The Role of a Minimum Immunohistochemical Antibody Panel in Confirming Undifferentiated Nasopharyngeal Carcinoma: A Cross-Sectional Study at the Muhimbili National Hospital, Dar-es-Salaam, Tanzania

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Abstract

Introduction: Nasopharyngeal carcinoma (NPC) is a malignant epithelial neoplasm arising in the nasopharyngeal mucosa that shows light microscopic and/or ultrastructural evidence of squamous differentiation. Immunohistochemistry (IHC) can be used to reliably distinguish undifferentiated NPC from other malignant tumors, and the technique may be a necessary tool toward the arrival of a definitive diagnosis, particularly when dealing with challenging cases. Materials and Methods: This was a cross-sectional hospital-based study which was conducted at Muhimbili National Hospital. The study involved 120 patients with NPC who were diagnosed on histopathological basis between 2009 and 2013. Results: The sensitivity and specificity of hematoxylin and eosin (H and E) stain in diagnosing NPC were 99% and 30.4%, respectively. The accuracy of H and E stain to diagnose NPC and lymphoma was 94.2% and 30.4%, respectively. CD45 antibody helped to confirm 16 cases which were diagnosed as NPC on H and E stain to be lymphoma. Further, AE1/AE3 antibody helped to confirm one case who was diagnosed as rhabdomyosarcoma on H and E stain to be NPC. Conclusions: The sensitivity and accuracy of H and E stains to diagnose NPC were very high whereas the specificity was very low. A significant proportion of previously diagnosed NPC cases by routine H and E stains were confirmed not to be so by a minimal IHC antibody panel of pan-cytokeratin cocktail (AE1/AE3) and leukocyte common antigen (CD45). This highlights the paramount importance of a minimum IHC panel in assisting to obtain a definitive diagnosis in challenging cases of NPC.

Keywords: AE1/AE3, CD45, nasopharyngeal carcinoma, undifferentiated

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a malignant epithelial neoplasm arising in the nasopharyngeal mucosa that shows light microscopic and/or ultrastructural evidence of squamous differentiation. The nasopharyngeal space being hidden allows for significant spread of nasopharyngeal cancer before any useful medical intervention is carried out. Lack of knowledge of the peculiar clinical features of disease allows the spread of this disease to advanced stage before diagnosis and medical intervention.

Examination of tissue sections stained with hematoxylin and eosin (H and E) stains is of paramount importance, in the diagnosis, classification, grading, and staging of malignancy. Besides, it is a daily practice in pathology laboratories. Challenges arise due to the fact that histological analysis is influenced by the practitioner’s experience, bias, adequacy, and quality of the sections as well as training of the one reporting the biopsies. Studies have shown that there is high

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interobserver and intraobserver variability in tumors which are undifferentiated. Immunohistochemistry (IHC) has significantly helped in the diagnosis of tumors that cannot be correctly diagnosed using routine H and E stains. In a study by Gatter et al., in which more than 100 anaplastic tumors were included, the H and E diagnosis of carcinoma or lymphoma was revised in approximately 50% of cases following IHC confirmatory analysis.

However, information in the literature regarding the accuracy or sensitivity of H and E stains in diagnosis of NPC is scanty, particularly in settings where there is absolute absence of immunostaining. The accuracy of routine H and E in diagnosing NPC is addressed in this study by correlating with a gold standard of a minimum IHC panel using only a pan-cytokeratin (CK) cocktail marker (AE1/AE3) and pan-leukocyte marker (CD45) monoclonal antibodies.

The former (AE1/AE3) is a combination of two different clones of anti-CK monoclonal antibodies, AE1 and AE3 both of which detect certain high- and low-molecular weight keratins, respectively. AE1 detects the high-molecular weight CKs 10, 14, 15, and 16 and also the low-molecular weight CK 19. Clone AE3 detects the high-molecular weight CKs 1, 2, 3, 4, 5, and 6 and the low-molecular weight CKs 7 and 8. By combining these two, a single reagent with a broad spectrum of reactivity against both high- and low-molecular weight CKs is obtained and is thus called “pan-CK.” Likewise, the pan-leukocyte marker CD45, also called leukocyte common antigen, is used to distinguish lymphomas from other tumors.

The aim of this study was to determine the role of a minimum IHC antibody panel in confirming undifferentiated NPC.

**Materials and Methods**

**Study design**

This was a cross-sectional hospital-based study. The study involved 120 cases of patients with NPC who were diagnosed between 2009 and 2013.

**Study settings**

The study was conducted at the Department of Pathology, Muhimbili University of Health and Allied Sciences (MUHAS), and the Muhimbili National Hospital (MNH), which receives referred patients from all over the country, and it also serves as a national consultant hospital, a zonal referral hospital for East and Southern Tanzania as well as a university teaching hospital for MUHAS. Patients’ biodata were obtained from the files and were also complemented with the clinical information from the laboratory requisition forms.

**Laboratory methods**

H and E histological sections of 4-μm thickness were prepared after retrieving the formalin-fixed paraffin-embedded (FFPE) tissue blocks from the histopathology laboratories at MNH. The sections were de-waxed by placing the slides in the microwave at 60°C for 50 min, and then cleared in two changes of xylene using 10 dips in each. Hydration was done by dipping the sections 10 times in the following changes of alcohol: absolute ethanol, 95%, 80%, and 70% ethanol followed by rinsing in running tap water and then stained with routine H and E stains. The slides were reviewed using a light microscope (Olympus Corporation, CX31RBSF Model, Tokyo, Japan) by two independent pathologists. The previous histopathological diagnoses of all the 120 cases with NPC were reviewed. All the cases were subjected to both AE1/AE3 and CD45 monoclonal antibodies staining.

**Immunohistochemistry**

The FFPE tissue blocks used for the original histopathological diagnoses of the 120 cases were then subjected to IHC staining. One representative section with thickness of 4 μm was prepared. The sections were de-waxed by placing the slides in the microwave at 60°C for 50 min, and then cleared in xylene. Hydration was done by dipping the sections in absolute ethanol, 95%, 80%, and 70% ethanol and then rinsed in distilled water. A ring was made around the section using Pap pen to limit the spread of antibody solutions. Two drops of 3% H₂O₂ solution was added to each section for 15 min to block endogenous peroxidase, and then, the slides were rinsed in distilled water. The slides were incubated in the antigen retrieval solution citrate buffer of pH 6.0 within a pressure cooker at 95°C, from which the slides were removed after 2 min of full pressure.

The slides were placed in distilled water waiting for addition of tris-buffered solution (TBS, Dako, Denmark) to the sections for 3 min. TBS was then drained from slides before adding the primary antibody of pan-CK (FLEX Monoclonal Mouse Anti-Human CK clone AE1/AE3 ready to use, Dako, USA) and allowed to incubate for 1 h. The slides were washed with TBS for 5 min, and then, two drops of horse rabbit peroxidase was added to each section for 30 min. The slides were again washed with TBS for 5 min. TBS was drained from the sections; then, chromogen diaminobenzidine was added to the sections for 5 min. The sections were washed in distilled water, counterstained in hematoxylin for 10 s, and differentiated by 2 dips into 1% acid alcohol. The sections were blued in warm water for 2 min, dehydrated through 70%, 80%, 95%, and 100% ethanol, and then cleared in xylene for 10 min. The sections were finally cover-slipped using Distyrene Plasticizer Xylene and were ready for assessment and interpretation. The similar protocol according to the manufacturer was used for CD45 (Flex Monoclonal Mouse Anti-Human CD45, Leukocyte Common Antigen, Clones 2B11 + PP7/26 ready to use, Dako, Denmark).

The immunoreactivity was assessed in hot spot areas which also were free from artifacts. The intensity of AE1/AE3 and CD45 antibodies staining was graded into four groups: 0: no staining of the tumor cells, +1: positive but <25% of tumor cells (weak positivity), +2: from 26%–60% of tumor cells (moderate positivity), +3: >60% of the tumor cells (strong positivity). Negative control for the immunostain was achieved by omitting the primary antibody and replacing it with TBS.
The positive control for AE1/AE3 was normal skin while that of CD45 was a normal tonsil.

Data analysis

Data entry was first done using EpiData Version 3.1. (Jens M. Lauritsen & Michael Bruus, Odense, Denmark). Then, data were exported to Statistical Package of the Social Sciences Version 20.0 (IBM SPSS Statistics, Chicago, USA) for analysis. Diagnostic accuracy of the H and E-stained sections was compared with that of the gold standard IHC panel consisting of AE1/AE3 and CD45 as the confirmatory tests. Sensitivity, specificity, and accuracy of histopathological diagnosis for undifferentiated (challenging) NPC as well as lymphomas were determined.

Ethical clearance

Ethical approval for conducting this study was obtained from the ethical clearance committee of MUHAS, and a reference number MU/PGS/SAEC/VOL.XII/44 was issued. In addition, permission for using the FFPE tissue blocks was sought from the management of MNH and reference number MNH/TRC/2014/80 was provided. All patients’ biodata in this study were treated as being strictly confidential and their identities were not revealed.

Results

This study involved 120 cases of patients with NPC. The median age of the patients was 39 years, with range of 9–88 years. Majority of the patients 65% (78/120) were males and 35% (42/120) were females. The male-to-female ratio of the patients in this study was 1.9:1.

Most of the cases in this study 60% (72/120) were undifferentiated NPC cases followed by differentiated and keratinizing types of NPC cases, which accounted for 24.5% (29/120). Table 1 represents the different diagnoses based on H and E staining before subjecting them on IHC staining. Among the 120 of NPC cases which were included in the study, 60% (72/120) were not straightforward cases and could not easily be diagnosed on routine H and E staining [Figure 1], making the NPC cases that could easily morphologically be substantiated to be NPC were 40% (48/120). Challenging cases (60%, 72/120) and those that were straightforward (40, 48/120) were subjected to IHC testing.

Table 2 shows the IHC staining of the 120 cases using AE1/AE3. Among the 112 cases which were previously diagnosed as NPC on H and E staining, 80% (96/120) of them were confirmed by AE1/EA3 truly to be NPC, whereas the remaining, 13.3% (16/120) which were previously diagnosed as NPC on H and E staining, turned to be lymphomas and not NPC. In all the six cases of lymphomas, none of them was positive for AE1/AE3 antibody testing.

Table 3 shows the staining of CD45 antibody for the cases which were initially diagnosed on H and E stains as either NPC or lymphoma. CD45 showed strong positivity in all the 13.3% (16/120) cases which were previously diagnosed as NPC on H and E stains [Figure 2]. These were negative for AE1/AE3, and hence, they were confirmed as lymphomas on CD45 monoclonal antibody. Likewise, CD45 showed strong positivity [Figure 3] for all the 6 (100%) cases which were also thought to be lymphomas even on routine H and E stains.

Table 4 shows the sensitivity and specificity of H and E stains for diagnosing NPC cases. The sensitivity of H and E diagnosis

| Hematoxylin and eosin diagnosis | Number of patients (%) |
|---------------------------------|------------------------|
| NPC                             | 112 (93.4)             |
| Lymphoma                        | 6 (5)                  |
| Rhabdomyosarcoma                | 1 (0.8)                |
| Plasmacytoma                    | 1 (0.8)                |
| Total                           | 120 (100)              |

NPC – Nasopharyngeal carcinoma

| Hematoxylin and eosin diagnosis | Number of cases (%) | AE1/AE3 staining | Total (%) |
|---------------------------------|---------------------|------------------|-----------|
| NPC                             | 112 (93.3)          | 96 (80)          | 120 (100) |
| Lymphoma                        | 6 (5)               | 0 (0)            | 6 (100)   |
| Rhabdomyosarcoma                | 1 (0.8)             | 1 (100)          | 1 (100)   |
| Plasmacytoma                    | 1 (0.8)             | 0 (0)            | 1 (100)   |
| Total                           | 120 (100)           | 97 (80.8)        | 120 (100) |

NPC – Nasopharyngeal carcinoma

Figure 1: A photomicrograph showing an undifferentiated nasopharyngeal tumor section. The tumor cells are mixed with small lymphocytes and plasma cells; this case was confirmed to be a lymphoma by CD45 antibody (H and E, ×400)
for NPC in the study was 97/98 × 100% = 99%, and the specificity was 6/22 × 100% = 27.3%. The positive predictive value (PPV) of H and E stains was 97/113 × 100% = 85.8% whereas the negative predictive value (NPV) was 1/7 × 100% = 14.3%. Therefore, the accuracy of H and E stains to diagnose NPC in this study was (97 + 6)/120 × 100% = 94.2%.

The sensitivity and specificity of H and E stains for diagnosing lymphoma cases were also determined as shown in Table 5. The specificity of H and E stains in diagnosing lymphoma cases was 6/16 × 100 = 27.3% and the specificity was 1/1 × 100 = 100%. The PPV and NPV for H and E stains was to diagnose lymphoma cases were 6/6 × 100 = 100% and 16/17 × 100 = 94.1%, respectively. The accuracy of H and E stains to diagnose lymphoma was 7/23 × 100 = 30.4%.

**Discussion**

The current study determined the role of H and E stains in diagnosing NPC, particularly cases that are undifferentiated which may easily mimic other nonepithelial malignancies including lymphoma. The role of an ancillary test, IHC comprising a minimum panel of only two antibodies (AE1/AE3 and CD45) was also determined as the method of confirming cases that are challenging. The sensitivity of H and E stains in diagnosing NPC in this study was as high as that has been reported in the previous studies.\(^{12,13}\) Adisa et al.\(^{12}\) reported a sensitivity of 97.1% close to 99% that was found in the current study for H and E stains in diagnosing NPC. In their study, the specificity was slightly higher (47.7%) than 30.4% that was found in this study. The observations from these two studies show that H and E stain is more sensitive than specific in diagnosing NPC, particularly undifferentiated ones. The specificity of H and E stains in diagnosing lymphoma of 98.4% in their study was very close to 100% obtained in the current study.

In another study by Tumwine et al.\(^{14}\) which determined the sensitivity and specificity of H and E stains in the diagnosis of Hodgkin’s lymphoma, they reported sensitivity and specificity of 76.61% and 92.75%, respectively, which were lower than the sensitivity and specificity which were found in the present study.

The diagnostic accuracy of H and E stains for NPC of 94.2% in this study is higher than 54.8% that was reported by Adisa et al.\(^{12}\) in Nigeria. In another study by Gouda et al.\(^{15}\) an accuracy of 100% higher than the 94.2% reported in the present study for H and E stains was reported for diagnosing undifferentiated NPC cases in Egypt. Taylor et al.\(^{16}\) also reported a slightly higher diagnostic accuracy of 100% of H and E stains in diagnosing NPC in a study which included

| CD45 IHC | Positive (%) | Negative (%) | Total (%) |
|----------|--------------|--------------|-----------|
| NPC      | 16 (25)      | 96 (80)      | 112 (93.3)|
| Lymphoma | 6 (100)      | 0 (0)        | 6 (100)   |
| Rhabdomyosarcoma | 0 (0) | 1 (100) | 1 (100) |
| Plasmacytoma | 0 (0) | 1 (100) | 1 (100) |
| Total    | 22 (29.7)    | 98 (81.2)    | 120 (100)|

**Table 3: CD45 immunohistochemistry staining for the cases**

NPC – Nasopharyngeal carcinoma, IHC – Immunohistochemistry

| Hematoxylin and eosin | IHC staining for AE1/AE3 |         |
|-----------------------|--------------------------|---------|
| Positive              | 96                       | 16      | 112     |
| Negative              | 1                        | 7       | 8       |
| Total                 | 97                       | 23      | 120     |

**Table 4: Sensitivity and specificity of hematoxylin and eosin for nasopharyngeal carcinoma in association with immunohistochemistry as the gold standard**

IHC – Immunohistochemistry

| Hematoxylin and eosin | IHC staining for CD45 |         |
|-----------------------|-----------------------|---------|
| Positive              | 6                      | 0       | 6       |
| Negative              | 16                     | 1       | 17      |
| Total                 | 22                     | 1       | 23      |

**Table 5: Sensitivity and specificity of hematoxylin and eosin for lymphoma in association with immunohistochemistry as the gold standard**

IHC – Immunohistochemistry

**Figure 2:** A photomicrograph showing a nasopharyngeal carcinoma section with strong cell membrane (brownish) staining of the monoclonal AE1/AE3 antibody (IHC, ×400)

**Figure 3:** A photomicrograph showing a nasopharyngeal tumor section with strong and diffuse cell membrane (brownish) staining of the monoclonal antibody CD45 in a lymphoma (IHC, ×400)
14 paraffin-embedded tissue of NPC. In their study, they also found that all the cases had positive staining reaction for CK and negative reactivity for lymphomas antibodies.

The higher diagnostic accuracy of H and E stains observed in the present study could be due to regular tumor board review of the cancer cases, including the NPC cases first by a panel of pathologists in the department and then the joint clinical meetings involving pathologists, surgeons, oncologists, and other stakeholders at MNH. In these two different settings, it has been observed that, when using H and E stains, a considerable number of NPC cases are diagnosed wrongly as it was found that 13.3% (16/120) of the cases which were previously diagnosed as NPC in the present study were finally confirmed by IHC as lymphoma. Another one case (0.8%) which was also diagnosed as rhabdomyosarcoma on H and E stains was later confirmed to be NPC. This means that, in most times, pathologists can diagnose NPC correctly on H and E-stained tissue slides. However, when pathologists are facing challenges in diagnosing undifferentiated cases of NPC, IHC should be incorporated in the diagnostic tests to avoid making wrong diagnosis, whose consequences may include wrong treatment, prolonged turnaround time, and unnecessary expenses, which altogether would culminate in poor clinical outcomes or even death.

The reason for the diagnostic discrepancy could be explained by the deceptive nature of NPC which may simulate histologically other lesions, such as lymphomas and other round cell malignant tumors. The deceptive histological features of undifferentiated NPC resembling histological morphology of malignant lymphomas suggest the possibility of the two entities to share some phenotypical and molecular basis. Therefore, due to this observed significant number of undifferentiated NPC mimicking other malignancies, the use of IHC stains ought to be regarded as an inevitable approach when handling such cases. The striking histological similarities between undifferentiated NPC and malignant lymphomas have been reported in the literature. For example, Kitcher et al. reported that serious problems on differential diagnosis with malignant lymphoma may occur and reviewed that a series of cases had been diagnosed as undifferentiated NPC, which were later found to be malignant lymphomas.

Although it is not possible for H and E stains to differentiate between undifferentiated NPC from other morphologically simulating malignancies, studies have shown that, with H and E staining, one can be able to diagnose lymphomas whose both clinical presentation and histopathology were deemed to be NPC; of course by also considering site of the lesion itself. In our series, 100% of lymphomas diagnosed by routine H and E stains were also confirmed by IHC using CD45 monoclonal antibody similar to what was communicated by Gouda et al. in their study. This is higher than 80% that was reported in the study by Adisa et al. in Nigeria. The present study demonstrates the role of affordable IHC panel that involves only the use of two markers in distinguishing between malignant lymphomas and undifferentiated NPC. This study, thus, agrees with others which suggest that IHC has a great value in differentiating undifferentiated tumors.

The staining properties of the AE1/AE3 antibody suggest that this may be the best method for differentiating undifferentiated NPC from nonepithelial tumors in diagnostic surgical pathology which are more likely to pose histomorphological diagnostic challenges. The AE1/AE3 and CD45 findings in the present study are particularly relevant in surgical pathology practice as nasopharyngeal biopsies are often small and considerably distorted by crush artifact. This means that the diagnostic dilemma as to whether a nasopharyngeal tumor is a carcinoma or a lymphoma, with its consequent effect on the treatment and prognosis of the patient, can be reliably solved by any pathologist augmenting the routine H and E-stained histological examination with IHC using such a panel of monoclonal antibodies, which comprises a minimum number of antibodies.

**Conclusion**

Although H and E stains can be used to diagnose NPC and even lymphoma from nasopharyngeal biopsies, still there is a need to include IHC stains to ensure that there is no case that may be diagnosed wrongly. From this study, it has been observed that, in settings with limited resources, a combination of one CK and CD45 antibodies may satisfactorily help to distinguish undifferentiated NPC from lymphoma.

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**Conflicts of interest**

There are no conflicts of interest.

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