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ARTICLE

Morphometric Study of Tumor Angiogenesis as a New Prognostic Factor in Nasopharyngeal Carcinoma Patients*

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The aim of this study was to determine the possible prognostic significance of tumor angiogenesis (TA) in nasopharyngeal carcinoma (NPC) patients. Fifty-five NPC patients were evaluated in relation to survival. Endothelial cells were immunohistochemically stained with anti-von Willebrand factor (F-VIII), CD-31 and CD-34 antibodies, and microvessels counted in the most active areas of tumor neovascularization or *hotspots* using both a manual and an automatic method. Overall survival analysis calculated by the *Kaplan–Meier* test revealed that both methods were correlated with a statistical significance between intratumoral microvessel density (IMD) and overall survival, using either manual ($p = 0.0141$) or automatic counting ($p = 0.0117$). Other angiogenic parameters studied were perimeter, roundness and accumulative area of the microvessels using a morphometric analyzer. Moreover, our results show that cases with high IMD demonstrated a prognostic significance in relation to the accumulative area ($p = 0.0072$). (Pathology Oncology Research Vol 6, No 3, 210–216, 2000)

Keywords: angiogenesis, morphometry, nasopharyngeal carcinona

Introduction

Nasopharyngeal carcinoma (NPC) is unique from other head and neck cancers because of its peculiar epidemiological and biological characteristics. NPC appears with a high incidence in some parts of the world like Southeast Asia and is relatively rare in others, such as occidental countries.[1] It is the most common epithelial malignancy of the nasopharynx and is strongly associated with EBV virus.[2,3,4] There are three histopathologic categories according to the WHO[5] classification: UCNT (undifferentiated carcinoma of nasopharyngeal type), NKC (nonkeratinizing squamous cell carcinoma), and SCC (squamous cell carcinoma). Its particular biologic behavior implies that patients in similar stages of NPC have opposite clinical courses and different responses to the same treatment. In order to achieve a more reliable prognostic evaluation of such patients, seeking other prognostic indicators that can be used in conjunction with other well-established factors (clinical stage, histological type, lymph node status) may prove of use.

The network of capillary blood vessels supplies oxygen and nutritional elements to the cell of every organism, including tumoral cells. Angiogenesis, the growth of new capillary blood vessels, is essential in normal processes, such as in ovulation and wound healing, but it is also involved in pathological processes such as inflammation and tumors.[6] In this latter case angiogenesis is an uncontrolled and dangerous process.

According to several previous studies, TA has proven to be a requirement for the growth and metastatic spread of solid neoplasms,[7] including head and neck cancer. Angiogenesis, in this case, would be like a signal to switch on the tumoral growth. Moreover, several observations have proposed that IMD is associated with overall survival in different tumor types. In fact, in the last

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decade, angiogenesis assessed by IMD has been incorporated as a new prognostic indicator in the evolution of many malignant tumors.[8-13]

Patients and Methods

Patients

In a retrospective study we examined tumor specimens from 55 patients (36 men and 19 women, mean age 49) with NPC morphologically diagnosed and treated between 1977 and 1994 at University Hospital La Fe. Cases were evaluated for tumor angiogenesis in relation to survival. The follow-up ranges from 2 to 120 months.

Seven cases were excluded because they contained metastatic tissue (13%), and also 5 cases (9%) because of scanty tissular material in original paraffin blocks. Hence, this study was performed with only 42 primary neoplasms (78%). Histological diagnosis of the 42 cases was performed according to the WHO classification[5] and Micheau's scheme,[14] which accept three and only two major microscopical types respectively. According to the WHO classification, patients were: UCNT (undifferenciated carcinoma of nasopharyngeal type) 21 cases (50%), NKC (non-keratinizing squamous cell carcinoma) 12 cases (29%), and SCC (squamous cell carcinoma) 9 cases (21%). On the other hand, 33 cases (79%) were considered as undifferentiated carcinoma and 9 (21%) as SCC according to Micheau's scheme.

Laboratory assay

All microvessels were highlighted by staining endothelial cells for anti-von Willebrand factor (factor VIII-related antigen) (*Biomeda Corp.*,USA). It was thus possible to identify the most intense vascularization areas of the tumors. Previously, we analyzed the immunohistochemically stained vessels with anti-von Willebrand factor (F-VIII), CD-31 and CD-34 antibodies in order to achieve the best result. The analyses were made on 15 of the 55 cases (27%) that we randomly chose. Finally, we chose anti-von Willebrand factor (F-VIII) antibody because of its minor unspecific staining and better highlighting of the microvessels in nasopharyngeal specimens.

Immunohistochemical staining was carried out by the streptavidin-biotin complex method. Formalin-fixed and paraffin-embedded 5 µm-thick sections from the 42 specimens were evaluated. Sections were dewaxed and microwave treated. Later they were incubated in 3% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase activity, followed by washing in distilled water and in phosphate-buffered saline PBS (*Biomeda Corp.*, USA). Predigestion of the tissue was performed by incubating the sections for 10 minutes at 37°C in trypsin 1mg/ml PBS, then washing in PBS for 5 min.

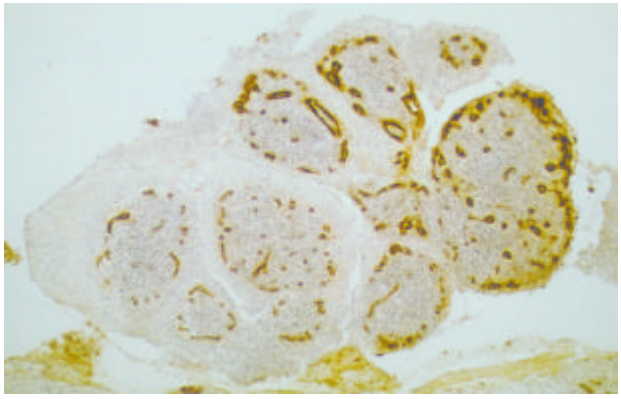

Figure 1. Microvessels were immunostained for anti-von Willebrand factor (factor VIII-related antigen). It was thus possible to identify the most intense vascularization areas of the tumors. Intratumoral microvessel density (IMD) was determined in areas of most intense neovascularization or hotspots. Single countable microvessels were then manually enumerated on a 200 x field (20 x objective lens and 10 x ocular lens, 0.7386 mm^2 per field).

Sections were then incubated in bovine serum albumin to reduce unspecific staining for 20 min., followed by incubation with the primary prediluted polyclonal antibody anti-von Willebrand factor (factor VIII-related antigen) (*Biomeda Corp*., USA) for 30 min. at room temperature. After washing in PBS for 5 min., sections were incubated with biotinylated secondary antibody for 30 min. (*Dako*, Denmark) and then incubated for 30 min. with streptavidin conjugated to horseradish peroxidase at room temperature (*Dako*, Denmark). The peroxidase reaction was developed using 3,3´Diaminobenzidine tetrahydrochloride 0.05% as chromogen (*Dako*, Denmark), and sections counterstained with hematoxylin.

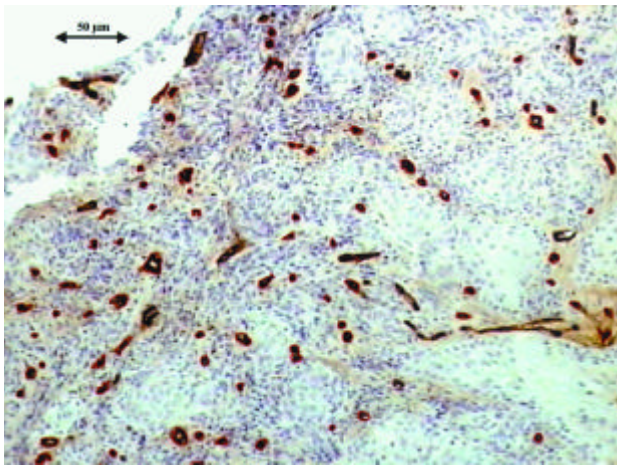

Figure 2. Screen displays a microscopically captured image in computer morphometry system showing highlighted microvessels (in red). The slide image captured by the computer (field 250 x) has a real field on TV screen of 0.5028 mm^2.

Negative controls were performed in every case by omitting the primary and secondary antibodies respectively from the immunohistochemical procedure.

Counting of vessels

Representative areas of the tumors were selected from sections stained with hematoxylin and eosin. Areas of invasive tumor containing the highest numbers of capillaries and small venules (microvessels) were examined by light microscopy. IMD was observed in areas of most intense neovascularization or *hotspots*, which, in turn, were detected by scanning the tumor sections at low power (40 x and 100 x) and identifying areas of invasive tumor with the greatest number of discrete microvessel staining per area.

After the highest neovascularization area was determined, single microvessels were manually counted on a 200 x field (20 x objective lens and 10 x ocular lens, 0.7386mm^2 per field) by two different observers (LR and FJVS) without knowledge of patient outcome. Both observers underwent a period of training before counting the study cases. The counts of the two observers matched (within a margin ± 5 microvessels) in the majority of cases; in case of discrepancies, these were reanalyzed and disagreement resolved by consultation. Any brown-staining endothelial cell or cell cluster that was clearly separated from adjacent microvessels was considered as a single, countable microvessel. A vessel lumen was not required for identification, nor was the presence of red cells, in accordance with Wiedner[8]. Distribution of high intratumoral microvascular density areas

Table 1. **The manual and automatic cut off point method**

Automatic Method				*Manual Method*			
No vessels	*Frequenc*	*Accumulative percentage (%)*	*Kapla–Meier (p-value)*	*No vessels*	*Frequenc*	*Accumulative percentage (%)*	*Kapla–Meier (p-value)*
9.00	1	2.4		15.00	1	2.4	
11.00	1	4.8		20.00	1	4.8	
12.00	1	7.1		24.00	1	7.1	
16.00	1	9.5		26.00	2	11.9	
18.00	1	11.9		27.00	2	16.7	
23.00	1	14.3		28.00	2	21.4	
24.00	1	16.7		29.00	1	23.8	
25.00	2	21.4		33.00	2	28.6	
27.00	2	26.2		34.00	3	35.7	
29.00	2	31.0		35.00	1	38.1	
30.00	2	35.7		36.00	1	40.5	
32.00	2	40.5		37.00	1	42.9	
33.00	1	42.9		41.00	1	45.2	
34.00	2	47.6		43.00	3	52.4	
35.00	2	52.4		44.00	4	61.9	
36.00	1	54.8		45.00	1	64.3	
37.00	2	59.5		46.00	1	66.7	
40.00	1	61.9		48.00	1	69.0	0.0129
41.00	3	69.0		49.00	1	71.4	0.0400
42.00	1	71.4	0.0182	50.00	1	73.8	0.0141
43.00	1	73.8	0.0117	*50.00*	*Cut off*	*75.0*	*0.0141*
43.00	*Cut off*	*75.0*	*0.0117*	52.00	2	78.6	0.0126
44.00	1	76.2	0.0180	54.00	1	81.0	
45.00	1	78.6	0.0100	55.00	1	83.3	
47.00	1	81.0	0.0465	59.00	1	85.7	
48.00	1	83.3		60.00	1	88.1	
49.00	1	85.7		69.00	1	90.5	
51.00	1	88.1		70.00	1	92.9	
55.00	1	90.5		72.00	1	95.2	
62.00	1	92.9		83.00	1	97.6	
66.00	1	95.2		97.00	1	100	
67.00	1	97.6					
78.00	1	100					
Total	42			Total	42		

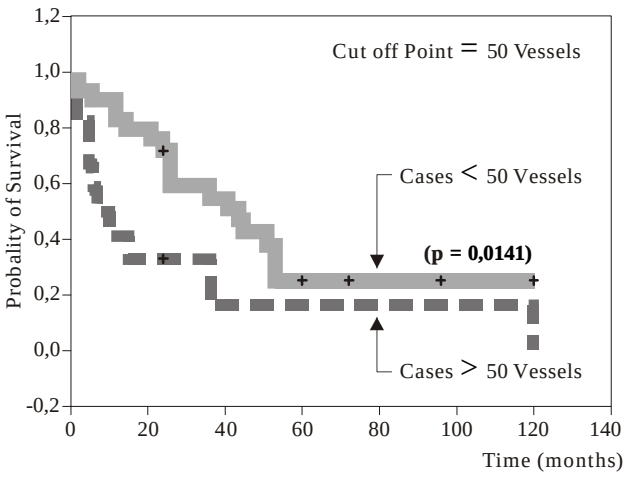

Figure 3. Survival curves (Kaplan–Meier) of patients in relation to manual vessel count.

was heterogeneous in each tumor, although usually these areas appeared at the periphery of tumor nests. Each count was expressed as the highest number of microvessels identified within any 200 x field (*Figure 1*).

Moreover, microvessels were automatically counted using an image computer analyzer a 250 x field. The highest neovascularization area or *hotspot* was determined as previously described. An image of each slide was captured digitally using a CCD camara, which was connected to an image computer analyzer (*MicroImage*®). The CCD camara was attached to an *Olympus CH-2* microscope. However, the slide image captured by the computer had a real field on the TV screen of 0.5028mm^2. We then proceeded with RGB digital color images (*Figure 2*).

In order to measure areas, perimeters and vessels count, we established the brown color range of the microvessel staining and selected a single, countable microvessel by computer. Microvessels with a greater diameter (>50 μm) were excluded from the study, according to Wiedner.[8] Additionally, we analyzed other angiogenic parameters such as perimeter, roundness and accumulative area of the microvessels in each case.

In both (manual and automatic methods) a single field for each case was utilized to determinate the microvessel count.

Statistical analysis

All statistical analyses were carried out using the SPSS-X statistical computer package (SPSS Inc, Chicago) for Windows 6.1. Univariate analysis by *Student's t test* was used to assess differences between angiogenesis in relation to clinicopathological variables. Non-parametric test (*Mann-Whitney*) was used to study other angiogenic parameters: perimeter, roundness and relative area. Survival curves of patients were established using the *Kaplan–Meier* method, and the statistical evaluation was performed using the log rank test; a *p* value less than 0.05 was considered significant.

Results

The cut off point

Mean values for IMD were 44 and 37 vessels by manual and automatic counting, respectively. Cases with low and high angiogenesis had to be separated so as to evaluate their possible prognostic value by comparing the two groups (low and high vascularization) in relation to survival. We ordered all 42 biopsic specimens with respect to their vessel numbers, from lowest to highest. Thereafter, we assigned, as a *cut off* point, the number of microvessels included in the sample, of which 75% of sample values were lower. The *cut off* point for the manual method was 50 vessels, and 43 for the automatic one (*Table 1*).

As a result of this *cut off* point we obtained a statistically significant difference between the low and high angiogenesis groups. Moreover, both microvessel counting methods (manual and automatic) were correlated with a statistical significance between IMD and overall survival, using either manual (Kaplan–Meier method, p=0.0141) or automatic counting (Kaplan–Meier method, p=0.0117) (*Figures 3 and 4*).

Clinicopathological variables in relation to survival

We analyzed certain clinicopathological variables such as tumoral type (UCNT, NKC and SCC), tumor size (T), stage (I, II, III, IV), metastasis (M), sex and age of the patients in relation to survival. Survival curves were established using the Kaplan–Meier method, the statistical evaluation by using the log rank test; a *p* value less than 0.05 was considered significant.

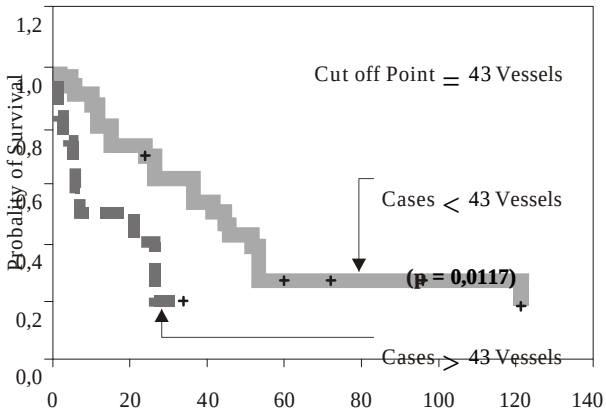

Figure 4. Survival curves (Kaplan–Meier) of patients in relation to automatic vessel count.

Table 2. **Correlation between clinicopathological variables and overal survival**

Variable	*Group*	*Survival (months)*	*p-value**
Histological	UCNT	57	p = 0.0001
Type	SCC	9	
Tumor	T = 1 / T = 2	68	p = 0.0484
Size	T = 3 / T = 4	35	
Tumoral	I, II, III	47	p > 0.05
Stage	IV	44	
Metastasis	negative	55	p > 0.05
	positive	28	
Sex	men	37	p > 0.05
	women	14	
Age	< 30 years	43	p > 0.05
	> 30 years	44	
	< 60 years	47	
	> 60 years	39	p > 0.05

**Kaplan–Meier method*

The statistical results show that only two clinicopathological variables, tumoral type and tumor size, have differences in relation to overall survival. UCNT tumoral type has a significantly better prognosis than SCC (p = 0.0001). On the other hand, small tumor size (T = 1, T = 2) has better prognosis than those with larger tumor size (T = 3, T = 4), (p = 0.0484) (*Table 2*).

Correlation between clinicopathological variables and angiogenesis

Here we analyzed the same clinicopathological variables in relation to angiogenesis. The statistical analysis was done using univariate analysis (Student's t test) and a nonparametric test (Mann–Whitney). Both methods failed to reveal differences between clinicopathological variables and microvessel count. Therefore, angiogenesis appears to be a factor independent from tumoral type, size, stage, metastasis, sex and age of the patients (*Table 3*).

Other angiogenic parameters

The computer image analysis provided us with an exact, objective and effective instrument to determine microvessel count. Moreover, other angiogenic parameters can be calculated by computer image analysis, that by manual method alone would prove impossible. These angiogenic parameters are perimeter (μm), accumulative area (μm^2) and roundness of the microvessels; thus, we compared same between the low and high angiogenesis groups. We observed that the cases above the *cut off* point had smaller perimeter (p = 0.0038), more roundness (p = 0.0108) and greater relative area (p = 0.0003) (Mann–Whitney test analysis) (*Table 4*).

Furthermore, cases over the *cut off* point (12 out of 42 cases) with high angiogenesis demonstrated a prognostic value in relation to the accumulative area (Kaplan–Meier method, p = 0.0072). Consequently, cases with a greater accumulative area had a poor prognosis vis-a-vis those with a lower relative area.

Discussion

There are certain prognostic factors (clinical stage, histological type, lymph node status) that are well-established and reliable indicators in different malignant neoplasms, including head and neck tumors. The primary aim of this study was to evaluate the possible significant correlation between angiogenesis and overall survival in order to establish a new prognostic indicator in NPC patients. Thus, angiogenesis was assessed as density of microvessels in the most intensive neovascularizated areas or *hotspots* so as to reflect the tumor activity and its development.

Several previous studies have described different endothelial markers such as *lectin Ulex europeaus*, anti-CD31, anti-CD34 and anti-von Willebrand factor (factor VIII-related antigen). In breast tumor studies, De Jong et al[15] demonstrated that CD-31 is more sensitive but less specific than F-VIII. On the other hand, Tomisaki et al[16] compared CD-34 and F-VIII in colorectal cancer, affirming that F-VIII is more specific than CD-34 because CD-34 also marks leukocytes and other inflammatory cells. In esophageal SCC, Tanigawa et al[17] showed that the microvessel count in *hotspots* with CD-34 is three times as large as with F-VIII, for the same reason. In 1997, Quian et al[18] used a computer analyzer for microvessel counts and studied the best marker for the nasopharynx

Table 3. **Correlation between microvessel count and clinicopathological variables**

Variable	*Group*	*Automatic*	*Manual*	*p-value**
Histological	UCNT	37	43	p > 0.05
Type	SCC	34	44	
Tumor size	T = 1 / T = 2	31	43	p > 0.05
	T = 3 / T = 4	39	44	
Tumoral	I, II, III	38	45	p > 0.05
Stage	IV	36	43	
Metastasis	negative	35	43	p > 0.05
	positive	40	45	
Sex	men	34	40	p > 0.05
	women	40	47	
Age	< 30 years	36	44	p > 0.05
	> 30 years	36	43	
	< 60 years	34	41	p > 0.05
	> 60 years	41	49	

**Mann–Whitney method and Student t test*

Table 4. **Statistical analysis of other angiogenic variables**

Angiogenic Variable		*Student t test*		*Mann–Whitney*	
low / high angiogenesis groups					
Relative area	*No. cases*	*Mean*	*SD*		*SE of the mean*
Cases < cut off	30	6380.8010 μm^2	2255.543		411.804
Cases > cut off	12	9848.0483 μm^2	2413.354		696.675
		(p = 0.001)		(p = 0.0003)	
Perimeter	*No. cases*	*Mean*	*SD*		*SE of the mean*
Cases < cut off	30	66.5110 µm	11.236		2.051
Cases > cut off	12	55.7193 µm	7.787		2.248
	(p = 0.001)	(p = 0.0038)			
Roundness	*No. cases*	*Mean*	*SD*		*SE of the mean*
Cases < cut off	30	1.7607	0.237		0.043
Cases > cut off	12	1.5258	0.191		0.055
		(p = 0.003)		(p = 0.0108)	

location. They decided on F-VIII in favor of CD-34 because of its greater sensitivity and specificity.

As a result thereof, we analyzed and compared anti-CD31, anti-CD34 and anti-von Willebrand factor to determine the best endothelial marker to our particular tumor location. We chose factor VIII-related antigen because of its better highlighting of vessels and its lesser non-specific staining. These two characteristics were essential because we worked with a morphometric analyzer, and a strong and clear staining is recommended to differentiate vessels from other structures with hematoxylin staining. Moreover, our results are in accord with previous NPC morphometric studies.[18,19]

When all microvessels were stained and both methods (manual and automatic) performed, two different angiogenesis groups had to be created. There are different forms to assess such angiogenic groups. Weidner et al[8] developed the way to obtain the measure of angiogenesis through the number of microvessels in the richest microvessel density areas or hotspots. The authors formed different angiogenesis groups in function of their microvessel count: 0-33, 34-67, 68-100 and >100. Their findings revealed that the probability of metastasis increases 1.7 fold when the number of microvessels increases to ten microvessels. Moreover, in 1992, the same authors carried out a similar study on breast carcinomas, showing that patients with a high microvessel count (>100) had a poor prognosis in relation to those with a low microvessel count (0-33).[9]

The first cut off point we chose to divide the two groups into low and high vascularity was 60 microvessels, as previously done by Roychowdhury in NPC patients.[19] In our study, however, we had no positive result with this particular cut off point and decided to use a new method.

The premises that we followed to obtain a new cut off point were that, first, it had to be stable, and second, and more important, it had to be wholly objective to separate cases with low and high angiogenesis. Thus, we ordered all 42 biopsic specimens with respect to their vessel numbers, from the lowest to the highest, and assigned as a cut off point the number of microvessels included in the sample, from which 75% of sample values were lower. The cut off point was 50 and 43 vessels for the manual and automatic methods, respectively. In our opinion, we believe we achieved the best possible cut off point, because other methods that use mean or median are subjective cut off points.

In clinicopathological variables (type, size, and stage of tumor, metastasis, sex and age), a direct relation with overall survival appeared only in tumoral type and size. Hence, SCC had a poor prognosis compared to UCNT. On the other hand, a greater tumor size implied a poor prognosis in relation to small size.

An attractive hypothesis would be that greater size is associated with a higher microvessel number; this would be the reason for its worse prognosis, but in our study we did not observe this relation and tumor size was not correlated with the microvessel number. Moreover, no statistical differences between clinicopathological variables and microvessel number were detected. As a result, angiogenesis can be taken as an independent prognostic factor from tumoral type, size and stage, as well as from metastasis, sex and age of patients.

The image analyzer affords us an objective tool to measure microvessels and, moreover, to study other angiogenic parameters such as perimeter or roundness, that are not easily quantified in direct observation. Morphometric results showed that there were differences between the low and high angiogenesis groups. Accordingly, the high angiogenesis group (cases over the *cut off* point) had smaller perimeter, more roundness and greater accumulative area than the low group. This morphological description revealed that cases with high vascularity have small and round vessels in contrast to cases with low vascularity, which have greater vessel size and irregular forms.

Additionally, only cases above the *cut off* point with high angiogenesis demonstrated a prognostic value in relation to the accumulative area. Hence, the cases with a greater accumulative area had a poor prognosis versus those with a lower accumulative area. As this direct correlation does not appear in low angiogenesis cases, perhaps other prognostic indicators can act in this situation.

In conclusion, measuring angiogenesis may be a further prognostic indicator that can be used in conjunction with other well-established factors (clinical stage, histological type, lymph node status) in order to achieve a more reliable prognostic evaluation in NPC patients.

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