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Original article

Correlation of microvascular density and proliferation index in undifferentiated nasopharyngeal carcinoma

Mana Taweevisit[a], Somboon Keelawat[a], Paul Scott Thorner[a,b]

[a]*Department of Pathology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.*
[b]*Department of Laboratory Medicine, Hospital for Sick Children and University of Toronto, Toronto, Ontario M5G 1X8, Canada*

Background: Undifferentiated nasopharyngeal carcinoma is a highly malignant tumor with an endemic distribution. Several histologic parameters have been studied to provide prognostic information for patient management. Both proliferation index and microvascular density are commonly determined on such tumors, but the relationship between these two parameters has not been studied fully.

Objectives: Determine the association between microvascular density and cellular proliferation in undifferentiated nasopharyngeal carcinoma.

Methods: A series of 60 cases were studied in patients of Southeast Asian origin. Cellular proliferation was determined using Ki67 immunostaining, and vessel proliferation using CD31 immunostaining in terms of areas of increased staining ('hot spots'). Ki67 results were scored on a scale of 0-4+ and CD31 results as a microvascular density/mm^2.

Results: The mean of the microvascular density was 22/mm^2 in the Ki67-negative group (25 cases). In the Ki67-positive group (35 cases), the mean was 35/mm^2. The difference between the positive and negative group was statistically significant ($p < 0.001$). Microvascular density significantly increased as the Ki67 score increased ($p<0.001$). However, the 'hot spots' for microvascular density in tissue sections did not correspond to areas of increased cellular proliferation.

Conclusion: Pathologists usually determine only one of these two prognostic factors when dealing with undifferentiated nasopharyngeal carcinoma. The proliferation index is suggested because it is easier to perform and can be done on small biopsies not to contain enough surface area for microvascular density determination.

Keywords: CD31, Ki67, microvascular density, morphometry, nasopharyngeal carcinoma, proliferation index

Nasopharyngeal carcinoma (NPC) is the most common epithelial malignancy of the nasopharynx, and is distinctive for both its epidemiological and biological characteristics. NPC is endemic in Southern China, South-East Asia, and North Africa, where the undifferentiated subtype is observed most frequently [1, 2]. Undifferentiated NPC is considered as a histological subtype of non-keratinizing NPC, according to classification by World Heath Organization (WHO) [1].

Correspondence to: Mana Taweevisit, MD, Department of Pathology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. E-mail: dr.mana4@gmail.com

The WHO staging system and histologic subtyping remain the main prognostic factors for NPC. This tumor is often highly malignant, with a five-year survival of only 10-40% [3]. Moreover, the biologic behavior is variable, such that patients with similar stages receiving similar therapy demonstrate differences in outcome. Thus, efforts have been made to identify additional prognostic markers for better patient management [4].

NPC, particularly the undifferentiated subtype, is strongly associated with Epstein-Barr virus (EBV) in endemic regions [5, 6], and pre- and post-treatment plasma EBV-DNA levels have been shown to correlate with outcome and survival [7]. However, this type of assay is expensive, and requires special

technology. Therefore, it is only available in larger medical centers [8, 9]. In addition, the prognostic value of this test is limited in low incidence areas such as Western countries where most cases are EBV-negative.

Because of these limitations, other tumour parameters that are less expensive and less labour intensive to determine have been examined including nuclear proliferation rates and tumor-related angiogenesis. Different studies have not yielded consistent results in terms of the prognostic values of such indices. For example, when studying the proliferation index for NPC cases based on positive immunostaining for Ki67 (MIB1), different groups have found that higher values correlated with decreased patient survival [10, 11], while others found no correlation [3, 12, 13]. One problem with these studies is that not all were studying the same subtypes of NPC, nor were sections scored in a consistent fashion.

The same problem exists for determinations of tumour-related angiogenesis. Angiogenesis is a basic requirement for nutrition and oxygenation of tumor cells. It is necessary for the cellular proliferation and metastatic spread of solid neoplasm including NPC [14-17]. Increased microvascular density has generally been linked to a worse prognosis in several cancers including breast, colon, melanoma, and the female genital tract [17-21]. However, in the case of NPC, vessels in different tumor subtypes have been counted either manually or with computer assistance, based on immunostaining with different antibodies marking endothelial cells. Then, prognostic values determined using different cutoff values. The results from the various studies showed a significant correlation with patient survival [13, 15] *vs*. none [3, 6, 22-24]; a significant correlation with metastatic spread [13, 14, 25-27] *vs*. none [22-24, 28]; and a significant correlation with disease recurrence [23, 26] *vs*. none [22, 24, 28].

Because of these inconsistencies, it is difficult to appreciate how these different parameters relate to each other. In Thailand, there is a high prevalence of the undifferentiated subtype of NPC, providing us an opportunity to carry out studies on a more uniform tumour group. In this study, we evaluated the proliferative index together with microvascular density in a series of sixty such cases. We then determined the correlation between these two indices, and addressed whether areas of high proliferation were related to areas of increased vascular density. We are not aware of this particular analysis being previously reported for NPC.

Materials and methods

Case series

Sixty cases diagnosed with undifferentiated nasopharyngeal carcinoma between 2001 and 2005 were collected from the surgical pathology files at King Chulalongkorn Memorial Hospital. The histology was reviewed on two micron-thick, hematoxylin and eosin-stained slides. Data about staging and metastatic spread were obtained from the patient records. The study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University.

Immunohistochemistry

Three-micrometer formalin-fixed, paraffin embedded tissue section were mounted on positively-charged slides and baked over night at 60°C prior to immunostaining. Proliferating cells were identified by Ki67 staining (Dako, Glostrup, Denmark; dilution 1:300), and vessels by CD31 (Dako; dilution 1:20). Immunostaining was performed on the Ventana Benchmark^{XT} auto-immunostainer (Tucson, USA) using the ultraVIEW Universal DAB (diaminobenzidine) Detection Kit (Ventana, Arizona, USA). Slides were counterstained with hematoxylin.

Morphometric assessment

Proliferation index was determined by counting the number of Ki67-positive cells among the neoplastic cells. The intent was to count those fields with the greatest number of Ki67 positive cells ('hot spots') as determined by the 10x objective. The proliferating cells were distributed evenly throughout the tumour, and one field was selected per slide and counted manually using a 40x objective. Then, results were categorized as follows: negative (<10% positive cells), 1+ (10-25%), 2+ (26-50%), 3+ (51-75%) and 4+ (>75%).

For assessment of microvascular density, CD31-stained slides were examined at low power (10x objective) for areas of increased numbers of vessels ('hot spots'). The 'hot spots' were photographed using a 40x objective on a Nikon Eclipse E600W microscope equipped with a digital Nikon camera (Tokyo, Japan) and DXM1200F software. Four fields were selected per slide providing a total area of 1 mm^2. Intratumoural and peritumoural microvessels (<50 μm in diameter without muscular wall as per Rubio et al. [29]) were counted in each of the four fields, and results combined to arrive at a count for microvessels/mm^2.

Statistical analysis

Data were analyzed by statistical program SPSS for Windows (Version 11.5). Student *t*-test and Spearman's rank correlation coefficient were used to test the differences of the mean of microvessel numbers between the group of neoplasms with negative Ki67 and positive Ki67 scores of varying levels. Confidence intervals (CI) were 95%. A p value of <0.05 was considered statistically significant.

Results

Clinical features

The sixty cases consisted of 45 males (75%) and 15 females (25%) with a mean age of 47 years (SD=13.7). Eight cases (13%) were stage I, 12 cases (20%) stage II, 19 cases (32%) stage III, and 21 cases (35%) stage IV, according to TNM staging of the head and neck tumors by World Health Organization (WHO 2005) [1]. Of the stage IV cases, there were 11 cases with stage IVA and 10 cases with stage IVB but no cases with metastatic spread (stage IVC).

Proliferation index

The nuclear proliferation index scored by Ki67 staining levels as related to tumor stage is shown in **Table 1**. Overall, there were 25 cases (42%) scored as 'negative', 16 cases (27%) as 1+, 5 cases (8%) as 2+, 3 cases (5%) as 3+, and 11 cases (18%) as 4+. No significant correlation was found between the proliferation index and stage, after statistical analysis. This was true regardless of whether stage IV was calculated as one group or divided into stages IVA and IVB.

Microvascular density

Overall, the mean microvascular density scored by CD31 staining was 29/mm^2. The density as related to tumour stage is shown in **Table 2**. The means of microvascular density according to tumor stage were stage I, 18/mm^2; stage II, 32/mm^2; stage III, 31/mm^2; and stage IV, 25/mm^2. No significant correlation was found between the microvascular density and tumor stage.

Table 1. Nuclear proliferation score by level of Ki67 staining according to tumor stage.

Stage	Number of cases (%)	Level of Ki67 staining				
		Negative (<10%)	1+ (10-25%)	2+ (26-50%)	3+ (51-75%)	4+ (76-100%)
I	8 (13%)	4	3	0	1	0
II	12 (20%)	4	3	2	1	2
III	19 (32%)	8	5	1	1	4
IV	21 (35%)	9	5	2	0	5
Total	60 (100%)	25 (42%)	16 (27%)	5 (8%)	3 (5%)	11 (18%)

Table 2. The ranges and means (with standard deviation) of microvascular density according to tumor stage.

Stage	Number of cases (%)	Microvascular density (1/mm^2)	
		Range	Mean (SD)
I	8 (13%)	6-28	18 (8.1)
II	12 (20%)	16-54	32 (13.2)
III	19 (32%)	4-60	31 (14.8)
IV	21 (35%)	4-43	25 (10.8)
Total	60 (100%)	4-60	29 (13.0)

Correlation of proliferative index and microvascular density

A comparison between the proliferation index and microvascular density results is presented in **Table 3.** The 25 cases of undifferentiated NPC scored as 'negative' for Ki67 staining showed a mean microvascular density of 22/mm^2. The remaining 35 cases, with Ki67 positive scores ranging from 1-4+, showed a mean microvascular density of 35/mm^2. The microvascular density in undifferentiated NPC with a positive Ki67 score was significantly higher than those with a negative score ($p < 0.001$). In addition, there was a significant correlation between the specific proliferative index score and microvascular density ($r = 0.508$, $p < 0.001$).

For the proliferation index used in this analysis, fields were selected based on examination of the Ki67-stained slide alone, without reference to the CD31-stained slide. To determine whether 'hot spots' for increased microvascular density were also 'hot spots' for proliferation or not, the same fields were located on both slides (**Fig. 1**). The Ki67 proliferation index on fields that were CD31 'hot spots' was comparable to the Ki67 proliferation index initially obtained (i.e., without knowledge of the CD31 results). Moreover, the fields initially assessed for the Ki67 counting (the results in Table 1) did not correspond to a CD31 hot spot.

Table 3. The distribution of nuclear proliferative levels and microvascular density for 60 patients with undifferentiated nasopharyngeal carcinoma.

Nuclear proliferation Level of Ki67 staining	Number of cases (%)	Microvascular density (1/mm^2) Range	Mean (SD)
Negative (<10%)	25 (41%)	4-60	22 (13.9)
1+ (10-25%)	16 (33%)	16-47	31 (10.2)
2+ (26-50%)	5 (10%)	22-54	33 (12.5)
3+ (51-75%)	3 (6%)	28-40	35 (6.2)
4+ (76-100%)	11 (10%)	25-52	36 (9.2)
Total	60 (100%)	4-60	29 (13.0)

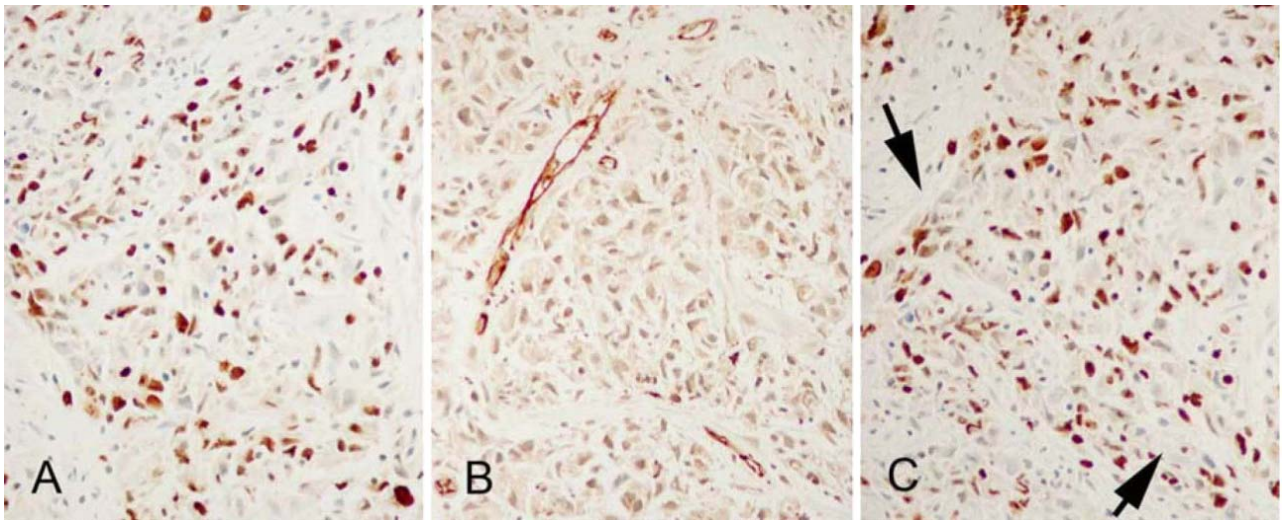

Fig. 1 (**A**) Cell proliferation as demonstrated by Ki67 immunostaining. (**B**) 'hot spot' for microvascular density as demonstrated by CD31 immunostaining. (**C**) same field stained for Ki67 showing comparable proliferation index to field shown in (**A**). Arrows mark microvessels that stain for CD31 in **B** because they do not stain for Ki67. (AC, original magnification x400).

Discussion

There have been many studies carried out on NPC measuring proliferation index and/or microvascular density, with different results and varying conclusions about the prognostic value of these parameters. Several different factors may affect these results. Not all studies examined a uniform group of NPC cases, but included all subtypes, that vary in their biologic behavior [1, 2] and/or included both EBV-positive and negative cases, because the latter are more common in Western countries [5, 6]. For this reason, we chose to study a more homogeneous group, namely 60 cases of undifferentiated NPC in Asian patients. Only a few other studies have included such numbers of undifferentiated NPC, ranging from 21-78 cases [3, 6, 11, 15]. Even within a uniform group of tumours, sampling can be a problem if tissue biopsies are small and 'hot spots' are not well represented [24, 30].

With respect to proliferation index, we found no correlation with tumor stage. It could not correlate with metastatic spread because none of our patients had metastases. Some studies have reported that higher indices correlate with decrease in patient survival [10, 11], while others have reported no correlation [3, 12, 13]. There were variations in their study designs. In fact, some studies included only high stage tumors [10], some counted 'hot spots' [11], and different cutoff values were used to assess prognostic significance. For example, using the value of <10% as 'low', a significant correlation was found with improved patient survival [11]. On the other hand, using 30% as 'low', no correlation was found [3]. However, in that same study, based on undifferentiated NPC in Asian patients (as is ours), 75% of cases had a proliferation index of 30% or greater. This contrasts with our study in which only approximately 25% of cases had proliferation indices in this range. These differences may account for the reason why this particular study did not find any correlation with patient outcome.

Studies on the microvascular density in NPC suffer from the same variability in results and significance as for the proliferation index. Similar concerns apply in terms of the tumor type studied and the methods for measuring microvascular density (reviewed by Erovic et al. [23]). Only two studies [5, 6] focused on undifferentiated NPC, while other studies included better-differentiated types of NPC. Most studies, including our work, have counted 'hot spots' rather than random fields [3, 6, 14, 23-26, 29]. Most have performed manual counts, as did we, but a few have used computerized-image analysis systems [23, 29]. The results were comparable to those obtained by manual counting. However, manual counting allowed better discrimination of false-positive staining. Thus, the method of counting is not likely to explain differences in results between various studies. Specialized-image analysis systems are not available in all medical centers, and are not part of daily practice. On the other hand, a manual method is suitable for any medical center to carry out assessment of microvascular density. For this reason, we selected to use a manual method for counting.

Another possible source of variation in previous studies is the antigen chosen for highlighting vessels in sections. Most studies have used antibodies directed against factor VIII-related antigen [3, 6, 14, 22, 24, 26, 28, 29], with others choosing anti-CD31 [25, 29] or anti-CD34 [23, 29]. Only one study compared all three and concluded factor VIII-related antigen gave the most specific results. This depends on the individual laboratory and how each antibody performs. In our hands, we found anti-CD31 was the best antibody for highlighting vessels with the least background staining plus no staining of other cell types.

The greatest source of variation in studies may be the cutoff values selected for microvascular density analysis. Some of these are based on a microscopic field (200x) with vessels counts in the range of 10-240/field [13, 24, 25, 29], and others on actual area measurements (mm^2) [3, 23]. Results were comparable in one study, with values of 50-60/130mm^2 (the calculated area of a 200x field) [23], whereas another study found much higher values, in the range of 200-1000/mm^2 [3]. We also chose to use an area measurement. This measurement is more reproducible, compared to microscope fields that can vary in area between microscopes. We could obtain vessel counts in the range of 4-60/mm^2. Thus, our values are higher than most other studies, but not as high as those obtained by Ma et al. [3]. We studied a more homogeneous series of undifferentiated NPC in Asian patients than any other investigators. There is no defined standard for microvascular density values in terms of low *vs*. high. Studies have variably designated as 'high' the top 25% of values [29], >60 vessels/200x field [13, 24], or arbitrary values derived from statistical analyses [26]. With these different approaches, it is not difficult to understand that some groups have found significant correlations between

vessel count and patient survival [13, 15] or presence of metastasis [13, 14, 25, 27], while others did not [3, 6, 22-24, 26, 28]. In our series, we found no correlation with tumor stage but could not address the issue of metastases.

None of the previous studies have specifically examined if there is a relationship between cellular (tumor) proliferation and vascular proliferation. We hypothesized that increased tumor proliferation would promote to increase number of vessels, that would be needed to supply the nutrient needs of the more rapidly dividing tumor cell populations. This association has been noted in other tumours such as colon and ovarian cancer [18, 31]. In our study, we demonstrated that the microvascular density in cases of undifferentiated NPC with a positive Ki67 index was significantly higher than those with a negative Ki67 index. Furthermore, the more positive the Ki67 index, the higher the microvascular density. These results may support the concept that neoplasm with high proliferation activity are likely to generate many neovessels for supplying the energy demands of the tumor, but they do not imply that the areas of greatest proliferation are those with the greatest vessel density. We found that the proliferating cells were restricted to tumor cells (i.e., endothelial cells were not proliferating) and were generally evenly distributed throughout the tumor. There were not 'hot spots' for proliferation, meaning that 'hot spots' for microvascular density were not 'hot spots' for proliferation. While increased cellular proliferation is associated with increased microvascular density, the latter is not a direct result of the former.

Determination of proliferation index and microvascular density both require extra time to carry out. In our opinion, the proliferation index is the simpler parameter to measure, and can be performed even on small biopsies that may not contain enough tissue for a microvascular density determination. Because a significant association was not found between these two parameters in undifferentiated NPC, it may be reasonable in daily pathology practice to determine only one of these for prognostic purposes.

In conclusion, the proliferation index is suggested because it is easier to perform and can be done on small biopsies not to contain enough surface area for determination of microvascular density.

The authors have no conflict of interest to declare.

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