ARTICLE

Cyclin D1 immunohistochemical stain as adjunct immunomarker in CD99 positive malignant small round blue cell tumors with primary consideration of primitive neuroectodermal tumor/ewing sarcoma (PNET/EWS) in a pediatric tertiary hospital

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OBJECTIVE: To evaluate the diagnostic accuracy of Cyclin D1 as an adjunct immunomarker in CD99 positive small round cell neoplasms with primary consideration of PNET/EWS.

MATERIALS AND METHODS: Tissue from 2017 to 2023 with a histopathologic diagnosis of CD99 positive small round blue cell tumors with primary consideration of Primitive Neuroectodermal Tumor (PNET)/Ewing Sarcoma were retrieved and Cyclin D1 immunohistochemical staining was done. Diagnostic accuracy of Cyclin D1 immunostaining was determined by calculating the sensitivity, specificity, positive predictive value, and negative predictive value.

RESULTS: Cyclin D1 immunohistochemical staining was performed in 19 specimens, of which 13 yielded a positive result. Of these, 8 had a final histopathologic diagnosis of CD99 positive small round blue cell tumor with primary consideration of PNET/Ewing Sarcoma, resulting in sensitivity of 61.54%, specificity of 100%, positive predictive value of 100% and negative predictive value of 50.0%. The overall accuracy is 72.2%.

CONCLUSION: Cyclin D1 can be used as an adjunct immunomarker to aid in the diagnosis of CD99 positive round cell tumor with primary consideration of PNET/Ewing Sarcoma specifically in resource limited settings where molecular testing is not readily available. Given the high specificity of Cyclin D1 in such cases, it can be used to rule out other small round blue cell tumors that can also stain positive for CD99 such as Rhabdomyosarcoma. However, interpretation must be done in conjunction with the results of other immunohistochemical stains in order to increase its diagnostic accuracy.

Keywords: *Pediatric small round blue cell tumor, Primitive Neuroectodermal Tumor/Ewing Sarcoma, Cyclin D1*

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INTRODUCTION

Malignant small round blue cell tumors are neoplasms composed of small undifferentiated, round cells. Tumors under this category include but are not limited to the following: primitive neuroectodermal tumors/ Ewing Sarcoma, lymphoma, neuroblastoma, rhabdomyosarcoma and nephroblastoma. There is significant overlap in the morphology of these various tumors and diagnosis is currently based on a combination of histomorphology, immunohistochemical and molecular findings as such, these tumors are often indistinguishable by histomorphology common immunohistochemical markers.

Primitive neuroectodermal tumor (PNET)/Ewing Sarcoma are small round cell tumors with neuroectodermal differentiation. They commonly arise in the long bones, pelvis, and ribs and may also occur outside of the skeletal system. Immunohistochemically, strong, diffuse membranous expression of CD99 is seen in 98% of cases. PNET/Ewing Sarcoma are associated with FET-ETS fusion genes with the most common translocation of t (11;22) (q24;q12) resulting in EWSR1-FL1 fusion transcript and protein. Genetic and molecular testing for such fusion proteins is required for the diagnosis [1]. In a study by Magro et. al, immunohistochemical staining was done on 128 cases of small round blue cell tumors in children wherein all cases of genetically confirmed Ewing Sarcoma/ Primitive Neuroectodermal tumor and undifferentiated neuroblastoma component of peripheral neuroblastic tumor showed strong, diffuse, nuclear staining (greater than 50% in neoplastic cells) for Cyclin D1. No immunoreactivity with Cyclin D1 is noted in Rhabdomyosarcoma and precursor T-cell and B-cell lymphoblastic lymphoma. Focal immunoreactivity (less than 50% in neoplastic cells) was noted in the blastemal component of Wilms Tumor and in cases of Desmoplastic Small Round Blue Cell Tumor (DSRCT) [2]. A study by Nahid et. al on the evaluation of CCyclin D1 expression in common pediatric small round blue cell tumors involving sixty four (64) cases of small round blue cell tumors diagnosed through immunohistochemical staining or histomorphology. All cases of Ewing Sarcoma and Neuroblastoma showed nuclear positivity of Cyclin D1 in tumor cells. 77% and 64% showed diffuse nuclear expression of Cyclin D1 in Ewing Sarcoma and Neuroblastoma cases, respectively. No immunoreactivity was noted in Rhabdomyosarcoma and Lymphoblastic Lymphoma cases. In Wilms Tumor cases, only 40% showed focal, moderate intensity in the blastemal component. [3] Microanalysis of cases of PNET/Ewing Sarcoma tumors show significant upregulation of Cyclin D1 in comparison with normal tissues and could be a novel marker in the diagnosis and prognosis of such cases. MicroRNA (miRNA) are small noncoding ribonucleic acids (RNA) composed

of an average of twenty-two (22) nucleotides in length. Most are transcribed from DNA sequences and converted into primary miRNAs to precursor miRNAs and eventually into mature miRNA. miRNAs can play a role in activating translation or regulating transcription and are also associated with cancers, metabolic diseases, and viral infections [3]. In a study by Marino et. al, absent expression of microRNA mir-34a in patients with Ewing Sarcoma are associated with higher incidence of tumor related deaths, adverse events and a decrease in 5-year event free survival. Mir-130b is overexpressed in institutional cases of Ewing Sarcoma (n=2) and was associated with invasion, proliferation, and higher metastatic potential in animal studies [4]. Cyclin D1 is a cell cycle regulator which controls the transition of the G to S phase of the cell cycle. It functions by inactivating RB, a tumor suppressor gene. The RB pathway plays a role in the progression of cell cycle from G1 to S phase. The dephosphorylation of RB renders it active by increasing its affinity to E2F transcription factor hence halting gene expression and cell cycle progression. Cyclin D1 activates Cyclin-dependent kinase 4 (CDK4) and inactivates the RB gene through phosphorylation hence continuing cell cycle progression [5]. Microarray analysis has shown a significant upregulation of Cyclin D1 in Ewing Sarcoma. In a retrospective study by Marino et al, mir-34a expression is lower in metastatic lesions compared to local tumors and is inversely correlated to Cyclin D1 [4].

This study aimed to evaluate the diagnostic accuracy of Cyclin D1 as an adjunct immunomarker in CD99 positive small round cell neoplasm with primary consideration of PNET/Ewing Sarcoma. Specifically, this is addressed by determination of distribution of immunohistochemical reactivity in cases with histopathologic diagnosis of CD99 positive round cell neoplasm with primary consideration of PNET/Ewing sarcoma in comparison with other CD99 positive round cell neoplasm. In addition, assessment of the sensitivity, specificity, positive predictive value, and negative predictive value of Cyclin D1 as adjunct immunomarker in detecting CD99 positive small round blue cell tumor with primary consideration of PNET/EWS was done. The demographic profiles (age, sex) of the two populations were also described.

Materials and Methods

A retrospective comparative diagnostic study was used to determine the sensitivity, specificity, positive predictive value and negative predictive value of immunohistochemical reactivity of Cyclin D1 among CD99 positive malignant small round blue cell tumor with primary consideration of Primitive Neuroectodermal Tumor (PNET)/Ewing's Sarcoma in comparison with other CD99 positive round cell neoplasm.

The target population of the study are surgical specimens from 2017 to 2023 with histopathologic diagnosis of CD99 positive malignant small round blue cell tumor with primary consideration of Primitive neuroectodermal tumor (PNET)/Ewing Sarcoma and other CD99 positive round cell neoplasm. The population included in this study are nineteen (19) cases which comprise eight (8) cases with final histopathologic diagnosis of CD99 positive small round blue cell tumor with primary consideration of PNET/Ewing Sarcoma and eleven (11) cases of CD99 positive small round blue cell tumor. Excluded are the following cases: referral from other institutions (i.e. slide review cases), bone marrow, liver, lymph node, cytology specimens, and specimens diagnosed to be metastatic.

This study did not utilize any form of medical intervention. The histopathological reports of the cases were retrieved, and the demographic profile of the patients were tabulated. Immunohistochemical staining techniques with Cyclin D1 (DAKO, Glostrup, Denmark) were performed in tissue sections 3 µm thick. The paraffin tissue sections were mounted in a charged slide along with a control tissue and deparaffinized in the oven for one hour. Antigen retrieval was done on the deparaffinized tissue section and incubated with the reagent antibody. Chromogen solution was added to directly visualize the target antigen. The chromogen solution formed an insoluble colored precipitate which signaled the presence of antigen in the tissue section. Xylene mounting medium was dropped on the prepared microscopic slide and a cover slip was added.

The immunohistochemical slides stained with *Cyclin D1 and the chromogenic reaction* were read microscopically and interpreted by the principal and supervising investigator. A positive result was noted by the presence of chromogenic reaction (brown precipitate color). The distribution of the chromogenic reaction in the neoplastic cells were categorized as focal (staining in less than 50% of the neoplastic cells) or diffuse (staining in greater than 50% of neoplastic cells). A negative reaction, in which the absence of the chromogenic reaction or no staining in the tumor cells was also noted.

The data was collated and tabulated in Microsoft Excel. The age, sex, tumor site, CD99 positive result and final diagnosis was used to illustrate the characteristics of the sample. Percentage of Cyclin D1 positivity was computed. The pattern of staining (nuclear, cytoplasmic) and distribution of Cyclin D1 staining (diffuse, focal) were also documented.

The research was developed in compliance to the Data Privacy Act (2012) and National Ethical Guidelines for Health and Health-Related Research. To ensure the protection of the study participants, each data

was treated with utmost confidentiality. No personal identifiable information was included, and each data set was coded with a control number. Only the investigators were allowed to retrieve and have access to the data. The hard copy and excel files used in this research were kept for 5 years from the time the last medical records were retrieved and disposed of by shredding the physical copy and deleting the electronic records. Approval was also obtained from the Institutional Review Board prior to the commencement of the study.

RESULTS

A total of 19 histopathologic specimens were obtained for this study, which are composed of eight cases of PNET/Ewing sarcoma and eleven cases of other CD99 positive round cell neoplasm comprising of the following: 3 Rhabdomyosarcoma, 3 Neuroblastoma, 2 Lymphoma, 1 Lymphoproliferative neoplasm, 1 Chloroma, and 1 Primitive Myxoid Mesenchymal Tumor of Infancy. The age ranges of the cases included in the study range from less than 1 year old up to 17 years old. Resulting median age is 6 years old while there are slightly more male (57.9%). Moreover, 68.4% of the cases are diffusely positive for CD99. All 8 cases with histopathologic diagnosis of PNET/Ewing sarcoma showed a positive result with Cyclin D1. Among the 11 cases of other CD99 positive round cell tumors, five show positive Cyclin D1 status. Among the 13 positive, most common staining patterns are 46.2% cytoplasmic while 38.5% are both nuclear and cytoplasmic. On distribution, the majority are diffuse (84.6%)(Table 1).

Table 1.0 Comparison of immunoreactivity among CD99 positive round cell tumors with primary consideration of PNET/Ewing Sarcoma and other CD99 positive round cell tumors and their Cyclin D1 status.

AGE	SEX	SPECIMEN	DIAGNOSIS	CD99 STATUS	CYCLIN D1 STATUS	STAINING PATTERN	DISTRIBUTION
17	F	PELVIC MASS	PNET/Ewing Sarcoma	POSITIVE DIFFUSE	POSITIVE	CYTOPLASMIC	DIFFUSE
1	M	PELVIC MASS	PNET/Ewing Sarcoma	POSITIVE DIFFUSE	POSITIVE	CYTOPLASMIC	DIFFUSE
3	M	RIGHT FEMUR	PNET/Ewing Sarcoma	POSITIVE DIFFUSE	POSITIVE	CYTOPLASMIC	DIFFUSE
8	M	SACROCOCCYGEAL MASS	PNET/Ewing Sarcoma	POSITIVE DIFFUSE	POSITIVE	CYTOPLASMIC	DIFFUSE
6	M	ILIAC BONE	PNET/Ewing Sarcoma	POSITIVE DIFFUSE	POSITIVE	CYTOPLASMIC	FOCAL
8	F	RIGHT PELVIS	PNET/Ewing Sarcoma	POSITIVE DIFFUSE	POSITIVE	CYTOPLASMIC	DIFFUSE
2	F	VERTEBRAL BODY	PNET/Ewing Sarcoma	POSITIVE DIFFUSE	POSITIVE	NUCLEAR CYTOPLASMIC	DIFFUSE
2	M	LEFT HAND MASS	PNET/Ewing Sarcoma	POSITIVE DIFFUSE	POSITIVE	NUCLEAR CYTOPLASMIC	DIFFUSE
17	M	ANT. MEDIASTINUM	Lymphoma	FOCAL POSITIVE	NEGATIVE	-	-

1	M	ALVEOLAR MASS	Rhabdomyosarcoma	FOCAL POSITIVE	NEGATIVE	-	-
10 M	M	INTRA ABDOMINAL MASS	Neuroblastoma	FOCAL POSITIVE	POSITIVE	NUCLEAR AND CYTOPLASMIC	DIFFUSE
7	F	ALVEOLAR MASS	Rhabdomyosarcoma	FOCAL POSITIVE	NEGATIVE	-	-
9	F	INTRA ABDOMINAL MASS	Neuroblastoma	DIFFUSE POSITIVE	POSITIVE	NUCLEAR AND CYTOPLASMIC	DIFFUSE
5	F	SUBMANDIBULAR MASS	Rhabdomyosarcoma	DIFFUSE POSITIVE	POSITIVE	NUCLEAR	FOCAL
11	M	ORBITAL MASS	Chloroma	DIFFUSE POSITIVE	NEGATIVE	-	-
3	F	ABDOMINAL MASS	Neuroblastoma	FOCAL POSITIVE	POSITIVE	NUCLEAR AND CYTOPLASMIC	DIFFUSE
14	M	TESTICULAR MASS	Lymphoma	FOCAL POSITIVE	NEGATIVE	-	-
7	M	SCALP MASS	Lymphoproliferative neoplasm	DIFFUSE POSITIVE	NEGATIVE	-	-
7D	F	ABDOMINAL MASS	Primitive myxoid mesenchymal tumor of infancy	DIFFUSE POSITIVE	POSITIVE	NUCLEAR	DIFFUSE

Among the 13 cases which were Cyclin D1 positive, 8 of them are also CD99 positive with primary consideration of PNET/ Ewing Sarcoma resulting in sensitivity of only 61.54% (95% CI 31.6 to 86.1%). All the six cases which tested negative for Cyclin D1 are all other CD99 positive small round blue cell tumors, resulting in 100% specificity (95% CI 47.8 to 100%). Also, positive predictive value is 100% (95% CI 63.1 to 100%) while negative predictive value is 50.0% (95% CI 33.5 to 66.5%). The overall accuracy is at 72.2% (95% CI 46.5 to 90.3%) (Table 2).

Table 2. Diagnostic Accuracy of Cyclin D1 as adjunct immunomarker in detecting CD99 positive small round blue cell tumor with primary consideration of PNET/EWS.

	Cyclin D1 Positive	Cyclin D1 Negative
CD99 positive neoplasm with primary consideration of PNET/EWS	**8**	**0**
CD99 positive small round blue cell tumor	**5**	**6**
	Values	**95% CI**
Sensitivity	61.54	31.6 to 86.1
Specificity	100	47.8 to 100
Positive Predictive Value	100	63.1 to 100
Negative Predictive Value	54.55	37.6 to 70.5
Overall accuracy	68.42	43.5 to 87.4
AUC	0.808	0.6 to 0.9
p value	0.002	

DISCUSSION

Primitive neuroectodermal tumor (PNET)/ Ewing Sarcoma is a group of small round blue cell tumors with varying degrees of neuroectodermal differentiation and share similar molecular translocations under the Ewing's family of tumors. CD99 is a transmembrane protein that has been used in the differentiation of small round blue cell tumors. Most PNET/Ewing Sarcoma are immunoreactive and are highly sensitive to CD99 but lack specificity with a sensitivity and specificity rate of 98.46% and 20%, respectively [6]. Hence, molecular testing is often required for the diagnosis of PNET/ Ewing Sarcoma. In a resource limited setting, such tests are not immediately available.

The study by Magro et. al which evaluated Cyclin D1 status on 128 cases of small round blue cell tumors in children and adolescents, all cases (30/30) of PNET/Ewing Sarcoma showed strong, diffuse, nuclear immunoreactivity for Cyclin D1. Moreover, only PNET/Ewing Sarcoma and neuroblastic tumors exhibited strong and diffuse (>50% staining in neoplastic cells) immunoreactivity for Cyclin D1. A similar study conducted by Nahid et.al showed all cases of Ewing Sarcoma (n=20) and Neuroblastoma (n=15) exhibited nuclear expression for Cyclin D1 with 70% and 66% showing a diffuse pattern, respectively [7]. This is similar to the results of our study where all 8 cases of PNET/Ewing Sarcoma had positive Cyclin D1 immunostaining, however 1 case among the 8 only showed focal immunoreactivity. Among the 5 other CD99 positive round cell tumors which tested positive for Cyclin D1, 3 are Neuroblastoma cases. With regards to the pattern of the staining, the majority of PNET/ Ewing Sarcoma cases showed cytoplasmic staining in contrast to nuclear pattern. In the study by Magro et. al and Nahid et al, all Rhabdomyosarcoma cases and Lymphoblastic Lymphoma cases tested negative for Cyclin D1 [2] [3] which is in contrast to our study, wherein 1 case of Rhabdomyosarcoma showed focal nuclear immunoreactivity. This similar in a cross sectional study by Shooshtarizadeh, et.al in comparing Cyclin D1 status in Rhabdomyosarcoma and Ewing Sarcoma cases, all cases of Ewing Sarcoma showed diffuse immunoreactivity and Rhabdomyosarcoma cases were mostly negative or focal [8].

The results of the study show that both PNET/Ewing Sarcoma and Neuroblastoma tumors are sensitive to Cyclin D1 and may be useful as an adjunct immunohistochemical stain in the histopathologic diagnosis of such tumors. The similarity of immunohistochemical expression of both may be due to their similar cell of origin, both being derived from neural crest cells [9] [10]. Cyclin D1 can also aid in the distinguishing between PNET/Ewing Sarcoma and Rhabdomyosarcoma cases as the pattern of staining in PNET/Ewing Sarcoma is diffuse and only focal or negative in the latter.

Conclusion

Cyclin D1 can be used as an adjunct immunomarker to aid in the diagnosis of CD99 positive round cell tumor with primary consideration of PNET/Ewing Sarcoma specifically in resource limited settings where molecular testing is not readily available. Given the high specificity of Cyclin D1 in such cases, it can be used to rule out other small round blue cell tumors that can also stain positive for CD99 such as Rhabdomyosarcoma. However, interpretation must be done in conjunction with the results of other immunohistochemical stains in order to increase its diagnostic accuracy. Limitations of this study are the low number of cases, due to irretrievable paraffin blocks and insufficient tissue samples left for immunohistochemical tests. The results of the study were not compared with results of molecular testing for mutations in PNET/Ewing Sarcoma which is often required for the diagnosis as such tests are not locally available.

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