMP+ve tumours. Another credible hypothesis might be pathogenesis relating to chronic premalignant conditions, for example, oral lichen planus. However our data argues against this, with a trend for CIMP+ve actually less likely (P = 0.084, NS) to be seen concurrent with premalignant conditions.

Comparing our data on the CIMP in oral squamous carcinoma with other previously described sites, it is perhaps not unexpected that we have found correlations between the CIMP and histological subtype and also clinical behaviour. However we have not found any sub-site differences, in contrast to the predilection for the CIMP in certain areas of the bowel in colorectal adenocarcinoma. Also, in contrast to much of the work on other sites, we have found that the CIMP+ve cases appeared to have tumours with more biologically favourable characteristics, although one recent study in gastric adenocarcinoma also found that CIMP+ve tumours had better prognosis[55]. The trend that we have identified towards improved disease specific survival did not reach statistical significance. However the finding of greater lymph node involvement and tumour thickness in CIMP-ve cases would routinely have triggered the prescription of post-operative radiotherapy or chemo-radiotherapy[56] and this may have obscured at least some of the survival effects. Clearly the histology, aetiology and pathogenesis of oral squamous malignancy contrasts greatly with that of bowel or haematological malignancy and it is perhaps not surprising that such contrasts were found with previously published studies. It would be of interest to see if our findings were replicated in studies of laryngeal or other aerodigestive tract epithelial malignancy.

In reviewing the results of this study, it is apparent that several themes for further research emerge, as well as implications for translational approaches. The genes investigated covered a broad range of cellular functions including cell cycle control, DNA repair, cell adhesion, differentiation, signal transduction and response to hypoxia. Clearly, the demonstrated association between methylation of these genes would not seem to be directly causal, i.e. methylation of MGMT does not directly cause methylation of cyclinA1. These associations presumably result from some underlying alteration in DNA methyltransferase activity, the investigation of which might be of considerable value in understanding the malignant process. Whether this abnormality has a genetic or environmental aetiology is also of interest. A highly active focus for current translational research is epigenetic therapy i.e. using histone deacetylase inhibitors or manipulating DNA methyltransferase using 5-azacytidine to reverse epigenetic silencing of tumour suppressor genes. It is not known whether CIMP+ve tumours are more sensitive to such epigenetic therapy. If this was the case, it would be paradoxical that this novel approach used in oral squamous cell carcinoma would be more effective in the less aggressive tumours. Another clinical exploitation of DNA methylation is the field of biomarkers i.e. to monitor DNA methylation in

plasma, saliva or surgical margins to aid diagnosis or staging of disease. Similarly, the finding that methylation seems to be concentrated in less aggressive tumours perhaps may impose a restriction on the clinical translation of epigenetic biomarkers.

HNSCC is often treated using primary surgery with adjuvant chemo- and radiotherapy being prescribed on the basis of pathological staging of the resected specimen. In particular, tumour thickness[54] and the presence of cervical lymph node metastases[55] are frequently cited as important criteria for radiotherapy. As CIMP has demonstrated associations with these prognostic indicators, it is possible that methylation analysis might form a useful component of risk modelling in future strategies of molecular grading. As the role of organ preservation in HNSCC expands, a relatively non-invasive assessment of tumour grading may also be useful. The CIMP+ve cases demonstrated a greater inflammatory reaction and this might shed new light on the host response to the tumour with its possible influence on outcome.

The authors are unaware of any previous attempts to describe the CpG island methylation phenotype in head and neck cancer. We have taken a multidisciplinary approach, bringing together clinical pathological staging and outcome data with analysis of both genetic and epigenetic aberrations. Quantitative methylation analysis seems to offer significant advantages to the field and, using this approach, we have demonstrated several novel findings and associations. These shed new light onto the role of methylation in carcinogenesis and offer new directions for further research.

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	P16	CYCA1	MGMT	ECAD	TIMP3	RARβ	CYGB
Unmethylated	73	44	71	59	97	27	57
Weakly Methylated	14	40	15	21	0	45	27
Strongly Methylated	14	15	14	20	3	28	16

Table 1: Percentage of tumours showing promoter methylation
N=74

Count of "strongly methylated "promoters	**Observed (O)**	**Expected (E) count** (assuming independence of 'strong' methylation between genes)	**O vs E** $(O-E)^2/E$
0	34	21.8	6.8
1	19	29.9	3.9
2	7	16.6	5.6
3	10	4.9	5.3
4	4	0.8	12.8
5	0		
6	0		
7	0		
Sum	74	74	CHI=33.4, 4df P<0.001

Table 2: Observed versus expected distribution of tumours with strongly methylated promoters

	CYGB					
RARß	0.46*	*RARß*				
CCNA1	0.13	0.15	*CCNA1*			
p16	0.16	0.29	0.13	*p16*		
MGMT	0.24	0.28	0.35*	0.12	*MGMT*	
CDH1	-0.22	0.01	0.28	-0.03	0.27	*CDH1*
TIMP3	-0.05	-0.01	0.26	0.09	0.08	0.21

Table 3: Spearman correlations between high levels of promoter methylation of individual gene

CYGB: cytoglobin; *CCNA1*: cyclin A1; *CDH1*: E-cadherin

- P<0.01

Anneroth Score	Number of cases	
	Strongly methylated	Weakly methylated
8	1	0
11	2	1
12	2	1
13	2	1
14	1	1
15	1	0
16	2	3
17	0	2
18	0	1
19	1	0

Table 4: Anneroth score in methylation subgroups

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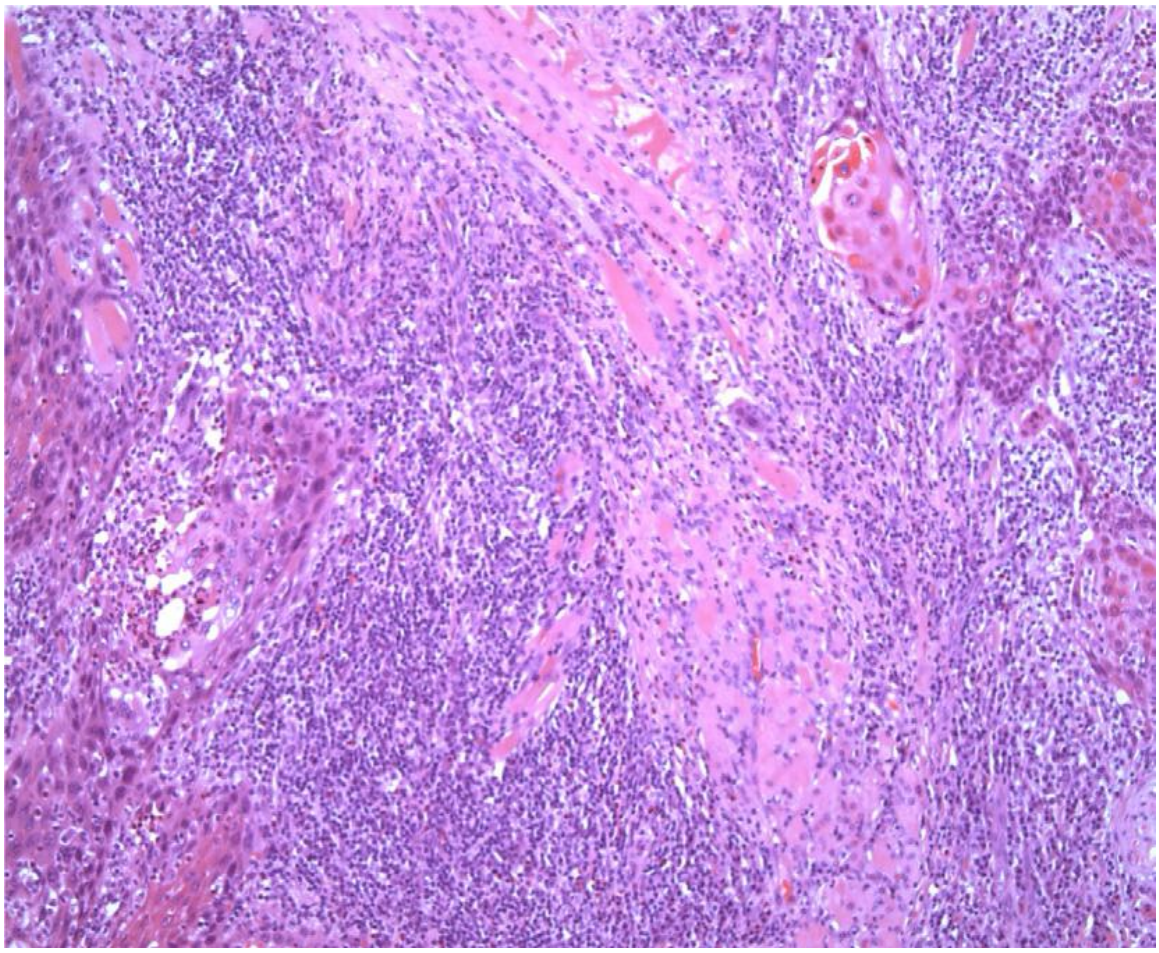

Figure 1 Photomicrograph demonstrating characteristics of deep invasive front (H&E ·20) of CIMP+ve tumour with profound inflammatory infiltrate. This SCC maxilla had pTNM staging: T4N0M0, with a depth of invasion 12 mm. Notably there was strong methylation of CYGB, RARb, cyclin A1 and MGMT. Latest clinical information is disease free survival at 25 months. The section shows a relatively cohesive advancing tumour front and obvious adjacent band of inflammation within underlying muscle.

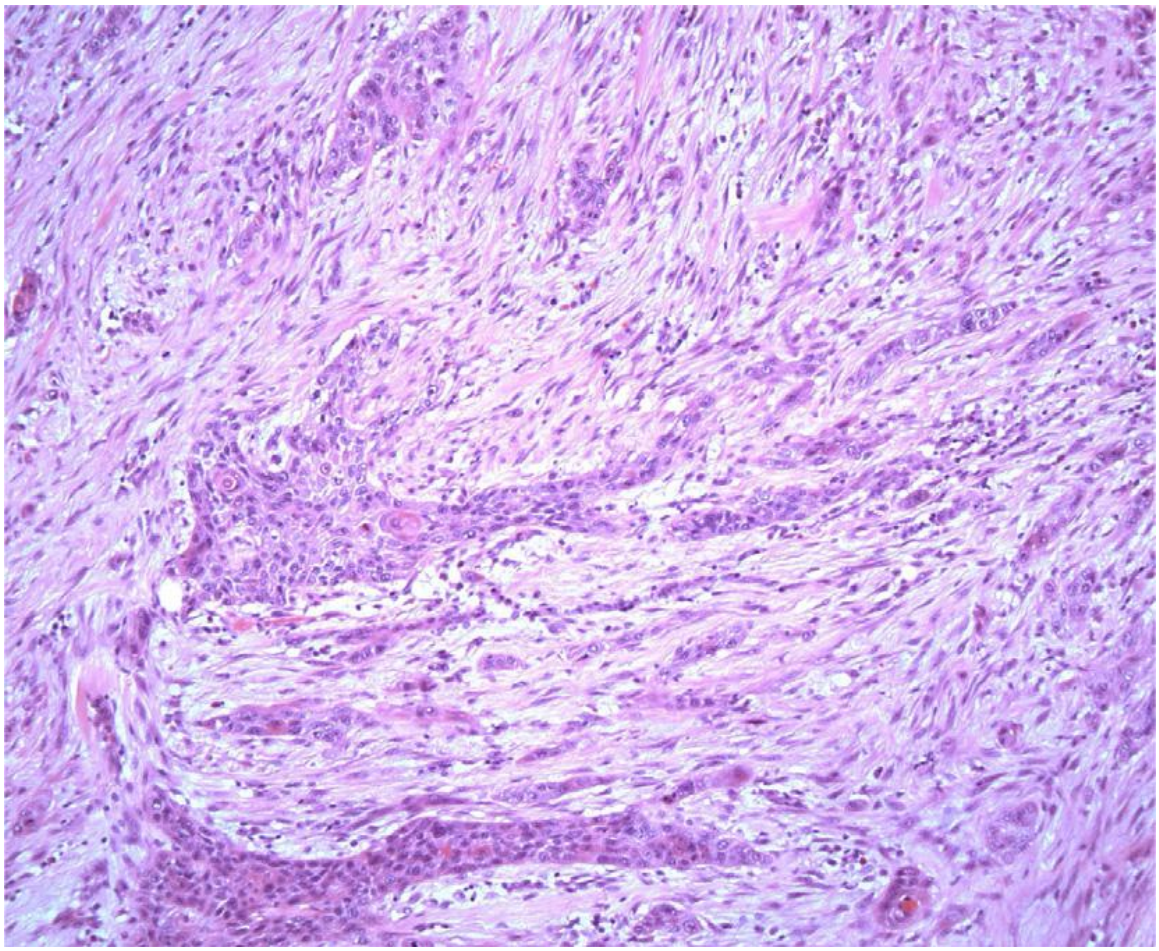

Figure 2 Photomicrograph demonstrating characteristics of deep invasive front (H&E ·20): CIMP_ve tumour with virtually absent inflammatory response. This case is from a tongue SCC pTNM stage T3N1M0 with a depth of invasion 30 mm. The MtI was below threshold or zero for all tested gene promoters. This patient had an aggressive tumour and unfortunately died of disease secondary to loco-regional recurrence at 7 months. A rather non-cohesive advancing front is seen with tumour cords in a desmoplastic but minimally inflamed stroma.