EDNRB and DCC Salivary Rinse Hypermethylation Has a Similar Performance as Expert Clinical Examination in Discrimination of Oral Cancer/Dysplasia versus Benign Lesions

Juliana Schussel1, Xian Chong Zhou2, Zhe Zhang2, Kavita Pattani1, Francisco Bermudez10, Germain Jean-Charles9, Thomas McCaffrey11, Tapan Padhya12, Joan Phelan4,5,6, Silvia Spivakovsky4,5,6, A. Ross Kerr4,5,6, David Sirois4,5,6, and Joseph A. Califano1,3

Introduction

Currently, in the United States, there will be more than 50,000 estimated cases of head and neck cancer in 2013 (1). However, there has been modest improvement in survival of head and neck cancer patients in the past 3 decades (2), only 50% of patients are cured with initial therapy (2). Early...
Late head and neck squamous cell carcinoma (HNSCC) diagnosis is responsible for disease morbidity and mortality. Oral screenings have been proposed as means of prevention. Our study compares oral lesion clinical risk assessment and molecular biomarkers. The presence of gene promoter methylation in salivary rinses compares well to examination by an expert clinician in risk classification of oral premalignant and malignant lesions. Given the current costs and availability of an expert health care provider trained and experienced in oral cancer diagnosis, risk assessment with a salivary biomarker is attractive as a cost-effective means to identify higher risk patients that should be referred for expert exam and biopsy. Also, these salivary biomarkers may be particularly useful in identifying patients with lesions that seem low risk by physical examination, but are identified as high risk by epigenetic salivary biomarkers.

Materials and Methods

DNA extraction

DNA obtained from salivary rinses was extracted by digestion with 50 μg/mL proteinase K (Boehringer) in the presence of 1% SDS at 48°C overnight followed by phenol/chloroform extraction and ethanol precipitation.

Bisulfite treatment

Sodium bisulfite conversion of DNA extracted from saliva was conducted using the EpiTect Bisulfite kit (Qiagen, Inc.), according to the manufacturer’s instructions. Bissulfite-converted DNA was stored at −80°C.

Gene selection

The genes used in this study are a result of a prior published biomarker discovery (11). A total of 21 informative genes were considered for this first study and were selected from 3 different sources: (i) genes with promoters that are reported as hypermethylated in HNSCC; (ii) genes with promoters that are reported as hypermethylated in other solid tumors; and (iii) gene discovery using expression microarray-based approach via unmasking of expression. The first step involved a screening evaluation, designed to eliminate targets that had an inappropriately high frequency of promoter hypermethylation in normal, control samples. It was conducted by comparing tumor samples.
logistic regression modeling were constructed for salivary
selected panels based on the method of multivariable
Receiver operating characteristic (ROC) curves for some
on salivary rinses in a limited cohort of HNSCC patients.
90% specificity or sensitivity were selected for further testing
absence of methylation) and an AUC
samples (control) for binary results (either presence or
methylation can be associated with age, race, or tobacco and
difference in methylation levels. Finally, promoter hyper-
levels were noted in HNSCC compared with controls but levels of methyla-
tion patterns as follows: (i) methylation was detected only in
methylation was noted in both groups (tumor and salivary
However, a quantitative difference
between groups was noted, and (v) methylation was noted in
both HNSCC and controls at a similar frequency with no
differences between groups were similar in both groups; (iv) a similar
frequency of methylation was noted in both groups (tumor
and salivary rinses); however, a quantitative difference
between groups was noted, and (v) methylation was noted in
both HNSCC and controls at a similar frequency with no
differing in methylation levels. Finally, promoter hyper-
methylation can be associated with age, race, or tobacco and
alcohol exposure. The results included the frequency distri-
butions area under curve (AUC), sensitivity, and specificity for each gene. Based on the above results, genes that
could distinguish tumor samples (case) from salivary rinse samples (control) for binary results (either presence or absence of methylation) and an AUC > 0.60 and at least 90% specificity or sensitivity were selected for further testing on salivary rinses in a limited cohort of HNSCC patients.
Receiver operating characteristic (ROC) curves for some
selected panels based on the method of multivariable
logistic regression modeling were constructed for salivary
rinses, where the single point represented the per-
formance of the panel with a positive panel being defined as
at least 1 gene of the panel presented methylation. From the
initial screening of 21 genes for salivary rinses, ultimately 8
genes were selected as part of a panel to distinguish salivary
rinses from HNSCC patients and healthy controls DAPK,
deleted in colorectal cancer (DCC), MINT-31, TIMP-3, p16,
MGMT, CCNA1, and PGP 9.5. Of note PGP9.5 and TIMP-3
have an identical methylation pattern, so PGP9.5 will be
omitted from the panel to simplify analysis.

### Quantitative methylation-specific PCR

Bisulfite-modified DNA was used as a template for fluo-
rescence-based real-time PCR, as previously described (15).
In brief, primers and probes were designed to specifi-
cally amplify the bisulfite-converted DNA for the ACTB gene and
all genes of interest (Table 2). Amplification reactions were
carried out in triplicate in a final volume of 10 μL containing
1.5 μL of bisulfite-modified DNA; 600 nmol/L of each primer;
200 μmol/L of probe; 0.75 unit of platinum Taq
polymerase (Invitrogen); 200 μmol/L of each dATP, dCTP,
dGTP, and dTTP; 200 nmol/L of ROX Reference Dye (Invi-
rogen); 16.6 nmol/L ammonium sulfate; 67 nmol/L
Trizma (Sigma); 6.7 mmol/L magnesium chloride; 10
mmol/L mercaptoethanol; and 0.1% dimethyl sulfoxide.
Thermal cycling started with denaturation step at 95°C for 3
minutes followed by 50 cycles at 95°C for 15 seconds and
60°C for 1 minute. Amplification reactions were carried out
in 384-well plates in a 7900H sequence detector (Perkin-
Elmer Applied Biosystems) and analyzed by a sequence
detector system (SDS 2.3; Applied Biosystems). Leukocyte
dNA from a healthy individual was methylated in vitro with
excess SssI methyltransferase (New England Biolabs, Inc.)
to generate completely methylated DNA, and serial dilutions of this DNA were used to construct a calibration curve for

<table>
<thead>
<tr>
<th>Study group</th>
<th>Case definitions</th>
</tr>
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<tbody>
<tr>
<td>Low risk for malignancy</td>
<td>Leukoplakia. Adherent white patch on any oral mucosal surface, which has no apparent explanation or etiology (i.e., frictional trauma, infection). Such lesions will NOT have associated erythroplakia, ulceration, erosion, or submucosal extension/induration.</td>
</tr>
<tr>
<td>High risk for malignancy</td>
<td>Any of the following features, alone or in combination. May be associated with other high-risk signs and symptoms such as history of oral cancer or other cancer with metastatic potential, regional adenopathy, submucosal extension/induration, ulceration. Leukoplakia: Any adherent white patch on any oral mucosal surface, which has no apparent explanation or etiology but DOES HAVE associated, ulceration, erosion, or submucosal extension/induration.</td>
</tr>
<tr>
<td>Known cancer</td>
<td>Biopsy-proven oral squamous cell carcinoma before study enrollment</td>
</tr>
</tbody>
</table>

(Leukoplakia, Erythroplakia, leukoplakia: adherent white patch on any oral mucosal surface, which has no apparent explanation or etiology. Erythroplakia: Mixed red and white patch on any oral mucosal surface, which has no apparent explanation or etiology. Ulceration: Any break in the oral epithelial surface, which has no apparent explanation or etiology. This may or may not be symptomatic and may be of undetermined duration. There is increased risk for malignancy with increased duration.)
Statistical analysis

Gene methylation was dichotomized at 0 (i.e., no methylation vs. any methylation), because we did not find any improvement of change in the performance of this test based on modeling with a continuous or binary variable. The cohort was divided into subcategories of histologic outcome including benign, premalignant, and malignant. Predictors associated with head and neck cancers were evaluated as well, including age, gender, race, smoking status, and alcohol consumption. Age was analyzed as a continuous variable, whereas all other variables were considered as categorical variables. Univariate and multivariate proportional odds modeling were constructed sequentially to explore the association of the variables with histologic outcome. Variables of significance based on the univariate models ($P < 0.20$) along with those deemed to be biologically and clinically important were retained for further analysis. Simultaneous effects expressed by these variables were studied using the multivariate proportional odds model. OR were reported with 95% CIs, which indicated the strength of the association and its uncertainty.

In a second analysis, we explored the independent association of methylation with histology, by excluding patients with known cancer at initial presentation ($n = 30$). The remaining 161 patients were categorized as having benign (with or without atypia) or dysplastic/cancerous lesions. Univariate and multivariate logistic regression analyses were conducted using the same biologically and clinically important covariates as described earlier. ROC analysis was conducted to estimate classification accuracy, sensitivity, specificity of the predictor along with 95% CIs, and also AUC as an index of predictive power. A similar analysis was made categorizing lesions as either benign-/mild-grade dysplasia or moderate- or severe-grade dysplasia/carcinoma in situ/cancer (Supplementary data).

Statistical analyses were done using SAS (v9.2; SAS Institute) and STATA software (v 8.2, SAS Institute), and all statistical tests were 2-sided with $P < 0.05$ considered statistically significant. Except for the univariate analysis where we accepted any $P < 0.20$, and then subjected those variables to multivariate analysis.

Results

Cohort

A total of 191 patients were included in this study. Most of them were males (69.9%) and Caucasian (69.6%) with a mean age of 54.1 years (ranging from 18 to 90 years). Alcohol or tobacco consumption (current or past) was found in 49.4% and 72.2%, respectively. When comparing baseline characteristics, benign, dysplasia, and cancer groups were similar. A total of 67.3% of patients presenting with a benign lesion were tobacco users (former and current), 69.8% of patients with epithelial dysplasia used tobacco, and 74.3% of patients with invasive cancer were tobacco users. For alcohol consumption, these values were 70.8%, 74.4%, and 77.1% for the histologic categories, respectively.

Risk classification

Dentists, based on WHO classification, conducted clinical risk assessment and lesions were categorized as low risk and high risk for dysplasia/cancer. After biopsy, risk classification was compared with histopathologic diagnosis (Table 3).

Nine-gene methylation status

A univariate analysis was done for association between individual genes and histopathology. At least 1 methylated gene was detected in 28.3% and 32.6% of benign and
premalignant lesions respectively, whereas 57.1% of the malignant lesions had at least 1 methylated gene. When analyzing genes separately, \textit{EDNRB} showed the highest relative risk of association with diagnosis of malignancy as a single biomarker (OR = 3.6, 95% CI = 2.0–6.4; \( P < 0.0001 \)), followed by \textit{DCC} (OR = 3.3, 95% CI = 1.7–6.6; \( P = 0.0005 \)) and \textit{HOXA9} (OR = 2.1, 95% CI = 1.2–3.7; \( P = 0.12 \)). Also \textit{CCNA1}, \textit{P16}, and \textit{MINT31} showed associations with histopathology, when analyzed separately (\( P = 0.0003; P = 0.031; P = 0.019 \)), however with large CIs (OR = 6.4, 95% CI = 2.4–17.4; OR = 6.9, 95% CI = 1.2–39.9; OR = 16.5, 95% CI = 1.6–171.9). \textit{DAPK}, \textit{TIMP3}, and \textit{MGMT} were not associated with histopathology in this cohort.

**Predictive factors**

Univariate analysis of predictors showed age as the only variable associated with histopathologic diagnosis in this cohort (OR = 1.3, 95% CI = 1.1–1.6; \( P = 0.014 \)). Neither tobacco nor alcohol consumption (\( P = 0.372 \) and \( P = 0.435 \), respectively) were significant predictors. In a multivariate analysis, after adjusting for covariates (age, race, gene methylation in any gene from the 7 gene panel, and tobacco and alcohol consumption), age remained associated with histopathologic diagnosis (OR = 1.3, 95% CI = 1.0–1.6; \( P = 0.034 \)). \textit{DCC} and \textit{HOXA9} were analyzed as individual biomarkers in the multivariate analysis adjusted for age, sex, race, tobacco, alcohol, along with \textit{DCC/EDNRB/HOXA9}, and these markers decreased the significance of age (\( P = 0.051 \) and 0.055, respectively). Although \textit{HOXA9} was no longer significantly associated with histopathologic diagnosis on multivariate analysis, \textit{DCC} and \textit{EDNRB} show strong independent associations (OR = 2.8, 95% CI = 1.4–5.7; \( P = 0.004 \); OR = 3.1, 95% CI = 1.7–5.8; \( P = 0.0003 \)), with histopathologic diagnosis.

**\textit{DCC} and \textit{EDNRB} exhibit similar performance to risk classification**

To analyze the predictive power of expert risk classification, the 161 samples were analyzed according to histopathologic diagnosis (benign \( n = 113 \) vs. epithelial dysplasia/cancer \( n = 48 \)) having excluded patients that had known cancer at first presentation (\( n = 30 \)). None of the predictors (e.g., age, sex, tobacco, and alcohol consumption) reached statistical significance on univariate analysis. Risk classification, described as low risk or high risk, was associated with histopathologic diagnosis (OR = 2.6, 95% CI = 1.1–6.1; \( P = 0.026 \); OR = 2.1, 95% CI = 1.0–4.4; \( P = 0.046 \), respectively).

A multivariate model analyzed risk classification and \textit{EDNRB}, \textit{HOXA9}, and \textit{DCC} methylation status. Risk classification was again independently associated with histopathologic diagnosis in three genes analysis (OR = 2.5, 95% CI = 1.3–5.1; \( P = 0.008 \)). Again, \textit{DCC} and \textit{EDNRB}, as single biomarkers, were associated with histopathologic diagnosis (OR = 2.6, 95% CI = 1.1–6.1; \( P = 0.026 \); OR = 2.1, 95% CI = 1.0–4.4; \( P = 0.046 \), respectively).

### Table 3. Clinical risk assessment vs. histological diagnosis

<table>
<thead>
<tr>
<th>Low risk</th>
<th>High risk</th>
<th>Cancer</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>75</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>16</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>2</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Cancer</td>
<td>0</td>
<td>6</td>
<td>29</td>
</tr>
<tr>
<td>Total</td>
<td>96</td>
<td>65</td>
<td>30</td>
</tr>
</tbody>
</table>

### Table 4. Predictive accuracy of risk classification and markers (\( n = 161 \))

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Sensitivity (%, 95% CI)</th>
<th>Specificity (%, 95% CI)</th>
<th>AUC (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risk classification</td>
<td>56 (41–71)</td>
<td>66 (57–75)</td>
<td>0.61 (0.53–0.70)</td>
</tr>
<tr>
<td>\textit{EDNRB}</td>
<td>38 (24–53)</td>
<td>78 (69–85)</td>
<td>0.58 (0.50–0.66)</td>
</tr>
<tr>
<td>\textit{DCC}</td>
<td>27 (15–42)</td>
<td>88 (80–93)</td>
<td>0.57 (0.50–0.64)</td>
</tr>
<tr>
<td>\textit{EDNRB} and risk classification</td>
<td>73 (58–85)</td>
<td>51 (42–61)</td>
<td>0.65 (0.56–0.74)</td>
</tr>
<tr>
<td>\textit{DCC} and risk classification</td>
<td>69 (54–81)</td>
<td>59 (50–68)</td>
<td>0.65 (0.57–0.74)</td>
</tr>
<tr>
<td>\textit{DCC} and \textit{EDNRB}</td>
<td>46 (31–61)</td>
<td>72 (62–80)</td>
<td>0.60 (0.51–0.69)</td>
</tr>
<tr>
<td>\textit{EDNRB, DCC, and risk classification}</td>
<td>75 (60–86)</td>
<td>48 (38–57)</td>
<td>0.67 (0.58–0.76)</td>
</tr>
</tbody>
</table>
of 0.60 (95% CI = 0.51–0.69). Risk classification, when analyzed as a single predictor for histopathologic diagnosis, had 56% (95% CI = 41–71) sensitivity and 66% (95% CI = 57–75) specificity, with AUC of 0.61 (95% CI = 0.53–0.70).

Using logistic regression analysis, we combined risk classification and EDNRB methylation status, and separately risk classification and DCC (sensitivity 73%; 95% CI = 58–85 and 69%; 95% CI = 54–81, respectively; specificity 51%; 95% CI = 42–61 and 59%; 95% CI = 50–68, respectively).

Finally, the combination of risk classification, DCC and EDNRB showed 75% of sensitivity (95% CI = 60–86) and 48% of specificity (95% CI = 38–57) with AUC of 0.67 (95% CI = 0.58–0.76; Fig. 1).

ROC curves corresponding to the use of gene signatures, clinical exam, and a combination of these are included (Supplementary Fig. S1).

Results categorizing lesions as either benign-/mild-grade dysplasia or moderate- or severe-grade dysplasia/carcinoma in situ/cancer are presented in Supplementary Tables S1 and S2.

Discussion

Late diagnosis with advanced-stage disease is the main cause of head and neck cancer morbidity and mortality (4). Although the oral cavity is an easy site for physical exam, often a delay in seeking medical care leads to the advanced stage of disease at time of diagnosis (16).

Detection of DNA methylation in salivary rinse samples is a potential non invasive method for early diagnosis of head and neck cancer. Our group has previously shown that it is possible to correlate methylation status with overall survival and prognosis using salivary rinses (11). Also, promoter CpG islands of KIF1A and EDNRB were shown to be methylated in primary HNSCC. These highly specific salivary biomarkers were shown to be potential biomarkers for HNSCC detection (13) as well as predictors of risk in oral cavity cancer and premalignancy (17). Furthermore, salivary rinse includes microorganisms, residual food, enucleated orthokeratinized cells and for that reason, extraction of nucleic acids, and purification must be carried out carefully for stable results. QMSP sensitivity allowed us to define methylated genes that were highly specific for tumor, and rarely or never present in any of the oral cavity sites that shed cells in salivary rinses. Also, the presence of cells from all epithelial surfaces may be helpful as potential predictor of malignant risk, as many studies show methylation as an early event on HNSCC carcinogenesis.

For this study, genes were selected using 3 criteria: (i) genes already reported in literature to be hypermethylated in head and neck cancers, (ii) genes reported as hypermethylated in other solid tumors, and (iii) genes identified via a methylation microarray based approach. The genes were tested in a pilot study with a limited cohort. Based upon specificity and sensitivity of tested genes, a panel was selected for analysis in an expanded cohort.

Our study used a cohort with mean age of 54.1 years with a history of tobacco and alcohol consumption, presenting with lesions epithelial oral lesions deemed suspicious for epithelial dysplasia or malignancy to study correlations between methylation status of select genes and cancer progression. Using univariate and multivariate analyses, only age, among predictors, was associated with histology (P = 0.014 and 0.034, respectively), although tobacco and alcohol exposure are well-known risk factors for oral cancer.

Although scalpel biopsy is the gold standard method for diagnosis, it may only represent a portion of the lesion and may not be representative of all pathologic changes. Many of the molecular alterations that may indicate early stages of malignant transformation cannot be seen in the morphological analysis (18). Such diagnostic testing requires training and proper equipment. For this study, salivary rinse samples were collected by untrained personnel without previous experience in this collection protocol. The amount of DNA collected was sufficient to perform the analysis with several genes. The results obtained in this study support an easy and efficient method for oral cancer screening and potentially for prevention.

Clinical risk assessment was conducted by dentists, based on WHO classification (14), classifying lesions as high risk or low risk. The risk classification assessment associated with methylation status was statistically significant, although not all histolopathological diagnoses matched the clinical risk classification (CRC; Table 3). Also in multivariate analysis risk classification was associated with histology, showing that the features of a detected oral lesion is important for early diagnosis.

Our study was able to find a significant correlation between histopathologic diagnosis and methylation status for DCC, EDNRB, and HOXA9, as single biomarkers. DCC is a putative tumor-suppressor gene at 18q21 that encodes a transmembrane protein with structural similarity to neural cell adhesion molecule (19), and is involved in both epithelial and neuronal cell differentiation (20). DCC hypermethylation has been detected in oral squamous cell carcinoma, other head and neck cancers, breast, gastric, and colon cancer (21–23). Our group has previously shown that DCC
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is epigenetically inactivated by promoter hypermethylation in a majority of HNSCC cases (21). In this study, DCC and EDNRB were hypermethylated in 40% of malignant salivary rinses samples and associated with malignant histopathologic diagnosis, independent of other predictors factors such as age and tobacco/ethanol exposure. These results suggest that they can be used as single biomarkers of malignancy.

EDNRB is a G protein–coupled receptor, which activates a phosphatidylinositol-calcium second messenger system. Its ligand, endothelin, consists of a family of 3 potent vasoactive peptides: ET1, ET2, and ET3. Studies suggest that the multigenic disorder, Hirschsprung disease type 2, can be due to mutations in EDNRB. Pattani and colleagues (17) showed that EDNRB promoter hypermethylation in salivary rinses is associated with increased risk of oral cancer and premalignancy.

The HOXA9 gene is part of the A cluster on chromosome 7 and encodes a DNA-binding transcription factor that may regulate gene expression, morphogenesis, and differentiation. A specific translocation event, which causes a fusion between this gene and the NIUP98 gene, has been associated with myeloid leukemogenesis. HOXA9 was found to be methylated in high-grade gliomas (24) and reported as a potential biomarker for prevention and early detection in oral squamous cell carcinoma using saliva samples (25).

Association of DCC and EDNRB hypermethylation with histopathologic diagnosis was discovered from a different approach in the attempt to identify a novel panel of promoter hypermethylation markers to improve the ability to detect epigenetic changes associated with HNSCC in salivary rinses (11, 13, 17). In combining the results from both DCC and EDNRB methylation assays, we observed improvement in performance, indicating their potential as biomarkers for HNSCC.

Clinical risk assessment also appeared as an important variable in this study. When we analyzed according to histopathology (benign vs. dysplasia/cancer), risk classification presented a strong association on univariate analysis (21, 26–28). Our study showed that salivary rinses can be obtained by untrained professionals and also that QMSP provides a cost-effective method that allows high-throughput and rapid analysis. The use of this technique as a means of early detection of premalignant and malignant lesions reinforces its usefulness as a screening and surveillance strategy. This low invasive approach allows easier high-risk population screening that may facilitate preventive medicine, therapeutic planning, and prognostic counseling.

The presence of EDNRB and/or DCC promoter methylation in salivary rinses compares well to examination by an expert clinician in risk classification of oral premalignant and malignant lesions. Given the current costs and availability of an expert health care provider trained and experienced in oral cancer diagnosis, it is simply not feasible to screen at risk populations via expert physical examination. Therefore, risk assessment with a salivary biomarker is attractive as a cost-effective means to identify higher risk patients that should be referred for expert exam and biopsy. Also, these salivary biomarkers may be particularly useful in identifying patients with lesions that appear low risk by physical examination, but are identified as high risk by epigenetic salivary biomarkers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: K.M. Pattani, J.D. Goldberg, J.A. Califano

Development of methodology: J.L. Schussel, K.M. Pattani, J.D. Goldberg, J.A. Califano

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.M. Pattani, F. Bermudez, G. Jean-Charles, T.V. McCaffrey, T. Padhye, J. Phelan, S. Spivakovsky, R.J. Li, H.Y. Bowne, M. Robbins, A.R. Kerr, D. Sirois, J.A. Califano

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.L. Schussel, X.C. Z.Z. Zhang, M. Brair, R.J. Li, L. Rolnitzky, A.R. Kerr, D. Sirois, J.A. Califano

Writing, review, and/or revision of the manuscript: J.L. Schussel, Z. Zhang, T. Padhye, J. Phelan, M. Brair, R.J. Li, J.D. Goldberg, L. Rolnitzky, D. Sirois, J.A. Califano

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.L. Schussel, K.M. Pattani, M. Brair, R.J. Li, J.A. Califano

Study supervision: J.A. Califano

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References

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