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Alterations of drug-resistant proteins in nasopharyngeal carcinoma after intensity-modulated radiation therapy with or without chemotherapy and correlation with clinicopathologic features and short-term prognosis

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Abstract

Objective The aim of this study was to investigate the alteration of drug-resistant proteins in nasopharyngeal carcinoma (NPC) after intensity-modulated radiation therapy (IMRT) with or without chemotherapy and its correlation with clinicopathologic features and short-term prognosis.

Methods Eligible NPC patients receiving IMRT with or without chemotherapy were studied. Tumor tissues from patients before treatment and at 2/3 of IMRT were obtained, and drug resistance proteins, P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), lung resistance protein (LRP), and glutathione S-transferase π (GST-π) were detected. Meanwhile, the clinicopathological data of the patients were collected and statistically analyzed. The short-term prognosis of the patients was observed and recorded, and the correlation between progression-free survival (PFS) and positive drug-resistant proteins was analyzed.

Results Before treatment, the positive rates of P-gp, MRP, LRP, and GST-π among the 43 NPC patients were 30.23%, 16.28%, 20.93%, and 34.88%, respectively; after treatment, the positive rates of P-gp and MRP were 65.12% and 41.86%, respectively (P-gp, $P < 0.001$; MRP, $P = 0.009$). Significantly higher positive rates of P-gp were observed only after treatment than before treatment in the IMRT-alone group (n = 18) and IMRT/chemotherapy group (n = 25). The intensity of P-gp, MRP and GST-π positive expression was increased in patients treated with IMRT/chemotherapy compared to pre-treatment. No correlation between positive expression of drug-resistant proteins and lymphatic metastasis was observed. Patients with P-gp and GST-π positivity had a more pronounced decrease in PFS.

Conclusion P-gp, MRP, and GST-π are increased in NPC patients after IMRT with or without chemotherapy and are closely associated with poor short-term prognosis of patients.

Keywords Intensity-modulated radiation therapy · Chemotherapy · Drug resistance · P-gp · Prognosis · GST-π · MRP

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1 Introduction

Originating from the mucosal epithelium of the nasopharynx, nasopharyngeal carcinoma (NPC) is a malignant tumor that mainly develops in the parietal and lateral walls, especially within the pharyngeal crypts [1]. In 2018, the International Agency for Research on Cancer recorded 129,079 new cases of NPC globally, which accounted for about 0.7% of all new cancer cases, and led to 72,987 deaths, making up approximately 0.8% of the total [2]. NPC occurs frequently in China, particularly in the southern provinces such as Guangdong, and in Southeast Asia [3].

Radiation therapy (RT) or RT-based comprehensive treatment is considered the standard radical treatment for NPC [4]. Intensity-modulated RT (IMRT) is currently one of the current major RT techniques for NPC [4]. In terms of treatment, for patients with NPC without distant metastases at the initial diagnosis, the National Comprehensive Cancer Network (NCCN) guidelines recommend [5] RT for early-stage NPC, while radiotherapy combined with chemotherapy for NPC without distant metastases [6]. Platinum-based concurrent radiochemotherapy is the predominant combination treatment modality. However, RT frequently leads to a rise in drug-resistant protein expression, negatively influencing therapy efficacy. RT can induce the activation of multiple resistance mechanisms that are mainly due to changes in the tumor microenvironment and remodeling of intracellular signaling pathways [7]. Radiation, while targeting tumor cells, unavoidably causes DNA damage and repair in these cells, which leads to the activation of damage resistance genes. One possible anti-damage response of tumor cells is multidrug resistance [8]. Currently, the more positively studied drug resistance proteins include P-glycoprotein (P-gp), multidrug resistance-related protein (MRP), lung resistance protein (LRP), and glutathione S-transferase π (GST-π).

As a membrane-associated efflux pump, P-gp efficiently expels chemotherapeutic drugs from the cell, lowering their intracellular concentration and resulting in drug resistance [9]. Through the activation of specific signaling pathways or transcription factors, radiation may activate multiple pathways, resulting in the enhanced expression of P-gp [10] and MRP [11]. In addition, RT can lead to changes in intracellular redox status, which may further affect the expression of P-gp [12] and MRP [13]. Notably, high expression of P-gp and MRP is associated with poor treatment prognosis, especially in patients receiving cisplatin-based adjuvant chemotherapy [14]. GST-π, a detoxification enzyme, has been shown to be expressed at significantly higher levels in NPC patients, which may be related to oxidative stress and inflammatory responses in the tumor microenvironment [15]. RT combined with inhibitors of P-gp or MRP may improve the effectiveness of chemotherapy and reduce drug resistance [16]. For LRP, its high expression is associated with poor prognosis in cancer, especially lung cancer [17].

Based on this, the following specific hypotheses were proposed in this study: compared with IMRT-alone, NPC patients who received IMRT combined with cisplatin chemotherapy (IMRT/chemotherapy) showed significantly higher expression levels and positivity rates of drug-resistant proteins (P-gp, MRP, LRP, and GST-π), and the combination therapy further exacerbated the resistance protein. The high expression of drug-resistant proteins after treatment may be associated with the shortening of patients' progression-free survival (PFS), suggesting that it serves as a potential biomarker for predicting short-term prognosis. By verifying the above hypotheses, the present study aims to elucidate the regulation of drug-resistant protein expression in NPC by IMRT with or without chemotherapy, and to explore its association with clinicopathological features and prognosis, so as to provide theoretical basis for optimizing the therapeutic strategy.

2 Materials and methods

2.1 Study population

Non-keratinizing differentiated or non-keratinizing undifferentiated NPC patients were diagnosed in The Fourth People's Hospital of Lin'an District between January 2022 and January 2023. Inclusion criteria: (1) Patients with II-III grade tumors according to the seventh edition of the UICC/AJCC staging system for nasopharyngeal carcinoma (i.e., T1N1M0, T2N0M0, T2N1M0, and T3N0-1M0); (2) Patients who have had no prior treatment for nasopharyngeal carcinoma, encompassing chemotherapy, radiotherapy, or surgical intervention.; (3) patients without distant metastases according to chest X-ray film/CT, abdominal B ultrasound/CT, and whole-body bone ECT; (4) patients treated with IMRT or IMRT + chemotherapy; and (5) patients with Karnofsky performance score of ≥ 70. Exclusion criteria: (1)

patients with previous or concurrent malignant diseases; (2) patients with severe hepatic or renal failure or pulmonary or cardiac dysfunction; (3) pregnant or breastfeeding patients; (4) patients without signing informed consent; and (5) patients with less than a 12-month follow-up after IMRT or IMRT + chemotherapy. The patient inclusion flowchart is shown in Fig. 1.

All patients underwent pre-treatment evaluations, including medical history, physical examination, nasopharyngoscopy, laboratory tests, enhanced MRI or enhanced CT of the nasopharynx and neck (only for patients with contraindications to MRI), nasopharyngoscopy, and enhanced CT of the thorax and abdomen, and whole-body imaging. Ultimately, 43 patients qualified, with 29 being male and 14 female, and their ages spanned from 30 to 74 years. Fifteen patients with chronic inflammation of the nasopharynx who were gender- and age-matched during the same period were also included as controls. Ethical review of the study was obtained from The Fourth People's Hospital of Lin'an District and the enrolled patients signed an informed consent form.

2.2 Treatments

All patients underwent IMRT: Patients were immobilized using a thermoplastic mask and scanned by 2.5 mm planned CT. Guided by fused MRI images, the target area was mapped on the planned CT. The gross tumor volume (GTV) was outlined on the enhanced CT sequences, including the GTV of the nasopharynx and lymph nodes (GTVnx and GTVnd). The clinical target volume, CTV1, covered the GTVnx and nearby lesions, usually extending 5 mm beyond, whereas CTV2 included CTV1 plus an extra 5 mm margin. PTV, encompassing PTV1 and PTV2, was expanded externally by 3–5 mm in each direction.

The radiation dose for each protocol was specified as follows: PTV for GTVnx, 68–72 Gy at 2.06–2.18 Gy per administration; PTV for GTVnd, 66–70 Gy at a dose of 2.00–2.13 Gy per administration; PTV for CTV1, 60 Gy at 1.82 Gy per administration; PTV for CTV2 and CTV-N, 54 Gy at 1.64 Gy per administration. Patients received 1 dose per day, 5 days per week, for 6 to 7 weeks.

Patients in the IMRT/chemotherapy group received cisplatin chemotherapy (70–80 mg/m^2) every 3 weeks along with RT. Each chemotherapy cycle, typically lasting 21 days, was administered on the first day. Chemotherapy was delayed if

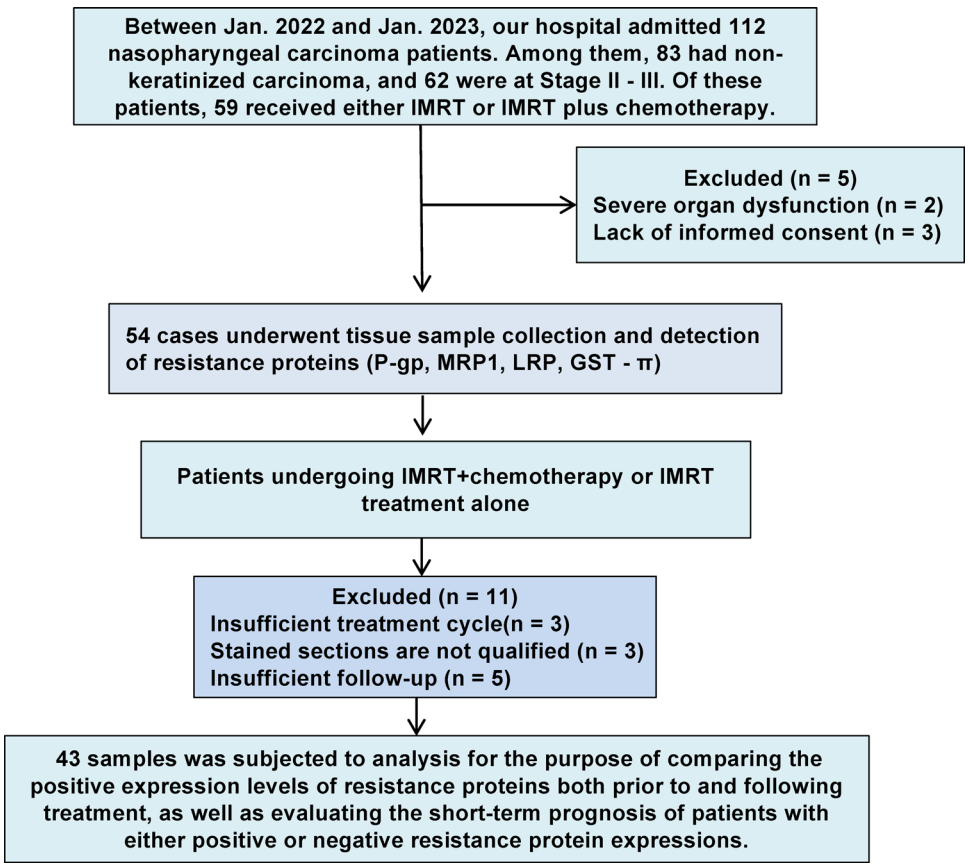

Fig. 1 A flowchart of the clinical workflow

patients had platelets < 100×10^9/L or neutrophils < 2.0×10^9/L; in patients suffering from acute nephrotoxicity, chemotherapy was delayed until adequate renal function was restored. In cases of severe hematologic or nonhematologic toxicity (including neutropenia and thrombocytopenia), nephrotoxicity, or neurotoxicity, chemotherapy dose adjustments were allowed during treatment. For patients experiencing Grade 3 neutropenia, Grade 2 neurotoxicity, Grade 2 thrombocytopenia, or a creatinine clearance between 40 and 60 mL/min, the cisplatin dose was decreased by one level (20 mg/m^2). Chemotherapy was permanently discontinued if grade 4 toxicity occurred.

2.3 Tissue sample collection

Nasopharyngeal samples were taken before RT and at 2/3 of RT, respectively. In a sitting or lying position, patients received a 1% ephedrine drip into the nasopharynx. At the same moment, 2% bupivacaine was administered to the nasopharynx to provide surface anesthesia and lessen pain and discomfort. The biopsy site was confirmed by the specialized physician using a nasopharyngoscope. Afterward, the nasopharyngeal lesion tissues were obtained with biopsy forceps. The tissue was divided into two parts for pathological examination and laboratory examination.

Before RT and at the stage of receiving two-thirds of the radical dose, nasopharyngeal biopsies were obtained and categorized according to positive pathology results. In cases of chronic inflammation in the nasopharynx, the sampling method was similar to the one previously described, but with more attention given to accurately sampling the inflamed tissue.

2.4 Immunohistochemistry

Tissues were embedded, dewaxed, placed in 0.01 M citrate buffer (pH = 6.0), and heated in a microwave oven at 92–98 °C for antigen retrieval. Hydrogen peroxide (3%) was added to remove endogenous peroxidase, and non-specific antigens were closed using 5% BSA. At 4 °C, rat anti-human primary antibodies P-gp, MRP, LRP, and GST-π (Invitrogen, Thermo Fisher Scientific) were incubated overnight, followed by biotin-labeled secondary antibodies (Invitrogen, Thermo Fisher Scientific) for 30 min. Streptavidin-peroxidase was incubated, and after color development using 3,3′-diaminobenzidine, tissues were sealed with neutral resin.

Ten random fields of view were counted at high magnification (× 400) for each section and their positive rates were calculated. The third quartile positive cell count was 10% based on 15 healthy controls. Therefore, a positive cell count of < 10% was specified as negative (–), 10% to 25% as (+), 25% to 75% as (++), and > 75% as (+++). A rating of (++) or greater was interpreted as positive, indicating that the tumor was resistant to the drug mediated by the drug resistance gene.

Drug-resistant protein IHC interpretation was assessed double-blind by two senior pathologists (15 years/18 years of experience in head and neck oncology, respectively), with divergent cases by consensus by co-viewing microscopy (Kappa value = 0.82). These specialists had no knowledge of the patient's clinical features. P-gp was expressed at the cell membrane, while MRP, LRP, and GST-π were found at the cell membrane and/or in the cytoplasm, with a brown color indicating a positive reaction.

2.5 Blood sample collection and EBV load measurement

Early in the morning of the day after admission, 3–5 mL of peripheral venous whole blood was collected in a fasting state and placed in a vacuum blood collection tube containing EDTA-K2 anticoagulant. The EBV DNA load was detected by the fluorescence PCR kit (Daan Gene, Guangzhou, China), with a positive result being a DNA load exceeding 1000 copies/mL. Briefly, the anticoagulated blood was subjected to erythrocyte lysis, followed by centrifugation to obtain the precipitate. The precipitate was added with nucleic acid extract, centrifuged, and mixed the EB reaction mix and the reaction enzyme, followed by PCR amplification using the ABIPRISM 7500 technique. The reaction parameters were set: 95 ℃ for 3 min, 94 ℃ for 15 s, and 60 ℃ for 30 s in 40 cycles. The samples were quantified according to the standard curve drawn from the negative and positive values.

2.6 Outcome and follow-up

Short-term progression-free survival (PFS) was assessed after RT and chemotherapy, with local recurrence, distant metastasis, or death from any cause (whichever occurred first) constituting the primary endpoint. After treatment,

patients were reviewed every 3 months. Patients with a follow-up period of less than 12 months were excluded, and the maximum follow-up period was 26 months. A dedicated follow-up team was established to contact patients on a regular monthly basis, and the lost visit rate was controlled at < 7% (3/43). When tumor progression was detected and confirmed, remedial treatment, including chemotherapy, re-radiation, surgery or other therapeutic measures, should be given whenever possible.

2.7 Statistical analysis

PASS 15.0 software was used for efficacy analysis, with change in P-gp positivity as the primary endpoint. The pre-treatment positivity rate was 30.2% and was expected to reach 55% after treatment, where α = 0.05 (bilateral) and power = 80%. The calculation yielded a need of 38 cases (actually met by 43 cases). Statistical analysis was performed using SPSS 20.0 software. Count data were expressed as frequencies (n) and ratios (%) and were tested using the chi-square test or Fisher's exact test. Kaplan–Meier curves were plotted using survival curves in the R software, and differences in survival curves were tested using the log-rank test.

Table 1 Baseline characteristics of patients in IMRT-alone group and IMRT/chemotherapy group

	IMRT-alone group (n = 18)	IMRT/chemotherapy group (n = 25)	P value
Gender			0.158
Male	10 (55.56)	19 (76.00)	
Female	8 (44.44)	6 (24.00)	
Age, years			
Median (range)	55.8 (30.9 ~ 68.3)	50.3 (33.6 ~ 73.2)	
≤ 45, n (%)	6 (33.33)	10 (40.00)	0.655
> 45, n (%)	12 (66.67)	15 (60.00)	
KPS			0.756
90–100	17 (94.44)	23 (92.00)	
70–80	1 (5.56)	2 (8.00)	
T stage			0.741
T1	3 (16.67)	2 (8.00)	
T2	14 (41.18)	20 (80.00)	
T3	1 (5.56)	3 (12.00)	
N stage			0.501
N0	6 (33.33)	6 (24.00)	
N1	12 (66.67)	19 (76.00)	
TNM stage			0.473
II	17 (94.44)	22 (88.00)	
III	1 (5.56)	3 (12.00)	
LNM	12 (66.67)	19 (76.00)	0.501
EB positive	6 (33.33%)	11 (44.00)	0.48
Chemotherapy cycles			
2		4 (16.00)	
3		21 (84.00)	

Data were expressed as frequency (n) and rate (%) and tested by chi- square test or Fisher's exact test. $P < 0.05$ was statistically significant

3 Results

3.1 Clinical characteristics of patients

This study included 43 patients who were eligible for this study (54 patients who fulfilled the clinical characteristics, of which 3 received only one cycle of IMRT + chemotherapy, 3 failed immunohistological staining, and 5 had less than 12 months of follow-up). The clinical characteristics of these NPC patients are shown in Table 1. Among the 43 patients, 29 (67.44%) were male. The vast majority, 39 (90.70%), were in stage II. More than half underwent both IMRT and chemotherapy (25, 58.14%), and there were 17 (39.53%) patients who were EBV positive. The patients were grouped according to their choice of treatment, IMRT-alone group (18 cases, 41.86%) and IMRT/chemotherapy group. There were no significant differences between the two groups in terms of gender, age, KPS score, clinical stage and number of EB-positive patients (all $P > 0.05$).

3.2 Positive expression of drug-resistant proteins before and after treatment

Before treatment, the positive rates of P-gp, MRP, LRP, and GST-π were 30.23% (12/43), 16.28% (7/43), 20.93% (9/43), and 34.88% (15/43), respectively, among all patients; after treatment, a significant increase in the positive rates of P-gp and MRP relative to pre-treatment was observed, which was 65.12% (28/43) and 41.86% (18/43), which were statistically significant (P-gp, 28 vs 12, $P < 0.001$; MRP, 18 vs 7, $P = 0.009$). LRP and GST-π positive rates were not statistically different between pre- and post-treatment ($P < 0.05$). No statistically significant difference was observed in the pre-treatment and post-treatment positive expression rates of drug-resistant proteins in the IMRT-alone group and the IMRT/chemotherapy group (all $P > 0.05$). In particular, the positive rate of P-gp was significantly higher post-treatment than pre-treatment (IMRT-alone group, 72.22% vs 33.33%, $P = 0.044$; IMRT/chemotherapy group, 57.14% vs 24.00%, $P = 0.004$) (Table 2).

In the IMRT-alone group, the intensity of positive expression of drug-resistant proteins was mainly dominated by + +, both before and after treatment. In addition, in the IMRT/chemotherapy group, it was also found that the intensity of positive expression of drug-resistant proteins was mostly + +. Notably, the intensity of P-gp, MRP and GST-π positive expression was more often + + + + in patients treated with IMRT/chemotherapy. Especially for GST-π, although its positive rate did not change between pre- and post-treatment (both 32.00%, 8/25), the intensity of GST-π positive expression was increased in all of these patients (elevated from + + to + + + +) (Table 3). Thus, elevated positive expression of P-gp and MRP resistance proteins after treatment was potentially correlated with RT and chemotherapy. In particular, the combination of cisplatin chemotherapy during RT exacerbated a further increase in the intensity of positive expression of P-gp, MRP, and GST-π in patients with positive expression of drug-resistant proteins before treatment.

Table 2 Positive expression of drug-resistant proteins in IMRT-alone group and IMRT/chemotherapy group before and after treatment

	IMRT-alone group (n = 18)	IMRT/chemotherapy group (n = 25)	P value
Before treatment			
P-gp positivity	6 (33.33%)	6 (24.00%)	0.501
MRP positivity	3 (16.67%)	4 (16.00%)	1
LRP positivity	5 (27.78%)	4 (16.00%)	0.455
GST-π positivity	7 (38.89%)	8 (32.00%)	0.64
After treatment			
P-gp positivity	13 (72.22%)*	16 (57.14%)**	1
MRP positivity	8 (44.44%)	10 (40.00%)	0.771
LRP positivity	5 (27.78%)	8 (32.00%)	0.766
GST-π positivity	7 (38.89%)	8 (32.00%)	0.64

Data were expressed as frequency (n) and rate (%) and tested by chi- square test or Fisher's exact test. $P < 0.05$ was statistically significant. * After treatment vs before treatment, $P < 0.05$; ** After treatment vs before treatment, $P < 0.01$

Table 3 Intensity of positive expression of drug-resistant proteins in the IMRT-alone and IMRT/chemotherapy groups before and after treatment

	Positive intensity	IMRT-alone group			IMRT/chemotherapy group		
		Before treatment	After treatment	P value	Before treatment	After treatment	P value
P-gp	+ +	6 (85.71%)	8 (66.67%)	0.603	6 (100.00%)	4 (25.00%)	0.003
	+ + +	1 (14.28%)	4 (33.33%)		0 (0.00%)	12 (75.00%)	
MRP	+ +	3 (100%)	7 (87.50%)	1	4 (100.00%)	2 (20.00%)	3 (100%)
	+ + +	0 (0.00%)	1 (12.50%)		0 (0.00%)	8 (80.00%)	0 (0.00%)
LRP	+ +	4 (80.00%)	3 (60.00%)	1	4 (100.00%)	7 (87.50%)	4 (80.00%)
	+ + +	1 (20.00%)	2 (40.00%)		0 (0.00%)	1 (12.50%)	1 (20.00%)
GST-π	+ +	7 (100.00%)	5 (71.43%)	0.462	7 (87.50%)	0 (0.00%)	7 (100.00%)
	+ + +	0 (0.00%)	2 (28.57%)		1 (12.50%)	8 (100.00%)	0 (0.00%)

Data were expressed as frequency (n) and rate (%) and tested by chi-square test or Fisher's exact test. $P < 0.05$ was statistically significant

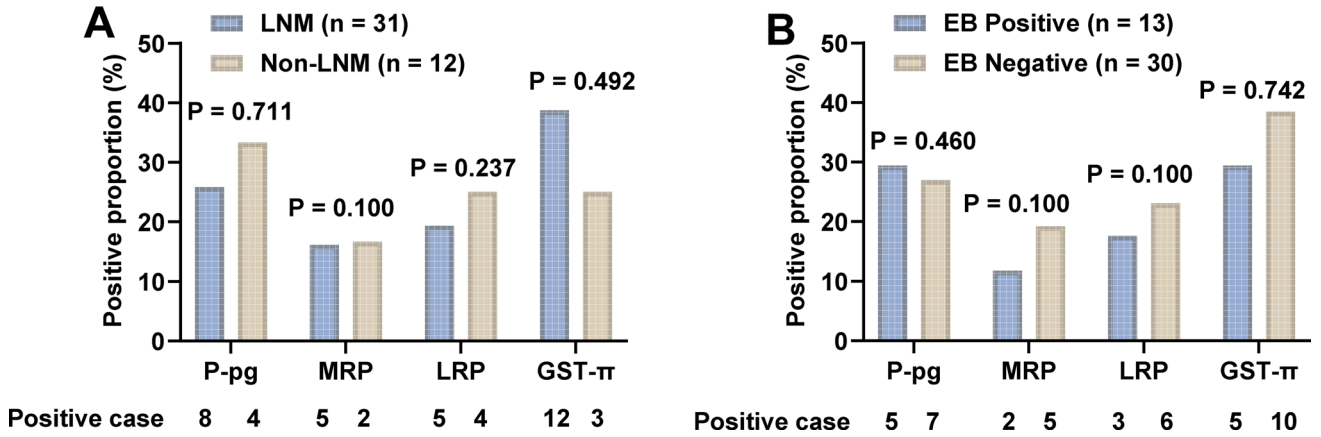

Fig. 2 Correlation of pretreatment resistance protein expression with LNM and EB positivity. **A** Positive rates of resistance proteins in NPC patients with LNM and Non-LNM; **B** Positive rates of resistance proteins in NPC patients with EB-positive versus EB-negative NPC

3.3 Correlation of patient drug-resistant proteins with clinical characteristics and short-term prognosis

The percentage of drug-resistant proteins in LNM and Non-LNM patients was plotted (Fig. 2A). A total of 31 patients had LNM events and 12 did not. In the LNM group, the positive rates of P-gp, MRP, LRP and GST-π were 25.81%, 16.13%, 19.35% and 38.71%, respectively. Compared with the Non-LNM group, there was no statistically significant difference in the positive rate of drug-resistant proteins between the two groups (all $P > 0.05$).

Among EBV-positive patients (n = 17), the positive rates of P-gp, MRP, LRP, and GST-π were 29.41%, 11.76%, 17.65%, and 29.41%, respectively. Compared with EB-negative patients, there was no statistically significant difference in the positive rate of drug-resistant proteins between the two groups (all $P > 0.05$) (Fig. 2B).

Follow-up for these patients lasted between 12 and 26 months, with a median follow-up of 21 months. The patients were grouped according to the positivity of drug-resistant proteins after treatment. Patients with P-gp-positive resistance and GST-π-positive resistance had a more pronounced decrease in PFS than those without resistance (both $P < 0.05$) (Fig. 3).

4 Discussion

This prospective study compared the positive expression of drug-resistant proteins P-gp, MRP, LRP, and GST-π and short-term prognosis in NPC patients treated with IMRT in combination with or without chemotherapy. P-gp and MRP positive expression in NPC patients was significantly increased after chemotherapy, and the combination of cisplatin

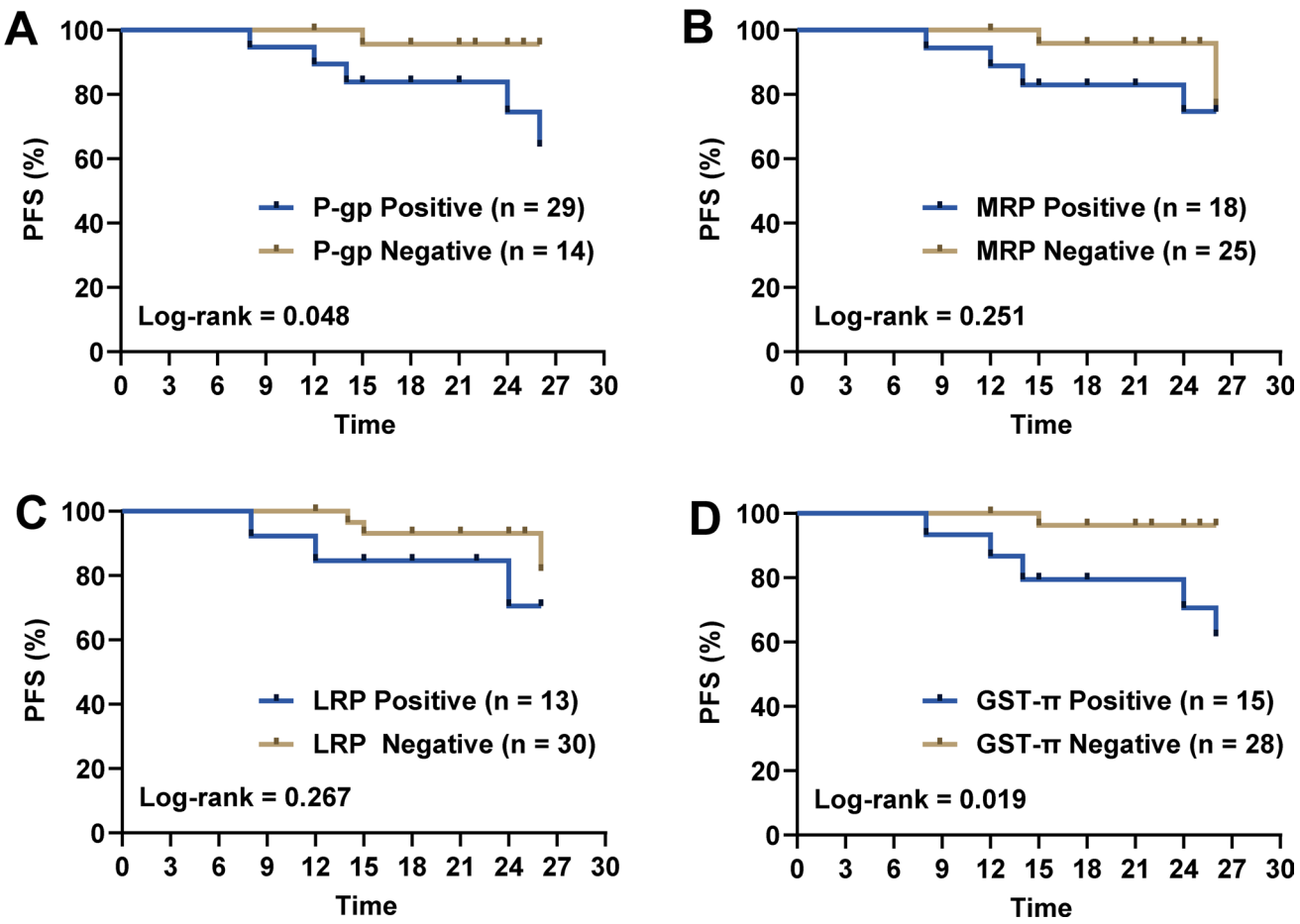

Fig. 3 Short-term PFS of NPC patients based on post-treatment resistant protein expression in positive and negative subgroups. **A** P-gp Positive and P-gp Negative; **B** MRP Positive and MRP Negative; **C** LRP Positive and LRP Negative; **D** GST- π Positive and GST-π Negative. log-rank < 0.05 was statistically significant

chemotherapy during RT exacerbated a further increase in the intensity of P-gp, MRP, and GST-π positive expression in patients. P-gp-positive resistant and GST-π-positive resistant NPC patients had worse PFS.

Concurrent chemoradiotherapy strengthens the local effects of RT more than RT alone, resulting in superior locoregional control and overall survival [5, 18]. However, the survival advantage of concurrent chemoradiotherapy seems to have disappeared as the advantage of high precision of IMRT has been widely applied in localized tumors [19, 20]. The primary treatment for NPC, a group of tumors sensitive to RT, remains IMRT, sometimes combined with chemotherapy [21]. This study grouped patients with intermediate-risk NPC, treated with IMRT alone or IMRT combined with chemotherapy and detected no significant difference between these patients in terms of their clinical characteristics (age, gender, KPS score, and clinical stage). Malignant tumor cells that acquire resistance due to complex drug resistance mechanisms are resistant not only to the antitumor drugs they have been exposed to but also to a wide array of untreated tumors and other drugs [22]. Some tumors exhibit natural resistance to antitumor medications because they express high levels of drug-resistant proteins, such as P-gp, even before treatment begins. Such tumors may be insensitive to a wide range of chemotherapeutic agents, resulting in poor treatment outcomes. Research on childhood acute lymphoblastic leukemia indicates that the expression of drug-resistant proteins varies significantly between initial and relapsed cases, implying that drug resistance might have been present before treatment [23]. In addition, a study has found that the positive expression of P-gp and MRP in gastric cancer tissues is 41.7% and 29.2%, respectively [24]. Based on this, we explored the positive expression of various drug-resistant proteins in the two groups of patients before treatment and observed that some patients did have drug resistance before treatment, especially P-gp and GST-π had a high pre-treatment positivity rate of 12 (30.23%) and 15 (34.88%), respectively. The expression of drug-resistant genes in tumors before treatment is associated with genetic mutations, genetic instability, tumor heterogeneity, and tumor microenvironment [25]. These are considered to be correlates of chemotherapy failure.

RT mainly damages cancer cells through high-energy rays, resulting in DNA breaks and cell death. However, cancer cells, when confronted with RT-induced DNA damage, activate a series of DNA damage repair mechanisms in response, and surviving cells are more resistant [26]. This damage repair mechanism may be associated with increased expression of drug-resistant proteins. In addition, radiology is associated with gene mutations and gene expression patterns [27, 28]. In an earlier in vitro study, hamster ovarian cancer cells showed a significant increase in cellular P-gp expression and resistance to chemotherapeutic agents after multiple X-ray irradiation [29]. In this study, NPC patients were found to have events of increased P-gp and MRP positive expression after treatment (IMRT with or without chemotherapy), although increased P-gp positive expression events were also observed in the cohort treated with IMRT alone. It is widely believed that tumor cells acquire resistance after receiving chemotherapeutic agents. This is due to the fact that, in general, tumor cells exclude chemotherapeutic drugs at interaction by up-regulating the drug efflux pump (i.e., P-gp), which reduces the effectiveness of the drugs [30]. Another important mechanism is the intracellular redox changes. In addition to destroying or interfering with the structure and function of DNA and thus inhibiting tumor cell division, cisplatin and IMRT can also damage the structure and function of cancer cells by promoting oxidative stress in tumor cells [31, 32], which results in the inhibition of cancer cell proliferation and the induction of cancer cell apoptosis. An increase in reactive oxygen species (ROS) can promote the expression of P-gp, thereby enhancing drug resistance in cells [33]. And increased ROS is a marker of oxidative stress [34]. For this reason, we observed the changes in the intensity of resistance protein expression after IMRT combined with or without cisplatin chemotherapy in patients and found that the intensity of P-gp, MRP and GST-π positive expression was more often + + + + in patients treated with IMRT/chemotherapy. Especially for GST-π, although its positive rate did not change before and after treatment (both 32.00%, 8/25), the intensity of GST-π positive expression was increased in all of these patients (elevated from + + to + + + +). This suggests that both RT and cisplatin chemotherapy increase the expression of P-gp and MRP, and that combining cisplatin chemotherapy during RT further increases the intensity of P-gp, MRP, and GST-π positive expression.GST-π degrades drugs mainly in a catalytic manner, thus decreasing cytotoxicity of antitumor drugs, and consequently, generating drug resistance [35].

It is well known that drug resistance leads to poor therapeutic efficacy, poor prognosis, etc. High expression of P-gp is closely associated with clinicopathological features and prognosis of tumor patients [36], especially in cases with lymph node metastasis. In this study, no association of various drug-resistant proteins with lymphatic metastases was observed. There is a correlation between EBV infection and the development of NPC, however, unfortunately, various drug-resistant proteins were observed to be associated with EB positivity. The genomic presence and expression of EBV was frequently detected in cancerous tissues of patients with NPC, but EB-positive expression was not detected in all patients. In the present study, most of the included were intermediate-risk NPC, and the lymph node metastatic load in such patients was relatively low, with an EB-positive rate of only 39.53%. In addition, the role of drug-resistant proteins may be more inclined to affect the drug efflux capacity of tumor cells rather than to be directly involved in metastatic or virus-related pathways [37, 38]. It has been shown that the efficacy of the IMRT combined with chemotherapy is comparable to that of the IMRT alone in intermediate-risk NPC [39]. Notably, although LNM and EB status were not shown to be associated with drug-resistant proteins, P-gp- and GST-π-positive patients had a more pronounced and significantly shorter PFS decline, providing direct evidence of the clinical prognostic value of drug-resistant proteins. P-gp, as an ATP-dependent drug efflux pump, can impair therapeutic efficacy by decreasing intracellular chemotherapeutic drug concentrations (e.g., cisplatin) [40]; whereas GST-π can neutralize the activity of platinum agents through glutathione-binding reactions while participating in ROS clearance to protect tumor cells [41]. The synergistic effect of the two may lead to the persistence of tiny residual lesions after treatment, which ultimately triggers early recurrence. The phenomenon of increased intensity of GST-π expression (+ + to + + + +) in patients in the IMRT/chemotherapy group in this study may reflect oxidative stress-induced adaptive resistance, suggesting that targeting the ROS-GST-π axis may improve prognosis. This study has some limitations. First, the sample size of this study was limited by tissue sampling and follow-up surveys, thus the sample size was small and may have some bias on the results. Although the efficacy analysis showed that 43 samples met the primary endpoint test (power = 80%), it should be noted that the subgroup analysis was not sufficiently potent: e.g., the EBV-positive subgroup was only 17 cases, which may mask the true association between viral status and drug-resistant proteins (risk of type II error). Small sample studies may amplify observed HR values (e.g., HR = 2.1 in the P-gp-positive group) with a 95% CI of 1.3–3.8, suggesting the need for cautious interpretation. In addition, a median follow-up of 21 months may introduce the following biases. [1] early event dominance: current PFS results mainly reflect the pattern of early recurrence within 1–2 years, whereas late recurrence (> 3 years) after radiotherapy may be associated with different resistance mechanisms. [2] Uncorrected competing risks: three non-tumor deaths (cardiovascular events) may underestimate true tumor-specific survival, but competing risk model analysis showed limited impact. This study focused on patients with stage II NPC (90.7%), and the results may not be applicable to patients with advanced (stage III-IV)

disease because of possible differences in their tumor microenvironment and response to treatment. Finally, the results of this study are applicable only to patients with intermediate-risk NPC, and caution is needed in NPC with more severe clinical stage or in other types of cancer. We are conducting a multicenter extension study (target sample size n = 120) to extend the follow-up to 5 years and to include subgroup analyses of advanced patients to validate the generalizability of the current findings. In addition, future multi-omics analyses are needed to further elucidate the molecular drivers of changes in the dynamics of drug-resistant proteins. For example, single-cell sequencing can reveal the clonal evolution pattern of P-gp high-expression subpopulations in the tumor microenvironment after radiotherapy, while spatial transcriptome technology can help to localize the spatial co-localization relationship between drug-resistant proteins and EBV latent membrane protein, thus complementing the limitations of conventional immunohistochemistry in this study. In addition, expanding the sample size and including stage III-IV patients will help to clarify whether the association of drug-resistant proteins with LNM/EB is altered in the late-stage population.

5 Conclusion

Alteration of drug-resistant protein P-gp is an important biological feature in NPC patients undergoing IMRT, and poorer PFS in NPC patients treated with IMRT combined with or without chemotherapy is correlated with the positive expression of P-gp and GST-π in tumor tissues. Therefore, the expression of drug-resistant genes should be closely monitored during the treatment of NPC, and individualized treatment plans should be developed according to the specific conditions of patients to enhance the therapeutic effect and improve the prognosis of patients.

Author contributions GaoFeng Wang designed the research study. GaoFeng Wang performed the research. Yuan Li provided help and advice. GaoFeng Wang and Yuan Li analyzed the data. GaoFeng Wang wrote the manuscript. Yuan Li reviewed and edited the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Declarations

Ethics approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All subjects was approved by The Fourth People's Hospital of Lin'an District (No.202104LA-08).

Competing interest The authors declare no competing interests.

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