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Sequential evaluation of DNA damage in patients with head and neck carcinoma receiving radiotherapy

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ABSTRACT

Background: Head and neck cancers account for about 30% of all cancers in India. Studies showed that there is an increased primary DNA damage even before the commencement of any modality of treatment in cancer patients which is further increased by the treatment. Chemo-radiation induced DNA damage is not repaired so effectively in patients with carcinoma which might pave way for secondary carcinoma. **Aims and Objectives:** The aim of this study was to assess the degree of DNA damage by comet assay technique in patients with head and neck carcinoma receiving radiotherapy. The degree of DNA damage was compared according to the age, gender, and associated risk factors of the patients. **Materials and Methods:** 35 patients with Stages II, III, and IVA, histopathologically confirmed Squamous cell carcinoma of head and neck with Karnofsky Performance Status >70 attending radiotherapy OPD for treatment were included in this study.1 ml of heparinized blood was collected from the study participants during various doses of radiation treatment. All the samples were processed immediately and analyzed for DNA damage by single cell gel electrophoresis assay - Comet assay technique. **Results:** The comet length parameter, head diameter, and tail length were found to be increased when compared to baseline sample. The percentage of DNA in head parameter of post-RT sample was decreased when compared to baseline sample All these findings are indicative of DNA damage following radiotherapy. **Conclusion:** Patients with locally advanced head and neck carcinoma following radiotherapy showed a sequential increase in the DNA damage. The co-existing risk factors and old age may increase the baseline DNA damage in the patients with head and neck cancers.

Key words: Comet assay; DNA damage; Head and neck carcinoma; Radiotherapy

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INTRODUCTION

Indian Subcontinent registers around 200,000 cases of head and neck carcinoma each year and nearly about 80,000 of them are diagnosed as oral carcinoma.[1] The pro-carcinogenic factors include alcohol in any form and consumption of tobacco in any form.[2] Patients are classified into anatomic groups that are categorized from I to IV based on the increasing severity of disease. Stage I denotes an early disease and Stage IV denotes a locally advanced disease with metastasis. Studies conducted in different types of cancers showed that there is an increased primary DNA damage even before the treatment in cancer patients. The treatment modality will further induce DNA damage in addition to the already existing DNA damage. Chemotherapy and radiotherapy being the mainstay of treatment for locally advanced cancers, induces DNA damage in the target cells as well as to some of the normal cells in the surroundings.[3,4] In normal healthy people, DNA damage is effectively repaired. However, in cancer patients,

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chemo-radiation induced DNA damage is not repaired so effectively. Consequently, there is a high risk of secondary cancer by unrepaired damaged DNA.[5,6] This study is taken up to assess the degree of DNA damage by comet parameters in patients with head and neck carcinoma who had complete tumor regression and residual tumor after receiving radiotherapy.

Aims and objectives

The aim of the study was to assess the correlation between DNA damage and tumor response in head and neck cancer patients receiving concurrent radiation.

The objectives of the study were to assess the DNA damage by comet parameters and to assess the tumor response by clinical/radiological examination.

MATERIALS AND METHODS

After approval from the Institutional Scientific and Ethics Committee, (Ethical clearance number [No. SEC/2011/4/2 dated 02.02.2014]), this study was performed in the department of anatomy in collaboration with the department of radiotherapy. The study design was a prospective cohort study. This cohort contained 35 subjects was selected from the radiotherapy department for a period of 3 years starting from April 2012 to March 2015.

Inclusion criteria

Patients with Stages II, III, and IVA, histopathologically confirmed squamous cell carcinoma of head and neck (lip, oral mucosa, nasal cavity, pharynx, paranasal sinuses, and larynx) with Karnofsky Performance Status more than 70 attending radiotherapy OPD for treatment were included in this study. Among the risk factors associated with head and neck carcinoma – smoking, alcohol, and tobacco chewing were included in the study.

Exclusion criteria

Patients with comorbid conditions such as severe infections, blood dyscrasias, other genetic disorders, patients with abnormal liver function test, and renal function test were excluded from this study. Patients with the previous H/O chemotherapy, radiotherapy to any part of the body before starting treatment were also exempted from this study.

After obtaining informed written consent by explaining to the study participants in the regional language, 1 ml of heparinized blood was collected from them under aseptic precautions. The blood samples were collected as follows:

a) Two hours before the first dose of radiation
b) Two hours after 10th fraction of radiation
c) Two hours after 20th fraction of radiation
d) Two hours after 30th fraction of radiation
e) During 1st follow-up (1 month after completion of treatment)

All the samples were processed immediately and analyzed for DNA damage by single cell gel electrophoresis assay (Comet assay). To assess the DNA damage, following comet parameters were used - Tail length, Head diameter, Comet length, Percentage of DNA in head, and Percentage of DNA in tail. The principle of alkaline comet assay is based on the movement of negatively charged damaged DNA fragments such as single strand breaks, double strand breaks, and alkali labile sites toward the anode during electrophoresis thereby forming a comet –like tail. Hence, the tail parameter and percentage of DNA in the tail in the comet play a significant role in assessing the DNA damage. The comet pictures were captured by Bright field microscope Olympus BX 43. The length of the comet tail was measured using ocular scale fitted to the microscope. To avoid bias these parameters were employed on randomly selected 40–50 cells per subject.

The tumor size was measured before starting radiotherapy and during follow-up to assess the tumor response to the radiation treatment. The size of the tumors in the region of lip and oral mucosa was measured by clinical examination using inch-tape and those tumors in the nasal cavity, pharynx, paranasal sinuses, and larynx were measured by radiological examination using MRI scan. Based on this tumor response, categorization of two sub-groups was done - complete tumor regression and residual tumor and analysis of the study was based on these subgroups.

Categorical data obtained from the study were presented as frequencies and percentages. Chi-square test was used to compare the categorical data. Normally, distributed continuous data are presented as mean with the standard deviation. Comparison of continuous data has been done using independent student's t-test. All statistical analysis carried out with $P<0.05$ was considered statistically significant.

RESULTS

Among the 35 study participants, 16 patients (46%) belonged to age group of 50–59 years, ten patients (29%) were more than 60 years of age, and nine patients (25%) were in the age group of 40–49 years. Considering the gender distribution, 21 were male and 14 were female participants. The details of the distribution of the cases in relation to the number of risk factors are shown in Table 1.

Age and baseline DNA damage

Comet parameters of total comet length, head diameter, percentage of DNA in head, tail length, and percentage of DNA in tail were recorded in all the age groups. The length of the comet and diameter of head and tail length were

measures in micrometer units (μM). The comet parameters of the baseline sample (before radiotherapy) among the various age groups are shown in Table 2. The percentage of DNA in tail was higher in age group > 60 years when compared to other age groups.

Risk factors and basal DNA damage

The percentage of DNA in tail parameter of patients with more than one risk factor was higher when compared with the other group.

Tumor response and basal DNA damage

The comet parameters of the baseline sample in relation to tumor response are shown in Table 3.

Table 1: Distribution of study participants in relation to risk factors among subgroups

Sub-group	Number of risk factors				Total number of cases
	1	2	3	No risk factors	
Complete tumor regression	3	9	1	1	14
Residual tumor	10	5	4	2	21
Total	13	14	5	3	35

Table 2: Comparison of comet parameters of baseline sample among the age groups

Comet parameters	Age group			P value
	40–49 years	50–59 years	>60 years	
Comet Length	76.6±31.3*	55.3±29.2*	54.5±25.5*	0.125
Head diameter	59.4±25.0*	46.5±23.6*	40.2±16.2*	0.018
% of DNA in head	87.1±5.9*	87.5±5.4*	83.2±8.2*	0.242
Tail length	17.8±16.4*	9.7±10.1*	15.0±13.6*	0.082
% of DNA in tail	12.9±5.9*	12.6±5.4*	16.8±8.2*	0.252

*Mean±SD

Table 3: Comparison of baseline comet parameters between the subgroups

Comet parameters	Subgroups		P value
	Complete tumor regression	Residual tumor	
Comet length	45.3 (38.6–77.8)#	56.4 (35.2–84.9)#	0.840
Head diameter	40.2 (37.6–55.6)#	37.5 (27.9–66.6)#	0.329
% of DNA in head	86.8±5.5*	85.7±7.1*	0.613
Tail length	6.5 (3.0–14.1)#	7.9 (3.7–23.0)#	0400
% of DNA in tail	13.3±5.5*	14.2±7.2*	0.652

*Mean±SD, #Median (Interquartile range)

The comet length, tail length, and percentage of DNA in tail parameters of the baseline sample were increased in residual tumor patients when compared to patients with complete tumor regression.

DNA damage after radiotherapy in patients with complete regression of tumor

The comet length parameter of post-RT sample 1 (114.5 [107.4–154.3] μM) was increased when compared to baseline sample (45.3 [38.6–77.8] μM) with P<0.05. There was a progressive increase in the comet length parameter of post-RT samples 1, 2, and 3. The comet length parameter of follow-up sample (67.9 [54.5–82.6] μM) was decreased when compared to post-RT sample 3 (116.9 [106.4–152.3] μM) with P<0.05. The tail length parameter of post-RT sample 1 (46.9 [41.1–76.0] μM) was increased when compared to baseline sample (6.4 [3.0–14.1] μM) with P<0.05. There was no progressive increase in the tail length parameter of post-RT samples 1, 2, and 3. The tail length parameter of follow-up sample (18.0 [10.8–26.0] μM) was decreased when compared to post-RT sample 3 (52.8 [45.5–73.4] μM) with P<0.05.The percentage of DNA in tail parameter of post-RT sample 1 (22.7±11.0) was increased when compared to baseline sample (13.3±5.5) with P<0.05. There was a progressive increase in the percentage of DNA in tail parameter of post-RT samples 1, 2, and 3. The percentage of DNA in tail parameter of follow-up sample (15.8±6.7) was decreased when compared to post-RT sample 3 (27.8±10.5) with P<0.05.

DNA damage after radiotherapy in patients with residual tumor

The comet length parameter of post-RT sample 1 (106.9 [97.5–129.0] Mm) was increased when compared to baseline sample (56.4 [35.2–84.9] μM) with P<0.05. There was no progressive increase in the comet length parameter of post-RT samples 1, 2, and 3. The comet length parameter of follow-up sample (56.6 [49.5–85.2] μM) was decreased when compared to post-RT sample 3 (108.2 [95.8–138.7] μM) with a P<0.05. The tail length parameter of post-RT sample 1 (47.0 [36.9–87.3] μM) was increased when compared to baseline sample (7.9 [3.7–23.0] μM) with P<0.05. There was no progressive increase in the tail length parameter of post-RT samples 1, 2, and 3. The tail length parameter of follow-up sample (16.5 [12.9–26.6] μM) was decreased when compared to post-RT sample 3 (47.5 [40.2–98.0] μM) with P<0.05.

The percentage of DNA in tail parameter of post-RT sample 1 (25.0±13.3) was increased when compared to baseline sample (14.2±7.2) with P<0.05. There was no progressive increase in the percentage of DNA in tail parameter of post-RT samples 1, 2, and 3. The percentage

of DNA in head parameter of follow-up sample (18.4±9.1) was decreased when compared to post-RT sample 3 (25.3±14.2) with a P<0.05.

Repair analysis and risk factors in patients with residual tumor

Comet parameters were compared in relation to smoking, alcoholic status, and tobacco chewing among the patients with residual tumor for repair analysis of DNA and the results are shown in Tables 4-6.

The repairing capacity of DNA was less in smokers when compared to non-smokers as evident by tail length parameter within subgroup-II.

The repairing capacity of DNA is less in alcoholics when compared to non-alcoholics as evident by tail length and percentage of DNA in tail parameters within subgroup-II, though it is statistically not significant due to less number of sample size.

Tail length parameter and % of DNA in tail parameter show that there is decrease in repairing capacity of DNA for tobacco users when compared to non-tobacco users within subgroup-II though it is statistically insignificant.

DISCUSSION

Around 200,000 cases of head and neck carcinoma are reported every year in India. Concurrent chemo-radiation forms the mainstay of treatment for locally advanced cancers.[7] Chemo-radiation induces DNA damage to the target cells as well as some of the normal cells. As the monitoring of repair of target tissue is practically not possible, lymphocytes are used as a surrogate marker. DNA repair capacity is measurable in various cell types as it has a genetic predisposition.[8] In earlier studies, comet assay has been documented as a reliable parameter to assess the DNA damage.[9]

Analysis of baseline characteristics

In the present study, most of the patients were distributed in the age group of 50–59 years. The total number of males was more when compared to females. Risk factors such as smoking, alcohol consumption, and tobacco chewing were documented among the study groups (Table 1). Most of the males were found to be associated with all the three risk factors whereas the females mostly with that of tobacco chewing.

Analysis of baseline DNA damage

The previous studies showed that there is baseline DNA damage in the patients with various carcinomas prior to radiotherapy.[10] The observations in this study were:

a. Increase in baseline DNA damage was noted as the age increases (Table 2).

Table 4: Comparison of comet parameters in relation to smoking in subgroup-II for repair analysis of DNA

Comet parameters	Smoking	Time period of sample collection	
		Baseline	Follow-up
Comet length	Yes	62.7±30.3*	68.9±25.9*
	No	58.7±30.4*	64.1±17.1*
Head diameter	Yes	45.4±21.5*	43.9±9.1*
	No	47.0±22.0*	45.2±15.6*
% of DNA in head	Yes	85.2±6.8*	82.7±9.8*
	No	86.2±7.8*	80.6±8.0*
Tail length	Yes	17.9±14.9*	24.7±20.4*
	No	12.5±12.9*	19.3±7.8*
% of DNA in tail	Yes	14.8±6.8*	17.3±9.8*
	No	13.8±7.8*	19.4±8.8*

*Mean±SD

Table 5: Comparison of comet parameters in relation to alcoholic status in subgroup-II for repair analysis of DNA

Comet parameters	Alcoholic status	Time period of sample collection	
		Baseline	Follow-up
Comet length	Yes	63.4±28.0*	70.8±30.5*
	No	59.5±30.9*	64.3±16.8*
Head diameter	Yes	43.5±20.8*	40.0±7.0*
	No	47.0±22.1*	45.6±14.4*
% of DNA in head	Yes	84.0±7.5*	80.9±12.0*
	No	86.4±7.2*	81.9±8.2*
Tail length	Yes	20.0±12.9*	28.9±25.8*
	No	13.0±14.1*	19.0±7.3*
% of DNA in tail	Yes	16.0±7.5*	19.1±12.0*
	No	13.5±7.2*	18.1±8.2*

*Mean±SD

Table 6: Comparison of comet parameters in relation to tobacco chewing in Subgroup-II for repair analysis of DNA

Comet parameters	Tobacco chewing	Time period of sample collection	
		Baseline	Follow-up
Comet length	Yes	57.9±30.7*	68.1±22.4*
	No	69.1±26.2*	59.8±15.8*
Head diameter	Yes	45.4±22.3*	45.4±13.1*
	No	48.1±19.7*	41.6±11.5*
% of DNA in head	Yes	85.6±7.6*	81.0±9.7*
	No	86.2±6.3*	83.5±7.6*
Tail length	Yes	13.1±13.1*	23.0±16.7*
	No	21.3±15.5*	18.3±7.9*
% of DNA in tail	Yes	14.4±7.6*	19.0±9.7*
	No	13.7±6.4*	16.5±7.6*

*Mean±SD

b. Patients with greater than one risk factor were found to have more baseline DNA damage when compared to the others (Table 7)
c. Baseline DNA damage is more in the patients with residual tumor when compared to the patients with complete response (Table 3).

Analysis of post-radiotherapy DNA damage

The second, third, and fourth samples were collected 2 h after the 10th, 20th, and 30th fraction of radiation respectively. Gamulin et al. observed an increase in the DNA damage in the post-radiotherapy period when compared to that of pre-radiotherapy and this study findings also confirmed the same in both the subgroups.[4] Significant sequential increase in the DNA damage was observed in the percentage of DNA in tail parameter throughout the course of the radiation in the subgroup-I (Table 8). Patients were followed up for 1 month after the completion of the treatment and the tumor response was analyzed. The follow-up samples of both subgroups revealed a decrease in the comet length and tail length and the percentage of DNA in tail parameters when compared to the post-radiotherapy values though the values had not returned to the baseline values (Tables 8 and 9) in both the subgroups and this was is in concordant with the findings of Terris et al.[9]

Table 7: Comparison of comet parameters of baseline sample in relation to risk factors

Comet parameters	Number of risk factors		P value
	Risk factors≤1	Risk factors>1	
Comet length	66.9±31.7*	55.2±27.1*	0.172
Head diameter	53.3±24.5*	43.6±20.7*	0.279
% of DNA in head	87.5±7.7*	85.1±5.3*	0.292
Tail length	14.4±14*	12.3±12.5*	0.317
% of DNA in tail	12.5±7.7*	15.0±5.3*	0.273

*Mean±SD

Repair analysis

In a study by Gamulin et al., who assessed radiotherapy induced DNA damage using comet assay in oropharyngeal cancer patients, the sample taken after the completion of the therapy showed decreased DNA damage when compared to the baseline level.[4] Our study did not show the values returning to the baseline level which was found to be in contrast to that of Gamulin et al. While Gamulin et al. recorded the post radiation values after 6 months of radiotherapy, our study recorded it 1 month following the completion of radiation. This could explain the possible reason for the follow-up sample values that were not returning to the baseline values, or it could be the patient's general condition mainly determining the repair capacity and thereby hindering the return of the values.

Risk factors and comet parameters

Smoking

Few authors studied the DNA damage in the smokers and the non-smokers and they observed higher DNA damage in the smokers than the non-smokers.[11,12] In our present

Table 8: Comparison of post-radiotherapy comet parameters in subgroup-I

Comet parameters	Time period of sample collection				
	Baseline	Post-RT1	Post-RT 2	Post-RT 3	Follow-up
Comet length	45.3# (38.6–77.8)	114.5# (107.4–154.3)	116.5# (107.5–146)	116.3# (106.4–152.3)	67.9# (54.5–82.6)
Head diameter	40.2# (37.6–55.6)	66.0# (54.7–81.9)	65.3# (54.0–73.5)	62.2# (51.6–85.7)	56.6# (39.3–61.5)
% of DNA in head	86.8±5.5*	79.6±7.8*	75.0±10.4*	73.2±10.3*	84.2±6.7*
Tail length	6.4# (3.0–14.1)	46.9# (41.1–76.0)	57.13# (44.6–67.3)	52.8# (45.5–73.4)	18.0# (10.8–26.0)
% of DNA in tail	13.3±5.5*	22.7±11.0*	25.7±10.2*	27.8±10.5*	15.8±6.7*

*Mean±SD, #Median (Interquartile range)

Table 9: Comparison of post-radiotherapy comet parameters in subgroup-II

Comet parameters	Time period of sample collection				
	Baseline	Post-RT1	Post-RT 2	Post-RT 3	Follow-up
Comet Length	56.4# (35.2–84.9)	106.9# (97.5–129.0)	104.0# (92.4–153.5)	108.2# (95.8–138.7)	56.6# (49.5–85.2)
Head diameter	37.5# (27.9–66.6)	57.5# (44.4–67.5)	60.8# (43.1–65.0)	57.5# (46.9–64.0)	39.0# (34.9–57.2)
% of DNA in head	85.7±7.1*	75.0±13.3*	73.3±16.0*	74.8±14.2*	81.6±9.1*
Tail length	7.9# (3.7–23.0)	47.0# (36.9–87.3)	45.0# (33.3–97.8)	47.5# (40.2–98.0)	16.5# (12.9–26.6)
% of DNA in tail	14.2±7.2*	25.0±13.3*	26.7±16.0*	25.3±14.2*	18.4±9.1*

*Mean±SD, #Median (Interquartile range)

study, the baseline values were higher in the patients with smoking when compared to the non-smokers but not statistically significant. The repairing capacity of DNA is less in the smokers when compared to the non-smokers within the subgroup-II (Table 4).

Alcohol consumption

In the present study, most of the alcohol consumption groups were males. The repairing capacity of DNA is less in the alcoholics when compared to the non-alcoholics as evident by the tail length and percentage of DNA in tail parameters within the patients of sub-group II (Table 5). This agreed with the findings of Rulten et al., who demonstrated that DNA damage is induced by the consumption of alcohol.[13]

Tobacco chewing

Tobacco chewing is documented as one of the major risk factors in the Indian population due to the cultural and ethnic variation. The comet parameters had increased baseline values in the tobacco chewers (Table 6) compared to the tobacco non-chewers which correlated with the findings of Pfeifer et al.[14] There is a decrease in the repairing capacity of DNA for the tobacco users when compared to the tobacco non-users within the patients with residual tumor.

Limitations of the study

The study was conducted at one centre. Hence, the results may not be generalized.

CONCLUSION

Patients with locally advanced head and neck cancers with complete tumor response following radiotherapy show a sequential increase in the DNA damage. The co-existing risk factors and old age may increase the baseline DNA damage in the patients with head and neck carcinoma. The repair mechanism is dependent on the patient's general condition, risk factors, and also the biology of the tumor.

Clinical relevance and scope of the study

There was a sequential increase in the DNA damage following radiation treatment to locally advanced head and neck cancers. Meanwhile, in the patients with residual tumor, no sequential increase in DNA damage is observed which may be attributed to the reduced radio-sensitivity of the tumor. The reason for the decreased radio-sensitivity of the tumor in the patients with residual tumor has to be investigated so that the treatment modalities can be modified.

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SKM - Concept and design of the study, prepared the manuscript; **VMS -** Interpretation of results, review of literature, preparation of manuscript; **LJT -** Statistical analysis and interpretation, preparation of manuscript; **SSKG -** Drafting and revision of manuscript, review of the literature.

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