Prospective Evaluation to Establish a Dose Response for Clinical Oral Mucositis in Patients Undergoing Head-and-Neck Conformal Radiotherapy

Samir Narayan, M.D.,* Joerg Lehmann, Ph.D.,* Matthew A. Coleman, Ph.D.,† Andrew Vaughan, Ph.D.,* Claus Chunli Yang, Ph.D.,* Danny Enepekides, M.D.,‡ Gregory Farwell, M.D.,‡ James A. Purdy, Ph.D.,* Grace Laredo, Ph.D.,* Kerry Nolan, A.S.,† Francesca S. Pearson, B.S.,† and Srinivasan Vijayakumar, M.D.*

*Department of Radiation Oncology, University of California Davis Medical Center, Sacramento, CA; †Lawrence Livermore National Laboratory, Livermore, CA; and ‡Department of Otolaryngology, University of California Davis Medical Center, Sacramento, CA

Purpose: We conducted a clinical study to correlate oral cavity dose with clinical mucositis, perform in vivo dosimetry, and determine the feasibility of obtaining buccal mucosal cell samples in patients undergoing head-and-neck radiation therapy. The main objective is to establish a quantitative dose response for clinical oral mucositis.

Methods and Materials: Twelve patients undergoing radiation therapy for head-and-neck cancer were prospectively studied. Four points were chosen in separate quadrants of the oral cavity. Calculated dose distributions were generated by using AcQPlan and Eclipse treatment planning systems. MOSFET dosimeters were used to measure dose at each sampled point. Each patient underwent buccal sampling for future RNA analysis before and after the first radiation treatment at the four selected points. Clinical and functional mucositis were assessed weekly according to National Cancer Institute Common Toxicity Criteria, Version 3.

Results: Maximum and average doses for sampled sites ranged from 7.4–62.3 and 3.0–54.3 Gy, respectively. A cumulative point dose of 39.1 Gy resulted in mucositis for 3 weeks or longer. Mild severity (Grade 1) and short duration (≤1 week) of mucositis were found at cumulative point doses less than 32 Gy. Polymerase chain reaction consistently was able to detect basal levels of two known radiation responsive genes.

Conclusions: In our sample, cumulative doses to the oral cavity of less than 32 Gy were associated with minimal acute mucositis. A dose greater than 39 Gy was associated with longer duration of mucositis. Our technique for sampling buccal mucosa yielded sufficient cells for RNA analysis using polymerase chain reaction.

Head-and-neck cancer, Radiation therapy, Mucositis, Buccal mucosa, RNA analysis.

Introduction

Understanding of the pathobiologic aspects of mucositis has increased greatly (1). Mucositis is understood as a complex interaction of mucosal injury, inflammatory response, ulceration, and healing. A frequent complication of head-and-neck radiation therapy (RT), mucositis causes such adverse outcomes as treatment breaks, dehydration, narcotic use, weight loss, hospitalization, and increased cost (2). The severity and duration of mucositis are variable, depending on field size and shape, total dose, dose per fraction, and duration of RT. Concurrent chemotherapy doubles the incidence of Grade 3 or higher acute mucosal effects compared with RT alone (3). A mucositis grading system incorporating both subjective and objective criteria is important to establish severity and guide therapy (4). The National Cancer Institute (NCI) Common Toxicity Criteria (CTC), Version 3.0, grades mucositis severity based on a clinical examination and functional/symptomatic scale.
Unexpected toxicities may be expected to occur in the oral cavity, especially given that use of highly conformal treatment techniques increases the irradiated volume, albeit at relatively low doses (5). Whether dose gradients in the oral cavity from intensity-modulated RT (IMRT) alter existing expectations of toxicity, such as mucositis, xerostomia, taste, swallowing, speech, and quality of life, are not well known.

Furthermore, in vivo dose verification and correlation of dose with biologic changes remain active areas of investigation with implications for radiation safety. As part of collaboration with Lawrence Livermore National Laboratory, we conducted a clinical study to correlate dose within the oral cavity with the presence of clinical mucositis, perform in vivo dosimetry, and determine the feasibility of obtaining buccal mucosal cell samples for molecular studies in patients undergoing head-and-neck RT. The main objective of this report is to establish a quantitative dose response for clinical oral mucositis and show the feasibility of RNA analysis from buccal epithelial cells.

METHODS AND MATERIALS

Patient eligibility

Adult patients with histologic confirmation of head-and-neck cancer requiring RT to the head-and-neck region at the University of California Davis Health System, Department of Radiation Oncology, were eligible as long as the treatment fields included a portion of the oral cavity. Karnofsky performance status of 60 or higher, platelets count of 100,000 per microliter or greater, and international normalized ratio of 2.0 or less were required. Concurrent chemotherapy was allowed. Exclusion criteria included active infection, fistula, or wound dehiscence of the buccal mucosa; surgical reconstruction of the buccal mucosa (making it impossible to obtain biopsy specimens from local native oral squamous mucosa); tumor involvement of the sampled buccal mucosa; previous cancer of the oral cavity; prior head-and-neck RT; and active bleeding disorders. All patients with teeth underwent a pretreatment dental evaluation. This prospective study was approved by the institutional review boards of the University of California, Davis, and the Lawrence Livermore National Laboratory.

Simulation, selection of sampling points, and treatment planning

Patients were positioned supine by using customized immobilization devices. Aquaplast masks with shoulder extension (Med-Tec Inc., Orange City, IA) or Aquaplast mask with shoulder retraction device and bite blocks also generally were used to ensure daily setup reproducibility. In addition, mouth guards routinely were placed over patients’ upper and lower gums and teeth. In the patient without teeth, an external fiducial device, such as a radiopaque marker, was placed on the outer surface of the mouth guard as a reference point. These points could be identified easily on the computed tomography (CT) scan. In patients with intact teeth, the teeth were used as a landmark that was also easily identified on CT. Four points (right and left and upper and lower gingivobuccal sulcus [A, B, C, and D]) were identified in the oral cavity (see Fig. 1 in the online version). All patients underwent a planning CT on a Picker CT simulator (Philips, Amsterdam, The Netherlands). Images were then transferred to the Philips AcQSim/AcQPlan workstation (Philips). Treatment planning was performed using the AcQPlan system or Eclipse IMRT treatment planning system (Varian Medical Systems, Palo Alto, CA). Treatment plans were generated by the treating radiation oncologist according to clinical practice.

Dosimetry

MOSFET dosimeters from Thomson Nielsen (Nepean, Ontario, Canada) were used for in vivo dose measurement. Before the second radiation treatment, four MOSFETs were secured to the mouth guard of the patient and placed adjacent to each preidentified location (see Fig. 2 in the online version). The measured dose of one fraction of treatment was compared with the calculated dose (6) from each point as identified from the dose distribution of the patient’s treatment plan (Fig. 1).

Sample collection and RNA processing

Buccal cell samples were collected before the first radiation dose and 24 hours after the initial exposure. Patients receiving concurrent chemotherapy had an additional sample collected before receiving chemotherapy. Patients were required to first rinse their mouths with water to flush the oral cavity of loose debris and bacteria. A Cytobrush (American Master* Tech Scientific, Inc, Lodi, CA) then was used to collect buccal cells from areas of the buccal mucosa that were selected based on the three-dimensional radiation treatment planning as receiving a defined dose. Samples were stored at −80°C in RNA Later (Qiagen, Valencia, CA). RNA was extracted using the manufacturer’s instructions. Total RNA samples were tested for DNA integrity by using gel electrophoresis or an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) to identify a 2:1 ratio for the 28S vs. 18S ribosomal RNA.

Complementary DNA synthesis and TaqMan analysis

Quantitative polymerase chain reaction (PCR) analyses were performed following the recommended protocols by the manufacturer, Applied Biosystems (Foster City, CA). Total RNA was collected as described, a two-step reverse-transcriptase PCR was executed using 5 μg of total RNA and primers for the first-strand complementary DNA (cDNA) synthesis (High Capacity Archive Kit; Applied Biosystems, Inc.). The cDNA generated from this step was diluted fourfold. TaqMan gene-specific primers (900 nM each) and fluorescent probes that are ready-to-use prevalidated assays (Applied Biosystems) were tested in-house by using universal human RNA. The 18S rRNA (4310893E) and β-actin (ACTB [4310855]) gene

![Fig. 1. Dose distribution of an intensity-modulated radiation therapy (IMRT) plan for a patient with nasopharynx cancer shows the dose gradient across the oral cavity and identification of sampled points B and D. PTV = planning target volume.](image-url)
transcripts were used as internal controls. Thirteen genes were chosen based on likely radiation response (7, 8). Primers for the two used here were as follows: GADD45A (Hs00169255_m1) and CDKN1A (Hs00355782_m1). Reactions were run on an Applied Biosystems Sequence Detection System 7700 Real-Time PCR System using a 96-well format. The PCR was performed following the manufacturer’s instructions and using 25 ng of input cDNA: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, during which time data were collected. Each sample was run in triplicate. Important controls included no-template controls and no-primer amplification controls.

Toxicity and mucositis assessment

Weekly assessments of clinical and functional mucositis at the selected points were performed by the radiation oncologist according to NCI CTC Version 3 (see Tables 1 and 2 in the online version). Overall oral pain and pain at the sampled sites also were assessed. The severity (grade) and duration (number of weeks of Grade ≥ 1) of mucositis and pain and the dose at the sampling sites were recorded. In the analysis, severity and duration of mucositis correlated with local radiation dose, and the relationship was described using a minimization squares method.

RESULTS

Patient characteristics

Patient characteristics are listed in Table 1. A total of 12 patients were enrolled, 9 men and 3 women. Median age was 61 years (range, 23–79 years). Histologic characteristics included squamous cell carcinoma in 8 patients, plasmacytoma in 2 patients, basal cell in 1 patient, and adenocarcinoma in 1 patient. Six patients underwent primary surgical resection. Two patients underwent concurrent chemotherapy.

RT characteristics

Six patients were treated using IMRT. Median total dose was 63 Gy (range, 30–70 Gy). Median number of fractions was 31 (range, 10–35 fractions). Median number of weekly evaluations was seven. Maximum and average doses for sampled sites ranged from 7.4–62.3 and 3.0–54.3 Gy, respectively.

Table 1. Patient and treatment characteristics

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Race</th>
<th>Histologic characteristics</th>
<th>RT technique</th>
<th>Total dose (Gy)</th>
<th>Fractions</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>Hispanic</td>
<td>Plasmacytoma</td>
<td>3D</td>
<td>45</td>
<td>25</td>
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<tr>
<td>2</td>
<td>F</td>
<td>Asian</td>
<td>SCCA</td>
<td>IMRT</td>
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<td>35</td>
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<tr>
<td>3</td>
<td>M</td>
<td>White</td>
<td>SCCA</td>
<td>IMRT</td>
<td>70</td>
<td>35</td>
</tr>
<tr>
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<td>Black</td>
<td>Large cell</td>
<td>3D</td>
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<td>30</td>
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<tr>
<td>5</td>
<td>F</td>
<td>White</td>
<td>Adenocarcinoma</td>
<td>3D</td>
<td>30</td>
<td>10</td>
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<tr>
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<td>3D</td>
<td>66</td>
<td>33</td>
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<tr>
<td>7</td>
<td>M</td>
<td>Black</td>
<td>SCCA</td>
<td>3D</td>
<td>60</td>
<td>30</td>
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<tr>
<td>8</td>
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<td>3D</td>
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</tr>
<tr>
<td>9</td>
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<td>IMRT</td>
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<tr>
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<td>SCCA</td>
<td>IMRT</td>
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<tr>
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<tr>
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<td>White</td>
<td>SCCA</td>
<td>IMRT</td>
<td>70</td>
<td>35</td>
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</tbody>
</table>

Abbreviations: RT = radiation therapy; SCCA = squamous cell carcinoma; 3D = three dimensional; IMRT = intensity-modulated radiation therapy.

Mucositis

The severity of mucositis along the buccal mucosa was mild, with most patients experiencing Grade 1 or no mucositis. Eight instances of Grade 2 oral mucositis at sampled points occurred in 2 patients (Fig. 2). The dose at these points was 32.8 Gy or greater. No patient experienced Grade 3 or higher oral mucositis. The most severe mucositis (Grade 3) was seen in the oropharynx and lateral tongue borders.

Eight patients had 3 weeks or longer of oral mucositis at the sampled points. Mucositis duration of 3 weeks or longer occurred with a dose of 39.1 Gy (Fig. 3). These patients had an average dose to the four oral cavity points of 32 Gy. Mild severity (Grade ≤ 1) and short duration (≤ 1 week) of mucositis were found at point doses less than 32 Gy. (Figs. 3 and 4).

Buccal sampling was well tolerated. No patient refused sampling and none reported discomfort at the sampled site lasting more than 1 day.

Dosimetry

Agreement within 25 cGy between measured and calculated doses occurred in 32 of 48 measurements. Reasons for disagreement were selection of points at the edge of a treatment field or within a steep dose gradient. Assessment of biologic results is ongoing and not part of this report.

RNA analysis

To show the ability to measure transcripts of interest in human buccal cells, we isolated total RNA for PCR analysis from unexposed buccal cells based on a previously published approach selected to maximize intact RNA from small sample sizes (9). The amount of total RNA isolated in a volume of 25 μL ranged from approximately 1 to 17 μg/μL, giving an average of 3.95 μg/μL per sampled area. All samples were deemed sufficient for PCR analysis based on yield and/or quality estimates. Quantitative PCR data were generated across four unexposed collection sites for three patient samples (Fig. 5). The selected internal reference control genes (18S rRNA and β-actin) showed relatively constant expression measured by using cycle threshold (Ct) values compared...
with GADD45A and CDKN1A within each patient. The Ct for each transcript gave relatively constant values for the sampled areas within any 1 patient. Baseline values across patients were variable. Importantly, PCR was able to consistently detect two known radiation-responsive genes within selected patients. All signals were the result of RNA-specific target amplification, shown by the absence of PCR products in nonirradiated controls (data not shown).

**DISCUSSION**

The development of mucositis using traditional head-and-neck RT techniques is predictable (10, 11). Use of opposed lateral beam arrangements and daily doses of 200 cGy result in mucosal erythema after 1 week (10 Gy), patchy pseudo-membranous formation after 2 weeks (20 Gy), and ulceration after 3 weeks (30 Gy). However, use of multiple nonuniform intensity beams results in a more heterogeneous dose distribution within both target volumes and normal tissues compared with opposed lateral beams (12). For example, a typical seven- or nine-field head-and-neck IMRT plan for tumors of the oropharynx uses beams that enter or exit through the oral cavity during the entire course of treatment. Conversely, conventional treatment planning with opposed lateral beams would block the majority of the oral cavity for most if not the entire duration of treatment.

As a consequence of conformal treatments, incidental dose to some normal tissues, such as the oral cavity, skin, and larynx, increased (13–15). Furthermore, heterogeneous dose distributions exist in normal tissues, necessitating the need for biologic equivalent dose corrections to determine both acute and late complications. Whether dose gradients in the oral cavity from IMRT alter existing expectations of stomatotoxicity is not well known. This prospective evaluation is the first to correlate oral cavity dose with mucositis by using in vivo dose verification and tissue sampling in patients undergoing head-and-neck RT.

Our study elucidates the relationship between radiation dose and oral mucositis severity and duration. In Figs. 2–4, we were able to use a sigmoid-type function because they are common in the response of living systems to radiation to fit data for grade and duration of mucositis as a function of radiation dose. Although this fitting is only qualitatively meaningful because of the limited number of data points, it is...
obvious that there is a threshold of dose range in each data set. Within the dose range of 7.4–62.3 Gy, the severity of mucositis at the sampled points of the buccal mucosa was mild, with only 2 patients experiencing Grade 2 or higher mucositis. Doses to sampled points less than 32 Gy did not result in mucositis severity of Grade 1 or higher. Duration of mucositis was associated with the maximum dose received of the four points within a patient. Durations of 3 weeks or longer and 4 weeks or longer occurred at maximum point doses within a patient greater than 39 Gy and greater than 41 Gy, respectively. The corresponding average point doses over the four locations were greater than 32 and greater than 32.9 Gy, respectively. Therefore, mild severity and short duration of oral mucositis were found with maximum point doses less than 39 Gy and average point doses less than 32 Gy.

The severity of mucositis along the buccal mucosa compares favorably with other series (16) of patients treated with RT using conventional techniques. Two major reasons could explain these results. Sensitivity to radiation-induced stomatitis depends on location within the oral cavity. Buccal mucosa, although relatively easy to sample reproducibly, might be less sensitive to radiation-induced mucositis than other mucosal surfaces, such as the nonkeratinized portions of the ventral tongue, floor of the mouth, and soft palate (1). We noted that both the most severe grade of overall mucositis and most painful site tended to be along the soft palate or lateral tongue, rather than the buccal mucosa.

Another explanation is that biologic correction of physical dose for time, dose per fraction, and repopulation needs to be considered. In other words, using uniform radiation of 30 Gy over 15 fractions is expected to result in ulcerative (Grade 3) mucositis. The biologically equivalent dose (17, 18) assuming normal tissue repopulation ($\alpha/\beta = 10$) during a 7-week course of treatment is 48 Gy. Therefore, the absence of Grade 3 or higher mucositis by using our treatment techniques is not unexpected. As such, our data lend support to the need to further study the relationship of dose, volume, and mucosal toxicity by using conformal treatment methods, which deliver heterogeneous dose distributions to normal tissues (19, 20).

In addition to making biologic corrections for dose, other issues hamper the ability to develop a robust normal tissue complication probability (NTCP) model for predicting acute oral mucositis. First, a standard method for delineating the oral cavity mucosa does not exist. Because the oral cavity is a virtual space similar to the rectum and esophagus (21–23), it is unclear whether the surface or the mucosa is a better predictor of radiation complications than cavity volume. Nevertheless, given the difficulty defining an actual mucosal surface, Sanguineti et al. (13) described a useful method for contouring the mucosa of the upper aerodigestive tract, for which an illustration can be found at www.utmb.edu/radoncology/mucosa.pdf. In our study, we chose points in the oral cavity for purposes of determining acute mucosal effects specifically at those points.

Second, lack of a universally accepted scale to report mucositis limits the ability to develop complication probabilities across studies (24). Mucosal toxicity reporting scales vary by grade, clinical description, and functional consequence and were summarized elsewhere (1, 4, 25, 26). The NCI CTC Version 3, which includes a scale for clinical and functional/symptomatic assessment of mucositis, was used in our study.

Third, the use of both concurrent chemotherapy (3, 16) and altered fractionation (27–30) were shown to increase acute mucosal reactions of RT and further limit the applicability of a predictive model. Finally, other confounding issues affecting mucosal sensitivity, including the use of tobacco and other drugs, dental artifacts, scatter effects, and immobilization techniques, need to be accounted for when determining a dose-and-effect relationship for mucositis.

Although there is currently no recognized dose constraint for the oral cavity for IMRT planning, Sanguineti et al. (13) showed the potential of using a dose objective during IMRT to achieve mucosal sparing. In their treatment planning study, IMRT (using a dose objective) resulted in a 20% mean absolute decrease in the percentage of mucosa receiving a dose equivalent of 30 Gy compared with conventional treatment techniques. The IMRT, without a specific dose objective for the mucosa, resulted in an increased amount of mucosa exposed to significant radiation doses vs. conventional treatment techniques. Although IMRT using a dose objective was able to spare normal mucosa, this study did not include clinical evaluation of mucositis.

Shogan et al. (31) showed a correlation of oral cavity dose with mucositis by using IMRT and concurrent chemotherapy. A statistically significant correlation between acute mucositis grade and percentage of volume of oral cavity receiving 15, 30, 40, and 45 Gy was found. However, this study did not directly correlate dose to a point with subsequent mucositis at that point.

We observed that limiting dose to less than 39 Gy or an average oral mucosa dose less than 32 Gy results in mild severity and only a short duration of mucositis. Such constraints could be used, especially for cases in which the oral cavity is not part of the planning target volumes. For patients with such disease as oral cavity primary tumor, base of tongue, and tonsil, in which the planning target volume expands into the oral cavity, we recommend assigning a lower weighting factor for oral cavity sparing. Furthermore, a more stringent dose objective resulted in greater dose to surrounding organs at risk (13). Therefore, we do not recommend sacrificing target volume coverage or increasing dose to other adjacent critical organs for purposes of oral cavity sparing. We recommend a prospective multicenter study to confirm the preliminary findings of our study.

Our molecular approach shows the plausibility of using cells from the oral mucosa in RT patients to apply RNA-based molecular biologic techniques for signature development. This is a very attractive technique because RNA samples can be obtained by using relatively noninvasive means. Previously published reports also showed minimal degradation of RNA by using similar approaches (9). This is important given the extreme labile nature of RNA isolated from the mucosal cavity. The RNA-specific method for quantitative PCR also illustrates that we can detect signals for
control, as well as target, transcripts of interest that will allow for the future data normalization to identify differential gene expression in RT patients. The two transcripts (GADD45A and CDKN1A) are well-established biomarkers of radiation exposure in multiple cell types in vitro and in vivo (7, 8). However, no study to date was done to show how differentially responsive GADD45A and CDKN1A are to ionizing radiation in buccal-derived cells. One limitation of our approach is that relatively small amounts of RNA were obtained and methods that use RNA amplification technologies may be needed to increase the number of transcripts we can interrogate within a single study (32). In the future, quantification of the amount of target in unknown samples can be accomplished by comparing Ct values within samples and between unexposed vs. exposed samples by using the Ct method (33).

CONCLUSION

Our study elucidates the relationship between dose and oral mucositis severity and duration by using in vivo dose verification and tissue sampling in patients undergoing conformational head-and-neck RT. In our study, cumulative doses to the oral cavity less than 32 Gy were associated with minimal acute mucositis. A dose greater than 39 Gy was associated with a longer duration of mucositis. Our method of sampling buccal mucosa cells from patients yielded sufficient cells for RNA analysis and was essentially painless. Our study shows that mucosal toxicity can be correlated with dose within the oral cavity by using conformal techniques. We believe that with further study, practical guidelines for minimizing mucositis within the oral cavity can be developed for patients undergoing head-and-neck IMRT.

REFERENCES


Supplementary Fig 1. Sampling points on the buccal mucosa (A, B, C, and D).
Supplementary Fig 2. MOSFETs placed on mouth guards before the second radiation treatment.
### Supplementary Table 1. National Cancer Institute Common Toxicity Criteria, Version 3, for clinical mucositis

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Erythema</td>
</tr>
<tr>
<td>2</td>
<td>Patchy ulcerations or pseudomembranes</td>
</tr>
<tr>
<td>3</td>
<td>Confluent ulcerations or pseudomembranes, bleeding with minor trauma</td>
</tr>
<tr>
<td>4</td>
<td>Tissue necrosis, significant spontaneous bleeding, life-threatening consequences</td>
</tr>
<tr>
<td>5</td>
<td>Death</td>
</tr>
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</table>
## Supplementary Table 2. National Cancer Institute Common Toxicity Criteria, Version 3, for functional/symptomatic mucositis

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Minimal symptoms, normal diet</td>
</tr>
<tr>
<td>2</td>
<td>Symptomatic, but can eat and swallow modified diet</td>
</tr>
<tr>
<td>3</td>
<td>Symptomatic and unable to adequately aliment or hydrate orally</td>
</tr>
<tr>
<td>4</td>
<td>Symptoms associated with life-threatening consequences</td>
</tr>
<tr>
<td>5</td>
<td>Death</td>
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</table>