The Role of Pyrosequencing in Head and Neck Cancer Epigenetics

Correlation of Quantitative Methylation Data With Gene Expression

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Objective: To evaluate promoter methylation quantitation using recently described pyrosequencing techniques by correlation with messenger RNA (mRNA) expression.

Design: DNA was extracted from tissue samples and was subjected to bisulphite conversion. Quantitative methylation data for multiple CpG sites in each of 9 gene promoters were obtained for tumors using pyrosequencing. RNA was extracted and converted to complementary DNA, and this formed the template for relative quantitation assays of the expression of each gene by real-time reverse transcription–polymerase chain reaction.

Setting: Academic research.

Patients: Thirty-seven patients with head and neck squamous cell carcinoma.

Main Outcome Measures: The genes studied were P16 (OMIM 600160), cyclin A1 (OMIM 604036), RARB (OMIM 180220), E-cadherin (OMIM 192090), MGMT (OMIM 156569), STAT1 (OMIM 600555), ATM (OMIM 607585), hMLH1 (OMIM 120436), and TIMP3 (OMIM 188826). Immunohistochemistry was also performed for p16.

Results: STAT1, TIMP3, ATM, and hMLH1 promoters were essentially unmethyalted in all cases. The data for cyclin A1 (Spearman rank correlation, $p=-0.53; P<.001$), MGMT ($p=-0.53, P<.001$), and RARB ($p=-0.34, P=.02$) showed the expected negative correlation between levels of methylation and mRNA expression. The data relating to E-cadherin were inconclusive. Surprisingly, P16 expression was statistically significantly greater in those cases with higher levels of methylation ($p=0.57, P<.001$), a finding at odds with assumptions usually made in the literature relating gene promoter methylation to reduced gene expression. The results from p16 immunohistochemistry were in keeping with the mRNA data, but the number of positive staining samples proved too few for statistical analysis.

Conclusions: These data present a novel perspective on head and neck cancer epigenetics and reveal new and some unexpected associations and findings. The advantages of pyrosequencing over nonquantitative techniques are discussed in analyses of this nature.


Although the role of gene promoter methylation in head and neck cancer is not in doubt,1,2 the methods available to detect and to quantify methylation are numerous, and opinions of the merits of each differ. The methods described might best be divided into those using and those not using bisulphite conversion. In the case of the former methods, methylation-sensitive and methylation-insensitive restriction enzymes3 are used to digest DNA followed by Southern blot hybridization, and derivations of this technique use restriction landmark genomic scanning4 to analyze the digested product. In the case of the latter methods, a landmark article describing bisulphite conversion was published by Herman et al5 in 1996. The DNA sample is treated using bisulphite, which specifically converts all methylated cytosine residues to uracil. Uracil is read by subsequent polymerase chain reactions (PCRs) as thymidine; hence, this method transfers a positive or negative methylation status to a C/T polymorphism, which is subsequently more readily amenable to measurement by several available techniques of DNA assay. Methylation-specific PCR (MSP), also described by Herman et al,3 uses 2 sets of PCR primers complementary to “methylated” or “unmethylated” sequences, the former to select for cytosine in the target sequence and the latter for thymidine. This method is cheap, rapid, and highly sensitive com-
pared with predecessors and provided the basis of most methylation reports in the oncology literature for the subsequent decade.

As technology has progressed and the limitations of MSP have become apparent, the resultant demand for other techniques has been met by numerous alternatives. Notable oversensitivity, with false-positive methylation results, can be the outcome of inadequate completeness of bisulphite treatment (inherent PCR specificity limitations) or the use of high numbers of PCR cycles. In addition, MSP usually gives results for 2 to 4 CpG sites in a gene promoter, and assumptions are generally made about comethylation of neighboring sequences. Some MSP modifications have been described, including the use of nested primers to control for bisulphite conversion or the addition of fluorescent dyes or radioactive nucleotides to primers to aid quantitative evaluation.

Bisulphite treatment followed by non–methylation-specific PCR and subsequent C/T assay has been a promising avenue. Although conventional sequencing techniques and restriction enzyme approaches (COBRA [combined bisulfite restriction analysis]) have been described, they have proved complex and cumbersome for large-scale analysis. The most recently described technique is pyrosequencing. Pyrosequencing offers a semi-quantitative, high-throughput, and reliable method with built-in internal control for adequacy of bisulphite treatment. Following PCR of bisulphite-converted DNA using primers that anneal to CpG-free regions within CpG islands, the target CpGs are evaluated by stepwise addition of bases to a cytosine-free pyrosequencing primer and by analysis of the resulting pyrogram. Guanine is incorporated during pyrosequencing if the template CpG was methylated, whereas adenine is incorporated if the template CpG was unmethylated. Therefore, the proportion of G/A incorporated is stoichiometrically proportional to the degree of methylation at that CpG site in the template DNA. The analysis of a non-CpG cytosine during pyrosequencing provides the internal control of the completeness of bisulphite treatment that critically underpins all of the analysis but is absent from many other techniques. In effect, pyrosequencing offers a high-throughput method of bisulphite sequencing that offers many logistic advantages.

The objective of this study was to evaluate promoter methylation quantitation using pyrosequencing in head and neck cancer by correlation with expression. The literature is now burdened with studies investigating methylation in isolation. The importance of methylation to gene expression, or to overall biological behavior of the tumor, is often overlooked. We aimed to address this problem using techniques we regard as gold standard. Although various methods exist, relative quantification of messenger RNA (mRNA) by real-time reverse transcription–PCR using fluorescent probes has become a widely accepted and valuable technique. A panel of 9 genes was formulated, and appropriate primers were designed to amplify CpG islands within their promoter regions. These genes were selected as representative of different cellular functions and as promising candidates for silencing in head and neck squamous cell carcinoma by promoter methylation based on published literature. P16 is a commonly studied gene in this context and is involved in cell cycle check point control. The cyclin A1 promoter has been shown to be frequently methylated in head and neck cancer using a pharmacologic unmasking array approach and is involved in regulation of the cell cycle. ATM, hMLH1, and MGMT, all of which have been suggested to be regulated by epigenetic means in cancer at this site, have functions in DNA mismatch repair. The literature describes methylation of the cell differentiation regulation gene RARB and of the cell adhesion molecule E-cadherin in head and neck cancer. Recently, methylation in the promoters of the cell-signaling molecule STAT1 and of the matrix metalloproteinase inhibitor TIMP3 has been suggested to be important in these cancers. By comparing paired pyrosequencing methylation assay (PMA) and expression data for the genes studied, further evaluation of the merits (or otherwise) of pyrosequencing is expected to shed more light on the contribution of methylation to carcinogenesis in head and neck cancer.

**METHODS**

**DESIGN**

Sample Preparation

DNA was extracted from 2-mm³ snap-frozen tissue samples using a tissue kit (DNeasy; Qiagen, Studio City, California). Thirty-seven tumor and 8 “normal” tissue samples from beyond the edge of the surgical resection were prepared for methylation analysis. Bisulphite conversion of 2 µg of each sample (for use in methylation assays) was undertaken using a kit (EZ DNA Methylation; Zymo Research, Orange, California), and the converted DNA was eluted in 50 µL of 0.1 x TE buffer (10 mM Tris hydrochloride, 1 mM EDTA, pH 8.0).

RNA was prepared from additional 2-mm³ specimens of the 37 tumors and 8 normal tissues using a tissue kit (RNeasy; Qiagen). Adequacy of RNA quality and concentration was confirmed (RNA Labchip with 2100 Bioanalyzer; Agilent Technologies Ltd, Santa Clara, California). Complementary DNA (cDNA) was prepared from 1 to 2 µg of RNA using 2-step reverse transcriptase (RETOscript; Ambion, Austin, Texas). The quality of resultant cDNA and the absence of contamination were confirmed by PCR using β-actin primers.

Quantitative PMA

Pyrosequencing methylation assays were performed as previously described. Briefly, hot-start PCR was carried out using 3 µL of bisulphite-treated DNA template in each reaction. Primer sequences, PCR conditions, and pyrosequencing primer sequences are available on request from the authors. Confirmation of PCR product quality and of freedom from contamination was established on 2% agarose gels using ethidium bromide staining. Pyrosequencing was performed (PSQ96MA System; Biotage, Uppsala, Sweden) according to the manufacturer’s protocol using single-strand binding protein (PyroGold reagents; Biotage Ltd, Uppsala, Sweden). A mean methylation index (MtI) was calculated from the mean of the methylation percentages for the CpG sites evaluated as previously described. Some PMA results were compared with those of a previously described semi-quantitative technique, namely, real-time methylation-specific PCR (RTMSP). Eighteen bisulphite-converted DNA samples with a spectrum of P16 promoter methylation were also...
subjected to RTMSP using techniques previously described and a thermal cycler (model 7500; Applied Biosystems, Foster City, California). Sequences for methylation-specific primers and probe are available on request from the authors.

### REAL-TIME PCR mRNA EXPRESSION ASSAY

Real-time PCR expression assays were performed for genes showing statistically significant promoter hypermethylation in the tumor samples. Commercially available intron-spanning probes (FAM/MGB TaqMan) were analyzed and compared with the TATA box-binding protein endogenous control “housekeeping gene” (TBP) using the thermal cycle. Each reaction was performed in duplicate, and the mean result was used. Relative quantification was by the 2−ΔΔCT method. The values for the tumor-derived samples were subsequently equated to an approximation for normal tissue by equating to the mean of the values for the 8 normal marginal samples. A nontemplate control assay was performed simultaneously in every PCR reaction to exclude contamination.

### IMMUNOHISTOCHEMISTRY FOR p16 EXPRESSION

Thirty-eight fixed-tissue specimens corresponding to the fresh tissue analyzed as already described were available and deemed suitable for immunohistochemistry (IHC). Sections were deparaffinized in xylene followed by dehydration in graded alcohols. Epitope retrieval was performed for 20 minutes using microwave retrieval solution. Following cooling and rinsing with wash buffer, sections were quenched of endogenous peroxidase by peroxidase block for 5 minutes and were rinsed again in wash buffer. Mouse antihuman p16 antibody (CINTEC histology kit; MTM Laboratories, Heidelberg, Germany) was applied to one slide and negative control to another slide from the same block, which were then incubated at room temperature for 30 minutes. Visualization reagent, consisting of secondary goat antimouse immunoglobulin and horseradish peroxidase molecules linked to a polymer backbone, was applied and incubated for an additional 30 minutes at room temperature. Localization of antigen was visualized using chromogen (DAB; DAKO, Carpinteria, California) for 10 minutes, and sections were then counterstained with hematoxylin, dehydrated in alcohol, cleared in xylene, and mounted. All sections were evaluated by 2 of us (G.L.H. and P.S.).

### STATISTICAL ANALYSIS

The paired methylation and expression data were tabulated and analyzed using commercially available software (SPSS, version 15; SPSS Inc, Chicago, Illinois). Correlations between the 2 methylation assays and expression with methylation values were performed using Spearman rank correlation (p) test.

### PATIENTS

The cohort for this study comprised 37 patients with squamous cell carcinoma of the oral cavity (30 cases [12 oral tongue, 6 floor of mouth, 5 maxilla, 3 buccal, 3 mandibular alveolus, and 1 retromolar] or oropharynx [7 cases [4 tonsil, 2 base of tongue, and 1 soft palate]). Selection criteria specified an intent to treat surgically and the absence of previous malignant neoplasms and curative treatments. Tumor samples were immediately snap-frozen in liquid nitrogen at the time of surgery. For comparison with each tumor, additional epithelial samples were obtained from beyond the surgical margin as best representative of normal tissue. These normal samples were subsequently used only if histopathologic staging confirmed mucosal margins free of malignancy exceeding 5 mm. Each sample was coded and subsequently stored at ~85°C for a maximum period of 22 months. All patients provided informed consent for use of their tissue for research, and ethical approval was obtained as per local protocols.

### METHYLATION DATA

Methylation analysis was undertaken on 37 tumor and 8 normal control samples and was obtained for gene promoters in more than 87% of specimens (Table). The promoters of the hMLH1, ATM, and STAT1 genes were unmethylated in all tumor and normal control samples (MtI < 0.05), while the TIMP3 promoter was methylated in only 2 tumors. No further data will be presented for these genes. Promoter methylation (MtI > 0.05) was observed for the remaining genes (P16 [26%], cyclin A1 [43%], MGMT [31%], RARB [75%], and E-cadherin [38%]).

Methylation data for 18 tumor samples were compared relative to PMA vs RTMSP. All of the PMA results exceeding an MtI of 0.05 reached threshold using RTMSP at between 31 and 35 PCR cycles. An additional 3 specimens that had an MtI of less than 0.05 using PMA reached threshold after 41, 43, and 46 cycles, demonstrating the predictable increased sensi-

### Table. Promoter Methylation (Based on the Mean Methylation Index [MtI]) and Messenger RNA (mRNA) Expression Data for 37 Tumor and 8 Normal Control Samples

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cyclin A1</th>
<th>MGMT</th>
<th>P16</th>
<th>RARB</th>
<th>E-Cadherin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter methylation, range (mean)</td>
<td>0.00-0.47</td>
<td>0.00-0.45</td>
<td>0.00-0.51</td>
<td>0.00-0.54</td>
<td>0.00-0.31</td>
</tr>
<tr>
<td>mRNA expression, normalized range (mean)</td>
<td>0.02-0.87</td>
<td>0.1-1.4</td>
<td>0.03-2.63</td>
<td>0.1-5.1</td>
<td>0.4-16.4</td>
</tr>
<tr>
<td>mRNA expression, fold change</td>
<td>935</td>
<td>47</td>
<td>8767</td>
<td>51</td>
<td>41</td>
</tr>
<tr>
<td>Spearman rank correlation between MtI and</td>
<td>-0.53 (P&lt;.001)</td>
<td>-0.53 (P&lt;.001)</td>
<td>0.57 (P&lt;.001)</td>
<td>-0.34 (P=.02)</td>
<td>0.06 (P=.73)</td>
</tr>
<tr>
<td>normalized mRNA expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a In a small number of samples (6 for E-cadherin, 3 for cyclin A1, and 2 for MGMT), pyrosequencing methylation assay data were not obtained.

b The MtI ranges from 0.00 (0% methylated alleles) to 1.00 (100% methylated alleles).

c Fold change between the lowest and highest normalized expression data points for that gene.
tivity offered by RTMSP. All 18 paired results for PMA and RTMSP were ranked and correlated with a Spearman $\rho$ value of 0.72 ($P < .001$).

mRNA EXPRESSION DATA

Messenger RNA expression data were obtained for all 5 genes (and $TBP$ endogenous control) that showed statistically significant promoter methylation (Table). Overall, cyclin A1 and $P16$ showed much greater expression in tumors than in normal controls, whereas $RARB$, E-cadherin, and $MGMT$ were expressed at similar orders of magnitude in tumors and in normal controls. Partly as a consequence, the ranges observed between the lowest and highest expression data points were much greater in cyclin A1 and $P16$ ($10^3$-$10^4$ fold) than in the other 3 genes ($40$-$50$ fold).

CORRELATION BETWEEN METHYLATION AND mRNA EXPRESSION DATA

Quantitative promoter methylation and mRNA expression data for all samples, including the 8 normal controls, were plotted on graphs. The cyclin A1, $MGMT$, and $RARB$ genes showed negative correlations between levels of methylation and mRNA expression (ie, the higher the MtI, the lower the expression) as shown by negative $\rho$ values for Spearman rank correlations (Figures 1, 2, and 3 and Table); these 3 negative correlations were statistically significant ($P < .001$, $P < .001$, and $P = .02$, respectively), and it was noted that an MtI of greater than 0.20 to 0.25 seemed necessary to statistically significantly reduce mRNA expression of each gene. Data from the E-cadherin gene showed no trend between the 2 variables (Figure 4). Surprisingly, the data for $P16$ showed a statistically significant positive correlation between the 2 data sets (ie, a greater degree of methylation correlated with a higher level of mRNA expression) (Figure 5 and Table).

IHC AND CORRELATIONS WITH METHYLATION AND mRNA EXPRESSION DATA FOR P16

Immunohistochemistry staining for p16 was graded absent (32 samples), weak (1 sample), moderate (2 samples), or strong (3 samples). Because of the small number of samples with statistically significant positive staining, subsequent statistical analysis proved difficult. In correlating with mRNA expression, it was notable that specimens with the highest mRNA values had strong or moderate staining; however, there was also a wide spectrum of mRNA expression values with no identifiable staining with IHC (Spearman $\rho$ mRNA expression vs IHC, $0.37$; $P = .02$). With regard to correlations between methylation and IHC results, 5 of 6 samples with positive staining had zero methylation (MtI, $<0.001$); however, the remaining sample (with strong staining) had an MtI of 0.27 (Spearman $\rho$ MtI vs IHC, $-0.13$; $P = .45$).
In this article, we describe the application of PMA, a new technique of quantitative methylation analysis that allows correlation with quantitative expression data. This technique seems to give results that are broadly comparable to those of a previously described semiquantitative technique. The strengths of PMA lie in providing quantitative methylation data\textsuperscript{11}: however, there is an MtI sensitivity threshold of approximately 0.05 (5% methylation).\textsuperscript{22} In applications requiring greater sensitivity, assays such as MSP, or derivations of MSP such as RTMSP or methylation enrichment pyrosequencing,\textsuperscript{25} have greater usefulness.

Four of 9 genes identified from the literature as being methylated in head and neck cancer were effectively unmethylated in our series. However, as might be expected, 3 of 5 of the remaining genes (RAR\textsubscript{B}, cyclin A\textsubscript{1}, and MGMT) showed strong and statistically significant negative correlations between the amount of promoter methylation and mRNA expression. The data for E-cadherin showed no statistically significant correlation but, remarkably, P16 expression positively correlated with promoter methylation, opposite to the relationship that has previously been assumed. These data present a novel perspective on head and neck cancer epigenetics and reveal new and some unexpected associations and findings. The advantages of pyrosequencing over nonquantitative techniques are apparent in analyses of this nature.

There was a small number of cases (2-6 samples) in which methylation data could not be obtained. We speculate that these failed because of excess DNA fragmentation during bisulphite conversion or because there were deletions or mutations at critical sites on the promoter. This reinforces our impression that methylation studies can be demanding of DNA in quality and in quantity. The effects of bisulphite conversion and the large amounts of PCR product required for successful pyrosequencing reactions lie behind this; this might be seen as a disadvantage of pyrosequencing, particularly if fixed specimens must be used as source material. All failures were repeated but with disappointing results. However, the real-time expression data were 100% complete, possibly relating to the high quality of tissue and RNA obtained, the meticulous preparation of the samples, and the use of fully optimized and validated commercial probes.

The finding that 4 of the genes selected from the literature were unmethylated in our series requires some explanation. In some cases, the previous sequences examined are unavailable; therefore, it may be that a different CpG island has been chosen or that the levels of CpG methylation vary within a single island. Another possibility is that the methylation results in the reports overestimated the number of tumors showing methylation because of limitations of the techniques used\textsuperscript{24} (eg, because of false-positive results due to incomplete bisulphite conversion).

The tissues used were not microdissected specimens and the tumor purity was unknown, although previous findings indicate that most of the tumor specimens are greater than 75% tumor cells (J. M. R. and J. K. F., unpublished data, 2003). It would be informative to repeat the study after microdissection and to compare the results. However, the amounts of DNA resulting from microdissection can be very low, and the total available after bisulphite conversion would not have been sufficient to study 9 gene promoters. In several genes, a minimum threshold MtI of approximately 0.25 (ie, 25% of the copies of the gene methylated) was required to statistically significantly downregulate expression. To what extent this threshold might relate to partial, monoallelic, or bi-allelic promoter methylation is not clear. These data seem to suggest that the levels of methylation required to suppress expression are high and that the percentage of tumors displaying this is lower than the usually quoted figures for methylation, particularly using MSP.

The mRNA data for P16 were particularly surprising. P16 is widely studied in the field of epigenetics, and it is an oft-repeated assumption, although seemingly rarely tested, that promoter methylation silences the gene.\textsuperscript{25,26}
In attempting to explore this correlation by the additional use of IHC for the same samples, we aimed to validate this finding. However, the results seemed inconclusive, although correlations have previously been attempted between methylation and protein expression in head and neck tumors, with similarly disappointing results.27 There seems to be some precedent in the recent literature that promoter methylation may paradoxically be associated with gene upregulation, a finding described by the authors as a "paradigm shift."28 Methylation may be initiated in a reaction to overexpression of the gene (ie, as a controlling arm of a negative feedback loop). It is also possible that, for some reason, the CpG island studied has the opposite downstream effect on histones than might normally be expected, although the CpGs studied herein include those originally described by Herman et al.3 This remains an intriguing finding that may be worthy of further investigation and validation in other tumor series.

As the various platforms available for molecular assays develop, it becomes possible to gain more accurate and comprehensive data about the aberrations found in cancer. For methylation assays, we believe that the benefits of pyrosequencing are notable in this regard. It is possible that, for some reason, the CpG island studied has the opposite downstream effect on histones than might normally be expected, although the CpGs studied herein include those originally described by Herman et al.3 This remains an intriguing finding that may be worthy of further investigation and validation in other tumor series.

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Author Contributions: Drs Shaw, Field, and Risk had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Shaw, Hall, Field, and Risk. Acquisition of data: Shaw, Hall, and Liloglou. Analysis and interpretation of data: Shaw, Lowe, Liloglou, Sloan, and Risk. Drafting of the manuscript: Shaw, Lowe, and Risk. Critical revision of the manuscript for important intellectual content: Shaw, Liloglou, Field, Sloan, and Risk. Statistical analysis: Shaw and Lowe. Obtained funding: Shaw and Liloglou. Administrative, technical, and material support: Shaw, Hall, and Field. Study supervision: Shaw, Liloglou, Field, and Risk.

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REFERENCES