

## Post-radiation changes in oral tissues - An analysis of cancer irradiation cases

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## Abstract

**Introduction:** Radiation, commonly employed as neoadjuvant, primary, and adjuvant therapy for head and neck cancer causes numerous epithelial and stromal changes, prominent among which is fibrosis with its early and late consequences. Very little is known about the true nature of the fibrosed tissue and the type of fibers accumulated. Radiotherapy affects the supporting tumor stroma often resulting in a worsening grade of tumor post-radiation. **Aim:** To study epithelial, neoplastic, stromal, and glandular changes in oral cavity induced by radiation therapy for oral squamous cell carcinoma (OSCC) using special stains. **Materials and Methods:** The study included 27 samples of recurrent OSCC following completion of radiotherapy (recurrence within an average span of 11 months), and 26 non-irradiated cases of OSCC. Patients with a history of combined radiotherapy and chemotherapy were not included in the study. The epithelial changes assessed included epithelial atrophy, apoptosis, necrosis, dysplasia, and neoplasia. The connective tissue was evaluated for amount of fibrosis, quality of fibers (using picosirius red staining), fibrinous exudate, necrosis, pattern of invasion, vessel wall thickening, and salivary gland changes. The aforementioned changes were assessed using light and polarizing microscopy and tabulated. **Statistical Analysis:** Epithelial and connective tissue parameters were compared between the irradiated and non-irradiated cases using chi square and t-tests. **Results:** Epithelial and connective tissue parameters were found to be increased in irradiated patients. Pattern of invasion by tumor cells varied from strands and cords between the two groups studied. The effect of radiation was seen to reflect on the maturity of fibers and the regularity of their distribution.

**Key words:** Fibrosis, pattern of invasion, radiation-induced changes, oral squamous cell carcinoma

## Introduction

Radiation is employed as neoadjuvant, primary, or adjuvant therapy to surgery and chemotherapy for head and neck cancer including oral squamous cell carcinoma (OSCC).<sup>[1]</sup> The use of radiotherapy to treat cancer inevitably involves exposure of normal tissues.<sup>[2]</sup> Though radiotherapy produces a significant increase in cure rates for many malignancies, higher doses of radiation in large areas, including the oral mucosa, skin, maxilla, mandible and salivary glands, brings about several undesirable reactions.<sup>[3]</sup> As a result, patients may experience symptoms associated with damage to normal tissue during the course of therapy, after the completion of therapy for months or years later.<sup>[2]</sup> This damage is caused by the ionizing radiation in the normal tissues located in the radiation field. Mucositis, candidosis, however dysphagia, radiation caries, fibrosis, osteoradionecrosis, soft tissue necrosis, progressive periodontal attachment loss, trismus and xerostomia are a few complications that are found significantly in irradiated patients, which affect their quality of life.<sup>[3]</sup> It has been established that the collagen content increases due to radiation-induced fibrosis, however, the histological nature of the fibers thus formed has not been assessed till now.

In the present study, an attempt has been made to assess the changes in epithelium and connective tissue following radiation therapy along with the evaluation of thickness and nature of collagen fibers using Picosirius red. Also, an attempt was made to assess the change in the grading pattern of the radiation-exposed tumor in relation to non-irradiated tumor.

## Materials and Methods

This study involved the retrieval of archival formalin-fixed, paraffin-embedded tissues from the Department of Oral Pathology, Manipal College of Dental Sciences, Mangalore, after due clearance from the Institutional Ethics Committee.

Twenty-seven cases of OSCC that recurred after radiation therapy of 6000cGy (recurrence within an average span of 11 months) were included in this study. The control group comprised 26 non-irradiated cases of OSCC. Two consecutive sections of 4- $\mu$  thickness of both cases (biopsy-proven cases of recurrent OSCC after radiation therapy) and control samples were taken. While one section of each case was stained with Hematoxylin and Eosin to assess various epithelial and connective tissue changes, the second section was stained with Picosirius red to assess collagen fiber thickness and nature.

The epithelial changes assessed were presence or absence of atrophy, degree of apoptosis and severity of dysplasia (Wahi *et al.* grading system). Connective tissue changes included presence or absence of fibrinous exudates, necrosis, vessel wall thickening, salivary gland changes (atrophy and ectasia) and pattern of neoplasia (Bryne's grading system).

## Assessment of picosirius red staining

The thickness of peri-tumoral collagen fibers of approximately 1375 fibers (average of 30 fibers per case) was assessed with an eyepiece reticule (40  $\times$  magnification; Olympus C  $\times$  21, NA of 0.65). Fibers were grouped as being "thick" (more than 1.5  $\mu$ m diameter) or "thin" (with diameter of 1.5  $\mu$ m or less).<sup>[4]</sup> The polarization colors varying from greenish-yellow to orange-red were noted for each fiber assessed (Leitz Wetzlar Aristoplan, Germany).

## Results

## Epithelial parameters

Chi square test showed significantly higher presence of atrophy and severity of dysplasia in irradiated cases of OSCC when compared to non-irradiated cases [ $P < 0.001$ , Table 1]. Application of t-test to the same groups showed significantly higher number of apoptotic bodies in irradiated cases [ $P < 0.001$ ,  $T = 6.458$ ; Table 2].

## Connective tissue parameters

Irradiated cases showed increase in presence of fibrinous exudates ( $P = 0.039$ ), necrosis ( $P = 0.010$ ), and vessel wall thickening ( $P < 0.001$ ) when compared to non-irradiated cases [Table 1].

The variables of Bryne's grading system (degree of keratinisation, nuclear polymorphism, number of mitosis, pattern of invasion and lymphoplasmacytic infiltration) were used in the assessment of neoplasia.

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**Table 1: Categorical variables-Chi square tests**

Parameters	Group (number of cases)		Total	Test value (Chi-square/Fisher's exact Chi-square test)	df	P value
	Irradiated tissue	Non irradiated tissue				
Epithelial atrophy						
Absent	7	25	32	27.306	1	<0.001
Present	20	1	21			
Necrosis						
Absent	12	21	33	7.438	1	0.010
Present	15	5	20			
Vessel wall thickening						
Absent	6	24	27	26.486	1	<0.001
Present	21	2	26			
Fibrinous exudate						
Absent	8	15	23	4.246	1	0.039
Present	19	11	30			
Colour of fibers (number of fibers)						
Dark green	197	426	623	203.583	1	<0.001
Orange red	528	224	752			
Size of fibers (number of fibers)						
Thick	389	141	530	147.813	1	<0.001
Thin	336	509	845			
Salivary gland ectasia						
Absent	10	17	27	1.568	1	0.274
Present	6	4	10			
Salivary gland atrophy						
Absent	4	16	20	9.582	1	0.002
Present	12	5	17			
Dysplasia						
Mild	0	11	11	17.386		<0.001
Moderate	14	12	26			
Severe	13	3	16			
Keratinization						
1	3	10	13	11.644		0.005
2	8	12	20			
3	14	4	18			
4	2	0	2			
Pleomorphism						
1	0	1	1	7.334		0.023
2	21	25	46			
3	6	0	6			
4	0	0	0			
Mitosis						
1	10	10	20	1.395		1.000
2	14	15	29			
3	2	1	3			
4	1	0	1			
Pattern of invasion						
1	0	5	5	22.340		<0.001
2	5	14	19			
3	11	7	18			
4	11	0	11			
Lympho-plasmacytic infiltrate						
1	4	7	11	4.738		0.045
2	14	17	31			
3	8	2	10			
4	1	0	1			

The degree of keratinisation and inflammation showed a significant decrease in irradiated cases when compared to primary OSCC (control cases) ( $P = 0.005, 0.045$ ), whereas nuclear pleomorphism was significantly increased ( $P = 0.023$ ). Mitosis, though found to be numerically higher in irradiated cases, was not statistically significant [ $P = 1.000$ ].

Comparison of the pattern of invasion between the two groups showed tumor infiltration principally in the form of small cords, groups and individual cells in irradiated cases in contrast to the non-irradiated cases, which showed predominantly solid cords, strands and bands [Table 1]. Overall, the irradiated cases had a combined higher score as compared to control

**Table 2: Statistical data of apoptosis and summation of Bryne's grading system variables: t test**

	Number of cases	Mean	Standard deviation	Test value	Degree of freedom	P value
Apoptosis						
Irradiated cases	27	8.26	1.318	6.458	51	<0.001
Non-irradiated cases	26	5.69	1.569			
Bryne's Grading system						
Irradiated cases	27	11.96	1.531	7.218	51	<0.001
Non-irradiated cases	26	9.27	1.151			

group, suggesting poorer differentiation using Bryne's grading system [ $P < 0.001$ , Table 2].

Both salivary gland atrophy and ectasia were found to be increased in irradiated cases, with statistically significant difference being noted only for glandular atrophy [ $P = 0.002$ , Table 1].

**Assessment of collagen fibers stained with picrosirius red**

Irradiated specimens showed dense fibrosis, with predominantly thick fibers ( $>1.5 \mu\text{m}$ ), in contrast to non-irradiated OSCC, which predominantly had thin fibers [ $<1.5 \mu\text{m}$ , Table 1].

When corresponding polarization of fibers was assessed, fibers of irradiated specimens mostly showed orange-red birefringence, indicating mature fibers, whereas majority of fibers in non-irradiated cases gave dark green birefringence, suggesting immaturity. The difference between the two groups was highly significant [ $P < 0.001$ ].

**Discussion**

The clinical sequelae following therapeutic irradiation include skin atrophy, soft tissue fibrosis, epithelial desquamation, ulceration, fistula formation and rupture of major vessels.<sup>[5]</sup>

The morbidity associated with radiation injury to skin, mucosa, subcutaneous tissues, bone and salivary glands in the course of radiotherapy for head and neck cancer affects the quality of life.<sup>[2]</sup> While some of the pathologies of radiation injury manifest immediately after exposure, some clinical and histological features may not be apparent for weeks, months, or even years after radiotherapy.<sup>[2]</sup>

Radiation effects may be acute, consequential, or late, based on the time of appearance of symptoms [Table 3].<sup>[1,2]</sup> However, there was no variation in the radiation dosage in the given cohort of patients, as all patients received a dosage of 6000 cGys, and the tissue specimen was evaluated with a mean time duration of 11 months. These alterations, which occur in a repetitive form in organs exposed to radiation, can also be categorized as those occurring in the epithelium, connective tissue stroma, salivary gland tissues and blood vessels.<sup>[1]</sup> Acute effects are those that are observed during the course of treatment or which appear within few weeks after radiotherapy. Radiation-induced DNA damage results in cell death during the first few cell divisions either as "mitotic death" or apoptosis.<sup>[2]</sup> We observed significantly higher number of apoptotic bodies in irradiated cases in comparison to the control cases as rapidly proliferating epithelial cells are known to show higher apoptosis as an acute effect of radiation.

The late effects develop months or years after exposure to radiation, more commonly in tissues with slow turnover rate. The diverse pathological effects reported include fibrosis, necrosis, atrophy and vascular damage.<sup>[2,6]</sup> Our study mirrored such findings where irradiated cases showed these parameters to

**Table 3: Radiation-induced changes**

Radiation-induced changes	Consequences
Acute	
Increased apoptosis	
• DNA damage because of ionization events and free radicals	• Reduced tumor load
Apoptosis of acinar cells in salivary gland	
• Serous cells are more radiosensitive	• Xerostomia
• Functional impairment	
Late effects	
Fibrosis	
• Increase in molecular markers like interleukin-2 and ICAM-1	• Fibrosis
	• Trismus
Necrosis	
• Subendothelial or adventitial fibrosis	• Abscess
• Hyalinization of tunica media	• Mucositis
• Accumulation of lipid laden macrophages in intima	• Ulceration
• Leukocyte adhesion and thrombi formation	• Vesicle formation
Atrophy	
• Death of epithelial cells	• Ulceration
• Focal and random	
Fibrin/Fibrinous exudate	
• Damage to vasculature	• Ulceration
• Cytokines	• Vesicle formation
• Decreased fibrinolysis/repeated formation of fibrin	
Vessel wall thickening	
• Repair of vascular damage	• Mucositis
	• Erythema

be significantly higher. Atypia and neoplasia are usually delayed alterations, affecting either the nucleus, or the cytoplasm or both.<sup>[1]</sup> The present study revealed higher severity of dysplasia (evidenced by hyperchromatic nuclei, altered nuclear cytoplasmic ratio, multinucleation, abnormal and atypical mitotic figures and cellular and nuclear pleomorphism) in cases who received radiotherapy, indicating genetic damage and abnormal ploidy as a result of ionization after radiotherapy.<sup>[1]</sup> Radiotherapy produces a wide spectrum of DNA changes which includes nuclear base damage, single and double strand breaks of various complexity and DNA cross links. These altered cells undergo repair, which if effective results in functionally normal cells. In case of misrepair or unrepaired cells, the progeny of a single irradiated cell will be expected to show radiation-induced genetic changes in descendant cells.<sup>[7]</sup> It has been observed that radiation doses of  $<0.2 \text{ Gy}$  fail to activate the G2/M cell cycle check point, leading to tumorigenesis.<sup>[8]</sup>

Fibrosis, while being one of the commonest delayed manifestations, varies in extent and severity from site to site and is dose- and time-dependent. Radiotherapy-induced fibrosis is non-homogeneous, with areas of very dense acellular collagen situated adjacent to areas with only few fibrous bands.<sup>[1]</sup>As is expected, as a sequelae of radiation, we observed

preponderance of thick and mature fibers (orange-colored) in cases of irradiated patients. An attempt was made to ascertain the histological composition of fibers in radiation-induced fibrosis using Picrosirius Red staining followed by polarized light analysis. Rich and Whittaker (2005) emphasized that fiber hue is not conclusive to permit identification of the type of collagen. Although type III collagen fibers are usually thinner than type I fibers, it would be presumptuous to state that green (thin) fibers and orange-red (thick) fibers are indicative of type III and type I collagen, respectively. The same authors also speculated the possibility of a green fiber being an immature, thin type I fiber or a thick type I fiber being “smeared” by a sectioning artifact, thereby decreasing its thickness.<sup>[9]</sup>

It has been suggested that fibroblastic secretion of interleukin-2 and subsequent upregulation of intercellular adhesion molecules (ICAM-1) and CD44 play a major role in fibrosis. In the course of tumor progression, neoplastic cells develop an immunosuppressive phenotype mainly due to secretion of cytokines such as IL-10, TGF-beta and TNF-alpha in low concentrations. Transforming growth factor beta (TGF-B) is acknowledged as an inducer of collagen synthesis contributing to fibrosis during radiation therapy. Furthermore, increased synthesis and secretion of macrophage-derived growth factor may play a role in fibrosis. Irradiation also induces terminal differentiation of fibroblasts resulting in fibrosis.<sup>[5,10]</sup>

Irradiation causes shrinkage of the tumor component by atrophy and increased apoptosis. These tumor cells invade the adjacent stroma by producing enzymes such as collagenase and matrix metalloproteinases that preferentially degrade thinner and immature fibers. As irradiated tissue has predominantly mature fibers, the tumor infiltrates in the form of nests and strands through areas having thinner and immature fibers. Irradiated tumor cells show greater pleomorphism and shrink in size because of increased apoptosis and increased fibrosis. This combination of smaller tumor cell groups infiltrating in the form of small nests and strands result in the award of a “poorer” grade of classification by the pathologist.

Consequential late effects occur when acute reactions fail to heal completely and persist in the late period, lending damaging chronicity to the tissue. Such effects are increasingly being observed in the skin and mucosa too as an adaptation to newer and aggressive treatment modalities.<sup>[2]</sup>

In addition to radiotherapy, the very presence of tumor alters the surrounding tissue, rendering physical distortion of tumor tissue that compounds the effects of radiation. Along with the release of proteolytic enzymes responsible for invasion and metastasis, there is leakage of fibrinogen, which gets converted into fibrin that contributes to inducing fibrosis, possibly by alteration of proinflammatory and profibrotic cytokines as seen in lung cancers.<sup>[2]</sup>

Advances in imaging and radio-technology have led to three-dimensional conformal radiotherapy and further, to intensity-modulated radiotherapy (IMRT). IMRT, the current standard in treatment of cancers of head and prostate, permits sparing of normal tissues with dose-escalation to tumors. Sculpted dose distributions, tumor motion and anatomical changes during radiotherapy make image-guided radiotherapy an integral part of modern radiation delivery, resulting in decreased radiation-associated toxicity.<sup>[11]</sup>

There is no established efficient treatment for radiation-induced fibrosis that either stabilizes or gradually worsens, with acute inflammatory periods. Several drugs including corticosteroids and nonsteroidal anti-inflammatory agents, hemorheologic and vasodilator drugs, zinc and interferon have proved to be effective when administered prophylactically or during early stages of fibrosis. A combination of pentoxifylline and tocopherol has proven to be effective in reversing radiation-induced fibronecrosis.<sup>[12]</sup> While supplementation with high doses of alpha tocopherol and beta-carotene may reduce the severity of adverse effects induced by radiation therapy, high doses of such antioxidants as adjuvant therapy might also reduce the efficacy of radiation treatment.<sup>[13]</sup>

## Conclusion

Radiotherapy, though a potent tool in cancer management, can unleash stromal and epithelial pandemonium if used without caution. The complex anatomy and geometry of the head and neck confers a greater risk of severe radiation-associated toxicity/damage by inducing necrosis, fibrosis, xerostomia, trismus and recurrent ulceration, all of which affect the quality of life in a cancer survivor. Hence, the optimal use of site-specific therapy may increase the therapeutic ratio by encompassing all cancer cells with sufficient doses of radiation while simultaneously sparing surrounding normal tissues.

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