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In Vitro Uptake of Bromodeoxyuridine by Human Nasopharyngeal Carcinoma (NPC) and its Relation to Clinical Findings

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A cell kinetic study of 27 newly diagnosed patients with nasopharyngeal carcinoma (NPC) using the *in vitro* bromodeoxyuridine (BrdU) technique was performed. The results were reproducible as demonstrated by three independent sections performed on each patient. No correlation between BrdU labelling index (LI) and Ho's clinical staging was found. A higher LI was associated with the development of distant metastases ($P = 0.057$). Statistically significant correlation was found between low LI and longer duration required to achieve complete remission in the primary site of disease ($P = 0.026$). This study suggests a potential role for *in vitro* BrdU labelling index as a prognosticator for NPC prior to treatment.

Keywords: nasopharyngeal carcinoma, *in vitro* bromodeoxyuridine, labelling index

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is unique amongst head and neck cancers. In southern China, over 95% of NPCs are pathologically WHO Grade 3 undifferentiated carcinoma [1]. Despite a high rate of locoregional control with primary radiotherapy (RT), a significant number of patients fail with distant metastases. The initial clinical staging is of paramount importance in prognosis [2]. Other factors that have independent prognostic significance include the patient's age, sex and absence of residual tumour after RT [3, 4]. Furthermore, antibody-dependent cellular cytotoxicity (ADCC), IgG antibody titre to early antigen (EA) of Epstein–Barr virus (EBV) and IgG antibody against recombinant Epstein–Barr virus BZLF-1 transactivator protein (ZEBRA) have also been claimed to be of prognostic value [5–8].

The cell proliferative fraction as determined by various cell kinetic parameters has been defined as an independent prognosticator in some malignant neoplasms [9–12]. *In vitro* incorporation of bromodeoxyuridine (BrdU) by DNA-synthesising cells has been used to determine the S phase fraction. This technique has been applied to tumours of the head and neck region with evidence to suggest prognostic significance [13, 14]. In this study the application of *in vitro* BrdU in a series of patients with newly diagnosed NPC is described and its results are correlated with clinical staging, treatment response and development of early distant metastasis.

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PATIENTS AND METHODS

27 newly diagnosed NPC patients were included in this study. Informed consent was obtained for nasopharyngoscopy and biopsy in every patient. Histological diagnosis was made using sections stained with haematoxylin and eosin. WHO grading for NPC was used [1].

Fresh biopsies were obtained during nasopharyngoscopy. The cell proliferation kit RPN20 was supplied by Amersham (Amersham, Buckinghamshire, U.K.). The fresh tissue sample was cut to yield slices approximately 1 mm thick and 2 mm^2 in area and put into cell culture medium containing BrdU diluted to 1:1000 concentration as the labelling reagent at 37°C. Thirty microlitres of 30% (v/v) hydrogen peroxide was added and the tube was sealed and incubated at 37°C for 2 h. The tissue slides were washed with phosphate-buffered saline (PBS) for 15 min at 37°C. Sections were cut and rehydrated by washing with PBS. This was followed by addition of reconstituted nuclease–anti-5-bromo-2-deoxyuridine and incubated for 75 min at room temperature. The specimen was rinsed with PBS and peroxidase antimouse IgG2a was added and incubated for 30 min at room temperature. The slides were washed with PBS and then immersed in 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution diluted with phosphate buffer for 10 min. After washing with distilled water the

slices were counterstained with methyl-green. The slices were then dehydrated and mounted and the cells examined by light microscopy. The labelling index (LI) of BrdU was determined by counting 1000–2000 tumour cells in randomly selected high-power fields and the LI was expressed as a percentage of the cells showing positive staining. A suspension of growing cells from the well-established NPC cell line CNE-2 [15] was used as the positive control. The same suspension without the addition of anti-BrdU served as the negative control of the assay.

In each patient three separate sections were stained for independent estimation of the BrdU LI by the same operator, thereby three separate labelling indices (LIs) were obtained to test the reproducibility within each patient.

Patients were staged clinically with computerised tomography and nasopharyngoscopy according to Ho's clinical staging classification [2, 16]. The patients entered into this study were prospectively assessed for response to treatment. The RT technique has been uniform and standardised as described previously [17]. Complete remission (CR) was defined as complete resolution of tumour endoscopically with negative histological biopsies of initial tumour site or suspicious areas. The time interval for achieving CR was calculated from the day RT was commenced to the day CR was documented.

RESULTS

The patient characteristics, treatment outcomes and BrdU labelling indices (LIs) are illustrated in Table 1. The median age was 42 years. Ninety-three per cent of the patients had undifferentiated carcinoma and 7% of the patients had poorly differentiated carcinoma. All except 1 patient, who had liver metastases at diagnosis, received a radical course of external RT. 7 patients received intracavitary treatment for residual local disease and 5 patients received lymph node boosting for residual nodal disease. For the 26 patients who received a radical course of RT, the median time required to achieve CR in the nasopharynx was 72 days.

The BrdU-positive nuclei stained brown and could be distinguished under the light microscope as illustrated in Figs 1 and 2. The mean LI varied from 0.90 to 97.3%, demonstrating a wide variation. High reproducibility of the *in vitro* BrdU LIs in NPC tissue of each individual patient was demonstrated by this study—three independent readings of the LI in each patient were compared using paired *t*-test and no statistical difference was seen comparing any two results with *P* values of 0.21 (tests 1 and 2), 0.21 (tests 1 and 3) and 0.62 (tests 2 and 3), respectively.

The median of the mean LI (average of tests 1, 2 and 3) in this series was 12.63%. The standard deviation was wide and non-parametric methods were used for statistical analysis.

Table 1. Patient characteristics, days to complete remission in primary tumour and BrdU labelling indices

Patient no.	Sex/age (years)	Ho's staging				Histology	Response in NPC (Days to CR)	BrdU labelling index			
		T	N	M	Overall			1	2	3	Mean
1*	M/37	2np3ab	0	0	III	U	68	98.9	96.5	96.5	97.30
2	F/35	2n	0	0	II	U	63	0.9	3.3	8.6	4.27
3	M/43	2np3ab	0	0	III	U	106	4.0	8.6	11	7.87
4	M/43	2n3b	0	0	III	U	131	5.7	7.2	6.5	6.47
5*	M/40	2nop3b	3	1	V	U	—	0	1	1.8	0.93
6	M/34	2n3abd	0	0	III	U	61	11	17.5	15.9	14.80
7	M/39	2p3ab	1	0	III	U	62	36	25.1	41.2	34.10
8	M/38	2nop	2	0	III	U	71	18.9	24.7	29.1	24.23
9	M/65	2np	0	0	II	U	72	0.0	6.4	12.1	6.17
10	M/50	2nop3ab	0	0	III	U	111	30.0	24.9	18.1	24.33
11	M/49	1	2	0	III	U	69	49.8	30.9	28	36.23
12	F/34	2np	1	0	II	U	25	35.7	35	12.6	27.77
13*	M/58	2p	2	0	III	U	80	60.4	48.2	41	49.87
14	M/33	2p	3	0	IV	U	86	4	5.8	11.4	7.07
15	M/61	2nop	3	0	IV	U	81	16	9.8	12.1	12.63
16*	M/43	2np	2	0	III	U	82	31.4	26.7	15.7	24.60
17	M/61	2n	0	0	II	U	59	21.9	30.7	14.9	22.50
18*	F/48	2np	2	0	III	U	—	44.1	36.9	39.6	40.20
19	M/63	2np	0	0	II	U	69	28.4	5.5	3.2	12.37
20	F/28	2np	1	0	II	U	102	5.6	8.7	3.4	5.90
21	F/42	2np	1	0	II	U	21	25.5	10	15	16.83
22	M/44	1	0	0	I	P	96	1.4	0.9	2.2	1.50
23	M/40	2np3a	3	0	IV	U	84	7.9	5.4	3.2	5.50
24	M/41	2np3a	0	0	III	U	64	0	0.3	2.4	0.90
25	M/34	2np	2	0	III	P	81	0	0	3.1	1.03
26	M/67	2p3abc	2	0	III	U	70	15.5	25.4	30.2	23.70
27	M/42	2np	2	0	III	U	81	0	2.9	0.1	1.00

*Patients who developed distant metastases within 12 months from diagnosis.

WHO Classification (1). U = undifferentiated carcinoma; P = poorly differentiated carcinoma.

Ho's Classification (2). T stage: T1 = confined to nasopharynx, T2n = nasal fossa involvement, o = oropharyngeal involvement, p = parapharyngeal involvement, T3a = bone involvement below base of skull, b = involvement of base of skull, c = involvement of cranial nerves, d = involvement of the orbits, laryngopharynx or infratemporal fossa. N stage: 0 = no LN, 1 = upper cervical LN, 2 = LN between skin crease and supraclavicular fossa, 3 = supraclavicular fossa LN.

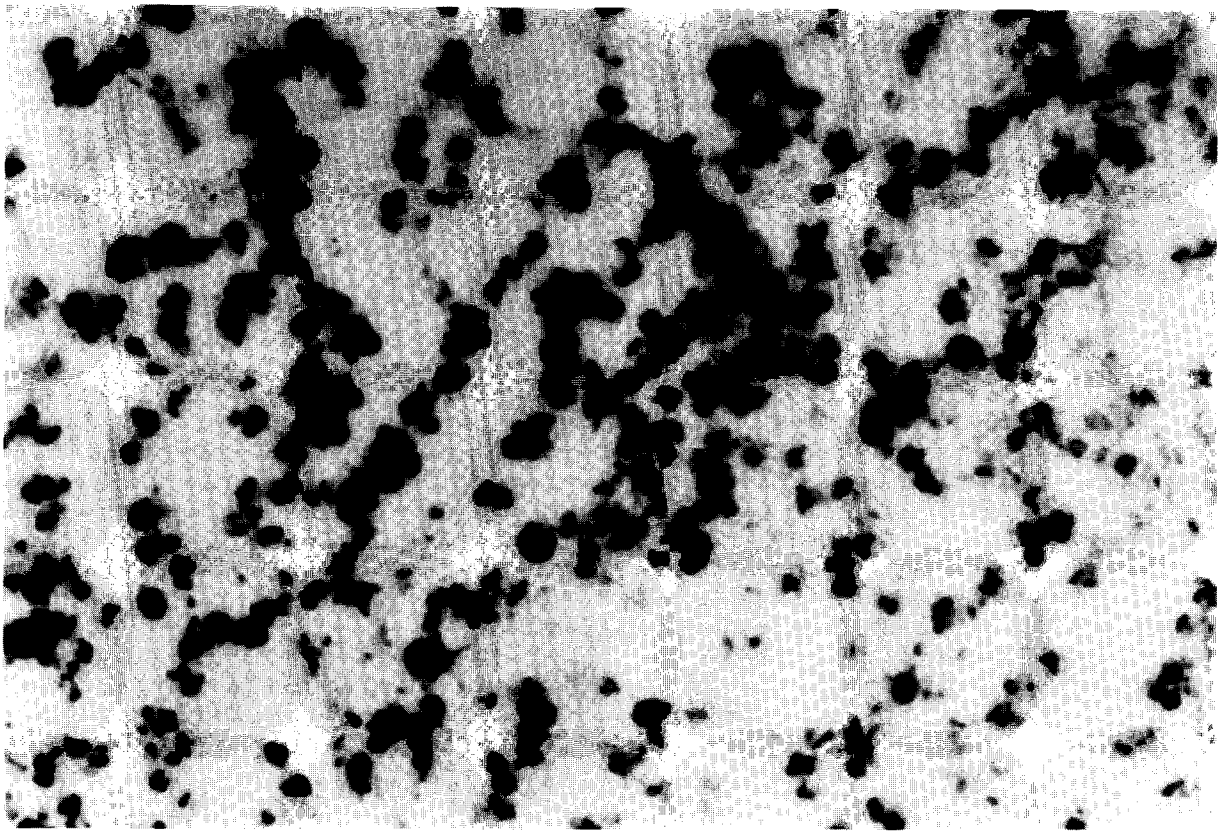

Fig. 1. High labelling index *in vitro* BrdU staining (96.5%).

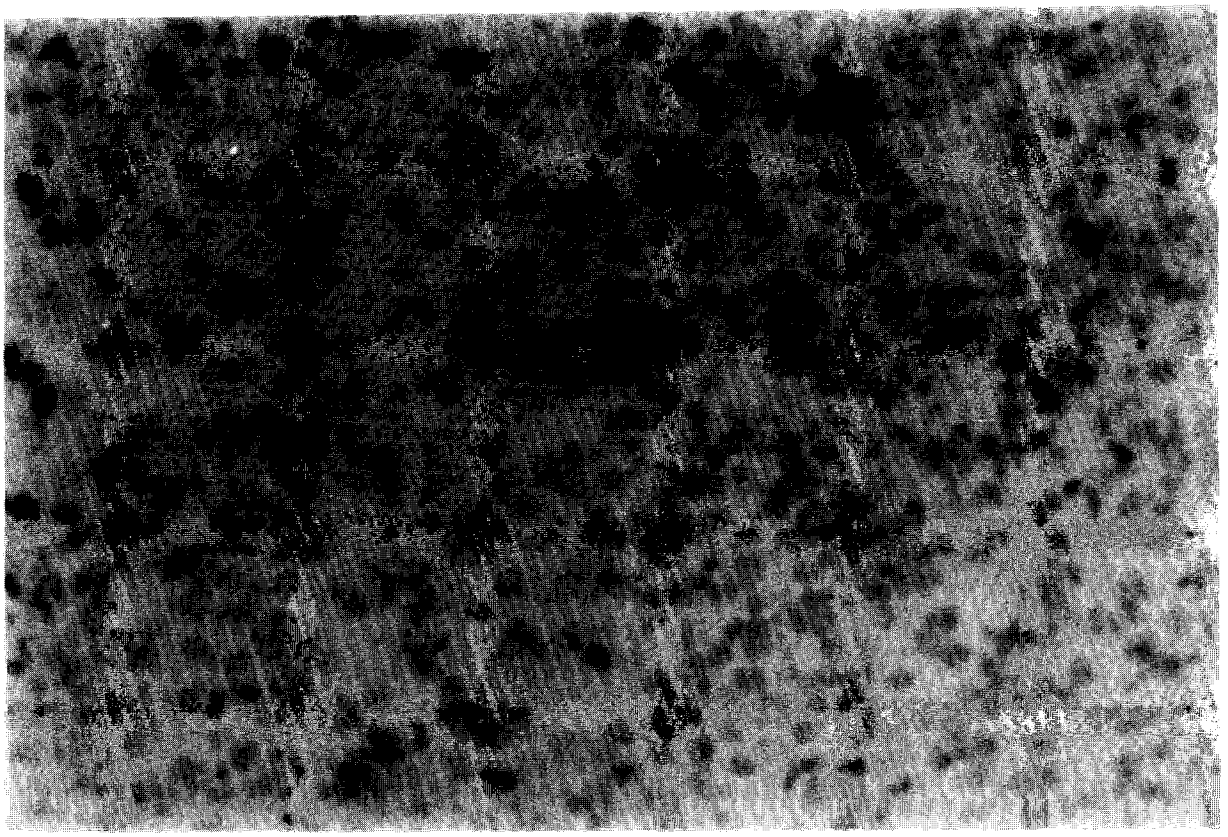

Fig. 2. Low labelling index *in vitro* BrdU staining (8.6%).

There was no significant correlation between BrdU LI and Ho's overall staging ($P=0.209$, Wilcoxin's rank sum test), Ho's T staging ($P=0.999$, Wilcoxin's rank sum test) or Ho's N staging ($P=0.198$, Wilcoxin's rank sum test). In the subgroup of 5 patients that developed distant metastasis within the first 12 months from diagnosis, the mean BrdU LIs were higher than the 22 patients with no evidence of distant metastasis with borderline statistical significance using Wilcoxin's rank sum test ($P=0.0569$). In the patient with distant metastasis at the time of diagnosis (case 5, Table 1), the biopsy obtained showed a high degree of necrosis histologically and this might account for the low BrdU LIs. 12 patients with mean BrdU LI below the median (low LI) were compared with 13 patients with mean BrdU LI above the median (high LI) for the number of days to achieve CR in the primary tumour after the start of RT. From Wilcoxin's rank sum test the group with low LI was found to require a significantly longer time to achieve CR compared to the group with high LI ($P=0.0209$).

DISCUSSION

Nasopharyngeal carcinoma is unique in its radiosensitivity and chemosensitivity. In southern China where this disease is endemic, over 95% of patients have undifferentiated carcinoma (WHO Grade 3) and most studies for this region did not report on the prognostic significance of NPC histology. The mainstay treatment for NPC is radical radiotherapy. Good locoregional control is achieved with the RT technique published previously [17]. In locoregionally advanced patients, a high rate of distant failure is seen and Ho's N staging has been demonstrated to be the strongest predictor of distant failure [3, 4]. However, even within staging subgroups, the prognosis of individual patients varies and no histopathological data accurately predict the behaviour of individual tumours. Attempts to identify serological factors with prognostic potential in NPC have made some progress in the past decade. De-Vathaire *et al.* [7] described the prognostic role of EBV markers in the clinical management of NPC. In patients who achieved clinical remission, high IgG and IgA titres to EBV EA at 1 year were found to be highly significant for prediction of relapse. However, no significant correlation with the antibody titres before treatment was found. Similarly, Yip *et al.* [8] reported a significant correlation between high ZEBRA/IgG titres at 10 months after radiotherapy and the development of distant metastasis to the lung or liver, but no correlation was found when antibody titres before treatment were analysed. Furthermore, Neel *et al.* [5, 6] described a significant correlation between low ADCC titres against EBV-specific membrane antigen at diagnosis and a shorter overall survival. In our institution, serum levels of soluble interleukin-2 receptors were found to correlate with clinical staging and the development of distant metastasis and may have prognostic significance in patients with NPC [18]. A recent study by Zheng *et al.* [19] showed elevated levels of epidermal growth factor (EGFR), Ki 67 antigen and the Epstein–Barr virus-encoded latent protein (EBV-LMP1) in NPC. An increased expression of EGFR and higher proportion of Ki 67-positive cells was correlated to the expression of EBV-LMP1 and this was more frequently found in NPC with advanced stages of disease.

As discussed above, thus far no single serological marker can be relied upon to predict the prognosis of an individual patient with NPC. The imperfections of traditional staging classifications to very accurately predict the clinical course of various malignanices have prompted the study of cell kinetic parameters using a variety of methods. In head and neck cancers, cell kinetic studies using *in vitro* BrdU labelling have been performed in oral squamous cell carcinomas. One study by Hemmer [13] showed T3 tumours to have significantly higher LIs than T1 and T2 tumours, and also LIs of primary tumours with cervical nodal metastases had significantly higher LIs than those without evidence of cervical lymph node involvement. Similarly, another study by Mukhopadhyay *et al.* [14] showed significantly higher LIs for higher nuclear grade carcinoma, higher LIs for stage III and IV tumours than stage I and II tumours, and higher LIs for patients with positive lymph nodes compared with patients with negative lymph nodes. Thus, *in vitro* BrdU LI in oral squamous cell carcinoma significantly correlates to cervical nodal metastasis which is a strong independent prognosticator for head and neck squamous cell carcinoma in general. However, its independent prognostic significance requires further evaluation.

In the present study using *in vitro* BrdU in 27 newly diagnosed NPC patients, the distribution of LIs was wide, demonstrating a heterogenous cell proliferation pattern. However, the results of *in vitro* BrdU staining is highly reproducible in each individual patient. In all 27 cases three independent readings were taken on three separate sections of the tumour tissue obtained, and variation within each patient was statistically insignificant. The results of this study found no correlation of BrdU LI with the Ho's clinical staging traditionally used in this region [2, 16]. However, correlation was found between high BrdU LI with the development of distant metastasis. This could be of clinical importance since distant metastasis and local failure are the two main causes limiting NPC survival. Since the patient numbers are too small to perform subgroup analysis or multivariate analysis, the BrdU LI and other known prognosticators have to be compared for independent significance in a larger prospective study.

Moreover, the rate of regression of primary tumour to radical RT was shown to correlate with a high BrdU LI in the present study. A higher BrdU LI represents a higher S-phase fraction, therefore the likely explanation for this correlation is that NPC cells in S-phase are more radiosensitive. The significance and therapeutic implications for slow-regressing primary lesions in NPC have not been clearly defined in the literature. A randomised study by Yan *et al.* [20] demonstrated a higher local recurrence rate in patients with pathologically proven residual primary lesion left unboosted. In other head and neck cancers, Bataini *et al.* [21] showed in a retrospective study that local failure rate was significantly higher for incomplete responders to RT.

Finally, it requires a multivariate analysis to determine the independent prognosticators governing survival, distant metastasis and local control in NPC. However, by virtue of the association of high BrdU LI with the development of distant metastasis, inclusion of BrdU LI as one potential prognosticator is indicated in future large-scale multivariate analysis.

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