Effect of Radiotherapy on Expression of Transmembrane Mucin MUC1 in Oral Mucosal Cells

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Abstract

Objective: Mucins form a protective surface called mucosal pellicle on oral epithelium. Mucin-1 (MUC1) is secreted from the oral mucosal squamous epithelium itself on the apical surface of the epithelial cell. The objective of this study was to determine the effect of radiotherapy (RT) on MUC1 expression of the oral epithelium in patients with head and neck cancer (HNC).

Methods: Oral mucosal tissue biopsies were obtained from 55 patients; Study group 1 consisted of 33 clinically healthy subjects as controls. The oncology group consisted of two subgroups: Group 2 consisted of 7 oral cancer patients treated with surgery without RT, and Group 3 consisted of 15 HNC patients treated with RT. To visualize MUC1 staining, HMFG1 antibody was used. In addition, microstructures of the specimens were studied under electron microscopies.

Results: The superficial layer of the oral epithelium had strong MUC1 staining in control samples compared to oncological groups (p=0.002). Intermediate layer showed the most expression of MUC1 in irradiated mucosa (p=0.02). In both oncological groups, the expression of MUC1 was detected on the basal layer (p=0.005). Morphological analysis with electron microscopies showed destruction in the microstructure of apical cells of the irradiated oral epithelium. Irradiated oral mucosa with strong MUC1 expression showed loose intercellular bonds.

Conclusion: Radiotherapy affects the expression of MUC1 in basal and intermediate layers of oral epithelium. Irradiation alters or hinders the intra and intercellular linkages which affects the normal apical transportation of MUC1 and hence, such alteration may play a role in promoting radiation-induced complications.

Keywords: mucosal pellicle, mucins, oral cancer, oral epithelium, radiotherapy
Introduction

Head and neck cancers (HNC), like other cancers, encompasses a diverse complex group with varied etiology and pathobiology. Radiotherapy (RT) is a very effective and widely used treatment modality for HNC, but despite enormous advances in RT, a significant number of patients will experience detrimental effects on the surrounding normal tissues or radiation associated toxicities. This adverse impact of developing tissue damage in irradiated patients negatively affect their quality of life (1). Unfortunately, the overall five-year survival rate has remained low between 50–60% for past 3 to 4 decades, due to intrinsic resistance of cancer cells to the existing treatment modalities (2).

In the head and neck region, RT causes both acute/early and long term/late complications. Acute side effects are mucositis, dysphagia, erythema and desquamation of the superficial epithelial layer. Late complications result chronic injury to vasculature, salivary glands, mucosa, connective tissue and bone, clinically presenting as osteonecrosis, subcutaneous fibrosis, trismus, loss of taste, thyroid dysfunction, esophageal stenosis, dental decay and damage to middle ear or inner ear (3–6). The side effects are directly related to radiation dosimetry, including total dose, fraction size and duration of the treatment. RT for HNC is conventionally given up to total doses of 66–70 Gy over 6 to 7 weeks, in the fractionation schedule along with chemotherapy for improved results (7–9).

Mucins are a group of highly glycosylated proteins on the oral mucosal surfaces, which along with saliva and microstructure of the apical cells, named microplicae (MPL) form a protective layer against bacteria from entering into the oral epithelium (10). The ‘Oral Mucosal Barrier Complex’ consisting of the oral epithelial defense system, the mucosal pellicle and the attached salivary film forms the first defense line of the oral mucosa. One major component of the mucosal pellicle is MUC1, which plays an important role in protection (by binding to secretory mucins) and maintenance of the pathophysiology of oral epithelium. It is confirmed that MUC1 was expressed on the apical surface of the healthy epithelial cells (11–13).

Despite being in first line of defense, MUC1 is also a part of an early epithelial response to infection and multiple function in carcinoma host interactions. The polymorphic nature of MUC1 is known to regulate cancer cell behavior both in vivo and in vitro (14). The aberrant localization and expression of MUC1 correlates with poor tumor differentiation and impaired prognosis, and depolarized MUC1 expression is correlated with tumor stage and patient outcome (15–17).

Irradiation might alter the pathophysiological ability of MUC1 and play a role in the aggressiveness and progression of the oral cancers. The aim of this study is to investigate the effect of RT on the MUC1 expression of the oral mucosa. The hypothesis is that irradiation alters the expression of MUC1 in the oral mucosa of cancer patients compared to healthy controls.

Materials and Methods

Subjects

Oral mucosal biopsies were harvested from 55 patients and they have divided into 3 study groups: Group 1 (controls), Group 2 and Group 3 (oncological groups) respectively (Table 1).

Group 1 consisted of 33 fully edentulous and clinically healthy patients without history of cancer or radiotherapy. Of 33 patients, 16 were male and 17 were females, with age range of 33 to 74 years. Group 2 (non-irradiated) consisted of seven (5 males, 2 female; age range 57–79 years) patients with history of oral cavity malignancy treated with ablative surgery without RT, and Group 3 (Irradiated) consisted 15 (10 males, 5 females; age range 54–84 years) patients with history of head and neck malignancy with RT. The local radiation dose at the biopsy site was estimated as follows: RT (intensity modulated radiotherapy=IMRT) planning CT-image was merged with postoperative cone beam-CT image. The patients with the history of bisphosphonate medication or systemic immunosuppressive medications up to three months prior to the oral surgery excluded from the study. All participants had blood HbA1c levels within the normal range.

Prior to the study, the Medical Ethical Committee of the Amsterdam University Medical Centers (location VU Medical Center), Amsterdam, the Netherlands (registration number 2011/220) provided approval for the research. The Research Ethics Committee of the Northern Savo Hospital District (754/2018) approved the study. The present study fulfilled the World Medical Association Declaration of Helsinki (Helsinki, Finland, 1964). All patients signed a written informed consent to participate in the study.
Biopsy retrieval

In the control group (Group 1), the biopsies were taken during vestibuloplasty from the anterior side of the anterior alveolar ridge. In the non-irradiated group (Group 2), mucosal biopsies were taken during dental rehabilitation with dental implants 6–12 months after cancer treatment and the samples did not include malignant lesion. Oral mucosal biopsies were obtained from irradiated patients (Group 3) during dental implant surgery after RT (see Table 2). Oral mucosal tissue specimen of size 10 x 5 mm was obtained by scalpel and washed gently with saline solution before fixation in 10 % phosphate buffered formalin. The tissue specimens were divided into two pieces, one for light microscopy (LM) and other for electron microscopic examinations. For LM, specimens were dehydrated in graded ethanol and embedded in paraffin blocks.

MUC1 staining

Routinely, 4-micron thick section were cut from the paraffin blocks for LM. The sections were stained with hematoxylin and eosin (HE). Immunohistochemical (IHC) MUC1 staining was done using Dako En Vision K 5007-kit (Dako, glostrup, Denmark). The method followed for IHC staining was as per manufacturer’s instructions (Abcam, Cambridge, UK). For, epitope retrieval, Tris-EDTA (pH 9) was used for 10 minutes in 98°C (Milestone). Dako Peroxidase–Blocking solution S2023 was used to remove endogenous peroxidase for 15 minutes to block non-specific staining. Samples were incubated with primary antibody for MUC 1 (concentration 2µg/ml, 1,500 dilution in Dako REAL Antibody diluent S2022) for 1 hour at room temperature. A MUC1 monoclonal antibody, human milk fat globule 1 (HMFG 1) purchased from Abcam (Cambridge, UK) was used. Labelled polymer horseradish peroxidase (HRP) was used for 30 minutes with conjugated secondary antibody at room temperature. DAB as chromogen was used for 5 minutes. The sections were subsequently washed with distilled water, counterstained with Mayer’s hematoxylin for 1 minute and bluing in ammonia water solution (0.75 %) for 1 minute. PBS-Tween wash followed in between the staining protocols. For negative control, we used NIS Dako mono, and a sample of minor salivary glands was used as a positive control for MUC1 staining.

Evaluation of the staining and statistics

Three researchers (HU, PP & AMK) did the examination and the scoring of MUC1 staining using light microscopy. The systematic recording carried out for staining distribution in basal, intermediate and superficial layers of the oral mucosal epithelium. The staining intensity in each layer was estimated with a three-level scoring (0=no staining, 1=some staining, 2=considerable staining) as mentioned by Siponen et al. (18). The statistical analysis was performed using IBM SPSS Statistic version 24 for windows (SPSS, Chicago, USA). Differences in staining intensity between different groups analyzed with cross tabulation and statistical significance of the differences calculated with Chi-square test. Probability values of less than 0.05 considered statistically significant. The slides were observed and photographed with a Zeiss AxioImager M2 (Carl Zeiss Microscopy GmbH, Jena, Germany).

Table 1. Demographic data and distribution of 55 patients

<table>
<thead>
<tr>
<th>Study group</th>
<th>Patients (n = 55)</th>
<th>Male/ Female</th>
<th>Age range (Mean age)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (G1) Controls</td>
<td>33</td>
<td>16/17</td>
<td>33 – 74 years (53.5 y)</td>
</tr>
<tr>
<td>Group 2 (G2) Oral cancer patients without RT (non-irradiated)</td>
<td>7</td>
<td>5/2</td>
<td>57 – 79 years (68.1 y)</td>
</tr>
<tr>
<td>Group 3 (G3) Head and neck cancer patients with RT (irradiated)</td>
<td>15</td>
<td>10/5</td>
<td>54 – 84 years (66.4 y)</td>
</tr>
</tbody>
</table>

Abbreviations: RT=radiotherapy
Scanning Electron Microscopy (SEM)

For SEM, the specimens were post-fixed for 2 hours in 2% osmium tetroxide. The fixed specimens were dried using ascending series of ethanol concentrations followed by critical point drying, covered with few microns thick gold using a sputter coater Polaron E 5100 (Polaron Equipment Ltd, UK). The gold-coated samples examined using Zeiss Sigma HD VP Scanning Electron Microscopy at 15kV (Carl Zeiss Microscopy GmbH, Jena, Germany).

Transmission Electron Microscopy (TEM)

The specimens were post-fixed in 2% osmium tetroxide with cacodylate buffer solution at 4°C for two hours. After rinsing with cacodylate buffer solution, the specimens were dehydrated in graded ethanol and embedded in epoxy resin. Thin sections were prepared with an ultramicrotome (Leica EM UC7; Leica Microsystems, Wetzlar, Germany), stained with 1% toluidine blue, and examined with light microscope. Ultrathin sections were cut with the ultramicrotome and stained with uranyl acetate and lead citrate. Sections were observed with JEOL JEM-2100F transmission electron microscopy (Jeol Ltd, Tokyo, Japan) equipped with a digital camera (Quemesa, Olympus Soft Imaging Solutions GmbH, Munster, Germany).

Results

The demographic data of 15 irradiated patients are summarized in Table 2. The mean local radiation dose was 51 Gy (range 15–66 Gy). The mean interval between RT and biopsy was 71 months (range 10-312 months).

Using light microscope, the epithelia of all samples demonstrated a stratified squamous epithelium with or without rete ridges. The superficial layer with non-kera-

Table 2. Patient, tumor site and treatment characteristics of the irradiated group (Group 3).

<table>
<thead>
<tr>
<th>Age/Sex</th>
<th>Tumor site</th>
<th>RT dose Total (Gy)</th>
<th>RT dose Local (Gy)</th>
<th>RT-biopsy interval (months)</th>
<th>MUC1 staining B/I/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>69/M</td>
<td>Nasopharynx</td>
<td>70</td>
<td>15</td>
<td>72</td>
<td>2/2/2</td>
</tr>
<tr>
<td>61/F</td>
<td>Uvula</td>
<td>70</td>
<td>32</td>
<td>16</td>
<td>1/0/0</td>
</tr>
<tr>
<td>58/M</td>
<td>Tonsil</td>
<td>70</td>
<td>34</td>
<td>13</td>
<td>1/0/0</td>
</tr>
<tr>
<td>64/M</td>
<td>Soft palate</td>
<td>70</td>
<td>49</td>
<td>23</td>
<td>2/2/2</td>
</tr>
<tr>
<td>70/M</td>
<td>Floor of mouth</td>
<td>66</td>
<td>51</td>
<td>17</td>
<td>1/2/1</td>
</tr>
<tr>
<td>74/M</td>
<td>Floor of mouth</td>
<td>55</td>
<td>55</td>
<td>199</td>
<td>2/2/2</td>
</tr>
<tr>
<td>63/F</td>
<td>Floor of mouth</td>
<td>70</td>
<td>56</td>
<td>18</td>
<td>1/2/2</td>
</tr>
<tr>
<td>82/F</td>
<td>Oral tongue (ORN)</td>
<td>70</td>
<td>57</td>
<td>90</td>
<td>0/0/0</td>
</tr>
<tr>
<td>67/F</td>
<td>Retromolar trigone</td>
<td>66</td>
<td>57</td>
<td>10</td>
<td>2/2/0</td>
</tr>
<tr>
<td>72/M</td>
<td>Base of tongue</td>
<td>70</td>
<td>57</td>
<td>90</td>
<td>2/1/2</td>
</tr>
<tr>
<td>69/M</td>
<td>Submandibular gland</td>
<td>66</td>
<td>57</td>
<td>10</td>
<td>2/2/2</td>
</tr>
<tr>
<td>72/F</td>
<td>Base of tongue</td>
<td>70</td>
<td>60</td>
<td>20</td>
<td>2/2/2</td>
</tr>
<tr>
<td>84/M</td>
<td>Floor of mouth (ORN)</td>
<td>66</td>
<td>66</td>
<td>94</td>
<td>1/2/0</td>
</tr>
<tr>
<td>54/M</td>
<td>Oral tongue</td>
<td>70</td>
<td>66</td>
<td>85</td>
<td>2/2/2</td>
</tr>
<tr>
<td>66/M</td>
<td>Soft palate</td>
<td>n.a.</td>
<td>n.a.</td>
<td>312</td>
<td>2/2/1</td>
</tr>
</tbody>
</table>

Abbreviations: B=basal cells; I=intermediated cell layer; S=superficial cell layer; ORN=osteoradionecrosis; n.a. = not available. A three-level scoring of MUC1 staining: 0=no staining; 1= some staining; 2= considerable staining.
tinization was observed in Group 1, whereas few samples with the erosion of superficial surface was observed in oncological groups.

**MUC1 overexpression in non-irradiated and irradiated epithelium**

MUC1 staining in controls was limited to the apical part of the cells in the superficial layer of oral epithelium, whereas basal cell layer did not express MUC1 staining (Figs. 1 and 3C). Irradiated group showed MUC1 expression in all layers of the oral epithelium, with most of the samples showing more of basal and intermediate layer of staining (Figs. 2B and 3D). Comparison of the MUC1 staining among groups revealed strong expression of MUC1 in irradiated samples (p=0.004). Intermediate layer showed the most expression of MUC1 only in irradiated mucosa. Superficial layer expression of MUC1 among groups were statistically significant (p=0.001). When the underlying connective tissue showed intense inflammatory cell infiltrate, the expression of MUC1 was considerably in all epithelial layers (Fig. 2). The correlation of MUC1 expression with the local radiation dose or with the time interval between RT and biopsy was not significant. No statistical difference was observed in the expression pattern of basal cell layer among irradiated and non-irradiated epithelium, solely, in two patients with osteoradionecrosis (ORN), the MUC1 expression was lower than in other cancer patients. The MUC1 staining of membrane and cytoplasm was observed among the both oncological groups.

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**Fig. 1.** Localization of MUC1 (HFMG 1) in the normal oral epithelium. Typical expression of MUC1 is seen mostly on the apical cell surface of the superficial part of the oral epithelium without basal cell expression (Bar=100 µm).

**Fig. 2.** Expression of MUC1 in an irradiated specimen with stromal inflammatory cells. HE staining reveals intense inflammation in the connective tissue stroma (A). Strong expression of MUC1 is seen in all the layers of epithelium - basal, intermediate and superficial (Bar=100 µm) (B).
MUC1 staining intensity increased in non-irradiated and irradiated epithelium

In both oncological groups, variable intensity and expression of MUC1 was noted in the epithelium, cytoplasm and on the cell membrane, and its polarization was lost, whereas MUC1 was confined to superficial layers not in basal layer in any of the controls. Statistical difference is observed in the staining intensity of MUC1 in basal (p=0.002), intermediate (p=0.029) and superficial layers of the epithelium between controls and irradiated group (p=0.002). Only 40 % of controls showed faint staining of basal cell layer. 80 % to 100 % of controls showed cytoplasmic granular staining pattern in the upper stratum intermedium and stratum superficiale. Staining intensities of MUC1 in Group 1, 2 and 3 are presented in Table 3.

Surface structural alteration among study groups

SEM examination of control group showed regular MPL structure at the apical cell surface and tight junctions between adjacent cells, which was confirmed also with TEM. The apical cells of irradiated epithelium showed irregular and even missing MPL structure and irregular or destroyed cell junctions. TEM images of irradiated sample presented loose intercellular junctions compared to normal (Fig. 3G and 3H).

Discussion

In the present study, MUC1 localization and expression in oral epithelium was investigated and studied among healthy, non-irradiated and irradiated subject’s tissue samples. Micromorphology of oral epithelium was investigated using SEM and TEM and the findings were compared with the difference in MUC1 staining expression.

The staining of MUC1 at superficial layers of the oral epithelium confirms its localization and function at the cell surface. In validation of earlier studies, the production of MUC1 by the oral epithelial cells is confirmed. MUC1 is produced by internal cell organelles i.e, rough endoplasmic reticulum and Golgi bodies and is transported towards the apical portion by exocrine secretion (11, 19, 20). The staining of MUC1 in normal epithelium differs, as membrane positivity alone or cytoplasmic positivity alone or membrane and cytoplasm positivity together, attributing towards its assembly. Immunolocalization by HFMG1 antibody demonstrated the presence of transmembrane MUC1 in the oral epithelium.

Some previous reports confirmed the presence of typical microplicae (MPL) on the apical surface of oral epithelial cells and transmembrane mucin MUC1 found to proj-
ect from the tips of MPL (11, 19, 21). MUC1 can exist both in membrane-associated and secreted forms, which forms complex with salivary pellicle to protect oral epithelium against noxious stimuli. Tight adherence of MUC1 to the epithelial cell is through its extracellular and intracellular domain, which remained constant despite numerous steps of laboratory procedures. SEM images revealed alteration of oral mucosal cells on microstructural level of irradiated compared to control. Irradiation caused changes in the MPL structure of the superficial cells. In all non-irradiated and irradiated samples, the MPL structure was destroyed compared to controls. In controls, oral mucosa presented typical parallel and branching MPL structure as previously shown (22).

Oral cancer produces abnormal, uncontrolled proliferation of cells causing architectural and hierarchical disturbances in the epithelial cell layers, cell–cell interaction and cell surface microstructure. Similar effect is observed in irradiated induced tissue damages. In the present study, MUC1 expressed homogeneously in all layers of irradiated tissue samples. Basal and intermediate layer expressed more staining and the localization and expression pattern were similar in both oncological groups. The strong staining intensity of MUC1 was detected in the basal layer of irradiated epithelium compared to control. However, the intermediate and superficial layers of the epithelium in irradiated showed intense intracellular expression but no difference in the MUC1 expression in-

<table>
<thead>
<tr>
<th>Table 3. MUC1 intensity in the oral epithelium of different study groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study group 1 (G1) (n=33)</td>
</tr>
<tr>
<td>--------------------------</td>
</tr>
<tr>
<td><strong>MUC1 in basal layer</strong></td>
</tr>
<tr>
<td>- Considerable staining</td>
</tr>
<tr>
<td>- Some staining</td>
</tr>
<tr>
<td>- No staining</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>MUC1 in intermediate layer</strong></td>
</tr>
<tr>
<td>- Considerable staining</td>
</tr>
<tr>
<td>- Some staining</td>
</tr>
<tr>
<td>- No staining</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>MUC1 in superficial cells</strong></td>
</tr>
<tr>
<td>- Considerable staining</td>
</tr>
<tr>
<td>- Some staining</td>
</tr>
<tr>
<td>- No staining</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: G1=Controls, G2=non-irradiated, G3=irradiated; p as compared to controls
*faint staining in 40%
**One patient with osteoradionecrosis
tensity between irradiated and non-irradiated. This difference in the MUC1 expression in basal, intermediate and superficial layers of the oral epithelium could hypothetically be due to aberrant MUC1 recycling process as Litvinov and Hilkens had previously shown (23).

Inflammatory cytokines directly or indirectly regulate the expression of MUC1 (24). In oncological samples, the sub-epithelial area and connective tissue papilla showed intense inflammatory cell infiltrate and intense MUC1 staining in all the layers of epithelium. Constant exposure of oral epithelial cells to pathogens or their secreted products leads to increased transcription of MUC1 by pro-inflammatory mediators/cytokines. These cytokines interact among themselves or with other biological active compounds to develop synergistic mucin induction (24). Some authors showed that MUC1 promotes migration and invasion via PI3K-AKT signaling (25–27). MUC1 translocation might detach adherence junctions between cells; reduce contacts between tumor cells and lead to basement membrane invasion. In present study, TEM showed enlarged intercellular spaces between oral epithelial cells of irradiated compared to control. Abnormal localization of MUC1 in non-irradiated and irradiated epithelium of cancer patients, act as an anti-adhesive molecule by interfering between adhesion molecule and its ligands or by reducing the interaction between integrin’s and extracellular matrix (13).

As already described, irradiation restricts proliferative ability and causes overlying epithelium to ulcerate (28). In this study, SEM & TEM images of irradiated epithelium presented increased breakdown of desmosomal adherence junction and loss of normal vitality of epithelial cells. These changes may be associated with underlying inflammatory infiltrates and resulting defective permeability barrier. In the view of our observation, MPL structure and MUC1 localization seems to give information about cell microstructural fate, architectural and cytological changes in cell and overall microenvironment of oral epithelial cells.

The uniqueness of this study is that it provides new information about the varying MUC1 expression and micro-structural changes of non-irradiated epithelium and irradiated oral epithelium in patients with HNC. The data detected in our study showed statistically significant increased MUC1 expression in non-irradiated and irradiated epithelium compared to control epithelium. No difference was observed between non-irradiated and irradiated groups due to similar architectural pattern of the oral epithelium. In addition, changes in the genetic, epigenetic and microenvironment of the individual cells began far-away than expected. Do the irregular and abnormal pattern of MUC1 expression in the oral epithelial cells, is a valuable tool to assess patient’s risk of developing radiation-induced lesions.

In summary, our study shows at first time the expression pattern of MUC1 and ultrastructural changes in the oral epithelium in normal, non-irradiated and irradiated patients. Understanding the role of MUC1, its association with MPL structure and mucosal pellicle formation might lead to the development of new topical drugs against radiation-induced lesions of the oral cavity. Further, studies involving the role of MUC1 in irradiation induced early and late adverse phase effects may aid in selection of optimal treatment for patients undergoing radiotherapy.

**Conflict of Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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