

Changes in c-Kit expression levels during the course of radiation therapy for nasopharyngeal carcinoma

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Received May 30, 2016; Accepted August 31, 2016

DOI: 10.3892/br.2016.757

Abstract. In the era of intensity-modulated radiotherapy, distant metastasis is currently the main cause of treatment failure for nasopharyngeal carcinoma (NPC). Additional therapeutic strategies are required to control the metastasis and improve survival. One strategy is targeted therapy, for example against c-Kit. In the current study, the frequency of c-Kit expression was determined immunohistochemically in 106 NPC patients. c-Kit expression changes during the course of radiation therapy were detected in 41 cases via weekly biopsy. Twelve cases (11.3%) had c-Kit expression scores of 3⁺ and 16 (15.1%) had scores of 2⁺. Thus, c-Kit overexpression (2⁺ or 3⁺) was observed in 28 (26.4%) patients. There were 35 (33.0%) and 43 (40.6%) patients with c-Kit expression scores of 1⁺ and 0, respectively. Furthermore, a trend of decreased c-Kit expression was observed after commencing radiotherapy according to the 41 NPC patients who were biopsied weekly. Therefore, c-Kit overexpression was identified to be common in NPC, and evaluating c-Kit as a therapeutic target for metastatic NPC via c-Kit overexpression subsequent to first line treatment may be of interest. To the best of our knowledge, the present study is the first to demonstrate a trend of decreased c-Kit expression during the course of radiotherapy.

Introduction

Nasopharyngeal carcinoma (NPC) has an incidence rate of 80,000 new cases per year worldwide, making up 0.7% of all cancers (1,2). However, NPC has a distinctive geographic

distribution, with a high prevalence in Southeast Asia, Northeast India, and North Africa, and in the Inuits of Canada and Alaska. The rates in Southeast China are particularly high (3), with the highest rate reported to be 35/100,000 in Guangdong province (4). At the time of diagnosis, >70% of NPC patients are at stage III and stage IV (5). Furthermore, ≤20% of newly diagnosed NPC patients exhibit occult distant metastases (6). Radiation therapy is the standard treatment option for NPC. With the advent of intensity-modulated radiotherapy (IMRT), local-regional control has been substantially improved and distant metastasis is currently the main cause of treatment failure (7). The 5-year distant metastasis rate has been reported to be 19% for all disease stages, and 25% for the stage III-IVB subgroup (8,9). Concurrent or adjuvant chemotherapy is administered to reduce the distant metastasis rate for locally advanced NPC patients. However, certain patients, particularly the elderly and those with poor performance status, cannot tolerate the toxicities. Additional therapeutic strategies are required to control the metastasis, and improve survival and the quality of life for patients with advanced NPC. One such strategy is targeted therapy.

Proto-oncogene c-Kit, also termed tyrosine-protein kinase it or CD117, is a protein encoded in humans by the KIT proto-oncogene receptor tyrosine kinase (*Kit*) gene (10). The c-Kit mediated signal transduction pathways appear to be involved in regulating cell differentiation and proliferation, and antibodies to c-Kit have been widely applied in immunohistochemistry to distinguish particular types of tumour, for example, malignant gastrointestinal stroma tumours (GISTs). c-Kit expression is of particular clinical interest, as it is one of the targets of the tyrosine kinase inhibitor, imatinib mesylate (termed Gleevec or Glivec). Imatinib has been shown to exert marked clinical activity in malignant GISTs containing a Kit gene mutation or c-Kit protein expression (11).

Studies concerning c-Kit expression in NPC are rare according to our search of the scientific literature from the NCBI PubMed database. To the best of our knowledge, whether c-Kit expression levels change during the course of radiation therapy has not yet been investigated. In the present study, the expression levels of c-Kit were investigated immunohistochemically in 106 NPC cases, and the changes of

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Key words: nasopharyngeal carcinoma, c-Kit, metastasis, targeted therapy, radiotherapy

c-Kit expression during the course of radiation therapy were evaluated in 41 of the 106 cases.

Materials and methods

Patients. The present study was approved by the Institutional Review Board of Zhejiang Cancer Hospital (Hangzhou, China). Between 2007 and 2009, a total of 106 pathologically confirmed NPC patients were treated in the same medical group of Zhejiang Cancer Hospital, including 73 males and 33 females, ranging in age from 17 to 75 years (mean age, 48 years; median age, 49 years). Ninety-seven patients received platinum-based concurrent chemoradiotherapy (38 cases with nedaplatin and 59 cases with cis-platinum) and nine cases received radiotherapy alone. Nasopharyngeal endoscopy was routinely performed at the time of diagnosis, and repeated weekly during the 6-7 week course of radiation therapy. In 41 out of the 106 NPC patients, biopsies were obtained weekly, until no tumour could be seen in the nasopharyngeal endoscopy examination (i.e., a complete response; CR). In total, 2-5 biopsies were performed during the course of radiotherapy for these 41 patients according to the status of tumour regression.

The stage of disease was determined according to the 7th edition of the Union for International Cancer Control and the American Joint Committee on Cancer staging system (12,13).

Clinical information was obtained from hospital records and included gender, age, pathological classification, disease stage, and concurrent chemoradiotherapy. Patient and tumour characteristics of the analysed cases are presented in Table I.

c-Kit staining. The tissue samples were fixed in 4% buffered formalin, dehydrated in graded alcohol, and fully embedded in paraffin. Subsequently, 4- μ m thick sections were sliced and deparaffinized in xylene at 60°C for 20 min, and hydrated through graded alcohol to distilled water. Enzymatic antigen retrieval was conducted in citric acid buffer solution (pH 6) for 20 min at 98°C. Endogenous peroxidase was blocked in 3% H₂O₂ in phosphate-buffered saline for 10 min. The slides were blocked in bovine serum (Sijiqing, Hangzhou, China), followed by incubation in rabbit anti-human c-Kit (cat. no. ab32363; Abcam, Cambridge, USA) diluted in 1:100 at 4°C overnight. After the sections were incubated in secondary antibody conjugated with AlexaFluor 488 (cat. no. A-11008; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 30 min at room temperature, the slides were finally counterstained with hematoxylin and mounted.

c-Kit scores. The c-Kit expression was scored using the scoring criterion as reported by Bar-Sela *et al* (14) with modifications as follows. All the slides were evaluated by investigators who were blinded to the patients' clinical data using an Eclipse E200 microscope (Nikon Corporation, Tokyo, Japan). The staining intensities were graded according to the percentage of stained tumour cells, using a scale of 0-3⁺. The positive staining appeared as cytoplasmic immunoreactivity with accentuation along the cell membranes. The score was based on a scale as follows: Positive staining of $\geq 50\%$ of the tumour cells was considered as 3⁺; positive staining of $\geq 10\%$ and $< 50\%$ of the

tumour cells was considered as 2⁺; 2⁺ and 3⁺ were considered to be overexpression. Positive staining of $< 10\%$ of the tumour cells was considered as 1⁺; No cellular staining was considered as 0; and 1⁺ and 0 were considered as low expression.

Follow-up. The patients were followed up every 3 months for the first 2 years, then every 6 months for a further 3 years and annually thereafter. Physical examination, including palpation of the neck, direct flexible fiberoptic nasopharyngoscopy, blood examination, magnetic resonance imaging scans of the nasopharynx and neck, chest radiography, and abdominal ultrasound were performed at each follow-up visit. Whole-body bone scans were obtained at least once per year.

Statistical analysis. The patients were divided into two groups; the overexpression group and low expression group, with respect to c-Kit expression. The Pearson χ^2 test was used to compare groups regarding categorical variables, such as gender, age, tumour stage, histology classification, and concurrent chemoradiotherapy (Table I). Progression-free survival was defined as the time from treatment to the time of disease progression. Overall survival was defined as the time from treatment to date of death from any cause. Progression-free survival, overall survival and the nasopharyngeal recurrence, regional recurrence, distant metastasis were analysed using the Kaplan-Meier curves. Data were analysed using SPSS 19.0 software (IBM SPSS, Armonk, NY, USA).

Results

Expression levels of c-Kit in NPC. In the current study, among the 106 analysed NPC cases, 12 (11.3%) had c-Kit expression scores of 3⁺, 16 (15.1%) had c-Kit expression scores of 2⁺, 35 (33.0%) had c-Kit expression scores of 1⁺. Negative c-Kit staining was observed in 43 cases (40.6%) of the analysed tumours. In total, c-Kit overexpression (2⁺ or 3⁺) was observed in 26.4% (28 of 106) of NPC cases, and low expression (1⁺ or 0) was found in 73.6% (78 of 106) of cases. As shown in Table I, no difference of c-Kit expression was identified among patients with different clinical staging. Examples of staining patterns for the different c-Kit expression levels are presented in Fig. 1.

Alteration of c-Kit expression during the course of radiotherapy in NPC. In the current study, weekly nasopharyngeal endoscopies were performed during the 6-7 week course of radiation therapy. In 41 out of the 106 NPC patients tumour biopsies were also performed weekly, until no tumour could be seen (complete response; CR) during nasopharyngeal endoscopy examination. Overall, 2-5 biopsies were made during the course of radiotherapy for these 41 patients according to the status of tumour regression. When c-Kit expression levels at different time points for the same patients were compared, a trend of decreased c-Kit expression was observed following the start of radiotherapy. For the five cases with 3⁺ c-Kit expression at diagnosis prior to radiotherapy, three exhibited reduced c-Kit expression (1⁺ and 0) in the second week of radiotherapy and, thereafter, one showed reduced c-Kit expression (2⁺) from the fourth week, and the other case showed stable 3⁺ c-Kit expression for the first 2 weeks of radiation and the tumour had disappeared under endoscopy in the third week. For the 8 cases

Table I. Tumour and patient characteristics (n=106).

Characteristics	Patients	c-Kit overexpression	c-Kit low expression	P-value
Gender				0.057
Male	73	15	58	
Female	33	13	20	
Histology	0.822			
Differentiated	64	16	48	
Non-differentiated	42	12	30	
T-stage				0.635
T1-T2	31	7	24	
T3-T4	75	21	54	
N-stage				0.378
N0-N1	55	17	38	
N2-N3	51	11	40	
M-stage				1.000
M0	102	27	75	
M1	4	1	3	
Stage at diagnosis				1.000
II-III	60	16	44	
IV	46	12	34	
Concurrent chemoradiotherapy				0.542
No CCRT	9	1	8	
Nedaplatin	38	11	27	
Cis-platinum	59	16	43	
Total	106	28	78	

The disease stage was determined according to the 7th edition of the Union for International Cancer Control and the American Joint Committee on Cancer staging system. T, primary tumour site; N, regional lymph node involvement; M, distant metastatic spread; CCRT, concurrent chemoradiotherapy.

with 2⁺ c-Kit expression at diagnosis prior to radiotherapy, all showed reduced c-Kit expression following radiotherapy (five cases exhibited reduced c-Kit expression from the second week of the course of radiotherapy, and the other three cases exhibited reduced c-Kit expression from the third week). Seven of the 10 cases (70.0%) with 1⁺ c-Kit expression prior to radiotherapy showed negative c-Kit expression (scored as 0). The other three cases retained low c-Kit expression (1⁺) during the course of radiation therapy. None of the cases with c-Kit expression scored as 1⁺ or 2⁺ demonstrated increased c-Kit expression subsequent to radiation. For the 18 cases with negative c-Kit expression, one case showed increased c-Kit expression (scored as 1⁺) after radiation, the other 15 cases maintained negative expression throughout. Examples of c-Kit expression alteration during radiation therapeutic course are presented in Fig. 2.

Treatment outcome. The median follow-up time was 50 months (range, 2-75 months). Local recurrence occurred in three patients, regional recurrence occurred in three patients and distant metastases in 15 patients: Four cases to the bone, five to the liver, four to the lung, one to the bone and lung, and the other case to the bone and liver. Fifteen patients had

succumbed at the time of this analysis; three from progression of residual disease, three from locoregional recurrence and 9 as a result of distant metastasis.

The results of Kaplan-Meier analysis are presented in Fig. 3. The 5-year progression-free survival rates were 83.4% for the cases with c-Kit overexpression compared with 74.7% for those with low c-Kit expression (P=0.769). Statistical analysis did not identify any significant differences. The overall survival rates at 5 years were 92.4 and 79.9% for the cases with c-Kit overexpression and low c-Kit expression, respectively; the difference was not statistically significant (P=0.199).

Discussion

In the present study, c-Kit expression levels of patients with NPC were examined, the dynamic changes of c-Kit expression during the course of radiation were evaluated, and the correlation between c-Kit expression and clinical characteristics was analysed.

The data regarding c-Kit expression in NPC are limited. In the study by Bar-Sela *et al* (14), 49 NPC tissue samples were analysed for c-Kit (CD117) expression. Overexpression of

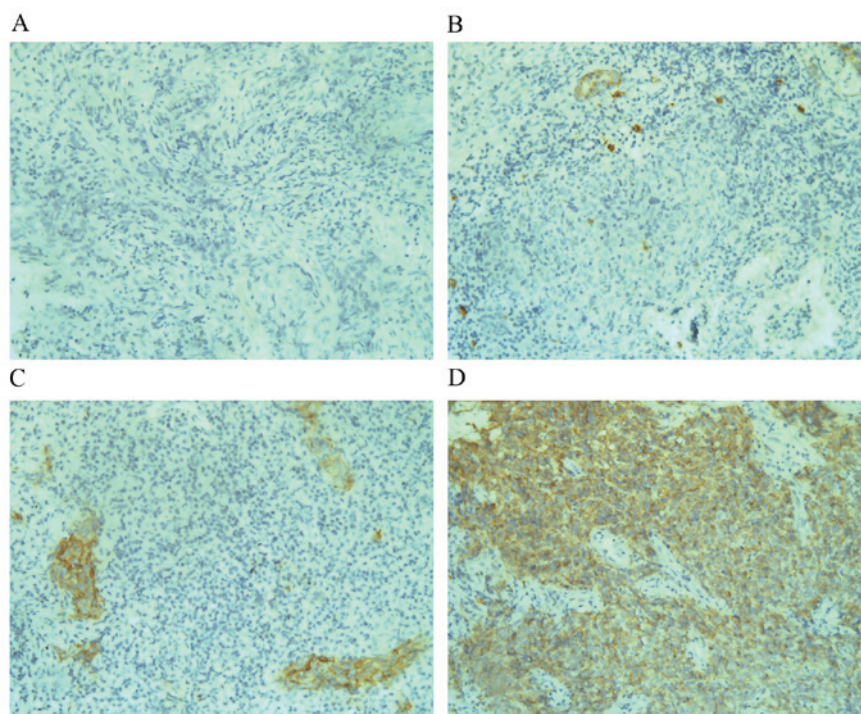


Figure 1. Immunohistochemical expression of c-Kit in nasopharyngeal carcinoma. Representative images of different c-Kit expression levels (0, 1⁺, 2⁺ and 3⁺) are presented. (A) 0, (B) 1⁺, (C) 2⁺ and (D) 3⁺. Magnification, x20.

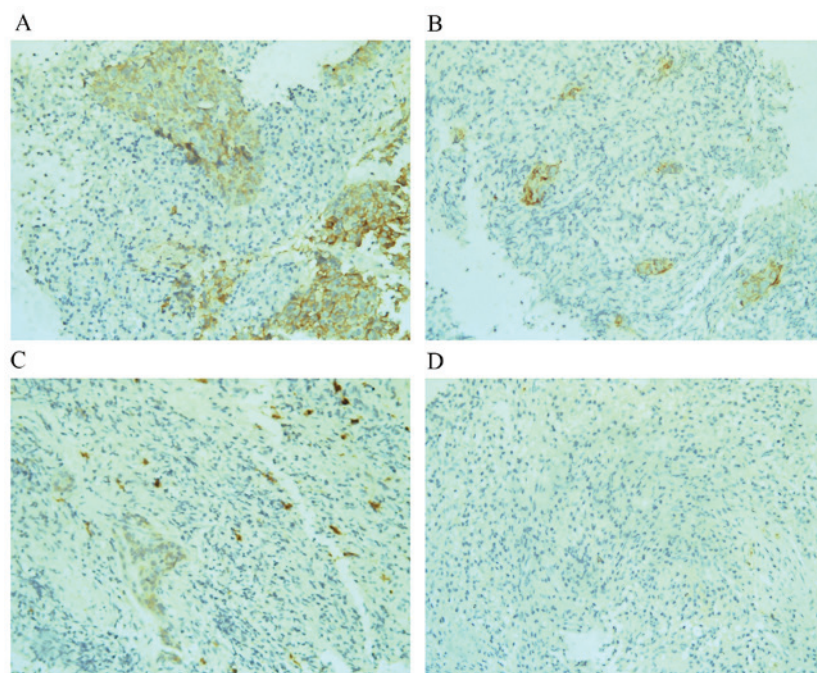


Figure 2. Weekly nasopharyngeal endoscopy and biopsy performed in one patient with nasopharyngeal carcinoma. A trend of decreased c-Kit expression was observed following radiation therapy. (A) Sample obtained prior to radiation: c-Kit staining was observed in $\geq 50\%$ of tumour cells, and the expression scored as 3⁺; (B) one week after commencing radiation: positive stained tumour cells were observed in $\geq 10\%$ and $< 50\%$ of tumour cells, c-Kit expression scored as 2⁺; (C) 2 weeks after commencing radiation: c-Kit staining was observed in $< 10\%$ of tumour cells, and the expression scored as 1⁺; (D) 3 weeks after commencing radiation: No cellular staining was observed, and c-Kit expression scored as 0. Magnification, x20.

c-Kit was identified in 33% (16/49) of the patients, with 18.4% (9/49) strongly positive for the c-Kit protein (staining of $> 50\%$ of the tumour cells). In another report by Bar-Sela *et al* (15), c-Kit overexpression was observed in 28% (9/32) of the adult NPC cases. In the present study, a relatively large population,

of 106 NPC cases were analysed in total, and similar c-Kit scoring criteria were used as in the study Bar-Sela *et al* (14). Twelve (11.3%) cases exhibited c-Kit expression that was scored as 3+ and 16 (15.1%) that was scored as 2⁺. Thus, c-Kit overexpression (2⁺ or 3⁺) was observed in 26.4% (28/106)

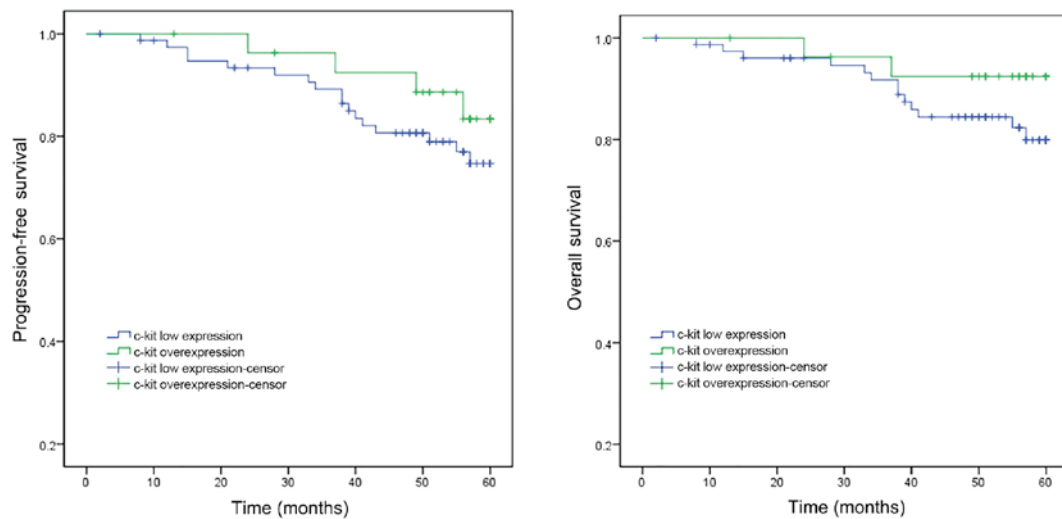


Figure 3. Kaplan-Meier curves of progression-free survival and overall survival of nasopharyngeal patients based on c-Kit staining.

of NPC cases, which is comparable to the former reports (28 and 33%, respectively) by Bar-Sela *et al* (14,15). However, higher expression rates of c-Kit have also been reported. In the study by Sheu *et al* (16), positive staining of >1% of the tumour cells was considered to be positive for c-Kit expression, and the proportion of NPC expressing c-Kit was 86% in that series.

Children with NPC demonstrate increased c-Kit expression when compared with adults with NPC. In a study by Charfi *et al* (17), expression of c-kit was detected in 79% of the cases for patients aged <30-years-old. Bar-Sela *et al* (15) reported on 16 NPC patients aged <20-years-old. Overexpression of c-Kit was found in 88% of the cases, as compared to 28% in adults (15). In the present study, only one patient was <20 years old. Furthermore, attempts were made to analyse the expression rates in different age ranges; however, no difference was identified between the younger adults and older patients.

To the best of our knowledge, the present study is the first to report whether c-Kit expression levels change during the course of radiation therapy. A trend of decreased c-Kit expression was observed subsequent to commencing radiotherapy when the c-Kit expression levels were compared at different time points for each individual patient. For the 13 cases exhibiting c-Kit overexpression (2⁺ and 3⁺) at diagnosis prior to radiotherapy, 12 cases demonstrated reduced c-Kit expression following radiotherapy, and the majority of patients with 1⁺ c-Kit expression showed negative staining subsequent to radiotherapy. Notably, none of the cases with c-Kit expression scored as 1⁺ or 2⁺ demonstrated increased c-Kit expression following radiation. For those cases exhibiting negative c-Kit staining, the majority maintained negative expression during radiation. Although the functional involvement of c-Kit in tumorigenesis and the therapeutic response of NPC remains to be elucidated in detail, the current study provides valuable data regarding dynamic changes of c-Kit expression during the course of radiation therapy.

C-kit expression has been reported as an independent prognostic factor in patients with other types of cancer, such as small cell lung cancer (18). However, in the current study, no association was identified between c-Kit expression and survival of NPC patients. The present results are consistent

with previous reports by Bar-Sela *et al* (14,15). In the present study, the majority of the cases were adults, with only one patient aged 17-years-old. For paediatric patients, tumours with strongly positive c-Kit expression were reported to have a lower recurrence rate (15). However, larger studies are required to evaluate c-Kit as a potential prognostic factor.

c-Kit overexpression had been reported in various NPC cell lines (19). In preclinical studies, imatinib and sunitinib induced a dose-dependent inhibitory effect on the proliferation of NPC cells (19,20). As yet, to the best of our knowledge, targeted therapy against c-Kit has not been investigated in NPC patients. As c-Kit overexpression has been reported in ~30% of adult NPC patients (14,15), it may be of interest to evaluate c-Kit as a therapeutic target for metastatic NPC patients with c-Kit overexpression, particularly for those whose first line treatment failed.

c-Kit overexpression was identified to be common in NPC, therefore, it may be of interest to evaluate c-Kit as a therapeutic target for metastatic NPC with c-Kit overexpression as a second line treatment. A trend of decreased c-Kit expression was observed during the course of radiotherapy. However, the prognostic value of c-Kit in patients with NPC remains to be elucidated.

Acknowledgements

The present study was supported by a grant from the National Natural Science Foundation of China (grant no. 81572952).

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