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ORIGINAL ARTICLE

The Association between Epidermal Growth Factor Receptor Single Nucleotide Polymorphisms and Radiochemotherapy Response in Cervical Cancer

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Abstract
Emerging data reveal that epidermal growth factor receptor (EGFR) single nucleotide polymorphisms (SNPs) can act as efficacy indicators for tumor treatment. Here, the association between EGFR R497K (rs11543848) and -216G/T (rs712829) SNPs and radiochemotherapy response in cervical cancer was investigated. EGFR R497K and -216G/T genotypes were analyzed by polymerase chain reaction-ligation detection reaction in 196 cervical cancer patients receiving radiotherapy alone, or in combination with chemotherapy. Compared with the 497G/G genotype, the A/A genotype significantly increased sensitivity to radiochemotherapy treatment (adjusted OR = 0.244, 95% CI = 0.087–0.680). Sensitivity to radiochemotherapy was not significantly different in carriers of the 'T' allele than that measured for the -216G/G genotype (adjusted OR = 2.412, 95% CI = 0.856–6.979). Additionally, the 497A/A genotype conferred a reduced risk of recurrence or metastasis than did the G/G genotype (adjusted OR = 0.248, 95% CI = 0.078–0.786, $P < 0.05$). Moreover, carriers of the 'T' allele did not have significantly modified risk of recurrence or metastasis compared with those with the -216G/G genotype (adjusted OR = 1.027, 95% CI = 0.324–3.253). Multivariate analysis revealed an association between clinical stage and treatment response (adjusted OR = 3.575, 95% CI = 1.662–7.692) and between age and the risk of recurrence or metastasis (adjusted OR = 0.319, 95% CI = 0.148–0.691). Our results show that, in patients with cervical cancer, the R497K polymorphism is correlated with treatment response and the risk of recurrence or metastasis. The R497K SNP might be a genetic marker for prediction of radiochemotherapy response and the risk of recurrence and/or metastasis in patients with cervical cancer.

Keywords Cervical cancer · Epidermal growth factor receptor · Single nucleotide polymorphism · Radiochemotherapy

Introduction

Second only to breast cancer, cervical cancer is one of the most common gynecological malignancies and presents a serious threat to women's health. Comprehensive treatment of cervical cancer includes surgery, radiotherapy, and chemotherapy. However, because of the tumor heterogeneity and the individuality of patients, different patients respond differently to radiotherapy and chemotherapy. Additionally, patients differ in their sensitivity and tolerance to radiotherapy and chemotherapy. However, the mechanisms underlying these differences are not yet clear. Clarification of such mechanisms can not only provide a theoretical basis for the implementation of precise cervical cancer treatments, but also provide new ideas for the development of treatments that enhance the sensitivity of cervical cancer to radiotherapy and chemotherapy [1–3].

Recent studies have shown that polymorphisms of the epidermal growth factor receptor (EGFR) are correlated with the sensitivity of tumors, and with the efficacy of treatment for some tumors, including non-small cell lung cancer and head and neck cancer. Therefore, EGFR SNPs could be efficacy indicators for tumor treatment. Several data have shown that over 90% of cervical cancer cases overexpress EGFR, and that this overexpression is associated with poor prognosis in patients with cervical cancer [4]. Studies showed that the expression of EGFR in cervical cancer tissues was significantly higher than that in

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adjacent tissues and normal cervical tissues [5, 6]. These results suggested that EGFR may play a role in promoting the occurrence and malignant transformation of cervical cancer. Increasing numbers of studies have shown that overexpression of EGFR leads to radiation resistance of tumor cells, and the EGFR downstream signaling pathway plays an important role in the response of tumor cells to radiation [7–9]. The SNP sites of EGFR may affect cell function by altering the transcriptional activity of the gene, affecting protein expression, or affecting the downstream signaling pathways [10, 11].

The extracellular domain codon 497 (R497K) is derived from the sub-structure IV of the EGFR gene. R497K is an EGFR polymorphism defined Arg (R) to Lys (K) replacement induced by a single, G to A, nucleotide change. In vitro experiments showed that, compared with wild-type 497R, mutated 497 K can weaken ligand binding function, growth stimulation, tyrosine kinase activity, and the induction of functions of proto-oncogenes*myc*, *fos*, and *jun* [12, 13]. In this study, we aimed to uncover the association between EGFR R497K (rs11543848) and -216G/T (rs712829) SNPs and radiochemotherapy response in patients with cervical cancer.

Materials and Methods

Study Subjects

The 196 patients with cervical cancer included in this study were hospitalized at the Fourth Hospital of Hebei Medical University from January 2010 to March 2014. The International Federation of Gynecology and Obstetrics criteria (2009) were applied for clinical staging and 77 patients were in phase II and 121 patients were in phase III. The clinical classification also identified 167 patients with exophytic tumors and 29 patients with non-exophytic tumors, 38 patients with tumor diameter ≤ 4 cm, and 158 patients with tumor diameter > 4 cm. Assessment of pathological type revealed that there were 163 patients with squamous cell carcinoma and 33 patients with non-squamous cell carcinoma. Treatment involved radiotherapy alone for 41 patients, and simultaneous radiotherapy and chemotherapy for 155 patients. A point radiotherapy doses ≤80 and > 80 Gy were received by 76 and 120 patients, respectively. Short-term responses of complete and partial remission were noted for 127 and 69 patients, respectively. During the follow-up period, 46 patients had recurrence and metastasis, 147 patients did not show any recurrence and metastasis, and three patients were lost to follow-up. The median age was 54 (rang: 29–83). Specimens were collected after obtaining informed consent from each patient.

Specimen Collection and Extraction of Peripheral White Blood Cell DNA

Sodium citrate was used for anticoagulation, and 5 ml of peripheral venous blood was extracted from the patients. The proteinase K-sodium chloride salting out method was used to extract white blood cell DNA, as described previously.

EGFR Gene Polymorphism Detection

R497K and -216G/T SNP genotype detection was performed using the polymerase chain reaction-ligation detection reaction (PCR-LDR) method. The PCR volume of 15 μL included 1 μL template DNA, 1.5 μL 10 × PCR buffer, 2 μL DMSO, 1.5 μL 25 mmol/L $MgCl_2$, 0.3 μL of *Taq* DNA polymerase, 0.3 μL 10 mmol/L dNTPs, 5 μmol/L R497K upstream primer (5′-CTG CTG TGA CCC ACT CTG TCT-3′) and downstream primers (5′-AAC AAC AAC CTG GAG CCT TAT −3 ′), 1 μL each of −216 G/T upstream primers (5′- GCC CGC GCG AGC TAG ACG TC −3′) and downstream primers (5′-GGG GCT AGC TCG GGA CTC CGG −3 ′), and 1.5 μL of 25 mmol/L $MgCl_2$. The PCR conditions were initial denaturation at 95 °C for 3 min, first cycle of denaturation at 94 °C for 15 s, annealing at 65 °C for 15 s, and extension at 72 °C for 30 s (which was then reduced by 0.5 °C for each subsequent extension until the completion of the 11th cycle), followed by denaturation at 94 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 30s. After 35 cycles, a final extension at 72 °C for 3 min was completed. The expected size of the PCR products for the EGFR R497K and -216G/T polymorphisms was 222 bp and 120 bp, respectively. The LDR system (10 μL) was composed of 2 μL PCR products, 1 μL 10 x TaqDNA ligation buffer, 1 μmol/L specific probe mix, and 5 U TaqDNA ligase. The LDR conditions were predenaturation at 94 °C for 2 min, 94 °C for 30 s, and 56 °C for 4 min, for 25 cycles. Once the LDR was completed, 1 μL of the reaction product and 2 μL ROX internal reference dye were denatured at 95 °C for 3 min and then immediately placed into a water-bath. An ABI1377 DNA sequencer was used for sequencing-based typing, and PCR products were randomly selected for direct sequencing to verify the LDR typing accuracy. All primers and probes were synthesized and labeled by Shanghai Generay Biotech Co., Ltd.

Statistical Methods

All data were analyzed using the SPSS21.0 software, and $P < 0.05$ was considered statistically significant. Chi-square tests were used to analyze differences in R497K and -216G/T genotype distribution between the groups.

Multivariate analysis and unconditional logistic regression were used to calculate the relative odds ratio (OR) and its 95% confidence interval (CI).

Results

Correlation between R497K Single Nucleotide Polymorphisms and Short-Term Efficacy of Radiotherapy and Chemotherapy in Patients with Cervical Cancer

No significant difference in the distribution of EGFR R497K genotypes was observed between the complete and partial remission groups ($\chi^2 = 5.522$, $P = 0.063$). There was no statistically significant difference in the sensitivity to radiotherapy of the G/A genotype compared with the G/G genotype. Compared with the G/G genotype, the A/A genotype had increased sensitivity to radiotherapy and chemotherapy, the former of which was statistically significant. After correction for age, clinical stage, tumor size, tumor clinical classification, pathological type, treatment method, and radiotherapy dose, the corrected OR value was 0.244 (95% CI = 0.087–0.680, Table 1).

Correlation between R497K Single Nucleotide Polymorphism and Recurrence or Metastasis in Patients with Cervical Cancer

No significant differences were observed in the distribution of R497K genotypes in the positive and negative groups when examining metastasis or recurrence during the follow-up period ($\chi^2 = 4.983$, $P = 0.083$). Compared with the R497K G/G genotype, the G/A genotype may not be associated with the recurrence and metastasis in patients with cervical cancer. Carrying the A/A genotype may reduce the risk of recurrence and metastasis in patients. After correction for age, clinical stage, tumor size, tumor clinical classification, pathological type, radiotherapy technology, and radiotherapy dose, the corrected OR value was 0.248 (95% CI = 0.078–0.786, Table 1).

Table 1 Correlation between R497K SNP and short-term efficacy of radiotherapy and chemotherapy in patients with cervical cancer

Genotype	Recent Efficacy			Recurrence or Metastasis		
	n	CR	PR	n	Positive	Negative
G/G	41	21 (51.22)	20 (48.78)	40	13 (32.50)	27 (67.50)
G/A	107	70 (65.42)	37 (34.58)	106	27 (25.47)	79 (74.53)
A/A	48	36 (75.00)	12 (25.00)	47	6 (12.77)	41 (87.23)

CR: complete remission; PR: partial remission

Correlation between -216G/T Single Nucleotide Polymorphisms and Short-Term Efficacy of Radiotherapy and Chemotherapy in Patients with Cervical Cancer

The distribution of -216G/T G/G and G/T + T/T did not differ significantly between the complete remission and partial remission groups ($\chi^2 = 3.290$, $P = 0.070$). Furthermore, there was no statistically significant difference in the distribution of G/G and G/T + T/T genotypes between groups positive and negative for recurrence or metastasis ($\chi^2 = 2.313$, $P = 0.128$). Compared with the G/G genotype, genotypes carrying the T allele (G/T + T/T) were correlated with radiochemosensitivity, with a corrected OR of 2.412 (95% CI = 0.856–6.797). However, these genotypes were not correlated with recurrence or metastasis in patients with cervical cancer, with a corrected OR of 1.027 (95% CI = 0.324–3.253, Table 2).

Correlation between Other Clinical Parameters and Short-Term Efficacy of Radiotherapy and Chemotherapy in Patients with Cervical Cancer

The results of univariate analysis showed that clinical stage, tumor size, radiotherapy techniques, and radiotherapy dose were significantly correlated with short-term efficacy (all P values <0.05) and logistic regression analysis OR values of 3.434 (95% CI = 1.758–6.708), 2.372 (95% CI = 1.225–4.595), 1.968 (95% CI = 1.068–3567), and 1.932 (95% CI = 1.032–3.618), respectively. Multivariate analysis results showed that only the correlation between clinical stage and short-term efficacy was statistically significant ($P < 0.05$), and the OR value of logistic regression analysis was 3.575 (95% CI = 1.662 to 7.692, Table 3).

Correlation between Other Clinical Parameters and Recurrence or Metastasis in Patients with Cervical Cancer

The results of univariate analysis showed a statistically significant correlation between age and recurrence or metastasis in

Table 2 Correlation between -216G/T SNP and short-term efficacy of radiotherapy and chemotherapy, and recurrence or metastasis in patients with cervical cancer

Genotype	Recent Efficacy			Recurrence or Metastasis		
	n	CR	PR	n	Positive	Negative
G/G	173	116 (67.05)	57 (32.95)	171	41 (23.98)	130 (76.02)
G/T + T/T	23	11 (47.83)	12 (52.17)	22	5 (22.72)	17 (77.28)

CR: complete remission; PR: partial remission

Table 3 Correlation between other clinical parameters and short-term efficacy and recurrence or metastasis in patients with cervical cancer

Clinical Parameter	CR	PR	Univariate analysis *P*	Multivariate analysis *P*	Positive	Negative	Univariate analysis *P*	Multivariate analysis *P*
Age (year)								
≤ 50	43	32			27	48		
> 50	84	37	0.086	0.067	19	99	0.001	0.004
Clinical Stage								
II	62	15			13	61		
III	65	54	0.000	0.001	33	86	0.080	0.062
Pathological type								
Squamous carcinoma	109	54			40	120		
Non-squamous carcinoma	18	15	0.179	0.457	6	27	0.524	0.815
Tumor diameter								
≤ 4	53	16			12	55		
> 4	74	53	0.010	0.148	34	92	0.140	0.708
Clinical classification								
Exogenous	111	56			38	128		
Non-exogenous	16	13	0.681	0.475	6	21	0.840	0.885
Treatment								
Radiotherapy alone	27	14			6	34		
Radiotherapy and chemotherapy	100	55	0.026	0.136	40	113	0.944	0.842
Point A dose								
≤ 80 Gy	56	20			17	57		
> 80 Gy	71	49	0.039	0.486	28	91	0.900	0.444

CR: complete remission; PR: partial remission

patients with cervical cancer ($P < 0.05$), and the OR value of logistic regression analysis was 0.331 (95% CI = 0.167–0.654). The results of multivariate analysis showed a statistically significant correlation between age and recurrence or metastasis of cervical cancer ($P < 0.05$), and the OR value of logistic regression analysis was 0.319 (95% CI = 0.148–0.691, Table 3).

Discussion

Radiotherapy and chemotherapy play important roles in the treatment of cervical cancer. Over the years, experts have devoted themselves to the optimization of treatment methods in the hope of realizing individualized treatment. However, clinical treatments have shown that equally advanced radiotherapy and chemotherapy regimens for the same disease condition result in individual differences in the responses and sensitivity of tumors to radiotherapy and chemotherapy [14]. Therefore, it is extremely important to identify the intrinsic factors in the patients that affect the efficacy.

Previously, Bandre´s et al. [15] performed a study in 78 patients with head and neck cancer undergoing surgery or radiotherapy and chemotherapy. The results showed that patients carrying the G/G genotype had a higher risk of mortality than those carrying the A/A genotype. Xue et al. conducted a preliminary study on the correlation between EGFR polymorphisms and radiosensitivity of nasopharyngeal carcinoma. The study showed that patients with the A/A genotype had higher radiosensitivity than did patients with the A/G and G/G genotypes ($P = 0.001$). The amino acid substitutions in the extracellular domain of EGFR may regulate ligand binding and affect transmembrane signal transmission to the intracellular domain, reducing the replenishing potency of the mutated EGFR intracellular enzyme substrate and causing changes in the activity of the downstream signaling pathways. By reducing the induction of proto-oncogenes or reducing growth stimuli, radiosensitivity is affected [13]. Our results show that, compared with patients with the G/G genotype, patients with the A/A genotype have good short-term responses to radiochemotherapy ($\chi^2 = 5.430$, $P = 0.020$), while there was no statistically significant difference between patients carrying the A/A genotype and patients carrying the G allele (A/G + G/G) ($\chi^2 = 2.902$, $P = 0.088$).

Studies performed in different countries produced different results. The studies by Williams et al. [16] showed that

compared with the G allele, the A allele was associated with short survival time, with a hazard value of 3.03 ($P = 0.045$). A gender-related study in 318 metastatic colorectal cancer patients treated with 5-fluorouracil-based chemotherapy by Press et al. [17]. Showed that the overall survival of males carrying the G/G genotype was shorter than that of female patients carrying the same genotype. The different results may be due to differences in the types of diseases of the study subjects, the treatment methods adopted for the disease, observation indicators, and genotype analysis methods, and errors in sample selection.

Recurrence refers to the discovery of new tumor lesions more than 6 months after the disappearance of clinical symptoms and signs. In spite of the improvement in radiotherapy techniques and the application of comprehensive therapies and the increase in the 5-year survival rate in each cancer stage, there are still 30% to 40% of patients with disease recurrence or progression [4]. In cervical cancer, about 42% to 50% of recurrences after treatment occurred within 1 year, and most recurrences occurred within 3 years. In this study, 196 patients with cervical cancer were followed up for 6 to 24 months. Among them, 46 patients had recurrence and the recurrence rate was 23.47%. The possible reason for the lower reported rate than that in the literature is that the follow-up period was shorter. The screening of intrinsic factors that can predict the risk of recurrence can allow clinicians to better assess the patient's prognosis and survival time after recurrence. Studies by Zhang et al. [18] showed that patients with locally advanced rectal cancer carrying the G/G genotype had a high risk of local recurrence. This suggests that, in patients with rectal cancer undergoing chemotherapy, the R497K single nucleotide polymorphism could be a predictor of recurrence. This is consistent with the results reported here.

EGFR-216G/T is located in an important EGFR promoter region and is involved in enhancing EGFR promoter activity. In vivo experiments show that compared with -216G, −216 T was associated with a 40% increase in EGFR expression [19, 20]. Liu et al. [21] found that carrying the T allele can improve progression-free survival. However, Bandre et al. showed that polymorphisms in the EGFR promoter region had no significant correlation with the clinical pathological features and mortality of the disease (head and neck cancer). In addition, studies by Costa et al. [22] on the risk of glioma showed that any genotype of -216G/T was not correlated with the risk of the disease. Here, we showed that, compared with the G/G genotype, the genotypes carrying the T allele (G/T + T/T) were not associated with sensitivity to radiotherapy and chemotherapy or with recurrence or metastasis in patients with cervical cancer. This could be related to the lower frequency of -216G/T polymorphisms in our study subjects. Therefore, we need to increase the sample size to further study the correlation between EGFR gene-216G/T SNP and the efficacy and prognosis of chemoradiotherapy in patients with cervical cancer in the future.

The efficacy and prognosis of radiotherapy and chemotherapy in cervical cancer are affected by many factors, often leading to inconsistent results. Retrospective studies showed that clinical stage, pathological type, histological classification, and pelvic lymph node metastasis were correlated with prognosis, while age and tumor size were not correlated with prognosis. After the multivariate analysis, only the clinical stage and histological type were independent risk factors affecting prognosis. In patients with early stage cervical cancer undergoing postoperative radiotherapy or chemoradiation, Demirci et al. [23] showed that tumors larger than 4 cm are important factors affecting prognosis. Our results showed that clinical stage, tumor size, radiotherapy techniques, and radiotherapy dose were correlated with chemotherapy efficacy, but following multi-factor analysis, only clinical stage correlated with radiotherapy and chemotherapy. Results inconsistent with those previously reported may result from factors such as sample size and clinical stage of the patients.

To date, there are no consistent reports on the clinicopathological features that affect the recurrence or metastasis of cervical cancer. In patients with locally advanced cervical cancer receiving radiotherapy or chemotherapy, Lee et al. [24] showed that age, clinical stage, and treatment are not correlated with central recurrence within 1 year. After multivariate analysis, only tumors ≥8 cm remained significantly correlated with central recurrence of cervical cancer. Huang et al. [25] showed that local recurrence of cervical cancer was correlated with young age, parametrial infiltration, adenocarcinoma/adenosquamous carcinoma, and stump positivity, while parametrial infiltration, lymph node positivity, and adenocarcinoma/adenosquamous carcinomas were correlated with distant metastases. In this study, univariate and multivariate analysis showed that only age was correlated with the recurrence or metastasis of patients with cervical cancer. Our results show that the onset of cervical cancer tends to occur at a younger age and should be taken seriously.

High expression of EGFR is one of the mechanisms that affect EGFR activity. In addition, mutations in EGFR can also lead to changes in its function. The most common EGFR somatic mutations are positioned in the TK domain, with exons 18 to 24 [26, 27]. Somatic mutations in the EGFR tyrosine kinase (TK) domain play a critical role in the development and treatment of non-small cell lung cancer (NSCLC). To identify the genetic factors conferring risk for the EGFR TK mutations in NSCLC, a case-control study was conducted in 141 Taiwanese NSCLC patients by focusing on three functional polymorphisms in the EGFR gene [−216G/T, intron 1(CA)n and R497K]. They found that the frequencies of the alleles -216 T and CA-19 are significantly higher in the patients with any mutation, in particular in those with exon 19 microdeletions, but not in the patients with L858R mutation [28]. The -216 T allele is favored to be amplified in both tumor DNA of lung cancer patients and cancer cell lines. We

conclude that the local haplotype structures across the EGFR gene may favor the development of cellular malignancies and thus significantly confer risk to the occurrence of EGFR mutations in NSCLC, particularly the exon 19 microdeletions. Notably, EGFR mutation status was not correlated with R497K EGFR genotype of lung cancers [29]. In this study, we focused on the effect of -216G/T and R497K on the high expression of EGFR, and the effect of its mutation warrants further investigation. Apart from mutations in the TK domain, extracellular domain (ECD) missense mutations comprise 10%–15% of transcripts and often accompany with focal EGFR amplification in glioblastoma [30]. Recently, the ECD variant EGFRA289V was identified as a molecular marker for responsiveness to therapy with EGFR-targeting antibodies in glioblastoma [31]. In addition, several polymorphisms (R451C and R521K) of EGFR extracellular domain predicts for cetuximab benefit in colorectal cancer [32, 33]. The relationship between gene polymorphism and EGFR mutation is well studied in NSCLC and other cancer types, however, related reports on cervical cancer are domestic. Therefore, it is worth further exploration in future research.

Our results suggest that the EGFR R497K SNP can serve as a predictor of the short-term efficacy of radiotherapy and of recurrence or metastasis of advanced cervical cancer. The -216G/T polymorphism was not correlated with the short-term efficacy and risk of recurrence or metastasis of cervical cancer in our study. Furthermore, our reports support the contention that clinical staging is an important factor affecting the short-term efficacy of radiotherapy and chemotherapy in patients with cervical cancer and show that age is an important factor that affects the recurrence or metastasis of cervical cancer.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All the human participants have been approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University.

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