Original Article

Karyoanomalic frequency assay during radiation therapy – A promising marker in the prognosis of oral and oropharyngeal carcinoma

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Abstract

Introduction: Radiotherapy is commonly used in the treatment of oral and oropharyngeal carcinomas, either alone or in combination with other modalities of treatment like surgery/chemotherapy. It is always essential to know the nature of tumor response to the irradiation for successful outcomes and prognosis. With this view, the study has been conducted to document the usefulness of nuclear changes, karyolysis (KL), and karyorrhexis (KR) in particular as prognostic markers during the treatment. **Materials and Method:** Sixty patients, aged between 28 and 73 years (56 males and 4 females) years, histopathologically confirmed cases of oral and oropharyngeal carcinoma of different degrees of differentiation, were included in the study. The mode of treatment for the patients was radiotherapy with a radiation dose plan of 4 Gy, 14 Gy, 24 Gy, and 60 Gy on the 2nd, 7th, 12th, 30th days, respectively. The mucosal scrapings obtained from the site of the lesion at each interval were stained with Giemsa and May-Grunwald's stain. The stained slides were studied to assess the frequency of KL and KR. **Results:** It was observed that there was no significant difference between the site of lesion and tumor differentiation with the frequency of KL or KR. However, there was a statistically significant difference in the KL and KR indices with each interval of treatment. The percentage of relative increment among both the studied parameters was also significant, indicating their efficiency as a promising prognostic marker in radiotherapy. **Conclusion:** Hence, assessment of KL and KR at different intervals of time during radiotherapy could be used as an efficient tool to determine the radiosensitivity and prognosis in oral and oropharyngeal carcinoma patients.

Keywords: Genotoxicity, karyolysis, karyorrhexis, radiotherapy, tumor response

Introduction

Oropharyngeal carcinomas are more common in developing countries than in developed countries. Its prevalence is particularly high among men and is the 8th most frequent carcinoma worldwide. In countries of South Central Asia, oral cavity cancers rank among the three most common types of cancer.[1,2] Oral cancers are an important public health concern in India.

India is considered the world capital for oral cancer cases as it shares one-third of the global burden.[3] Worldwide, it has been estimated that 43% of cancer deaths are because of tobacco,

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unhealthy diet, physical inactivity, and infections.[2] Tobacco use and excessive alcohol consumption have been estimated to account for about 90% of cancers in the oral cavity; the risk increases when tobacco is used in combination with areca nut or alcohol.[4]

They are usually squamous cell carcinoma (90%), which presents as unexplained growth or ulcer in the oral mucosa, often extending into the oropharynx.[5] The mainstay of the current modality of treatment for oral and oropharyngeal carcinoma is surgery or radiotherapy. The intrinsic property of malignant cells to respond to radiation plays a key role in the prognosis and better outcome of the treatment.[6-8]

Evaluation of cellular changes like micronucleus, nuclear budding, multinucleation, karyorrhexis (KR), and karyolysis (KL) with respect to radiation dates back to 1957.[9] Nuclear changes like micronuclei assay have been widely studied.[10-13] The pattern of nucleoproteins of irradiated malignant cells, both exfoliated and persistent cells in smears after completion of therapy, were also proposed to correlate with clinical outcome.[14]

KL refers to the complete dissolution of chromatin of a dying cell, which is usually followed by KR.[15] However, limited studies are emphasizing the usefulness of KL and KR as prognostic markers in the treatment of oral and oropharyngeal carcinomas. So, this study aimed to determine the dose-dependent effect of radiation on nuclear fragmentation.

Material and Method

Sixty patients aged between 28 and 73 years, referred from the Departments of Surgery and E.N.T. for radiotherapy in All India Institute of Medical Sciences (AIIMS), Rishikesh were included in the study. All were histopathologically confirmed cases of oral and oropharyngeal carcinoma, being treated by radiotherapy alone with a radiation dose plan of 4, 14, 24, and 60 Gy, respectively, on the 2nd, 7th, 12th, and 30th day. Patients who were treated by chemotherapy or a combination of treatment modalities were excluded from the study.

The study protocol was carried out after obtaining the approval from institute human ethical committee (28/IEC/Ph.D./2018 dated 8/3/2018) and consent from each participant of the study. Standard proforma was used to collect basic information of the patients including the diagnosis.

To study the nuclear changes, i.e., KL and KR, mucosal scrapings were carefully collected from the site of the lesion using aseptic precautions at 0, 2, 7, 12, and 30 days. Air-dried smears were fixed with methanol and stained with Giemsa and May-Grunwald's stain. After staining, slides were mounted in DPX and observed under the microscope. A total of 500 cells were assessed from each prepared smear at 4, 14, 24, and 60 Gy.[16] Cell clumps, poorly preserved cells, were excluded. Observed changes in the cells were tabulated and statistical analysis was done using ANOVA test. Results were considered significant if the p value is less than 0.05. Relative increment of KL and KR were calculated using the below formula for each interval.[12]

Relative increment % was calculated for the KR

$$KR\ \% = \frac{\text{No. of cells with KR after radiation}}{\text{No. of cells with KR before treatment}} X\ 100$$

Observations and Results

In the study, there were 56 male and 4 female patients, and distribution with their age group is as shown in [Table 1]. In total, 72% cases were of oropharyngeal carcinoma and 28% cases were of oral carcinoma. The distribution of cases with stages of differentiation is as shown in Figure 1.

Response to radiotherapy was seen in the form of KL and KR. KR denotes nuclear break up into smaller fragments [Figure 2], while KL denotes a progressive dissolution of chromatin [Figure 3]. With each dose of radiation, there was a significant response of the tumor to the treatment in terms of the above-said parameters, as shown in Tables 2 and 3.

There was a significant difference in the relative increment of KL and KR with each interval of treatment [Tables 4 and 5].

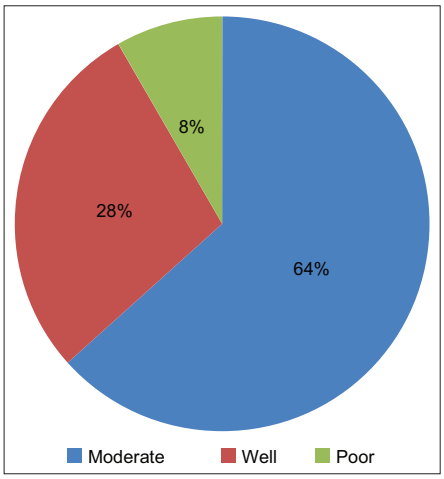

Figure 1: Pie chart representing the percentage of cases with different stages of the disease

Table 1: Distribution of patients wrt age and sex

Age group	*n*		
	Male	Female	Total
≤40 yrs	11	0	11
41-50 yrs	10	1	11
51-60 yrs	22	2	24
>60 yrs	13	1	14
Total	56	4	60

However, there was no significant difference in terms of KR and KL in response to each dose of radiation, with respect to oral and oropharyngeal carcinoma [Tables 6 and 7].

Discussion

In the recent past, studies on cancers have evidenced that even carcinomas labeled under the same category by pathologists were significantly heterogeneous in their biological behavior.[17] The time required for DNA fragmentation in a cell undergoing apoptosis varies depending on the organism, cell type, and the type of inducing agents.[18] Hence, rapid and reliable methods to evaluate the tumor response to the dose-dependent radiation are of utmost importance for the prognosis of the disease.[19]

Radiation used in the treatment of such cancers causes series of atomic reactions and results in alteration of various nuclear parameters because of free radicals generated by irradiation.[15] In the present study changes resulting from radiation, the damage was evaluated in malignant cells at the 2nd, 7th, 12th, and 30th day of radiotherapy at fraction 4, 14, 24, and 60 Gy, respectively.

Radiation-induced damage to the nuclear membrane results in nuclear breakup into smaller Fragments – KR followed by the progressive dissolution of chromatin – KL. KR takes place in a dynamic nuclear envelop and the transfer of DNA material to the cytoplasm happens by the rupture.

There was a progressive increase in the mean value of KR from 35 to the maximum of 245 at 24 Gy, suggesting the genotoxic effect of radiation on the cancer cells. Similarly, the mean value of KL raised from 58 to the maximum of 195 at 24 Gy fraction. At 24 Gy, the increase in KR was about 6–7 times and KL was about 3–4 times compared with pretreatment values. Similar results have been reported by Bindu *et al.*[15] However, in their study, they did not include the 30th day of treatment, which in the present study is included to evaluate the degree of maintenance of the effect of radiotherapy. This suggests that

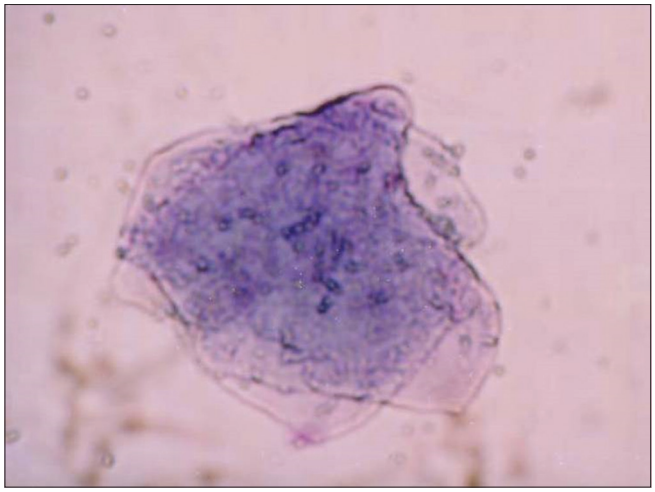

Figure 2: Malignant cell showing karyorrhexis

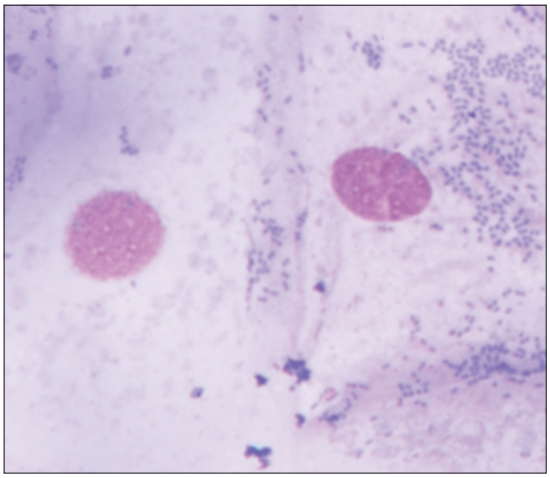

Figure 3: Malignant cell showing karyolysis

Table 2: Altered nuclear parameter (KR) in relation to radiation dose

Day	Mean	Std Dev	SE of mean	Median	95% CI for Mean		*F*	*P*
					Lower bound	Upper bound		
Day 0	35.08	6.12	0.79	35.5	33.50	36.66	3568.404	<0.001*
Day 2	104.28	9.26	1.20	105.5	101.89	106.68		
Day 7	223.28	17.31	2.23	222.0	218.81	227.75		
Day 12	245.17	10.90	1.41	244.0	242.35	247.98		
Day 30	202.50	10.92	1.41	202.0	199.68	205.32		

Table 3: Altered nuclear parameter (KL) in relation to radiation dose

Day	Mean	Std Dev	SE of mean	Median	95% CI for Mean		*F*	*P*
					Lower bound	Upper bound		
Day 0	58.45	4.56	0.59	58.5	57.27	59.63	2222.938	<0.001*
Day 2	82.10	4.75	0.61	81.0	80.87	83.33		
Day 7	152.00	8.93	1.15	151.0	149.69	154.31		
Day 12	195.62	13.30	1.72	197.0	192.18	199.05		
Day 30	105.73	10.06	1.30	106.0	103.13	108.33		

Table 4: Relative increment of karyolysis with each dose of radiation

Day	Mean	Std Dev	SE of mean	Mean difference	% Increment	*t*	*P*
Day 0	58.45	4.56	0.59	-23.650	-40%	-28.797	<0.001*
Day 2	82.10	4.75	0.61				
Day 2	82.10	4.75	0.61	-69.900	-85%	-55.658	<0.001*
Day 7	152.00	8.93	1.15				
Day 7	152.00	8.93	1.15	-43.617	-29%	-21.067	<0.001*
Day 12	195.62	13.30	1.72				
Day 12	195.62	13.30	1.72	89.883	46%	38.364	<0.001*
Day 30	105.73	10.06	1.30				

Table 5: Relative increment of karyorrhexis with each dose of radiation

Day	Mean	Std Dev	SE of mean	Mean difference	% Increment	*t*	*P*
Day 0	35.08	6.12	0.79	-69.200	-197%	-51.982	<0.001*
Day 2	104.28	9.26	1.20				
Day 2	104.28	9.26	1.20	-119.000	-114%	-46.670	<0.001*
Day 7	223.28	17.31	2.23				
Day 7	223.28	17.31	2.23	-21.883	-10%	-8.042	<0.001*
Day 12	245.17	10.90	1.41				
Day 12	245.17	10.90	1.41	42.667	17%	20.930	<0.001*
Day 30	202.50	10.92	1.41				

Table 6: Comparison of karyorrhexis between the sites of the lesion at different time intervals

Time interval	Site	*n*	Mean	Std Dev	SE of mean	Mean difference	*t*	*P*
Day 0	Oral	17	36.41	7.39	1.79	1.854	1.058	0.294
	Oropharynx	43	34.56	5.55	0.85			
Day 2	Oral	17	107.12	9.79	2.37	3.955	1.506	0.137
	Oropharynx	43	103.16	8.91	1.36			
Day 7	Oral	17	226.53	18.80	4.56	4.529	0.912	0.365
	Oropharynx	43	222.00	16.74	2.55			
Day 12	Oral	17	246.59	11.01	2.67	1.984	0.632	0.530
	Oropharynx	43	244.60	10.93	1.67			
Day 30	Oral	17	205.53	12.02	2.91	4.227	1.361	0.179
	Oropharynx	43	201.30	10.36	1.58			

Table 7: Comparison of karyolysis between the sites at different time intervals

Time interval	Site	*n*	Mean	Std Dev	SE of mean	Mean difference	*t*	*P*
Day 0	Oral	17	59.47	3.62	0.88	1.424	1.093	0.279
	Oropharynx	43	58.05	4.85	0.74			
Day 2	Oral	17	81.41	3.95	0.96	-0.960	-0.703	0.485
	Oropharynx	43	82.37	5.04	0.77			
Day 7	Oral	17	152.59	10.16	2.46	0.821	0.318	0.751
	Oropharynx	43	151.77	8.51	1.30			
Day 12	Oral	17	195.88	12.43	3.02	0.371	0.096	0.923
	Oropharynx	43	195.51	13.77	2.10			
Day 30	Oral	17	105.35	10.47	2.54	-0.531	-0.183	0.856
	Oropharynx	43	105.88	10.02	1.53			

with increasing doses of radiation, the percentage of cells with distinct nuclear damage increases. Thereafter, the mean value of the above-said parameters decreased suggesting that the cells affected by radiation lost their proliferative ability and abnormal DNA profile was merely seen after 4 weeks, which is attributed to the possibility of initiation of DNA repair or cessation of lethal damage or genetic inactivity.[20]

Similar to micronuclear assay, estimations of KL and KR are noninvasive, which do not require the complicated process of cell culture and metaphase preparation. They can be done in interphase cells and the process is cost effective. In the literature, there are a plethora of studies evaluating the regularly studied parameters like micronuclear assay, multinucleation, nuclear budding. But not many established studies evaluating KR and KL as malignant cell responses to irradiation.[10-12] Hence, the assessed parameters can be used for monitoring and evaluating the clastogenic effects of chemicals, radiation, and many other genotoxins.[21]

Conclusion

Thus, the present results suggest that among the various quantifiable changes occurring in irradiated oral cancer cells, parameters such as KL and KR may have potential use as predictive tests for radiosensitivity and also prognostic markers in oral and oropharyngeal carcinoma. Compared to KL, KR is the more sensitive marker and shows a significant difference with each incremental dose of radiation. Not only as prognostic markers these parameters also suggest the response of the cancer cells to radiation suggesting their radiosensitive or radioresistant behavior.

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Conflicts of interest

There are no conflicts of interest.

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