KI67 AS A SURROGATE MARKER FOR MITOTIC KARYORRHECTIC INDEX IN NEUROBLASTOMA

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ABSTRACT

Objective: To evaluate the use of Ki67 as a surrogate marker for Mitotic Karyorrhectic Index (MKI) in Neuroblastoma.

Study Design: Cross-sectional study.

Place and Duration of Study: This was a retrospective study conducted at Department of Histopathology, Shaukat Khanum Memorial Cancer Hospital and Research Centre, Lahore, Pakistan. 41 cases of neuroblastoma from 2010 to 2014 were retrieved.

Methodology: Forty one cases of neuroblastoma were evaluated. Clinical and morphological features like age, gender and subtype of tumor were quantified. For each case, Mitotic Karyorrhectic Index was calculated and categorized as low, intermediate and high. This was followed by immunostaining for Ki67 on each case. Using linear regression model an equation was derived to calculate ki67 from Mitotic Karyorrhectic Index. The equation was as follows: Mitotic Karyorrhectic Index = (Ki67 x 0.115) ± 1.355. Using this equation, the cut-off values for low, intermediate and high Ki67 were <5.60%, 5.60% - 23.0% and >23.0% respectively.

Results: Twenty four cases had low Mitotic Karyorrhectic Index and in 18/24 cases, Ki67 was also low. There were 5 cases with intermediate Mitotic Karyorrhectic Index with two cases showing correspondingly intermediate Ki67 index. There were 12 cases with high Mitotic Karyorrhectic Index and 10/12 cases showed a high Ki67 index as well.

Conclusion: Ki67 is a useful technique and may be used as an adjunct to Mitotic Karyorrhectic Index for better evaluation of cases.

Keywords: Ki67, Mitotic karyorrhectic index, Neuroblastoma.

INTRODUCTION

Neuroblastoma is the most common extra-cranial tumor in childhood and accounts for 8-10% of all childhood tumors[1]. This tumor is responsible for 15% of cancer-related deaths in paediatric population[2,3]. The median age of diagnosis is 22 months with most patients being under the age of 5 years[1,3]. Primary sites of origin include paraspinal ganglia or the adrenal medulla. Metastatic disease has been seen in bone marrow (17%), bone (56%), lymph nodes (31%), lung (3%) and other internal organs (15-45%)[4]. There are 4 histopathologic types of neuroblastoma as recommended by the International Neuroblastoma Classification including (i) Neuroblastoma (Schwannian stroma-poor); (ii) Ganglioneuroblastoma, nodular (composite, Schwannian-stroma-rich/stroma-dominant and stroma-poor); (iii) Ganglioneuroblastoma, intermixed (Schwannian stroma-rich); and (iv) Ganglioneuroma (Schwannian stroma dominant)[5,6]. Neuroblastoma is a tumour showing cellular heterogeneity and variable clinical outcomes[7,8,9]. Important prognostic markers in neuroblastoma include age at diagnosis, morphologic features, tumor stage, genetic factors (n-myc) as well as Mitotic Karyorrhectic Index (MKI).

The MKI is one of the useful prognostic marker for neuroblastomas. MKI is the combined number of cells in mitosis and/or undergoing karyorrhexis based on evaluation of 5000 tumor cell nuclei of neuroblastoma[5,6]. There are 3 prognostic categories including low (L-MKI) = (<2% or <100/5000 cells), Intermediate (I-MKI): (2-4% or 100-200/5000 cells) and High (H-MKI)

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(>4% or 200/5000 cells)[5,12]. However, the evaluation of MKI requires manual counting of adequate microscopic fields to count 5000 cells. This is time-consuming and sometimes, depending on the observer, it might just be estimated rather than accurately measured. Additionally, a fair amount of expertise and a trained eye is needed to identify cells that show mitosis and karyorrhexis for accurate assessment.

Ki67 is commonly used in routine pathology practice as a proliferation marker. Ki67 is a proliferation associated nuclear antigen only present in dividing cells (and not in quiescent cells (G0 phase)[14]. Ki67 protein expression indicates proliferative activity of intrinsic cell population in malignant tumors and is used as a marker of tumor aggressiveness. It has the potential to be used as a reliable marker in cancers of the breast, soft tissue, lung, prostate, cervix and CNS[9,10].

Few studies have assessed ki67 immunohistochemistry in neuroblastoma and its prognostic role. However, only rare studies have attempted to relate Ki67 index to MKI[13]. The aim of this study is to evaluate the use of Ki67 as a surrogate marker for determination of MKI.

METHODOLOGY

This study is a descriptive cross sectional study. This study was conducted at Shaukat Khanum Cancer Memorial Hospital and Research Centre Lahore after approval from Institutional Review Board (IRB).

A total of 41 cases of neuroblastomas diagnosed between 2010 to 2014 were retrieved from histopathology records at Shaukat Khanum Memorial Cancer Hospital and Research Centre (SKMCH). Cases were included irrespective of age, gender and site. Autolyzed, scanty, poorly preserved samples were excluded from the study.

MKI is calculated by two methods. Manual counting of number of nuclei with mitosis and/or karyorrhexis or by calculating a percentage. We calculated MKI by assessing nuclei of 5000 cells and the combined number of mitosis and/or karyorrhexis in these cells was counted. When mitosis and/or karyorrhexis was shown by less than 100 cells out of the 5000 cells examined, then MKI was considered low. It was considered to be in intermediate category when mitoses and/or karyorrhexis noted was between 100-200. When more than 200 out of 5000 cells showed evidence of mitotic activity and/or karyorrhexis, then MKI was considered to be high[19]. MKI was re-evaluated by another experienced histopathologist to decrease inter-observer variability. In cases where more than one block was available, a representative block with maximum viable area was taken.

Ki67 Immunohistochemistry

Ki67 was then calculated for all 41 cases. Paraffin embedded-formalin fixed tissue was sectioned at 4 micrometer thickness and was deparaffinised. Ki67 FLEX Monoclonal Mouse Anti-human Ki67 Antigen (Clone MIB-1), Dako immunostaining was used.

Determination of Ki67 Index

For each case, 2-4 foci with high proliferation index (hotspots) were identified. A total of thousand cell nuclei were assessed for Ki67 nuclear staining. A percentage of ki67 for each case was taken out by counting the number of positive nuclei out of 1000 nuclei and taking out a percentage. Any amount of nuclear staining was considered as positive. Absence of nuclear staining was considered as negative. It is important to emphasize that in our study Ki67 was calculated blindly without knowing the manual MKI value for that case. In addition, Ki67 was confirmed by evaluation by another histopathologist and an average value taken where there was any difference.

Statistical analysis was carried out using the SPSS statistical software package (version 20.0; SPSS, Chicago, IL, USA). The Shapiro wilk test was used to check the normality of MKI values. Pearson correlation was used to determine the relationship between the Ki67 index and MKI, and simple linear regression was performed to predict the MKI value from the Ki67 index. The

linear regression model was then developed, and the co-efficient of determination (r2) was evaluated to define how well the data points fit with the model. Additionally, the 2-tailed, paired t-test was used to determine whether the manually obtained MKI differed significantly from the calculated MKI. *p*-values (0.05) were regarded as statistically significant.

Simple linear regression analysis revealed high significance levels (analysis of variance; $p<0.01$) with Ki67. Statistically significant levels for the intercept value and slope value of the linear regression were 1.355 and 0.115, respectively ($p<0.01$). In addition, the R2-value was moderate at 0.60. Using ki67 index as the x variable, the formula could be written using the following equation MKI = (Ki67 index x 0.115) ± 1.355.

Using the well-known published and established values of 2% and 4% to categorize low, intermediate and high MKI, the above equation calculated Ki67 Index of 5.60% for the 2% cutoff value and 23.0% for the 4% cutoff value. Thus, a case with low MKI would have a Ki67 index of <5.60%, an intermediate MKI would have a Ki67 index between 5.60% and 23.0% and a high MKI, a Ki67 index >23.0% table-I.

RESULTS

There were a total of n=41 cases. Age of patients ranged from one month to 25 years with a median age of 3 years for primary tumors and median age of 4 years for metastatic neuroblastomas. There were 22 males (53.7%) and 19 (46.3%) females with a male to female ratio of 1.16:1. Most lesions were from primary tumor sites (n=25) including suprarenal/renal (n=8),

Table-I: Comparison of MKI and Ki67.

	Low(%)	Intermediate(%)	High(%)
MKI	<2	2-4	>4
Ki67 index	<5.60	5.60-23.0	>23.0

Table-II: Various primary and metastatic sites of ongin of neuroblastoma cases of neuroblastoma cases with frequencies.

Primary Sites	Number of Cases	Metastatic Sites	Number of Cases
Mediastinum	2	Liver	3
Supra Renal/Renal	8	Extra Dural	1
Retro-Rectal	2	Scalp	1
Retro-Peritoneum	3	Hemi-thorax	1
Abdomen (NOS)	8	Axillary Lymph Node	1
Iliac Fossa	1	Mesenteric Lymph Node	2
Spinal Cord	1	Abdominal Lymph Node	1
		Inguinal Lymph Node	1
		Lymph Node (NOS)	5

Table-III: Correlation between low, intermediare and high MKI with correspondingly low, intermediate and high Ki67 values.

MKI (No of Cases, n=41)	Ki67	No of Cases	Percentage
Low MKI (n=24)	Low	18	75
	Intermediate	5	21
	High	1	4
Intermediate MKI (n=5)	Low	2	40
	Intermediate	2	40
	High	1	20
High MKI (n=12)	Low	1	8
	Intermediate	1	8
	High	10	83

spinal (n=1), retro-rectal (n=1), retroperitoneal (n=3), iliac fossa (n=1), spinal cord (n=1) and mediastinum (n=2). There were 16 cases of metastatic neuroblastoma which in addition to metastasis to lymph nodes (n=10) also included liver (n=3), scalp (n=1), thorax (n=1) and extradural region (n=1). In our series, in terms of degree of differentiation, 8% were undifferentiated type, 42% were poorly differentiated and 50% were differentiating type (fig-1).

The Ki67 indices ranged from 0.81% to 60.12% (mean 17.26 ± 19.89). A significant correlation between Ki67 index and MKI was

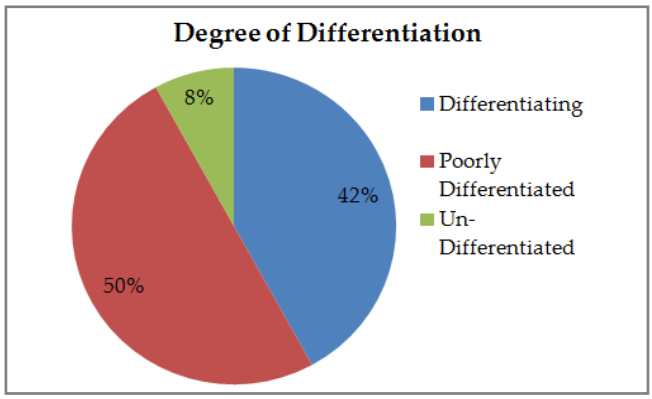

Figure-1: Degree of Differentiation.

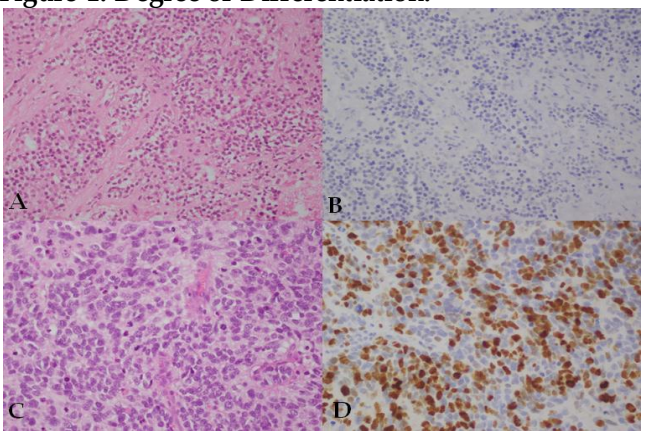

Figure-2: MKI and Ki67 in neuroblastoma tumor. A: Low-MKI tumor. B: Low-Ki67 in the same tumor as in A. C: High-MKI tumor. D: High-Ki67 in the same tumor as in C.

obtained (Pearson co-relation co-efficient; r= 0.78; p<0.001). MKI was calculated for each of the 41 cases. There were 24 (58.5%) cases with low MKI, 5 (12.2%) cases with intermediate MKI and 12 (29.3%) cases with high MKI. Ki67 was low proliferation index in 21 (51.2%) cases, intermediate proliferation index in 7 (17.1%) cases and high proliferation index in 13 (31.7%) cases. Out of 41 cases, majority (n=24) cases had low MKI. Out of these n=24 cases with low MKI, Ki67 was also low in 18/24 (75%) number of cases. However in the remaining 6 of 24 cases, Ki67 did not correspond. Out of these cases where Ki67 did not match, 5 cases had an intermediate Ki67 and 1 case showed high Ki67 proliferation index.

There were 5 (12.2%) cases with intermediate MKI with correspondingly 2 cases showing intermediate Ki67 index. However, in the remaining 3 cases, Ki67 proliferation index was high in 1 case and low in 2 cases. There were 12 (29.3%) cases with high MKI. In these 12 cases, Ki67 proliferation index was also high in 10 (83.3%) cases, low in 1 (0.8%) case and intermediate in 1 (0.8%) case.

DISCUSSION

Neuroblastomas are heterogeneous group of tumors. Internationally, efforts have been targeted at risk stratification of patients on the basis of prognostic factors including clinical stage, age at diagnosis, histologic features and molecular properties[11-15].

International Neuroblastoma Pathology Classification was introduced in 1999. It was later modified in 2003. This classification has prognostic implications. According to the INPC, peripheral neuroblastic tumors include (i) neuroblastoma (Schwannian stroma-poor), (ii) ganglioneuroblastoma, intermixed (Schwannian stroma-rich), (iii) ganglioneuroma (Schwannian stroma-dominant) and (iv) ganglioneuroblastoma, nodular (composite, Schwannian stroma rich/stroma-dominant and stroma poor). In this system, MKI is one histologic parameter which impacts prognosis[16-20].

Traditionally, MKI is determined by manually counting 5000 cells in multiple microscopic fields to evaluate mitosis and karyorrhexis. MKI refers to the total combined number of nuclei of cells showing mitosis and/or undergoing karyorrhexis. It is classified as low (>2% or,

100/5000 cells), intermediate (2% to 4% or 100-200/5000 cells) and high (>4% or >200/5000 cells).

MKI is an independent predictive factor for outcome in neuroblastoma. In a study using one of the largest cohort of neuroblastoma patients (n=11, 54; 1980-2002), 1860 patients were assessed for MKI and its effect on outcome. Overall survival was 73% in those with low MKI compared to 45% in those with high MKI[22].

A recent study of more than 4000 cases showed that 55% of tumors had low MKI, 25% with intermediate MKI and 20% with high MKI[21]. In our study, 58.5% of tumours had low MKI, 12.2% had intermediate MKI and 29.3% had high MKI. Calculating MKI is a tedious task. It is especially more difficult for tumors that are closer to the cutoff values between low and intermediate or intermediate and high usually need a very careful evaluation. This can be time consuming and a trained eye is needed for this. This limits this task to the histopathologist and technician or technologist cannot perform this[13].

Previous studies have shown that high risk cases of neuroblastoma which have advanced stage disease usually do not respond well to different therapeutic approaches, leading to poor survival[23,24]. This suggests that there is a strong need to find new molecular markers in neuroblastoma to improve targeted therapy and increase survival. Ki67 is a commonly used proliferation marker to determine growth fraction of cells in various malignancies[25]. Although several studies have examined Ki67 staining in neuroblastoma, only two studies have attempted to link Ki67 to the MKI. In both studies, there was a correlation between MKI and Ki67. In the study by Atikankul *et al* in 2015, MKI was correlated with Ki67[13]. They concluded that Ki67 expression permits reliable detection of the cellular proliferation fraction and provides useful prognostic information when associated with other biologic factors. In that study, Ki67 was calculated manually and also by automated image analyzers. A formula was devised by using the linear regression model to calculate MKI from the Ki67 index. The formula was MKI = MIB-1-index x 0.128 + 1.393. Using this formula, they were able to establish a correlation between the two variables and establish that Ki67 could be used as a surrogate marker for MKI. We devised a similar formula based on our results. The formula turned out to be MKI= (Ki67 index x 0.115) ± 1.355. The cut-off values for low, intermediate and high Ki67 varied slightly. Thus, it can be concluded that Ki67 can be used as an adjunct to MKI.

In another study by Mejia *et al*[19]. MKI was correlating with Ki67 analysis (p<0.05), revealing strong correlation especially amongst low MKI and low Ki67 (26/39, 66.7%). 31/45, (68.9%) cases matched for intermediate MKI and Ki67 and 11/15 (73.3%) cases for high MKI and correspondingly high Ki67. Our study revealed a strong correlation between low MKI with low Ki67 (75%) and even stronger for high MKI with high Ki67 index(83%). Our results for intermediate MKI did not correlate well with Ki67 with only 40% cases with a correspondingly intermediate Ki67 in contrast to results of study by Mejia *et al* which showed 68.9% concordance between MKI and Ki67. This may have several explanations. The number of cases was limited for the intermediate category (n=5). Also degree of differentiation may have a role with varying differentiations of neuroblastomas showing variable Ki67 index. This needs to be further validated by using a greater sample size and correlating with degree of differentiation in further projects.

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CONCLUSION

In conclusion, Ki67 is a useful technique and may be used as a complementary adjunct to MKI for better evaluation of cases.

CONFLICT OF INTEREST

This study has no conflict of interest to be declared by any author.

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