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Salivary Parameters as Predictive Markers for Radiation-induced Treatment Response in Head and Neck Cancers: An Investigational Study

Dipika Jayachander*, Arnadi Ramachandrayya Shivashankara, Mamidipudi Srinivasa Vidyasagar***, Raees Tonse****, Sarita Pais*****, Ashwin DL Lobo** Sanath Kumar Hegde******, Suresh Rao******, Manjeshwar Shrinath Baliga******♦**

*Department of Radiation Oncology, Apollo Hospital, Bengaluru, Karnataka, India
**Department of Biochemistry, Father Miller Medical College, Kankanady, Mangalore, India
***Department of Radiotherapy, Kasturba Medical College, Manipal University, Manipal, Karnataka, India
****Department of Radiation Oncology, Tata Memorial Hospital, Mumbai, Maharashtra, India
*****Department of Radiation Oncology, Narayana Hrudayalaya, Bengaluru, Karnataka, India
******Department of Radiation Oncology, Mangalore Institute of Oncology, Pumpwell, Mangalore, Karnataka, India

Abstract

Background: This study evaluates the predictive significance of salivary amylase, glutathione, lipid peroxides, and lactate dehydrogenase in the treatment of head and neck cancer patients who undergo curative radiotherapy/chemoradiotherapy.

Methods: The volunteers for the study included head and neck cancer patients that required curative radiotherapy/chemoradiotherapy. Patients provided saliva and blood samples before the start of radiation treatment and 24 hours after the first radiation fraction of 2 Gy (before the start of the second fraction). Samples were assessed for the levels of blood and salivary amylase, glutathione, lipid peroxides, and lactate dehydrogenase by standard laboratory methods. Clinical tumor radioresponse was assessed one month after the completion of treatment as complete responders, partial responders, and nonresponders.

Results: The results indicated a significant increase in the levels of amylase, lactate dehydrogenase, and lipid peroxides; and a concomitant decrease in the levels of glutathione $P<0.05$ - $P<0.0001$ in saliva and blood. The correlation between the differences in each biochemical parameter with that of the treatment response showed a significant correlation only for the salivary lactate dehydrogenase (R2=0.25; $P<0.02$).

Conclusion: The results indicate that salivary lactate dehydrogenase can be a useful predictive marker to ascertain radiation-induced tumor regression in head and neck cancers.

♦Corresponding Author:
Manjeshwar Shrinath Baliga, PhD
Senior Radiobiologist
Incharge Head of Research-Mangalore Institute of Oncology, Pumpwell, Mangalore, Karnataka, India
Tel: 91-9945422961
Email: msbaliga@gmail.com

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Introduction

Head and neck cancers are the leading cause of cancer related deaths in many parts of the world.[1] Surgery and/or radiotherapy and/or chemotherapy is used for cancer control and possible cure depending on the patient's general health, stage, and localization of the tumor.[2,3] Of these, radiotherapy is especially useful in the treatment of inoperable tumors and estimates are that approximately 70% of all patients receive radiation therapy at some point during the course of their disease for either curative or palliative purposes.[4]

With respect to cancer of the head and neck, when radiation therapy is employed for curative intent, a total dose of 65-70 Gy in 6-7 weeks can produce local control rates of 80%-90% in the early stages (T1 and T2 lesions).[5-7] However, this is not effective for advanced cancers (T3 or T4) and doses that range from 75 to 80 Gy or more may be needed to obtain meaningful results.[5-7] Additionally, radiation is also combined with small doses of chemotherapy (chemoradiation) to enhance the therapeutic effects. Concurrent platinum-based (cisplatin, carboplatin) chemoradiation is shown to be effective in increasing absolute survival.[8,9] However the use of chemoradiation is shown to compromise quality of life by increasing non-hematological side effects that include mucositis, dysphagia, and xerostomia.[5,6]

From clinical perspective, though radiation treatment protocol is almost same for all head and neck cancers, the inter-individual differences in the tumor intrinsic factors (like as DNA aneuploidy; S-phase fraction and proliferation

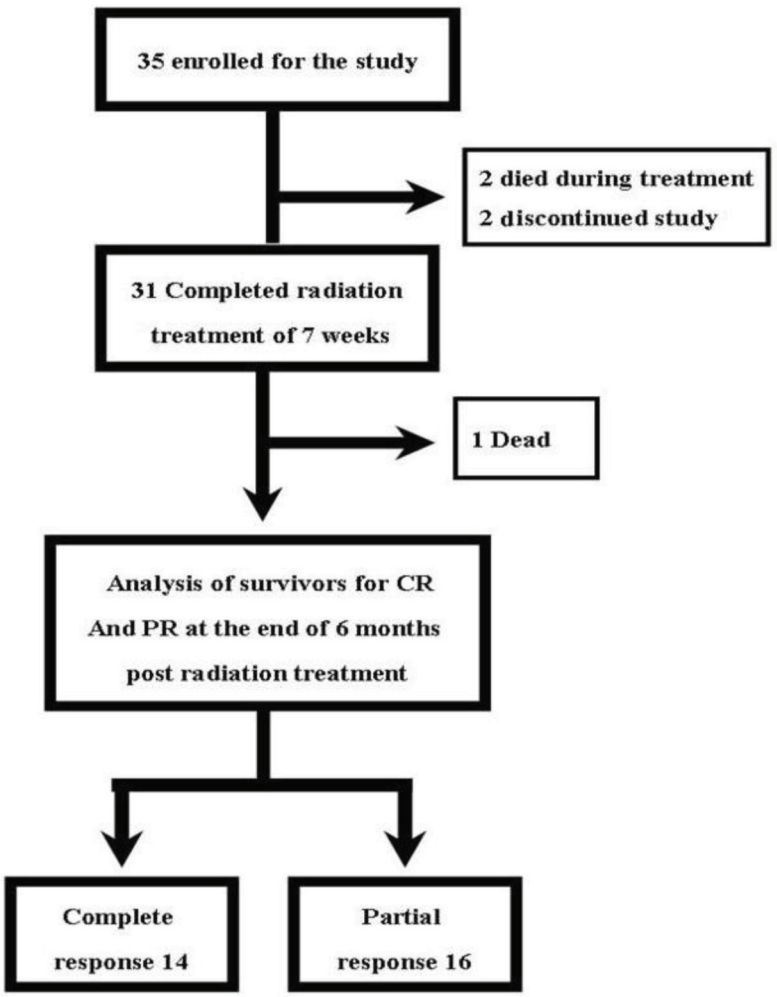

Figure 1. Flow chart of the study emphasizing the death and loss of follow up during the course of treatment and during the follow up.

Table 1. Details on the criteria used for selection of the study patients.

Inclusion criteria	Exclusion criteria
1. Age >18 years 2. Patients with a definitive diagnosis of head and neck cancer. 3. Patients scheduled to receive radiotherapy (> 60 Gy) or chemoradiation either as primary treatment or postoperative treatment of the head and neck. 4. Patients whose general health condition according to Karnofsky's scale was above 80% at the start of the treatment.	1. Patients unwilling to be a part of the study. 2. Women who were pregnant. 3. Patients who had oral surgery within the previous six weeks. 4. Received chemotherapy or radiation treatment previously to the head and neck region. 5. Patients who used high doses of non-steroidal anti-inflammatory drugs. 6. Patients with co-morbid conditions such as poorly controlled diabetes mellitus and hypertension. 7. Patients with existing mental illnesses like schizophrenia or bipolar disorders. 8. Patients with pre-existing ulceration or open wounds in the treatment area.

kinetics; tumor vascularity-related hypoxia; intracellular low-molecular-weight thiol; and glutathione (GSH); as well as alterations in the genes responsible for detoxification, drug resistance, apoptosis, angiogenesis, and cell growth) may result in a diverse response to the cytotoxic effects of ionizing radiation and negate the radiotherapeutic outcome.[9,10] This would necessitate a need for predictive methods to detect tumor radioresponse prior to treatment or

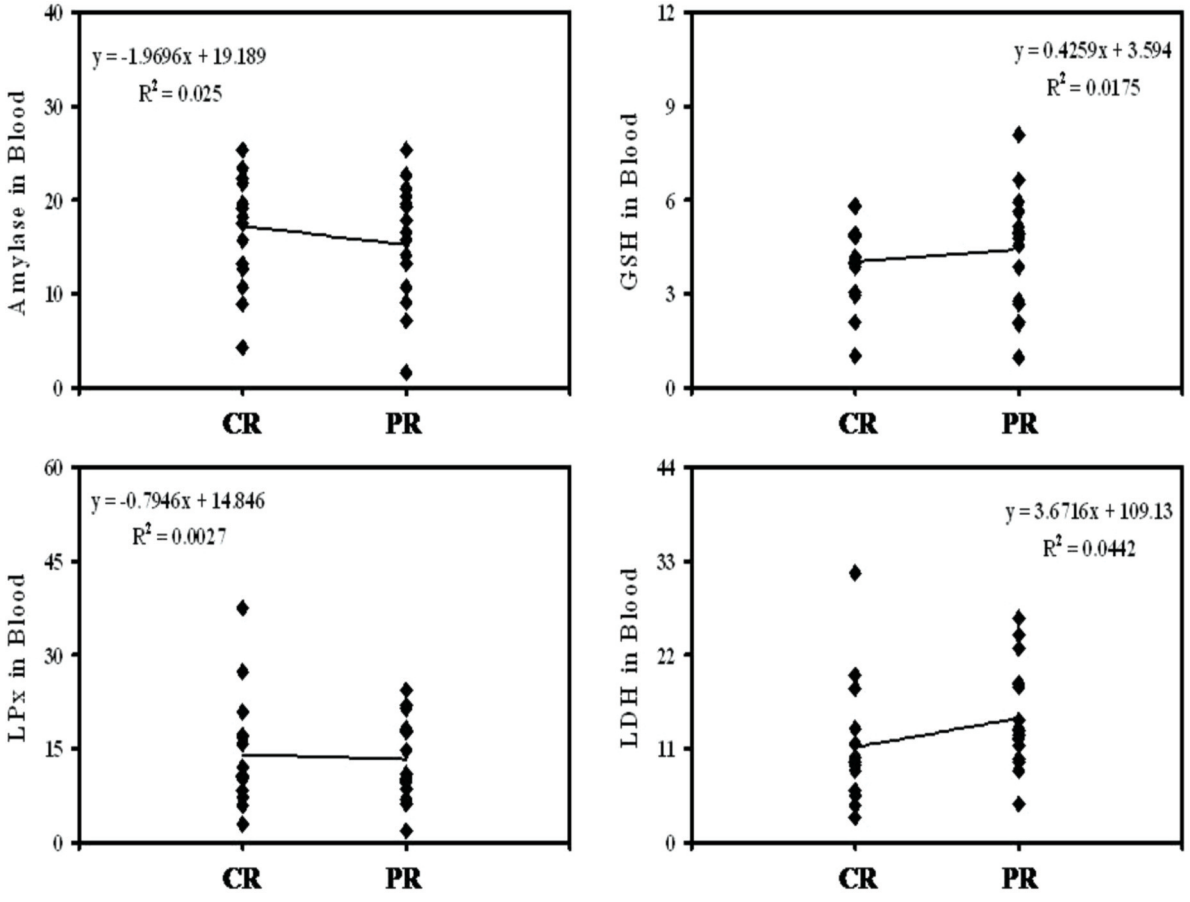

Figure 2. Graphs show correlation between various differences in the pre post biochemical parameters of blood with treatment response. (CR = Complete; PR = partial response)

Table 2. Age, gender and habits of the complete and partial responders as ascertained 4 weeks after the completion of the treatment.

	Complete responders (n = 14)	Partial/No responders (n = 16)
Age (years)	**53.71±4.4**	**58.31±1.9**
Gender		
Male	11	14
Female	3	2
Alcohol consumption		
Male	5	11
Female	0	0
Beedi smokers		
Male	11	12
Female	0	0
Tobacco/betel chewers		
Male	4	9
Female	3	1

immediately after a few fractions. In this regard, the development and use of molecular techniques like microarray that predict clinical outcome on the basis of gene expression appear to be useful.[11] However, these techniques are either unavailable in developing countries and, if available, are extremely expensive. In lieu of these observations, the alternatives that are cost effective and confirm radiation cell kill and tumor regression during the early stage of cancer treatment is important.[9]

With respect to the evaluation of the radiation treatment outcome, the extent of tumor regression is ascertained one month after the completion of the last treatment and, depending on the decrease in tumor size, is categorized as either complete response (CR), partial response (PR), or no

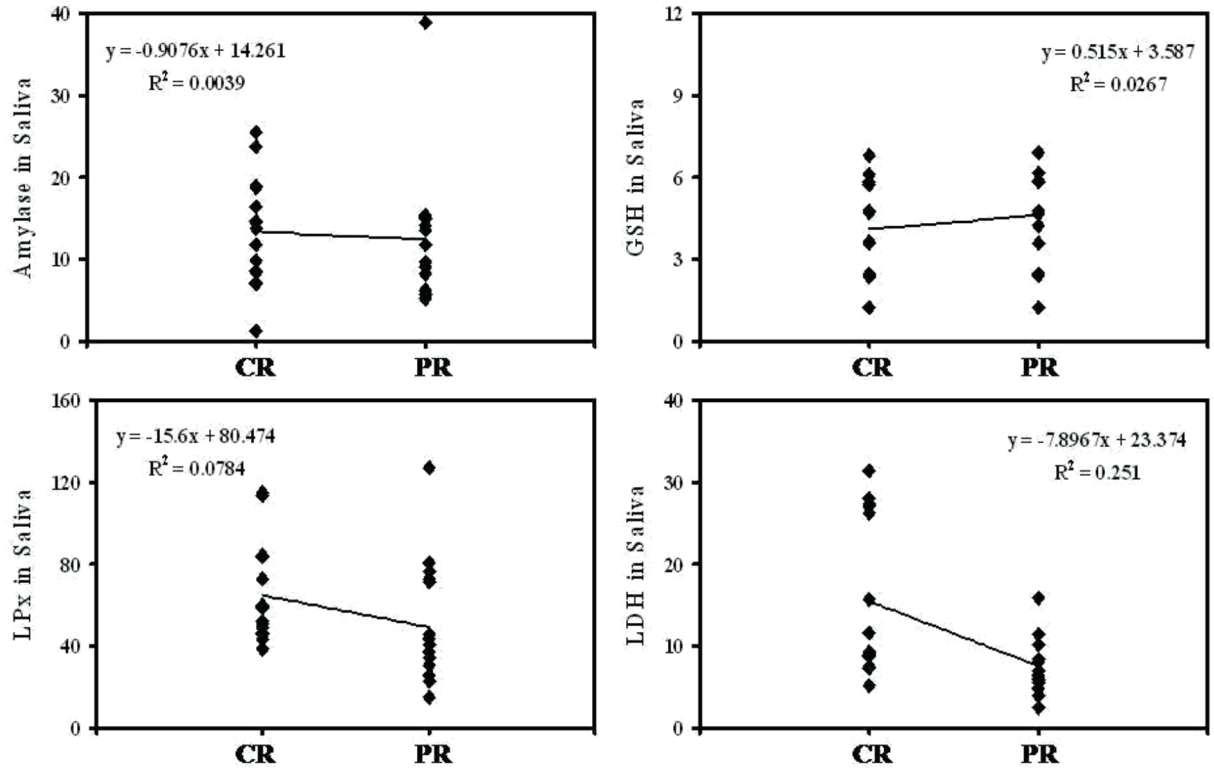

Figure 3. Graphs show correlation between various differences in the pre post biochemical parameters of saliva with treatment response. (CR = Complete; PR = partial response)

Table 3. Tumor site, tumor node, and metastasis (TNM) grading, age, gender, habits, treatment modality, and previous treatment details of the complete and partial responders.

	Complete responders	Partial responders
Site		
Tongue	5	4
Buccal mucosa	3	3
Palate	0	1
Gingivobuccal sulcus	0	2
Tonsil	3	2
Vallecula	1	2
Floor of mouth	1	1
Salivary gland	1	1
Primary		
T1	2	1
T2	6	6
T3	4	3
T4	0	5
Tx	2	1
Regional lymph nodes		
N0	8	5
N1	2	3
N2a	3	3
N2b	3	3
N2c	0	1
N3	1	0
Nx	0	1
Distant metastases		
M0	14	16
Treatment modality		
Prior surgery	7	6
Radiotherapy only	3	4
Chemoradiation	11	12

response (NR).[7] When compared to most cancers, the advantage of most head and neck cancers are its easy access for detailed inspection. Another recently recognized important aspect is that saliva is an important body fluid in the diagnosis and prediction of the severity of various ailments such as oral cancers.[12-17] In lieu of these observations, the present study was undertaken to assess the usefulness of salivary amylase (a vital functional enzyme involved in digestion of carbohydrates), GSH (a major cellular antioxidant and factor for radioresistance), lipid peroxides (an end product of radiation damage on lipids), and lactate dehydrogenase (LDH; an enzyme known to be released on cell death) before and after the first fraction (2 Gy) of radiation/chemoradiation. We compared these findings to plasma levels to assess the use of saliva as a possible predictive marker.

Materials and Methods

The study was conducted from October 2011 to May 2013 in the Departments of Radiation Oncology and Biochemistry at Father Muller Medical College, Mangalore, Karnataka, India. The subjects comprised histopathologically confirmed adult oral cancer patients scheduled to receive curative radiotherapy or chemoradiotherapy. Table 1 lists details of the exclusion and inclusion criteria. The Father Muller Charitable Institutional Ethical Committee approved the study.

During the first visit, eligible patients who satisfied the inclusion criteria received explanations of the nature and purpose of the study in either English or their mother tongue (Kannada, Tulu or Malayalam) by one of the investigators. The subjects were informed that they had the right to withdraw from the study at

Table 4. Alterations in the levels of various biochemical parameters prior to and after exposure to 2 Gy of radiation in saliva samples from complete and partial responders.

Saliva	Complete response (CR)			
	Pre-treatment	**Post-treatment**	**Difference**	**P-value**
Amylase (U/L)*	146.35±16.05	160.16±12.26	13.80±6.64	<0.04
Glutathione (GSH) (µmol/L)	11.96±1.43	8.05±0.14	3.91±1.47	<0.001
Malondialdehyde (MDA) (nmol/L)	59.71±11.56	123.99±23.57	64.27±25.04	<0.0001
Lactate dehydrogenase (LDH) (U/L)*	341.57±21.49	355.59±13.78	14.03±9.64	<0.0001
Saliva	**No response (NR)/Partial response (PR)**			
	Pre-treatment	**Post- treatment**	**Difference**	***P*-value**
Amylase (U/L)*	148.62±12.69	160.74±12.11	12.11±8.07	<0.05
GSH (µmol/L)	12.69±1.86	7.95±0.34	4.74±1.69	<0.001
MDA (nmol/L)	61.56±12.89	112.33±35.69	50.77±30.27	<0.0002
LDH (U/L)*	352.75±19.88	360.45±22.49	7.70±3.26	<0.001

*One unit of enzyme activity (U/L) is defined as the amount of enzyme required to convert one µmol of substrate to product in one min under standard assay conditions.

any time during the course of the study and that their non-willingness to be a part of the study would not deprive them of the necessary planned treatment. The willing patients were then included in the study after they provided written informed consent, which was collected by one of the authors.

Radiation treatment and follow-up

All patient participants received external beam irradiation from a linear accelerator (Varian) at a 6 MV energy level. The patients were treated daily with no more than one fraction of 2 Gy per day, five times per week without any intended gaps for a planned target dose of 70 Gy (seven consecutive weeks). Whenever chemoradiation was planned, a cisplatin infusion (50 mg/m2 intravenous) was administered on a weekly basis four hours before exposure to the first weekly radiation procedure.

Collection of blood and saliva, and their biochemical analyses

Volunteers provided 5 ml of blood and 15 ml of saliva at two time points - before start of treatment (usually during the mold preparation) and after the first radiation fraction of 2 Gy (on day 2 before the second fraction of radiation) . On both occasions, blood samples (5 ml) were collected in vacutainers by a trained phlebotomist with the necessary aseptic precautions. The blood was kept at room temperature for the serum to separate and then centrifuged at 5000 g in a refrigerated centrifuge. The plasma was separated and stored in pre-labeled tubes in a deep freezer (-20°C). Saliva for the study was collected as previously described by Navazesh.[18] Briefly, the volunteers were asked by the investigator to thoroughly rinse their mouths with clean water. This was to ensure elimination of most food debris from the oral cavity. After 10 min, the patients were then requested to expectorate into a sterile plastic container by not exerting any form of force. The collected saliva samples were then centrifuged at 3000 rpm for 10 min. The supernatant was carefully collected in a pre-marked sterile 15 ml plastic tube and immediately transferred to a deep freezer (-20°C) and kept until further analysis. The stored plasma and saliva samples were thawed and assayed for amylase activity,[19] LDH,[20] Thiobarbituric acid reactive substances (TBARS),[21] and GSH22 by standard validated procedures using appropriate blanks, controls, and standards in a UV-visible spectrophotometer (Shimadzu, Japan) by investigators unaware of the patients' clinical conditions.

Clinical evaluation and follow-up of the patients

Patients were examined twice every week during the seven week treatment and specific examinations were performed whenever signs and/or symptoms possibly related to either the side effects or disease were suspected. The response to radiotherapy was assessed during the first follow-up (i.e., four weeks after completion of treatment).

Table 5. Alterations in the levels of various biochemical parameters prior to and after exposure to 2 Gy of radiation in the blood samples from complete and partial responders.

Blood		Complete response (CR)		
	Pre-treatment	**Post- treatment**	**Difference**	***P*-value**
Amylase (U/L)*	119.07±10.31	135.84±10.28	16.77±6.26	<0.001
Glutathione (GSH) (µmol/L)	13.55±1.30	9.65±0.22	3.89±1.26	<0.002
Malondialdehyde (MDA) (nmol/L)	267.5±17.92	279.87±15.81	12.38±6.61	<0.001
Lactate dehydrogenase (LDH) (U/L)*	209.35±27.8	220.67±24.93	11.31±7.52	<0.001
Blood	**No response (NR)/Partial response (PR)**			
	Pre-treatment	**Post- treatment**	**Difference**	**P-value**
Amylase (U/L)*	125.37±21.41	141.11±23.33	15.73±6.74	<0.001
GSH (µmol/L)	14.29±1.96	9.76±0.46	4.52±1.93	<0.001
MDA (nmol/L)	259.06±55.80	273.74±58.30	14.68±8.87	<0.001
LDH (U/L)*	197.37±29.97	211.68±31.73	14.30±6.16	<0.001

*One unit of enzyme activity (U/L) is defined as the amount of enzyme required to convert one µmol of substrate to product in one min under standard assay conditions.

The clinical assessment was done according to World Health Organization guidelines.[7] The degree of tumor volume shrinkage was considered an index of radio responsiveness and confirmed by clinical and radiological methods. Patients with 100% tumor regression at the primary site were considered to be CR, whereas PR patients had a higher than 50% regression, and NR patients had a lower than 50% regression.[7]

Statistical analysis

The values were expressed as mean with standard deviation. Significance of the difference of the values between the groups was evaluated by analysis of variance (ANOVA), Bonferroni multiple comparison. The correlation between the differences in the levels in blood and cell death with the treatment response (CR, PR, and NR) was analyzed by Pearson's correlation analysis. A value of $P<0.05$ was considered statistically significant.

Results

This study selected a total of 35 patients (27 males and 8 females) that required curative radiotherapy of >60 Gy. Of these, two patients discontinued treatment for personal reasons and two succumbed to their medical condition during the course of the seven week treatment. Of the 31 surviving patients who completed the treatment, one died before the first follow up, which left 30 evaluable individuals (25 males and 5 females) to ascertain the treatment response (Figure1). Clinical evaluation by 3 independent investigators confirmed 14 patients with a CR, 15 that had PR, and 1 had NR to the therapeutic effects of radiation (Figure1). For statistical purposes, the biochemical observations for the NR patients were grouped with PR patients (Table 2).

The average age of patients in the CR group was 53.71±4.4 years while in the PR group it was 58.31±1.9 years. The CR group included 11 males and 3 females, while the PR group included 14 males and 2 females. A total of 11 patients in the PR group had a history of alcohol consumption compared to 5 patients in the CR group, while an almost equal number of patients in both the groups were beedi smokers (11 vs. 12) and 10 patients in the PR group had a history of tobacco/betel nut chewing compared to 7 patients in the CR group (Table 2). Table 3 lists the details on tumor site, staging, treatment modality, and previous surgery. The most important observation was that all five patients with T4 tumor classification responded poorly to treatment (Table 3).

Tables 4 and 5 depict the results of the biochemical analysis in saliva and blood before and after exposure to radiation. Pearson's correlation analysis was carried out to evaluate correlation between changes in blood and saliva by tabulating the difference between the first and the second values of the respective assays. This analysis was conducted with respect to tumor response (partial and complete) 4 weeks after completion of radiation in patients who finished the planned treatment (Figures 2, 3). There was a

significant correlation in the difference between salivary LDH values before and after treatment with cancer treatment response (R2=0.25; $P<0.02$).

Discussion

Various intrinsic factors of tumors contribute to the observed interindividual difference in radiation-induced cell kill. This has necessitated the need for predictive markers that assist doctors to plan effective radiation treatment before initiation or during the early treatment stages.[9,10] In this study, we investigated whether estimating the alterations in the levels of salivary and blood levels of amylase, GSH, Lipid peroxidation (LPx), and LDH before and after the first 2 Gy radiation fraction could be useful as a predictive marker to ascertain the radiation-induced cell kill and tumor regression.

We observed that the amylase levels statistically increased in both saliva and blood after exposure to 2 Gy of radiation (Tables 4 and 5). Estimation of salivary amylase is considered important in orodental biology.[14] Studies have shown that the levels of salivary amylase are altered in patients with oral cancers and during the course of treatment.[15,23] Additionally studies have also shown that amylase is a sensitive biomarker for stress-related changes in the body, which reflects activity of the sympathetic nervous system[24] and in chronic stress.[25] However, the lack of a correlation between the differences in their levels during the two assay time points with tumor response discourages its usefulness as a predictive marker for radiation-induced cell kill.

With respect to GSH, previous studies have shown that this abundant cellular antioxidant, correlate with tumor sensitivity and to be a prospective predictive marker in assessing radiation-induced cell kill and tumor regression.[9,26-31] GSH is shown to play a cardinal role in a multitude of cellular processes that include regulation of mutagenic mechanisms, DNA synthesis, growth, cell differentiation, proliferation, and apoptosis,[32-34] and to confer protection against the cytotoxic effects of chemotherapy and radiation when at high concentrations.[32-34]

Exposure to one fraction of radiation caused a significant decrease in GSH levels in both saliva and blood (Tables 4 and 5). The effect was more prominent in CR individuals, and is in agreement to previous reports.[9,26] However, we did not observe any correlation between the decrease in both blood saliva with treatment response, which was possibly due to differences in sample size, cancer type (cervical cancer), assay time points (before start of radiotherapy and 24 h after exposure to 4 Gy and 10 Gy), and selection of tissues (blood and cervical) for the study.[9] Studies are underway with a large sample size and various time points during the course of the treatment to ascertain the usefulness of salivary, blood, and tissue GSH with treatment response.

Exposure to ionizing radiation causes auto-oxidation of polyunsaturated fatty acids present in cell membranes. Malondialdehdye, one of the end products of the reaction, is a sensitive marker of lipid peroxidation and is increased in both cancers and after exposure to radiation in serum.[35-37] On a comparative note, unlike blood, saliva has not been investigated in detail in the past. However, a series of reports published in the past decade, indicate that salivary LDH is a useful end point assay in myriad medical conditions and is altered in ailments like diabetes mellitus, periodontitis, dental caries, premalignant oral lesions, and oral cancer.[13, 38-42] In this study, we have observed that exposure to radiation enhanced the levels of lipid peroxides in saliva (Tables 4 and 5) and changes seen in blood agreed with an earlier study.[37] However, we did not observe any association with treatment response for both saliva and blood.

The enzyme LDH is a highly conserved, ubiquitous protein with an evolutionally link in various species. In humans, LDH is a universal, sensitive marker of cell turnover. Increased serum LDH levels are shown in various cancers, including oral cancer,[14] and researchers have reported elevated levels of the LDH isoenzymes (LDH-4 and LDH-5) in oral mucosal tissue in oral cancer when compared to normal mucosa.[15]

Earlier reports have shown that the levels of salivary LDH increased in oral cancer.[15-17] In the current study we observed an increase in the level of both salivary and blood LDH. However, the difference in the levels of LDH was more prominent in the saliva than blood, and in patients who had CR to the therapeutic effects of radiation. This finding was statistically significant (P<0.02). Based on these observations, it can be concluded that salivary LDH is a prospective assay to evaluate the response of radiation therapy in head and neck cancers, and that it could be a useful predictive marker. Studies are planned to understand the most effective saliva sampling time point for more precise information.

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Authors' Contribution

Conceived and designed the experiments: ARS, SR, MSV, SKH and MSB

Recruitment of subjects, explaining the purpose of the study and collection of consent, saliva and blood samples from volunteers: DJ, RT, SP.

Conducted patient care, clinical care and radiation treatment assessments: DJ, RT, SP, MSV.

Provided chemical reagents for the study: ARS and MSB

Conducted biochemical estimations: ARS, ADLL

Interpretation of results: DJ, ARS and MSB

Contributed to writing of the manuscript: DJ, ARS and MSB. All authors have read the manuscript and approved for publication.

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Conflict of Interest:

No conflict of interest is declared.

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