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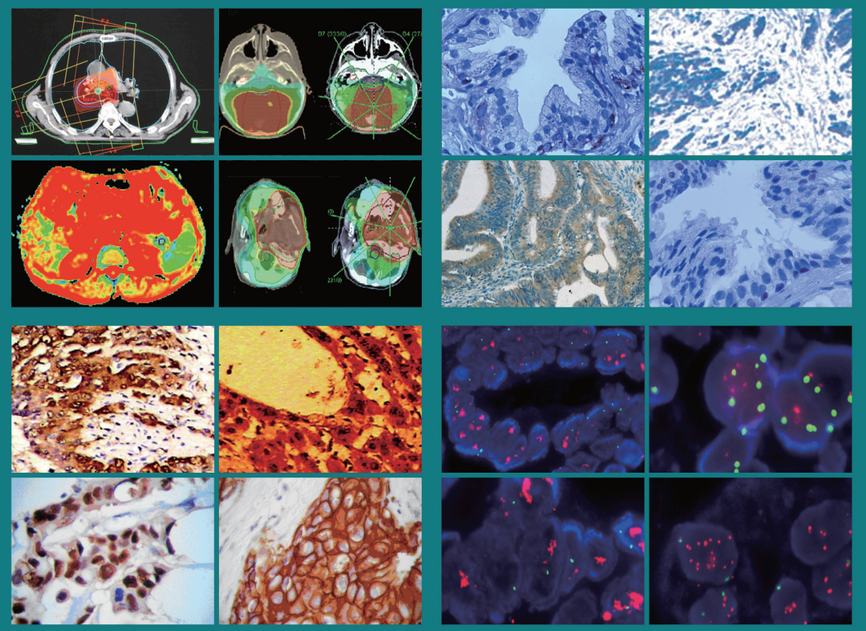

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Aims & Scope

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ORIGINAL ARTICLE

Implications of the autophagy core gene variations on brain metastasis risk in non-small cell lung cancer treated with EGFR-TKI*

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Abstract

Objective The brain is the main site of failure in cancer patients with epidermal growth factor receptor (EGFR) mutations undergoing treatment. However, identifying patients who may develop brain metastases (BM) is difficult. Autophagy is critical for cancer initiation and progression. We hypothesized that genetic variants in autophagy core genes might contribute to BM risk of non-small cell lung cancer (NSCLC) following treatment with EGFR tyrosine kinase inhibitor (EGFR-TKIs).

Methods We systematically examined 16 potentially functional genetic polymorphisms in seven autophagy core genes among 105 TKI-treated NSCLC patients. Kaplan-Meier curves were plotted to assess the cumulative BM probability. Univariate and multivariate Cox proportional hazard regression analyses were utilized to calculate hazard ratios (HRs) and 95% confidence intervals (CIs). We evaluated the potential associations of these genes with subsequent BM development.

Results We found that ATG16L1: rs2241880, ATG10: rs10036653, rs3734114, and ATG3: rs7652377 are significantly associated with NSCLC treated with EGFR-TKIs (all $P < 0.05$). BM developed more often in patients with ATG3 rs7652377 CC genotype (33%), ATG10 rs10036653 AA genotype (43%), ATG10: rs3734114 CT/CC genotype (46%), and ATG16L1 rs2241880 AA genotype (37%) compared to patients with AA genotypes at rs7652377 (12%), AT/TT genotypes at rs10036653 (16%), the TT genotype at rs3734114 (13%), or AG/GG genotypes at rs2241880 (17%).

Conclusion These associations may be critical for understanding the role of autophagy in BM risk. Future prospective studies are needed to determine if prophylactic cranial irradiation (PCI) could offer a survival benefit in this group of patients.

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Key words: autophagy; non-small cell lung cancer (NSCLC); brain metastasis (BM); single nucleotide polymorphism; predictive biomarker

Brain metastases (BM) are a common problem in patients with lung cancer, and they are associated with poor prognoses. The reported incidence of BM in non-small cell lung cancer (NSCLC) ranges from 17% to 54% [1]. Mutations in the epidermal growth factor receptor (EGFR) tyrosine kinase domain occur in approximately 15% of advanced non-squamous NSCLC patients [2]. Studies have shown that EGFR tyrosine kinase inhibitor (EGFR-TKI) is an effective treatment option for lung cancer patients with EGFR mutations [3]. However, advances in the development of targeted therapy against (NSCLC) mean that patients are more likely to develop BM due to

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prolonged survival. Patients whose tumors harbored an EGFR mutation had nearly a two-fold increase in the risk of BM [4]. The incidence of central nervous system (CNS) disease in patients with EGFR mutant (EGFRm) is high and is associated with increased use of health resources. Similarly, there is financial toxicity associated with CNS disease. Prophylactic cranial irradiation (PCI) for patients with locally advanced NSCLC decreases the incidence of BM [5], but it is not routinely used because it causes toxicity without improving survival. Differences in tumor biology may impact the pattern of metastases to the brain, putting some patients at greater risk than others [6]. However, PCI might be advantageous for an identifiable subgroup of patients with a high risk of developing BM.

Prior studies have identified the clinical features (including increased size of primary tumor, higher nodal stage and histology) that are associated with increased incidence of BM in patients with NSCLC [7–9]. However, not all studies have shown a significant correlation. In one study, the expression levels of three genes, *CDH2* (N-cadherin), *KIFC1*, and *FALZ*, were found to be highly predictive of BM in early and advanced lung cancer [10]. However, none of these studies specifically addressed NSCLC treated with EGFR-TKI. Considering autophagy's essential role in cancer development, we hypothesized that genetic variants in autophagy core genes might contribute to BM risk of NSCC treated with EGFR-TKIs.

Autophagy plays important roles in various organismal processes like development and aging. Abnormal autophagy leads to pathologies like cancer [11–14]. During cancer development, autophagy facilitates tumorigenesis in most contexts [15–16]. The autophagosome is a spherical organelle with double layer membranes that is formed during autophagy. Establishment of the autophagosome is controlled by several autophagy core genes [17], which might be involved in cancer initiation and progression [18]. Single-nucleotide polymorphisms (SNPs) have been found to be associated with risk and/or prognosis in numerous cancer types, including breast, thyroid, prostate, colorectal, and gastric cancer [19–20]. However, to our knowledge, there are no studies examining the relationship between autophagy-associated gene (ATG) SNPs in NSCLC patients undergoing EGFR-TKI therapy. In this study, we hypothesized that genetic variants of autophagy core genes may contribute to differential BM risk of NSCLC patients treated with EGFR-TKI. To test this hypothesis, we systematically examined the clinical implications of 16 potentially functional polymorphisms in seven autophagy core genes (*ATG3*, *ATG5*, *ATG7*, *ATG10*, *ATG12*, *ATG16L1*, and *LC3*) in NSCLC patients who received EGFR-TKI therapy.

Materials and methods

Study population and data collection

A total of 105 patients with advanced lung adenocarcinoma who had been treated with EGFR-TKI were included in this study (Table 1). Patients were recruited between July 2008 and July 2012, at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Eligible patients had at least one measurable lesion with a minimum size of at least one diameter of ≥10 mm for liver, lung, brain, or lymph node metastases. No restrictions on age, gender, or disease stage were applied, but all patients were required to have blood samples available for analysis. The Karnofsky performance status (KPS) of all patients was at least 70, and all of them had a life expectancy of at least 6 months. CT or MRI scans had been obtained from each patient before treatment as part of the disease staging process. All patients were asked to return to the hospital for examination (which included CT scans of the chest and abdomen) every two months. Repeat brain CT or MRI scans were obtained only in the event of clinical indications like neurological symptoms per the standard of care. This study was approved by the Review Boards of Tongji Hospital, Tongji Medical College. Written informed consent was obtained from each patient for the use of his/her DNA and clinical information. All procedures were performed in accordance with the approved guidelines.

Polymorphism selection of autophagy core genes

Single nucleotide polymorphisms (SNPs) of autophagy core genes were selected as previously described [21]. Briefly, common SNPs (MAF ≥ 0.05 in Chinese Han population) in six core autophagy genes (*ATG3*, *ATG5*, *ATG7*, *ATG10*, *ATG12*, and *LC3*) were screened in the 10-kb upstream region of each gene based on the HapMap database. A total of 16 potentially functional SNPs were finally selected according to linkage disequilibrium analyses with an r^2 threshold of 0.80 and predictions from SNP info Web Server (http://snpinfo.niehs.nih.gov/). The *ATG3* rs2705507 polymorphism was excluded since it cannot be analyzed by the MassArray system (Sequenom Inc., USA). Other SNPs previously reported as being associated with survival or metastasis in general were also included, including *ATG16L1*: rs2241880 (Table 1).

Genotyping

The SNPs were genotyped as previously described [22]. Briefly, the SNPs were genotyped using MALDI-TOF mass spectrophotometry to detect allele-specific primer extension products with the Mass ARRAY platform (Sequenom, Inc., USA). Assay data were analyzed using

Table 1 Patient- and disease-related characteristics and their association with brain metastasis

Characteristic	No. of patients (%)	Univariate analysis			Multivariate analysis		
		HR	95% CI	*P* Value	HR	(95% CI)	*P* value
Sex							
Female	41 (39)	1.000			1.000		
Male	64 (61)	1.426	0.615–3.305	0.408	1.016	0.346–2.981	0.977
Age (years)							
≥ 60	39 (37)	1.000			1.000		
< 60	66 (63)	1.217	0.525–2.822	0.646	1.047	0.421–2.608	0.921
Median (range)	57 (26–82)						
Disease stage at diagnosis							
I–III	48 (46)	1.000			1.000		
IV	57 (54)	3.008	1.201–7.538	0.019	2.756	1.051–7.233	0.039
Tumor histology							
Squamous cell	13 (12)	1.000			1.000		
Adenocarcinoma	85 (81)	0.974	0.290–3.266	0.966	0.787	0.217–2.863	0.717
NSCLC, NOS	7 (7)	0.589	0.061–5.661	0.646	0.461	0.046–4.645	0.511
KPS Score							
> 80	12 (11)	1.000			1.000		
80	83 (79)	2.584	0.382–21.326	0.307	1.379	0.168–11.309	0.765
< 80	10 (10)	7.955	0.928–68.205	0.059	3.960	0.392–40.023	0.244
Tobacco Smoking Status							
Current	36 (34)	1.000			1.000		
Former	11 (11)	0.831	0.237–2.916	0.772	1.191	0.309–4.587	0.800
Never	58 (55)	0.381	0.162–0.891	0.026	0.371	0.129–1.064	0.065

Multivariate analyses were adjusted for all of the factors listed in this table

the Sequenom TYPER software (version 4.0). The individual call rate threshold was at least 95%. To assess reproducibility, 5% of the DNA samples were blindly and randomly analyzed in duplicates, and the results revealed a reproducibility of 99%.

Statistical analysis

Patients were grouped according to their genotype. Statistical analyses were performed using the SPSS software (version 16.0). Univariate and multivariate Cox proportional hazard regression analyses were utilized to calculate hazard ratios (HRs) and 95% confidence intervals (CIs). Gender, age, disease stage, tumor histology, KPS, and smoking status were used as adjustment factors for the multivariate analyses. Kaplan-Meier curves were plotted to assess the cumulative BM probability. Differences between the BM risks were examined using the log-rank test. All *P* values were two-sided, and *P* values of < 0.05 were considered statistically significant.

Results

Patient characteristics and clinical outcomes

Associations according to univariate and multivariate analyses between patient and tumor-related characteristics with BM are shown in Table 1. The median age of all patients was 57 years (range, 26–82 years). Of all the cases, 54% were diagnosed at stage IV, and the others were recurrence cases from stage I, II, and III. 45% had smoked tobacco (68.7% of men and 7.3% of women). Overall, the median time from NSCLC diagnosis to detection of BM was 11 months, and median follow-up time was 25 months.

Clinical characteristics and BM risk

The association between six clinical characteristics and brain metastasis risk were studied (Table 1). Fig. 1 illustrated the cumulative BM rates for all patients according to clinical characteristics. We found that disease stage at diagnosis and KPS were associated with the risk of brain metastasis. Patients with stage IV disease were more likely to develop BM. The patients with stage IV disease also had an increased cumulative BM hazard of 33% compared to 13% in patients with state I, II, or III (log-rank $P < 0.01$, Fig. 1a). Patients with KPS > 80 were associated with an decreased BM risk (log-rank $P = 0.039$, Fig. 1b). In multivariate analysis, only stage is still associated with the risk of brain metastasis (HR = 2.756, 95% CI = 1.051–7.233, $P = 0.039$). All of these variables were adjusted in the subsequent multivariate analyses.

Individual SNPs and BM risk

A total of 16 SNPs from six genes in the autophagy pathway were analyzed (Table 2). Four SNPs from three

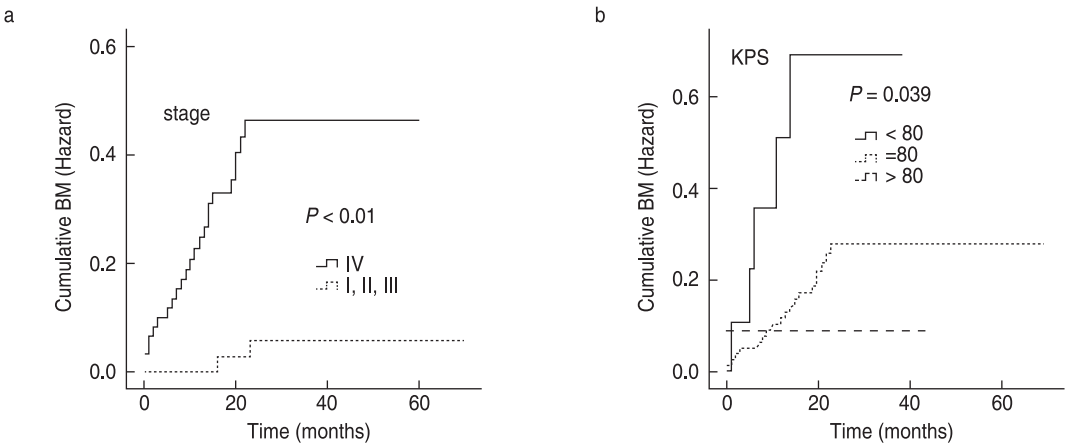

Fig. 1 Kaplan-Meier estimates of the cumulative probability of BM among patients with NSCLC according to the clinical characteristics. (a) stage at diagnosis; (b) KPS. The patients with stage IV disease had an increased cumulative BM hazard. Patients with KPS > 80 were associated with decreased BM risk.

genes showed significant associations with BM risk: two SNPs with $P < 0.05$ and two SNPs with $P < 0.01$ (Table 3). Fig. 2 illustrates the cumulative BM rates for all patients according to their genotype. The most significant association between BM risk and SNPs in this study was found with ATG10: rs3734114. The CT/CC genotype of ATG10: rs3734114 was associated with increased BM risk among all the patients in this group. The patients with the CT/CC genotype of ATG10: rs3734114 had an increased cumulative BM hazard of 46% compared to 13% in patients with the TT genotype (log-rank $P = 2.448E\text{-}4$; Fig. 2c). Multivariate Cox proportional hazard analyses showed that the CT/CC genotype of ATG10: rs3734114 was associated with a significantly higher BM risk [hazard ratio (HR) 4.593, 95% confidence interval (CI) 1.956–10.783, $P = 4.642E\text{-}4$] after adjusting for gender, patient age, disease stage, tumor histology, Karnofsky performance status (KPS), and smoking status (Table 3).

We also found three other SNPs, ATG3: rs7652377, ATG10: rs10036653 and ATG16L1: rs2241880, that were associated with BM risk. BM rates were higher for patients with the CC genotype of ATG3: rs7652377 (log-rank $P = 0.013$; Fig. 2a), the AA genotype of ATG10: rs10036653 (log-rank $P = 0.003$; Fig. 2b), and the AA genotype of ATG16L1: rs2241880 (log-rank $P = 0.018$; Fig. 2d). Multivariate Cox proportional hazard analyses showed the same results. The ATG3 rs7652377 A allele, ATG10 rs10036653 T allele, and ATG16L1 rs2241880 G allele proved to be protective alleles, which were significantly associated with lower BM risk. (HR = 0.267, 95% CI = 0.095–0.750, $P = 0.012$ for ATG3 rs7652377; HR = 0.254, 95% CI = 0.112–0.574, $P = 0.001$ for ATG10 rs10036653; HR = 0.417, 95% CI = 0.181–0.963, $P = 0.040$ for ATG16L1 rs2241880). The remaining 12 selected SNPs showed no associations between their genotype and BM risk (Table 4).

Table 2 Genes and single nucleotide polymorphisms selected for analysis

Gene (Number of SNPs)	SNP	Allelic change
ATG3 (1)	rs7652377	C > A
ATG5 (3)	rs510432	G > A
	rs688810	T > C
	rs3804338	C > T
ATG7 (3)	rs8154	T > C
	rs1375206	C > G
	rs1470612	G > A
ATG10 (5)	rs1864183	A> G
	rs1864182	T > G
	rs10514231	T > C
	rs10036653	A > T
	rs3734114	T > C
ATG12 (3)	rs26532	A > C
	rs26534	G > A
	rs26538	C > T
ATG16L1 (1)	rs 2241880	T > C

Discussion

EGFR-TKIs have proved to be promising in NSCLC treatment, especially in lung adenocarcinoma patients harboring *EGFR* mutations. However, these patients seem more inclined to develop BM. Here, we determined whether genetic variations in the autophagy core genes are associated with brain metastasis risk. Multiple genetic variations in autophagy core genes, including *ATG3*: *rs7652377*, *ATG10*: rs10036653, *ATG10*: rs3734114 and *ATG16L1*: rs2241880, were found to be significantly associated with brain metastasis. To the best of our

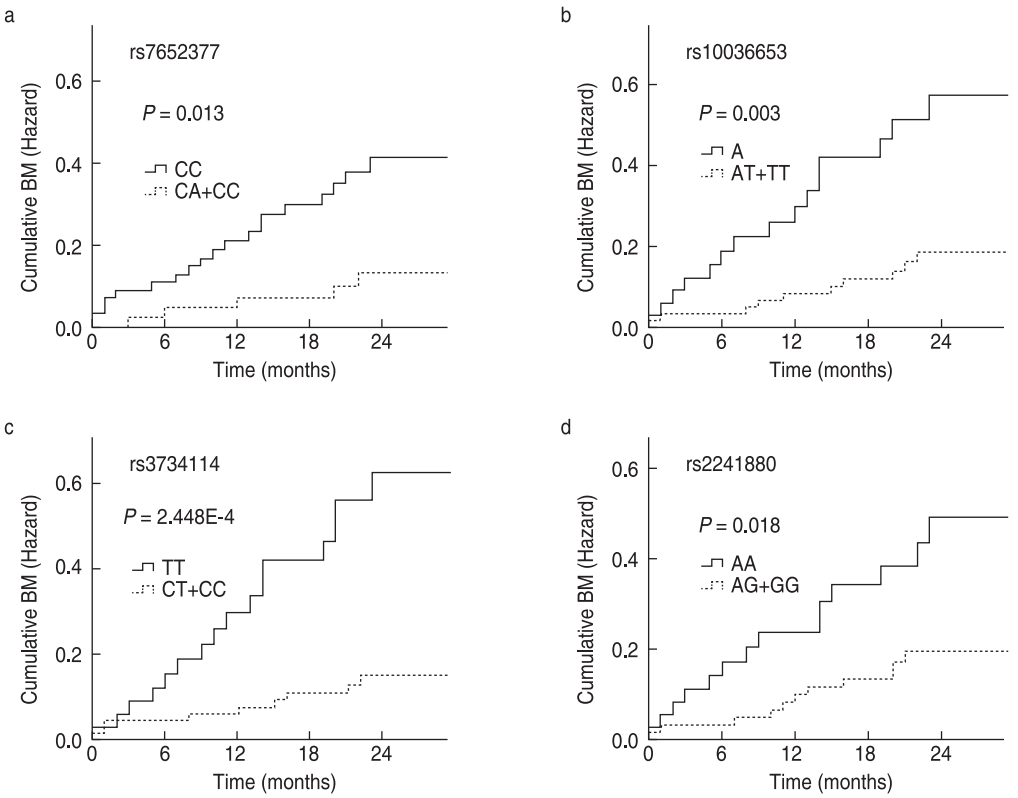

Fig. 2 Kaplan-Meier estimates of the cumulative probability of BM among patients with NSCLC according to the following genotypes: (a) ATG3: rs7652377; (b) ATG10: rs10036653; (c) ATG10: rs3734114; (d) ATG16L1: rs2241880. The CC genotype at rs7652377, the AA genotype at rs10036653, the CT/CC genotype at rs3734114, and the AA genotype at rs2241880 were associated with higher cumulative probability of brain metastasis than the other genotypes

Table 3 Associations between genotypes and BM

Characteristic	No. of patients	No. of events (%)	Univariate analysis			Multivariate analysis		
			HR	95% CI	*P* value	HR	95% CI	*P* value
ATG3: rs7652377								
CC	58	19 (33)	1.000			1.000		
AC + AA	43	5 (12)	0.309	0.115-0.828	0.019	0.267	0.095–0.750	0.012
ATG10: rs10036653								
AA	35	15 (43)	1.000			1.000		
AT + TT	63	10 (16)	0.312	0.140-0.694	0.004	0.254	0.112–0.574	0.001
ATG10: rs3734114								
TT	68	9 (13)	1.000			1.000		
CT + CC	35	16 (46)	4.094	1.807-9.277	0.001	4.593	1.956–10.783	4.642E-4
ATG16L1: rs2241880								
AA	38	14 (37)	1.000			1.000		
AG + GG	64	11 (17)	0.399	0.181-0.880	0.023	0.417	0.181–0.963	0.040

Multivariate analyses in this table were adjusted for sex, patient age, tumor histology, disease stage, Karnofsky Performance Status, and smoking status. HR, hazard ratio; CI, confidence interval; BM, brain metastases

knowledge, this is the first study to show this association in NSCLC patients who received EGFR-TKI.

Here, we also found one SNP (rs2241880) in ATG16L and two SNPs (rs10036653 and rs3734114) in ATG10 that were associated with BM risk in NSCLC patients treated with EGFR-TKI. Variations in genes in the autophagy pathway are detected in several human cancers. Autophagy in cancer is context-dependent, acting as both a tumor suppressor and tumor promotor, depending on the tumor development stage [23]. The SNPs

Table 4 Associations between genotypes and BM (the other 12 selected SNPs)

Characteristic	No. of patients	No. of events (%)	Univariate analysis				Multivariate analysis		
			HR	95% CI	*P* value	[a]*P* value	HR	95% CI	*P* value
ATG5: rs510432									
GG	30	3 (10)	1.000				1.000		
AG + AA	73	22 (30)	3.323	0.994–11.104	0.051	0.061	3.581	0.949–13.515	0.060
ATG5: rs688810									
TT	37	10 (27)	1.000				1.000		
CT + CC	65	15 (23)	0.844	0.379–1.880	0.678		1.506	0.621–3.656	0.365
ATG165: rs3804338									
CC	77	19 (25)	1.000				1.000		
CT + TT	27	6 (22)	0.882	0.352–2.210	0.790		0.655	0.255–1.683	0.380
ATG7: rs8154									
TT	79	17 (22)	1.000				1.000		
CT + CC	21	8 (38)	2.050	0.884–4.754	0.094		2.185	0.905–5.273	0.082
ATG7: rs1375206									
CC	45	11 (24)	1.000				1.000		
CG + GG	55	12 (22)	0.896	0.395–2.030	0.792		0.911	0.381–2.180	0.835
ATG7: rs1470612									
GG	56	13 (23)	1.000				1.000		
AG + AA	48	12 (25)	1.105	0.504–2.422	0.803		1.171	0.504–2.724	0.713
ATG10: rs1864183									
AA	81	21(26)	1.000				1.000		
AG + GG	22	4 (18)	0.657	0.225–1.914	0.441	0.580	0.711	0.223–2.266	0.564
ATG10: rs1864182									
TT	86	21 (24)	1.000				1.000		
GT + GG	16	4 (25)	1.014	0.348–2.954	0.980	1.000	1.078	0.347–3.342	0.897
ATG10: rs10514231									
TT	84	19 (23)	1.000				1.000		
CT + CC	19	6 (32)	1.525	0.609–3.818	0.368		1.455	0.535–3.957	0.463
ATG12: rs26532									
AA	36	5 (14)	1.000				1.000		
CA + CC	66	20 (30)	2.216	0.831–5.904	0.112		1.798	0.656–4.931	0.254
ATG12: rs26534									
GG	53	14 (26)	1.000				1.000		
AG + AA	49	11 (22)	0.827	0.375–1.821	0.637		0.843	0.362–1.963	0.692
ATG12: rs26538									
CC	56	13 (23)	1.000				1.000		
CT + TT	47	12 (26)	1.133	0.517–2.483	0.756		1.025	0.430–2.442	0.956

Multivariate analyses in this table were adjusted for sex, patient age, tumor histology,disease stage, Karnofsky Performance Status, and smoking status. HR, hazard ratio; CI, confidence interval; BM, BM. [a] *P* values were calculated by the Fisher exact test

investigated in our study are located in the genes that are critical in the early stage of the autophagy pathway, and they are necessary for autophagosome formation [24]. ATG10 is essential for the conjugation of ATG12 to ATG5 and ultimately to ATG16L. Previously, variants in ATG genes have been associated with risk and/or prognosis in other cancers [20, 25]. Huang *et al.* observed an association between *ATG16L1* rs78835907 and recurrence of localized disease, which was replicated in more advanced disease [26]. In head and neck squamous cell carcinoma, Fernández-Mateos *et al.* observed an association between ATG10 rs1864183 and a higher susceptibility to develop laryngeal cancer and an association between *ATG16L1* rs2241880 and oral carcinoma [27]. A nonsynonymous polymorphism in *ATG16L*, rs2241880 (T300A), has been extensively studied in Crohn's disease [28]. This ATG16L SNP (GG) creates a caspase 3 and caspase 7 cleavage site, reducing protein stability and resulting in decreased autophagy. The presence of this variant is clinically associated with increased risk of ileal Crohn's disease in adults and decreased survival [28]. *ATG10* is an E2-like enzyme involved in E2 ubiquitin-like modifications, and it is essential for autophagosome formation. Jo *et al.* found that ATG10 was increased in colorectal cancer and

associated with lymphovascular invasion and lymph node metastasis [29]. Qin *et al*. demonstrated that potentially functional polymorphisms in *ATG10* were associated with breast cancer risk in the Chinese population [20]. These results indicate that *ATG10* and its genetic polymorphisms might be an important component during carcinogenesis.

Furthermore, we found *ATG3*: rs7652377 polymorphisms to be associated with brain metastasis risk. ATG3, an E2-like enzyme, catalyzes ATG8 phosphatidylethanolamine conjugation, which is essential for autophagy [30]. Wang *et al*. observed that ATG3 knockdown together with oncogenic RAS activation achieved a synergistic effect in inducing epithelial mesenchymal transformation (EMT) [31]. Another study revealed that ATG3 was significantly upregulated in patients with NSCLC [32]. Cells expressed high basal autophagy-related 3 protein (ATG3) in erlotinib-resistant lung adenocarcinoma [33]. ATG3-mediated autophagy also plays an important role in apoptotic cell death of NSCLC cells [33].

The brain is the main site of treatment failure in EGFR mutant patients. Although we have shown that PCI can decrease the incidence of BM and prolong disease-free survival in patients with high risk of BM in a randomized phase III trial [34], it remains to be determined whether conducting early intervention on patients with EGFR mutations who received a first-line EGFR-TKI with PCI could prolong disease-free survival or even overall survival of these patients.

Here, the incidence of BM was 24% (25 of 105 patients), which is slightly lower than in some other studies. We obtained post-treatment computed tomography (CT) or magnetic resonance imaging (MRI) scans only if clinical evaluation revealed suggestive findings like neurological symptoms. As is true in other studies analyzing BM risk factors, this could limit the accuracy of a putative molecular marker of BM risk. These differences may explain the relatively low incidence of BM in our population. As with all retrospective analyses, interpretation of these results is limited by bias. Another limitation is the small size of some subgroups, which could have affected some analyses. Further studies will be needed to determine if PCI in this group of patients will offer a survival benefit.

In conclusion, to our knowledge, this study is the first to evaluate the associations between genetic variations in the autophagy pathway and BM risk. We found that four SNPs (ATG16L1: rs2241880, ATG10: rs10036653, rs3734114, and ATG3: rs7652377), are significantly associated with NSCLC treated with EGFR-TKI. Further studies will be needed to determine if PCI in this group of patients will offer a survival benefit.

Conflicts of interest

The authors declared that they have no conflicts of interest.

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ORIGINAL ARTICLE

A missense variant of MASP2 is associated with increased risk of radiation pneumonitis in lung cancer patients treated with radiation therapy*

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Abstract

Objective In this study, mannan-binding lectin-associated serine protease 2 (*MASP2*) gene variant was evaluated to assess the risk of radiation pneumonitis (RP) in patients with pulmonary malignancies.

Methods A total of 169 lung cancer patients with radiotherapy were included in our prospective study (NCT02490319) and genotyped using the Sanger sequencing method. Multivariate Cox hazards analysis and multiple testing were applied to estimate the hazard ratio (HR) and 95% confidence intervals (CIs) of all factors possibly associated with RP risk.

Results Patients with mean lung disease ≥ 15 Gy and V_{20} ≥ 24% had higher risk of RP ≥ grade 2 compared with their counterparts (HR = 1.888, 95% CI: 1.186–3.004, P = 0.007; HR = 2.126, 95% CI: 1.338–3.378, P = 0.001, respectively). Importantly, CC + CA genotype of MASP2: rs12711521 was strongly associated with an increased occurrence of RP ≥ grade 2 (HR = 1.949, 95% CI: 1.278–2.971, P = 0.002).

Conclusion *MASP2: rs12711521* was found to be significantly associated with RP ≥ grade 2 in our cohort and may thus be one of the important predictors of severe RP before radiotherapy, if further validated in larger population.

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Key words: radiation pneumonitis; lung cancer; mannan-binding lectin-associated serine protease 2 (MASP2); Single Nucleotide Polymorphisms (SNP)

Currently, lung cancer remains as one of the greatest health threats against humans. According to the latest summary of cancer data, in 2020, there will be approximately 228,820 new lung cancer cases and 135,720 deaths in the USA [1]. As a state-of-the-art therapeutic intervention, radiotherapy (RT), with or without the combination of chemotherapy, acts as an effective imaging tool used to treat this deadly disease. However, due to RT-related complications that cause patients intolerant of the RT dosage, the overall efficacy of RT is suppressed.

Pathologically, radiation pneumonitis (RP) generally causes inflammatory response of the lung tissue after irradiation treatment. Subsequently, fibrotic process occurs after acute inflammation is no longer observed. Because of its devastating effects against normal lung tissue and its prevalence in lung cancer patients receiving RT, RP is considered as one of the most common complications and major dose-limiting toxicity factors. Since the radiation dose and size of the irradiated volume have to be adjusted according to the patients' condition, the occurrence of RP hinders the tumor-controlling effects of RT [2–3]. RP results in poor quality of life or life-threatening symptoms in approximately 15%–40% of all patients who are irradiated for lung cancer [4]. Based on the facts mentioned above, to maximize the therapeutic effects and to minimize the adverse effects of RT, identifying reliable biomarkers for RP occurrence is considered significant. Up until now, several patient- and treatment-related factors [5], including Karnofsky Performance Status (KPS), chronic lung disease [6], smoking status, chemotherapy [7–8], dosimetric parameters,

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and plasma values of tumor growth factor (TGF)-β [9–10], have been demonstrated to be associated with RP risk. Additionally, some genetic variants were recently found to be associated with the occurrence and development of RP [11–16].

Complement system is one of the major components of human innate immunity [17]. Complement system generally promotes body clearance of invading microorganisms or damaged cells. There are several pathways that trigger complement activations, including the lectin pathway. The lectin pathway is initiated through the binding of mannan-binding lectin (MBL) with microorganism carbohydrate structures [18], whereas MBL-associated serine protease (MASP), specifically MASP2, acts as a pathway activator by cleaving into C2 and C4 [19]. As MASP2 possesses a crucial role in human defense mechanism, multiple genetic variants of MASP2 have been demonstrated to be associated with increased susceptibility to infection and sepsis [20]. However, up until now, little is known about the impact of variants of MASP2 on radiation-induced damage and tissue fibrosis. Therefore, to identify the clinically significant single-nucleotide polymorphisms (SNPs) on RP occurrence and severity, in this study, we investigated the association between MASP2 SNP *rs12711521* and RP risk in our cohort.

Materials and methods

Patient population

In this prospective study (NCT02490319), a total of 190 lung cancer patients were included. Patients enrolled received radiation therapy at Tongji Hospital, Huazhong University of Science and Technology (Wuhan, China) between 2009 and 2015. The inclusion criteria were as follows: patients with a radiation dose of at least 45 Gy, patients aged > 18 years, patients with KPS score > 60, and patients with a life expectancy of at least 6 months. Patients with previous thoracic irradiation or severe cardiopulmonary diseases were excluded from our study. Of the 199 patients, 169 [114 with non-small-cell lung cancer (NSCLC) and 55 with small-cell lung cancer (SCLC)] were eventually included for the final genotyping analysis. Samples from 169 patients were initially used to genotype the candidate SNP using the Sanger sequencing method. This study was approved by the Institutional Review Board of Tongji Hospital. Written informed consents were obtained from all patients for the use of their clinical information and for obtaining their blood and DNA samples.

Treatment and follow-up

All patients received RT with 6-MV X-rays from a linear accelerator (Elekta Synergy, Elekta, Sweden). The median total radiation dose was 56 Gy (range, 45 to 66 Gy), with 1.5 to 2 Gy administered per radiation treatment. Intensity-modulated radiation therapy was administered to 46.7% of patients (n = 79). Computed tomography simulation (CT/e, GE, USA) was performed before the RT treatment was planned. The target volumes and critical normal organs were delineated using the three-dimensional planning system (Pinnacle version 9.2). The baseline clinical characteristics and treatment details of the patients are shown in Table 1.

All patients enrolled in this study were examined during and 1 month after RT. Subsequently, the patients were followed up every 3 months for the first year and every 6 months thereafter. At each follow-up visits, all patients were instructed to undergo a chest X-ray or CT, and patients' clinical information, including symptoms, was collected. RP was graded by two radiation oncologists according to the Common Terminology Criteria for Adverse Events version 4.0 as follows: Grade 0, no change; Grade 1, asymptomatic and diagnosed by radiographic findings only; Grade 2, symptomatic, not interfering with daily activities; Grade 3, symptomatic, interfering with daily activities or oxygen required; Grade 4, assisted ventilation required; and Grade 5, fatal.

Genotyping methods

Genomic DNA was extracted using a PureLink Genomic DNA Mini Kit (Invitrogen, K1820-01) from the patients' peripheral blood. Subsequently, the *MASP2: rs12711521* was genotyped by the Sanger sequencing method in the remaining 169 patients. The primer pairs for *MASP2: rs12711521* were F: 5'-GATTCCTCCCTCCCATGCTTC -3' and R: 5'-ATGTACTCCACTCGGCCACT-3'. The polymerase chain reaction products were subsequently subjected to DNA sequencing to detect mutations.

Statistical analyses

The end point for this study was the development of RP ≥ grade 2. The time to the end point was calculated from the start of RT. Patients who did not experience RP ≥ grade 2 within 12 months of RT were censored. The Statistical Package for the Social Sciences (SPSS) version 21.0 statistical software (SPSS, Inc., USA) was used for the statistical analysis. Patients were divided into groups according to their genotypes, and Cox proportional hazards analysis was performed to estimate the hazard ratio (HR) and 95% confidence intervals (CIs) of all factors possibly associated with RP risk. Moreover, multivariate Cox regression analysis was used for the adjustment of covariates. The influences of the genotypes on RP risk were assessed using the Kaplan-Meier analysis and compared using the log-rank tests.

Table 1 Patient characteristics (n = 169)

Characteristics	No. of patients	%
Sex		
Male	125	74.0
Female	44	26.0
Age (years)		
Median	58	
Range	28–78	
Histology		
SCLC	55	32.5
NSCLC	114	67.5
Stage		
I–II	24	10.2
III–IV	145	85.8
KPS		
80–100	123	72.6
< 80	46	27.4
Smoking		
Smoker	106	62.0
Non-smoker	63	38.0
Chemotherapy		
Yes	160	94.7
No	9	5.3
CRT		
Yes	44	26.0
No	125	74.0
Surgery		
Yes	86	50.9
No	83	49.1
IMRT		
Yes	79	46.7
No	90	53.3
Radiation dose (cGy)		
Median	5600	
Range	4500–6600	
MLD (cGy)		
Median	1368	
Range	178–2017	
V_{20}		
Median	24.82	
Range	0–42.00	
COPD		
Yes	19	11.2
No	150	88.8

CRT, concurrent chemoradiation; IMRT, intensity-modulated radiation therapy; V20, volume of normal lung receiving 20 Gy or more radiation; COPD, chronic obstructive pulmonary disease

Results

Patient characteristics and RP

A total of 169 (125, men; 44, women) patients were included in this study. Their characteristics were listed in Table 1. The median age of the patients was 58 years (range, 28–78 years); moreover, 114 and 55 patients had NSCLC and SCLC, respectively. In the study cohort, 85.5% of patients had stage III–IV disease, 50.9% underwent surgery before RT, almost all patients (94.7%) received induction chemotherapy followed by RT, and 26.0% received concurrent chemoradiation. The median radiation dose was 56 Gy (range, 45–66 Gy), the median mean lung disease (MLD) was 13.68 Gy (range, 1.78–20.17 Gy), and the median V_{20} was 24% (range, 0%–42.00%).

Within 12 months of RT, 99 patients (58.6%) had RP ≥ grade 2. The associations between patient-, tumor- and therapy-related characteristics and RP ≥ grade 2 were listed in Table 2. The univariate and multivariate analyses by Cox regression model revealed that MLD and V_{20} were significantly associated with RP ≥ grade 2. Patients with older age, MLD ≥ 15 Gy, and V_{20} ≥ 24% had higher risk of RP ≥ grade 2 compared with their counterparts (H1.888, 95% CI: 1.186–3.004, P = 0.007; HR = 2.126, 95% CI: 1.338–3.378, P = 0.001, respectively) (Table 2), which were consistent with the results of other publications.

MASP2 single-nucleotide polymorphisms and RP

MASP2: rs12711521 was found to be significantly associated with the occurrence of RP ≥ grade 2 (Table 3). Fig. 1 presented a plot of the RP-free survival percentage for RP ≥ grade 2 for each genotype of *MASP2: rs12711521* determined using the Kaplan-Meier method. Patients with the CC + CA genotype of *MASP2: rs12711521* had significantly higher risks of RP ≥ grade 2 than patients with AA genotype (P = 0.003). Furthermore, multiple Cox proportional hazards analyses with adjustments for all of the characteristics listed in Table 1 revealed that the

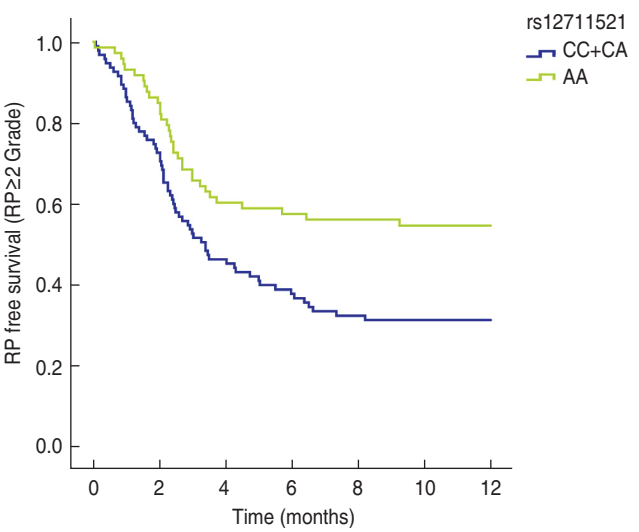

Fig. 1 Kaplan-Meier estimates RP-free survival (RP ≥ grade 2) for each genotype. Patients with CC genotype of *MASP2: rs12711521* had significantly higher risks of RP ≥ grade 2 (P < 0.0001)

Table 2 Association between patient-, tumor-, and therapy-related characteristics and grade ≥ 2 radiation pneumonitis (*n* = 169)

Parameter	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*	HR	95% CI	*P*
Sex						
Male	1			1		
Female	1.216	0.773–1.915	0.398	1.379	0.758–2.511	0.292
Age (years)						
< 58	1			1		
≥ 58	1.413	0.951–2.098	0.087	1.541	0.997–2.393	0.052
Histology						
SCLC	1			1		
NSCLC	1.195	0.791–1.804	0.398	1.251	0.727–2.153	0.418
Stage						
I–II	1	1		1		
III–IV	1.100	0.601–2.013	0.758	1.132	0.593–2.163	0.707
KPS						
80–100	1			1		
< 80	1.341	0.877–2.052	0.176	1.566	0.993–2.470	0.054
Smoking						
Smoker	1			1		
Non-smoker	0.926	0.619–1.386	0.708	0.964	0.337–2.192	0.435
Surgery						
Yes	1			1		
No	1.014	0.684–1.504	0.945	0.690	0.390–1.223	0.204
Chemotherapy						
Yes	1	1		1		
No	0.500	0.203–1.233	0.132	0.473	0.189-1.187	0.111
CRT						
Yes	1			1		
No	0.843	0.529–1.344	0.472	0.956	0.575–1.588	0.861
IMRT						
Yes	1			1		
No	1.077	0.726–1.598	0.712	1.098	0.710–1.699	0.675
Radiation dose (cGy)						
< 5600	1			1		
≥ 5600	1.083	0.729–1.610	0.692	1.139	0.633–2.535	0.737
MLD (cGy)						
< 1500	1			1		
≥ 1500	1.510	1.093–2.235	0.045	1.888	1.186–3.004	0.007
V_{20} (%)						
< 24	1			1		
≥ 24	1.730	1.138–2.631	0.010	2.126	1.338–3.378	0.001
COPD						
Yes	1			1		
No	0.639	0.246–1.661	0.358	0.780	0.339–1.998	0.472

Multivariate analyses were adjusted for all of the factors in this table.*Either MLD or V_{20} was used in the multivariate analyses, but not both

CC + CA genotype of *MASP2: rs12711521* was strongly associated with an increased occurrence of RP ≥ grade 2 (HR = 1.949, 95% CI: 1.278–2.971, *P* = 0.002; Table 3).

MASP2: rs12711521 and dosimetric factors

Patients were divided into four groups based on the dosimetric factors, V_{20} or MLD and *MASP2: rs12711521* genotypes, to evaluate the impact of the *MASP2: rs12711521* genotypes on RP in different dosimetric groups. Patients with CC + CA genotype of *MASP2: rs12711521* and MLD ≥ 15 Gy or V_{20} ≥ 24% had higher risk of RP grade ≥ 2 compared with the other groups (*P* = 0.003 and *P* = 0.002, respectively; Fig. 2a and 2b). Interestingly, patients with *MASP2: rs12711521* AA genotype and

Table 3 Association between genotypes and grade ≥ 2 RP

Polymorphism and Genotype	No.of event	No.of total	Univariate analysis			Multivariate analysis		
			HR	95% CI	*P*	HR	95% CI	*P*
MASP2: rs12711521								
AA	33	73	1			1		
CC+CA	65	95	1.856	1.220–2.824	0.004	1.949	1.278–2.971	0.002

Multiple analyses in this table were adjusted for MLD listed in Table 1. HR, hazard ratio; CI, confidence interval

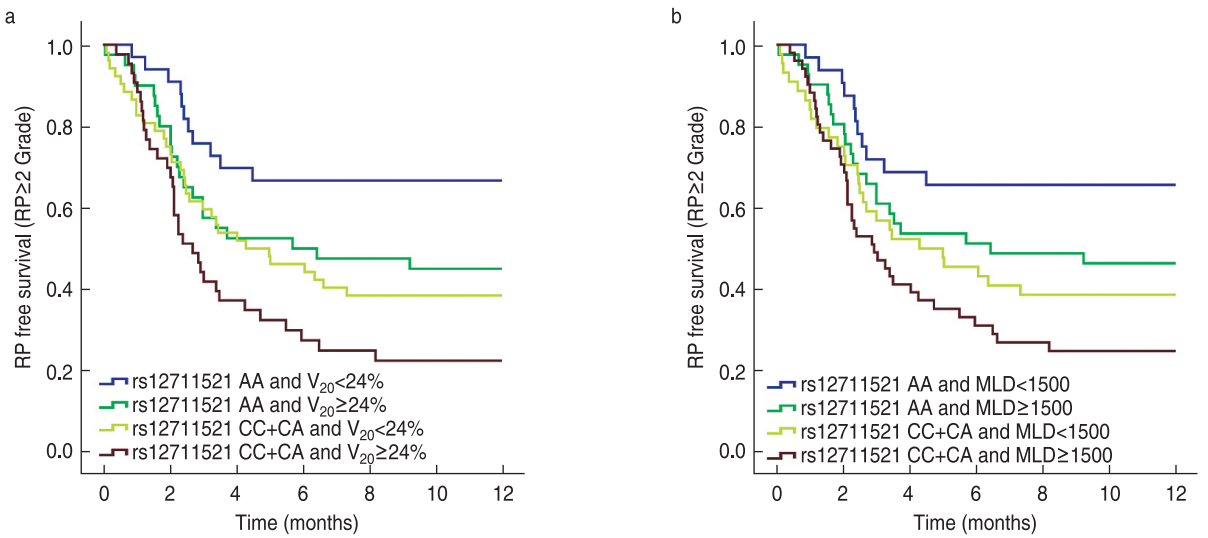

Fig. 2 Kaplan-Meier estimates effect of genotype in *MASP2: rs12711521* and dosimetric parameters on RP-free survival (RP ≥ grade 2). (a) *MASP2: rs12711521* and MLD; (b) *MASP2: rs12711521* and V_{20}

MLD ≥ 15 Gy or V_{20} ≥ 24% had similar incidence of RP ≥ grade 2 with patients with CC/CA genotype who received MLD less than 15 Gy or V_{20} less than 24%, suggesting the dominant and independent role of rs12711521 genotypes in severe RP.

Discussion

In this study, *MASP2: rs12711521* was found for the first time to be significantly associated with the occurrence of RP ≥ grade 2. Patients with the CC + CA genotype of *MASP2: rs12711521* had a significantly increased risk of RP after RT for lung cancer. We also discovered that the association between *MASP2: rs12711521* and RP grade≥ 2 was independent of MLD and V_{20}.

In this study, the incidence rate of RP ≥ grade 2 was 58.6%, which was similar to the incidence rates reported in previous studies. Due to the prospective nature of our study, the incidence rate of RP was relatively higher than the incidence rates in some retrospective studies. We also confirmed that age, MLD, and V_{20} were closely associated with RP risk. In our cohort, patients with MLD ≥ 15 Gy and V_{20} ≥ 24% had a greater risk of developing RP grade ≥ 2 than their counterparts, which verified the associations between the radiation dosimetric-related factors and the occurrence of RP.

Up until now, the exact role of MASP2 in tissue inflammation and fibrosis is largely unknown. However, since MASP2 functions as a critical molecule in the complement pathway activation, investigating the influences of complement pathway on pulmonary fibrosis was considered significant. A study utilizing complement C5-deficient bleomycin-induced pulmonary fibrosis mice model indicated that C5-deficient mice exhibited increased inflammatory response during the acute phase of bleomycin treatment. However, in chronic phase of bleomycin injury, C5-deficient mice showed lower tissue fibrosis compared with the C5-sufficient group. Further study indicated that such pro-fibrotic effects of C5 were possibly associated with the expression of TGF-β1 and matrix metalloproteinase-3 [21]. Additionally, another genome-wide and linkage region-specific association study utilizing bleomycin treatment mice model indicated that MASP2 E384Q and V172I polymorphisms were significantly associated with bleomycin-induced lung fibrosis [22]. Our study presented additional evidence of MASP2 participation in radiation-induced lung fibrosis.

Moreover, as a missense variant, with *rs12711521* being located in the coding region of MASP2, *in silico* prediction using PolyPhen (http://genetics.bwh.harvard.edu/pph2/) demonstrated possibly damaging impact (0.51), which indicated that *rs12711521* might be associated with MASP2 normal protein structure or expression value. Detailed molecular and biological effects of *rs12711521* require further functional analysis.

Moreover, we demonstrated for the first time the prevalence and clinical value of *MASP2: rs12711521* on RP in independent Chinese Han cohort, and based on the result of our study, *MASP2: rs12711521* may be one of the important predictors of severe RP before RT in addition to radiation dosimetric factors. Patients with RP susceptibility genotypes will significantly benefit from the early prediction and prevention of RP by genotyping before the initiation of RT. Furthermore, this study is considered beneficial in selecting patients without RP susceptibility genotypes and increasing their radiation dose appropriately for a better tumor control. Specifically for patients with favorable genotypes, increased MLD and V_{20} will not increase their incidence of severe RP, which could assist the oncologist in individually adjusting patients' radiation dose. On the contrary, our results still require further validation in expanded cohorts from different races since the substantial ethnic variation exists in SNP frequencies.

Furthermore, our findings suggest the possible role of MASP2 in the pathogenesis of RP. According to the latest report, MASP2 monoclonal antibody (narsoplimab, OMS721) has been developed, and its recent clinical trial demonstrated promising result in the treatment of thrombotic microangiopathy in patients undergoing hematopoietic stem cell transplantation [23]. If MASP2 biological function was confirmed in RP, targeted inhibition of MASP2 via narsoplimab might provide novel therapeutic value in future RP treatment.

In summary, this is the first study to present the associations between RP risk and MASP2: rs12711521 and thus indicated that in addition to radiation dosimetric factors, MASP2 SNP can be used as a useful predictive biomarker of RP risk before RT. Thus, patients will significantly benefit from the early prediction and prevention of RP by genotyping before the initiation of RT. Moreover, this study will benefit lung cancer patients receiving RT since appropriately tailored radiation dose might result in better control of their diseases and lower occurrence and severity of RP.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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ORIGINAL ARTICLE

Elevated pretreatment plasma fibrinogen level is associated with metastasis of non-small cell lung cancer (NSCLC)

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Abstract

Objective The aim of this study was to investigate the correlation between pretreatment fibrinogen levels and metastasis in non-small cell lung cancer (NSCLC).

Methods The study included 503 NSCLC patients with a clear pathological diagnosis and 168 patients diagnosed with benign lung diseases by histological examination. Pretreatment plasma fibrinogen values were quantified, and the relationship between plasma fibrinogen level and clinical variables comprising tumor size, metastasis, and clinical stage was examined using Kruskal-Wallis test, Wilcoxon rank sum test, and Chi-square test.

Results The median plasma fibrinogen values were statistically higher in NSCLC patients with metastasis than patients with benign lung diseases and NSCLC patients without metastasis (Kruskal-Wallis test; $P < 0.001$). Plasma fibrinogen values were also significantly higher in advanced clinical stages (Wilcoxon rank sum test; $P < 0.001$). A significant relationship was observed between elevated fibrinogen (> 2.974 g/L) and metastasis, clinical stage, and tumor size (Chi-square test; $P < 0.001$).

Conclusion This correlation suggests that elevated pretreatment plasma fibrinogen levels can predict metastasis and advanced tumor stage in NSCLC patients.

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Key words: fibrinogen; metastasis; NSCLC; tumor stage

Lung cancer is the most frequently diagnosed cancer and the leading cause of cancer deaths, with non-small cell lung cancer (NSCLC) representing 80% of all cases [1]. Majority of these cases are advanced or have metastasized by the time of diagnosis. Fibrinogen, which is synthesized mainly by the liver epithelium, plays overlapping roles in blood clotting, fibrinolysis, cellular and matrix interactions, inflammatory response, wound healing, and neoplasia [2–3]. Recently additional evidence has demonstrated that serum fibrinogen is associated with operable malignant cell growth, progression, and metastasis, such as in esophageal cancer [4], gastric cancer [5], renal cell carcinoma [6], colon cancer [7], and hepatocellular cancer [8]. In NSCLC, there is evidence that fibrinogen contributes to tumor progression, metastasis, and poor survival in operable NSCLC patients [9] and is related with the chemotherapy outcomes in advanced NSCLC patients [10]. However, as far as we know, the association of pretreatment plasma fibrinogen levels with metastasis in patients has not been well defined. Therefore, in this study, we investigated the correlation between pretreatment plasma fibrinogen levels, clinicopathological parameters, and metastasis in NSCLC patients.

Patients and methods

Patients

A retrospective study on patients with NSCLC or benign lung disease was conducted at the Harbin Medical University Cancer Hospital from January to December 2018. We retrospectively collected data from medical records and obtained oral informed consent. All data were collected and analyzed anonymously. The inclusion

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criteria were as follows: (1) all patients have a clear pathological diagnosis; (2) in operable patients, surgical specimens (lung tumor tissue or lymph nodes) were used to confirm the pathological diagnosis; (3) in inoperable patients, fine needle aspiration biopsy or bronchoscopic biopsy were used to confirm the pathological diagnosis. Patients who met the following criteria were excluded from the study: (1) received preoperative chemotherapy or radiotherapy; (2) clinical evidence of infection or other bone marrow, hematological, or autoimmune disease; (3) history of another cancer; (4) thrombosis in lower limbs. Based on the inclusion and exclusion criteria, a total of 503 NSCLC and 168 benign lung disease patients were analyzed in this study. All patients were Chinese. The pretreatment evaluation included a detailed clinical history and physical examination along with a series of biochemistry tests, complete blood cell count, and coagulation tests. Further investigations included radiography, flexible bronchoscopy, chest and upper abdominal computed tomography (CT), radionuclide bone scan, and CT or magnetic resonance imaging (MRI) of the brain. NSCLC stages were based on the 8th edition of the TNM Classification.

Fibrinogen measurement

Blood samples were collected before the initial diagnosis and invasive detection techniques. Venous blood sampling was performed one week prior to treatment, and collected in ethylenediaminetetraacetic acid (EDTA) containing tubes. Fibrinogen values were measured by an automatic coagulation analyzer.

Statistical analysis

All statistical analyses were carried out using SPSS 18.0 software (SPSS Inc., Chicago, USA). Differences between the analyzed categories were identified using the Chi-square test, Kruskal-Wallis test, and the Wilcoxon rank sum test. In order to classify the patients into two groups, the cut-off values of clinicolaboratory variables were determined using receiver operating characteristic (ROC) curve analyses. The value of the maximum combined sensitivity and specificity on the ROC plot was defined as the recommended cut-off value. Differences were considered significant when the *P* value was less than 0.05.

Results

Clinical characteristics of patients

Patient characteristics are shown in Table 1. Out of the total 671 enrolled patients, 503 patients (75.0%) were diagnosed with NSCLC with clear pathology, and the remaining (25%) were diagnosed with benign lung disease, including hamartoma, pulmonary sclerosing hemangioma, and atypical hyperplasia of the alveolar epithelium. Among all patients, 274 (40.83%) were women, and 397 (59.17%) were men. The median age was 57.24 years, with an age range from 15 to 81 years. We defined people who smoked currently or had in the past as smokers, and there were 391 smokers in this study. A total of 360 patients were diagnosed with adenocarcinoma, 127 were diagnosed with squamous cell carcinoma, and the rest were diagnosed with other types of NSCLC. The distribution of pathological stages was as follows: stage I, 205; stage II, 18; stage III, 120, and stage

Table 1 Characteristics of the 671 patients

	Benign lung disease (*n*=168)	NSCLC without metastasis (*n*=210)	NSCLC with metastasis (*n*=293)	*P*
Sex				< 0.001
Male	103	98	196	
Female	65	112	97	
Age (years)	53.95 ± 11.937	59.10 ± 8.989	57.80 ± 9.152	< 0.001
Smoking history				0.004
Yes	86	97	178	
No	82	113	115	
Histological types of NSCLC				< 0.001
Adenocarcinoma	–	164	196	
Squamous cell carcinoma	–	35	92	
Others	–	11	5	
Tumor size (cm)				< 0.001
Median	–	2	3.5	
Range	–	0.6–8.0	0.6–9.5	
Fibrinogen (g/L)				< 0.001
Median	2.695	2.509	3.620	
Range	1.203–5.506	1.530–5.223	1.577–6.539	

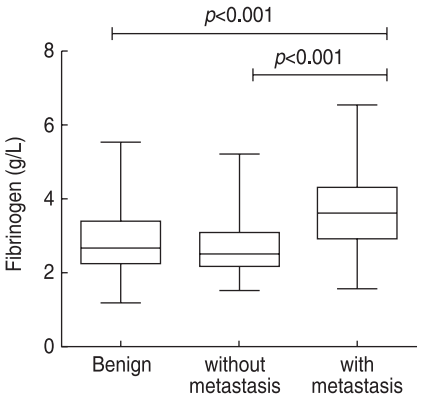

Fig. 1 Median fibrinogen values were higher in NSCLC patients with metastasis compared with the other groups

IV, 160. Among the NSCLC patients, 293 (43.67%) were diagnosed with metastasis, with 133 patients (19.82%) diagnosed as NSCLC with only lymph nodes metastasis (TxN1-3M0) and the remaining 160 patients diagnosed with at least one distant metastatic lesion (TxNxM1). NSCLC patients had a mean tumor size of 3.12 cm (range, 0.6 to 9.5 cm). Patients diagnosed with metastasis showed higher fibrinogen level than patients without metastasis and patients with benign lung disease.

All patients were divided into three groups: patients with benign lung diseases (n = 168), NSCLC patients without metastasis (n = 210), and NSCLC patients with metastasis (n = 293). As shown in Fig.1, the median fibrinogen value of patients with benign lung disease, NSCLC patients without metastasis, and NSCLC patients with metastasis was 2.695, 2.509, and 3.620 g/L, respectively. Statistically higher median fibrinogen values were exhibited by the metastasis group (Kruskal-Wallis test, P< 0.001). This result indicated that the median fibrinogen values of NSCLC patients with metastasis were higher than patients without metastasis.

Kruskal-Wallis test was used to compare the fibrinogen values among the benign, NSCLC without metastasis, and NSCLC with metastasis groups. The Wilcoxon rank sum test was used to compare the fibrinogen values between the NSCLC patients with and without metastasis. P< 0.05 was considered as statistically significant. The results demonstrated that the fibrinogen values in NSCLC patients with metastasis were higher than in the other groups, and the difference showed statistical significance.

To exclude the interference of age, sex, smoking history, and histological types of NSCLC, the difference in the fibrinogen values between cancer patients 60 years and older versus those younger than 60 years, smokers versus non-smokers, men versus women, and adenocarcinoma versus squamous cell carcinoma were compared in Fig. 2. We found that the median fibrinogen values were higher in the metastasis group regardless of age, sex, smoking history, and histological types of NSCLC.

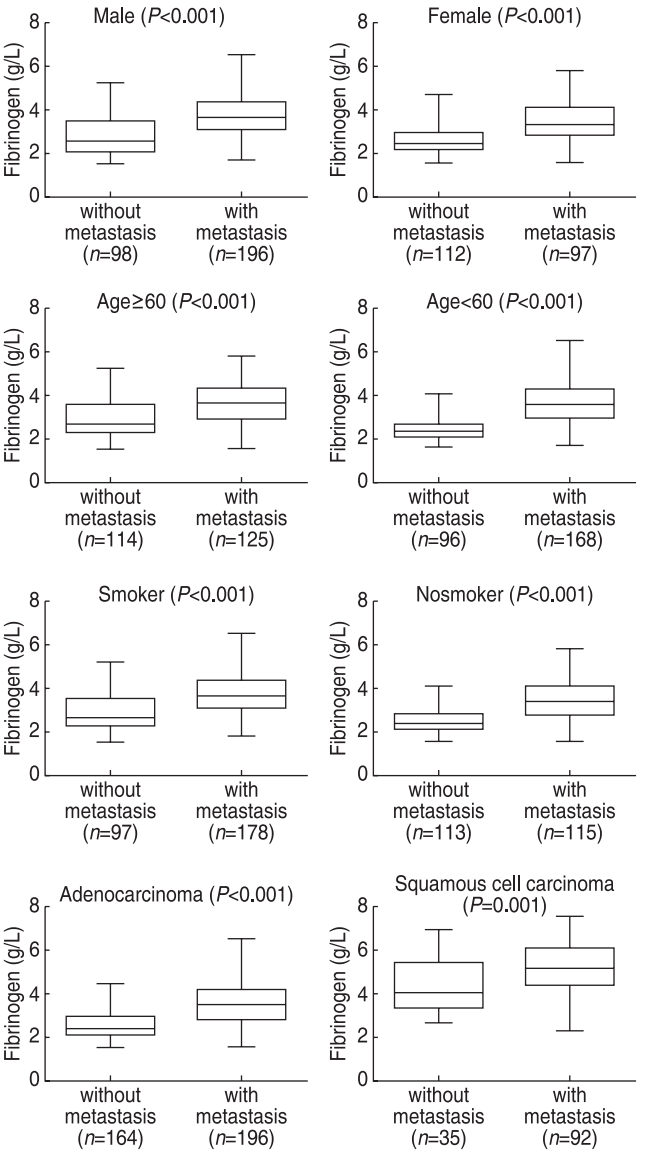

Fig. 2 Fibrinogen values were higher in NSCLC patients with metastasis than those without metastasis regardless of age, sex, smoking history, and histological types of NSCLC. The Wilcoxon rank sum test was used to compare the fibrinogen values between the two group. P < 0.05 were considered as statistically significant. A Male NSCLC patients, B Female NSCLC patients, C NSCLC patients aged 60 years and older, D NSCLC patients aged less than 60 years, E Smoker NSCLC patients, F Non-smoker NSCLC patients, G Adenocarcinoma patients, H Squamous cell carcinoma patients

Fibrinogen values in different stages of lung cancer

Since metastasis affects the TNM stage, we concluded that fibrinogen values may also have a statistically significant impact on the different TNM stages. Spearman rank correlation test was used to analyze the correlation

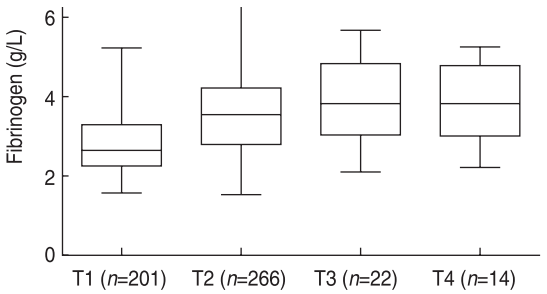

Fig. 3 Fibrinogen values in different T stages. Median fibrinogen values were 2.607, 3.522, 3.814, and 3.805g/L

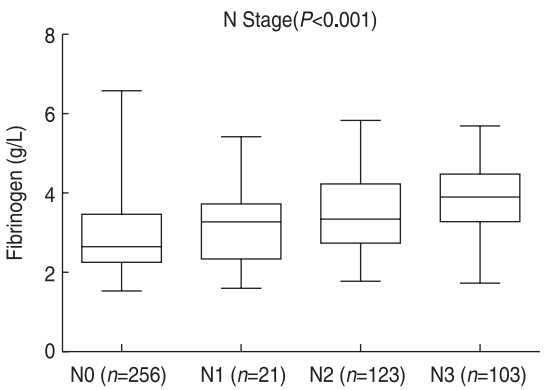

Fig. 4 Fibrinogen values in different N stages. Median fibrinogen values were 2.643, 3.267, 3.321, 3.878g/L. A statistically significant difference in the fibrinogen values was observed between the different N stages (Kruskal-Wallis test, $P < 0.001$) and the highest fibrinogen value was obtained in N3 patients

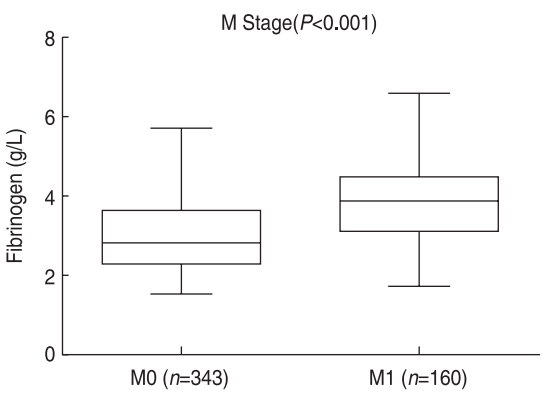

Fig. 5 Fibrinogen values in different M stages. Median fibrinogen values were 2.816 and 3.866 g/L in M0 and M3, respectively. M1 patients showed higher fibrinogen values (Wilcoxon rank sum test, $P < 0.001$)

between fibrinogen values and the TNM stage. The results of Spearman rank correlation test indicated that fibrinogen values correlated significantly with T, N, and M stages (Spearman's rho was 0.401, 0.418, and 0.378,

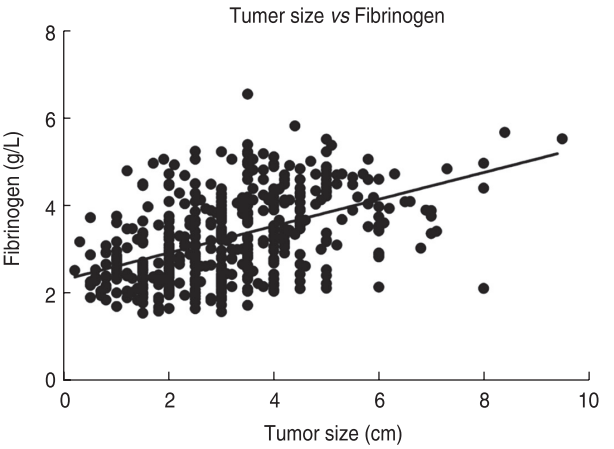

Fig. 6 Correlation between fibrinogen values and tumor size
Pearson correlation test indicated that the fibrinogen values correlated positively and significantly with tumor size (Pearson correlation coefficient = 0.480, $P < 0.001$)

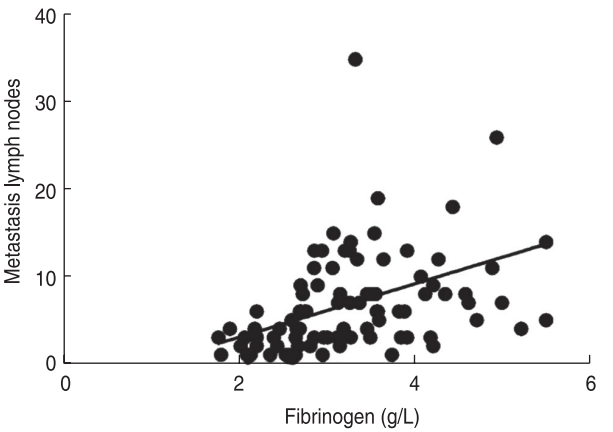

Fig. 7 Correlation between fibrinogen values and number of metastases
A total of 97 operable patients were confirmed to have lymph nodes metastasis, and Pearson correlation test was used to evaluate the relationship between fibrinogen values and lymph metastasis (Pearson correlation coefficient = 0.445, $P < 0.001$)

respectively; Fig. 3–5). Moreover, the results of the Pearson correlation coefficient analysis indicated that fibrinogen values correlated positively and significantly with the tumor size (Pearson correlation coefficient = 0.480, $P < 0.001$; Fig. 6). A total of 97 operable patients were confirmed to have lymph nodes metastasis, and Pearson correlation was used to detect the relationship between fibrinogen values and lymph metastasis. The results are shown in Fig. 7. The Pearson correlation coefficient of lymph node metastasis and fibrinogen values was 0.445 ($P < 0.001$) Although a statistically significant difference was demonstrated between the M0 and M1 patients (Wilcoxon rank sum test, $P < 0.001$; Fig. 5), neither distant metastatic lesions (Kruskal-Wallis test,

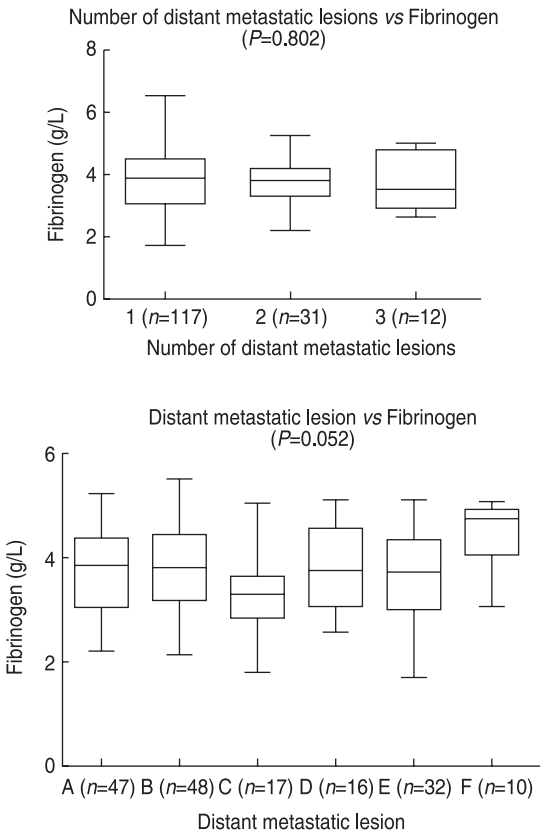

Fig. 8 Fibrinogen values in different distant metastatic lesions and the number of distant metastatic lesions. Fibrinogen values in different distant metastatic lesions (Kruskal-Wallis test, $P = 0.052$) and the number of distant metastatic lesions (Kruskal-Wallis test, $P = 0.802$) showed no statistically significant difference (A: pleural metastasis; B: lung metastasis; C: brain metastasis; D:liver metastasis; E: bone metastasis; F:adrenal gland metastasis)

$P = 0.052$), nor the number of distant metastatic lesions (Kruskal-Wallis test, $P = 0.802$) showed a statistically significant difference (Fig. 8). Overall, with regard to the TNM stage, the fibrinogen values in advanced lung cancer patients (stage III–IV) were significantly higher than those in early lung cancer patients (stage I–II) (Wilcoxon rank sum test, $P<0.001$, Fig. 9).

Fibrinogen values as a predictor of metastasis

Statistically significant difference in the fibrinogen values was observed between patients with metastasis and patients without metastasis, as shown in Fig. 1. Furthermore, fibrinogen values showed the highest correlation with the TNM stage. Therefore, we hypothesized that fibrinogen values may be used to predict metastasis and (ROC) curve analysis was used to prove this hypothesis.

The area under the curve (AUC) was 0.788 ($P < 0.001$). The value of the maximum combined sensitivity and specificity on the ROC plot was defined as the

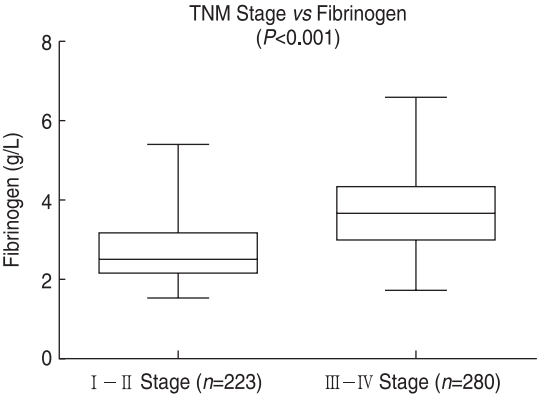

Fig. 9 Fibrinogen values and TNM stage. With regard to the TNM stage, the fibrinogen values in advanced lung cancer patients (stage III–IV) were significantly higher than in early lung cancer patients (stage I–II) (Wilcoxon rank sum test, $P < 0.001$)

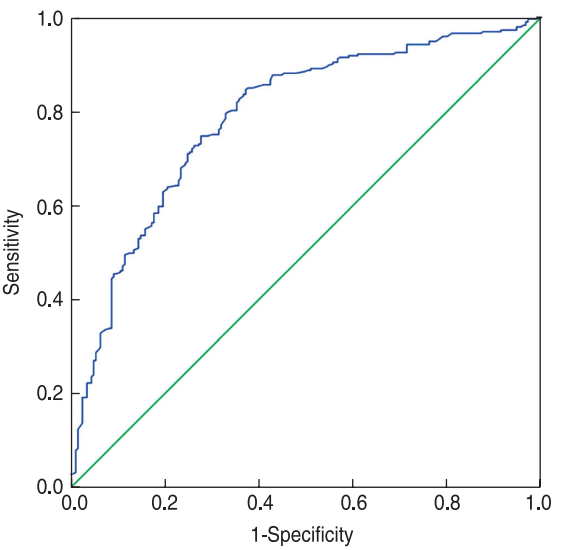

Fig. 10 Receiver operating characteristic (ROC) curve of fibrinogen values for predicting metastasis. Area under the receiver operating characteristic curve (AUC) values were used for predicting metastasis (AUC= 0.788; 95 % confidence interval [CI], 0.748–0.829; $P<0.001$)

recommended cut-off value. Therefore, we defined the cut-off value as 2.974 g/L, with a sensitivity of 0.747 and specificity of 0.724 (Fig. 10).

As a dichotomous variable (≤ 2.974 or > 2.974 g/L), a significant correlation was observed between fibrinogen values and NSCLC metastasis, tumor stage, tumor size, and histological types of NSCLC (Table 2).

Discussion

In other studies, fibrinogen values were shown to correlate with chemotherapy efficacy and prognosis in lung cancer [9, 11–16]. However, these studies were mainly

Table 2 Relationship between Fibrinogen values and NSCLC metastasis, tumor stage, tumor size

Variables	≤2.974 g/L (*n* = 226)	>2.974 g/L (*n* = 277)	*P*
Sex			< 0.001
Male	106	188	
Female	120	89	
Age	57.62 ± 9.13	58.93 ± 9.05	0.110
Tumor size (cm)			< 0.001
Median	2	3.5	
Range	0.6–8	0.6–9.5	
Histological types of NSCLC			< 0.001
Adenocarcinoma	187	173	
Squamous cell carcinoma	27	100	
others	12	4	
Metastasis			< 0.001
None	152	58	
Lymph node only	43	90	
Distant metastasis	31	129	
T stage			< 0.001
T1	137	64	
T2	81	185	
T3	5	17	
T4	3	11	
N stage			<0.001
N0	162	94	
N1	7	14	
N2	43	80	
N3	14	89	
M stage			< 0.001
M0	195	148	
M1	31	129	
TNM stage			< 0.001
Stage I–II	159	64	
Stage III–IV	67	213	
Number of metastasis lymph nodes			< 0.001
Median	3	8	
Range	1–35	1–13	

focused on the relationship between the fibrinogen value and survival rate or chemotherapy efficacy in patients with lung cancer. Additionally, these studies only investigated the operable or advanced patients, and none of the studies included patients of all stages.

In this study, the clinicopathology of 671 lung disease patients, including 503 NSCLC patients, were retrospectively analyzed and the association with pretreatment fibrinogen values in NSCLC patients was examined. The fibrinogen values in NSCLC patients were found to be significantly associated with metastasis and tumor stage. The median fibrinogen value in NSCLC patients with metastasis was significantly higher than in patients with benign lung diseases and NSCLC patients without metastasis. However, there was no statistical difference in fibrinogen values between patients with benign diseases and NSCLC patients without metastasis. When we reviewed the literature [17–18], there were no reports exploring the difference in the fibrinogen values between NSCLC patients without metastasis and patients with benign lung diseases. A study by Chen found that the plasma D-dimer level showed no statistical difference between NSCLC patients without metastasis and benign lung disease patients [19]. We concluded that after the development of NSCLC metastasis, the cancer cells can cause systemic reactions, thus impacting the fibrinogen level. However, in NSCLC patients without metastasis, the influence of the tumor is confined to the primary site, and it cannot cause increase in the fibrinogen level. Therefore, this result also indicates that metastasis is the critical event responsible for an increase in the fibrinogen level. Moreover, these results were not influenced by age, sex, smoking history, and histological types of NSCLC.

In case of tumor stage, significant statistical differences were observed in the fibrinogen levels between different T, N, and M stages. For N stage, median fibrinogen values increased from N0 to N3, and the number of metastatic lymph nodes was higher in patients with high fibrinogen values. The relationship between lymph node metastasis and fibrinogen values is still controversial, but it has confirmed that lymph metastasis are always accompanied by high fibrinogen values in gastric and esophageal cancers [4, 20]. With regard to M stage, fibrinogen values in distant metastasis patients (M1) were clearly higher than patients without distant metastasis (M0). However, in distant metastasis patients, the location of distant metastasis and the number of distant metastatic lesions did not have an influence on the fibrinogen values. Based on these results, we concluded that once the metastasis occurs, the cancer becomes a systemic disease, and therefore, the number and location of distant metastatic lesions does not affect the fibrinogen level. Surprisingly, patients with metastasis to the liver did not show different fibrinogen values when compared with patients with metastasis to others organs that cannot produce fibrinogen, and this may be related to the sample size of this study. In advanced NSCLC patients (Stage III–IV), higher fibrinogen values were observed when compared to early stage NSCLC patients (Stage I–II). Tumor size also exhibited a positive correlation with the fibrinogen values.

In addition, our study showed that a plasma fibrinogen cutoff value of 2.974 g/L was associated with a specificity of 0.724 and sensitivity of 0.747 for predicting metastasis. When the fibrinogen values were higher than 2.974 g/L, there were statistically significant differences in tumor size, tumor stage, metastasis, and histological types of NSCLC. Our results were in agreement with previous studies that demonstrated that high plasma fibrinogen values correlated with metastasis and advanced stage in

patients with NSCLC [9, 12–15]. Moreover, some previous research studies about NSCLC have reported similar results, showing that most of the lung squamous cell carcinoma patients had high fibrinogen values, and the results showed statistical significance [21]. Previous research has also revealed that squamous cells can biosynthesize fibrinogen and increase the fibrinogen produced by hepatocytes [22].

Fibrinogen is a modest acute-phase response protein that increases in concentration in response to most physiological and pathological conditions, such as acute infection, tissue injury, shock, hypercoagulable state, acute myocardial infarction, and malignant tumor [2]. Nevertheless, the exact mechanism underlying the association between elevated fibrinogen values and metastasis of NSCLC remains unknown. At present, two leading theories may explain this. One of them is that an inflammatory reaction to tumor growth, hypercoagulable state, and hypoxia associated with cancer may induce the elevated plasma fibrinogen values. Amrani advocated for the first time the ability of purified human recombinant interleukin-6 (originally BSF-2) to stimulate fibrinogen production in primary chicken hepatocytes [23]. Lawrence further suggested that extrahepatic fibrinogen biosynthesis evoked only during inflammation plays a role in localized injury and repair to restore tissue homeostasis [24]. The other leading theory is that fibrinogen is produced by the tumor itself. When immunohistochemistry was used to detect fibrinogen in resected pancreatic cancer in Bloomston's study, fibrinogen gamma was found to be overexpressed and discriminated cancer sera from normal sera [25]. Sahni's research also indicated that endogenously synthesized fibrinogen promotes the growth of lung and prostate cancer cells through interaction with FGF-2 [26]. Furthermore, Simpson-Haidaris's study indicated that fibrinogen functioned as an extracellular matrix protein in breast carcinoma, and affected tumor cell growth and metastasis. Close relationship between fibrinogen and metastasis has been shown in studies. In two studies by Palumbo, it was suggested that fibrinogen plays an important role in spontaneous metastasis, facilitating the stable adhesion and/or survival of metastatic emboli after tumor cell intravasation. However, in tumor models, fibrinogen deficiency strongly diminished but did not prevent the development of lung metastasis [27, 28]. In Shu's study, an *in vitro* experiment showed that fibrinogen induced Epithelial-Mesenchymal Transition (EMT) in gallbladder cancer by increasing the expression of vimentin and reducing expression of E-cadherin [29]. With regard to clinical treatment, Kuderer's in vitro study and animal findings have demonstrated that anticoagulants, in particular low molecular weight heparins (LMWHs), exert an antineoplastic effect through multiple mechanisms including interference with tumor cell adhesion, invasion, metastasis formation, angiogenesis, and immune system [30]. A recent study of Park revealed that anticoagulant use (LMWH in particular) is an independent predictor of improved survival in men with metastatic castration-resistant prostate cancer receiving docetaxel [31]. In contrast, in a randomized phase III trial of standard therapy plus low molecular weight heparin in patients with lung cancer, the results showed that LMWH did not improve overall survival in patients with lung cancer [32]. Additionally, in a meta-analysis by Che and Sanford, the use of LMWH did not show a survival benefit in cancer patients [33–34]. Currently, the relationship between fibrinogen level and cancer, along with the effectiveness of anticoagulants, is still open to dispute. However, we can confirm that fibrinogen is closely correlated with metastasis on the basis of our results.

Some studies have indicated that coagulation could be activated by tissue factor (TF) expressed by NSCLC cells [35]. A limitation of the present study is its retrospective nature; therefore, all data were collected from clinical examination, and the TF (tissue factor) and plasmin concentration were not included. However, in the inclusion and exclusion criteria, we excluded patients with clinical evidence of infection or other bone marrow, hematological, or autoimmune diseases. Furthermore, lower limb vascular ultrasound was used to exclude the influence of phlebothrombosis on the fibrinogen values.

There were some limitations of this study: single medical center, small number of participants, and retrospective research might potentially lead to an inappropriate conclusion, and the association between plasma fibrinogen level and prognosis in patients with NSCLC was not evaluated. Therefore, a large-scale multi-center prospective validation study is needed to further investigate the results. On the other hand, further basic medical research and clinical trials are also needed to identify the mechanism by which elevated plasma fibrinogen level might function to enhance metastasis, and to evaluate the usefulness of anticoagulants as a new therapy in cancer.

In conclusion, this study supports the role of elevated pretreatment plasma fibrinogen levels in predicting metastasis and advanced tumor stage in NSCLC patients.

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ORIGINAL ARTICLE

Combination of TACE and FOLFOX4 in the treatment of unresectable advanced hepatocellular carcinoma: a prospective cohort study*

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Abstract

Objective The aim of the study was to assess the effectiveness and safety of a combined therapy with transcatheter arterial chemoembolization (TACE) and FOLFOX4, in patients with unresectable advanced hepatocellular carcinoma (HCC).

Methods In this study, patients with advanced HCC, that received treatment between November 2015 and October 2017, were recruited. Among these, 30 patients were treated with TACE only (TACE group); whereas 33 patients were treated with a combination of FOLFOX4 chemotherapy and TACE (combination group). Survival analyses, including overall survival (OS) and progression free survival (PFS) analysis, were performed for both groups. Following this, the responses of patients to treatment were evaluated every 3 months, and the toxic and adverse events were observed.

Results The median follow-up time was 9.2 months (3–36 months). In the combination group, at 3 months, a disease control rate (DCR) of 60.6%, and a median OS of 9.1 months was obtained [95% confidence interval (CI) 6.5–11.7]. In the TACE group, the DCR and OS were 33.3% and 5.5 months (95% CI 4.3–6.7), respectively. On the other hand, the PFS in the combination and TACE groups were observed as 5.6 months (95% CI 3.6–7.6) and 2.6 months (95% CI 2.0–3.2), respectively. Both these findings indicate a statistically significant difference (P = 0.01) between both the groups. Similar TACE associated adverse events were observed in both groups. In the combination group, frequently observed FOLFOX4 related adverse effects included nausea (90.9%), leukopenia (75.8%), thrombocytopenia (69.7%), and vomiting (69.7%). Most adverse reactions were between grades I–III and were alleviated after symptomatic treatments.

Conclusion The combination of TACE with FOLFOX4 therapy has better effectivity and safety than TACE alone.

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Key words: advanced hepatocellular carcinoma; transcatheter arterial chemoembolization (TACE); FOLFOX4

Hepatocellular carcinoma (HCC) represents a serious threat to public health and is a medical burden worldwide. This is especially true for China, with its high rate of hepatitis B and C infection [1–2]. Globally, HCC is one of the five most common types of cancer. In addition, approximately 50% of all new cases are diagnosed in China [3]. The diagnosis of HCC is often difficult, due to its insidious onset and atypical early symptoms. Most patients reach an advanced stage or have distant metastases by the time HCC is identified. As a result, less than 20% of the diagnosed cases are eligible for surgical treatment [4]. Currently, transcatheter arterial chemoembolization (TACE) is one of the most commonly used methods for the treatment of advanced HCC. TACE has been proven to delay tumor progression and vascular invasion. It can also prolong patient survival through several years of clinical application [5–6]. Moreover, TACE can selectively destroy HCC tissues and is believed to be a suitable option for patients with cirrhosis [7]. Molecular targeted drugs, such as sorafenib or regorafenib, are still the only choice for drug treatment of patients that are not surgical candidates. However, the efficiency of

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these agents is far from satisfactory. In addition, there are significant obstacles to the widespread use of these agents [8–10]. First, patients with advanced HCC often have compromised gastrointestinal function, that influences the absorption of orally administered drugs [11]. Second, the daily dose of sorafenib is very high (over 800 mg, p.o.) and it produces serious side effects during clinical usage. In addition, this agent is expensive, and its usage places a heavy financial burden on the patient or their family. Third, only a small proportion of patients are observed to have neoplasms that are initially sensitive to sorafenib. In some cases, the tumor developed a resistance to sorafenib during treatment [12]. Some of the newly approved molecular targeted agents, such as regorafenib or lenvatinib, are used clinically for only a short period of time. The deficiencies of these agents, including high cost, rapid onset of drug-resistance, and high toxicity are gradually becoming apparent following their widespread applications. Therefore, developing novel and effective anti-tumor drugs or strategies will not only help achieve improved clinical outcomes, but will also provide patients with better treatment choices.

The role of traditional chemotherapy in advanced HCC may be controversial. However, traditional chemotherapies offer more choices at a lower cost than the molecularly targeted drugs [13]. Therefore, a breakthrough involving the use of chemotherapies for advanced HCC may be of great significance. Qin *et al* evaluated the effectivity and safety of FOLFOX4 chemotherapy in the treatment of advanced HCC, over a single drug therapy with doxorubicin [14]. This study revealed that FOLFOX4 treatment significantly improved the objective response rate (ORR) and disease control rate (DCR) in Chinese patients with HCC and significantly prolonged their survival [14]. Based on this breakthrough, the "Guidelines for the diagnosis and treatment of primary liver cancer (2017)", recommended that FOLFOX4 chemotherapy be made available as a therapy for advanced HCC [15]. Moreover, the use of combination treatments with agents that employed various mechanisms, was viewed as the future of anti-tumor therapy [16–18]. As a targeted therapy, the use of TACE can induce tumor necrosis and reduce the tumor burden. It is observed that approximately 30%–50% of patients develop extensive tumor necrosis after TACE treatment [19]. Similarly, FOLFOX4 chemotherapy is a systemic treatment that can delay tumor progression via synergistic anti-proliferative activity [20]. Thus, hepatic arterial chemoembolization combined with FOLFOX4 chemotherapy may achieve better therapeutic effects against advanced HCC than the use of TACE alone. Studies assessing the effect of TACE combined with FOLFOX4 in the treatment of advanced HCC are limited. Therefore, the present study was designed to prospectively analyze the effectiveness and safety of TACE combined with FOLFOX4 chemotherapy in patients with advanced liver cancer.

Materials and methods

General information

This prospective cohort study enrolled 63 consecutive patients, that were diagnosed with unresectable advanced hepatocellular carcinoma, and received treatment between November 2015 to October 2017, at our hospital (The Fifth Medical Center of PLA General Hospital, Beijing, China). The diagnoses were performed via histology or via dynamic enhancement magnetic resonance imaging (MRI)/computed tomography (CT), in accordance with the Barcelona Clinic Liver Cancer Staging (BCLC) Classification [21]. As per their wishes, the patients received either TACE alone (TACE group, n = 30), or TACE plus FOLFOX4 regimen (combination group, n = 33). All patients enrolled refused to be treated with molecular targeted drugs. The study protocol was approved by the Ethics Committee of our center (The Fifth Medical Center of PLA General Hospital, Beijing, China). The protocol number is 2014185D. All patients signed a written-informed consent prior to their enrollment in the study.

Inclusion criteria

The inclusion criteria were as follows: (1) age 18 to 75 years; (2) Karnofsky performance status (KPS) score ≥ 70; (3) dynamic enhanced MRI/CT or pathological diagnosis as advanced HCC (BCLC C Stage); (4) having lesions that could be evaluated objectively but could not be treated surgically; (5) Child-Pugh scores ≤ 7; (6) expected survival times > 3 months; (7) neutrophil counts ≥ 1.5×10^9 /L and platelet counts ≥ 75×10^9 /L.

Exclusion criteria

The exclusion criteria were as follows: (1) presence of other systemic diseases, such as coronary heart disease, cerebrovascular accidents, and mental and neurologic diseases; (2) liver dysfunction (Child-Pugh C grade), with coagulation disorders that were untreatable, combined with untreatable active infections; (3) pregnancy or active menstruation for women; (4) allergy to chemotherapeutic drugs included in the FOLFOX4 regimen; (5) presence of tumors in other tissues; (6) dyscrasias or multiple organ failure; (7) parallel use of other drugs such as chemotherapeutic drugs or treatment with traditional Chinese medicine.

Treatment protocols

All patients were routinely treated with TACE. Once the liver functions recovered 3–5 days after completion of the TACE treatment, the FOLFOX4 regimen was

administered.

For TACE, routine preoperative preparation was performed. The Seldingers method was employed to catheterize the femoral artery, and to allow the arteriography of the hepatic and superior mesenteric arteries. This was followed by an assessment of tumor staining, and the filling of the portal veins. The vessel supplying the tumor was selectively catheterized, followed by the slow injection of fluorouracil 0.5–1.0 g, epirubicin 20–40 mg, and lipiodol emulsion embolization of 5–25 mL into the target vessel. The patients with extrahepatic metastasis were treated with chemoembolization. The amount of iodized oil was determined according to tumor size. Finally, the artery supplying the tumor was embolized using Gelfoam particles. TACE was performed according to the tumor response and patients' health status and was usually repeated no more than four times per six months.

FOLFOX4 chemotherapy was performed with oxaliplatin 85 mg/m^2 (intravenous infusion for 2 h on the first day), leucovorin 200 mg/m^2 (intravenous infusion for 2 h on the first and second days), and fluorouracil (400 mg/m^2, intravenous injection; 600 mg/m^2, continuous intravenous infusion for 22 h on the first and second days). An interval of two weeks was defined as one cycle The treatment was continued until it was completed, or until the patient was intolerant due to toxic side effects, or died. The treatment lasted for no more than 12 cycles.

Follow-up

The duration of this study was from the day of commencement of the treatment to April 30th, 2019, or until the death of the patient. During the follow-up period, there existed no events that were considered as competing risks significantly affecting the patients mortality and prognoses.

Observation indicators and evaluation methods

Routine blood tests including liver and renal function test, tests for alpha fetal protein (AFP) levels, and abdominal enhanced MRI/CT and chest CT scans were performed before and after the treatment. The patient's condition was reviewed and evaluated every 6 weeks. Effectiveness outcomes included the median or 95% CI of overall survival (OS), progress-free survival (PFS) and 3-month tumor response, including ORR and DCR. OS was defined as the period from commencement of treatment to the end of the follow-up period or death. PFS was defined as the time interval between commencement of treatment and tumor progression or death. Tumor response was evaluated according to the modified guidelines for Response Evaluation Criteria in Solid Tumors [22], and included complete response (CR, in which arterial enhancement disappeared in all target lesions), partial response [PR, reduction of the diameter of the target lesion (shown by enhanced imaging at the arterial phase) by no less than 30%], stable disease (SD, reduction of the diameter of the target lesion not reaching PR, or the increase in the diameter of the target lesion not reaching PD), and progressive disease (PD, diameter increase of the target lesion by no less than 20% and/or the occurrence of new lesions). The ORR was defined as the percentage of CR and PR among all patients. The DCR was defined as the percentage of total cases indicating CR, PR, and SD.

According to the Common Terminology Criteria for Adverse Events (CTCAE) v3.0 [23], the toxicity of chemotherapy was divided into degrees 0–4. According to the oxaliplatin specialized Levi neuropathy grading criteria [24], the toxicity of the nervous system was divided into grades 0 to 4: grade 0 indicated no response; grade 1 indicated abnormal feeling or insensitivity (caused by cold) that resolved completely within 7 days; grade 2 indicated abnormal feeling or insensitivity that resolved completely within 21 days; grade 3 indicated abnormal feeling or insensitivity with no recovery within 21 days; and grade 4 indicated abnormal feeling or insensitivity, accompanied by dysfunction.

Statistical analysis

Count data were expressed as number and percentages and were analyzed by the Chi-square test or Fisher's exact test as appropriate. Measurement data were expressed as mean and standard deviation (SD), and were analyzed by Student's *t*-tests. Survival was estimated by the Kaplan-Meier method. The log-rank test was used to compare the survival rates between the two groups. Software from the SPSS 23.0 statistical package (IBM Corp, Armonk, NY, USA) was employed for data analysis.

Results

Baseline characteristics of patients

As represented in Table 1, 33 and 30 cases of unresectable advanced HCC were treated with TACE + FOLFOX4 and TACE only, respectively. There were no significant differences between patient age (P = 0.378), gender distribution (P = 0.461), tumor number (P = 0.993), vascular involvement (P = 0.288), metastases (P = 0.942), AFP and cholinesterase levels (P = 0.271 and 0.102, respectively), tumor diameter (P = 0.919), Child-Pugh classification (P = 0.646), TACE number (P = 0.288), and the various pathologies (P = 0.224). The median number of chemotherapy cycles utilized in the combination group was 5 (range, 2–13 cycles). Therefore, no significant differences in these parameters between these two groups of patients were identified.

Table 1 Baseline characteristics of patients [*n* (%)]

Parameter	TACE + FOLFOX4 (*n* = 33)	TACE (*n* = 30)	*P* value
Age (years), mean ± SD	53.94 ± 8.79	55.3 ± 12.4	0.378
Sex			0.461
Male	29 (87.9)	28 (93.3)	
Female	4 (12.1)	2 (6.7)	
Tumor number			0.993
1	8 (24.2)	7 (23.3)	
2	2 (6.1)	2 (6.7)	
≥ 3	23 (69.7)	21 (70.0)	
Vascular involvement			0.288
None	16 (48.5)	9 (30.0)	
Branch of portal vein	10 (30.3)	14 (46.7)	
Main portal vein	7 (21.2)	7 (23.3)	
Metastasis			0.963
No	14 (42.4)	13 (43.3)	
Yes	19 (57.6)	17 (56.7)	
Lung	12	10	
Lymph node	7	7	
AFP (ng/mL)			0.271
< 20	6 (18.2)	9 (30.0)	
≥ 20	27 (81.8)	21 (70.0)	
Cholinesterase (U/L)			0.102
≤ 5000	13 (39.4)	18 (60.0)	
> 5000	20 (60.6)	12 (40.0)	
Tumor diameter (cm), mean± SD	7.55 ± 3.72	7.59 ± 3.68	0.919
Child-Pugh classification			0.646
A	26 (78.8)	25 (83.3)	
B	7 (21.2)	5 (16.7)	
Number of TACE			0.288
1	7 (21.2)	2 (6.7)	
2	14 (42.4)	9 (30.0)	
≥ 3	12 (36.4)	19 (63.3)	
Pathology			0.224
Hepatitis B	25 (75.7)	29 (96.7)	
Hepatitis C	4 (12.1)	1 (3.3)	
Alcoholic liver disease	2 (6.1)	1 (3.3)	
Others	2 (6.1)	0	

Positive tumor response

In the combination group, ORR and DCR at 3 months were 39.4% (95% CI: 22.8%–56.0%) and 60.6% (95% CI: 48.1%–73.1%), respectively. In comparison, the ORR and DCR were 13.3 % (95% CI: 7.5%–19.1%) and 33.3% (95% CI: 22.8%–43.8%) in the TACE group, respectively (Table 2). These findings indicate that the ORR (P = 0.045) and DCR (P = 0.030) were significantly higher in the combination-therapy group than in the TACE group. Moreover, PD was markedly less common in the combination group as compared to that in the TACE group (P = 0.014; Table 2). And indicated in Fig. 1 and 2, combination therapy can significantly reduce the intrahepatic and intrapulmonary lesions in patients with advanced hepatocellular carcinoma and pulmonary metastasis.

Table 2 Treatment response of patients [n (%)]

	TACE + FOLFOX4 (*n* = 33)	TACE (*n* = 30)	*P* value
CR	0	0	
PR	13 (39.4)	4 (13.3)	0.045
SD	7 (21.2)	6 (20.0)	0.040
PD	13 (39.4)	20 (66.7)	0.014
ORR	13 (39.4) (95% CI: 22.8%–56.0%)	4 (13.3) (95% CI: 7.5%–19.1%)	0.045
DCR	20 (60.6) (95% CI: 48.1%–73.1%)	10 (33.3) (95% CI: 22.8%–43.8%)	0.030

Note: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; ORR, objective response rate = CR + PR; DCR, disease control rate = CR + PR + SD

Table 3 TACE related adverse events [n (%)]

Adverse event	TACE + FOLFOX4 (*n* = 33)	TACE (*n* = 30)	*P* value
Fever	28 (84.8)	26 (86.7)	0.095
Nausea and vomiting	18 (54.5)	15 (50.0)	0.718
Abdominal pain	19 (57.6)	14 (46.7)	0.387
Liver function damage	27 (81.8)	26 (86.7)	0.857
Leukopenia and thrombocytopenia	1 (3.0)	1 (3.3)	1.000
Puncture point bleeding	2 (6.1)	1 (3.3)	1.000

Prolonged survival

The follow-up periods ranged from 3 to 36 months, with a median value of 9.2 months. In the combination-therapy group, 28 patients died and 5 survived. In the TACE group, 26 died and 4 survived. In the combination group, the median OS was 9.1 months (95% CI: 6.5–11.7 months), while in the TACE group the median OS was 5.5 months (95% CI: 4.3–6.7 months). This represents a significant difference in OS between the two groups (P = 0.006; Fig. 3a). Moreover, the PFS values were observed as 5.6 months (95% CI 3.6–7.6) and 2.6 months (95% CI 2.0–3.2) in the combination and TACE groups, respectively, also representing a significant difference (P = 0.01; Fig. 3b). The patients were further divided into two groups, portal vein tumor thrombus (PVTT) group and no-PVTT group. There is no statistically significant difference in OS and PFS of no-PVTT group treated with TACE or combination-therapy. However, the combination-therapy may have influenced and increased the OS and PFS of no-PVTT group (Fig. 4a–4b). As compared with TACE alone, the combination-therapy may increase the OS and PFS of patients with PVTT (Fig. 4c–4d).

Similar adverse events

TACE associated adverse events, including fever (P = 0.095), nausea and vomiting (P = 0.718), abdominal pain

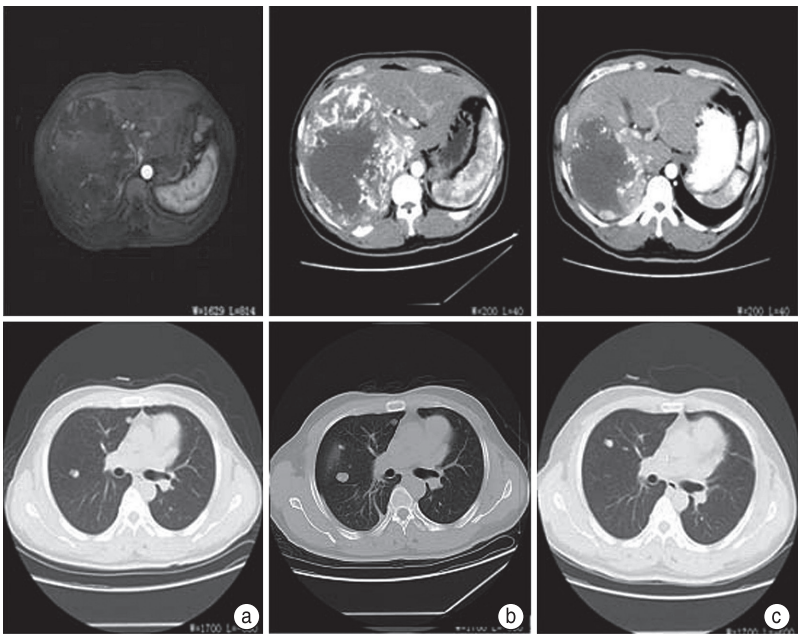

Fig. 1 Tumor response in a patient with massive hepatocellular carcinoma and double lung metastases. (a) Diagnosis of massive hepatocellular carcinoma with double lung metastases. (b) After the first TACE, lipiodol accumulation at the site of liver cancer, enlarged intrahepatic tumor, and a significant increase in double lung metastases compared with (a); following which the patient received systemic chemotherapy. (c) The patient received systemic chemotherapy for four times after the second TACE, intrahepatic tumor reduced (17 cm to 10 cm) and double lung metastases declined

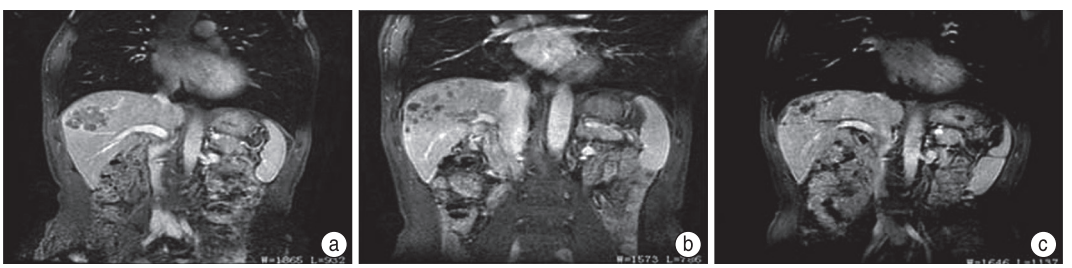

Fig. 2 Tumor response in a patient with PVTT. (a) Before the first TACE, imaging indicated a tumor in liver right lobe, with incomplete capsule and no obvious PVTT (AFP was 13 ng/mL). (b) After the third TACE, intrahepatic tumor was enlarged and was accompanied with tumor thrombus in the right branch of the portal vein (AFP, 159 ng/mL); following this, the patient received systemic chemotherapy and a fourth TACE. (c) The patient then received systemic chemotherapy for three cycles after the fourth TACE; tumor and tumor thrombus were reduced (AFP, 71 ng/mL).

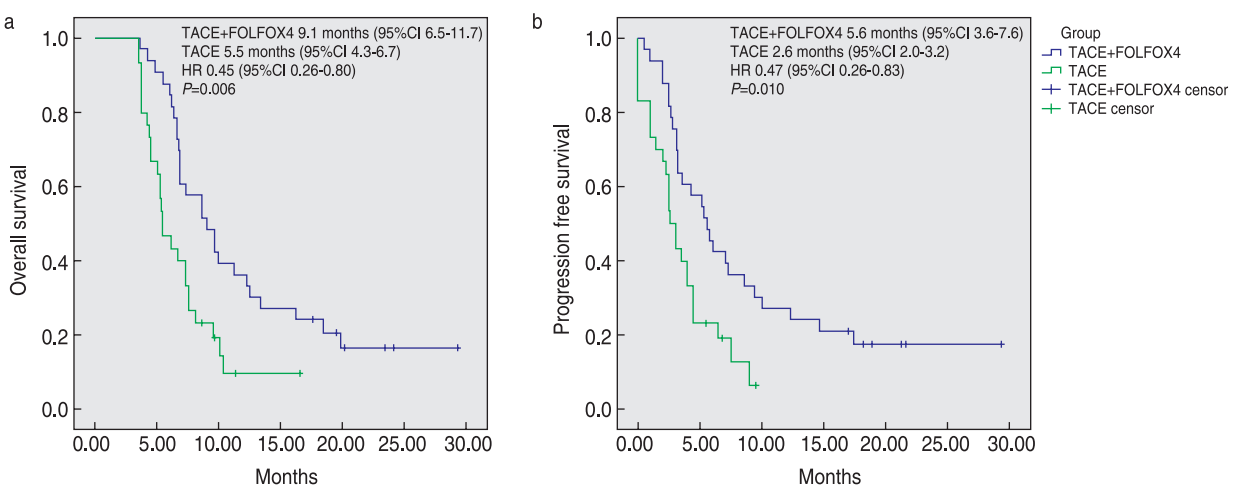

Fig. 3 OS and PFS of patients in both groups. (a) Overall survival, (b) Progression free survival

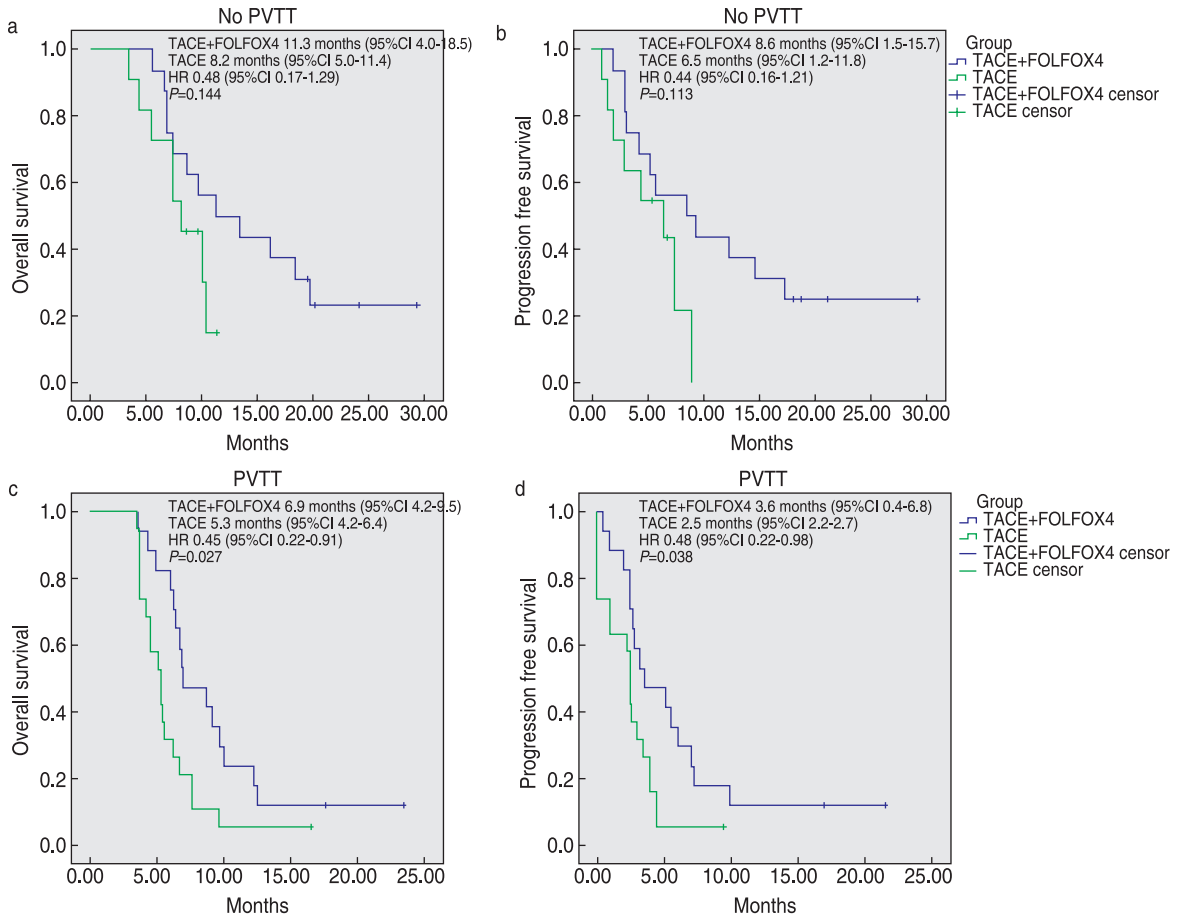

Fig. 4 OS and PFS of patients with or without PVTT. (a and c) OS; (b and d) PFS

(P = 0.387), liver function changes (P = 0.857), leukopenia and thrombocytopenia (P = 1.000), and puncture site bleeding (P = 1.000), were similar in both groups (Table 3). In the combination group, the adverse events frequently observed after treatment with FOLFOX4 included nausea (90.9%), leukopenia (75.8%), thrombocytopenia (69.7%), and vomiting (69.7%). Allergies (3%) and peripheral neuropathy (15.2%) were observed in some cases. However, the adverse events mostly ranged between grades I–III, and alleviated after symptomatic treatments (Table 4).

Discussion

This study compared the safety and effectiveness of TACE and a combination of TACE and FOLFOX4

Table 4 FOLFOX4 related adverse events [*n* (%)]

Adverse event	TACE + FOLFOX4 (*n* = 33)					
	All grades	Grade 1	Grade 2	Grade 3	Grade 4	Grade 3–4
Leukopenia	25 (75.8)	9 (27.3)	10 (30.3)	4 (12.1)	2 (6.1)	6 (18.2)
Thrombocytopenia	23 (69.7)	7 (21.2)	11 (33.3)	5 (15.2)	0	5 (15.2)
Nausea	30 (90.9)	20 (60.6)	7 (21.2)	2 (6.1)	1 (3.0)	3 (9.1)
Vomiting	23 (69.7)	15 (45.5)	7 (21.2)	1 (3.0)	0	1 (3.0)
Bilirubin elevation	8 (24.2)	4 (12.1)	3 (9.1)	1 (3.0)	0	1 (3.0)
Transaminase elevation	9 (27.3)	8 (24.3)	1 (3.0)	0	0	0
Allergy	1 (3.0)	1 (3.0)	0	0	0	0
Peripheral nerve toxicity	5 (15.2)	5 (15.2)	0	0	0	0
Pigmentation	14 (42.4)	14 (42.4)	0	0	0	0

regimen, in the treatment of HCC patients with unresectable lesions. We observed that the combination of the FOLFOX4 regimen with TACE resulted in improved clinical outcomes including increased OS, PFS, ORR, and DCR, and reduced PD.

TACE is an effective local treatment for advanced HCC. As compared to controls, it leads to a significantly improved 1-year survival rate [25]. There have been studies comparing the combination therapy of TACE and molecularly targeted drugs, including sorafenib, and therapy with TACE alone. From these studies it was indicated that the combination group experienced improved overall 1-year survival and disease control rate [26]. However, studies combining TACE and chemotherapy, for example FOLFOX4, have not been performed in the past. In the present study, an OS of 9.1 months (95%CI 6.5–11.7) and 5.5 months (95% CI 4.3–6.7) were obtained for the combination group and TACE group, respectively These findings indicate that combining the FOLFOX4 regimen with TACE is superior to TACE alone. Other treatment outcomes followed a similar trend.

In addition, we carried out a subgroup analysis based on PVTT. The results indicate that patient survival outcomes (OS and PFS) were more favorable in the no-PVTT subgroups as compared with those in the PVTT groups (Fig. 4). However, our findings also demonstrate that, PVTT patients treated with the FOLFOX4 + TACE combination showed a higher survival than those treated with TACE alone. The same is true for patients with extrahepatic metastasis. Two cases that were treated by this combination are represented in Figs. 1 and 2. We found that 81.3% (13/16) of the patients with PVTT died from upper gastrointestinal hemorrhaging. This indicates that portal hypertension followed by cirrhosis and PVTT may be the major cause of death in patients with advanced HCC. We believe that with the application of portal vein stenting and radiotherapy, the goal of reducing portal vein thromboses and decreasing portal pressure can be achieved [27–29]. Moreover, incorporating FOLFOX4 chemotherapy would better control of tumor progression. Previously, we combined TACE with sorafenib treatment in 51 patients with advanced HCC, and observed that the median OS rates for the PVTT and non-PVTT groups were 6 months and 10.3 months [30], respectively. These findings indicate that the efficacy of TACE combined with FOLFOX4 chemotherapy (OS of 6.9 months and 11.3 months for the PVTT and no-PVTT, respectively) was similar to that of TACE combined with sorafenib treatment in patients with advanced hepatocellular carcinoma. It also had the advantages of lower cost and fewer side effects.

For TACE combined with FOLFOX4 in the treatment of advanced HCC, the main toxicities were gastrointestinal reactions (nausea, anorexia, and vomiting) and myelosuppression (leukopenia and thrombocytopenia). The rare side effects of this treatment included liver damage, drug allergies, and mild peripheral neurotoxicity. Grade 3–4 adverse effects were not commonly observed. Compared with the EACH study [14], it was observed that nausea, vomiting, leukocytopenia, and thrombocytopenia all increased in the combination group. This was because FOLFOX4 chemotherapy was administered for more cycles in this study (5 versus 4 times). Gastrointestinal symptoms such as nausea and vomiting could be alleviated after FOLFOX4 chemotherapy. In addition, myelosuppression was significantly relieved by treatment with granulocyte colony-stimulating factor and interleukin-11 [31–32]. Both, this study and the EACH study indicated that FOLFOX4 chemotherapy had little effect on liver function tests. It was observed that ALT increased by 27.3% and 21.86% in this and the latter studies, respectively. Additionally, the bilirubin increase rates were 24.2% and 20.22%, respectively. The main adverse effects following sorafenib treatment include hand and foot skin reactions, diarrhea, fatigue, hypertension, and liver function damage. Serious side effects often led to drug dose reductions or withdrawal. Following the development of sorafenib intolerance, up to 44% of dose reduction or drug withdrawal was required [33–34]. Of the 33 patients that were administered with FOLFOX4 and TACE, only 3 (9.1%) withdrew as a result of FOLFOX4 side effects. Two of them continued chemotherapy after symptomatic treatment. Only one patient was unable to continue treatment due to overt nausea. Interestingly, previous reports have suggested that in advanced HCC, the hepatic arterial infusion of FOLFOX4 therapy better ameliorates survival with acceptable toxicity and elevated quality of life [35–36], as compared to treatment with sorafenib. As a result, FOLFOX4 therapy could be a novel treatment for advanced HCC. Moreover, all patients in this study had presented with HCC of BCLC stage C. This is similar to the patients in the Oriental experiment (BCLC stage C: 143/150 of sorafenib group; 73/76 of placebo group). A few patients included in the SHARP experiments also presented with BCLC stage B HCC (54/299 in sorafenib group or 51/303 in placebo group).

This study has certain limitations. It was carried out in a single center with a small sample size. Therefore, larger multicenter, randomized control trials are needed to confirm these findings.

In summary, TACE combined with FOLFOX4 therapy has good efficacy, in that it prolongs the survival and improves the quality of life, with limited toxicity and adverse events. Moreover, most patients were able to tolerate this treatment. This suggests that it may be able to significantly improve the clinical approach to HCC.

Ethics approval and consent to participate

Patient data were used after obtaining approval from the Ethics Committee of the Fifth Medical Center of PLA General Hospital, Beijing, China. All research was performed in compliance with the Helsinki Declaration, with the informed written consent of patients.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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ORIGINAL ARTICLE

Induction of apoptosis in renal cell carcinoma by cinobufotalin through inhibition of Notch1 signal activation*

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Abstract

Objective The aim of this study was to investigate the effect of cinobufotalin on apoptosis in renal cell carcinoma and its possible mechanism of action.

Methods The expression levels of Notch1 in renal cancer cells, as well as in adjacent and normal tissues were assessed in 64 patients with renal cellcarcinoma. The 769-P cells were treated with 0, 10, 20 and 40 mg/L cinobufotalin and the proliferation activity and apoptotic rate of the cells were measured. The expression levels of Notch1, Bcl-2, and Pro-caspase 3 were detected by RT-PCR and Western-blot.

Results (1) The rates of Notch1 expression in renal cancer cells, adjacent tissues, and normal tissues were 75.0%, 45.3%, and 9.4%, respectively. Notch1 expression had significant effects on tumor, node and metastasis (TNM) staging, Fuhrman grade, and tumor size in patients with renal cell carcinoma ($P < 0.05$); (2) The inhibition rates of cinobufotalin on 769-P cells were 0%, 6.85%, 11.37%, and 16.33% at 24 h; 0%, 13.57%, 20.14%, and 31.69% at 48 h; 0%, 19.97%, 28.53% and 51.42% at 72 h. At 24 h, the apoptotic rates were 8.2 ± 3.1%, 19.8 ± 5.6%, 33.7 ± 5.0%, and 51.5 ± 6.8%.The effect of cinobufotalin on apoptosis of 769-P cells was dose-dependent; (3) RT-PCR assay showed that protein expression levels for Notch1, Bcl-2, and Pro-caspase 3 were significantly decreased with the increase of drug concentration. Western-blot analysis also showed that Notch1, Bcl-2 and Pro-caspase 3 protein levels showed a significant downward trend with the increase of drug concentration.

Conclusion Cinobufotalin inhibits the growth of renal cancer cells and induces apoptosis in renal cell carcinoma, which may be related to the inhibition of Notch1 signal activation.

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Key words: renal cell carcinoma; cinobufotalin; cell line 769-P

Renal cell carcinoma is one of the most common malignant tumors of the urinary system in China [1]; however, its pathogenesis is not completely understood, although it is generally considered to be related to smoking, chronic urinary tract infection, and kidney stones [2]. According to traditional Chinese medicine practice, kidney cancer is caused by impaired kidney functions such asexcessive dampness or heat, qi stagnation, and blood stasis [3]. Cinobufotalinis a water-soluble extract obtained from the skins and parotid venom glands of the toad Bufo gargarizans Cantor and has been widely used in China as an effective traditional Chinese medicine to treat conditions such asswelling, pain, and heart failure for thousands of years [4]. Previous studies by the current and other authors have demonstrated that the major pharmacologic constituents of Cinobufotalinare bufodienolides (which primarily include bufalin, cinobufagin, resibufogenin, bufotalin, and lumichrome), biogenic amines, alkaloids, peptides, and proteins [5]. Currently, Cinobufotalinis widely administered through injectionin various dosagesin clinical cancer therapy in China. Cinobufotalin has been reported to have a variety of biological effects, such as immunomodulatory and antineoplastic effects [5–6]. In the treatment of liver, gastrointestinal, and esophageal cancer, as well asother malignant tumors, cinobufotalin inhibits the

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progression of cancer, improves patient symptoms such as cachexia, fever, fatigue, weight loss, and loose stools. Some researchers have reportedthat cinobufotalin has inhibitory effects on the proliferation and promotion of apoptosis in renal cancer cells *in vitro* and *in vivo*, though the mechanism is still unclear.

Materials and methods

Reagents and cell line

Human kidney cancer cell line 769-P was purchased from Wuhan Boster Biotechnology Co. Ltd. (China). The reverse transcription and RT-PCR kits were purchased from Takara Corporation (China). Monoclonal antibodies for Rabbit anti-human Notch1, rabbit anti-human Bcl-2, and rabbit anti-human Pro-caspase3, respectively,were purchased from Abcam (UK). Bicinchoninic acid (BCA) protein concentration determination and enhanced chemiluminescence (ECL) kits were purchased from Tianjin Yuyang Biological Products Technology Co. Ltd. (China).

Cell culture and 3-(4, 5) dimethylthiahiazo (-z-yl)-3, 5-diphenytetrazoliumromide (MTT) assay

The 769-P cells were inoculated into a culture medium containing 10% fetal bovine serum and penicillin and streptomycin. Cells were cultured at 37 °C with 5% CO_2 and passaged once every two days. Cells in the growth phase were used. The 769-P cells were seeded in 96-well plates at a density of approximately 2000 cells/well and cultured until the cells were adherent. The supernatant was discarded and replaced with 200 μL of cell culture medium containing 0 mg/mL, 10 mg/L, 20 mg/L, and 40 mg/L of cinnamycin according to the four group schemes. The blank group was added as control. All plates were cultured for 24 h, 48 h, and 72 h. Upon completion of the culture, 20 μL of MTT solution was added to each well, and the cells were further incubated for 4 hand the absorbance at a wavelength of 570 nm (*A* value) wasmeasured. This experiment was repeated three times.

Reverse transcription-polymerase chain reaction (RT-PCR) detection

Total RNA in the cells was extracted according to the manufacturer's protocol. The cellular RNA was reverse transcribed into cDNA, and then amplified into DNA, and all steps were performed in strict accordance with the manufacturer's instructions on the reverse transcription and the amplification kits. Notch1 primer sequences: upstream 5'-CTGTATCAAAAGGCCAACTGAA-3', d ownstream 5'-GTGTCTATCCTTATGAATCGCCA-3' , Bcl-2 primer sequences: upstream 5'-AACTGTGCTGA ACTGGATCAAA-3', downstream 5'-AGTCGTTCTCC TTATGGCATACA-3', Pro-caspase 3 primer sequences: upstream 5'-AAAAGCGCCTGCCTGAA-3', downstream 5'-GACTTCTGAATCGCTGTCTATCA-3'. All primer sequences were synthesized by Shanghai Bioengineering Technology Co. Ltd. (China). The RT-PCR assay conditions were as follows: predenaturation at 95 °C for 10 min, then 40 cycles of denaturation at 95 °C for 30 s and annealing at 30 °C for 30 s, and finalextension at 70 °C for 10 min.

Protein detection by Western-blot analysis

Total cellular protein was extracted. Then 25 μg of the extracted proteins were resolved by SDS-PAGE, before transferring onto ablotting membranein 5% skim milk for 2 h. Subsequently, the membrane was incubated with primary antibodies such as Notch1 monoclonal antibody (1:2000), Bcl-2 monoclonal antibody (1:2000), and Pro-caspase 3 monoclonal antibody (1:5000) overnight at 4 °C. Then, after washing with phosphate buffer saline (PBS) for three times, secondary antibody was added in a dropwise manner to conjugate with the bound primary antibodies. The β-actin protein was used as an internal reference, incubated in a greenhouse, washed with tris buffer saline tween (TBST) buffer, exposed by chemiluminescence, and the gray color of the strip was measured by an image-J image analysis system. Transplanted apoptotic tumor cells were detected by TUNEL method. The tumor is routinely dehydrated, embedded, and sectioned. The staining process was carried out according to the kit instructions. After the filming, five fields of view were randomly selected and the number of positive cells was counted. Positive criteria: blue particles in the nucleus.

Clinical sample source and data collection

A total of 64 patients with renal cell carcinoma who underwent surgery in our hospital from October 2016 to January 2019 were enrolled. Inclusion criteria: (1) Diagnosed as patients with renal cell carcinoma by pathological evidence and for the first time; (2) Patients undergone surgical treatment, and the surgical resection specimens were preserved in our pathology department laboratory. Exclusion criteria: (1) Perioperative deaths; (2) A history of other malignant tumors; (3) Incomplete clinical data. Of the 64 patients, 48 were males and 16 were females, ranging from 38 to 81 years old with a median age of 56 years. Data on tumors were also collected, including tumor, node and metastasis (TNM) staging, Fuhrman grade, and tumor size.

Statistical analysis

All data analysis was performed using SPSS 20.0 software. Descriptive analysis of the results was performed based on frequency, rate, mean ± standard, and statistical

inference using chi-square test. The significance level is $\alpha = 0.05$.

Results

Expression of Notch1 in renal cancer cells, adjacent and normal tissues

Immunohistochemical staining showed that Notch1 was mainly expressed in the cell membrane or cytoplasm of the cells, and appeared yellowish or yellowish brown. The positive rates of Notch1 in renal cell carcinoma, adjacent tissues and normal tissues were 75.0% (48/64), 45.3% (29/64), and 9.4% (6/64), respectively, and the difference was statistically significant ($P < 0.05$; Fig. 1).

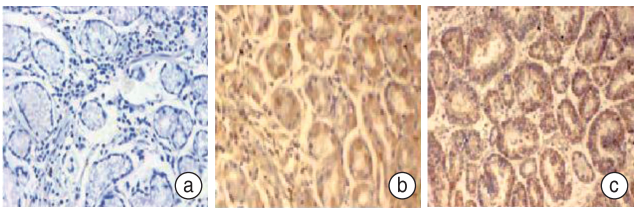

Fig. 1 Expression of Notch1 in renal cancer cells (a), adjacent (b) and normal (c) tissues

The effect of Notch1 expression on the clinical features of renal cancer cells

Table 1 showed that univariate analysis revealed that Notch1 expression was correlated to TNM stage, Fuhrman grade, and tumor size in patients with renal cell carcinoma ($P < 0.05$), but not with gender and age ($P > 0.05$).

Table 1 Effect of Notch1 expression on clinical features of renal cancer cells

Index	Negative ($n = 16$)	Positive ($n = 48$)	χ^2	P
Gender			0.444	0.505
Male	11	37		
Female	5	11		
Age (years)			2.351	0.125
< 60	10	39		
≥ 60	6	9		
TNM			7.528	0.006*
I + II	13	20		
III + IV	3	28		
Fuhrman			8.466	0.004*
1 + 2	14	22		
3 + 4	2	26		
Tumor size (cm)			6.206	0.013*
< 5	9	11		
≥ 5	7	37		

*Statistical significance at $P < 0.05$

The effect of different concentrations of cinobufotalinon the proliferation of renal cancer cells

After MTT assay, the cancer cell inhibition rates of cinobufotalin at 0 mg/L, 10 mg/L, 20 mg/L and 40 mg/L were 0%, 6.85%, 11.37%, 16.33%, 48 h after intervention of 769-P cells at concentrations of 0 mg/L, 10 mg/L, 20 mg/L and 40 mg/L, respectively. The cell inhibition rates were 0, 13.57%, 20.14%, and 31.69%, respectively, and the cell inhibition rates at 72 h were 0%, 19.97%, 28.53%, and 51.42%, respectively (Table 2). The inhibitory effect of cinobufotalin on the growth of 769-P cells was dose- and time-dependent.

Table 2 The effect of different concentrations of Cinobufotalinon the proliferation of renal cancer cells

Cinobufotalin (mg/L)	24 h (%)	48 h (%)	72 h (%)
0	0	0	0
10	6.85	13.57	19.97
20	11.37	20.14	28.53
40	16.33	31.69	51.42

Effects of different concentrations of cinobufotalin on apoptosis of renal cell carcinoma

After treatment with cinobufotalin for 0 h, 10 mg/L, 20 mg/L, 40 mg/L for 24 h, the apoptotic rate of cells was 8.2 ± 3.1%, 19.8 ± 5.6%, 33.7 ± 5.0%, 51.5 ± 6.8%. The effect of cinobufotalin on the promotion of 769-P cell apoptosis was dose-dependent (Fig. 2).

Effect of cinobufotalin on mRNA expression

After interfering 769-P cells with cinobufotalin at concentrations of 0 mg/L, 10 mg/L, 20 mg/L and 40 mg/L for 24 h, RT-PCR showed that the expression of Notch1 mRNA decreased with the increase of drug concentration (Table 3). Bcl-2 mRNA and Pro-caspase 3 mRNA increased significantly.

Table 3 Effect of cinobufotalin on mRNA expression of cell-related molecules

Cinobufotalin (mg/L)	769-P cell		
	Notch1 mRNA	Bcl-2 mRNA	Pro-caspase3 mRNA
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
10	64.7 ± 10.52	47.13 ± 9.85	26.48 ± 8.48
20	44.8 ± 6.36[a]	62.30 ± 12.03[a]	52.13 ± 14.75[a]
40	28.3 ± 5.41[ab]	75.95 ± 14.26[ab]	74.87 ± 15.94[ab]
F*	63.35	13.57	21.79
P	0.000	0.004	0.000

* One-way analysis of variance; [a] Compared with 10 mg/L, $P < 0.05$; [b] Compared with 20 mg/L, $P < 0.05$

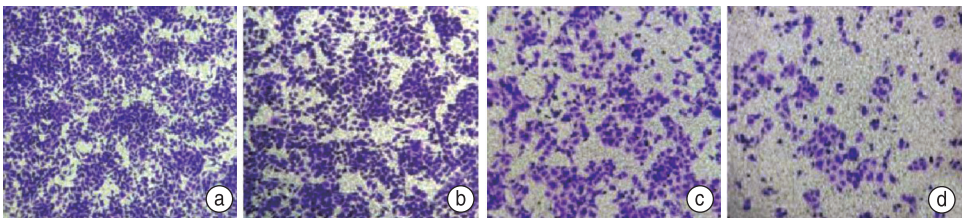

Fig. 2 Effect of different concentrations of cinobufotalin on apoptosis of renal cell carcinoma. (a) 0 mg/L; (b)10 mg/L; (c) 20 mg/L; (d) 40 mg/L

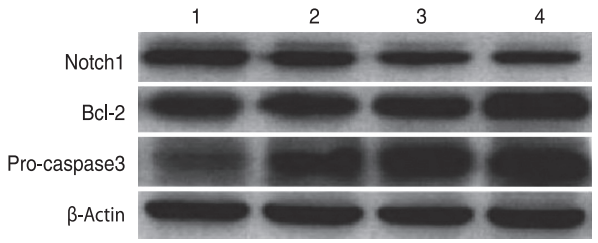

Fig. 3 Western-blot detection of Notch1 signaling pathway-associated proteins. 1: 0 mg/L; 2: 10 mg/L; 3: 20 mg/L; 4: 40 mg/L

Effect of cinobufotalin on the expression levels of proteins relative to signaling pathway

After interfering 769-P cells with cinobufotalinat concentrations of 0, 10 mg/L, 20 mg/L and 40 mg/L for 24 h, Western blot analysis showed that the expression level of Notch1 protein decreases with the increase of drug concentration. However, Bcl-2 and Pro-caspase3 proteinssignificantlyincreased.

Discussion

Renal cell carcinoma is a very common urological malignant tumor in China. Surgery is the one of the most important treatmentapproaches. At present, traditional Chinese medicine has a well-documented cancer therapy regime for the treatment of kidney cancer. In addition to the treatment efficacy, traditional Chinese medicine plays a significant role to complement Western medical practices [7]. Post operation, tumor burden in a patient's body is significantly reduced, but the risk of hidden malignancy cannot be completely eliminated, since the internal environment of tumorigenesis in the body remains. Furthermore, the host immunity is often compromisedfollowing the surgical intervention and hence, there is always a risk of cancer relapse. In view of the these reasons, traditional Chinese medicine is often sought to replenish qi and reinvigorate the spleen, dissipate the phlegm and clear the lungs, and synergistically restores the digestive functionto strengthen the host immunityand qi. When the functions of the spleen and stomach are restored, the residual disease-causing agents in the body can be removed too [8]. Thus, traditional Chinese medicine, as a treatment modality for kidney cancer, helps ward off all the disease-causing agents without affecting host immunity while also benefiting postoperative recovery.

Cinobufotalinis often made from dried mink and its main effects include detoxification, reducedswelling, and pain relief. In the treatment of malignant tumors, it can synergize with other chemotherapeutic drugs such as cyclophosphamide, cisplatin, etc. to enhance the efficacy of chemotherapy drugs. In humans, four Notch genes have been identified (Notch1, Notch2, Notch3, and Notch4), which exhibit diversity in tissue distribution and function. Gain or loss of Notch signaling is associated with multiple human disorders, including a variety of cancers [9]. The Notch gene encodes a highly conserved cell surface receptor whose signal affects multiple processes of normal morphogenesis, including differentiation of pluripotent progenitor cells, apoptosis, cell proliferation, and cell boundary formation [10]. Among these Notch genes, Notch1 is most closely related to renal cell carcinoma [11–12]. The activation of Notch1 signaling pathway is closely related to tumor formation and development. The main mechanisms include: (1) Induction of growth-promoting genes such as cyclin D1 and C-myc expression [13]; (2) Triggering anti-apoptosis such as P13 kinase/AKT pathway [14]; (3) Promotion ofgrowth and self-renewal in tissues and maintenanceof the undifferentiated state of stem cells [15]. In this study, by retrospective analysis of clinical data, Notch1 expression in renal cancer cells, adjacent tissues, and normal tissues were found to be 75.0% (48/64), 45.3% (29/64), and 9.4%, respectively; (4) Notch1 expression in renal cancer cellsis significantly higher. At the same time, Notch1 expression has a remarkablenegative impact on TNM staging, Fuhrman grading, and tumor size in patients with renal cell carcinoma.

In the *in vitro* experiment using the 769-P cell line, we found that the inhibitory effect of cinobufotalin on the growth of 769-P cells was dose- and time-dependent, and the effect on apoptosis was dose-dependent. These findings indicate that cinobufotalinhas significant inhibition ability onthe growth and apoptosis of renal cancer cells. Apoptosis is the autonomous and programmed cell death controlled by genes to maintain

homeostasis. Apoptosis is an active and complex process involving the activation, expression, and regulation of a series of genes. Defects or obstruction in apoptosis can disrupt the proliferation, differentiation, and death of normal cells, which is critical in tumorigenesis [16]. The effect of cinobufotalinon the apoptosis of renal cancer cells indicates that itcan delay the course of tumor malignancy to some extent. Both RT-PCR and Western-blot assays showed that the expression levels of Notch1 at the molecular and protein levels decreased with the increase of drug concentration, while Bcl-2 and Pro-caspase3 showed an upward trend. The main substrate for Pro-caspase-3 and poly (ADP)ribose polymerase (PARP), which is involved in DNA repair and gene integrity monitoring [17]. At the initiation of apoptosis, PARP is cleaved into two fragments by caspase-3and the two zinc finger structures bound to DNA in PARP are separated from the catalytic region at the carboxy terminus and become denatured. This indicates that cinobufotalin can induce tumor cell apoptosis by promoting the expression of Bcl-2 and Pro-caspase3.

This study found that cinobufotalinexerts anti-tumor effects by inhibiting the growth and inducing apoptosis of renal cancer cells, which may be related to the inhibition of Notch1 signaling activation.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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ORIGINAL ARTICLE

Effects of enteral nutrition intervention on immune and nutritional indexes of patients with gastric malignant cancer during postoperative chemotherapy

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Abstract

Objective The aim of this study was to investigate changes in nutritional status and related indexes in patients with Nutritional Risk Score (NRS) ≥ 3 gastric cancer after nutritional support treatment.

Methods A total of 50 patients with gastric cancer were divided into two groups according to the different nutritional support treatment they received during postoperative chemotherapy: immune-enhanced enteral nutrition group (n = 25) and conventional enteral nutrition group (n = 25). Changes in patient' body mass index (BMI), hemoglobin (HB), serum total protein (TP), serum albumin (ALB), and immune indexes (CD3+, CD4+/CD8+, CD3+/CD8+) were monitored before and after chemotherapy. At the same time, the incidence and classification of gastrointestinal adverse reactions after chemotherapy were assessed.

Results Compared with the conventional enteral nutrition group, the nutritional and immune indexes in the immune-enhanced enteral nutrition group were significantly improved. After chemotherapy, the incidence of adverse reactions in the digestive tract was relatively lower and the grade was reduced.

Conclusion Immune-enhanced enteral nutrition support can significantly improve the nutritional status of patients, improve immune function, increase the susceptibility of cancer patients to chemotherapy, reduce toxicity and adverse effects, and improve the quality of life of tumor patients compared with conventional enteral nutrition support.

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Key words: gastrointestinal malignancy; enteral nutrition; immune-enhanced nutrition support therapy

Cancer is a widespread disease with extremely high mortality, which seriously threatens human health. In recent years, the incidence of cancer has increased year by year. Gastrointestinal cancer is a common malignancy seen in clinics and surgery is the standard of radical treatment. However, the quality of life of patients after surgery is greatly affected. Surgery of gastrointestinal tumors is often accompanied by short-term and long-term complications. The five-year survival rate of these patients is no more than 61% [1–4]. Nutritional risk refers to the risk of adverse effects on a patients' clinical outcomes (hospitalization time, complications of infection, etc.) due to existing or potential nutritional factors [5]. Malignant tumor patients are prone to malnutrition and have a poor prognosis due to their unique stress state and high basal metabolic rate [6–7]. Gastrointestinal tumors involve the digestive tract, affect the digestion and absorption of food, and the risk of malnutrition is higher than that of other tumors [8]. Over 60% of patients with upper gastrointestinal cancer suffer from malnutrition while around 30% of patients with hepatobiliary and colorectal tumors have malnutrition [9]. More than 15% of patients with malignant tumor experience a 10% weight loss at the time of presentation [10] and around 40% of cancer patients die from malnutrition, rather than the cancer itself [11].

Tumor cells have the capacity of malignant uptake, even if no nutrition is provided, tumor cells can still absorb nutrients from the hosttissue and subsequently show increased tissue consumption, anorexia, skeletal muscle atrophy, fatigue, anemia, and hypoalbuminemia. This can eventually lead to the occurrence of cancer anorexia-cachexia syndrome (CACS), caused by both tumor metabolism and the host immune response. CACS

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manifests as malignant consumption of the body (10% body weight loss), reduced food intake (6.276 KJ/d), and systemic inflammation (c-reactive protein > 10 mg/L) as well as other malignant depletion manifestations [12]. More than 50% of patients with advanced tumors suffer from CACS. It is noteworthy that some patients with early tumors may also have CACS [13]. Early nutrition support therapy (NST) for cancer patients can meet the daily basic metabolic needs of the body, promote the recovery of intestinal barrier function in patients with digestive tract surgery, regulate intestinal flora, promote visceral protein synthesis, improve chemotherapy tolerance, and reduce adverse reactions caused by radiotherapy and chemotherapy [14–15].

In this study, 50 patients with gastrointestinal cancer with Nutritional Risk Score (NRS) ≥ 3 were collected and divided into the immune-enhanced enteral nutrition treatment group and the conventional enteral nutrition treatment group according to the different nutritional support treatments. The effects of nutritional support therapy on the nutrition-related laboratory indexes and immune function as well as the tolerance to chemotherapy and chemotherapy-related adverse reactions of the two groups were compared and analyzed.

Materials and methods

Patient population and data collection

A total of 50 patients [31 male and 19 female, aged 18–72 years, average age 51 years, Karnofsky Performance Status (KPS) score > 60, stable condition, and clinical stage II–III] with gastric malignant tumors after surgery were included in the study. All patients were treated with the XELOX regimen. All were diagnosed as poorly differentiated adenocarcinoma of the stomach by pathology and were receiving chemotherapy. Baseline examination showed no distant metastasis. Patients with cardiopulmonary, liver, and kidney disease were excluded from the study. Changes in nutritional status indicators, including body mass index (BMI), serum total protein (TP), serum albumin (ALB), and hemoglobin (Hb) were assessed as well as immune indicators, including CD3+, CD4+/CD8+, CD3+/CD8+. The incidence and grading of gastrointestinal adverse reactions after chemotherapy were also monitored and analyzed.

Methods

According to the NRS 2002, a total score ≥ 3 is classified as nutritional risk and < 3 as non-nutritional risk [16]. Focus on patients with nutritional risks. Patients were divided into the immune-enhanced enteral nutrition therapy group (receiving whey protein powder supplemented with glutamine, arginine, omega-3 fatty acid, and total nutrition formula powder) or the conventional enteral nutrition support group (receiving full nutrition formula powder rich in protein, fat, carbohydrate, vitamins and minerals) depending on the nutritional support treatment they received. Analysis was carried out of each subject's nutritional status and clinical outcomes before and after 3 weeks of chemotherapy. Body mass index (BMI) ranged from 18.5 to 23.9 kg/m^2 in the subjects, with a BMI of 17.0–18.4 kg/m^2 indicating mild malnutrition, moderate dystrophy at 16.0–16.9 kg/m^2, and severe malnutrition at < 16.0 kg/m^2 [17]. World Health Organization (WHO) criteria for toxicity and side effects of chemotherapeutic drugs are shown in Table 1. Changes in Hb, ALB, TP, and BMI before chemotherapy were compared with those after 3 cycles of chemotherapy. Immune indexes defined as the changes of T lymphocyte subsets CD3+, CD3+/CD8+, and CD4+/CD8+ before and after chemotherapy were also collected.

Statistical analysis

Data processing was performed using SPSS 20.0 statistical software. Count data was expressed as frequency and rate, and measurement data was expressed as mean ± standard deviation ($\bar{\chi} \pm s$). Comparisons between groups was performed using independent sample *t*-tests. Differences were considered significant at *P*-values < 0.05.

Results

After analysis, there were no significant differences in the patient characteristics, including age, sex, and duration of disease, between the two groups ($P > 0.05$; Table 2), suggesting that the two groups of patients were comparable. The absolute value of BMI in the immune-enhanced group increased more than that in the conventional enteral nutrition group, but there was no significant difference between the two groups. There

Table 1 WHO chemotherapeutic side reaction degree

Gastrointestinal reaction	Grade 0	Grade I	Grade II	Grade III	Grade IV
Oral cavity	None	Erythema, pain	Small ulcers, edible	Large ulcers, liquid food only	Inability to eat
Nausea and vomiting	None	Nausea	Temporary vomiting	Vomiting, need treatment	Uncontrolled vomiting
Diarrhea	None	Transience (< 2 h)	Tolerable (> 2 h)	Intolerance, treatment	Bloody diarrhea
Astriction	None	Occasionally or intermittently	Continuous constipation	Serious constipation, affecting daily life	Life-threatening conditions (such as intestinal obstruction, toxic megacolon)

Table 2 General information comparison between two groups

	Immune-enhanced group (n = 25)	Conventional group (n = 25)	P
Age (year)	51 (35–72)	51 (18–69)	0.540
Gender			
Male	15 (51.7%)	16 (55.2%)	0.574
Female	10 (34.5%)	9 (31.0%)	
BMI	19.08 ± 2.79	19.40 ± 2.31	0.206
ALB	35.04 ± 5.99	34.89 ± 5.37	0.344
TP	56.20 ± 5.32	55.54 ± 5.40	0.679
HB	120.12 ± 17.07	121.48 ± 19.10	0.638

Notes: By one-way ANOVA, $P > 0.05$, there is no significant differences among two groups

Table 3 Comparison of nutritional status in two groups before and after chemotherapy

	Before chemotherapy	After chemotherapy	P
BMI			
I	19.08 ± 2.79	20.43 ± 2.01	0.060
E	19.40 ± 2.31	19.21 ± 1.65	0.126
ALB			
I	35.04 ± 5.99	38.64 ± 2.04	0.000
E	34.89 ± 5.37	36.91 ± 3.89	0.188
TP			
I	56.20 ± 5.32	59.33 ± 1.75	0.000
E	55.54 ± 5.40	57.66 ± 2.94	0.015
HB			
I	120.12 ± 17.07	130.04 ± 10.13	0.007
E	121.48 ± 19.10	127.44 ± 16.70	0.348

Table 4 Comparison of immune related indexes in two groups before and after chemotherapy

	Before chemotherapy	After chemotherapy	P
CD3+			
I	52.4 ± 5.1	60.9 ± 3.5	0.021
E	51.7 ± 5.1	53.5 ± 5.1	0.932
CD3+/CD8+			
I	38.04 ± 3.83	44.77 ± 5.62	0.016
E	38.16 ± 4.96	41.39 ± 5.22	0.390
CD4+/CD8+			
I	26.56 ± 2.19	29.20 ± 3.43	0.027
E	25.21 ± 2.00	29.90 ± 2.54	0.373

were significant differences in Hb, TP, and ALB (P < 0.05; Table 3). The percentage of CD3+, CD3+/CD8+, and CD4+/CD8+ T cells in the peripheral blood of the two groups before chemotherapy was at a low level, and there was no significant difference between the two groups before treatment. After 3 cycles of chemotherapy and enteral nutrition support, the percentage of CD3+, CD3+/CD8+, and CD4+/CD8+ cells was higher in the immune-enhanced group than that in patients receiving conventional enteral nutrition support, and the difference was statistically significant (P < 0.05; Table 4), indicating an improvement inimmune status.

The incidence of adverse reactions, such as anorexia, nausea, vomiting, abdominal pain, and diarrhea, after chemotherapy in the two groups was analyzed and the incidence of adverse reactions of the digestive tract in the immune-enhanced group was significantly lower than that of the conventional enteral nutrition group. The incidence of grade I–II digestive tract adverse reactions in the immune-enhanced group was 80% and grade III–IV adverse reactions were present in 20% of participants, while the incidence of grade I–II adverse reactions in the conventional enteral nutrition group was 44% and grade III–IV was 56%, which was statistically significant (P = 0.008).

Discussion

The metabolism of various nutrients that provide energy in cancer patients is altered compared with that in a healthy human body. The energy supply needed by a healthy individual in daily living is mainly supplied by the aerobic decomposition of sugar. In the case of hypoxia, energy can be obtained by anaerobic glycolysis. When a tumor occurs in the body, anaerobic glycolysis is predominantly utilized, even under aerobic conditions. Approximately 50% of ATP in tumor cells is obtained by glycolysis. Most patients with tumors have changes such as the reduction of glycogen stores, increased gluconeogenesis, and insulin resistance. Most tumor patients have reduced fat reserves and body weight, resulting in increased endogenous fat hydrolysis and fatty acid oxidation, increased triglyceride conversion, and ultimately an increased plasma free fatty acid concentration [18]. Meanwhile, protein catabolism is increased, anabolism decreased, and protein conversion rate is increased. This leads to an abnormal plasma amino acid spectrum, skeletal muscle atrophy, hypoalbuminemia, and a negative nitrogen balance [19–20]. Malnutrition can lead to decreased active ability, reduced responsiveness to anti-tumor therapy, increased incidence of adverse reactions to treatment, and can affect the quality of life and survival time of patients.

As one of the primary treatment methods of cancer, chemotherapy kills tumor cells by inhibiting the growth and reproduction of tumor cells, so as to reduce the risk of tumor recurrence and metastasis and to prolong the survival time of patients. Chemotherapy drugs not only kill tumor tissue, but also have a certain effect on the growth of normal tissue cells, resulting in malnutrition. Long-term malnutrition leads to problems in the absorption, distribution, metabolism, and excretion of drugs in the body, which affects the pharmacokinetics of chemotherapeutic drugs, causing an accumulation

of chemotherapeutic drugs in the body, and increasing the toxicity and side effects. The incidence of adverse reactions, such as anorexia, fatigue, nausea, vomiting, diarrhea, and constipation, is significantly increased with malnutrition, which in turn increases the risk of malnutrition in patients and forms a vicious cycle. Ultimately this leads to reduced sensitivity and tolerance of patients to chemotherapy, affecting the quality of life and increasing the mortality rate [21].

Malnutrition is a very important factor affecting the prognosis of patients with gastrointestinal malignant tumors during the management and treatment of these tumors [22]. The common cause of malnutrition in these patients may be that for the tumor to grow, it competes with the healthy tissue to bind raw material, resulting in the normal metabolism of the body being affected [23]. Digestive tract tumors also directly compress or obstruct the digestive tract, hindering the digestion and absorption of nutrients, leading to an increased incidence of malnutrition [24]. In addition, a patients' own psychological factors and anorexia factors secreted by tumors themselves act on the hypothalamus, which can also lead to loss of appetite [25–26]. Therefore, early nutritional support therapy plays an important role in improving the prognosis and the tolerance of patients to treatment.

Current nutritional support therapy includes enteral nutritional support therapy and parenteral nutritional support therapy. In recent years, early enteral nutrition has been recommended for postoperative patients with gastrointestinal tumors [27]. Studies have shown that parenteral nutrition alone can lead to intestinal flora translocation, intestinal mucosal atrophy, decreased intestinal barrier function, and increase the incidence of infection and metabolic complications. Severe cases can lead to complications such as sepsis and multiple organ dysfunction [28–30]. Early detection of patients with nutritional risks and early nutritional support can reduce the intestinal inflammatory response, stimulate hormone and digestive fluid secretion, promote intestinal mucosal barrier repair, prevent intestinal flora translocation, improve immunity, shorten hospitalization time, and improve the quality of life of patients [10, 31–33]. Therefore, early enteral nutrition intervention is essential for patients with digestive tract tumors [34].

Current studies have found that soluble immunosuppressive factors can be secreted during the development and progression of malignant tumors, resulting in low cellular immune function, characterized by a reduction in CD3+, CD4+/CD8+, and CD3+/CD8+ T cells, leading to tumor development, metastasis, and ultimately, a poor prognosis [35–37]. It has been shown that T cell subsets are considered to be the main effect or cells of cancer treatment. A variety of immune cells play an important role in preventing tumor growth and metastasis, which is the theoretical basis of the clinical application of immunotherapy for a variety of tumors, including gastrointestinal tumors [36–37]. When the number and function of T cell subsets change, the ability to eliminate tumor cells is reduced, which directly affects tumor development and prognosis [38–39]. Dynamic detection of T cell subsets in the peripheral blood of digestive tract tumors before and after treatment can indirectly reflect the immune status of the body and have a certain suggestive significance for the evaluation of disease prognosis.

The main role of nutritional support is to meet the needs of patients to recover metabolism and immune responses on the basis of daily necessary energy for the body [40]. The timely addition of immune-enhanced nutrition therapy in the clinic can play a significant role in treatment. Nutritional therapy, adding a full-nutrition formula to immune-enhanced whey protein, can fully supplement the body's energy supply, improve immunity, maintain organ function, and reduce the occurrence of complications and adverse reactions [41]. Immune nutrients such as arginine, glutamine, and omega-3 fatty acid are immunomodulators and intestinal mucosal nutrient substrates, which can remove toxic substances and promote intestinal mucosal growth [42–43].

Glutamine is an essential amino acid, which can be obtained by healthy individuals by eating a normal diet. It plays a role in promoting cell growth and protein synthesis [44]. Some researchers have also shown that glutamine plays an indispensable role in the treatment of digestive tract tumors [45], acting on the intestinal mucosa, reducing the patient's inflammatory response, and reducing the incidence of infection [46–47]. It has been reported that glutamine supplementation can reduce the incidence of vomiting and gastrointestinal discomfort after surgery by improving intestinal immune function, reducing the stimulation and damage of treatment to the gastrointestinal mucosa, and is conducive to the recovery of gastrointestinal function. Moreover, glutamine has no obvious promoting effects on the growth of tumors, nor does it increase the incidence of metastasis of tumors [48–49].

Arginine can promote the progression of tumor cells from the G0 phase to the S phase and can be combined with chemotherapy drugs that specifically act on the S phase to improve the sensitivity of chemotherapy [50]. Arginine can also promote the release of growth hormone, prolactin, and insulin as well as stimulating the differentiation and proliferation of T cells, enhancing the function of T cells, and modulating immune regulation by effecting macrophages, NK cells and monocytes [51–52].

Omega-3 fatty acids, including eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and flax oil, are polyunsaturated fatty acids. They are important

components of biofilms and can play a role as anti-inflammatory agents as well as regulating immunity, modulating the bodies energy, thereby enhancing the ability to eliminate bacteria and inhibit tumor growth. These acids also have a certain anti-tumor effect and can inhibit the growth of a variety of tumor cells. Domestic scholars have suggested that EPA and DHA can inhibit the growth of gastric cancer cell lines [53] in a dose and time dependent manner. EPA has a synergistic effect on epirubicin, improving the therapeutic effect and reducing the side effects of chemotherapy. Comprehensive research has found that exogenous glutamine, arginine, and omega-3 fatty acids may reduce macrophage phagocytosis and superoxide production by reducing prostaglandin E2 synthesis [54–55], thus reducing the incidence of mucositis and diarrhea as well as other adverse effects of chemotherapy and increase the uptake of chemotherapeutic agents by tumor tissues, increasing the local concentration of chemotherapeutic drugs, which can inhibit tumor and synergistic chemotherapy [41].

This study found that early enteral nutrition support can prolong progression-free survival (PFS), and immune-enhanced enteral nutrition support therapy may be a valuable method to improve a patient's long-term quality of life [56]. Research has shown that gastrointestinal symptoms and protein reduction can reflect the nutritional status of cancer patients after chemotherapy, and this plays an important role in detecting early malnutrition and can guide the evaluation of therapeutic effects after intervention [57]. In this study, by screening patients with NRS ≥ 3 after gastrointestinal tumor surgery, the effects of early immune enhanced enteral nutrition support after adding the above-mentioned immuno-nutrients on nutritional indicators (BMI, TP, ALB, and Hb), immune indicators, and digestive tract reactions after chemotherapy were compared with that of conventional enteral nutrition support. It was found that compared with conventional enteral nutrition support, early enteral nutrition support with immune enhancement significantly increased the nutrition related and immune indexes, and the difference was statistically significant. The incidence of adverse gastrointestinal reactions was also lower after chemotherapy in this group. This indicates that for patients undergoing gastrointestinal tumor surgery, early immune-enhanced enteral nutrition not only promotes the recovery of nutritional status and immunity post-surgery, but also plays a role in improving a patients' tolerance to chemotherapy and reducing the related side effects. In turn, the quality of life of patients and overall prognosis will be improved. This provides a theoretical basis for the recovery of early nutritional status and the improvement of tolerance and sensitivity of subsequent adjuvant chemotherapy in patients with gastrointestinal cancer.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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ORIGINAL ARTICLE

High level of preoperative serum fibrinogen is a predictor of poor prognosis in patients with esophageal squamous cell carcinoma

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Abstract

Objective This study aimed to elucidate the association between the level of preoperative serum fibrinogen (PSF) and the prognosis of patients with esophageal squamous cell carcinoma (ESCC).

Methods From January 2010 to December 2016, all patients diagnosed with ESCC who underwent surgery in Qingdao Municipal Hospital were analyzed retrospectively. Moreover, the fibrinogen levels of all patients were assessed before surgery, and hyperfibrinogenemia was diagnosed when the fibrinogen level was ≥4.0 g/L. The impact of PSF on disease-free survival (DFS) and overall survival (OS) was analyzed using the log-rank method and Cox proportional hazards regression model. *P* value less than 0.05 was considered statistically significant.

Results A total of 336 patients were finally analyzed, and approximately 102 patients (30.36%) were diagnosed with hyperfibrinogenemia before surgery. Hyperfibrinogenemia was associated with older age (≥ 70 years) (*P* = 0.012), advanced pathological T stage (*P* = 0.003), and lymph node involvement (*P* = 0.024). Univariate analysis showed that patients with hyperfibrinogenemia had shorter DFS (1.96 years *vs.* 3.64 years, *P* = 0.001) and OS (2.27 years *vs.* 4.15 years, *P* < 0.001) than patients without hyperfibrinogenemia. Multivariate analysis confirmed that PSF was an independent factor affecting DFS (risk ratio [RR]: 1.35, 95% confidence interval [CI]: 1.02–1.79, *P* = 0.038) and OS (RR: 1.37, 95% CI: 1.03–1.83, *P* = 0.034) in patients with ESCC.

Conclusion For patients with operable ESCC, hyperfibrinogenemia had poor prognosis. Moreover, PSF is an independent prognostic factor for operable ESCC.

Key words: esophageal squamous cell carcinoma; biomarker; prognosis; serum fibrinogen

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Esophageal squamous cell carcinoma (ESCC), with high morbidity and mortality, is prevalent in East Asia [1–2]. Although patients diagnosed with early-stage ESCC underwent radical resection, their 5-year survival rate was still poor [3]. Postoperative recurrence and metastasis were the main factors affecting the survival of ESCC [3]. At present, a significant prognostic biomarker for ESCC is not yet available in clinical practice; thus, it was dispensable to determine the potential prognostic biomarkers for patients with ESCC.

Fibrinogen, which is a coagulation factor, is mainly synthesized by hepatocytes. A previous study had reported that tumor escape involving fibrinogen was an important pathway for tumor recurrence and metastasis [5]. Some studies demonstrated that preoperative serum fibrinogen (PSF) can be used as a prognostic factor in common malignant tumors, such as in gastric cancer [6], lung cancer [7], and colorectal cancer [8]. However, the significance of PSF in predicting the prognosis of ESCC remains unclear. Some studies indicated that PFS was an independent prognostic predictor of esophageal cancer; however [9], some studies had contradicting results [10]. Therefore, this study was designed to analyze the association between the PSF and the pathological characteristics of ESCC and elucidate the association between the PFS and prognosis of ESCC.

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Materials and methods

Study design

From January 2010 to December 2016, all patients with esophageal cancer who underwent surgery in Qingdao Municipal Hospital were included in this study. Pathological stage was determined according to the 8th edition American Joint Committee on Cancer (AJCC) staging system[10]. The inclusion criteria were as follows: (1) patients with pathologically diagnosed ESCC, (2) patients with serum fibrinogen level assessed before surgery, (3) patients undergoing radical resection, and (4) patients with complete follow-up data after surgery. On the contrary, the exclusion criteria were as follows: patients who died during the perioperative period and patients diagnosed with concomitant disease influencing serum fibrinogen levels. Patients were followed up until December 2019.

A 5 mL fasting venous blood collected before surgery was provided for testing, and fibrinogen level test was performed within 30 min after blood collection using the Clause method. According to the test, hyperfibrinogenemia was diagnosed if the fibrinogen level was ≥4.0 g/L, and patients with fibrinogen level < 4.0 g/L were considered to have normal fibrinogen level.

Statistical analyses

The association between the level of PSF and clinicopathological characteristics was compared using the chi-squared test and Mann-Whitney *U* test. Moreover, univariate survival analysis was performed using log-rank test, and the Kaplan-Meier curve was used to draw the survival curve. Multivariate analysis was performed using Cox proportional hazards regression model. *P* value less than 0.05 was considered statistically significant. All statistical analysis was performed using the Statistical Package for the Social Sciences software version 22.0.

Results

Patients' characteristics

Finally, a total of 336 patients diagnosed with ESCC were included in this study. The oldest and youngest patients were aged 82 and 39 years, respectively, and the median age was 63 years. Among these patients, 249 were male and 87 were female. According to the 8th edition of the AJCC staging system, 56, 136, and 144 patients were categorized as stage I, stage II, and stage III, respectively.

The fibrinogen levels of all patients were assessed before operation, and the median preoperative fibrinogen level of all patients was 3.4 g/L (range, 1.1–9.6 g/L). Moreover, 102 patients (30.36%) were diagnosed with hyperfibrinogenemia (≥4.0 g/L), and 234 patients (69.64%) were diagnosed as normal according to the diagnostic criteria.

Preoperative serum fibrinogen level and clinical characteristics

In this study, we found that older patients (≥ 70 years) had higher PSF than younger patients (43.48% *vs.* 26.97%, $P = 0.012$), as shown in Table 1. Further analysis confirmed that pathological T stage ($P = 0.003$) and N stage ($P = 0.024$) as prognostic factors were significantly associated with PSF. Fig. 1 also shows that patients with hyperfibrinogenemia were more likely to be diagnosed with advanced pathological T stage and to had more metastatic lymph nodes compared to patients without hyperfibrinogenemia.

Survival analysis

The median time for disease-free survival (DFS) was 2.81 years, and univariate analysis indicated that patients with hyperfibrinogenemia had poorer DFS than patients without hyperfibrinogenemia (1.96 years *vs.* 3.64 years, $P = 0.001$), as shown in Table 2 and Fig. 2. Multivariate analysis confirmed that patients with hyperfibrinogenemia had 35.0% higher risk of disease progression than patients without hyperfibrinogenemia (risk ratio [RR]: 1.35, 95% confidence interval [CI]: 1.02–1.79, $P = 0.038$), as shown in Table 3.

Table 1 Clinical characteristics and level of PSF in 336 patients with ESCC

Variables	*n*	Preoperative serum fibrinogen level		*P*
		≥ 4.0 g/L (*n*,%)	< 4.0 g/L (*n*,%)	
Gender				0.104
Male	249	82 (32.93)	167 (67.07)	
Female	87	20 (23.50)	67 (76.50)	
Age (years)				0.012
< 70	267	72 (26.97)	195 (73.03)	
≥ 70	69	30 (43.48)	39 (56.52)	
Tumor location				0.300
Upper	27	9 (33.33)	18 (66.67)	
Middle	221	61 (27.60)	160 (72.40)	
Lower	88	32 (36.36)	56 (63.64)	
Differentiation				0.421
G1	97	30 (30.93)	67 (69.07)	
G2	158	52 (32.91)	106 (67.09)	
G3	81	20 (24.69)	61 (75.31)	
Pathological T stage				0.003
T1 + T2	115	23 (20.00)	92 (80.00)	
T3 + T4	221	79 (35.75)	142 (64.25)	
Pathological N stage				0.024
N0	171	42 (24.56)	129 (75.44)	
N1 + N2 + N3	165	60 (36.36)	105 (63.64)	
Pathological TNM stage				0.003
Stage I	56	12 (21.43)	44 (78.57)	
Stage II	136	32 (23.53)	104 (76.47)	
Stage III	144	58 (40.28)	86 (59.72)	

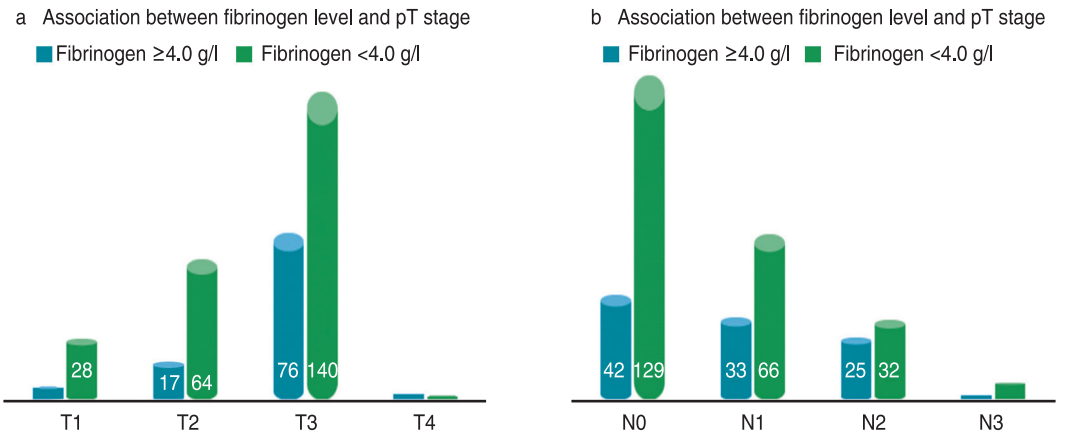

Fig. 1 Association between PSF and pathological T/N stage. (a) Association between PSF and pathological T stage; (b) Association between PSF and pathological N stage

Table 2 Univariate analysis of prognosis in patients with ESCC

Variables	DFS			OS		
	Median	95% CI	*P*	Median	95% CI	*P*
Gender			0.086			0.104
Male	2.71	2.10–3.31		3.33	2.68–3.98	
Female	3.45	1.30–5.60		3.64	0.88–6.40	
Age (years)			0.514			0.412
< 70	3.00	2.26–3.75		3.64	2.72–4.56	
≥ 70	2.39	1.52–3.25		2.88	2.18–3.58	
Tumor location			0.968			0.967
Upper	2.26	2.04–2.49		3.40	0.79–6.02	
Middle	2.96	2.30–3.61		3.49	2.99–3.99	
Lower	2.31	0.77–3.86		3.23	1.15–5.32	
Differentiation			0.317			0.399
G1	3.04	1.56–4.51		3.58	1.64–5.52	
G2	2.29	1.66–2.93		3.06	2.24–3.88	
G3	3.64	2.14–3.48		4.13	3.05–5.20	
Pathological T stage			< 0.001			0.001
T1	7.16	1.92–12.40		7.47	3.15–12.17	
T2	5.39	2.07–8.72		4.22	2.71–9.18	
T3	2.10	1.62–2.58		2.56	1.85–3.28	
T4	1.19	0.58–1.81		1.66	1.22–2.11	
Pathological N stage			< 0.001			< 0.001
N0	4.64	2.65–6.63		5.10	4.67–5.53	
N1	2.96	2.02–3.89		3.33	2.77–3.89	
N2	1.11	0.94–1.29		1.49	1.32–1.67	
N3	0.98	0.25–1.71		1.04	0.86–1.22	
Pathological TNM stage			< 0.001			< 0.001
Stage I	5.39	2.24–8.55		5.84	4.41–9.18	
Stage II	3.80	2.16–5.43		5.56	3.90–7.23	
Stage III	1.53	0.97–2.09		1.80	1.34–2.26	
Preoperative serum fibrinogen level			0.001			<0.001
≥ 4.0 g/L	1.96	1.29–2.62		2.27	1.44–3.09	
< 4.0 g/L	3.64	2.82–4.46		4.15	3.03–5.28	

For patients with ESCC, the median time for overall survival (OS) was 3.40 years. Survival analysis revealed that the median OS for patients with hyperfibrinogenemia was 2.27 years, which was significantly shorter than

that of patients with normal PSF (4.15 years) (Fig. 3). Multivariate analysis confirmed that PSF was an independent factor affecting OS (RR: 1.37, 95% CI: 1.03–1.83, $P = 0.034$) in patients with ESCC.

Discussion

Hyperfibrinogenemia was observed in esophageal cancer, and whether PSF can be an independent prognostic predictor for esophageal cancer remains controversial [6, 9]. In this study, approximately 30.4% of patients with ESCC were diagnosed with hyperfibrinogenemia before surgery, indicating that PSF might be a potential biomarker for operable ESCC.

The molecular mechanism of fibrinogen in tumorigenesis is still unclear. Fibrinogen is normally secreted by liver cells and is also recognized as a coagulant and inflammatory factor [12]. The recent study demonstrated that cancer cell can also synthesize fibrinogen, and fibrinogen has been shown to participate in the process of tumorigenesis, development, metastasis, and implantation [13–14].

The serum tumor biomarkers were widely used to predict the survival of patients with ESCC and to assess the therapeutic effects of certain treatments. It has been reported that cytokeratin 19 fragment (CYFRA 21-1) level was significantly associated with T stage ($P = 0.019$) and N stage ($P = 0.019$). Some studies also confirmed that CYFRA 21-1 can be used to assess the effect of radiotherapy [15]. Our study indicated that PSF would be useful for predicting the survival of patients with ESCC, and the level of PSF was significantly associated with pathological T stage, pathological N stage, and pathological TNM stage.

Moreover, whether PSF can be used to predict the prognosis of esophageal cancer remains controversial. This study was designed to investigate the prognostic value of coagulation in patients with ESCC. Based on the result of the recent study, the level of PSF was significantly higher in patients with ESCC than that in normal individuals, but it was not a prognostic indicator for ESCC (hazard ratio [HR]: 1.165, 95% CI: 0.790–1.717, $P = 0.441$) [5]. Another study, which comprised 1305 patients with ESCC, regarding this issue yielded a positive result. According to the study's survival analysis, patients with hyperfibrinogenemia had poor DFS ($P \leq 0.001$) and OS ($P \leq 0.001$), and the study's multivariate analysis revealed that PSF was an independent prognostic factor for patients with ESCC [9]. A meta-analysis comprising 2865 patients with esophageal cancer from 11 studies was designed to analyze the prognostic role of PSF in esophageal cancer. This meta-analysis demonstrated that high PSF was significantly associated with poor DFS (HR: 1.51, 95% CI: 1.16–1.97, $P < 0.001$) and OS (HR: 1.76, 95% CI: 1.28–2.42, $P < 0.001$) based on multivariate

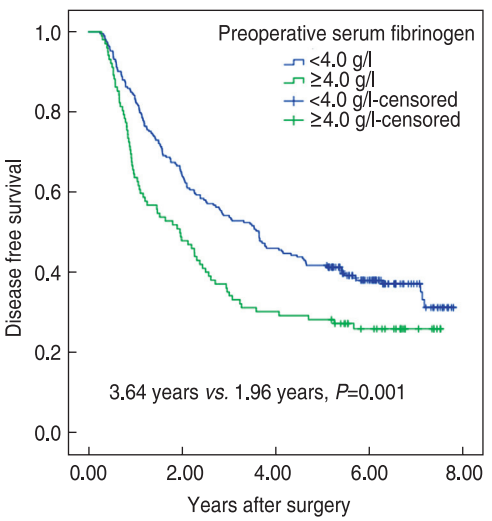

Fig. 2 Survival curve of DFS in patients with ESCC based on PSF

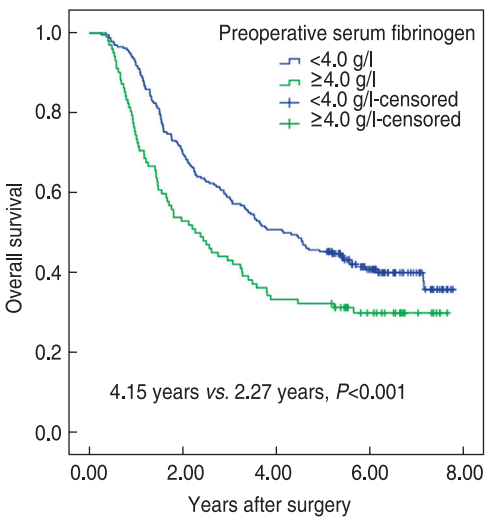

Fig. 3 Survival curve of OS in patients with ESCC based on PSF

Table 3 Multivariate analysis of prognosis in patients with ESCC

Variables	DFS			OS		
	RR	95% CI	*P*	RR	95% CI	*P*
Pathological T stage	1.31	1.05–1.64	0.017	1.35	1.07–1.71	0.011
Pathological N stage	1.54	1.32–1.80	< 0.001	1.57	1.35–1.84	< 0.001
Preoperative serum fibrinogen level	1.35	1.02–1.79	0.038	1.37	1.03–1.83	0.034

analysis [16]. Our study confirmed once again that PSF was a prognostic factor for patients with ESCC.

In conclusion, these results suggested that hyperfibrinogenemia was significantly associated with pathological T stage and N stage in patients with ESCC. PSF may be a serum prognostic biomarker for patients with ESCC.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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REVIEW ARTICLE

Multi-disciplinary treatment for hepatocellular carcinoma in primary hospitals in China during the COVID-19 epidemic

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Abstract

Hepatocellular carcinoma (HCC) is a common malignant tumor in the Chinese population. Due to its high degree of malignancy, rapid progression, and poor prognosis, it mainly requires multi-disciplinary treatment (MDT) in the clinic. In December 2019, COVID-19, a novel coronavirus pneumonia, broke out in Wuhan, China. It has rapidly spread across the country, with various places launching a level I response to major public health emergencies and traffic being restricted. Most patients with HCC were only able to attend primary hospitals, while the MDT model for HCC in provincial hospitals was restricted. Therefore, it was a huge task for clinicians in primary hospitals to ensure MDT was given to patients with HCC during the level I response to major public health emergencies. How to formulate a reasonable MDT mode for patients with HCC according to local conditions was worthy of consideration by hepatobiliary surgeons in primary hospitals.

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Key words: COVID-19; primary hospital; hepatocellular carcinoma; multi-disciplinary treatment

In December 2019, COVID-19, a novel coronavirus pneumonia, broke out in Wuhan, China[1]. The epidemic spread across the country with unprecedented momentum, with various places launching a level I response to major public health emergencies and traffic being restricted, which caused challenges for primary hospitals. HCC is a common malignant tumor in the Chinese population. Due the high degree of malignancy and rapid progression, most patients with HCC are already in the mid-late stages when diagnosed and prognosis is poor. In recent years, the rapid development of biomedicine, imaging medicine, interventional medicine, and surgical technology, has promoted the birth of new model treatment for HCT, multi-disciplinary treatment (MDT) [2].

During the level I response to major public health emergencies, most patients with HCC were only able to attend primary hospitals, while the MDT model for HCC in provincial hospitals was restricted. In addition, HCC patients were susceptible to COVID-19 due to their low immunity. Therefore, it was a huge task for clinicians in primary hospitals to ensure the MDT mode was given to HCC patients during this level I response to major public health emergencies. The formulation of a reasonable MDT mode for patients with HCC according to local conditions was worthy of consideration by hepatobiliary surgeons in primary hospitals.

Particularity of patients with HCC during the COVID-19 epidemic

Patients with HCC are susceptible to COVID-19 due to their low immunity. It was shown that a history of cancer was the highest risk factor for COVID-19, resulting in a patient's condition deteriorating more quickly once infected by COVID-19[3]. Moreover, patients who have recently received chemotherapy or surgery have a higher risk of serious illness than those who have not received treatment. Patients with cancer will consume more nutrients than the healthy population due to the coexistence of tumor cells and the ability to fight pathogens

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is relatively weak. Moreover, the immune function of these patients has been in a state of immunodeficiency for a long period of time due to surgery, chemotherapy, biological agents, immunosuppressive therapy, and so on, meaning the defense ability against pathogens has been significantly reduced. In addition, most patients with HCC have a background of viral hepatitis and cirrhosis, and therefore the outcome may be worse than the general population once infected by COVID-19 [4]. Hence, it is very important to strengthen personal protection.

MDT model for HCC in primary hospitals of China during the COVID-19 epidemic

It is feasible to wait for a short period of time for patients with HCC in very early stage of the disease, then surgical resection (SR) or radiofrequency ablation (RFA) can be performed after the epidemic situation is stable. As HCC presents as asymptomatic with a single lesion less than 2 cm in diameter, without vascular invasion and distant metastasis [5], the efficacy of treatment will not be effected by a short postponement.

The early stage of HCC refers to a single cancer nodule with a diameter less than 5 cm or no more than 3 cancer nodules each with a diameter less than 3 cm [6]. For patients with early HCC (Child-Pugh A or B) and good hepatic reserve function, the choice of treatment depends on the degree of liver dysfunction, portal hypertension, as well as the systemic state of the patient. RFA should be considered for the patients with tumors smaller than 3 cm as the overall survival (OS) and cancer-specific survival (CSS) have been reported to be similar between patients receiving RFA and SR [7]. RFA also has a lower incidence of complications and requires a shorter hospital stay, which is a good option during the epidemic period. For patients with a single tumor lesion and no portal hypertension, SR should be considered after excluding COVID-19. One randomized clinical trial comparing RFA and SR for early-stage HCC showed that RFA is not superior to hepatic resection in terms of tumor recurrence, overall survival, and disease-free survival[6]. SR may be a superior treatment option with better long-term outcomes compared to RFA in patients with tumors measuring 3.1-5 cm[7]. The protection of patients during the perioperative period is very important. RFA can be selected for patients with tumors which cannot be resected.

Patients with compensated liver cirrhosis, no tumor symptoms, no vascular infiltration, but a single large lesion or multiple lesions are considered to have middle-stage HCC. Transcatheter arterial chemoembolization (TACE) is a good choice for these individuals [8]. TACE is one of the most safe, effective, and commonly used interventional therapies at present and is widely recommended by domestic and foreign guidelines for the treatment of HCC [9]. Compared to conservative treatment, the 2-year survival rate with TACE can be increased by 20% to 25%. Furthermore, it is also suitable for the epidemic situation due to the advantages of reduced trauma, quick recovery, and the convenient and short operation time. In addition, TACE should be combined with molecular-targeted agents and PD-1/PD-L1 in the early phases of disease, which can result in improved survival benefits for patients [10].

Patients with mild tumor-related symptoms, vascular invasion, or extrahepatic tumor metastasis are considered to be in the late stage of disease and are no longer suitable for more aggressive treatments. In recent years, studies have found that hepatic artery infusion chemotherapy (HAIC) for the treatment of advanced liver cancer shows great advantages [11]. HAIC not only has an improved objective remission rate and longer survival benefits, but can also significantly reduce the size of the tumor and reduce the stage of disease, providing a new option for the treatment of advanced HCC [12]. In addition, the approval of molecular-targeted agents and PD-1/PD-L1 has brought new hope to these patients [10].

End-stage HCC is often accompanied by liver failure, vascular invasion, extrahepatic tumor spread, and so on. The 1-year survival rate is less than 10% and they are unable to benefit from the above treatment [13]. For patients with ruptured live cancer, emergency SR, RFA, and TACE can be used when required, and the secondary protection from COVID-19 must be done well.

The conventional MDT mode for HCC has been greatly affected during the epidemic period, and primary hospitals should play a practical role in the whole process of diagnosis and treatment of HCC. Primary hospitals are duty-bound not only to do a safe job in the prevention and control of COVID-19, but also to give effective MDT treatment to patients with HCC.

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