Association between XRCC1 G399A Polymorphism and Late Complications to Radiotherapy in Saudi Head and Neck Cancer Patients

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ABSTRACT

Background: It has been hypothesized that patient to patient variation in normal tissue reactions to radiotherapy is associated with the presence of polymorphic variations in genes involved in DNA repair.

Purpose: To test for a possible association between two single-nucleotide polymorphisms (SNPs), XRCC1 399 G>A Arg/Gln and XRCC3 241 C>T Thr/Met and late reactions to radiotherapy.

Patients and Methods: In this case control study, 50 Head and Neck cancer patients were retrospectively recruited. The grade (G) of fibrosis, a late complication to radiotherapy, was scored using the RTOG/EORTC grading system. Radiosensitive patients with moderate to severe subcutaneous and deep tissue fibrosis (cases, G2-3, n=25) were matched with patients with minimal fibrotic reactions (control, G0-1, n=25). The two nonsynonymous SNPs were genotyped by direct sequencing of DNA extracted from blood or cultured fibroblasts.

Results: Allelic frequency showed significant association with grade of fibrosis for XRCC1 399 G/A (p=0.05), but not for XRCC3 241 C>T (p=0.10).

Conclusions: This pilot study corroborates the association between XRCC1 399 G>A and risk of late normal tissue complications following radiotherapy in our patients. Large studies are required to unravel more SNPs that can influence radiosensitivity and ascertain the associations with reactions to radiotherapy in order to be used as genetic predictive biomarkers of individual radiosensitivity.

Key Words: Single nucleotide polymorphism – Radiosensitivity – Late reactions to radiotherapy – XRCC1 – XRCC3.

INTRODUCTION

Ionizing irradiation is an effective modality to kill neoplastic tissues and about 50% of cancer patients receive curative radiotherapy (RT) during the management of their disease. The chances to achieve local tumor control are improved with increasing the radiation doses; however normal tissues in the irradiation field are also affected and their limits of tolerance pre-set deliverable doses. Furthermore, patients vary considerably in their normal tissue response to RT even after similar treatment [1]. Although many factors could influence tissues radiosensitivity, large parts of inter-individuals variations remain unexplained (Fig. 1). The limits of tolerance of normal tissues are the dose limiting factor in radiotherapy. This patient-to-patient variations in normal tissue reactions to radiotherapy raise the possibility of developing biomarkers or predictive assays for radiosensitivity. The rational is that if the radiosensitivity of normal tissue is known, radiotherapy doses can be tailored to each individual patient. Since hypersensitive patients who keep doses low are only a minority of cancer patients (about 5%), the individualized treatment should allow escalating the radiation dose to improve local tumor control while reducing overall complications. This goal has been supported by the demonstration of a possible positive therapeutic gain from applying knowledge of radiosensitivity to treatment planning [2-3]. This assumption is sustained
by case-report studies where RT doses prescriptions were successfully reduced to account for the increased radiosensitivity of the patients [4-5]. Although many factors could influence the severity of reactions to RT, large parts of interpatient variability is attributed to individual differences in radiosensitivity, which is determined by genetic variations among patients [6].

The well-known examples are the mutations in the ataxia telangiectasia (ATM), the NIBRIN (Nijmegen Breakage Syndrome), the DNA ligase IV and also MRE11 genes, which are components of cell cycle control and DNA repair [7-10]. However, gene mutations are rare and can only explain a minority of exquisitely sensitive patients. Recently, attention was focused on the more common polymorphic variations in candidate genes to explain the wide range of radiosensitivity observed in patients treated with RT [11]. The hypothesis is that single nucleotide polymorphisms (SNPs) could account for a proportion of such genetic component [12]. The SNPs can influence the rate of mRNA transcription, mRNA stability, its rate of translation to protein and/or the protein-protein interactions leading to sub-optimum protein function leading to different degrees of clinical radiation sensitivity.

To test this hypothesis, investigators targeted SNPs in candidate genes assumed to be involved in the response to radiation injury such as ataxia telangiectasia mutated (ATM), superoxide dismutase 2 (SOD2), tumor growth factor beta 1 (TGFβ1), X-ray repair cross-complementing group 1 (XRCC1), X-ray repair cross–complementing group 3 (XRCC3) and apurinic / apyrimidinic exonuclease (APEX) (Reviewed in [13]). Preliminary results suggest possible association between certain SNPs and the risk to develop normal tissue complications. In this pilot case control study, we sought confirmation of these observations in 50 Head and Neck cancer patients treated with definitive radiotherapy. We assessed the association between the grade of fibrosis, a late complication to RT and 2 selected non-synonymous SNPs (XRCC1 codon 399 G>A Arg/Gln and XRCC3 codon 241 C>T Thr/Met) previously described as potentially associated with clinical radiosensitivity [12].

Fig. (1): Schematic representation of factors involved in response to radiation treatment with emphasis on those that can potentially be used for predictive/prognostic testing.
MATERIAL AND METHODS

Patients' population and clinical data:
A total of 50 Head and Neck cancer patients with nasopharyngeal carcinoma were retrospectively recruited for this study during the follow-up of their disease. The patients were treated at the Radiation Oncology Department at the King Faisal Specialist Hospital and Research Centre. The standardized radiation treatment was planned using CT-based (computerized tomography) 3D conformal technique and involved definitive RT with no surgery. Locally advanced stages also received neoadjuvant and concurrent chemotherapy consisting of cisplatinum and epirubicin [14]. Total radiation doses were 66 or 70Gy delivered using 2Gy per fraction per day. The primary tumor site received the maximum dose using 6MV photon linear accelerator. The grade (G) of subcutaneous and deep tissue fibrosis, a late radiation-induced side effect, was jointly scored by the two participating physicians at the recruitment visit according to the RTOG/EORTC grading system. In this pilot case-control study, 25 patients with moderate to severe subcutaneous or deep tissue fibrosis (G2-3) were selected and matched for age, gender and treatment characteristics with 25 patients with no or mild fibrotic reactions (G0-1). For groups comparison, the patients with no or minimal fibrotic reactions (G0-1) were referred to as control (25 patients) and the patients with moderate to severe fibrosis (G2-3) were referred to as the radiosensitive group (cases, 25 patients). An effort was made to balance selected patients between these two groups taking into account the total radiation dose and the chemotherapy received. The institutional basic and ethics research committees approved the study and all patients signed an informed consent.

DNA extraction, amplification, sequencing and genotyping of polymorphisms:
The selected SNPs along with the PCR primers are listed in Table (1). DNA was extracted from cultured fibroblasts established from punch skin biopsies [15] or from cells contained in 5 ml peripheral blood samples, using Puregene DNA Purification Kit (Gentra System, USA) according to the manufacturer’s instruction. Relevant segments of DNA were amplified by thermal cycling (95°C for 15min, 39 rounds of 95°C for 1min, 56°C for 1min and 72°C for 1min and final extension at 72°C for 7min) using HotStarTaq DNA polymerase (Qiagen), and 50ng template DNA in 25 microliter volume with standard reaction conditions. The quality of the PCR product was checked by running 5 microliter of the reaction on 1% agarose gel. The amplified fragment was directly sequenced using the DYEEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences) according to the manufacturer’s instruction and were run on the MegaBase 1000 sequencer (Applied Biosystems). Sequencing results were aligned to the corresponding reference sequence and the SNPs were genotyped using SeqManII sequence analysis software (DNASTAR Inc.).

Table (1): Assessed SNPs and primers used for PCR amplification and DNA sequencing.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Codon</th>
<th>SNP</th>
<th>AA substitution</th>
<th>PCR primers Forward</th>
<th>PCR primers Reverse</th>
<th>NCBI dbSNP id/Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>XRCC1</td>
<td>399</td>
<td>G/A</td>
<td>Arg/Gln</td>
<td>GCCCCTCAGATCGC</td>
<td>GATAAAGCGGCATT</td>
<td>rs25487</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CACCTAA</td>
<td>TCACAGACG</td>
<td></td>
</tr>
<tr>
<td>XRCC3</td>
<td>-strand</td>
<td>241</td>
<td>C/T</td>
<td>GGTTAGGCAACG</td>
<td>CTTGCGACCAAGC</td>
<td>rs861539</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Thr/Met</td>
<td>GCTGCTAC</td>
<td>ATAGACAA</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations:
SNP = Single-nucleotide polymorphism.  NCBI = National Center for biotechnology information.
PCR = Polymerase chain reaction. ID = Identification.
AA = Amino acid. XRCC = X-ray repair cross-complementing.

Data analysis:
The association between grade of fibrosis and SNPs genotype and allelic frequencies were measured by the odds ratio (OR) and its 95% confidence interval. The degree of significance was calculated using the Chi-Squares method, except in case of small sample size (<5), the Fisher’s Exact test was used. A p-value of 0.05 or less is considered statistically significant.
RESULTS

Patients and treatment:
The age of patients at RT ranged between 18 and 71 years with a median of 49 years. There were 43 males and 7 females. Only patients who completed 24 months of follow-up (range: 24-84 months, median: 39 months) were included. This is in our experience largely sufficient for the appearance and the intensification of subcutaneous and deep tissues fibrosis following radiotherapy. Control (G0-1 fibrosis) and radiosensitive (G2-3 fibrosis) groups were comparable for total radiation dose and chemotherapy received (Fig. 2). Therefore, the average dose received in the control group (67.12Gy, SD=1.8) was similar to that of the radiosensitive group (67.00Gy, SD=1.7). Likewise, the number of patients who received chemotherapy in the control group was the same as in the radiosensitive group (15 patients each). Comorbid diseases were infrequent: Overall 5 patients had diabetes and/or hypertension, 2 controls and 3 radiosensitives.

Genotyping analysis:
The genotype and allelic distributions of the assessed polymorphisms are listed in Table (2) and depicted in Fig. (3). Those were compared to the genotype and allelic frequency in blood samples of 50 volunteers of similar composition (Table 2). Overall, the frequencies in the cancer patients were comparable to those in the volunteers without cancer. Furthermore, the frequencies were quite comparable to those available at the National Center for Biotechnology Institute Website, suggesting similar distribution to the global human genotype frequencies. Fig. (3) showed variable genotype distributions according to the grade of fibrosis. However, apparent trends were observed between the allelic distribution and the grade of fibrosis (Fig. 3). Compared with the control group (G0-1); there was relative abundance of the wild-types XRCC1 399 G allele and the variant allele XRCC3 241 T in the radiosensitive (G2-3) patients. The association study showed that the variant XRCC1 399 A allele is associated with decreased risk (protective) while the variant XRCC3 241 T allele is associated with increased risk to develop fibrotic reactions following radiotherapy (odds ratios were 0.31 and 1.99; respectively, Table (2)). Interestingly, significant association was observed for XRCC1 399 Arg/Gln G>A (p=0.05), whereas no significant association with radiosensitivity was detected for XRCC3 241 Thr/Met C>T (p=0.10). This indicates that the variant XRCC1 399A (Gln) allele was significantly less frequent in the radiosensitive G2-3 group of patients.

Table (2): Genotype and allele frequencies of 2 assessed polymorphisms in 50 nasopharyngeal carcinoma patients treated at the Radiation Oncology section at KFSHRC. Patients either developed minimal (control: G0-1) or substantial (radiosensitive: G2-3) late reactions (fibrosis) after radiotherapy.

<table>
<thead>
<tr>
<th>Gene, genotype and allele</th>
<th>Grade of fibrosis n (%)</th>
<th>Odds Ratio (95% CI)</th>
<th>Significance level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G2-3 (n=25)</td>
<td>G0-1 (n=25)</td>
<td></td>
</tr>
<tr>
<td>XRCC1 codon 399 G&gt;A Arg/Gln</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>21 (84)</td>
<td>17 (68)</td>
<td>0.65 (0.15-2.79)</td>
</tr>
<tr>
<td>G/A</td>
<td>4 (16)</td>
<td>5 (20)</td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td>0</td>
<td>3 (12)</td>
<td>0.31 (0.09-1.04)</td>
</tr>
<tr>
<td>G</td>
<td>46 (92)</td>
<td>39 (78)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>4 (8)</td>
<td>11 (22)</td>
<td>0.19*</td>
</tr>
<tr>
<td>XRCC3 codon 241 C&gt;T Thr/Met</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>7 (28)</td>
<td>12 (48)</td>
<td></td>
</tr>
<tr>
<td>C/T</td>
<td>13 (52)</td>
<td>11 (44)</td>
<td>2.03 (0.59-6.93)</td>
</tr>
<tr>
<td>T/T</td>
<td>5 (20)</td>
<td>2 (8)</td>
<td>4.29 (0.65-28.26)</td>
</tr>
<tr>
<td>C</td>
<td>27 (54)</td>
<td>35 (70)</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>23 (46)</td>
<td>15 (30)</td>
<td>1.99 (0.87-4.52)</td>
</tr>
</tbody>
</table>

KFSHRC: King Faisal Specialist Hospital & Research Centre
NA: Not available because one value is equal to zero.
* 2-tailed Fisher’s Exact test.
Fig. (2): Distribution of the 50 nasopharyngeal carcinoma patients according to the total radiation dose received. The patients developed either minimal (control: G0-1) or substantial (radiosensitive: G2-3) fibrotic reactions following radiotherapy.

Fig. (3): Genotype and allelic distribution of XRCC1 codon 399 G>A Arg/Gln and XRCC3 codon 241 C>T Thr/Met polymorphisms in 50 nasopharyngeal cancer patients who developed different grade of radiation-induced fibrosis.
DISCUSSION

The aim of this pilot study was to evaluate in our cancer patients treated with radiation whether nonsynonymous SNPs in XRCC1 and XRCC3 genes are associated with the grade of fibrosis, a late reaction to radiotherapy. The selected SNPs (XRCC1 codon 399 G>A Arg/Gln and XRCC3 codon 241 C>T Thr/Met) were previously described, although none unequivocally, as potentially associated with clinical radiosensitivity in Caucasian patients [12]. These are nonsynonymous SNPs that lead to amino acid change in the translated protein and therefore, they have the potential to alter protein function and contribute to variations between patients. The 50 cancer patients included in this study followed standardized radiation treatment that was delivered using 6MV photon linear accelerator. The total radiation doses were either 66 or 70Gy given as 2Gy per fraction. Locally advanced tumors are also treated with neoadjuvant and concurrent chemotherapy consisting of cisplatinum and epirubicin [14]. Patients were carefully selected for this study during the follow-up of their disease. All patients completed at least 2 years of follow-up which is in our experience largely sufficient for the appearance and the intensification of subcutaneous and deep tissues fibrosis following radiotherapy. The patients were well balanced between the radiosensitive (G2-3) and the control (G0-1) groups taking into account the total radiation dose and the chemotherapy received (Fig. 2). Therefore, overall no differences could be attributed to treatment related factors.

Results presented here showed significant association between clinical radiosensitivity and the allelic frequency for XRCC1 399 G>A polymorphism ($p=0.05$; Table 2). These are encouraging results and suggest that this genetic variation is contributing factor to the severity of fibrotic reaction following radiotherapy. As compared to the control (G0-1), the radiosensitive group (G2-3) harbored relatively lower number of the variant XRCC1 399 A alleles that appeared to have protective effect ($OR=0.31$) and therefore the wild-type allele could be considered as a risk factor.

It is important to notice that in line with a number of other studies, our results established significant associations between clinical radiosensitivity (grade of fibrosis) and XRCC1 399 G>A Arg/Gln polymorphism. In agreement with other studies we found that the wild-type XRCC1 399 G allele (Arg) is associated with increased risk to develop late reactions to radiotherapy (Reviewed in [13]). These results suggest that the variant (or minority) allele could confer higher radiation resistance which would be advantageous for normal tissues in radiation treatment. At the molecular level XRCC1 protein is required for efficient DNA single-strand breaks repair to maintain genomic stability in human cells and its reduction leads to increased sensitivity to cell killing by ionizing radiation [16]. The codon 399 is situated in the BRCT I active domain of the protein and both wild-type and variant alleles were found to be in vitro equally functional [17]. Nevertheless, the results of present and similar clinical studies seem to be counter intuitive to in vitro studies. However a study by Brem et al., suggested that it is the haplotype in the XRCC1 gene (i.e. segregation with other SNPs) rather than the 399 G>A SNP per se that is possibly associated with cellular or clinical radiosensitivity [18].

As for XRCC3 241 Thr/Met C>T, the association study did not reach statistical significance and more patients are required to reach robust conclusion. However, the results showed a trend ($p=0.10$) toward association with the XRCC3 241 T variant allele being the risk factor ($OR=1.99$, CI: 0.81-4.92). A study by Andreassen et al. [12] suggested that the XRCC3 241 wild-type C (Thr) is the risk factor. However, previously published reports indicated that the XRCC3 wild-type allele is associated with less bulky DNA adducts than the variant allele suggesting more efficient DNA repair in vitro [19]. This indicates that we are still far from having an exhaustive understanding of the relationship between genetic variations and clinical normal tissue radiosensitivity. These results, however, are encouraging and warrant large scale multicenter studies of cancer patients to clarify the value of these findings and uncover more polymorphic variations in susceptible genes that can be used as predictive biomarkers to individualize radiation therapy on genetic bases.

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