Int J Clin Exp Pathol 2016;9(11):11868-11875
www.ijcep.com /ISSN:1936-2625/IJCEP0032514

Original Article

ERCC5 single nucleotide polymorphism (rs2296147) predicts the risk of acute radiation pneumonitis in lung cancer patients undergoing radiotherapy

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Received May 20, 2016; Accepted August 4, 2016; Epub November 1, 2016; Published November 15, 2016

Abstract: We explored the correlation between DNA damage repair gene polymorphisms (rs4462560 and rs7402844 locus of *NEIL1* gene, h*OGG1* rs1052133 and rs293795 locus, *FEN1* gene rs174538 and rs4246215, *ERCC2* rs238406 locus, *ERCC5* rs2296147 locus) and radiation pneumonitis (RA) in lung cancer patients treated with radiotherapy at the Zhangjiagang First People's Hospital and Aoyang hospital. Out of 167 patients who received chest radiotherapy for lung cancer, 34 patients (20.4%) had RP grade ≥ 2. We detected eight kinds of DNA repair-based SNPs in these 34 patients. The results showed an association of *ERCC5* rs2296147 CC, CT and CT/CC genotypes with ≥ grade 2 acute RP, which suggests that rs2296147 locus of *ERCC5* gene may be associated with risk of RP after radiotherapy for lung cancer.

Keywords: Single nucleotide polymorphism, *ERCC5,* acute radiation pneumonitis

Introduction

Incidence of lung cancer, which is one of the most common malignant tumors, has shown an increasing trend [1, 2]. Radiation therapy is one of the main treatment modalities for lung cancer. Radiation pneumonitis (RP) is the most common dose-limiting factor in patients with lung cancer receiving chest radiotherapy or concurrent chemoradiotherapy, which seriously impacts their quality of life and survival [3]. Dosimetric parameters, smoking history, the average amount of radiation received by lung, and lung V20 are significant correlates of radiation-induced lung injury [4-7]. However, considerable inter-individual variability in radiation-induced injury among patients with comparable clinical parameters, and who are treated with equivalent doses of radiation, has been documented. This suggests an individual's susceptibility to radiation side effects may have a genetical basis. Identification of genetic markers that may help optimize radiation therapy is a key imperative.

Mammalian cells have evolved distinct pathways to repair different types of DNA damage in order to maintain genome stability. Nei endonuclease VIII-like 1 (*NEIL1*), 8-hydroxyguanine DNA glycosylase (*hOGG1*) and flap structure-specific endonuclease 1 (*FEN1*) are responsible for the base-excision repair (BER) pathway, which repairs DNA damage caused by reactive oxygen species and alkylating agents. Similarly, *ERCC2* and *ERCC5* are involved in the nucleotide excision repair (NER) pathway, which repairs DNA damage caused by ultraviolet light. Single nucleotide polymorphisms (SNPs) alter the function or expression of DNA repair genes, and are thus thought to be closely associated with radiation damage. In this study, we investigated the association between SNPs of DNA repair genes and RP in lung cancer patients undergoing radiotherapy. The aim is to provide a theoretical basis for developing personalized preventive, diagnostic and therapeutic strategies for patients with lung cancer.

Materials and methods

Clinical data

A total of 167 (105 men and 62 women) patients with lung cancer undergoing radiotherapy at

Table 1. Primers for detection of SNPs of DNA repair genes

Gene symbol	SNP sites		Primer Sequence	Product
NEIL1	rs4462560	Up	5'-CCCCATCTTCCACATCTTT-3'	762 bp
		Down	5'-TCCCTGTTGCTCGTGCTT-3'	
NEIL1	rs7402844	Up	5'-GAATCTCCCTATGTTGACC-3'	430 bp
		Down	5'-ATCTCATTGTCCCACTTTA-3'	
hOGG1	rs1052133	Up	5'-TGGATTCTCATTGCCTTCG-3'	498 bp
		Down	5'-CACCTGCTTCCCTACCACT-3'	
hOGG1	rs293795	Up	5'-AGAAGGGCATGGGTGGTC-3'	592 bp
		Down	5'-CGCAGAAAGGGTTCCAAA-3'	
FEN1	rs174538	Up	5'-GCATTGTAGGATGGGCACG-3'	517 bp
		Down	5'-CCTTCTCCACCGCTTGTC-3'	
FEN1	rs4246215	Up	5'-GCAAGGAGCCAGAACCCA-3'	630 bp
		Down	5'-CAGCCAGTAATCAGTCACAAAC-3'	
ERCC2	rs238406	Up	5'-ACTGAGTATCGAGCAAGGAAG-3'	382 bp
		Down	5'-GTTTGAAGAGTGGTTGGGTT-3'	
ERCC5	rs2296147	Up	5'-AAAGCCCGTTACTCCACC-3'	553 bp
		Down	5'-AAGCCCTCCCTGCTCCTAC-3'	

SNP, single nucleotide polymorphism.

Zhangjiagang First People's Hospital and Aoyang Hospital, between January 2013 and March 2015, were enrolled in the study. The diagnosis of lung cancer was confirmed on histopathology in all patients. Further, all patients had normal lung function prior to radiotherapy, and an ECOG score between 0-2. The histopathological types of lung cancer were: squamous cell carcinoma (N=75), adenocarcinoma (N=54), adenosquamous carcinoma (N=5) and small cell carcinoma (N=23). Sixty eight patients had central lung cancer while 99 had peripheral lung cancer. Clinical stage (as per 2009 UICC staging system): 13 cases were stage II, 58 were stage III A, 76 were stage III B and 20 were stage IV cases.

Chronic obstructive pulmonary disease (COPD) was present in 67 cases (grade I in 16 cases, grade II in 2 cases). Radiotherapy alone was administered to 34 patients, and sequential chemoradiotherapy to 132 patients. The chemotherapy regimens were EP (VP-16+Cisplatin) (N=23); TP (Taxel+Cisplatin) (N=81); NP (Navelbine+Cisplatin) (N=23); or PP (Pemetrexed+Cisplatin) (N=40). Concurrent chemoradiotherapy was employed in 68 patients using DDP (cisplatin) 20 mg/m^2 qw during radiotherapy.

Radiotherapy

The gross tumor volume (GTV, lung window) and mediastinal window shortest diameter ≥ 10 mm mediastinal lymph nodes (GTVnd); clinical target volume (CTV) for the GTV extended (adenocarcinoma 8 mm, squamous 6 mm), mediastinal lymph node metastasis according to site conditions prevent selective lymph node irradiation; planning target volume (PTV) to expand outside the CTV direction around, 5 mm, up and down outside the expansion 10~15 mm. 6 MV using linear accelerator 1.80-2.00 cGy/time, 1 time/d, 5 times/week, GTV total radiation dose of 50~70 Gy.

Evaluation of radiation pneumonitis

The patients were evaluated for RP after the start of radiotherapy and within three months of completion of radiotherapy as per American College of Radiology and Radiation Therapy Oncology Group (RTOG) radiation-induced lung injury criteria [8, 9]. The diagnostic criteria for RP included: 1) history of pulmonary radiotherapy; 2) clinical symptoms, such as irritating cough, chest pain, shortness of breath, and fever; 3) line on X-ray shows the dense shadow of a large radiation area.

The grading of RP was performed using the toxicity assessment criteria NCICTC 3.0 (jointly developed by the European and American Association). Level 1: asymptomatic, only imaging changes; Level 2: symptoms present but do not affect basic daily activities; Level 3: symptoms present and affect the basic daily activities, require oxygen supplementation; Level 4: life-threatening, require assisted ventilation; and Level 5: death.

We chose the end point of ≥ 2 level RP since this level shows persistent cough and/or breathing difficulties, and the need for clinical treatment. Depending on whether radiological pneumonia was present or not, patients were divided into two groups.

Experimental method

Detection of SNPs: Single nucleotide polymorphisms of eight DNA repair genes (*NEIL1* rs4462560 and rs7402844, *hOGG1* rs1052133

Table 2. Univariate analysis: Clinical correlates of radiation pneumonitis in lung cancer patients (N=167)

Parameters	Classification	Patients (N)	Cases of radiation pneumonitis, N (%)	*P*-value
Gender	Male	105	23 (21.9)	
	Female	62	11 (17.7)	0.519
ECOG	0-1	130	27 (20.7)	
	2	37	6 (16.2)	0.539
Weight loss	≥ 5%	32	5 (15.6)	
	< 5%	135	29 (21.5)	0.459
Tumor location	Central	68	14 (20.6)	
	Peripheral	99	20 (20.2)	0.951
Smoking history	Yes	117	25 (21.3)	
	No	50	9 (18.0)	0.621
Complications (COPD)	Yes	67	15 (22.4)	
	No	100	19 (19.0)	0.594
Pathological type	Squamous cell CA	75	16 (21.3)	
	Adenocarcinoma	54	13 (24.1)	0.655
	SCLC	23	4 (17.4)	0.722
	Adenosquamous CA	5	1 (20.0)	0.967
Clinical stage	II	13	2 (15.4)	
	IIIA	58	12 (20.1)	0.664
	IIIB	76	16 (21.1)	0.638
	IV	20	4 (20.0)	0.737
Treatment modality	Radiotherapy	35	5 (14.3)	
	Sequential ± chemoradiotherapy	132	29 (22.0)	0.316
Radiotherapy	IMRT	118	23 (19.5)	
	3D CRT	49	11 (22.4)	0.666

ECOG, Eastern Cooperative Oncology Group; COPD, chronic obstructive pulmonary disease; CA, carcinoma; SCLC, small cell lung cancer; IMRT, Intensity-modulated radiation therapy; 3D CRT, 3D conformal radiation therapy.

and rs293795, *FEN1* rs174538 and rs4246215, *ERCC2* rs238406, and *ERCC5* rs2296147) were detected by polymerase chain reaction and DNA sequencing.

Genomic DNA extraction: Blood samples (2 mL) were obtained from all patients for extraction of genomic DNA using blood genomic DNA extraction kit (DP348, Tiangen Technology (Beijing) Co., Ltd.). The extracted DNA was stored at -20°C for later use.

PCR assay: PCR amplification primers for 8 SNP loci were designed based on corresponding gene sequences from NCBI. Primer sequences are shown in **Table 1**. PCR reaction system was 20 μL total as following: 10 μL 2 × PCR reaction buffer, 1 μL upstream and downstream of each primer, 1.5 μL genomic DNA, 6.5 μL double distilled water. PCR reaction conditions were as follows: 94°C for 3 min; 94°C for 30 s, 57°C for 30 s, 72°C for 30 s for 35 cycles; 72°C for 10 min. PCR products were sequenced by China Suzhou genewiz Biological Technology Co.

Primers for construction of PGL3/ERCC5 5' UTR: Upstream: 5'-GGGGTACCCGGAAACCGAGCGGGCC-3', downstream: 5'-GAAGATATCTTCGTGGTATACGGAGGA-3'. Mutagenesis primers: upstream- 5'-GGCCATTCTCTGGACCCGTCTTTCTTC-3', downstream- 5'-GGGTCCAGAGAATGGCCGTTGGCGGGGA-3'. Mutagenesis was performed made in accordance with the rapid mutation kit instructions (Beijing Gold Biotechnology Co., Ltd.) (**Table 1**).

Transfection and dual fluorescence assay: Transfection and dual fluorescence assay was performed according to FuGENE 6 Transfection Regent [E2693, Promega (Beijing) Biotechnology Co., Ltd.]. A549 cells per well were dosed

Table 3. Comparison of DVH parameters in patients with and without radiation pneumonitis

Variable	Radiation pneumonitis		*P* value
	No (N=133)	Yes (N=34)	
GTV			
Average dose	62.1 ± 5.1	63.7 ± 6.4	0.867
Maximum dose	64.2 ± 3.9	65.5 ± 4.7	0.913
Minimum dose	54.6 ± 4.3	53.8 ± 5.2	0.721
Average amount received by lung (Gy)	15.8 ± 2.9	14.9 ± 2.7	0.526
V_5 (%)	50.4 ± 2.9	51.3 ± 2.6	0.655
V_{20} (%)	22.6 ± 4.6	21.8 ± 5.3	0.578

DVH, dose-volume histogram; GTV, gross tumor volume; V_5, Volume of lung receiving at least 5 Gy; V_{20}, Volume of lung receiving at least 20 Gy.

with plasmid pGL3-basic, pGL3/ERCC5 5' and pGL3/ERCC5 5' UTR mutant (350 ng each), with pGL3-Bsaic plasmid as a negative control. Each well was transfected with 50 ng of pRL-TK vector. The cells were harvested 24 hours after transfection in accordance with the instructions of the subject dual fluorescence value [E1910, Promega (Beijing) Biotechnology Co., Ltd.]. Relative fluorescence value (firefly luciferase luminescent value divided by renilla luciferase luminescence value) was calculated.

Statistical analyses

All statistical analyses were performed using SPSS 18.0 software (IBM, U.S). Differences in baseline clinical characteristics between RP and non-RP groups were assessed by Ch-squared test. DVH (*dose-volume histogram*) parameters were compared using the *t* test. The distribution of genotypes and allele frequency was compared using Chi-squared test. Cox regression analysis was performed to assess the relationship of genotype and allele frequencies with RP and patient survival. Odds ratios with 95% Confidence Interval (CI) are presented. Sub-group analysis by gender, radiation dose and other variables were performed. Associated *P* values < 0.05 were considered indicative of a statistically significant between-group difference.

Results

Correlation of radiation pneumonitis with clinical and physical parameters

All 167 patients with lung cancer received complete chest radiation treatment according to radiotherapy planning. There were no missing data on clinical and physical parameters. Median duration of follow-up was 13.6 months (min, max: 3.6, 26). No significant difference was observed with respect to clinical and physical parameters of patients with and without RP ($P > 0.05$) (**Tables 2** and **3**).

Correlation between SNP and ≥ grade 2 acute radiation pneumonitis

On univariate analysis, rs-2296147 gene loci *ERCC5* CC, CT/CC genotype showed a significant association with ≥ grade 2 acute RP (P=0.022 and 0.045, respectively), while the other SNPs at other sites did not show any significant association with RP.

On multivariate Cox regression analysis, rs2296147 gene locus *ERCC5* CC, CT genotype and CT/CC genotype were independent predictors of ≥ grade 2 acute RP (**Table 4**).

ERCC5 rs2296147 locus genotype CC reduce gene transcription ERCC

The Construction of pGL3/ERCC5 5' UTR and pGL3/ERCC5 5' UTR mutant sequencing results shown in **Figure 1A** and **1B**, in A549 cells were co-transfected the plasmid pGL3-Bsaic, pGL3/ERCC5 5' UTR, pGL3/ERCC5 5' UTR mutant and pRL-TK vector, which pGL3-Bsaic role of negative control, pRL-TK vector as internal control, after 24 hours of dual fluorescence detection of cell values, the results showed that pGL3/ERCC5 5' UTR rs2296147 of TT to CC mutation significantly reduced the relative fluorescence values ($P < 0.01$) which showed that rs2296147 locus CC genotype affect mRNA transcription activity of ERCC5 (**Figure 1**).

Discussion

In this study, we explored potential correlation between DNA damage repair gene polymorphisms (rs4462560 and rs7402844 locus of *NEIL1* gene, h*OGG1* rs1052133 and rs293795 locus, *FEN1* gene rs174538 and rs4246215, *ERCC2* rs238406 locus, *ERCC5* rs2296147 locus) and radiation pneumonia in lung cancer patients treated with radiotherapy. Out of 167 patients with lung cancer who received chest radiotherapy, 34 (20.4%) patients developed

Table 4. Association of SNPs with radiation pneumonitis ≥ 2

Genotype	Cases N (%)	Controls N (%)	Univariate analysis		Multivariate analysis	
			P-value	HR (95% CI)	*P*-value	HR (95% CI)
NEIL1, rs4462560						
GG	8 (23.5)	31 (23.3)				
CG	19 (55.9)	85 (63.9)	0.334	0.791 (0.312-1.594)	0.231	0.598 (0.351-1.214)
CC	7 (20.6)	17 (12.8)	0.442	0.687 (0.331-1.714)	0.564	0.542 (0.311-1.509)
CG/CC	26 (76.5)	102 (76.7)	0.542	0.765 (0.439-1.569)	0.464	0.654 (0.301-1.068)
NEIL1, rs7402844						
GG	8 (23.53)	36 (27.07)				
CG	19 (55.88)	78 (58.65)	0.945	0.956 (0.512-1.743)	0.898	0.881 (0.512-1.669)
CC	7 (20.59)	19 (14.29)	0.498	0.762 (0.353-1.594)	0.457	0.745 (0.312-1.523)
CG/CC	26 (76.47)	97 (72.93)	0.793	0.912 (0.558-1.723)	0.771	0.952 (0.569-1.573)
hOGG1, rs1052133						
GG	3 (8.82)	39 (29.32)				
CG	5 (14.71)	85 (63.91)	0.853	1.113 (0.372-3.513)	0.833	1.112 (0.357-3.143)
CC	26 (76.47)	9 (6.77)	0.367	0.578 (0.264-1.472)	0.315	0.573 (0.228-1.249)
CG/CC	31 (91.18)	94 (70.68)	0.385	0.765 (0.542-2.661)	0.336	0.714 (0.552-2.432)
hOGG1, rs293795						
TT	29 (85.29)	125 (93.98)				
CT	3 (8.82)	7 (5.26)	0.143	0.532 (0.208-1.245)	0.123	0.425 (0.152-1.397)
CC	2 (5.88)	1 (0.75)	0.554	0.876 (0.413-1.549)	0.346	0.763 (0.513-1.659)
CT/CC	5 (14.71)	8 (6.01)	0.278	0.765 (0.263-2.167)	0.258	0.697 (0.245-2.25)
FEN1, rs174538						
GG	12 (35.29)	50 (37.59)				
AG	20 (58.82)	71 (53.38)	0.630	0.858 (0.534-1.495)	0.643	0.748 (0.432-1.392)
AA	2 (5.88)	12 (9.02)	0.786	1.244 (0.414-3.142)	0.758	1.172 (0.432-2.504)
AG/AA	22 (64.71)	83 (62.41)	0.714	0.936 (0.569-1.552)	0.729	0.829 (0.412-1.694)
FEN1, rs4246215						
TT	15 (44.11)	59 (44.36)				
TG	18 (52.94)	67 (50.38)	0.157	1.508 (0.824-3.283)	0.124	1.549 (0.750-2.867)
GG	1 (2.94)	7 (5.26)	0.361	1.362 (0.895-2.150)	0.246	1.269 (0.643-3.209)
TG/GG	19 (55.88)	74 (55.64)	0.312	1.524 (0.693-3.394)	0.282	1.369 (0.593-2.925)
ERCC2, rs238406						
TT	17 (50.59)	62 (46.62)				
TG	15 (43.53)	62 (46.62)	0.484	1.231 (0.710-1.938)	0.381	0.945 (0.572-1.747)
GG	2 (5.58)	9 (6.77)	0.937	1.157 (0.355-3.085)	0.865	0.919 (0.295-2.774)
TG/GG	17 (49.41)	71 (53.38)	0.512	1.141 (0.692-1.884)	0.389	1.201 (0.551-1.662)
ERCC5, rsrs2296147						
TT	9 (26.47)	64 (48.12)				
CT	14 (41.18)	51 (38.35)	0.052	0.513 (0.274-0.886)	0.046	0.474 (0.283-0.819)
CC	11 (32.35)	18 (13.53)	0.022	0.215 (0.097-0.463)	0.019	0.252 (0.099-0.423)
CT/CC	25 (73.53)	69 (51.88)	0.045	0.317 (0.195-0.564)	0.029	0.278 (0.138-0.563)

SNP, single nucleotide polymorphism; HR, hazard ratio; CI, confidence interval.

RP grade ≥ 2. We detected eight kinds of DNA repair-based SNPs in these 34 patients. Our results showed an association of *ERCC5* rs2296147 CC, CT and CT/CC genotypes with ≥ grade 2 acute RP, which suggests that rs2296147 locus of ERCC5 gene may be associated with risk of RP after radiation treatment for lung cancer.

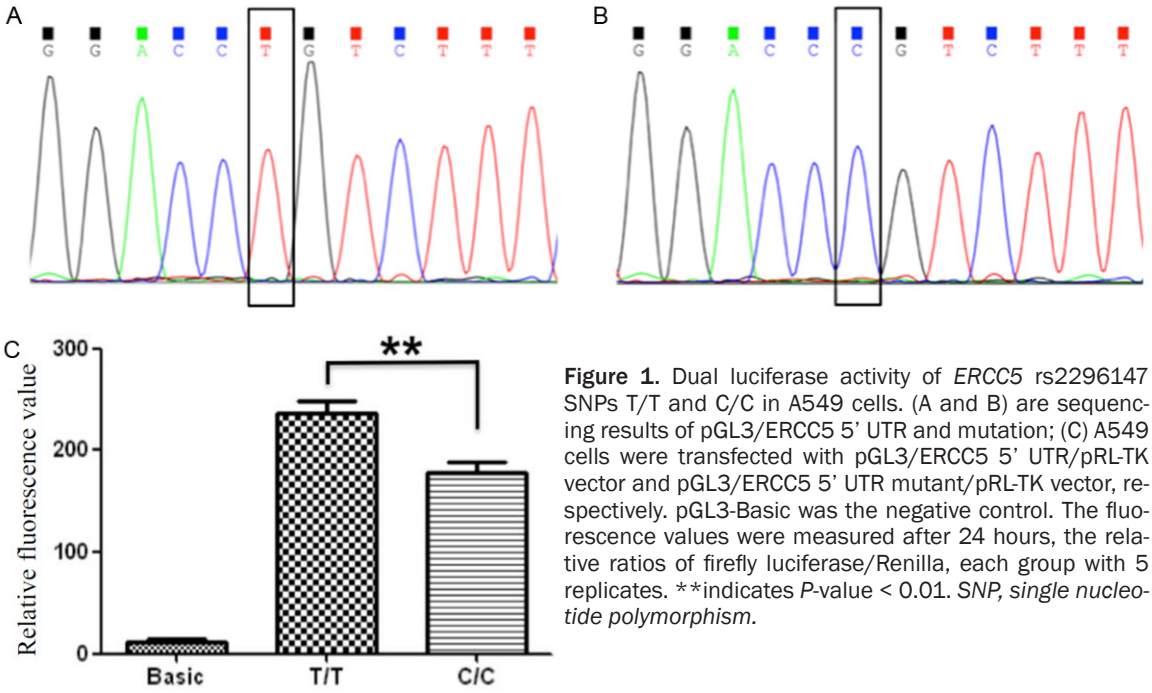

Figure 1. Dual luciferase activity of *ERCC5* rs2296147 SNPs T/T and C/C in A549 cells. (A and B) are sequencing results of pGL3/ERCC5 5' UTR and mutation; (C) A549 cells were transfected with pGL3/ERCC5 5' UTR/pRL-TK vector and pGL3/ERCC5 5' UTR mutant/pRL-TK vector, respectively. pGL3-Basic was the negative control. The fluorescence values were measured after 24 hours, the relative ratios of firefly luciferase/Renilla, each group with 5 replicates. **indicates *P*-value < 0.01. *SNP, single nucleotide polymorphism.*

Radiotherapy causes DNA single or double-stranded breaks to the body by high energy rays, which leads to cell death in the absence of repair. The DNA repair ability of an individual determines the sensitivity of the irradiated tissue to radiation; the cellular repair mechanisms are key determinants of the effect of radiation injury [10, 11].

Single nucleotide polymorphism are liable to affect the structural or functional integrity of genes involved in the repair of DNA damage, which in turn may affect the susceptibility to RP. Correlation between SNPs of genes (e.g., *TGFbeta1, APEX1, LIG4, MTHFR, ATM, P53* genes) and RP has been demonstrated in patients with lung cancer radiotherapy and chemotherapy since 2009 [12-17]. Radiation therapy causes direct DNA damage, which includes base damage and both single- and double-strand breaks. Repair of these injuries occurs in four different ways: base excision repair (BER), nucleotide excision repair (NER), DNA double-strand break repair (DSBR), mismatch repair (MMR). BER and NER pathways are the main pathways for repair of DNA damage caused by ionizing radiation [18-20]. *NEIL1* and *hOGG1* are involved in short repair pathways of oxidative damage, while *FEN1* genes act through long-range repair works. *NEIL1* was shown to be associated with RP in patients with esophageal cancer [6]. *hOGG1, FEN1, ERCC2* and *ERCC5* are also involved in a variety of DNA damage repair, in the regulation of signaling in cancer of and radiation damage [21-23]. These studies suggest that gene SNPs may be key determinants of radiation-induced lung injury.

Excision repair cross complementation group 5 (*ERCC5*), also known as Xeroderma pigmentosum complementary group G (*XPG*), which belongs to RAD2/XPG family, is one of the key factors in the repair pathway NER; its gene is located on chromosome 13q33. The gene encodes for a structure-specific nuclease, an enzyme responsible for the 3' nucleotide excision repair, also involved in XPF/ERC1 complex-mediated 5' nucleotide excision repair [20]. SNPs of *ERCC5* are associated with susceptibility to a variety of tumors such as, lung cancer and breast cancer [24]. *ERCC5* gene polymorphism was shown to be associated with advanced colorectal cancer related platinum-based drug efficacy [20]. Our study revealed a significant association of *ERCC*5 rs2296147 with ≥ 2 level RP.

On dual luciferase reporting assay, mutation of *ERCC5* rs2296147 site to base T C significantly reduced the relative fluorescence ratio, which indicated that the site of gene transcription ERCC5 plays an important role [25], ERCCC5

rs2296147 is located nearby 5' gene. Since 5' UTR non-coding region connects the first exon and the promoter region (and plays an important role in the regulation of gene transcription and translation), rs2296147 may affect ERCC5 gene transcription activity and reduce ERCC protein expression. This may compromise the mechanisms involved in repair of radiation injury, and increase susceptibility to RP. However, we did not examine ERCC protein expression in this study and further exploration is expected.

Though several studies have investigated the association of SNPs with radiotherapy-induced injury, the results have not been entirely consistent. This may be due to differences in sample size, ethnicity of the study population, heterogeneity with respect to lung cancer sub-type, possible involvement of multiple genes, and potential interaction between multiple genetic polymorphisms. Due to the small sample size in our study, further large scale studies are required to validate our findings.

Acknowledgements

Supported by Zhangjiagang science and technology grant: ZKS1316.

Disclosure of conflict of interest

None.

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