Discrepant expression and prognostic role of epidermal growth factor receptor and icotinib radiosensitization for nasopharyngeal carcinoma

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Abstract

Background: Epidermal growth factor receptor (EGFR) expression was observed in many tumors. Icotinib (IH), an EGFR tyrosine kinase inhibitor, could enhance the radiosensitivity, but few studies have focused on nasopharyngeal carcinoma (NPC).

Materials and Methods : One hundred fifteen NPC lesions and 30 nasopharyngitis lesions were enrolled for EGFR expression evaluation with immunohistochemical staining. The correlation of EGFR expression and clinical parameters was analyzed. Survival analysis was calculated using univariate and multivariate analysis. A radioresistant NPC cell line, CNE-2R, was established with a gradient irradiation schedule of 60 Gy from CNE-2. Cell viability of CNE-2/2R was detected using microculture tetrazolium assay and colony-forming assay. The IH radiosensitization effect and EGFR expression were also evaluated in CNE-2/2R.

Results: High EGFR expression was more frequently detected in NPC lesions (73/115) than chronic nasopharyngitis lesions (5/30, $p = 0.000$). EGFR expression was correlated with tumor stage ($p = 0.000$) and tumor-node-metastasis (TNM) stage ($p = 0.006$). EGFR expression was an independent prognostic factor of disease-free survival ($p = 0.047$) and overall survival ($p = 0.016$). The optical density value of CNE-2R was higher than CNE-2 on days 1, 2, 4, and 6 after radiation of 4 Gy ($p < 0.005$). CNE-2 colony numbers clearly decreased compared with CNE-2R ($p < 0.05$), and IH enhanced the radiosensitizing effect of CNE-2R with an apparently lower survival fraction (1.414 times, $p < 0.05$). Higher EGFR expression was observed in CNE-2R cells and decreased accordingly when exposed to IH.

Conclusion: High EGFR expression was more frequently detected as a poor prognostic factor in NPC patients. Higher radioresistance and EGFR expression were observed in CNE-2R, and IH could reduce it. These results provided a new theoretical basis for clinical application of icotinib radiosensitization in NPC radiotherapy.

1. Introduction

Nasopharyngeal carcinoma (NPC) is a common malignant tumor with regional distribution in southern China with an annual incidence of 3.0 per 100,000 persons[1]. Radiotherapy with or without chemotherapy is the predominant treatment modality for NPC, especially local advanced NPC. It is well-known that distant metastasis and local failure contribute largely to poor outcome[2, 3]. Residual irradiated NPC cells with high radioresistance are the most common cause. In addition, chemotherapy drugs are regarded as radiosensitizers in the concurrent chemoradiotherapy of local advanced NPC[4, 5]. However, severe therapeutic side effects, including myelosuppression and mucositis lead to reduced treatment tolerance and quality of life[6]. Thus, it is urgent to explore effective therapeutic strategies for increasing radiosensitivity with fewer side effects.

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein that belongs to the ErbB receptor tyrosine kinase family, which enhances cancer cell proliferation, differentiation, metastasis, and angiogenesis[7]. EGFR overexpression has been demonstrated in NPC, which involves chemo-/radioresistance, malignant progression, and poor survival, making it possible to block the EGFR signal pathway to improve the radiosensitivity[8, 9]. EGFR inhibitors include monoclonal antibodies and small molecule tyrosine kinase inhibitors. For the former, Huang et al. demonstrated that concurrent administration of nimotuzumab and celecoxib cooperatively enhanced the cytotoxicity and radiosensitivity of NPC cells (CNE-2) in vitro[10]. Clinical studies have shown that cetuximab or nimotuzumab, combined with radiotherapy can enhance the efficacy of radiotherapy with a better safety profile[11–13].

Icotinib hydrochloride (IH), a small molecule inhibitor of EGFR, has shown radiosensitivity in several malignant tumors. Zhang et al. found that pretreatment with IH reduced clonogenic survival, inhibited EGFR activation, and

increased radiation-induced apoptosis of lung cancer cells[14]. Similar results were also observed in colorectal cancer and cervical cancer cells[15, 16]. However, very little is known about the radiosensitization of IH on NPC. In our previous study, IH (125 mg/day) plus intensity-modulated radiation therapy (IMRT) in patients with locally advanced NPC had an acceptable safety profile and was well tolerated[17]. Hence, we check EGFR expression and its correlations with clinical pathological features in NPC patients, and then explore IH radiosensitization efficacy to NPC cells.

2. Materials And Methods

2.1 Tissue samples

According to the Helsinki Declaration, this study was approved by the Institutional Review Board committee of Taizhou Central Hospital (Taizhou University Hospital) and Taizhou Hospital of Zhejiang Province. All the enrolled patients signed an informed consent form for the potential use of biopsy specimens for scientific research. To protect their privacy, only de-identified patient data were used.

On hundred fifteen primary NPC paraffin-embedded specimens were collected from the two hospital tissue banks between January 2011 and October 2017 with the approval of the Ethics Committee. Chronic nasopharyngitis lesions (n = 30) were collected between August 2017 and October 2017. All the patients were pathologically diagnosed with immunohistochemistry, and NPC patients with distant metastasis were excluded at the time of diagnosis. According to the 7th edition of the American Joint Committee on Cancer staging system, tumor-node-metastasis (TNM) staging score was determined. All the patients received standard treatment with radiotherapy, chemotherapy, or chemoradiotherapy. There was also no another malignant disease history for the selected patients. The median age of 115 patients was 54 years (ranging from 30 to 82 years), and other detailed characteristics are summarized in Table 1. The average follow-up was 53.7 ± 25.1 months (range: 10.6–99 months).

Table 1

Correlation of EGFR expression in NPC lesions with clinicopathological parameters.

Clinical features	Case numbers	EGFR expression		p-value
		Low(42)	High(73)	
Gender				
Male	80	30	50	0.742
Female	35	12	23	
Age (years)				
≤ 54	63	18	43	0.510
> 54	52	24	28	
Tumor differentiation				
Keratinizing	15	6	9	0.764
Non-Keratinizing	100	36	64	
Tumor stage				
T1 + T2	74	36	38	0.000
T3 + T4	41	6	35	
Lymph node status				
N0	56	26	30	0.328
N1-3	59	16	43	
TNM stage				
I + II	39	21	18	0.006
III + IV	76	21	55	
Abbreviation: EGFR, Epidermal growth factor receptor; TNM, tumor-node-metastasis.				

2.2 Immunohistochemical staining and score for EGFR

Paraffin-embedded slides of 4 μm were dewaxed in xylene and rehydrated through a graded series of ethanol. Antigen retrieval was performed by boiling the slides with citrate buffer (pH = 6) at 120 °C for 5 min, then blocked by normal goat serum. Internal peroxidase activity was blocked using a 3% hydrogen peroxide solution at room temperature for 15 min. After that, the samples were incubated with antibodies against EGFR (18986-1-AP, Proteintech Group, Wuhan, China) at 4 °C overnight. After washing with 0.01 M of phosphate-buffered saline (PBS) solution, the samples were incubated with labeled goat anti-rabbit secondary antibody (SA00001-2, Proteintech Group, Wuhan, China) for 60 min at 37°C. Following washing with PBS again, the samples were stained with diaminobenzidine solution, counterstained with hematoxylin, and mounted with glycerol gelatin.

EGFR expression was semi-qualitatively assessed by two pathologists according to positive staining intensity and percentage in cell membrane with or without cytoplasm. EGFR expression was graded as follows: negative; > 5–25%,

(1+); > 25–50%, (2+); and > 50%, (3+). Furthermore, negative and 1 + grades were classified as low EGFR expression, and 2 + and 3 + grades were classified as high EGFR expression.

2.3 Cell lines and cultures

Human NPC cell line CNE-2 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), Cells were maintained in Roswell Park Memorial Institute (RPMI-1640, Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin, and cultured in a humidified incubator in 37°C/5% CO₂. IH was purchased from Zhejiang Beta Pharma, Ltd. (Hangzhou, China) and dissolved in 100% dimethyl sulfoxide (DMSO) to a final concentration of 40 mg/ml and stored at -20°C.

2.4 Generation of radioresistant subclone

When CNE-2 cells reached approximately 60% confluence, the gradient irradiation schedule was initiated: The flask surface was covered by tissue equivalent filler with a thickness of 1.5 cm and then subjected to 2 Gy using Siemens clinical linear accelerator (Primus H, 6MV-X, dosage rate of 200 cGy/min, source skin distance 100 cm, and irradiation field of 10*10 centimeter). Following radiation exposure, cells were further cultured in a new flask. When they reached 60% confluence again, cells were then irradiated to 2 Gy a second time. These procedures were repeated three times with a gradient model of 2, 4, 6, and 8 Gy to the total dose of 60 Gy in 12 months, and the surviving cells were cultured and named CNE-2R. Another flask of CNE-2 cells was cultured synchronously as a control cell line with no radiation exposure.

2.5 Microculture tetrazolium (MTT) assay

CNE-2/2R cells in the exponential phase were seeded and incubated overnight in 96-well micro-titer plates at a density of 5,000 cells/well with a volume of 100 µl/well. CNE-2/2R cells were irradiated with the dose of 4 Gy according to the above irradiation scheme. MTT assay was performed to test optical density (OD) value on days 1, 2, 4, and 6 after irradiation. Briefly, CNE-2/2R cells were treated with 20 µl/well of 3-(4, 5-diethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (5 mg/ml) for 4 h at 37°C, resulting in the generation of formazan crystals. After aspirating the medium, DMSO (150 µl/well) was added to dissolve the formazan for 10 min, and then OD value was measured in a microplate reader (Safe Heart, SHE 3000) at a wavelength of 490 nm. Each group had three repeated wells on different days.

CNE-2 cells in the exponential phase were seeded in 96-well microtiter plates at a density of 10,000 cells/well and incubated overnight. CNE-2 cells were then treated by IH with different final concentrations (0.013, 0.025, 0.05, 0.1, 0.2, and 0.4 mg/ml) in a total volume of 100 µl/well. After 24 h of IH addition, MTT assay was performed, and the percentage inhibition rate was calculated as: $(1 - \text{OD value of experimental group} / \text{control group OD}) \times 100\%$. The IC₂₀ (20% inhibitory concentration) was selected as the subsequent experimental concentration.

2.6 Colony-forming assay

CNE-2/2R cells in the exponential phase were seeded in 6-well plates with appropriate densities (300–3,000/well). Three cohorts were conducted: CNE-2, CNE-2R, and CNE-2R + IH. NPC cells were incubated in the medium containing IH (IC₂₀ concentration) or DMSO for 24 h. After substituting with complete growth medium, the plates were correspondingly irradiated with 0 Gy, 2 Gy, 4 Gy, 6 Gy, 8 Gy, and 10 Gy depending on cell numbers and continuously incubated for approximately 10 to 14 days. The incubation ended until a macroscopic colony (> 50 cells per colony) appeared. Plates were subjected to Giemsa staining and counted under an inverted microscope. The plating efficiency (PE) was calculated as the fraction of colonies counted divided by the numbers of cells. The survival fraction (SF) was then calculated as colonies counted / (cells seeded x PE/100). Eventually, the data were fitted to a single hit

multiple targets equation, which was expressed as $SF = 1 - [1 - \exp(-D/D_0)]^N$ using GraphPad Prism 6.0 software. The cell survival curve was obtained, and radiosensitivity parameters (D_0 , D_q , N , SF_2) were compared.

2.7 Western Blot assay

The CNE-2, CNE-2R, and CNE-2R + IH groups in the exponential phase were treated with or without IH for 24 h. The medium containing IH or DMSO was substituted with complete growth medium, and then cells were irradiated with 4 Gy. After 24 h, the protein lysates of the three cell groups were harvested and centrifuged, and then the supernatants were collected. Protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% protease-free bovine serum albumin for 1 hour, the membranes were incubated with EGFR antibody (18986-1-AP, Proteintech Group, Wuhan, China) overnight at 4°C. Following washing with tris-buffered saline with Tween (TBST) three times, the membranes were continually incubated with secondary antibody labeled with horseradish peroxidase. The bands of EGFR were finally visualized. GAPDH was used as an internal control to balance equal loading.

2.8 Statistical analysis

Statistical analysis was performed with PASW Statistics V18.0 software. All quantitative data are represented as mean values ($\pm$ SEM), and statistical differences between groups were analyzed using the t-test. GraphPad Prism software V6.0 was used for graphing the data. The correlation between EGFR expression and clinical parameters was calculated with the Pearson's chi-squared (χ^2) test. Survival analysis was conducted according to the Kaplan-Meier method and Cox proportional hazards regression analysis. $P < 0.05$ was considered to be significant.

3. Results

3.1 EGFR expression of NPC lesions and chronic nasopharyngitis lesions

Positive staining was observed in the membrane and cytoplasm region (Fig. 1). Overall, EGFR positive expression was observed in 96 of 115 (83.5%) NPC lesions, which EGFR intensity in 20.0% (23/115) was 1+, 49.6% (57/115) was 2+, 13.9% (16/115) was 3+, respectively. As for chronic nasopharyngitis patients, there were 7 of 30 (23.3%) lesions with EGFR positive expression, of which EGFR intensity in 6.7% (2/30) was 1+, 10.0% (3/30) was 2+, and 6.7% (2/30) was 3+, respectively. According to the grade definition, high and low levels of EGFR expression were noted in 73 (63.48%) and 42 (36.52%) NPC patients, 5 (16.7%) and 25 (83.3%) chronic nasopharyngitis patients, with a significant statistical difference ($p = 0.000$).

3.2 Association of EGFR expression and clinical parameters in NPC patients

The association between EGFR expression and clinical parameters of NPC patients was shown in Table 1. The results demonstrated that EGFR expression level in NPC lesions was significantly correlated with T stage ($p = 0.000$) and TNM stage ($p = 0.006$). No correlation was observed between EGFR expression and gender, age, tumor differentiation, and lymph node status.

3.3 Association of EGFR expression and NPC patients' survival

The 5-year disease-free survival (DFS) and overall survival (OS) rate in 115 NPC patients was 72.7% and 75.3%, respectively. Patients with high EGFR expression had a poorer DFS (62.6% and 87.1%, $p = 0.049$) and OS (69.2% and

87.9%, $p = 0.042$) than those with low expression (Table 2, Fig. 2). After adjusting for prognostic factors, univariate and multivariate Cox proportional regression analysis showed that EGFR expression (HR: 3.10; 95% confidence interval (CI) [1.02–9.44], $p = 0.047$) and TNM stage (HR: 3.31; 95% CI [1.06–10.31], $p = 0.039$) were independent prognostic factors for DFS (Table 3). Similarly, EGFR expression (HR: 3.05; 95% CI [1.23–7.59], $p = 0.016$), age (HR: 3.16; 95% CI [1.37–7.31], $p = 0.007$) and TNM stage (HR: 3.16; 95% CI [1.17–8.52], $p = 0.023$) were found to be independent prognostic factors for OS.

Table 2

Cox proportional regression analysis of variables affecting disease free survival (DFS) and overall survival (OS) in NPC patients.

Variables	Subsets	Disease free survival				Overall survival			
		Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
		HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p
EGFR expression	High VS low	3.00 (1.00–8.98)	0.049	3.10 (1.02–9.44)	0.047	2.56 (1.04–6.31)	0.042	3.05 (1.23–7.59)	0.016
Gender	Male VS female	0.51 (0.21–1.23)	0.134	0.71 (0.28–1.79)	0.471	1.09 (0.48–2.49)	0.830	1.30 (0.56–3.02)	0.540
Age (years)	≤ 54 VS > 54	1.00 (0.41–2.41)	0.999	1.03 (0.40–2.64)	0.947	3.09 (1.36–7.02)	0.007	3.16 (1.37–7.31)	0.007
Tumour differentiation	Keratinized VS non-keratinized	0.50 (0.18–1.37)	0.177	0.38 (0.13–1.09)	0.072	0.70 (0.27–1.85)	0.471	0.72 (0.27–1.94)	0.517
TNM stage	(I + II) VS (III + IV)	3.36 (1.12–10.07)	0.030	3.31 (1.06–10.31)	0.039	3.29 (1.25–8.67)	0.016	3.16 (1.17–8.52)	0.023
Abbreviation: EGFR, Epidermal growth factor receptor; HR, hazard ratio; CI, confidence interval; TNM, tumor-node-metastasis.									

Table 3

The main parameters of cell survival curves of CNE-2 and CNE-2R treated irradiation with or without icotinib.

parameters	CNE-2	CNE-2R + IH	CNE-2R
D0	2.088	1.898	2.695
Dq	1.995	1.466	3.487
N	2.600	2.165	3.647
SF2	0.715	0.604	0.854
SER		1.414	
D0, mean lethal dose; Dq, quasi-threshold dose; N, extrapolation number; SF2, surviving fraction at 2 Gy; IH, Icotinib.			

3.4 Radiosensitivity differences between CNE-2/2R cells and IH radiosensitization efficacy on CNE-2R cells

The CNE-2R cell line was generated by graded doses of irradiation. The morphology of CNE-2/2R cells were distinguished under an inverted microscope (Fig. 3A, 3B). CNE-2 cells grew in a multilateral manner, while the CNE-2R cells had a spindle or fusiform shape. CNE-2R cells exhibited an advantage in cell proliferation compared with CNE-2 cells. After radiation of 4 Gy, the OD value of CNE-2R was higher than CNE-2 at day 1 (0.510 ± 0.0346 , 0.367 ± 0.0351 , $p = 0.007$), day 2 (1.070 ± 0.151 , 0.623 ± 0.031 , $p = 0.007$), day 4 (1.157 ± 0.227 , 0.713 ± 0.091 , $p = 0.035$), and day 6 (1.367 ± 0.083 , 1.117 ± 0.055 , $p = 0.036$) (Fig. 3C).

MTT assay was performed to detect the effect of IH on cell viability and IC20 value (0.04 mg/ml) was obtained for subsequent experiments (Fig. 3D). In clonogenic assay, the radiosensitivity difference between CNE-2/2R was obvious (Table 3 and Fig. 4A). In response to radiation, there was a dose-dependent reduction of clonogenic cell survival. CNE-2 colony numbers decreased compared with CNE-2R ($p < 0.05$), and IH enhanced the radiosensitizing effect of CNE-2R with an apparently lower survival fraction (1.414 times, $p < 0.05$).

3.5 EGFR expression profiles in CNE-2/2R cells.

In RT-PCR assay, the EGFR expression level was 1.343 times higher in CNE-2R cells than CNE-2 cells ($p < 0.05$). In western blot assay, GAPDH was used as an internal control. Compared with CNE-2 cells, EGFR protein expression was higher in CNE-2R cells (Fig. 4B). When IH was added, EGFR expression was decreased accordingly. These results demonstrated that EGFR expression was higher in the radioresistant cell lines than the parental cell line and IH could reduce EGFR expression.

4. Discussion

Although NPC is sensitive to radiotherapy, local recurrence and distant metastasis remain the leading causes resulting in treatment failure, which is attributed primarily to the inherent and acquired radioresistance. Enhancing the radiosensitivity with chemotherapy drugs is a conventional method for locally advanced NPC treatment[4, 5, 18]. However, severe side effects may affect and even interrupt the chemoradiotherapy procedure. A number of studies have demonstrated that EGFR inhibitors can improve the radiosensitivity in many malignant tumors[14–16, 19, 20]. Few studies have focused on the radiosensibility of IH in NPC, which is the aim of the study based on our previous study[17].

EGFR expression was presented in 83.5% of NPC patients, in contrast to 23.3% of chronic nasopharyngitis patients, with a significant statistical difference. Further analysis showed that EGFR expression was significantly associated with TNM stage and T stage, but not gender, age, tumor differentiation, and lymph node status. In univariate and multivariate Cox proportional regression analysis, two parameters (EGFR expression and TNM stage) and three parameters (EGFR expression, age and TNM stage) were identified as independent prognostic factors for DFS and OS, respectively, while EGFR expression was the common factor. Zhang et al. reported that EGFR expression was 89.5% in 96 NPC patients and correlated with T stage[21]. Zafar et al. reported 86.35% of EGFR expression in head and neck squamous cell carcinoma[22].

In addition, EGFR expression is an independent prognostic factor, three-year survival rates were lower in EGFR-positive (75.8%) patients than in EGFR-negative patients (91.2%). A meta-analysis including 15 studies and 1,225 NPC patients demonstrated that the pooled HRs for both OS (2.11 [1.23, 3.60]) and DFS (2.17 [1.41, 3.35]) were higher than 2, indicating a strong prognostic value of EGFR[23]. Another meta-analysis including 22 studies produced similar results [8]. Hashmi et al. showed a significant association of EGFR expression with tumor stage and DFS[24]. Inhibiting EGFR expression might be feasible to reverse the radioresistance of NPC cells.

Radioresistant cell lines can be established via cumulative exposure to irradiation[25–27]. In our study, the radioresistant cell line CNE-2R was successfully obtained using a gradient irradiation schedule with accumulated doses of 60 Gy. Compared with CNE-2, CNE-2R cells had stronger ability of proliferation and colony forming, which means a higher radioresistance. There was a higher EGFR expression in CNE-2R cells than CNE-2 cells. When CNE-2R was exposed to IH (IC20 value, 0.04 mg/ml) plus irradiation, EGFR expression and colony-forming ability were reduced. These results demonstrated that IH improved the radiosensitivity of CNE-2R. Ma et al. evaluated the effect of IH on radiosensitivity and showed that IH more effectively sensitized colorectal cancer cells (HT29 and HCT116) to radiation[15]. Wang et al. reported IH can significantly inhibit cell proliferation, redistribute cell cycle, enhance apoptosis and impair DNA damage response of Hela S3 cells following radiation[16].

In conclusion, high EGFR expression is a poor prognostic factor for NPC patients, CNE-2R cells obtained by gradient irradiation are distinguished from the CNE-2 cells in viability, radiosensitivity, and EGFR expression. IH is capable of enhancing the radiosensitivity of CNE-2R. The study provides a new theoretical basis for clinical application of IH radiosensitization in NPC radiotherapy.

5. Conclusions

High EGFR expression was more frequently detected as a poor prognostic factor in NPC patients. Higher EGFR expression and radioresistance were observed in radioresistant cell line CNE-2R, which was reduced by IH. These results provided a new theoretical basis for clinical application of IH radiosensitization in NPC radiotherapy.

Declarations

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Availability of data and material

Raw data may be available on request from the corresponding author.

Authors' contributions

Bo Wu and Hua-cai Xiong contributed equally to this work. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics committee of Taizhou Cnetral Hospital approved the study. Written informed consent was obtained from the patients for the participation.

Conflict of publication

Not applicable

Competing interests

The authors declare that there are no conflicts of interest.

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Figures

Figure 1

Different immunohistochemical staining intensity (1+, 2+, 3+) of EGFR under 10X and 40X magnification. (A) Primary NPC lesions; (B) chronic nasopharyngitis lesions.

Figure 2

Kaplan–Meier survival analysis for NPC patients: (A) comparison of disease-free survival (DFS) between high and low EGFR expression ($p=0.049$). (B) Comparison of overall survival (OS) between high and low EGFR expression ($p=0.042$).

Figure 3

(A) CNE-2 cells with a multilateral manner; (B) CNE-2R cells with the shape of spindle or fusiform; (C) cell viability at different time points was determined by MTT assay; (D) cell viability of CNE-2 under different icotinib concentration.

Figure 4

(A) The three cohorts were exposed to different dose and continuously incubated until macroscopic colony (>50 cells per colony) appeared. Survival fraction curve was conducted; (B) expression level of EGFR protein in CNE-2/2R cells was detected by western blot for the three treatment groups.