

Friend Leukemia Virus Integration 1 Expression Has Prognostic Significance in Nasopharyngeal Carcinoma¹

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Abstract

This study aimed to investigate the expression pattern and prognostic value of friend leukemia virus integration 1 (FLI-1) in nasopharyngeal carcinoma (NPC). Immunohistochemistry (IHC) staining of FLI-1 was performed in specimens from 198 untreated NPC patients. Ninety-nine patients were randomly assigned to the training set to analyze the prognostic value of FLI-1 and other clinicopathological characteristics, while the others were assigned to the testing set for validation. Clinicopathological data were compared using the Pearson chi-square test. Univariate and multivariate analyses were performed using the Cox proportional hazards model to test independent prognostic factors and calculate the hazard ratio (HR) and 95% confidence interval (CI). Cytoplasmic FLI-1 expression positively correlated with N stage, distant metastasis and death ($P < 0.05$) and also predicted poorer overall survival (OS) ($P = 0.014$), distant metastasis-free survival (DMFS) ($P = 0.010$), progression-free survival (PFS) ($P = 0.031$). In multivariate analysis, FLI-1 expression and clinical stage were both independent prognostic factors of poor OS and DMFS. Prognoses of patients in the training set, the testing set, and the entire set were clearly divided into four risk subgroups by supplementing FLI-1 with clinical stage. These results indicate that FLI-1 expression is an independent prognostic factor for NPC patients and suggest that supplementing FLI-1 with clinical stage could be helpful for more accurate risk definition.

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Introduction

Nasopharyngeal carcinoma (NPC), the most common cancer originating from nasopharynx, is a unique type of head and neck malignancy in terms of its unbalanced distribution, poor differentiation, strong propensity to metastasize to regional lymphatic and/or distant organs, and chemo-radiosensitivity. NPC is most prevalent in the Guangdong Province of the southern China and universally associated with Epstein-Barr virus infection, with most classified as the undifferentiated non-keratinized carcinoma [1]. With the improve-

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ment of diagnosis techniques, irradiation and chemo-radiotherapy, while locoregional control rate has increased greatly in the past few decades, however, the incidence of distant metastasis has not decreased significantly, as high as 16% to 30% [2,3], which becomes the leading cause of treatment failure nowadays. Currently, prediction of NPC survivals is mainly based on the TNM staging system. However, different outcomes are observed in NPC patients with the same clinical stage of tumors after receiving similar standard treatment, indicating a pressing need of prognostication utilizing some biomarkers and the TNM staging to guide individualized treatment.

Friend leukemia virus integration 1 (FLI-1), which was first identified in erythroleukemia induced by Friend Murine Leukemia Virus (F-MuLV) [4], is a new member of the E26 transformation-specific (ETS) transcription factor family. FLI-1, which is localized within the 240 kb of the ETS-1 locus on mouse chromosome 9 and on human chromosome 11q23 [4,5], is activated through retroviral insertion mutagenesis in most F-MuLV-induced erythroleukemias. Activated FLI-1 can alter EPO/EPO-receptor signaling pathway and the Ras pathway, blocking erythroid differentiation and apoptosis and leading to massive EPO-independent proliferation of erythroblasts [6]. In addition, FLI-1 is also involved in various malignancy formation and progression in vitro and/or in vivo, including Ewing's sarcoma [7], melanoma [8], breast cancer [9], lymphoma [10,11] and head and neck squamous cell cancer (HNSCC) [12], and tumor micro-angiogenesis [13].

Studies on the role of FLI-1 expression in NPC are rare. FLI-1 was found to be over-expressed in the metastatic NPC cell line, the 5-8F cell line, in the research by Yang et al [14]. However, little is known about the FLI-1 expression and prognostication of NPC patients. Therefore, this study aims to detect FLI-1 expression in NPC tissue samples by immunohistochemistry (IHC), analyze the associations between FLI-1 expression and clinicopathological characteristics, and evaluate the prognostic value of FLI-1 for NPC patients.

Materials and Methods

Patients Selection and Samples Collection

This study was approved by the Clinical Ethics Review Board of Sun Yat-sen University Cancer Center. All the patients signed informed consent documents before participating in the study. Patients were recruited according to the following criteria: histologically diagnosed NPC with available biopsy sample; newly proven and non-metastatic NPC; no other malignancy or prior anti-cancer treatment; continuously finished at least radiotherapy at the Cancer Centre of Sun Yat-sen University with complete and detailed medical records and regular follow-ups. A total of consecutive 198 patients were eligible, who were diagnosed between May 2005 and December 2006. Medical files were reviewed retrospectively and patients were restaged based on the American Joint Committee on Cancer (AJCC) staging system 2010 clinical classification (the seventh edition). All 198 patients were histologically diagnosed with differentiated non-keratinized carcinoma or undifferentiated non-keratinized carcinoma. The tumor specimens were obtained by biting biopsy from primary NPC, prior to treatment, and processed through formalin fixation for at least 8 hours and paraffin embedment.

Pretreatment Evaluation

Patients underwent a routine pretreatment evaluation including history, physical examination of the head and neck, optic fiber nasopharyngoscopy, nasopharynx and neck magnetic resonance imaging (MRI), chest X-ray, the abdominal ultrasonography, bone scanning, a complete blood count and biochemical profile. The serological titer of Epstein-Barr virus immunoglobulin A antibodies against viral capsid antigen (EBV VCA-IgA) was measured using an immunoenzymic assay. The serum titer of Epstein-Barr virus immunoglobulin A antibodies against early antigen (EBV EA-IgA) was further measured using an immunoenzymic assay by Raji cell line. The serologic antienzyme rate of

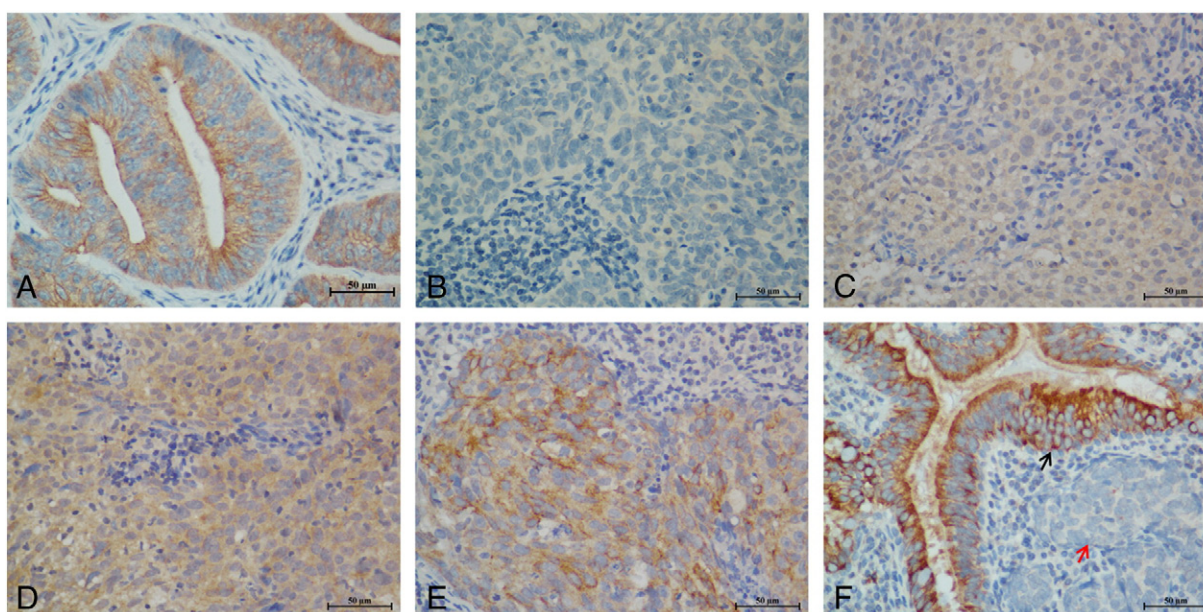


Figure 1. Immunohistochemical (IHC) staining for FLI-1 expression in nasopharyngeal carcinoma (NPC). Positive FLI-1 expression was mainly observed in the cytoplasm of NPC cells. (A) Positive control for FLI-1 IHC staining in rectal cancer (SP×400); (B) Negative expression (SP×400); (C) low expression (SP×400); (D) moderate expression (SP×400); (E) high expression (SP×400); (F) the adenoid-like differentiated tumors highly expressed FLI-1 (black arrows), with negative expression in the carcinoma nests (red arrows) (SP×400).

Table 1. The clinicopathological characteristics of nasopharyngeal carcinoma patients in the training and testing sets and their correlations between FLI-1 expressions

Characteristics	No. of patients (n=198)	The training set			The testing set		
		Positive, n (%) (n=33)	Negative, n (%) (n=66)	<i>P</i>	Positive, n (%) (n=33)	Negative, n (%) (n=66)	<i>P</i>
Gender							
Male	148	26 (17.6)	44 (29.7)	0.212	27(18.2)	51(34.5)	0.602
Female	50	7 (14.0)	22 (44.0)		6(12.0)	15(30.0)	
Age (years)							
≤50	130	26 (20.0)	44 (33.8)	0.212	20(15.4)	40(30.8)	1.000
>50	68	7 (10.3)	22(32.4)		13(19.1)	26(38.2)	
T classification ^a							
T ₁₋₂	98	14 (14.3)	33 (33.7)	0.477	13(13.3)	38(38.8)	0.088
T ₃₋₄	100	19 (19.0)	33 (33.0)		20(20.0)	28(28.0)	
N classification ^a							
N ₀₋₁	125	13 (10.4)	49 (39.2)	0.001	15(12.0)	48(38.4)	0.008
N ₂₋₃	73	20 (27.4)	17 (23.3)		18(24.7)	18(24.7)	
Clinical stage ^a							
I-II	64	8 (12.5)	22 (34.4)	0.353	9(14.1)	25(39.1)	0.295
III-IVb	134	25 (18.7)	44 (32.8)		24(17.9)	41(30.6)	
Histological type							
U	189	32 (16.9)	63(33.3)	1.000 ^c	30(15.9)	64(33.9)	0.417 ^c
D	9	1 (11.1)	3 (33.3)		3(33.3)	2(22.2)	
EBV VCA-IgA titre							
<1:480	131	22 (16.8)	42 (32.1)	0.915	19(14.5)	48(36.6)	0.059
≥1:480	60	11 (18.3)	20 (33.3)		14(23.3)	15(25.0)	
EBV EA-IgA titre							
<1:30	96	18 (18.8)	30 (31.3)	0.568	12(12.5)	36(37.5)	0.053
≥1:30	95	15 (15.8)	32 (33.7)		21(22.1)	27(28.4)	
AER							
≤49%	94	15 (16.0)	25 (26.6)	0.205	17(18.1)	37(39.4)	0.639
>49%	81	12 (14.8)	36 (44.4)		12(14.8)	21(25.9)	
Diameters of lymph node ^b							
< 2.0cm	91	16 (17.6)	29 (31.9)	0.669	11(12.1)	35(38.5)	0.064
≥ 2.0cm	107	17 (15.9)	37 (34.6)		22(20.6)	31(29.0)	
Lymph node extracapsular spread							
Yes	46	9(19.6)	14(30.4)	0.501	9(19.6)	14(30.4)	0.501
No	152	24(15.8)	52(34.2)		24(15.8)	52(34.2)	
Chemotherapy							
Yes	138	22(15.9)	49(35.5)	0.430	27(19.6)	40(29.0)	0.033
No	60	11(18.3)	17(28.3)		6(10.0)	26(43.3)	
Locoregional failure							
Yes	32	9 (28.1)	8 (25.0)	0.060	6(18.8)	9(28.1)	0.552
No	166	24 (14.5)	58 (34.9)		27(16.3)	57(34.3)	
Distant metastasis							
Yes	55	16 (29.1)	14 (25.5)	0.005	15(27.3)	10(18.2)	0.001
No	143	17 (11.9)	52 (36.4)		18(12.6)	56(39.2)	
Death							
Yes	60	18 (30.0)	17 (28.3)	0.005	13(21.7)	12(20.0)	0.022
No	138	15 (10.5)	49 (34.3)		20(14.0)	54(37.8)	

^a the American Joint Committee on Cancer (AJCC) staging system 2010 clinical classification; ^b minimal axial diameter of lymph node based on magnetic resonance imaging; ^c continuity correction; AER denotes serologic antienzyme rate of Epstein-Barr virus DNase-specific neutralizing antibody; EBV VCA-IgA titer denotes the serological titer of Epstein-Barr virus immunoglobulin A antibodies against viral capsid antigen; EBV EA-IgA titer denotes the serum titer of Epstein-Barr virus immunoglobulin A antibodies against early antigen; U, undifferentiated non-keratinized carcinoma; D, differentiated non-keratinized carcinoma.

Epstein-Barr virus DNase-specific neutralizing antibody (AER) was tested by an automatic liquid scintillation counter, using the mixture of patient sera and the lysate of an EBV-infected Raji cell line which was previously treated with croton oil and n-butanoic acid to induce DNase antigen production.

Treatment

All patients received continuous definitive radiotherapy: 177/198 (89.4%) patients were treated with two-dimensional radiotherapy (2D-RT) and 21/198 (10.6%) patients received intensity-modulated radiotherapy (IMRT). Total dose delivered to the primary tumor site was 64-80 Gy, with a mean of 70.85 Gy (standard deviation, SD: ±4.27 Gy) and a median of 70 Gy respectively. Dose for positive lymph node was 56-70 Gy, with a mean of 63.87 Gy (SD: ±3.93 Gy) and a median of 64 Gy respectively. The prophylactic dose was 50-56 Gy.

One hundred and thirty-eight patients received platinum-based chemotherapy: 72 patients received neoadjuvant chemotherapy, 100 patients received concurrent chemotherapy and 11 patients received adjuvant chemotherapy. The institutional guidelines for NPC during this research period recommended no chemotherapy for T₁₋₂N₀M₀ (the AJCC staging system 2002 clinical classification, the sixth edition) patients, whose diseases were classified as stage I and stage II with no enlarged lymph nodes. However, concurrent chemoradiotherapy was required for stage II disease with positive lymph nodes and concurrent chemoradiotherapy with or without neoadjuvant/adjuvant chemotherapy was necessary for stage III to IV_{a-b} patients. Neoadjuvant chemotherapy consisted of two cycles of cisplatin (80 mg/m²) by intravenous drip and 5-fluorouracil (5-Fu) (4 g/m²) by continuous intravenous infusion for 120 hours every three weeks. Concurrent chemotherapy consisted of two to three cycles of cisplatin (80 mg/m²) by intravenous drip on weeks 1, 4 and/or 7 during

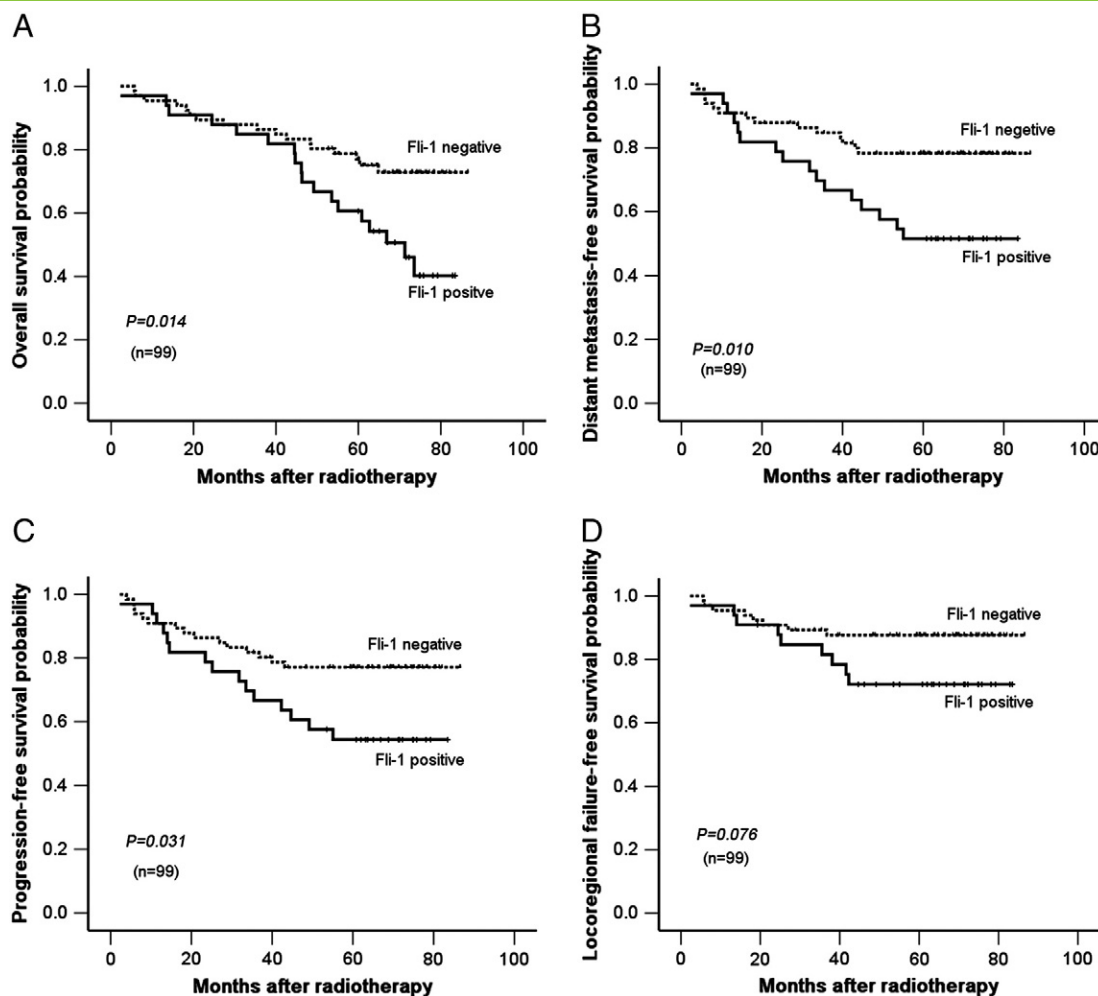


Figure 2. Survival curves of nasopharyngeal carcinoma patients in the training set stratified by FLI-1 expression. FLI-1 was associated with prognoses. (A) Overall survival curves of patients with negative and positive FLI-1 expression; (B) Distant metastasis-free survival curves of patients with negative and positive FLI-1 expression; (C) Progression-free survival curves of patients with negative and positive FLI-1 expression; (D) Locoregional failure-free survival curves of patients with negative and positive FLI-1 expression.

radiotherapy. Adjuvant chemotherapy consisted of cisplatin (80 mg/m^2) by intravenous drip and 5-Fu (4 g/m^2) continuous intravenous infusion for 120 hours every four weeks.

Follow-up

Patients were advised to attend follow-up visit every three months for the first three years, every six months for the fourth and fifth years, and every year thereafter. The primary end point was overall survival (OS), and the secondary end points were distant metastasis-free survival (DMFS), progression-free survival (PFS) and locoregional failure-free survival (LRFS). The up-mentioned end points were defined as followed: OS, the time from finishing radiotherapy to the date of death or the latest visit date if patients were still alive; DMFS, the time from finishing radiotherapy to the date of distant metastasis or the latest visit date when censored; LRFS, the time from finishing radiotherapy to the date of failure in nasopharynx and/or cervical lymph nodes or the latest visit date when censored; PFS, the time from finishing radiotherapy to the date of relapse at any site or the latest visit date when censored.

Immunohistochemistry (IHC)

The paraffin-embedded tumor tissues were cut into $4\mu\text{m}$ slices, dried at 60°C for at least six hours, deparaffinized in xylene for four times, rehydrated in graded alcohols, microwaved in EDTA buffer solution to retrieve tissue antigen, and incubated in 3% H_2O_2 solution for ten minutes to eliminate endogenous peroxidase activity. The slices were incubated in anti-FLI-1 rabbit polyclonal antibody (1:100 dilution, SC-356, SANTA CRUZ BIOTECHNOLOGY, Inc.) at 37°C for 60 minutes and next in anti-rabbit secondary immunoglobulin G antibody solution labeled by horse radish peroxidase (DAKO, Denmark) at 37°C for 30 minutes in the same humidified chamber. Staining was visualized using diaminobenzidine (DAKO, Denmark) staining, followed by hematoxylin nuclear counterstaining. Finally, the slices were dehydrated by graded alcohols and mounted by neutral transparent gum. Negative controls were performed by omitting the primary antibody. Positive controls were done in rectal cancer sections (Figure 1A).

Histological and IHC staining were evaluated by two independent pathologists who were blind to clinicopathological and survival data of the patients. Any different evaluation was discussed until a consensus

Table 2. Univariate analysis with the Cox proportional hazards model for OS, DMFS, PFS and LRFS of nasopharyngeal carcinoma patients in the training set (n=99)

Prognostic factors	P	HR	95% CI for HR	
			Lower	Upper
OS				
Age (years) (>50 vs.≤50)	0.636	1.188	0.582	2.427
Gender (female vs. male)	0.299	0.658	0.299	1.449
Pathology (U vs. D)	0.112	1.878	0.864	4.082
T stage (T ₃₋₄ vs. T ₁₋₂)	0.078	1.874	0.932	3.766
N stage (N ₂₋₃ vs. N ₀₋₁)	<0.001	5.571	2.669	11.626
Clinical stage (III-IVb vs. I-II)	0.004	5.731	1.754	18.724
Fli-1 (positive vs. negative)	0.017	2.240	1.154	4.348
AER (>49% vs. ≤49%)	0.105	1.876	0.876	4.013
Chemotherapy (yes vs. no)	0.178	1.768	0.772	4.048
DMFS				
Age (years) (>50 vs.≤50)	0.806	0.904	0.402	2.030
Gender (female vs. male)	0.032	0.316	0.110	0.905
Pathology (U vs. D)	0.182	1.813	0.757	4.343
T stage (T ₃₋₄ vs. T ₁₋₂)	0.284	1.491	0.718	3.095
N stage (N ₂₋₃ vs. N ₀₋₁)	<0.001	4.343	2.025	9.314
Clinical stage (III-IVb vs. I-II)	0.043	2.687	1.031	7.001
Fli-1 (positive vs. negative)	0.013	2.481	1.210	5.087
AER (>49% vs. ≤49%)	0.660	1.200	0.533	2.703
Chemotherapy (yes vs. no)	0.095	2.259	0.867	5.885
PFS				
Age (years) (>50 vs.≤50)	0.805	0.903	0.402	2.028
Gender (female vs. male)	0.015	0.228	0.069	0.752
Pathology (U vs. D)	0.185	1.811	0.753	4.352
T stage (T ₃₋₄ vs. T ₁₋₂)	0.036	2.310	1.058	5.044
N stage (N ₂₋₃ vs. N ₀₋₁)	<0.001	4.968	2.267	10.883
Clinical stage (III-IVb vs. I-II)	0.012	4.638	1.406	15.295
Fli-1 (positive vs. negative)	0.035	2.157	1.054	4.416
AER (>49% vs. ≤49%)	0.409	1.417	0.620	3.239
Chemotherapy (yes vs. no)	0.115	2.166	0.829	5.660
LRFS				
Age (years) (>50 vs.≤50)	0.896	1.072	0.377	3.043
Gender (female vs. male)	0.054	0.137	0.018	1.034
Pathology (U vs. D)	0.835	1.187	0.237	5.943
T stage (T ₃₋₄ vs. T ₁₋₂)	0.052	3.039	0.991	9.321
N stage (N ₂₋₃ vs. N ₀₋₁)	0.132	2.080	0.802	5.394
Clinical stage (III-IVb vs. I-II)	0.044	3.476	1.037	11.658
Fli-1 (positive vs. negative)	0.084	2.313	0.892	5.996
AER (>49% vs. ≤49%)	0.986	0.991	0.359	2.734
Chemotherapy (yes vs. no)	0.068	3.085	0.920	10.347

Abbreviations: OS, overall survival; DMFS, distant metastasis-free survival; PFS, progression-free survival; LRFS, locoregional failure-free survival; HR, hazard ratio; CI, confidence interval; U, undifferentiated non-keratinized carcinoma; D, differentiated non-keratinized carcinoma; vs., versus. AER denotes serologic antienzyme rate of Epstein-Barr virus DNase-specific neutralizing antibody.

was reached. Each slice was observed in its entirety in a light microscope (original magnification was 400 multiples). FLI-1 IHC staining was evaluated using a semiquantitative scoring system incorporating the percentage of positively stained cancer cells and the staining intensity. The criteria were detailed as followed: 0% (0), 1%-25% (1), 26%~50% (2), 51%-75% (3), 76%-100% (4); no staining (0), light yellow weak staining (1), yellow brown moderate staining (2), brown strong staining (4). The synthesizing evaluation score ranged from 0 to 7. Tumors with scores ≥ 4 were defined as high FLI-1 expression, tumors with scores = 3 were considered as moderate FLI-1 expression, tumors with scores ≤ 2 were designated as low FLI-1 expression and tumors with scores = 0 were regarded as negative FLI-1 expression.

Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences, version 13.0 (SPSS, Chicago, IL, USA) and two-tailed P values < 0.05 were considered statistically significant. A random number table was generated for assigning patients to either the training

set or the testing set. The Pearson chi-square test of independence was used to analyze the associations between clinicopathological characteristics and FLI-1 IHC expression. Differences of actuarial survival rates were determined by the Kaplan-Meier method and the log-rank test. The life-tables method was employed to calculate cumulative survival rates. Univariate and multivariate analysis were both performed using the Cox proportional hazards model (enter method) to test independent prognostic factors and calculate the hazard ratio (HR) and 95% confidence interval (CI) as well. Ninety-nine patients were randomly assigned to the training set, which was used to analyze prognostic factors and establish a prognostic model. The remaining patients were assigned to the testing set for validation.

Results

Patient Clinical Characteristics and Follow-up Outcomes

The follow-up visit data were updated in July 2012, and the range was between 2.3 and 86.6 months with a median of 62.8 months. For all the patients, the median age was 46 years (range: 16-75 years). A summary of clinical characteristics was presented in Table 1.

Sixty patients died during follow-up, with a median time to death of 38.2 months (range: 2.3-73.5 months). The 3, 5 and 7 year OS for all the patients were 78%, 67% and 65%, respectively. Fifty-five patients developed distant metastasis, with a median time of 22.3 months (range: 5.1-56.4 months). The 3, 5 and 7 year DMFS for the whole patients were 73%, 71% and 71%, respectively. Thirty-two patients experienced nasopharyngeal and/or cervical lymph nodes failure, with a median time to failure of 23.6 months (range: 2.3-64.9 months). The 3, 5 and 7 year LRFS for all the patients were 85%, 82% and 80%, respectively.

Immunohistochemical Expression of FLI-1 in NPC

Positive FLI-1 expression was mainly localized in the cytoplasm of NPC cells in sixty-six patients (33.3%): seventeen patients (8.6%) with high expression, twenty patients (10.1%) with moderate expression and twenty-nine (14.6%) patients with low expression. Negative FLI-1 expression was observed in 66.7% (132/198) of the tumors. Representative images of FLI-1 IHC staining in NPC tissues are shown in Figure 1B-E. The adenoid-like differentiated tumors, which constituted small portion of differentiated or undifferentiated non-keratinized carcinoma, highly expressed FLI-1 (5/198, 2.5%), as shown in Figure 1F.

Correlation between FLI-1 Expression and Clinicopathological Characteristics

The impact of FLI-1 expression levels on OS was analyzed using the Kaplan-Meier method and the log-rank test to identify that positive FLI-1 expression was predictive of poor OS (Figure 2A). So the status of positive or negative expression was chosen for the subsequent binary variable analysis. In the training set, gender, age, T classification, clinical stage, histological type, EBV EA-IgA titre, EBV VCA-IgA titre, AER, axial diameter of lymph node (<2.0cm versus ≥ 2.0cm), lymph node extracapsular spread, chemotherapy, or locoregional failure was not associated with FLI-1 expression. However, positive FLI-1 expression correlated with advanced N classification (P=0.001), metastasis (P=0.005) and death (P=0.005). A similar association was verified in the testing set except for chemotherapy (Table 1).

FLI-1 Expression in Predicting the Survival of NPC

In the training set, the 6-year OS rates for the positive FLI-1 expression group and the negative FLI-1 expression group were 37% and

Table 3. Multivariate analysis with the Cox proportional hazards model for OS and DMFS of nasopharyngeal carcinoma patients in the training set (n=99)

Prognostic factors	P	HR	95% CI for HR		Prognostic factors	P	HR	95% CI for HR	
			Lower	Upper				Lower	Upper
I OS					I DMFS				
Age (years) (>50 vs. ≤50)	0.468	1.351	0.599	3.051	Age (years) (>50 vs. ≤50)	0.883	1.070	0.432	2.651
Gender (female vs. male)	0.499	0.747	0.321	1.739	Gender (female vs. male)	0.054	0.297	0.086	1.020
Pathology (U vs. D)	0.139	1.929	0.808	4.605	Pathology (U vs. D)	0.401	1.509	0.578	3.941
T stage (T ₃₋₄ vs. T ₁₋₂)	0.144	1.686	0.837	3.395	T stage (T ₃₋₄ vs. T ₁₋₂)	0.370	1.408	0.667	2.974
N stage (N ₂₋₃ vs. N ₀₋₁)	0.011	2.389	1.221	4.671	N stage (N ₂₋₃ vs. N ₀₋₁)	0.008	3.054	1.341	6.955
Clinical stage (III-IVb vs. I-II)	0.045	5.545	1.041	29.541	Clinical stage (III-IVb vs. I-II)	0.069	2.569	0.928	7.111
FlI-1 (positive vs. negative)	0.031	2.076	1.068	4.037	FlI-1 (positive vs. negative)	0.042	2.445	1.032	5.793
AER (>49% vs. ≤49%)	0.082	2.175	0.906	5.220	AER (>49% vs. ≤49%)	0.233	1.745	0.698	4.362
Chemotherapy (yes vs. no)	0.907	0.941	0.339	2.609	Chemotherapy (yes vs. no)	0.186	2.261	0.675	7.580
II OS					II DMFS				
Age (years) (>50 vs. ≤50)	0.377	1.432	0.646	3.173	Age (years) (>50 vs. ≤50)	0.823	1.108	0.452	2.713
Gender (female vs. male)	0.414	0.707	0.308	1.624	Gender (female vs. male)	0.048	0.290	0.085	0.991
Pathology (U vs. D)	0.166	1.825	0.780	4.272	Pathology (U vs. D)	0.420	1.463	0.580	3.691
Clinical stage (III-IVb vs. I-II)	0.014	5.074	1.386	18.572	Clinical stage (III-IVb vs. I-II)	0.035	2.820	1.074	7.401
FlI-1 (positive vs. negative)	0.039	2.017	1.037	3.922	FlI-1 (positive vs. negative)	0.045	2.337	1.020	5.355
AER (>49% vs. ≤49%)	0.072	2.155	0.933	4.976	AER (>49% vs. ≤49%)	0.233	1.696	0.712	4.039
Chemotherapy (yes vs. no)	0.990	0.994	0.370	2.668	Chemotherapy (yes vs. no)	0.188	2.236	0.675	7.410

Abbreviations: OS, overall survival; DMFS, distant metastasis-free survival; HR, hazard ratio; CI, confidence interval; U, undifferentiated non-keratinized carcinoma; D, differentiated non-keratinized carcinoma; vs., versus. AER denotes serologic antigenzyme rate of Epstein-Barr virus DNase-specific neutralizing antibody.

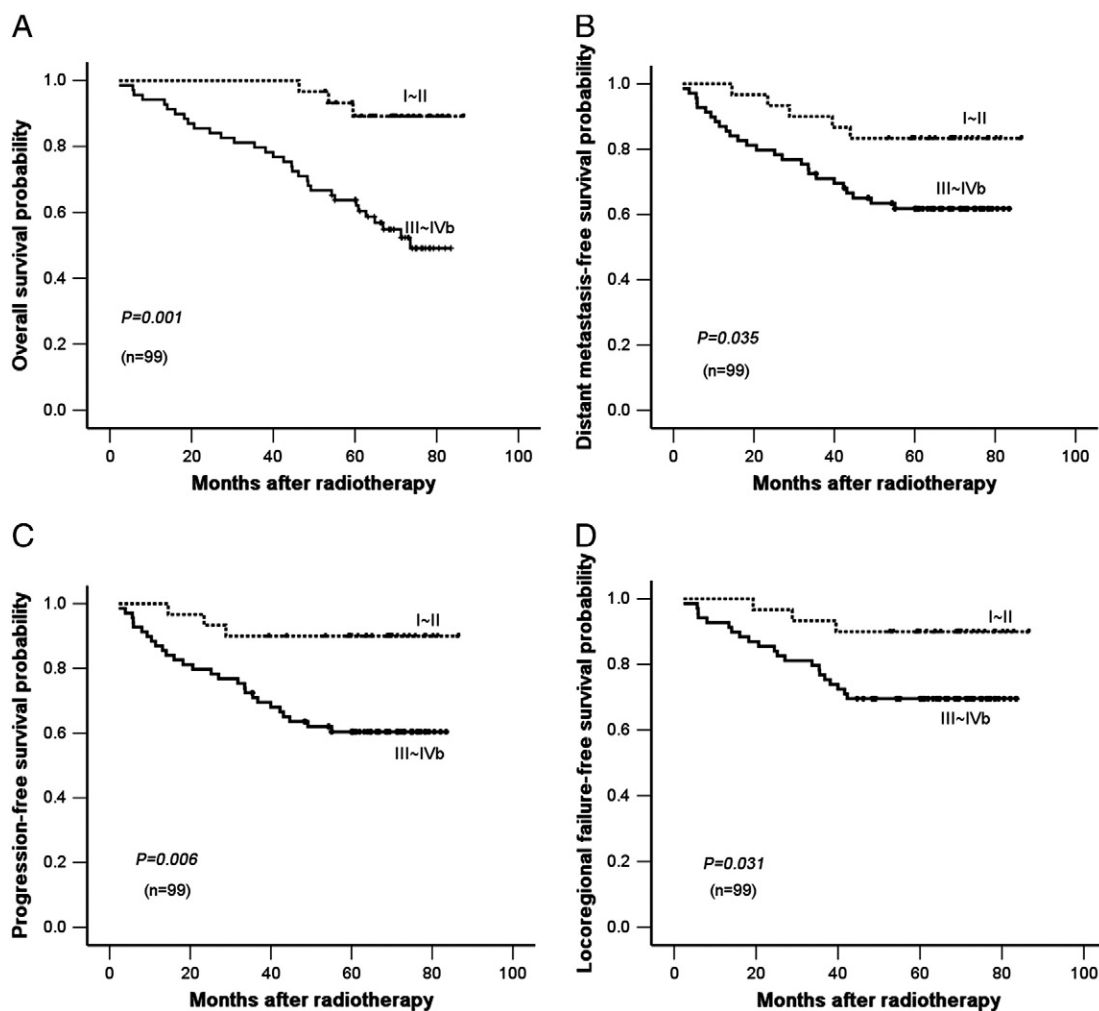


Figure 3. Survival curves of nasopharyngeal carcinoma patients in the training set stratified by clinical stage. All survival rates were well separated by clinical stage. (A) Overall survival curves of patients with I~II stage and III~IVb stage; (B) Distant metastasis-free survival curves of patients with I~II stage and III~IVb stage; (C) Progression-free survival curves of patients with I~II stage and III~IVb stage; (D) Locoregional failure-free survival curves of patients with I~II stage and III~IVb stage.

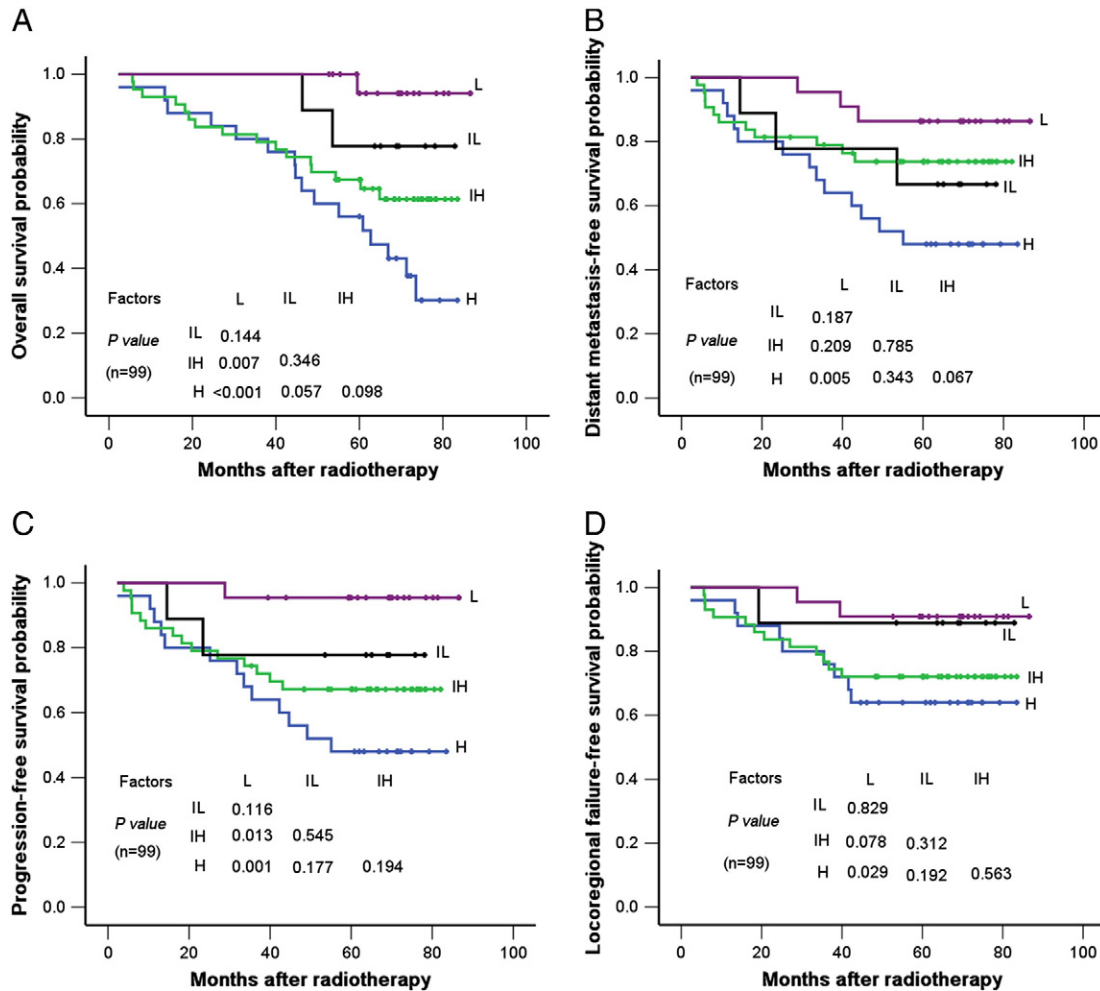


Figure 4. Survival curves of nasopharyngeal carcinoma patients in the training set stratified by supplementing FLI-1 with clinical stage. The prognoses of patients in the training set were clearly discriminated by FLI-1 expression and clinical stage. (A) Overall survival curves for the L, IL, IH and H subgroups; (B) Distant metastasis-free survival curves for the L, IL, IH and H subgroups; (C) Progression-free survival curves for the L, IL, IH and H subgroups; (D) Locoregional failure-free survival curves for the L, IL, IH and H subgroups. Abbreviation: L, low risk; IL, intermediate-low risk; IH, intermediate-high risk; H, high risk.

72% ($P=0.014$), respectively. The 6-year DMFS rates for the positive FLI-1 expression group and the negative FLI-1 expression group were 52% and 78% ($P=0.010$), respectively. The 6-year PFS rates for the positive FLI-1 expression group and the negative FLI-1 expression group were 54% and 77% ($P=0.031$), respectively. However, no significant differences in the 6-year LRFS rates were indicated, with 72% and 88% ($P=0.076$) for the positive and negative FLI-1-expression groups, respectively. The survival curves were shown in Figure 2A-D.

Univariate and Multivariate Analysis with the COX Proportional Hazards Model

Univariate analyses was performed by the COX proportional hazards model to test if gender, age, pathological type, T stage, N stage, clinical stage, AER, chemotherapy or FLI-1 expression was associated with OS, DMFS, PFS or LRFS in the training set. The results showed that N stage, clinical stage and FLI-1 expression were prognostic factors for OS, DMFS and PFS. Gender was a prognostic factor for both DMFS and PFS. T stage, which had a borderline significance in LRFS, was significantly associated with PFS. Advanced clinical stage was also associated with poor LRFS (Table 2).

In the training set, multivariate analyses was performed by the COX proportional hazards model to determine the independent prognostic factors of NPC, including all the factors analyzed in the univariate analysis. The results indicated that N stage, clinical stage and FLI-1 expression were independently significant for OS. N stage and FLI-1 expression were independent predictors for DMFS. Further COX proportional hazards model analysis was required because of the interactive effects between clinical stage and T/N stage, which included clinical stage and the rest clinical characteristics except T stage and N stage. The results showed that both clinical stage and FLI-1 expression were independent predictors for both OS and DMFS (Table 3).

FLI-1 Expression Discriminates the Prognoses of a Subgroup with Similar Clinical Stage

Patients were divided into two groups according to clinical stage (I-II versus III-IVb). Survival analysis was performed to the training set, with the result indicating that clinical stage distinguished all survival curves well (Figure 3A-D). Patients in the training set were further stratified based on FLI-1 expression. Survival analysis with Kaplan-Meier method and log-rank test showed that the prognoses of

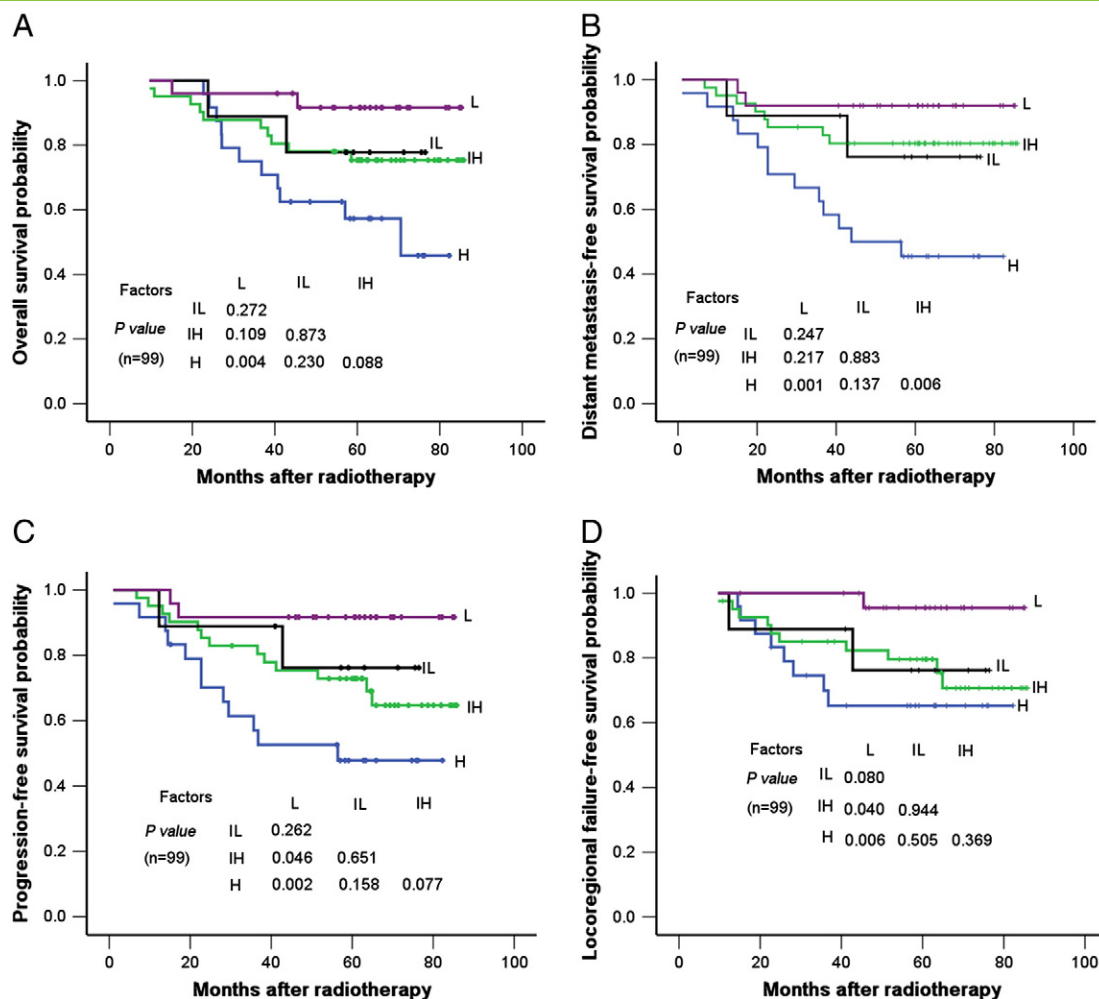


Figure 5. Survival curves of nasopharyngeal carcinoma patients in the testing set stratified by supplementing FLI-1 with clinical stage. The prognoses of the patients in the testing set were separated by FLI-1 expression and clinical stage. (A) Overall survival curves for the L, IL, IH and H subgroups; (B) Distant metastasis-free survival curves for the L, IL, IH and H subgroups; (C) Progression-free survival curves for the L, IL, IH and H subgroups; (D) Locoregional failure-free survival curves for the L, IL, IH and H subgroups. Abbreviation: L, low risk; IL, intermediate-low risk; IH, intermediate-high risk; H, high risk.

NPC were further discriminated by FLI-1 expression (Figure 4A-D). There were four subgroups: low risk (L), with I-II stage and negative FLI-1 expression; intermediate-low risk (IL), with I-II stage and positive FLI-1 expression; intermediate-high risk (IH), with III-IVb stage and negative FLI-1 expression; high risk (H), with III-IVb stage and positive FLI-1 expression. Similar results were obtained both in the testing set (Figure 5A-D) and in the whole patients (Figure 6A-D). These results conformed that supplementing FLI-1 with clinical stage led to more accurate prognostication of NPC.

Discussion

In this study, we observed that cytoplasmic positive expression of FLI-1 correlated significantly with advanced N classification and survival of NPC patients. In addition, OS and DMFS of NPC patients with positive FLI-1 expression were significantly poorer than those with negative FLI-1 expression in the multivariate analysis. Incorporating the clinical stage and FLI-1 expression, by which NPC patients were classified into four risk subgroups, was more effective and accurate in predicting prognosis for NPC than clinical stage alone, especially for patients with III-IVb stage diseases. Thus, FLI-1 has potential as a biomarker to facilitate individualized treatment of NPC. To our

knowledge, we were the first to evaluate FLI-1 expression in NPC tissue and analyze the associations between FLI-1 expression and clinicopathological characteristics and prognosis.

Various benign and malignant neoplasms, especially Ewing's sarcoma/primitive neuroectodermal tumor (EWS/PNET) [11], positively expressed FLI-1, a proto-oncogene, which was negatively expressed in most normal tissues except lymph node, spleen and blood vessel endothelium. FLI-1 is still considered as a sensitive and specific biomarker for diagnosing EWS/PNET currently. This study indicated that FLI-1 protein was localized in the cytoplasm of NPC cells, consistent with the study by Shintani et al [12], who observed cytoplasmic FLI-1 expression in the oral squamous cell cancer (OSCC). In our study, the incidence of positive FLI-1 expression was 33.3% (66/198), higher than previously reported 5.3% (27/508) in the total squamous cell carcinoma [11], but lower than 53.8% (14/26) in OSCC [12]. NPC is a kind of malignant tumor originating from nasopharyngeal mucosa stratified squamous epithelium. All patients in this study were diagnosed as undifferentiated non-keratinized carcinoma (189/198) or differentiated non-keratinized carcinoma (9/198), but in NPC tissue specimens, small portion of adenoid-like differentiated tumor was occasionally observed (5/198). In addition, all the adenoid-

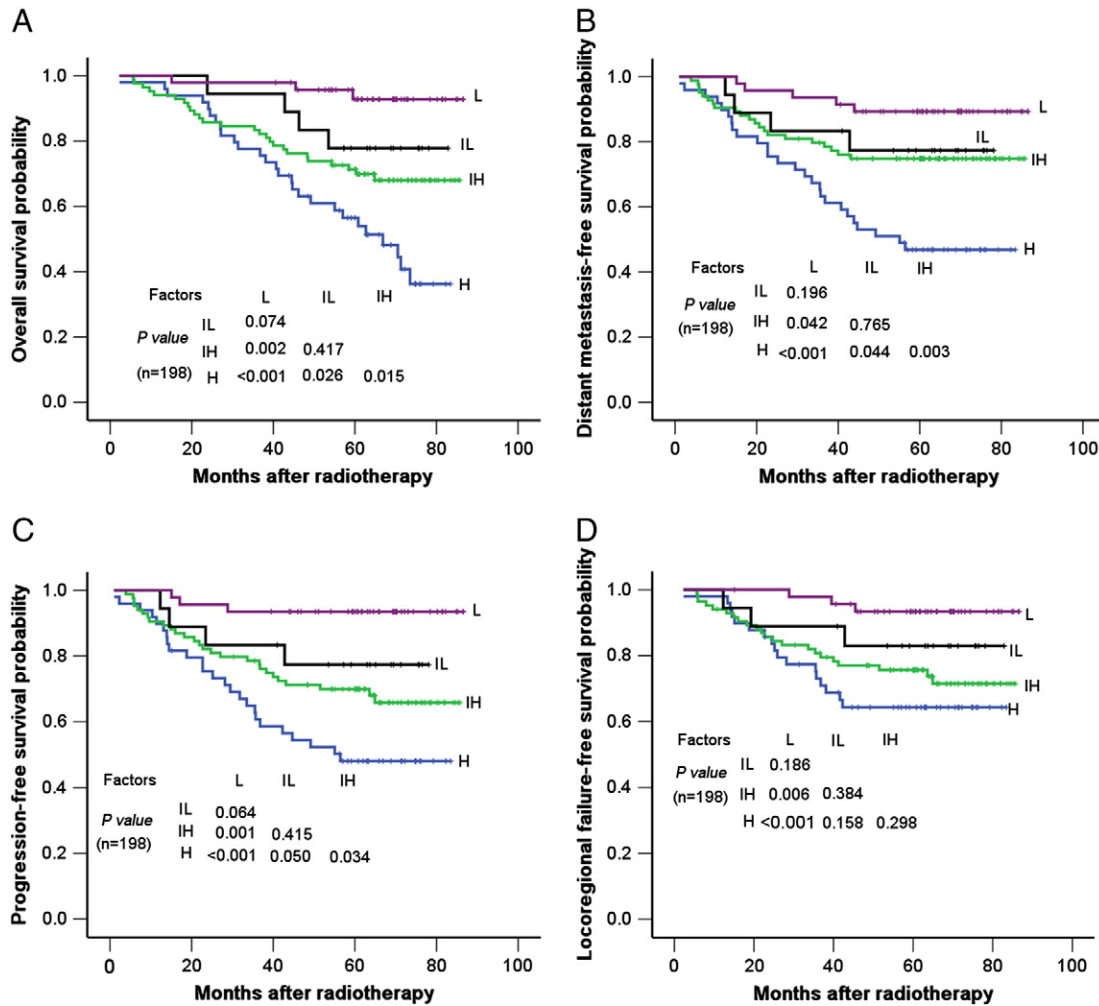


Figure 6. Survival curves of all nasopharyngeal carcinoma patients stratified by supplementing FLI-1 with clinical stage. The prognoses of the 198 patients were clearly discriminated by FLI-1 expression and clinical stage. (A) Overall survival curves for the L, IL, IH and H subgroups; (B) Distant metastasis-free survival curves for the L, IL, IH and H subgroups; (C) Progression-free survival curves for the L, IL, IH and H subgroups; (D) Locoregional failure-free survival curves for the L, IL, IH and H subgroups. Abbreviation: L, low risk; IL, intermediate-low risk; IH, intermediate-high risk; H, high risk.

like differentiated portion of NPC highly expressed FLI-1 protein, with negative expression in the peripheral carcinoma nests, which was similar to the previous result that adenocarcinoma strongly expressed FLI-1 [11]. These findings suggested that FLI-1 might play an important but unclear role in the development and progression of NPC. FLI-1 expression correlated with advanced N classification and metastasis. Patients with FLI-1 positive expression tended to have lower or bilateral neck lymph node metastasis or large lymph nodes, and were likely to be afflicted by distant metastasis after definitive radiotherapy. These results suggested that cancer cells might have acquired the capacity of proliferating faster and higher malignancy degree when FLI-1 was positively expressed. Our findings were previously confirmed in melanoma and a NPC metastatic cell line, respectively. Torlakovic et al found that FLI-1 expression was detected in all melanoma cell lines and higher in metastatic tumors than in the primary ones. FLI-1 expression also positively correlated with Ki-67 expression and the presence of an ulcer in the primary tumor, which were both the independent adverse prognostic factors for melanoma [8]. Yang et al discovered that FLI-1 were differentially expressed in the metastatic 5-8F and non-metastatic 6-10B NPC cell lines, and confirmed positive expression of FLI-1 in 5-8F cells through subtractive suppression

hybridization, reverse Northern blotting and cDNA fragments analysis [14]. These up-mentioned studies hinted FLI-1 might be involved in the tumor progression and metastasis.

One of the leading causes of tumor-related death is distant metastasis, which is closely associated with abnormal angiogenesis, hypoxia and radioresistance [15]. ETS family plays a key role in the endothelial-specific gene expression regulation, as its family members have binding sites in many known endothelial-specific enhancers, including the endothelial enhancers in the human genome [16]. The expression of FLI-1 has been detected in the hematopoietic cells and endothelial cells at the very early development stage. FLI-1 binds to specific enhancers, activates endothelium-related gene expression and induces embryonic stem cells differentiate towards endothelial progenitor cells [17]. In our study, typical tumor angiogenesis and FLI-1 expression in the vascular endothelium were difficult to evaluate because of the insufficiency of biopsy NPC specimen on most tissue sections. However, the finding that FLI-1 was highly expressed in the adenoid-like differentiated NPC suggested that NPC cancer cell might had developed like adenoid-like endothelium through FLI-1 related gene expression. EWS/FLI-1 fusion gene promotes tumor angiogenesis through upregulating VEGF-A expression [13]. Disorganized angiogenesis exacerbates tumor hypoxia,

which mediates cancer cells invasion, metastasis [18] and resistance to radiotherapy and cytotoxic drugs [19]. In our study, FLI-1 expression was associated with poorer OS, DMFS and PFS; multivariate analysis further confirmed the independent prognostic value of FLI-1 in NPC in the training set.

Accurate prognostication is urgently needed for individualized treatment. The TNM staging system is the mainstay for survival prediction, although it can not always meticulously distinguish the risk. Several biomarkers have been recognized as valuable prognostic factors of NPC patients. For example, Zhou et al identified that baseline serum lactate dehydrogenase level was a reliable predictor of inferior survival and subsequent liver metastasis for locally advanced NPC patients [20]. In the study by Xu et al, supplementing pretreatment serologic antienzyme rate of Epstein-Barr virus DNase-specific neutralizing antibody with TNM staging system further accurately defined the risk of metastasis, local failure, progression and death in NPC patient subgroups [21]. Herein, FLI-1 expression segregated two distinguished subgroups within similar clinical stages in the training set, comparing the OS, DMFS, PFS and LRFs. The testing set was used to verify the accuracy of FLI-1 in risk grouping for OS, DMFS, PFS and LRFs. The disease progression and survival of NPC patients were also better predicted with FLI-1 and clinical classification in the testing set. The results were further validated in a set containing all the NPC patients. The findings suggested that FLI-1 expression, complementing clinical classification, had potential as a novel biomarker in prognostication of NPC.

Conclusions

In summary, our study revealed the expression of FLI-1 in NPC, and found that FLI-1 expression significantly correlated with advanced N classification, distant metastasis and death. Multivariate analysis showed that FLI-1 was also an independent prognosticator for poor OS and DMFS. Incorporation FLI-1 with clinical stage enabled accurate stratification of NPC patients into four subgroups with different risk levels of death, distant metastasis and progression in the training, testing and whole set. Before FLI-1 is eventually applied in clinical practice, the mechanism by which FLI-1 is involved in the carcinogenesis and progression of NPC should be clarified and all results need to be replicated in a different NPC population.

Authors' Contributions

Wuguo Deng and Fangyun Xie both designed the study and help to draft the manuscript. Xuexia Liang and Dingbo Shi carried out the immunohistochemical staining work and interpreted the data. Xuexia Liang analyzed the data and drafted the manuscript. Xuexia Liang, Yanping Mao, Jingping Yun, Puyun Ouyang and Zhen Su collected the data. Jia Fu and Jinghui Hou evaluated the immunohistochemical staining. All authors read and approved the final manuscript.

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