Association between genetic polymorphisms and carotid atherosclerosis in patients treated with radiotherapy for nasopharyngeal carcinoma

Chuang Yuan¹,³, Shea Ping Yip¹, Vincent WC Wu¹, Dora LW Kwong², Isabella WY Cheuk¹ and Michael Ying¹

Abstract

Background: Radiotherapy (RT) of the neck is commonly given to nasopharyngeal carcinoma (NPC) patients for preventing cervical lymph node metastasis. However, neck RT may induce the development of carotid atherosclerosis. The mechanisms of radiation-induced carotid atherosclerosis are still unclear and no previous study has investigated the genetic involvement of radiation-induced carotid atherosclerosis. The present study aims to determine the association between genetic polymorphisms and carotid atherosclerosis in patients treated with RT for nasopharyngeal carcinoma.

Methods: The present study recruited 128 post-RT NPC patients. Carotid plaque score was assessed using ultrasonography. Thirteen single nucleotide polymorphisms (SNPs) that affect the function of anti-atherosclerotic genes, including SOD2, SOD3, CAT, PON1, PPARG, ADIPOQ, IL10, TGFB1 and NOS3, were genotyped. Association between the 13 SNPs and carotid atherosclerosis was evaluated using multiple regression after adjustment for covariates (PLINK). Multiple testing was corrected using Benjamini-Hochberg step-up false discovery rate controlling procedure.

Results: rs662 and rs705379 of PON1 were close to be significantly associated with carotid plaque score (Corrected P value, P<cor> = 0.0528 and P<cor> = 0.0842). When the two SNPs were combined together, TC haplotype in rs662-rs705379 of PON1 was significantly associated with higher carotid plaque score (P<cor> < 0.05). None of the other SNPs showed significant association with carotid plaque score.

Conclusions: TC haplotype in rs662-rs705379 of PON1 is likely to be a genetic risk factor of carotid plaque score. Post-RT NPC patients with the TC haplotype may need earlier and more frequent carotid ultrasound examinations for early detection of carotid atherosclerosis.

Keywords: Radiation, Nasopharyngeal carcinoma, Carotid atherosclerosis, Carotid plaque score, Single nucleotide polymorphisms, Paraoxonase

Background

Nasopharyngeal carcinoma (NPC) is a common head and neck malignancy in Southeast Asia and Southern China [1]. Radiotherapy (RT) is the standard strategy for treating nasopharyngeal carcinoma (NPC). Owing to the high prevalence of cervical lymph node metastasis in NPC patients, RT of the neck is usually given to the patients for preventing or treating the nodal metastasis [2]. However, ionizing radiation in neck RT damages the carotid artery and may induce carotid atherosclerosis, which may lead to cerebrovascular events [3-6].

The mechanisms of radiation-induced carotid atherosclerosis are still unknown. However, the mechanisms of spontaneous atherosclerosis are well established, which provide baseline information for the understanding of the mechanisms of radiation-induced carotid atherosclerosis. Different pathways in the regulation of oxidative stress, lipid metabolisms, and inflammation may protect the carotid artery from atherosclerosis. There are many genes whose encoded proteins are involved in these protective pathways. Superoxide dismutases (SODs) are the primary enzymes in the defense of oxidative stress, which

* Correspondence: htimying@polyu.edu.hk
1Department of Health Technology and Informatics, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong, SAR, China
Full list of author information is available at the end of the article
convert the toxic superoxide anions to the less toxic hydrogen peroxide ($H_2O_2)$ [7]. Catalase (CAT) further scavenges the toxic $H_2O_2$ by converting it into water ($H_2O$) and molecular oxygen ($O_2$) [8]. Paraoxonase 1 (PON1) prevents the oxidation of low intensity lipoprotein (LDL), and inhibits the uptake of oxidized LDL by and cholesterol synthesis in macrophages [9]. Peroxisome proliferators-activated receptor $\gamma$ (PPARG) is a pivotal nuclear receptor that regulates the expression of genes involved in lipid metabolisms and inflammatory responses [10]. Adiponectin (ADIPOQ) suppresses the inflammatory responses and the uptake of oxLDL by macrophages [11]. Interleukin-10 (IL10) and transforming growth factor-$\beta$1 (TGFB1) are the most important anti-inflammatory cytokines in immune cells [12,13]. Endothelial nitric oxide synthase (NOS3) catalyzes the production of nitric oxide, which prevents platelet aggregation, adhesion molecule expression in endothelial cells and vascular SMC proliferation [14]. Some single nucleotide polymorphisms (SNPs) that affect the expression of these genes (SOD2, SOD3, CAT, PON1, PPARG, ADIPOQ, IL10, TGFB1 and NOS3) and/or the functions of their corresponding proteins have been shown to be associated with spontaneous atherosclerosis [15-24]. However, the association of these SNPs with radiation-induced carotid atherosclerosis is still unknown. Therefore, the present study was undertaken to investigate the association between the SNPs in these nine genes and the severity of carotid atherosclerosis in post-RT NPC patients. The findings will offer potential genetic markers of radiation-induced carotid atherosclerosis, which might facilitate the selection of high-risk patients with carotid atherosclerosis so that timely diagnosis and treatment can be given to the patients.

**Methods**

**Subjects**

Post-RT NPC patients were recruited from the Department of Clinical Oncology of Queen Mary Hospital from March 2013 to March 2014. The inclusion criteria of subjects were local residents, Han Chinese NPC patients, older than 18 years, and completed RT for at least four years, whilst the exclusion criteria of subjects were more than one course of RT, history of carotid atherosclerosis prior to RT, previous carotid endarterectomy and carotid stenting.

This study was approved by the Human Subject Ethics Subcommittee of the Hong Kong Polytechnic University and the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. Written consent was obtained from all patients before the commencement of the interview and ultrasound examination.

**Clinical information**

Archived clinical records were reviewed and individual face-to-face interviews were conducted. The information of post-RT duration, radiation dose, chemotherapy and history of carotid atherosclerosis was obtained from archived clinical records. The presence of cardiovascular risk factors was identified as follows: 1) DM, diagnosed with DM in the clinical record, taking medications to lower blood glucose and/or fasting plasma (blood) glucose $\geq 7.0$ (6.1) mmol/L [25]; 2) hypertension, diagnosed with hypertension in the clinical record, undergoing anti-hypertensive medications and/or the measured blood pressure $\geq 140/90$ mmHg [26]; 3) hypercholesterolemia, diagnosed with hypercholesterolemia in the clinical record, undergoing medications to lower the cholesterol level and/or fasting total cholesterol $\geq 5.2$ mmol/L [27]; 4) CHD, diagnosed with coronary vascular disease in the clinical record and/or had coronary stenting [28]; 5) smoking, current smoker consuming 10 cigarettes per day for at least six months [28].

**Selecting and genotyping of SNPs**

A literature research was performed for the selection of candidate genes and relevant SNPs in the present study (PubMed). Candidate genes involving oxidative stress, lipid metabolism and inflammation and having association with atherosclerosis were reviewed. Only the genes, in which SNPs influence the expression of these genes or the function of the encoded proteins, and have minor allele frequency $>0.1$ in Han Chinese and evidences for association with spontaneous atherosclerosis, including carotid atherosclerosis, coronary atherosclerosis, and ischemic cardiovascular and cerebrovascular diseases, were selected. In total, 13 SNPs in the 9 genes, SOD2, SOD3, CAT, PON1, PPARG, ADIPOQ, IL10, TGFB1 and NOS3, were included in the present study (Table 1) [15-24]. Genomic DNA was extracted from 6 ml of peripheral blood for genotyping. Restriction fragment length polymorphism (RFLP) and unlabeled probe melting analysis (UPMA) were used for genotyping as described previously [29-31]. The primers and probes used in genotyping are shown in Table 1.

**Ultrasound examinations**

Carotid ultrasound examinations were performed in a 22°C air-conditioned examination room using the Esaote MyLab Twice ultrasound unit in conjunction with a 4–13 MHz linear transducer (Esaote, Genoa, Italy). Subjects lied supine on the examination couch with the neck slightly extended and the head turned away from the side under examination. Using gray-scale ultrasound, the extra-cranial carotid artery was screened longitudinally and transversely. Carotid plaque was identified as a focal thickening $>50\%$ of the adjacent intima-media layer [32].
Once a carotid plaque was identified, transverse gray-scale images of the plaque were obtained and the degree of carotid stenosis was expressed as a percentage reduction of the lumen diameter at the most stenotic site. Carotid plaque score was evaluated using an adjusted plaque scoring system [28]. In the scoring system, the carotid artery was divided into five segments: 1. Proximal common carotid artery (≥2 cm proximal to carotid bifurcation); 2. Distal common carotid artery (<2 cm proximal to carotid bifurcation); 3. Carotid bulb and bifurcation; 4. Internal carotid artery; and 5. External carotid artery. The degree of carotid stenosis in each segment was measured and carotid plaque score was expressed as the summation of the degree of carotid stenosis of all segments in both carotid arteries (Figure 1).

Statistical analysis
Data of carotid plaque score was transformed logarithmically because it was not normally distributed. Testing of genotypes for Hardy-Weinberg equilibrium (HWE) in all subjects was determined by exact test as executed in PLINK (version 1.07, [33]). The threshold for significant deviation from HWE was set as 0.01 [34]. Only markers fulfilling HWE were included in association analyses.

In the potential covariates, such as age, gender, radiation dose, chemotherapy, post-RT duration and cardiovascular risk factors, the significant predictors in regression models were adjusted in association analyses. Linear regression executed in PLINK was used for assessing the association between single SNP and carotid plaque score with adjustment for post-RT duration and number of cardiovascular risk factors (significant predictors in regression models). The regression analysis was performed under additive, dominant or recessive models. FDR correction was used for correcting multiple testing. $P_{cor} < 0.05$ was considered as significant for association analysis.

Three genes, $PON1$, $ADIPOQ$ and $TGFB1$, had more than one SNP examined in the present study. The haplotypes in each of these three genes were determined for the association with carotid plaque score in post-RT NPC patients. Sliding window (2 or 3 SNPs per window) using linear regression in PLINK was utilized for the association analysis with adjustment for post-RT duration and number of cardiovascular risk factors. FDR correction was also used for correcting multiple testing. Linkage disequilibrium (LD) statistics $D'$ and $r^2$ in paired SNPs were calculated using Pairwise LD in PLINK. $P_{cor} < 0.05$ was considered as significant for association analysis.

Results

**Demographic information**
A total of 128 post-RT NPC patients were included in the present study. All patients were treated conventional 2D RT of the neck. The mean age of the patients was 55.2 ± 8.8 years with a range of 33 to 86 years. There were 86 males and 42 females. The mean radiation dose
was 66.82 ± 3.20 Gy with a range of 58.44 to 73.72 Gy. Of the 128 patients, 63 were also treated with chemotherapy. The mean post-RT duration was 12.8 ± 6.0 years with a range of 4 to 37 years. The most common cardiovascular risk factor was hypercholesterolemia (n = 39), followed by hypertension (n = 35) and then by DM (n = 14). Only 5 patients were current smoker, and 5 patients had CHD and 7 patients developed stroke or transient ischemia attack (Table 2).

Association analysis

Genotype proportions were all in HWE for 13 SNPs (P > 0.01, Table 3). In the 13 SNPs, only rs662 and rs705379 in PON1 were close to be significantly associated with carotid plaque score in post-RT NPC patients (rs662, P_cor = 0.0842 in additive model and P_cor = 0.0528 in dominant model; and rs705379, P_cor = 0.0842 in additive and dominant models, Table 3). T allele of rs662 and C allele of rs705379 were the risk alleles for higher carotid plaque score (rs662, TT + TC vs CC: 1.76 ± 1.60 vs 1.07 ± 0.97; rs705379, TT + TC vs CC: 1.22 ± 1.01 vs 1.79 ± 1.82). When the two SNPs were combined, the haplotype window rs662-rs705379 in PON1 had a significant association with carotid plaque score (P_cor < 0.05, Table 4). TC haplotype of rs662-rs705379 posed a higher risk for higher carotid plaque score (unstandardized coefficients = 0.0873, P_cor < 0.05). None of other SNPs and haplotypes showed significant association with carotid plaque score (P_cor > 0.05, Tables 3 and 4).

Discussion

Carotid atherosclerosis is a common complication in post-RT NPC patients. However, the mechanisms of radiation-induced carotid atherosclerosis are still unclear and no previous study has reported the association between genetic polymorphisms and radiation-induced carotid atherosclerosis. The present study comprehensively investigated the association between 13 SNPs in anti-atherosclerotic genes and radiation-induced carotid atherosclerosis. Results showed that SNPs in PON1 tended to

### Table 2 - Demographic information of post-RT NPC patients

| Parameters                        | Total n = 128 |
|-----------------------------------|--------------|
| Age, years                        | 55.2 ± 8.8   |
| Gender (female/male), n           | 42/86        |
| Chemotherapy, n (%)               | 63 (49.2%)   |
| Radiation dose, Gy                | 66.82 ± 3.20 |
| Post-RT duration, years           | 12.6 ± 6.0   |
| Hypercholesterolemia, n (%)       | 39 (30.5%)   |
| Hypertension, n (%)               | 35 (27.3%)   |
| Diabetes mellitus, n (%)          | 14 (10.9%)   |
| Current smoker, n (%)             | 5 (3.9%)     |
| Coronary heart disease, n (%)     | 5 (3.9%)     |
| Stroke or transient ischemia attack, n (%) | 7 (5.5%) |
| Presence of carotid plaque, n (%) | 114 (89.1%) |
| Carotid plaque score              | 1.41 ± 1.37  |
be genetically associated with carotid plaque score in post-RT NPC patients.

PON1 is one of the important enzymes for hydrolyzing LDL oxidation, playing a pivotal role against carotid atherosclerosis. The SNP rs662 (T > C) locating in the coding region of PON1 gene replaces glutamine (Q) by arginine (R) at codon 192 (Q192R). This variation affects the activities of PON1 in the hydrolysis of different substrates. The 192Q allozyme has higher hydrolytic activity toward diazoxon, soman, and sarin, while the 192R allozyme is more efficient for hydrolyzing paraoxon and fenitroxon [35,36]. Another important variation, rs705379, is located at position −107 of the promoter region (−107 T/C), which contributes to a decrease in the PON1 expression level and PON1 circulating concentration [37].

In the present study, significant observed P values were found in the association analyses between carotid plaque score and rs662 as well as rs705379 in additive and dominant models (rs662, P = 0.0054 and 0.0014 respectively; rs705379, P = 0.0077 and 0.0086 respectively). The significant association failed to survive in the correction for multiple testing by FDR, but it was close to be significant (P_{corr} = 0.0528 and 0.0842 respectively). In the association analyses with rs662 and rs705379, the statistical power was 0.524 and 0.302 respectively. To achieve a statistical power of 0.8, at least 194 and 283 patients would be needed respectively. Therefore, small sample size in the present study (n = 128) may account for the non-significant findings. Future studies with larger sample sizes are needed for investigating the association. Nevertheless, the two SNPs had a cumulative effect on carotid plaque score. Patients carrying T allele in rs662 (QR + QQ, n = 64) had 1.76 ± 1.60 of carotid plaque score, whilst those with CC genotype in rs705379 (n = 44) had 1.79 ± 1.82 of carotid plaque score. In patients carrying both T allele in rs662 and CC genotype in rs705379 (n = 38), the plaque score was 1.94 ± 1.89. TC haplotype in rs662-rs705379 showed significant association with the plaque score after the correction for multiple testing (P_{corr} = 0.0158). Thus, rs662 and rs705379 in combination were more powerful to detect the association with carotid plaque score in the present study. TC in rs662-rs705379 would be the risk haplotype for carotid plaque score in post-RT NPC patients.

D’ and r² of the two SNPs were 0.871 and 0.294 respectively. The high D’ indicated that the two SNPs were in LD and were co-inherited most of the time. However, the different frequencies of alleles in the two SNPs (minor allele frequency = 0.3086 and 0.4648 respectively) resulted in the low r². Therefore, the two SNPs cannot predict for each other.

In contrast to previous studies in which the R variant of the Q192R polymorphism (C allele in rs662) was a risk factor for spontaneous atherosclerosis diseases [21,38], the present study found that the R variant would be protective for radiation-induced carotid atherosclerosis. Patients carrying RR had lower plaque score as compared to those with QR and QQ genotypes (RR vs QR + QQ: 1.07 ± 0.97
호르몬적 역할을 하는 것은 아니지만, 특히 동물실험에서 관찰된 사례는, 이러한 플래크 조성에 대한 연구의 중요성을 보여준다. 이는 치료 후의 적혈구의 변화, 이온화 방사선 치료에 의한 동맥 장애의 역할을 이해하는 데 중요한 역할을 한다. 따라서 이러한 연구는 치료 후의 적혈구의 변화와, 이온화 방사선 치료에 의한 동맥 장애의 역할을 이해하는 데 중요한 역할을 한다.
