Quantitative Promoter Hypermethylation Analysis of Cancer-Related Genes in Salivary Gland Carcinomas: Comparison with Methylation-Specific PCR Technique and Clinical Significance

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Abstract  Purpose: To compare the methylation status of tumor-associated genes by quantitative pyrosequencing and qualitative methylation-specific PCR (MSP) techniques and to correlate the results with clinicopathologic features and patients outcome to determine which method might have greater clinical utility.

Experimental Design: The hypermethylation status of the retinoid acid receptor \( \beta \)2 (\( RAR\beta 2 \)), \( RAS \) association domain family 1A (\( RASSF1A \)), \( O^6 \)-methylguanine-DNA methyltransferase (\( MGMT \)), and \( E-cadherin \) genes was analyzed in five salivary carcinoma cell lines and 69 human salivary gland carcinoma specimens by pyrosequencing and MSP techniques. The two datasets were compared by linear regression. Correlations between methods and with clinicopathologic characteristics were assessed by Pearson’s \( \chi^2 \) test or the two-tailed Fisher exact test, as applicable, using cutoff points determined from the regression curves and empirical fitting. We also investigated the effect of demethylating agents on methylated genes in cell lines to assess their effect on the expression of these genes.

Results: Overall, regression analysis indicated high degrees of correlation of the two methods for measurement of methylation for the \( RAR\beta 2 \), \( RASSF1A \), and \( MGMT \) genes (adjusted \( R^2 = 0.319, 0.835, \) and 0.178; \( P < 0.001, <0.001, \) and 0.0002, respectively) among the 69 tumors tested. However, the pyrosequencing technique yielded four more instances of methylation above background levels than MSP for \( RAR\beta 2 \) and three more for \( RASSF1A \). Methylation of both \( RAR\beta 2 \) and \( RASSF1A \) alone or both by pyrosequencing were correlated with tumor type (\( P = 0.027, 0.014, \) and 0.012, respectively). Methylation of \( RAR\beta 2 \) alone and in combination with \( RASSF1A \) by pyrosequencing were also significantly correlated with tumor grade (\( P = 0.014 \) and 0.011, respectively) and 3-year survival (\( P = 0.002 \) and 0.004, respectively). The survival curves of patients who had hypermethylation at both \( RAR\beta 2 \) and \( RASSF1A \) were significantly lower than those of patients who had hypermethylation at neither or just for the \( RASSF1A \) (\( P = 0.008 \) and 0.007, respectively). 5-Aza-2-deoxycytidine treatment of methylated cell lines led to the reactivation of \( RAR\beta 2 \) expression in only one of the five cell lines.

Conclusions: (a) Although the methylation status of \( RAR\beta 2 \), \( RASSF1A \), and \( MGMT \) genes by both techniques were significantly correlated, pyrosequencing is generally more sensitive and its results correlate better with the clinical variables than those of MSP. (b) The methylation level of \( RAR\beta 2 \) and/or \( RASSF1A \) by pyrosequencing is significantly associated with aggressive tumor phenotypes and patients survival.

Salivary gland carcinomas (SGC) are relatively uncommon, comprising ~10% of all head and neck cancers. Known for their marked phenotypic heterogeneity and wide range of biological behavior (1), these tumors can be categorized on the basis of their cytologic and architectural features into two broad classes, high and low to intermediate categories (2). Conventional primary therapy for SGCs is complete surgical resection, but for advanced surgically unresectable and recurrent tumors, the therapeutic options are limited and patient mortality is high (3). In search of objective variables for the biological stratification of patients with such advanced tumors, we and others have studied several biomarkers (4–6), but none of them has been validated for clinical utility.

Hypermethylation of CpG-enriched sites, CpG islands, at the promoter and the first exon of genes is an important
mechanism in the functional suppression of genes associated with mammalian development and cancer (7–9). This process entails the enzymatic addition of a methyl group to the fifth position of the deoxythymidine residue in each affected CpG dinucleotide (8–10). A number of reports have revealed an association between the aberrant hypermethylation of these CpG islands in several tumor suppressor genes and tumor development and thus implicated methylation as an alternative epigenetic mechanism of gene inactivation (11–15). In contrast to the integral nature of DNA-related alterations, epigenetic modification is a dynamic and reversible process of considerable therapeutic implications (16–20).

Techniques for assessing promoter methylation status vary considerably. Several methods that assess this feature have been reported (21–27), but the most widely used is the methylation-specific PCR (MSP) analysis of DNA after bisulfite treatment (24). However, the popularity of the MSP methods (whether nested or unnested) is tempered by notable shortcomings, including its qualitative nature, the limited CpG sites targeted, lack of internal control, and false-positive signals at high PCR cycles (25, 28). Recent attempts to remedy some of these deficiencies have led to the development of an alternative high-throughput technique for methylation analysis, known as pyrosequencing (27, 29, 30). Pyrosequencing, a sensitive real-time sequencing-by-synthesis method is based on the conversion of DNA by pyruvate kinase enzymatic treatment and a stepwise luminescence incorporation of nucleotide for the quantitative measurement of CpG islands methylation (31–33).

Several studies have reported certain tumor suppressor genes to be frequently methylated in epithelial malignancies, including those originating in the salivary glands. Among these genes are retinoic acid receptor α2 (RARα2), RAS association domain family 1 (RASSF1A), O6-methylguanine-DNA methyltransferase (MGMT), and E-cadherin genes (7–14). All of which are known for their ability to suppress vital cellular processes, including cell-cycle regulation, apoptosis, DNA repair, differentiation, and metastasis (34–38). In a previous study, we used the MSP method to analyze the methylation status of some of these genes in these tumors (39). However, because of the arbitrary and qualitative nature of the MSP technique, the need for more objective and quantitative assessment of methylation has recently been raised (26).

In the present study, we evaluated by means of MSP and pyrosequencing the methylation status of the promoters for RARα2, RASSF1A, MGMT, and E-cadherin in a large number of human SGC tissue specimens and in several SGC tumor cell lines and then compared the two datasets to determine which method might have greater potential clinical utility.

**Materials and Methods**

**Cell lines and culture conditions.** Five cell lines derived from human SGCs were used. These included four lines derived from salivary gland adenocarcinoma A253, HSG, HSY (Dr. Kaye, National Cancer Institute/National Naval Medical Center), and RET (Dr. Rao, Texas Children’s Cancer Center) and one line derived from an adenoid cystic carcinoma ACC3 (Dr. Frierson, University of Virginia). Cells from line A253 were grown in McCoy’s media containing 10% fetal bovine serum and penicillin-streptomycin. Cells from lines HSG and HSY were grown in high-glucose DMEM containing 10% fetal bovine serum, penicillin-streptomycin, and L-glutamine. The RET cell line was grown in RPMI 1640 containing 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, and penicillin-streptomycin. The ACC3 cell line was grown in RPMI 1640 containing 15% fetal bovine serum and penicillin-streptomycin. All cells were cultured and maintained in monolayers at 37°C in a humidified incubator under 95% O2 and 5% CO2.

**Tissue specimens.** A total of 69 fresh-frozen tissue specimens from human SGCs harvested by medical oncologists and pathologists and stored in the Head and Neck Pathology Section at University of Texas M.D. Anderson Cancer Center, in accordance with institutional policies, formed the materials for this study. All of the patients whose specimens were used had presented to M.D. Anderson Cancer Center for surgical treatment of their tumors between 1994 and 2006. In each case, tumor and matching nonneoplastic salivary gland tissues were harvested separately. A frozen section evaluation of each tumor specimen was done at the acquisition and before storage. Frozen section evaluation was used as a quality control step and insured that all specimens contained an excess of 80% neoplastic cells. Harvested tissues were immediately frozen in liquid nitrogen and stored at -80°C until use. Each specimen was classified histopathologically according to WHO histologic classification system for SGC and was staged according to the tumor-node-metastasis (TNM) classification system for malignant tumors defined by the International Union Against Cancer. To establish the baseline methylation status of targeted genes, 12 samples of normal salivary gland tissue were also analyzed.

**DNA extraction and bisulfite treatment.** Genomic DNA was extracted from fresh-frozen tissue specimens and cultured cells using DNeasy (Molecular Research Center, Inc., Cincinnati, OH) in accordance with the manufacturer’s instructions. Human genomic DNA (5 µg) was artificially methylated as a positive control using SssI (CpG) methylase (New England Biolabs). Extracted DNA samples were modified by treatment with bisulfite using a CpGenome DNA modification kit (Intergen) in accordance with the manufacturer’s instructions. In brief, 2 µg of genomic DNA were denatured by treatment with 2 mol/L NaOH and modified by treatment with 3 mol/L sodium bisulfate for 16 h. DNA samples were purified using Wizard DNA purification resin (Promega), treated with 3 mol/L NaOH, precipitated with ethanol, and resuspended in 20 µL water. Finally, 2-µL aliquots were used as templates for PCRs.

**MSP methylation analysis.** Because our materials consisted of fresh-frozen specimens, where the quality of extracted DNA is not a limiting factor, we used the conventional MSP method instead of the two-step nested MSP. The latter is more efficient in yielding better DNA quality from archived materials (40, 41). The PCR primer sequences of each gene and the annealing temperatures for pyrosequencing, MSP, and reverse transcriptase–PCR (RT-PCR) are listed (see electronic files).

**Pyrosequencing.** Pyrosequencing analysis was done using PCR assays to amplify specific CpG islands in the promoter of each target gene. Pyrosequencing primers were designed to amplify five or six target CpG dinucleotides and to not amplify CpGs within the primer sequence. Methylation of target CpGs was assessed by determining the ratio of cytosine to thymine incorporated during pyrosequencing; cytosine incorporation indicated a methylated CpG and thymine incorporation, an unmethylated CpG. This guaranteed that the ratio of incorporated cytosine to thymine would be proportional to the degree of methylation at a particular CpG site in the template DNA. Analysis of non-CpG cytosines provided an internal control for the completeness of bisulfite treatment. Hot-start PCRs were carried out in a volume of 30 µL containing Taq Gold polymerase (Applied Biosystems). Each reaction mixture contained 2 µg bisulfite-treated DNA, 0.25 mmol/L nucleotide triphosphates, 2.5 mmol/L MgCl2, 5 µL PCR buffer, and 2 units Taq Gold polymerase. For analysis of RASSF1A and MGMT, 2 pmol of forward primer and 2 pmol of reverse primer were used; for analysis of RARα2 and E-cadherin, 2 pmol
of forward primer, 0.8 pmol of reverse primer, and 1.8 pmol of universal primer were used. The quality and purity of the PCR products were established by their electrophoresis through 2.5% agarose gels and subsequent ethidium bromide staining. Pyrosequencing was carried out using single-strand binding proteins (PyroGold reagents) and the PSQ96MA System (Biotage) in accordance with the manufacturer’s protocol. All pyrosequencing experiments were done twice.

Methylation-specific PCR. Bisulfite-modified DNAs were amplified using primer sets specific for unmethylated and methylated sequences in each gene. Each PCR was done in a final volume of 25 μL containing template DNA, 1× PCR buffer, 2.5 mmol/L MgCl₂, 0.25 mmol/L deoxynucleotide triphosphate, 2 μmol/L each primer, and 1 unit AmpliTaq Gold (Applied Biosystems). PCR amplification was done as follows: at 94°C for 10 min (one cycle); at 94°C for 45 s (35 cycles), at the specific annealing temperature for each gene for 45 s (one cycle), at 72°C for 1 min (one cycle); and, in a final extension step, at 72°C for 5 min. The resulting PCR products were electrophoresed on 2% agarose gels. DNA isolated from normal salivary gland tissue and modified by SssI methylase was used as a positive control. To assess methylation semi-quantitatively, SssI-treated control DNA diluted stepwise (90-5%)
with untreated DNA was used as a reference. On the basis of the dilution results, a distinct methylated band whose signal was at least 10% of the ratio of the signals for the methylated and unmethylated bands was identified and used as a cutoff point for scoring methylation (data not shown). The extent of methylation (i.e., methylation status) was recorded as the percentage of the relative ratio of the signals for the methylated and unmethylated bands (Fig. 1).

**Treatment with demethylating agents.** To evaluate the potential functional effect of chemically induced demethylation in reverting these changes cultured cells were treated with the RARβ2 demethylating agent 5-azadeoxycytidine (DAC; Fig. 2). Moreover, because histone deacetylation is mechanistically linked to gene silencing through CpG islands methylation, we reasoned that adding Trichostatin A may elucidate the involvement of this modification in the inactivation of these genes (42).

Cells were seeded at densities of 1 × 10⁶ cells in T75 flasks with fetal bovine serum–containing medium, incubated for 24 h at 37°C, and then treated with or without 5 mmol/L DAC (Sigma) as follows. In a subset of cultures (groups A-E), the culture medium was replaced with medium containing DAC at concentrations of 0.1, 0.2, 0.5, 1, or 5 μmol/L, after which the cells were incubated for 3 d and harvested with TRIzol reagent (Invitrogen) on day 4. In another subset (group F), the culture medium was replaced with medium containing Trichostatin A at a concentration of 200 nmol/L, after which the cells were incubated for 20 h and then harvested. In yet another subset (group G), the culture medium was replaced with medium containing DAC at a concentration of 1 μmol/L from day 1 and Trichostatin A at a concentration of 200 nmol/L from day 3. Cells were incubated and harvested on day 4. In a final subset (group H), which also served as a control, the culture medium was replaced with plain medium and cells were incubated for 3 d and harvested on day 4. Harvested cells were stored at -80°C until evaluation by RT-PCR analysis.

**RNA preparation and RT-PCR analysis.** RNA was isolated from the eight groups of cultured cells by treating the cells with TRIzol reagent in accordance with the manufacturer’s instructions. RT-PCR analysis of RARβ2 mRNA expression was done under the following conditions: at 45°C for 45 min (one cycle); at 94°C for 2 min (one cycle); at 94°C for 30 s, 60°C for 1 min, and 68°C for 2 min (40 cycles); and, in a final extension step, at 68°C for 7 min. The resulting RT-PCR products were electrophoresed through 2.5% agarose gel, stained with ethidium bromide, and examined under UV light. GADPH mRNA expression in each RNA sample was also analyzed and used as an internal loading control.

**Statistical analysis.** To compare the results of the pyrosequencing-based and MSP-based methylation assessments, regression coefficients were calculated via linear regression methods. Scatter plots were drawn with least-squares regression lines bounded by 95% confidence limits. Cutoff values above which samples were coded as being hypermethylated were established by comparison with values from normal tissues and inspection of the scatter plots of values obtained from the two techniques and two-by-two tables of correlations between the two techniques at various cutoff points. Cutoff points for pyrosequencing and MSP, respectively, were as follows: RARβ2, ≥30% and ≥20%; RassF1A, ≥30% and ≥20%; MGMT, ≥15% and ≥10%; E-cadherin, pyrosequencing, >15%.
Correlations between methylation variables and clinicopathologic and end point variables were assessed by Pearson’s χ² test or, where there were <10 subjects in any cell of a 2 × 2 grid, by the two-tailed Fisher exact test. Comparison of Kaplan-Meier product limit survival curves was done by application of the log-rank test. Statistical analyses were done using a commercially available software program (Statistical, version 7.1; StatSoft, Inc.).

**Results**

**Clinicopathologic findings.** The patients in this study were composed of 39 males (57%) and 30 females (43%) who ranged in age from 8 to 89 years, with a mean age of 55.7 years (SD, 18.5 years; median age, 58.5 years). Tumor sites composed of 52 (69%) in the parotid, 20 (27%) in minor salivary glands, and 3 (4%) in the submandibular glands. Histopathologically, the tumors were classified as acinic cell (ACC; n = 13, 19%), adenoid cystic (n = 25, 36%), mucoepidermoid (n = 17, 25%), and salivary duct carcinomas (n = 8, 12%) or carcinoma ex pleomorphic adenoma (n = 6; 9%). Most of the tumors were classified as low to intermediate grade (40 of 69, 58%) the remaining were high grade (29 of 69, 42%). It should be noted that, in this study, all differentiated salivary duct and grade III of mucoepidermoid carcinomas were categorized as high-grade tumors. The tumor size was ≥2 cm in 35 of the 66 cases (53%) for which it could be determined. Metastasis to regional cervical lymph nodes was noted in 21 of 69 patients (30%). The disease stage was I or II in 31 of 68 patients (46%) and III or IV in 37 (54%). The mean follow up time, from the date of presentation at our institution for this tumor until last date of presentation at our institution for this tumor until last contact or death was 56.4 months (SD, 41.8 months; median, 47.2 months with a range of 1.3-174.3 months). The overall 3-year survival rate was 65% (41 of 63). Six of the patients were lost to follow up before 3 years had elapsed. The 5-year survival rate was 46% (24 of 52).

**Methylation findings.** In normal salivary tissue specimens, pyrosequencing analysis revealed low levels of target gene methylation (i.e., ≤10% in RASSF1A and MGMT and ≤15% in RARβ2 and E-cadherin). Because these were considered to represent background levels of methylation, they were used as cutoff points for analyzing methylation in the cell lines (data not shown). By both pyrosequencing and MSP techniques, only the RARβ2 promoter methylation was observed in all five cell lines (100%); in contrast, methylation of the RASSF1A, MGMT, and E-cadherin promoters was detected in only one cell line each. The pyrosequencing analysis of human tumor tissue specimens revealed a widely distributed variation in methylation levels among methylated tumors (n = 69; Fig. 3), but the great majority of assays gave a result of <50% methylation (248 of 256, 96.9%). The most frequently methylated genes were RARβ2 (46%) and RASSF1A (35%). MGMT and E-cadherin were infrequently methylated. Twenty-seven of the patients (39%) showed no hypermethylation at any of the four sites. Methylation of multiple target gene promoters was relatively rare; only one promoter was methylated in 38% (26 of 69) of tumors, two promoters in 22% (15 of 69), three promoters in 1% (1 of 69), and all four in none (0%).

**Correlation of pyrosequencing and MSP data.** Pyrosequencing and MSP analysis of methylation in the five cell lines produced similar and concordant results. In contrast, pyrosequencing and MSP analysis of methylation of RARβ2, RASSF1A, and MGMT in the 69 human tumor tissue specimens using background levels as the cutoff points showed variable results (Table 1 and Fig. 3). In 206 instances of recorded methylation, the results of the two methods were concordant in 174 (84.5%) and discordant in 32 (15.5% P < 0.001). The pyrosequencing method, however, detected 23 more instances of RARβ2 methylation and seven more instances of RASSF1A methylation than the MSP method. Regression analysis, shown in Fig. 3, showed high correlations between the methylation values obtained by pyrosequencing and MSP. The adjusted R² values were 0.319 (P < 0.000001), 0.835 (P > 0.000001), and 0.178 (P = 0.0002) for RARβ2, RASSF1A, and MGMT, respectively.

At cutoff levels chosen to maximize concordance between methylation of the RARβ2, RASSF1A, and MGMT promoters as detected by both methods, there was agreement between the methods in 94% (64 of 68), 96% (66 of 69), and 97% (67 of 69) of tumors, respectively (Table 1).

**Correlation of methylation status and clinicopathologic factors.** Table 2 presents the correlations between target gene methylation status the cutoff values listed (as determined by pyrosequencing and MSP) and clinicopathologic factors. Data for E-cadherin are not shown because only one patient had hypermethylation at that site. There were no statistically significant correlations between methylation status and sites, gender, or stage of their disease. There was a significant correlation between RARβ2 methylation status by pyrosequencing and tumor categories (P = 0.027; only one patient with ACC or adenoid cystic carcinoma had hypermethylation at RARβ2).

### Table 1. Comparison of methylation status in RARβ2, RASSF1A, and MGMT promoters as determined by pyrosequencing and MSP

<table>
<thead>
<tr>
<th>Genes</th>
<th>PS/MSP +/+</th>
<th>PS/MSP -/-</th>
<th>PS/MSP +/+</th>
<th>PS/MSP -/+</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RARβ2</td>
<td>6/68 (8.8%)</td>
<td>58/68 (85.3%)</td>
<td>4/68 (5.9%)</td>
<td>0/74 (0%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>15/69 (21.7%)</td>
<td>51/69 (73.9%)</td>
<td>3/69 (4.4%)</td>
<td>0/69 (0%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MGMT</td>
<td>1/69 (1.5%)</td>
<td>66/69 (95.7%)</td>
<td>1/69 (1.5%)</td>
<td>1/69 (1.5%)</td>
<td>0.0575</td>
</tr>
</tbody>
</table>

**NOTE:** Cutoff points for pyrosequencing and MSP, respectively, were as follows: RARβ2, ≥30% and ≥20%; RASSF1A, ≥20% and ≥20%; MGMT, ≥15% and ≥10%.

Abbreviations: PS, pyrosequencing; +, methylation level exceeded the cutoff point; -, indicates that the methylation level did not exceed the cutoff point; +/+ , positive for both; -/-, negative for both; +/+, positive pyrosequencing/negative MSP; -/+ negative pyrosequencing/positive MSP.
tumor grade ($P = 0.014$), tumor size ($P = 0.008$), 3-year survival ($P = 0.002$), and 5-year survival ($P = 0.028$). There was a significant correlation between hypermethylation at RASSF1A, as measured by pyrosequencing, and diagnosis ($P = 0.014$). It is interesting to note that over half of patients with ACC had high methylation of this gene. Concurrent hypermethylation at RAR/β2 and RASSF1A, correlated with diagnosis, high tumor grade, lymph node metastasis, and death within 3 years, was observed. The correlation with lower survival was confirmed by log-rank analysis of Kaplan-Meier survival curves (Fig. 4).

**Reactivation of RAR/β2 expression.** Treatment with DAC at concentrations of 0.5, 1.0, and 5.0 μmol/L led to the reactivation of RAR/β2 gene in the A253 cell line only (Fig. 2) at a concentration of 0.5 μmol/L with maximum activation achieved at 5.0 μmol/L.

**Discussion**

In this study, we compared two techniques, quantitative pyrosequencing and qualitative MSP, for their ability to assess the methylation status of several tumor-associated genes in a large number of human SGCs and in several tumor cell lines. Because fresh-frozen instead of archival materials were used in our study, the traditional one-step MSP method was considered adequately sensitive for analysis (40, 41). In general, the

<p>| Table 2. Clinicopathologic characteristics of study population (n = 69) when stratified by target gene hypermethylation status as determined by pyrosequencing or MSP |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>RAR/β2</th>
<th>RASSF1A</th>
<th>MGMT</th>
<th>RAR/β2 + RASSF1A</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n = 39)</td>
<td>3/39 (7.7)</td>
<td>3/39 (7.9)</td>
<td>8/39 (20.5)</td>
<td>6/39 (15.4)</td>
</tr>
<tr>
<td>Female (n = 30)</td>
<td>7/30 (23.3)</td>
<td>3/30 (10.0)</td>
<td>10/30 (33.3)</td>
<td>9/30 (30.0)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
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<tr>
<td>&lt;50 y (n = 26)</td>
<td>3/26 (11.5)</td>
<td>1/26 (3.9)</td>
<td>5/26 (19.2)</td>
<td>3/26 (11.5)</td>
</tr>
<tr>
<td>&gt;50 y (n = 43)</td>
<td>7/43 (16.3)</td>
<td>5/42 (11.9)</td>
<td>13/43 (30.2)</td>
<td>12/43 (27.9)</td>
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<tr>
<td><strong>Diagnosis</strong></td>
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<tr>
<td>ACC (n = 13)</td>
<td>1/13 (7.7)</td>
<td>0/13 (0)</td>
<td>7/13 (53.9)</td>
<td>6/13 (46.2)</td>
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<td>ACC (n = 25)</td>
<td>0/25 (0)</td>
<td>0/25 (0)</td>
<td>4/25 (16.0)</td>
<td>4/25 (16.0)</td>
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<tr>
<td>MEC (n = 17)</td>
<td>4/17 (23.5)</td>
<td>3/17 (17.7)</td>
<td>1/17 (5.9)</td>
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<tr>
<td>SDC (n = 8)</td>
<td>3/8 (37.5)</td>
<td>2/7 (28.6)</td>
<td>3/8 (37.5)</td>
<td>2/8 (25.0)</td>
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<tr>
<td>CexPA (n = 6)</td>
<td>2/6 (33.3)</td>
<td>1/6 (16.7)</td>
<td>3/6 (50.0)</td>
<td>2/6 (33.3)</td>
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<td><strong>Tumor grade</strong></td>
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<tr>
<td>Low to intermediate (n = 40)</td>
<td>2/40 (5.0)</td>
<td>0/40 (0)</td>
<td>9/40 (22.5)</td>
<td>8/40 (20.0)</td>
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<tr>
<td>High (n = 29)</td>
<td>8/29 (27.6)</td>
<td>6/28 (21.4)</td>
<td>9/29 (31.0)</td>
<td>7/29 (24.1)</td>
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<tr>
<td><strong>Sex</strong></td>
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<tr>
<td>&gt;50 y (n = 55)</td>
<td>3/55 (9.1)</td>
<td>3/55 (5.5)</td>
<td>17/55 (30.9)</td>
<td>15/55 (27.3)</td>
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<tr>
<td><strong>LN metastasis</strong></td>
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<tr>
<td>+ (n = 48)</td>
<td>5/48 (10.4)</td>
<td>1/48 (2.1)</td>
<td>11/48 (22.9)</td>
<td>9/48 (18.8)</td>
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<td>- (n = 21)</td>
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<td>5/20 (25.0)</td>
<td>7/21 (33.3)</td>
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<tr>
<td><strong>Disease stage</strong></td>
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<tr>
<td>I (n = 6)</td>
<td>3/6 (50.0)</td>
<td>1/6 (16.7)</td>
<td>0/6 (0)</td>
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<td>0/25 (0)</td>
<td>8/25 (32.0)</td>
<td>7/25 (28.0)</td>
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<tr>
<td>III (n = 33)</td>
<td>4/33 (12.1)</td>
<td>4/32 (12.5)</td>
<td>6/33 (18.2)</td>
<td>3/33 (9.1)</td>
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<tr>
<td>IV (n = 4)</td>
<td>1/4 (25.0)</td>
<td>1/4 (25.0)</td>
<td>2/4 (50.0)</td>
<td>2/4 (50.0)</td>
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<tr>
<td><strong>P</strong></td>
<td>0.019</td>
<td>0.007</td>
<td>0.385</td>
<td>0.363</td>
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<tr>
<td><strong>Survival</strong></td>
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<tr>
<td>1-yr survival</td>
<td></td>
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</tr>
<tr>
<td>Yes (n = 41)</td>
<td>2/41 (4.9%)</td>
<td>0/41 (0)</td>
<td>8/41 (19.5)</td>
<td>7/41 (17.1)</td>
</tr>
<tr>
<td>No (n = 22)</td>
<td>8/22 (36.4%)</td>
<td>6/21 (28.6)</td>
<td>8/22 (36.4)</td>
<td>6/22 (27.3)</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>0.002</td>
<td>0.001</td>
<td>0.224</td>
<td>0.390</td>
</tr>
<tr>
<td>5-yr survival</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (n = 24)</td>
<td>1/24 (4.2%)</td>
<td>0/24 (0)</td>
<td>5/24 (20.8)</td>
<td>4/24 (16.7)</td>
</tr>
<tr>
<td>No (n = 28)</td>
<td>8/28 (28.6%)</td>
<td>6/27 (22.2)</td>
<td>8/28 (28.6)</td>
<td>6/28 (21.4)</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>0.028</td>
<td>0.024</td>
<td>0.749</td>
<td>0.736</td>
</tr>
</tbody>
</table>

NOTE: Cutoff points for pyrosequencing and MSP, respectively, were as follows: RAR/β2, 30% and 20%; RASSF1A, 20% and 20%; MGMT, 15% and 10%. Abbreviations: ACC, adenoid cystic carcinoma; CexPA, carcinoma ex pleomorphic adenoma; LN, lymph node; MEC, mucoepidermoid carcinoma; SDC, salivary duct carcinoma.

*In three cases, information on tumor size was not available.*
pyrosequencing technique detected all of those identified by MSP method, as well as several additional instances in tumors that were negative by the latter method. We, however, encountered only one instance in which a single gene was scored positive for methylation by MSP but negative by pyrosequencing. This occurrence was attributed to the incomplete conversion of all nonmethylated cytosine residues to thymine by the sodium bisulfite treatment of tumor DNA (28). This possibility was verified by repeating DNA treatment and MSP analysis of additional tumor sample where no methylation was detected.

Moreover, a faint shadow band may not be infrequently observed on gel electrophoresis of MSP analysis, and this may potentially lead to interpretive difficulties if only present in tumor specimen. We also encountered these faint bands in some cases on MSP analysis. In all of these instances, no methylation was detected in the corresponding pyrosequencing analysis. These artifacts, together with the semiquantitative and the subjective nature of the MSP scoring, provide further support for the application of the pyrosequencing.

Although our results show that, at a certain level of methylation, MSP is a simple qualitative technique for screening and detecting hypermethylated genes in tumors, it is less sensitive at low level methylation and is prone to false-positive results. Although the two-step MSP method may enhance sensitivity, the method fundamentally shares many of the shortcomings of the MSP and may render similar results on fresh-frozen materials; further comparative studies are recommended (40, 41).

Our results also indicate that certain genes may be selectively methylated during salivary gland carcinogenesis. RARβ2 and RASSF1A, in contrast to MGMT and E-cadherin, were found to be frequently methylated in cell lines, as well as in human tumor tissue specimens, suggesting an association between the functional status of both genes and the development and/or progression of some SGCs. Contrary to previous reports, however, we found the E-cadherin promoter to be infrequently methylated in our tumor samples, a discrepancy that can be attributed to differences in the nature of the materials analyzed (archival versus fresh frozen), the interpretation of results, or both (41). In our study, we used fresh-frozen tissue, which yields better results than those obtained from archival materials (41). We also noted that concurrent methylation of RARβ2 and RASSF1A genes correlated significantly with certain clinicopathologic factors and patients survival, suggesting a synergistic effect of both genes on salivary gland tumorigenesis. Other investigators have implicated methylation at both RARβ-2 and RASSF-1 in the tumorigenesis of different human malignancies (13, 14, 34–39, 43–47).

In our study, RARβ2 was the only gene found to be highly methylated in all five cell lines and in most of the SGC tissue specimens tested. Moreover, regardless of the method of detection, RARβ2 methylation correlated significantly with the adenocarcinoma phenotype, tumor size, high tumor grade, and 3-year and 5-year survival, thus implicating this gene in the progression of certain types of SGC. A similar association between gene methylation and aggressive tumor phenotypes has been reported for other malignancies as well (38, 39, 43–47). Located on chromosome 3p24, the RARβ gene encodes multiple retinoid receptor isoforms (β1, β2, β4) by two different promoters and alternative splicing (38). The RARβ2 isoform has specifically been shown to be involved in carcinogenesis of several malignant tumors (40, 41, 43–50). Altered expression of retinoid receptors including RARβ2 has been reported in various cancer cell types, premalignant oral lesions (7–10) and head and neck squamous cell carcinoma (11–16). These alterations have in turn been linked to resistance to retinoids (7).

We also noted significant hypermethylation of the RASSF1-A in salivary duct and acinic cell carcinomas compared with other
tumor types. Also, methylation of the same gene was found more in high grade, large size, and lymph node–positive tumors, but this did not reach a statistical significance. The RASSF-1 gene is located on chromosome 3p21.3 and encodes two major isoforms of a Ras-associated oncoprotein (51). RASSF-1A and RASSF-1C via alternative splicing and promoter usage. RASSF-1 protein has been shown to heterodimerize with other family members to induce Ras gene activation and tumorigenesis (43). Thus, suppression of RASSF-1 activity, whether dependent or not on the Ras pathway, seems to be a prerequisite for the induction of cell cycle arrest and apoptosis during tumorigenesis (50). Interestingly, our data revealed frequent concomitant RASSF1A and RARβ2 methylation in both ACCs and salivary duct carcinomas phenotypes. This finding suggests that the two genes may synergistically involve in the carcinogenesis of these two entities.

Our in vitro analysis of salivary cell lines showed that DAC-treatment led selectively to the reactivation of RARβ2 expression in only one of the five cell lines. However, the addition of Trichostatin either alone or in combination with DAC failed to show any appreciable changes. This finding indicates a minor role for histone deacetylation in the functional suppression of the RARβ2 gene in this cell line (42). Moreover, the inability of DAC to reactivate RARβ2 expression in the remaining cell lines suggests variable response of tumor cells to demethylating agents (38). Further studies with other demethylating agents are required to determine the differential response to these agents. Nonetheless, attempts are under way to delineate the functional consequences of reactivating these genes as a novel therapeutic target for patients with advanced disease (19, 20). The potential therapeutic application of demethylating agents in salivary carcinomas patients may await validation for prospective clinical trials.

In conclusion, the results of our present study (i.e., the first comprehensive and quantitative comparison of the gene methylation profiles of four major types of SGC) have revealed that the pyrosequencing method is more sensitive than the MSP method and its results correlate significantly with clinicopathologic factors and patients survival. The study also shows that the RARβ2 and RASSF1A genes are frequently methylated in ACC, adenoid cystic carcinoma, and salivary duct carcinoma and thus may warrant further study as potential therapeutic targets in that setting. Our findings also imply that future methylation-based investigations of SGC may provide useful information in the assessment and management of salivary gland tumors.

References

Clinical Cancer Research

Quantitative Promoter Hypermethylation Analysis of Cancer-Related Genes in Salivary Gland Carcinomas: Comparison with Methylation-Specific PCR Technique and Clinical Significance

Eung-Seok Lee, Jean-Pierre Issa, Dianna B. Roberts, et al.


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