Polymorphisms in Radio-Responsive Genes and Its Association with Acute Toxicity among Head and Neck Cancer Patients

Goutham Hassan Venkatesh¹, Vadhiraja Bejadi Manjunath³, Kamalesh Dattaram Mumbrekar¹, Hitendra Negi², Donald Jerard Fernandes⁵, Krishna Sharan⁵, Sourjya Banerjee⁴, Satish Rao Bola Sadashiva¹

¹ Division of Radiobiology & Toxicology, School of Life Sciences, Manipal University, Manipal, Karnataka, India, ² Division of Biotechnology, School of Life Sciences, Manipal University, Manipal, Karnataka, India, ³ Manipal Hospital, Bangalore, Karnataka, India, ⁴ Department of Radiotherapy & Oncology, Kasturba Medical College and Hospital, Mangalore, Karnataka, India, ⁵ Department of Radiotherapy & Oncology, Shiridi Sai Baba Cancer Hospital and Research Centre, Kasturba Hospital, Manipal, Karnataka, India

Abstract

Cellular and molecular approaches are being explored to find a biomarker which can predict the development of radiation induced acute toxicity prior to radiation therapy. SNPs in radiation responsive genes may be considered as an approach to develop tools for finding the inherent basis of clinical radiosensitivity. The current study attempts to screen single nucleotide polymorphisms/deletions in DNA damage response, DNA repair, profibrotic cytokine as well as antioxidant response genes and its predictive potential with the normal tissue adverse reactions from 183 head and neck cancer patients undergoing platinum based chemoradiotherapy or radiotherapy alone. We analysed 22 polymorphisms in 17 genes having functional relevance to radiation response. Radiation therapy induced oral mucositis and skin erythema was considered as end point for clinical radiosensitivity. Direct correlation of heterozygous and mutant alleles with acute reactions as well as haplotype correlation revealed NBN variants to be of predictive significance in analysing oral mucositis prior to radiotherapy. In addition, genetic linkage disequilibrium existed in XRCC1 polymorphisms for reactions as well as haplotype correlation revealed NBN variants to be of predictive significance in analysing oral mucositis and skin reaction indicating the complex inheritance pattern. The current study indicates an association for polymorphism in NBN with normal tissue radiosensitivity and further warrants the replication of such studies in a large set of samples.

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Introduction

Precise radiation delivery methodologies have significantly improved tumor cure and survival rate owing to recent developments, however it has been at the expense of significant increase in normal tissue toxicity. Heterogeneity in normal tissue radioresponse is observed among patients treated with identical doses of radiation, which further leads to a dynamic and cumulative process of normal tissue toxicity. In head and neck cancer (HNC) patients, oral mucositis and skin erythema are the major complications during the course of chemoradiotherapy. It affects pain control and adequate treatment delivery, thereby leading to unanticipated radiotherapy (RT) breaks, compromising treatment efficacy [1]. Earlier clinical experiences have indicated that only 20% of the variability was because of stochastic or random events, whereas the rest 80% were because of patient related genetic factors [2]. The association between severe radiosensitivity and genetic syndromes like Ataxia-telangiectasia, Fanconi’s anemia, and Bloom syndrome, etc., provides us a proof of the principle about the involvement of genetic component behind normal tissue acute reactions [3]. Also, previous findings demonstrate genotype-dependent cause for acute and late effects of RT seen in normal tissues [4] [5]. Apart from molecular aspects, our earlier studies suggest that there exist a cellular basis for normal tissue radiation sensitivity [6]. Two-third of the studies conducted till date report the association of genetic variation in candidate genes with radiation induced toxicity, but most of these studies are with small patient numbers and lacks independent validation [7]. Although studies have been conducted to associate the polymorphism in selected candidate genes with clinically observed normal tissue adverse effects, its clinical applicability as biomarker/s is still questionable [8] [9]. Therefore, well designed clinical studies with hundreds of samples are needed to seek a biomarker for developing individual treatment protocols [10] [11].

In the present study, single nucleotide polymorphisms/deletions in selected candidate genes related to DNA damage and repair, antioxidant response and detoxification enzymes and profibrotic cytokine were analysed. SNPs in candidate radiation responsive genes like ATM, XRCC1, XRCC3, XRCC4, Ku70, Ku80, LIG4, OGG1, NBN, RAD51, TGFB1, SOD2, CAT and GST were selected. The severity of oral mucositis and skin reaction was recorded according to Radiation Therapy Oncology Group
(RTOG) criteria [12] and the association between genetic polymorphism and oral mucositis and skin reaction was evaluated for the increased risk of developing these normal tissue adverse reactions.

**Materials and Methods**

**Patients and clinical data**

The study was conducted from 183 HNC patients undergoing chemoradiotherapy at Kasturba Hospital, with a prior approval by the University Ethical Committee (UEC/15/2007) and a written informed consent from the patients before collecting blood prior to RT. All patients were treated using 3-Dimensional Conformal Radiotherapy. Gross tumor volume (GTV), Clinical Target Volume (CTV) and Planning target volume (PTV) were defined by using these planning CT scan. Gross tumor volume encompassed all known gross disease as defined by clinical physical examination and imaging findings. Patients with gross disease were treated using Linac 6-MV X-ray linear accelerator (Elekta Precise digital, Stockholm, Sweden) with the total tumor dose of 70 Gy (2 Gy per day for 5 days per week). Patients after surgical resection having positive margins were given a dose of 66 Gy in 33 fractions. Patients with no positive margins were given 60 Gy in 30 fractions. Dose to parotid gland, submandibular salivary glands, constrictor muscles and other structures were not restricted in view of 3-Dimensional treatment planning in all the patients. Cisplatin chemotherapy (100 mg/m² for once in 3 weeks) was given to majority of the patients when serum creatinine was normal. Elderly patients received a weekly dose of 40 mg/m² for 6 weeks and patients having borderline elevation of serum creatinine received carboplatin (area under curve (AUC) @ 1.5) on a weekly basis for 6 weeks. A total of 148 patients received concurrent chemoradiotherapy and the remaining patients were received radiotherapy alone. Patients with recurrent tumour and distant metastasis were excluded. Acute adverse events (oral mucositis and skin reaction) were recorded during and after completion of treatment. Elderly patients received a weekly dose of 40 mg/m² for 6 weeks and patients having borderline elevation of serum creatinine received carboplatin (area under curve (AUC) @ 1.5) on a weekly basis for 6 weeks. A total of 148 patients received concurrent chemoradiotherapy and the remaining patients were received radiotherapy alone. Patients with recurrent tumour and distant metastasis were excluded. Acute adverse events (oral mucositis and skin reaction) were recorded during and after completion of therapy according to RTOG criteria [10]. The details of HNC patient characteristics are described in table 1.

**Genotyping, haplotype and linkage disequilibrium analysis**

Genomic DNA isolation was performed by employing the conventional phenol chloroform extraction and ethanol precipitation procedure. Genotyping was performed by Polymerase Chain Reaction based Restriction Fragment Length Polymorphism. The details are available in table 2. Five percent of the samples were randomly selected and re-genotyped to assess the consistency in results.

**Statistical analysis**

Each polymorphism was tested for deviation from Hardy-Weinberg equilibrium. Statistical significance was analysed by Fisher exact test. Odds ratio was estimated to test whether any association exist between the grade of acute toxicity and selected SNP/haplotypes. Haplotype analysis and linkage disequilibrium estimates were done using SHEsis software [13]. All statistical tests were performed using Prism v.5.0 (GraphPad Software, San Diego, California, USA) and Statistical Package for Social Science (Version 16.0, Chicago, USA).

**Results**

The distribution of patients based on histopathological grading, tumor stage and acute toxicity grades is provided in table 1. The mean age group of the patients considered in the study was 54.74 years. Radiation doses ranging from 60 to 70 Gy (median = 66 Gy) in 30 to 35 fractions were given to the patients. A total of 148 patients underwent platinum-based chemoradiotherapy, while the remaining was given radiotherapy alone. Out of 183 patients, 71 (38.79%) patients experienced severe mucositis (grade 3 and 4) and 44 (24.04%) experienced severe skin reactions. We analysed the presence of confounding factors like diabetes, hypertension, smoking, alcohol, surgery and chemotherapy and found that patients with alcoholism was associated with grade ≤2 mucositis (p = 0.02) (Table 3).

According to radiation oncologists, toxicity up to grade 2 is usually tolerated by patients without any therapeutic intervention but grade 3 and 4 requires intervention with therapeutic agents. Based on this, and considering reports from earlier studies [14][15], we grouped the patient normal tissue toxicity data as grade ≤2 or >2. Since the dose exposed to oral cavity/pharyngeal region varies from 0–40% in hypopharynx, larynx, thyroid and region of

**Table 1. Demographic and clinical details of head and neck cancer patients.**

| Patient clinical details | Number of patients | Mean age | Males | Females | Smoking/Tobacco chewing | Alcohol consumption | Region |
|--------------------------|--------------------|----------|--------|---------|------------------------|-------------------|--------|
| Number of patients       | 183                | 55 (26–80)| 157    | 26      | 122                    | 66                |
| Mean age                 |                    |          |        |         |                        |                   |
| Males                    |                    |          |        |         |                        |                   |
| Females                  |                    |          |        |         |                        |                   |
| Smoking/Tobacco chewing  |                    |          |        |         |                        |                   |
| Alcohol consumption      |                    |          |        |         |                        |                   |
| Region                   |                    |          |        |         |                        |                   |
| Hypopharynx              | 32                 |          |        |         |                        |                   |
| Oropharynx               | 85                 |          |        |         |                        |                   |
| Nasopharynx              | 5                  |          |        |         |                        |                   |
| Larynx                   | 27                 |          |        |         |                        |                   |
| Oral cavity              | 15                 |          |        |         |                        |                   |
| Para nasal region        | 11                 |          |        |         |                        |                   |
| Parotid                  | 4                  |          |        |         |                        |                   |
| Thyroid                  | 3                  |          |        |         |                        |                   |
| Unknown origin of region | 1                  |          |        |         |                        |                   |
| Tumor staging            |                    |          |        |         |                        |                   |
| T1                       | 9                  |          |        |         |                        |                   |
| T2                       | 32                 |          |        |         |                        |                   |
| T3                       | 48                 |          |        |         |                        |                   |
| T4                       | 65                 |          |        |         |                        |                   |
| Tx                       | 12                 |          |        |         |                        |                   |
| Treatment                |                    |          |        |         |                        |                   |
| Chemoradiotherapy        | 148                |          |        |         |                        |                   |
| RT alone                 | 35                 |          |        |         |                        |                   |
| Skin reaction (RTOG Grading) |            |          |        |         |                        |                   |
| Grade 0                  | 3                  |          |        |         |                        |                   |
| Grade I                  | 27                 |          |        |         |                        |                   |
| Grade II                 | 97                 |          |        |         |                        |                   |
| Grade III                | 36                 |          |        |         |                        |                   |
| Grade IV                 | 3                  |          |        |         |                        |                   |
| Mucositis (RTOG Grading) |                    |          |        |         |                        |                   |
| Grade 0                  | 1                  |          |        |         |                        |                   |
| Grade I                  | 8                  |          |        |         |                        |                   |
| Grade II                 | 57                 |          |        |         |                        |                   |
| Grade III                | 48                 |          |        |         |                        |                   |
| Grade IV                 | 6                  |          |        |         |                        |                   |

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unknown origin, data from cancers in these regions were excluded for analysing oral mucositis. Of all the polymorphisms tested, RAD51 (rs1801321), Ku70 (rs2267437) and XRCC4 (rs1805377) were found to deviate from Hardy-Weinberg equilibrium. Univariate analysis showed that none of the polymorphisms presented any significant association to skin reaction (Table 4). However, it indicated that odds of patients experiencing severe oral mucositis (grade ≥ 2) with recessive allele of NBN (rs1805794) was 3.75 times higher having a confidence interval of 1.201–11.70 and p = 0.023. Also, heterozygous variants in CAT (rs7943316) displayed 0.452 (odds value) times lesser prone to experience severe oral mucositis (grade ≥ 2) with a confidence interval of 0.206–0.993 and p = 0.048. In continuation, multivariate analysis indicated that odds of patients experiencing severe oral mucositis

| Table 2. The list of candidate genes selected in the present study. |
|---------------------------------------------------------------|
| **Gene** | rs number | Amino acid/nucleotide change | Chromosome | PCR product size (bp) | Enzyme | Dominant (wild type) | Heterozygous | Recessive (mutant) |
|---------|-----------|-----------------------------|------------|----------------------|--------|---------------------|--------------|-------------------|
| DNA damage and repair genes | | | | | | | | |
| XRCC1 | rs25487 | Glu399Arg (A>G) | Chr 19 | 615 | Msp I | 615 | 615, 377, 238 | 377, 238 |
| XRCC1 | rs1799782 | Arg194Trp (C>T) | Chr 19 | 491 | Msp I | 491 | 292,178,21 | 313, 292, 178, 21, 313, 178 |
| XRCC1 | rs25489 | Arg280His (G>A) | Chr 19 | 280 | Rsa I | 280 | 280, 140 | 140 |
| XRCC1 | rs3213245 | −77 (C>T) | Chr 19 | 219 | BseI | 219 | 173, 46 | 173, 116, 57, 46 | 116, 57, 46 |
| XRCC3 | rs861359 | Thr241Met (C>T) | Chr 14 | 336 | Nol III | 336 | 336, 231, 105 | 231, 105 |
| XRCC4 | rs1805377 | 894–7 (A>G) | Chr 5 | 170 | Tsp509 I | 170 | 170, 88, 82 | 88, 82 |
| XRCC5 (Ku80) | rs3835 | 2110–2408 (G>A) | Chr 2 | 170 | Tsp509 I | 170 | 170, 88, 82 | 88, 82 |
| XRCC6 (Ku70) | rs2267437 | 88–57 (C>G) | Chr 22 | 170 | Tsp509 I | 170 | 170, 88, 82 | 88, 82 |
| LG4 | rs1803388 | Thr916, 26C>T | Chr 13 | 121 | HpyCH4 III | 121 | 121 | 121, 65, 56 | 121, 65, 56 |
| NBN | rs1805794 | Glu158Gln (G>A) | Chr 19 | 174 | Hinf I | 174 | 174 | 174, 125, 49 | 125, 49 |
| NBN | rs1805787 | 1125–520 G>C | Chr 8 | 197 | Ear I | 197 | 197 | 197, 172, 25 | 172, 25 |
| RAD51 | rs1801320 | 298G>C | Chr 19 | 131 | BstN I | 131 | 131 | 131, 71, 60 | 71, 60 |
| RAD51 | rs1801321 | −61G>T | Chr 19 | 131 | BstN I | 131 | 131 | 131, 71, 60 | 71, 60 |
| ATM | rs3218698 | 3285-10delT | Chr 13 | 200 | Fnu4HI | 200 | 200 | 200, 176, 24 | 176, 24 |
| OGG1 | rs1052133 | Ser326Cys (C>G) | Chr 3 | 156 | BseI | 156 | 156 | 156, 100, 56 | 100, 56 |
| Profibrotic and inflammatory cytokine | | | | | | | | |
| TGF-β1 | rs1800469 | 509C>T | Chr 19 | 418 | Bsu36I | 418 | 418 | 418 | 418 |
| Antioxidant genes | | | | | | | | |
| CAT | rs7943316 | −21A>T | Chr 11 | 250 | Hinf I | 250 | 250 | 250, 177, 73 | 177, 73 |
| SOD2 | rs4880 | 41G>C | Chr 6 | 207 | BsaWI | 207 | 207 | 207, 167, 40 | 167, 40 |
| NQO1 | rs1131341 | C>T | Chr 16 | 194 | Msp I | 194 | 194 | 194,102,94 | 102,94 |
| Detoxification genes | | | | | | | | |
| GSTP1 | rs1695 | Ile105Val (G>A) | Chr 11 | 433 | BsmAI | 433 | 433 | 222,106,105 | 222,106,105 |
| GSTT1 | - | Present/Null | Chr 12 | 480 | | | | |
| GSTM1 | - | Present/Null | Chr 1 | 240 | | | | |

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| Table 3. Effect of confounding factors on the set of samples analysed. |
|---------------------------------------------------------------|
| **Confounding factors** | **Grade ≤2 Skin reaction (n = 139)** | **Grade >2 Skin reaction (n = 44)** | **p-value** | **Grade ≤2 Mucositis (n = 66)** | **Grade >2 Mucositis (n = 54)** | **p-value** |
|---------|--------------------------|--------------------------|----------|--------------------------|--------------------------|----------|
| Age | 54.55 ± 11.041 | 54.41 ± 13.191 | 0.948 | 51.98 ± 11.48 | 54.15 ± 12.49 | 0.326 |
| Gender | 120m, 19f | 37m, 7f | 0.805 | 57m, 9f | 45m, 9f | 0.798 |
| Diabetes | 19% | 4% | 0.035 | 7% | 7% | 0.778 |
| Hypertension | 14% | 9% | 0.114 | 7% | 8% | 0.583 |
| Alcohol | 53% | 13% | 0.369 | 29% | 12% | 0.020* |
| Smoking | 93% | 29% | 1.000 | 47% | 35% | 0.055 |
| Surgery | 51% | 15% | 0.858 | 27% | 18% | 0.451 |
| Chemotherapy | 111 | 37 | 0.148 | 52 | 47 | 0.429 |

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Table 4. Univariate analysis of candidate single nucleotide polymorphisms and radiation-induced oral mucositis and skin reactions in head and neck cancer patients.

| Gene name | Genotype | Skin reaction ≤ 2 (n = 139) | Skin reaction > 2 (n = 44) | Odds ratio | 95% CI | p-value | Oral Mucositis ≤ 2 (N = 66) | Oral Mucositis > 2 (N = 54) | Odds ratio | 95% CI | p-value |
|------------|----------|-----------------------------|-----------------------------|------------|-------|---------|-----------------------------|----------------------------|------------|-------|---------|
| XRCC1 (rs25487) | AA | 50 | 17 | Reference | 29 | 23 | Reference |
| | GA | 62 | 14 | 0.411 | 0.154–1.096 | 0.076 | 28 | 25 | 1.339 | 0.418–4.295 | 0.623 |
| | GG | 15 | 8 | 0.545 | 0.204–1.458 | 0.227 | 9 | 6 | 1.190 | 0.370–3.829 | 0.771 |
| XRCC1 (rs1799782) | CC | 95 | 31 | Reference | 48 | 40 | Reference |
| | CT | 30 | 6 | 0.624 | 0.253–1.357 | 0.305 | 15 | 12 | 0.960 | 0.403–2.285 | 0.927 |
| | TT | 2 | 2 | 1.962 | 0.315–12.228 | 0.470 | 3 | 2 | 0.800 | 0.127–5.026 | 0.812 |
| XRCC1 (rs25489) | AA | 97 | 30 | Reference | 52 | 41 | Reference |
| | GA | 30 | 8 | 0.727 | 0.307–1.722 | 0.469 | 14 | 13 | 1.087 | 0.454–2.603 | 0.851 |
| | GG | 0 | 1 | 3.000 | 0.183–49.239 | 0.442 | 0 | 0 | 2.049 | 0.000 | 1.000 |
| XRCC1 (rs3213245) | TT | 58 | 21 | Reference | 28 | 27 | Reference |
| | TG | 25 | 11 | 1.891 | 0.842–4.247 | 0.123 | 12 | 14 | 1.750 | 0.712–4.299 | 0.222 |
| | CC | 3 | 2 | 0.452 | 0.122–1.677 | 0.235 | 11 | 4 | 0.377 | 0.107–1.330 | 0.129 |
| RAD51 (rs1801321)* | GG | 81 | 20 | Reference | 45 | 30 | Reference |
| | GT | 21 | 11 | 1.891 | 0.842–4.247 | 0.123 | 12 | 14 | 1.750 | 0.712–4.299 | 0.222 |
| | TT | 25 | 8 | 1.164 | 0.466–2.909 | 0.745 | 9 | 10 | 1.667 | 0.606–4.586 | 0.323 |
| RAD51 (rs1801320) | GG | 90 | 29 | Reference | 48 | 35 | Reference |
| | CT | 34 | 9 | 1.000 | 0.377–1.880 | 0.674 | 17 | 17 | 1.371 | 0.616–3.055 | 0.440 |
| | CC | 3 | 2 | 0.452 | 0.122–1.677 | 0.235 | 11 | 4 | 0.377 | 0.107–1.330 | 0.129 |
| NBN (rs1805794) | GG | 44 | 13 | Reference | 24 | 16 | Reference |
| | CG | 62 | 13 | 0.906 | 0.409–2.010 | 0.808 | 36 | 23 | 0.958 | 0.422–2.178 | 0.919 |
| | CC | 21 | 13 | 2.110 | 0.853–5.223 | 0.106 | 6 | 15 | 3.750 | 1.201–11.70 | 0.023 |
| NBN (rs1805787) | GG | 87 | 30 | Reference | 44 | 40 | Reference |
| | GC | 36 | 8 | 0.599 | 0.254–1.411 | 0.241 | 22 | 12 | 0.550 | 0.237–1.275 | 0.164 |
| | CC | 4 | 1 | 0.554 | 0.063–4.911 | 0.596 | 0 | 2 | 1.777 | 0.000 | 0.999 |
| OGG1 (rs1052133) | CC | 57 | 13 | Reference | 31 | 26 | Reference |
| | CG | 53 | 19 | 1.235 | 0.586–2.605 | 0.579 | 25 | 23 | 1.097 | 0.508–2.368 | 0.814 |
| | GG | 17 | 7 | 1.560 | 0.583–4.176 | 0.376 | 10 | 5 | 0.596 | 0.181–1.955 | 0.396 |
| GSTP1 (rs1695) | AA | 63 | 21 | Reference | 37 | 30 | Reference |
| | AG | 52 | 14 | 0.737 | 0.355–1.528 | 0.412 | 25 | 18 | 0.888 | 0.410–1.925 | 0.764 |
| | GG | 12 | 4 | 0.933 | 0.275–3.162 | 0.912 | 4 | 6 | 1.850 | 0.478–7.163 | 0.373 |
| GSTM1 | 1 | 88 | 28 | Reference | 47 | 33 | Reference |
| | 0 | 39 | 11 | 0.866 | 0.422–1.778 | 0.695 | 19 | 21 | 0.635 | 0.296–1.363 | 0.244 |
| GSTT1 | 1 | 100 | 32 | Reference | 48 | 46 | Reference |
| | 0 | 27 | 7 | 1.394 | 0.563–3.446 | 0.473 | 18 | 8 | 2.156 | 0.854–5.442 | 0.104 |
| Gene name | Genotype | Skin reaction ≤ 2 (n = 139) | Skin reaction > 2 (n = 44) | Odds ratio | 95% CI | p-value | Oral Mucositis ≤ 2 (N = 66) | Oral Mucositis > 2 (N = 54) | Odds ratio | 95% CI | p-value |
|-----------|----------|-----------------------------|-----------------------------|------------|-------|--------|-----------------------------|-----------------------------|------------|-------|--------|
| CAT (rs7943316) | TT | 55 | 14 | Reference | 21 | 25 | Reference |
| | TA | 62 | 17 | 0.532 | 0.532–2.356 | 0.766 | 39 | 21 | 0.452 | 0.206–0.993 | 0.048 |
| | AA | 10 | 8 | 0.874 | 0.874–7.151 | 0.087 | 6 | 8 | 1.120 | 0.335–3.745 | 0.854 |
| ATM (rs3218698) | T/T | 113 | 36 | Reference | 56 | 49 | Reference |
| | T/−T | 14 | 3 | 0.718 | 0.228–2.528 | 0.571 | 10 | 5 | 0.571 | 0.183–1.787 | 0.336 |
| Ku80 (rs38335) | AA | 96 | 27 | Reference | 53 | 39 | Reference |
| | GA | 25 | 12 | 1.543 | 0.701–3.400 | 0.282 | 11 | 14 | 1.730 | 0.709–4.218 | 0.228 |
| | GG | 6 | 0 | 0.000 | 0.000 | 0.999 | 2 | 1 | 0.679 | 0.059–7.763 | 0.756 |
| KU70 (rs2267437)* | CC | 82 | 27 | Reference | 41 | 37 | Reference |
| | GC | 33 | 10 | 0.812 | 0.370–1.784 | 0.605 | 19 | 13 | 0.758 | 0.329–1.745 | 0.515 |
| | GG | 12 | 2 | 0.401 | 0.086–1.865 | 0.244 | 6 | 4 | 0.739 | 0.193–2.824 | 0.658 |
| XRCC4 (rs1805377)* | GG | 94 | 28 | Reference | 44 | 40 | Reference |
| | GA | 28 | 9 | 1.028 | 0.455–2.325 | 0.947 | 20 | 11 | 0.605 | 0.258–1.417 | 0.247 |
| | AA | 5 | 2 | 1.062 | 0.204–5.526 | 0.943 | 2 | 3 | 1.650 | 0.262–10.386 | 0.594 |
| XRCC3 (rs861539) | CC | 87 | 29 | Reference | 42 | 32 | Reference |
| | CT | 46 | 15 | 0.978 | 0.477–2.007 | 0.952 | 22 | 22 | 1.313 | 0.621–2.775 | 0.477 |
| | TT | 6 | 0 | 0.000 | 0.000 | 0.999 | 2 | 0 | 0.000 | 0.000 | 0.999 |
| UG1 (rs1805388) | CC | 100 | 32 | Reference | 52 | 48 | Reference |
| | CT | 26 | 6 | 0.724 | 0.292–1.794 | 0.486 | 13 | 4 | 0.333 | 0.102–1.093 | 0.070 |
| | TT | 1 | 1 | 1.500 | 0.132–17.03 | 0.744 | 1 | 2 | 2.167 | 0.190–24.66 | 0.533 |
| SOD2 (rs4880) | CC | 29 | 11 | Reference | 15 | 18 | Reference |
| | CT | 71 | 17 | 0.688 | 0.307–1.542 | 0.364 | 35 | 24 | 0.571 | 0.242–1.350 | 0.202 |
| | TT | 27 | 11 | 0.992 | 0.386–2.548 | 0.987 | 16 | 12 | 0.625 | 0.227–1.724 | 0.364 |
| TGF β1 (rs1800469) | CC | 62 | 16 | Reference | 30 | 23 | Reference |
| | CT | 50 | 16 | 1.361 | 0.646–2.869 | 0.417 | 29 | 23 | 1.034 | 0.478–2.237 | 0.931 |
| | TT | 15 | 7 | 1.855 | 0.686–5.015 | 0.224 | 7 | 8 | 1.491 | 0.472–4.711 | 0.496 |
| NQO1 (rs1131341) | CC | 103 | 33 | Reference | 55 | 45 | Reference |
| | CT | 21 | 6 | 0.818 | 0.309–2.168 | 0.687 | 9 | 8 | 1.086 | 0.388–3.045 | 0.875 |
| | TT | 3 | 0 | 0.000 | 0.000 | 0.999 | 2 | 1 | 0.611 | 0.054–6.959 | 0.692 |

* Not in HWE.
Haplotype analysis was done for 4 SNPs in XRCC1, 2 polymorphisms in RAD51 as well as 2 polymorphisms in NBN to explore association of the combinatorial effect of these variants with increased normal tissue radiosensitivity. Haplotype analysis of NBN (rs1805787, rs1805794) gene demonstrated G-C haplotype to be associated with development of oral mucositis (odds ratio of 1.687 and 95% CI of 1.005–2.831 with p = 0.047) (Table 6).

Further, to determine if any multiple SNP has an additive effect on the oral mucositis as well as skin reaction, the average number of variant alleles per patient in each RTOG group was analysed (Figure 1). Based on the allele frequency reported in the dbSNP database, we have reported the wild type, heterozygous and recessive genotypes. We considered the minor allele in this group as the risk allele and counted the number of risk alleles in each patient. The results suggest that the number of variant alleles has no effect on severity of normal tissue toxicity.

Furthermore, we analysed the linkage disequilibrium pattern for SNPs in XRCC1 and found that rs3213245, rs1799782, rs25489 and rs25487 were linked with severe oral mucositis while, rs1799782 and rs25489 were linked with severe skin reaction. Also, to validate this linked SNPs were analysed for linkage disequilibrium pattern separately in normal tissue overreactor and non-overreactor phenotypes. The $D_{9}^2$ values were strikingly higher in normal tissue radiosensitive phenotypes (Figure 2).

Discussion

Although, the growing volume of SNP data suggests the genetic basis for susceptibility to radiotherapy induced acute effects, it is

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### Table 5. Multivariate analysis for CAT and NBN polymorphisms with radiation-induced oral mucositis in presence of alcohol among head and neck cancer patients.

| Gene name | Genotype | Oral mucositis ≤2 (n = 66) | Oral mucositis >2 (n = 54) | Adjusted Odds ratio | 95% CI | p-value |
|-----------|----------|----------------------------|---------------------------|---------------------|--------|---------|
| CAT (rs7943316) | TT       | 21                         | 25                        | Reference           |        |         |
|           | TA       | 39                         | 21                        | 0.463               | 0.199–1.076  | 0.074   |
|           | AA       | 6                          | 8                         | 1.675               | 0.427–6.575  | 0.460   |
| NBN (rs1805794) | GG       | 24                         | 16                        | Reference           |        |         |
|           | CG       | 36                         | 23                        | 1.275               | 0.531–3.062  | 0.587   |
|           | CC       | 6                          | 15                        | 4.728               | 1.384–16.151 | 0.013   |

All those haplotypes with frequency <0.03 were ignored during the analysis.

### Table 6. Haplotype analysis for XRCC1 (rs3213245, rs1799782, rs25489 and rs25487), RAD51 (rs1801320, rs1801321) and NBN (rs1805787, rs1805794) and radiation-induced oral mucositis and skin reactions in head and neck cancer patients.

| Gene   | Haplotype | Skin reactions | Oral mucositis |
|--------|-----------|----------------|----------------|
|        | OR frequency | NOR frequency | Fisher’s p-value | Odds ratio (95% CI) | OR frequency | NOR frequency | Fisher’s p-value | Odds ratio (95% CI) |
| XRCC1  | C-C-A-A    | 20.00 (0.256)  | 72.86 (0.287)  | 0.406               | 0.782 (0.438–1.397) |        |        |    |
|        | C-C-A-G    | -              | -              | -                   | 28.05 (0.260)  | 43.53 (0.330)  | 0.195               | 0.687 (0.389–1.215) |        |
|        | T-C-A-A    | 9.07 (0.116)  | 33.72 (0.133)  | 0.573               | 0.799 (0.365–1.747) | 32.20 (0.298)  | 39.81 (0.302)  | 0.873               | 0.955 (0.544–1.676) |        |
|        | T-C-A-G    | 28.93 (0.371) | 76.49 (0.301)  | 0.414               | 1.250 (0.731–2.136) | 16.05 (0.149)  | 12.94 (0.098)  | 0.252               | 1.576 (0.719–3.453) |        |
|        | T-C-G-A    | 10.0 (0.128)  | 25.78 (0.101)  | 0.627               | 1.213 (0.556–2.648) | -              | -              | -                   | -                   |        |
|        | T-C-G-G    | -              | -              | -                   | 12.75 (0.118)  | 9.71 (0.074)  | 0.258               | 1.654 (0.687–3.986) |        |
|        | T-T-A-A    | 8.93 (0.114)  | 26.28 (0.103)  | 0.918               | 1.043 (0.465–2.338) |        |        |    |
|        | T-T-A-G    | -              | -              | -                   | 13.90 (0.129)  | 17.38 (0.132)  | 0.897               | 0.951 (0.445–2.033) |        |
| RAD    | G-C        | 7.97 (0.102)  | 32.60 (0.128)  | 0.537               | 0.773 (0.340–1.754) | 16.65 (0.154)  | 16.70 (0.127)  | 0.538               | 1.258 (0.605–2.616) |        |
|        | G-G        | 43.03 (0.552) | 150.40 (0.592) | 0.526               | 0.848 (0.508–1.418) | 57.35 (0.531)  | 85.30 (0.646)  | 0.070               | 0.620 (0.369–1.043) |        |
|        | T-C        | 3.03 (0.039)  | 7.40 (0.029)   | 0.667               | 1.347 (0.345–5.258) | 4.35 (0.040)  | 2.30 (0.017)  | 0.282               | 2.371 (0.470–11.968) |        |
|        | T-G        | 23.97 (0.307) | 63.60 (0.250)  | 0.319               | 1.328 (0.760–2.322) | 29.65 (0.275)  | 27.70 (0.210)  | 0.242               | 1.425 (0.786–2.583) |        |
| NBN    | C-G        | 39.0 (0.500)  | 103.99 (0.409) | 0.158               | 1.442 (0.867–2.401) | 16.00 (0.148)  | 21.99 (0.167)  | 0.696               | 0.870 (0.431–1.753) |        |
|        | G-C        | 10.0 (0.128)  | 43.99 (0.173)  | 0.346               | 0.702 (0.335–1.470) | 53.00 (0.491)  | 47.99 (0.364)  | 0.047               | 1.687 (1.005–2.831) |        |
|        | G-G        | 29.0 (0.372)  | 106.01 (0.417) | 0.473               | 0.826 (0.490–1.393) | 39.00 (0.361)  | 62.01 (0.470)  | 0.089               | 0.638 (0.379–1.074) |        

All those haplotypes with frequency <0.03 were ignored during the analysis.

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less clear whether the SNPs can serve as a biomarker for predicting the normal tissue toxicity. Identifying the genetic profiles associated with an enhanced or reduced risk for radiotherapy complications seems to be a most promising factor to improve the efficacy of radiotherapy [9] [16]. The amount of human genetic diversity is immense, and we are just now beginning to understand how such changes influence the specific phenotypic expression. Remarkable genetic variations exist among populations and understanding this variation will help us to tailor the therapy with a personalised approach for safer and effective outcome.

Based on the functional significance of genes in radiation response, we analysed the association of some of the important gene variants belonging to DNA damage response, DNA repair, profibrotic cytokine, antioxidant genes with normal tissue over-reactor phenotype (grade 2 toxicity). We did not find any significant association for either the selected SNPs or for the haplotypes with the risk of developing oral mucositis and skin reaction in HNC patients. However, we observed an association for NBN (rs1805794) polymorphism in univariate as well as multivariate model of analysis. In addition, one of the NBN haplotype was associated with severe oral mucositis. NBN is a component of MRE complex (MRE11-RAD50-NBN) which is

![Figure 1. Association of average number of risk allele with the increasing RTOG grades of normal tissue toxicity.](image1)

The results of Kruskal-Wallis test demonstrate that the comparison between the groups are non-significant (p>0.05). The error bars represent the minimum to maximum values of risk allele represented in each group. doi:10.1371/journal.pone.0089079.g001

![Figure 2. Linkage disequilibrium analysis for XRCC1 polymorphisms (a = rs3213245, b = rs1799782, c = rs25487, d = rs25489) for skin reaction (A and B) and oral mucositis (C and D).](image2)

The numbers inside every box represent r2 values (%) of the linkage disequilibrium. doi:10.1371/journal.pone.0089079.g002
involved in damage sensing, signaling and responding to DSBs [17]. This polymorphism brings about the change in Nibrin protein at 185th codon position from glutamic acid to glutamine and the functional significance of this change still remains unclear [10]. It was reported that rs1805794 was not associated with acute side effects of radiotherapy in breast cancer patients [10] [19]. Also, there are studies which report no association for rs1805794 and late radiation toxicities [20] [21].

Currently there are only few studies related to normal tissue toxicity and genotype analysis in HNC patients, and it is less clear whether the SNPs can serve as a biomarker for predicting the normal tissue toxicity. Werbrouck and co-workers [22] report that SNPs in DNA repair genes XRCC3 (rs861539) and Ku70 (rs2267437) may help in determining the risk for acute dysphagia. Study conducted by Pratesi et al. [23] has suggested that patients with XRCC1 (rs25487) and RAD51 (rs1801320) have higher likelihood of developing oral mucositis and dysphagia in HNC patients. Unlike previous reports, null variants of GSTM1 and GSTT1 also showed no association with the development of acute reactions [24]. Also, a large-scale analysis for screening 3,144 SNPs from 156 breast cancer patients has revealed that ABCA1 and IL12RB2 polymorphism are highly susceptible to radiation-induced dermatitis [25]. Conversely, several studies aimed at validating the effect of TGFβ1 [26], [27], ATM, GSTP1, SOD2, TGFβ1, XPD and XRCC1 [28] have indicated no such association for the risk of developing normal tissue toxicity. Earlier reports with the approach of haplotype analysis have revealed several haplotypes with significant evidence for predicting acute reactions of radiotherapy [4] [29] [30]. Our analysis for XRCC1 haplotype did not associate with the risk of increased acute reactions, but NBN haplotype had an association for oral mucositis. Studies suggest that SNPs or haplotypes in functionally important candidate genes alone may not contribute to radiation induced acute reactions. In addition, extensive literature review [8], [31] has indicated a contradictory association for susceptibility to radiation induced toxicity, which supports the need for further studies.

In conclusion, we report that gene variants and haplotypes of NBN are associated with the risk of developing oral mucositis in head and neck cancer patients undergoing chemoradiotherapy/radiotherapy. In addition to the screening for rare variants having large effects and common variants with small effects, we need to identify other types of variation and explore gene environment interactions for developing predictive models. Further replication of our results in large data sets with pathway-based approach and large genome wide association studies with methodological approach can be of great use to develop predictive biomarkers for this complex trait and the findings must be independently confirmed in different populations.

Supporting Information

Table S1 Univariate analysis of candidate single nucleotide polymorphisms and radiation-induced skin reactions after categorising the samples based on chemoradiotherapy and radiotherapy alone. (DOCX)

Table S2 Univariate analysis of candidate single nucleotide polymorphisms and radiation-induced oral mucositis after categorising the samples based on chemoradiotherapy and radiotherapy alone. (DOCX)

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Author Contributions

Conceived and designed the experiments: VBM DJF SRBS. Performed the experiments: GHV KDM HK. Analyzed the data: GHV KDM HK SRBS. Contributed reagents/materials/analysis tools: VBM SB DJF KS SB. Wrote the manuscript: GHV KDM SRBS.

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