Genomic determinants of normal tissue toxicity after radiotherapy for head and neck malignancy: a systematic review

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Short running title: Systematic Review of Radiogenomics in Head and Neck Cancer

Introduction

Radiotherapy is an integral component of a multimodality treatment approach in head and neck (HN) cancer [1]. Previous interest has primarily focused on tumour radiocurability but a shift towards cancer survivorship in recent years has seen growing interest in understanding radiation-induced complications in normal tissues [2, 3]. Irradiated patients demonstrate variable normal tissue responses to radiotherapy despite apparently uniform treatments [4]. While some of this may be due to stochastic effects, evidence supports the influence of deterministic variations in radioresponsiveness [5]. Evidence of genetic and protein polymorphism underlying inter-individual differences in adverse responses of normal tissues to radiation is rapidly increasing [6,7]. Unsurprisingly, much work has focused on the role of single nucleotide polymorphism (SNP) because it is the most common cause of the differences observed in DNA sequence among individuals [8].

Many HN cancer patients achieve survivorship at the cost of treatment complications occurring in normal tissues. The ability to predict a predisposition for severe radiotherapy-induced adverse effects in normal tissues could potentially aid treatment decision-making, particularly in those with 'intermediate risk' disease [9,10]. Avoiding or reducing radiation in these patients could lessen the likelihood of radiotoxicity-related morbidities and may also potentially reduce the burden of healthcare costs incurred for the supportive care required for these conditions.

Genetic association studies (GAS) have been employed to identify causal functional SNPs in normal tissue radiotoxicity [8]. Most genetic association studies have relied on the candidate gene approach (CGA), where postulated causal genes in radiobiological processes were selected for evaluation of associations. The genome-wide approach (GWA) is an alternative method, where the entire genome is screened for significantly altered allele frequencies based on the linkage disequilibrium concept. Irrespective of the approach chosen, methodological considerations in GAS of radiotoxicity are critical to enable reliable interpretation of study findings, especially if these findings are to be translated into biomarkers of oncological treatment [5,11].

Due to the lack of critical literature review on the association of SNPs with the occurrence of HN radiotoxicity

normal tissues, we undertook the first systematic review in this subject based on the PRISMA statement [12], to gain a perspective of current knowledge as a basis to chart further work in this area. The specific aims are to (a) evaluate the reported relationship between genetic variants and adverse radiotherapy effects in HN cancer and (b) address the possibility of undertaking a meta-analysis of the genetic risk of various SNP in predicting HN radiotoxicity.

Methods

Research question

Is there an association between gene polymorphisms and the occurrence of HN radiotoxicity?

Definitions

Radioresponsiveness is defined as the clinical features associated with the response to radiotherapy. 'Radiotoxicity' is the temporary or permanent adverse changes/effects in normal tissue and/or related symptoms resulting from radiotherapy. 'Radiosensitivity' is the sensitivity of cells to irradiation *in vitro*, which is usually indicated by the surviving fraction at 2 Gy or by the parameters of the linear-quadratic or multitarget equations [13,14]. This systematic review only considers normal tissue radiotoxicity and also in instances where the published study refers to this condition as 'clinical radiosensitivity'.

Search strategy

A literature search of PubMed, Embase (1950-February 2012) and the Cochrane Reviews (to February 2012) was undertaken using various combinations of keywords and MeSH terms related to the subject. Searches were limited to human studies and the English language. The detailed search strategy used is available in supplementary information. Additionally, potential articles were also screened from the citation lists of retrieved articles and identified from expert source.

Inclusion criteria

(i) All prospective, cross-sectional and retrospective studies reporting on adverse effects involving radiotherapy in HN cancer with genetic polymorphism.

(ii) Studies with sufficient data for estimating odds ratio (OR) and 95% confidence interval (95% CI).

Exclusion criteria

(i) Studies evaluating radiotoxicity in thyroid, oesophageal and other non-HN cancers.

(ii) Eligible studies that provided insufficient information.

(iii) Studies of cellular radiosensitivity derived from HN tumour and/or normal cell lines or animal studies.

Study selection

The list of retrieved articles was examined. Duplicates and obviously unrelated articles were eliminated. Abstracts of remaining articles were examined to determine if the full-text article should be obtained.

Data extraction

Relevant data were extracted from all eligible publications by one author using a standardized data extraction form. The following items were collected: author, year of publication, country of origin, ethnicity, type of study, type of radiotoxicity, total number of cases and controls, and confounding/covariates. Treatment and genotype distributions were also extracted.

Types of outcome and measurement

(i) Clinical endpoints of acute HN radiotoxicity e.g., mucositis, dysphagia, dermatitis.

(ii) Clinical endpoints of late HN radiotoxicity e.g., subcutaneous fibrosis, osteoradionecrosis (ORN).

(iii) Surrogate markers of HN radiotoxicity e.g., dependence on enteral tube feeding for dysphagia.

Methodological quality assessment

All eligible publications were subjected to methodological quality assessment based on the STrengthening the REporting of Genetic Association studies (STREGA) recommendations on reporting of genetic association studies [15].

Data synthesis

Meta-analysis was not performed due to the substantial clinical heterogeneity of the data in the included studies.

Results

The search found 692 articles (Figure 1). After excluding 652 articles (12 duplicates; 640 unrelated and/or not GAS), 40 abstracts were considered. A further 29 abstracts were excluded based on the exclusion criteria and 11 full text articles were retrieved. On examining the full text articles, 5 articles were excluded based on exclusion criteria. One article was sourced through expert knowledge resulting in the final total of 7 articles eligible for further evaluation (Table 1).

Results of the STREGA statement assessment are available as supplementary material. All HN radiotoxicity studies were first reports, declared the limitation of a small sample size and stated the need for validation in replication studies. Only 2 from 7 studies were undertaken prospectively. The number of patients included per study ranged from 32-140. There were 5/7 case-control and 2/7 cohort studies. Only three studies evaluated one primary site exclusively (oropharynx, 1; nasopharynx, 2). The mean age at treatment or diagnosis ranged from 50-61 years. Multimodality treatment was used in all studies (Table 2). A single ethnic group was considered in 3/7 studies involving Arab [18,21] and white American (non-Hispanic) [22] ancestries. Potentially confounding patient- and treatment-related factors were variably considered in all studies. Genotyping technique was described adequately in all studies but internal validation was not reported. Univariate analysis was performed in 3/7 studies [17,20,22] and when significant factors were identified, 1/3 study performed multivariate analysis [22]. The issue of population stratification was considered in three studies by using the Hardy-Weinberg equation [16,17,22], estimation of genotype frequency from a sample of 50 volunteers of similar age and gender [21] and consideration of allele frequency relating to the particular ethnic group [22].

The clinical endpoints evaluated were acute mucositis, acute dysphagia, acute dermatitis/erythema, subcutaneous fibrosis and osteoradionecrosis. These were graded using the Common Terminology Criteria for Adverse Events, CTCAE [23] (3 studies) and the Radiotherapy Oncology Group/European Organization for Research and Treatment of Cancer, RTOG/EORTC [24] (2 studies). Gastrostomy tube dependence was used as a clinical surrogate marker of radiation-induced dysphagia in one study [22]. Eleven polymorphisms in 8 genes were evaluated for association with acute radiotoxicity endpoints, and 6 polymorphisms in 4 genes were evaluated for late radiotoxicity endpoints (Table 2).

Acute mucositis: Three studies [16,19,20] evaluated this endpoint, consisting of two case-control studies, where severe acute mucositis (CTCAE, ≥ G2) was controlled against grades 0 or 1 [16,20] and one cohort study of patients demonstrating various RTOG grades of acute mucositis [19]. Cumulatively, there were 225 patients of various HN cancer subsites. IMRT (range mean tumour dose, 62-70 Gy; fractionated) was administered in three studies [16,19,20]. Chemotherapy was used variably in all studies. Cisplatin alone was used in 2 studies [16,20] while a platinum-derived agent was used in multiagent combination protocol in 1 study [19] (Table 2). The impact of chemotherapy was considered in all three studies by univariate and/or multivariate analyses. The risk of severe acute mucositis (≥ G2) was associated with the G allele of XRCC1 (c.1196 A > G) in patients treated with both radiotherapy alone and chemoradiotherapy (OR, 4.02; p = 0.025; CI=1.16–13.90) [18] (Table 4). Other assayed variants of DNA repair genes [16,20] and TGFβ1 [19] were not associated with severe acute mucositis (Table 4).

Acute dysphagia and acute dermatitis: Two case-control studies [16,20] evaluated these endpoints. IMRT was administered in all patients in one study [20] while the other study had an undisclosed proportion receiving IMRT with SIB [16]. While the mean tumour dose administered ranged between 62-70 Gy, attempts were made in both studies to provide a more accurate dose parameter in relation to the clinical endpoint. Both studies also considered the impact of chemotherapy using chi-squared test. Severe acute dysphagia (CTCAE, ≥ G2) was associated with the T allele in XRCC3 (722 C>T; OR=3.2; p=0.07) and the G allele in XRCC6 (1310 C>G; OR=4.08; p=0.014) and severe acute dermatitis (CTCAE, ≥ G2) was associated with the T allele of RAD51 (3392 G>T; OR= 2.02; p = 0.216) [20].

Tube dependence >180 days: Tube dependence >180 days as a surrogate marker of persistent dysphagia from radiotoxicity [22]. This prospective study consisted of a homogenous cohort of American white, non-Hispanics with oropharyngeal cancers treated by radiotherapy (with concomitant boost fractionation, 75/110 cases) and chemotherapy (57/110 cases). Variants of ERCC4 gene (T2505C and G1244A) were evaluated against tube dependence >180 days. The C allele of ERCC4 T2505C SNP was found to significantly reduce the requirement of long-term gastrostomy tube placement in irradiated and chemo-irradiated patients. Following adjustment for significant factors on univariate analysis, the adjusted OR was 0.20 (95% CI, 0.06–0.67).

Subcutaneous skin fibrosis: Two case-control retrospective studies [18,21] from the same centre evaluated subcutaneous fibrosis in a single ethnic group (Arab) with nasopharyngeal carcinoma treated using 3D radiotherapy (total neck dose, 66-70 Gy; fractionated) with chemotherapy employed in locally advanced diseases. Association between the risk of severe grade subcutaneous fibrosis with variants of TGFβ1 (-509 C>T), XRCC1 (1196 A > G), XRCC3 (722 C>T) and mitochondrial DNA (mtDNA) were evaluated. No significant association was found between the risk of severe subcutaneous skin fibrosis with both XRCC3 and TGFβ1 [18]. However, the G allele of XRCC1 (1196 A > G) was associated with a lower grade of subcutaneous fibrosis (OR 0.30, 95% CI: 0.10–0.89, P = 0.02), suggesting that wild-types were the risk alleles [18].

The possible association between mtDNA coding for mitochondrial respiratory activity with subcutaneous fibrosis was investigated [21]. This study found a significantly higher number of nonsynonymous genetic variations in the severe fibrosis group (RTOG, ≥ G2) as compared with the control (G0-G1) groups (p=0.003). The nonsynonymous A10398G variation in the NADH dehydrogenase subunit 3 gene was significantly associated with fibrotic reaction (p=0.01). Radiosensitive patients had a 7-fold (95% CI, 1.16-51.65) higher risk of developing moderate to severe fibrosis (RTOG, ≥ G2) postradiotherapy.

Osteoradionecrosis: The risk of developing ORN with TGFβ1 (-509 C>T) variant was evaluated in a multicenter, retrospective case-control study (n_{case} *vs* $n_{control}$: 39 *vs* 101) with heterogeneous diagnoses and

treatment regime (total radiotherapy dose, 50-65 Gy; fractionated with 2/39 cases of brachytherapy; chemotherapy given in some) [17]. Although univariate analyses of covariants were performed, the potential confounding factor of pre-extraction prophylactic measures, was not considered. ORN was found significantly associated with the T allele of TGFβ1-509 C>T polymorphism (OR, 4.2; 95% CI, 1.7-10.9), while the CC genotype was significantly associated with post-extraction related ORN. The positive and negative associations are summarized in Tables 3 and 4, respectively.

Discussion

Overall, this review found only a limited number (n=7) of normal HN tissue radiotoxicity GAS in comparison to the plethora of studies in other cancers [6]. Due to case heterogeneity, a meta-analysis was not undertaken. All studies used the CGA method in cohort and case-control studies with small numbers of subjects, which are often characteristic of exploratory research phase. Therefore, the results only offer, at best, hypothesis-generating findings needing validation in replication studies before any significant conclusions can be made. Bearing this caution in mind, the positive associations reported in these studies, and their biological pathways, were represented diagrammatically in Figure 2.

The DNA damage response is essential for the maintenance of genomic integrity following irradiation and consists of specific DNA repair pathways that are initiated based on the type of DNA damage present [25]. Double-strand DNA breaks occur frequently following irradiation and are repaired via homologous recombinant repair (HRR) and/or non-homologous end joining repair (NHEJ) [25]. Non-end joining repair (NER) is rarely utilized in radiation-induced DNA damage although it is influential in the repair of DNA adducts induced by platinum chemotherapy and where irradiation occurs in the presence of hypoxia [26]. DNA damage response genes were evaluated in 4/7 HN cancer studies based on the hypothesis that SNPs in these genes may alter the cellular capacity, particularly of cells showing high turnover, to repair sublethal damage following irradiation resulting in a more severe reaction. Unsurprisingly, these genes have been evaluated predominantly in acute HN radiotoxicity endpoints (3/4 studies) where variants of genes of the HRR pathway i.e. XRCC2, XRCC3 and RAD-51 paralogues [27] were found positively associated with acute mucositis [16,20].

The significant association of XRCC6 gene variants with severe dysphagia [20] may be due to the role of its gene product, Ku, as a double-strand DNA break sensor in NHEJ repair [28]. The ERCC1-XPF-ERCC4 complex performs a critical incision step in NER, and is also involved in the repair of DNA interstrand crosslinks [29]. This may be the underlying cause of the C-allele ERCC4 T2505C being significantly associated with a reduction in requiring long-term gastrostomy tube placement in irradiated and chemo-irradiated patients [24].

Radiation-induced fibrosis, a late radiotoxicity response, results from dysregulation of inflammation and regeneration. TGFβ1 retains a central role through its activation of fibroblasts into myofibroblasts [30]. Once activated, myofibroblasts within irradiated tissues become unregulated to produce abundant collagen types I and III, which are the hallmarks of fibrosis. TGFβ1 also mediates various other biological pathways including angiogenesis [31] and bone formation [32,33]. However, it is the role of TGFβ1 in radiation-induced fibrosis that provides the rationale for evaluating its genetic polymorphisms in radiotoxicities of normal HN soft and hard tissues [17-19] and led to the observations that the T-allele of TGFβ1 -509 C>T was significantly associated with ORN while the CC genotype of the -509 C>T was found significantly associated with ORN related to post-radiation extraction [17] (Table 3). In addition, the T allele of TGFβ1 -509 C>T was associated with a lower grade of skin fibrosis, suggesting that the wild-type of this allele was possibly related with late subcutaneous fibrosis in the Arab ethnic group [18].

Radiation-generated reactive oxygen radicals (ROS) cause critical imbalances in cellular redox state, leading to significant cellular damage from oxidative stress [34,35]. The mtDNA codes for components of the mitochondrial electron transport machinery, including NADH dehydrogenase, an energy-transducing enzyme [34]. mtDNA variants may impair energy conversion and promote tissue accumulation of ROS. Nonsynonymous A10398G variation in the NADH dehydrogenase subunit 3 gene was significantly associated with fibrotic reaction ($p=0.01$) [23]. No associations were identified between GSTP1 a peptide anti-oxidant that prevents ROS-induced metabolic oxidative stress, with HN radiotoxicity [16]. Unfortunately, the oxidative metabolism pathway-related genes have not been evaluated more extensively in HN radiotoxicity studies despite the influence of radiation-induced ROS in multiple biological pathways including DNA damage repair, radiation-induced fibrosis and chronic inflammatory responses [30,34,35].

With CGA, the selection and prioritization of candidate genes [36] directly impacts upon the results obtained, particularly in late radiotoxicity, where thought must be given to other genes that influence biological processes beyond cell survival [37]. Multiple biological processes are engaged in normal tissues following irradiation [30,34,35], culminating in a particular clinical radiotoxicity phenotype. Accordingly, multiple risk SNPs could contribute towards this phenomenon [38], including SNPs in biological pathways that have not been previously deduced. Furthermore, site-specific biological responses may occur in different tissue-types due to the unique tissue constituent and its interaction with the immediate environment. A compendium of site-specific factors and particular SNPs are associated with normal tissue radiotoxicity in lung [39], breast [40] and prostate [41,42] cancers, suggesting the possible influence of site-specific elements on selected SNPs in these circumstances. The incomplete understanding of mechanisms responsible for many complex traits (including radioresponsiveness) means that biological candidacy is inevitably speculative and could account for why this approach has so far yielded disappointing results in many common complex traits evaluated [43,44]. Thus, a move towards GWA in normal tissue radiotoxicity GAS is advocated [6,8,45]. To date, only one such study undertaken in a post-radiation prostate cancer cohort has been published, where GWA was used to evaluate the association of SNP with erectile dysfunction in African-American men [46].

Common methodological issues in GAS influence both CGA and GWA with sample size being a major determinant of quality. Studies with a small sample size, e.g. the HN studies reviewed here, are frequently under-powered to detect a correct result and also run the risk of over-estimating the effect size when a positive result is obtained. With the remote chance of finding common genes with significant effects, studies must be powered to detect variants that are either common but have low relative risk or variants that are rare but with higher relative risk, which entails massive samples sizes in the order of thousands [6,43]. Multicenter studies may overcome a small sample size problem by case pooling and this should be aimed for, particularly when replication studies are considered.

A case-control design is the mainstay of GAS because it allows comparison between two groups that are expected to differ in their SNPs prevalence [43]. In 5/7 HN radiotoxicity case-control studies reviewed, controls

were obtained from a larger HN cancer cohort, where the control group generally consisted of patients who developed comparatively milder grades of toxicity (3/5 studies) or those that did not exhibit the toxicity (2/5 studies). Defining the phenotype (i.e. radiotoxicity endpoint) is a fundamental methodological issue in radioresponsiveness since all irradiated patients are affected to some degree. Scoring systems for adverse events can help with phenotypic characterization [6]. Established radiation-specific scoring systems include the RTOG/EORTC classification [24] and the LENT/SOMA scale [47,48]. The CTCAE, which incorporates chemotherapy-related toxicities with RTOG/EORTC classifications [23], is increasingly used. However, its reliability could be undermined by dependence upon the clinician's subjective interpretation of the severity of toxicity present [49]. This may introduce the error of misclassification. Alternatively, distinct clinical endpoints of severe HN radiotoxicity can be used instead e.g., ORN, trismus and proximal oesophageal strictures. These clinical endpoints provide a more objective measure of radiotoxicity because of diagnostic unambiguity and in some instances, the prospect of quantitative assessment. This approach is also valuable when case pooling is considered in multicenter studies, where phenotype definition can be problematic when different scoring systems are used in different centres [40]. One HN study has used this approach in choosing ORN [17]. Other possible endpoints that may be considered in future HN radiotoxicity studies include imaging-based quantification of salivary gland function [50,51] and endoscopically-defined oesophageal strictures [52-54].

Establishing uniformity within case and/or control group is essential to reduce the confounding effects of other factors that may contribute towards HN radiotoxicity. Heterogeneity within case and/or control group is an overwhelming problem highlighted in this review, mainly due to multimodality treatment and also variations in treatment protocols. Patient-related factors could introduce heterogeneity [39,40]. Patient-related factors were considered variably in all studies evaluated in this review (Table 1). When it might not be possible to control for all these factors, employing multivariate statistical analysis could determine the level of significance of potentially confounding factors [43].

Radiation dosimetry and dose-volume differences can directly influence severity of radiotoxicity [5]. This problem may be addressed by homogenising radiation dose-volume parameters in critical areas (e.g. bone, pharyngeal muscle, skin and oral cavity mucosa) [52-54]. This is increasingly considered in radiotoxicity GAS in

other cancers [43,55,56]. In this review, one study determined homogenized doses based on the dose-volume histogram [20] while another study used biologically effective dose (BED) values [16]. BED accounts for the impact of radiation delivery and tissue tolerance to the biological effects observed [57] and is particularly used in other radiotoxicity GAS with fractionation protocols [4, 58-62].

Many HN cancer patients undergo surgery, but it is impossible to standardize the surgical procedures received by individual patients. Clinical endpoints common to surgery and radiotherapy e.g., trismus, esophageal strictures and skin fibrosis/scarring, should be quantified at the completion of one treatment modality before the addition of another. Recording of post-surgical morbidity at a specified time point e.g., 6 weeks post-surgery or 1 week pre-radiotherapy using standardized, valid and reliable definitions is fundamental to accurate measurement and monitoring of surgical adverse events. There is need for considered research and consensus in this area before it is possible to fully appreciate the range and degree of toxicity experienced by multimodality-treated HN cancer patients.

Genotyping methods were described adequately in the 7 assessed papers, but internal validation was not reported in any HN studies. This may reflect the small numbers of cases available. Future larger studies using GWA with high-throughput screening should be performed in accredited laboratories with standard operating protocols [63], considered as critical factors in the quality of GAS as assessed by STREGA [15]. All the HN cancer papers reviewed provided OR which was calculated individually for various genotypes or combinations of genotypes. The OR is presented for heterozygotes, homozygotes and for the combined group of heterozygotes and homozygotes rather than genotype relative risk values. Future studies ought to consider utilizing genetic models e.g., as suggested by Andreassen and Alsner [6], which accounts for the relationship between allele frequency and relative risk for genetic variants associated with normal tissue radiotoxicity.

Learning from the experiences of other cancer sites [6,42], future HN normal tissue radiotoxicity studies should focus on conducting well-designed pilot study and validating these findings in larger studies. A suggested model is a case-control study design with subjects of defined ancestry, who are recruited prospectively. Careful characterization of cases and controls that limits heterogeneity is paramount. Regarding approach,

GWA is a preferable platform over CGA due to its unbiased approach to the genome. When using the CGA, judicious selection of SNPs, quality control of genotyping and astute statistical analyses can optimize their usefulness and information [36].

Still, there is an opportunity in the present to undertake a robust GAS study using existing data. There is a potentially large repository of data available from various randomized control trials in HN cancer involving radiation and/or chemoradiation. These studies may provide a large sample size from case pooling with high-quality documentation on treatment parameters, toxicity and potential comorbidities. Other cancer sites have already moved towards multi-trial case pooling for validation of normal tissue radiotoxicity GAS, where 92 SNPs from 46 genes were evaluated in 1613 patients with breast and prostate cancers recently [63]. The amalgamation of trial data in HN cancer has started with the evaluation of the late complications in combined RTOG studies [64] and analyzing similarly accrued data in GAS of normal tissue radiotoxicity seems the logical next step forward.

Another consideration is the incorporation of GAS as part of on-going prospective HN cancer studies featuring tissue collection. In the United Kingdom, the Head and Neck 5000 study [65] may provide an excellent opportunity for a GAS for radioresponsiveness because of the expected large sample size, prospective recruitment and tissue banking. However, potential issues could stem from case heterogeneity due to variations in treatment received at different centers, the influence of ethnicity and perhaps the target of 5000 patients may yet provide adequate power to show for statistical relation between individual SNPs and radiotoxicity.

Conclusions

The association of common SNPs in normal tissue radiotoxicity following HN cancer treatment remains unproven. This is due to a combination of methodological issues. Preliminary results from these studies suggest the association of certain SNPs in genes involved in DNA damage response and radiation-induced fibrosis in the development of acute and late radiotoxicity endpoints. These findings require validation through

replication studies. Future HN radiotoxicity genetic association study design must incorporate critical methodological issues and technological improvements, including using GWA. However, there is an opportunity to make headway in the present through case pooling of existing clinical trial data, creating a larger sample size consisting of patients with well-characterized treatment and endpoints. Also, HN cancer clinical trials that are currently running should consider extending their toxicity evaluation to include genetic association studies. These avenues could increase the likelihood of finding useful biomarkers of treatment, and may provide new ways in approaching supportive care of HN cancer survivors in the future.

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Table legends:

Table 1. Summary of eligible studies.

Table 2: Summary of the treatment received (exposure), outcomes and genetic variants evaluated.

Table 3. Studies reporting positive associations.

Table 4. Negative associations obtained.

Figure legends:

Figure 1: Flow chart showing the literature search.

Figure 2: Diagram showing summary of genetic variants showing association with radiotoxicity.