Usefulness of PTAH stain in the diagnosis of rhabdomyosarcoma in low resource setting in reference to myogenin immunoreactivity

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Abstract: Rhabdomyosarcomas (RMS) are a group of soft tissue tumors that share a propensity to undergo myogenesis. They present a wide range of differential diagnoses which renders them difficult to diagnose using routine H&E stain alone. Immunohistochemistry (IHC) method with myogenic markers is highly sensitive and specific in the diagnosis of RMS, but rather expensive. Phosphotungstic Acid Hematoxylin (PTAH) stain is significantly cheaper and easy method to use but its accuracy has not adequately been studied. The aim of this study was to ascertain the diagnostic value of PTAH histochemistry method in RMS diagnosed by routine H&E stain with reference to myogenin IHC method. A cross-sectional retrospective hospital based study was conducted at Muhimbili National Hospital from January 2010 up to April 2014. All cases diagnosed as RMS and cases suspected to be RMS were retrieved and stained with myogenin IHC to confirm the diagnosis, then all positive cases were subjected to PTAH stain to determine its accuracy and usefulness. There were 36 cases of RMS confirmed by IHC. The age range was 2 to 72 with median age of 16 years. The male to female ratio was 1.2:1. Children (<18 years) were 52.8% and adults (≥18 years) were 47.2%. Head and neck tumors were more predominant in children compared to adults (p value=0.006) and embryonal RMS type was more predominant in children compared to adults (p value=0.000). PTAH stain was positive in only 8 cases (22.2%) and the positivity was not associated to any particular histological type of RMS. This study revealed that PTAH stain does not have any added value to confirm the diagnosis of RMS. Therefore, IHC with myogenin should be routinely used as a confirmation test for all cases suspected to be RMS.

Keywords: PTAH, myogenin, rhabdomyosarcoma

Introduction

Rhabdomyosarcomas (RMS) constitute a group of soft tissue tumors where tumor cells show tendency to undergo myogenesis and express myogenic markers, therefore giving morphology of interrupted normal skeletal muscle development [1]. Although relatively rare in adults, RMS is the most common soft tissue malignancy in children and adolescents, in whom they comprise approximately 60% of sarcomas reported worldwide per annum [2]. Some authors in USA have reported a relatively stable incidence with 4.5 cases/million children/adolescents per year [3]. The age predilection of RMS depends on the histologic type. Embryonal RMS mostly affects children younger than 10 years, alveolar RMS affects mainly adolescents and young adults. Pleomorphic RMS is commonly found in older patients with 40 years of age and above [4].

Rhabdomyosarcoma may arise from a wide variety of locations. Axial tumors often arise from the head and neck region the most common site being the orbit. Other sites are mouth, pharynx nasal passages, paranasal sinuses, parotid region, pterygoid region, temporal region, and cheek, the paraspinal region, and the genitourinary system namely vagina, cervix, uterus, urinary bladder, prostate, perineum, and paratesticular soft tissues. Another considerable number of tumors arise in extremities. In general, the head and neck is the most com-
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mon anatomical location, followed by genitourinary system and extremities [4].

The molecular biology aspects of RMS particularly the alveolar RMS has been studied and a translocation t (2; 13) (q35; q14) has been discovered, which results in a fusion between the PAX3 gene on chromosome 2q35 (encoding a DNA transcription factor playing a role in initiation of embryonic myogenesis) and the FKHR gene on chromosome 13q14 (encoding a molecule that controls the myoblast conjunction) [5]. The resultant PAX3-FKHR fusion gene can activate events leading to cell cycle and myogenesis. These fusion positive tumors are known to be aggressive. A second group of alveolar RMS presents at (1; 13) (p36; q14) translocation involving PAX7 gene (which is also an activator of myogenesis) and FKHR gene. A third, more aggressive group of alveolar rhabdomyosarcomas that lack either PAX3-FKHR or PAX7-FKHR fusions has also been described [6].

Histological features of RMS may resemble developing muscle; however majority of tumors offer no evidence of differentiation by routine H&E stain. The key cell is a rhabdomyoblast, a cell with an eccentric round nucleus and variable amounts of brightly eosinophilic cytoplasm. The shape of a rhabdomyoblast may resemble tadpoles, tennis racquets, or spiders. According to the WHO classification [7], histological types of RMS include embryonal RMS with its variants: botrioid and anaplastic, alveolar RMS, with its solid variant and mixed embryonal-alveolar variant and pleomorphic RMS. Spindle cell rhabdomyosarcoma is no longer considered as a variant of embryonal RMS but a separate entity with NCOA2 gene rearrangements [8].

RMS presents a wide variety of morphology, which gives it many differential diagnoses especially when the tumor is not well differentiated. These include small round cell tumors of childhood (ES/PNET, lymphoma, desmoplastic small cell tumor, and neuroblastoma); myogenic tumors of various types (ectomesenchymoma, malignant triton tumors) and spindle cell sarcomas (fibrosarcomas, leiomyosarcomas, and malignant peripheral nerve sheath tumors). This wide range of differential diagnoses renders RMS difficult to diagnose using routine H&E stain alone and other advanced methods are often required to confirm the diagnosis.

In resource limited settings, the confirmation of the diagnosis of RMS is commonly made by using relatively cheap histochemical methods such as PTAH and PAS. Phosphotungstic acid hematoxylin is used for demonstration of muscle cross-striations and fibrin. Cross-striations are a diagnostic feature of rhabdomyosarcoma or tumors arising from striated muscle. In well resourced laboratories, powerful diagnostic tools such as immunohistochemistry with various myogenic regulatory protein markers are routinely used. One of the most sensitive and specific immunohistochemistry methods is myogenin stain [9]. Myogenin expression may also correlate with different histologic subtypes of rhabdomyosarcomas (strong positivity with alveolar subtype) as it has been demonstrated by Palmer [10]. Although immunohistochemistry with myogenin (myf-4 antibody) is specific for RMS, it is relatively expensive to be used routinely in most of resource limited pathology laboratories. PTAH is cheap and fast but its utility has not been documented. The aim of this study was to determine the utility of PTAH histochemistry in the diagnosis of various histological subtypes of RMS, and whether it can serve as a reliable tool in the diagnosis of RMS.

Materials and methods

This was a retrospective and descriptive hospital based study. It was conducted at Muhimbili National Hospital and Muhimbili University of Health and Allied Sciences in Dar es Salaam Tanzania, from January 1st 2010 to April 30th, 2014. All paraffin embedded tissue blocks for cases diagnosed as RMS and cases suspected to be RMS were retrieved, sectioned and stained with H&E to determine histological subtypes. Myogenin immunohistochemistry stain was done to confirm the diagnosis of RMS and later PTAT stain was performed to ascertain its diagnostic value in different histological variants of confirmed cases of RMS. The data entry was done through Epidata 3.1 computer software, analyzed using IBM SPSS Statistics 20 software. Associations between variables and difference between categorical variables were determined using the Chi-square or Fisher’s...
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Table 1. Diagnostic accuracy of H&E in diagnosing RMS

<table>
<thead>
<tr>
<th></th>
<th>Myogenin positive</th>
<th>Myogenin negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMS by H&amp;E</td>
<td>25 (TP)</td>
<td>46 (FP)</td>
<td>71</td>
</tr>
<tr>
<td>Non RMS by H&amp;E</td>
<td>11 (FN)</td>
<td>30 (TN)</td>
<td>41</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>76</td>
<td>112</td>
</tr>
</tbody>
</table>

exact test and a p-value less than 0.05 was considered statistically significant. Mean, median and standard deviation were used to describe continuous variables.

Results

A total of 71 cases were diagnosed by H&E as RMS among 1012 soft tissue sarcomas in 4 years and 4 months study period. On the other hand, additional 41 cases were isolated from the 1012 cases as suspected to be RMS by H&E, making a sample size of 112 cases. Among the 71 cases initially diagnosed as RMS by H&E, only 25 (35%) were confirmed to be RMS by immunohistochemistry, whereas among the 41 cases suspected by authors to be RMS by H&E, 11 (27%) were positive for myogenin stain. This made a total of 36 immunohistochemically confirmed cases of RMS. The age range of the 36 cases of RMS was 2 to 72 years with a median age of 16 years. The male to female sex ratio was 1.2:1. Children (<18 years) were 52.8% (19/36) and adults (≥18 years) were 47.2% (17/36). The common tumor site was the head and neck with 44.4% followed by extremities with 36.1%. The sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of H&E in diagnosing RMS were 69.4%, 39.5%, 35.2%, 73.2% and 49.1%, respectively (Table 1). The age distribution for the 36 confirmed RMS was right-skewed with a median age of 16 years (Figure 1).

Based on the 2013 WHO classification of soft tissue tumors, cases were classified as embryonal, pleomorphic, alveolar, or spindle cell RMS. Embryonal RMS was the most common with 47.2% of cases (n=17). These were tumors with mainly small round primitive rhabdomyoblasts growing in a diffuse pattern alternating with hypocellular spaces sometimes exhibiting a myxoid change (Figure 2E). Pleomorphic RMS was the second most common with 22.2% (n=8). These were tumors presenting a marked cellular pleomorphism with abundant multinucleated tumor giant cells and areas of necrosis as well as abnormal mitotic figures (Figure 2F). The third type was alveolar RMS with 16.6%. They presented sheets of small round hyperchromatic primitive rhabdomyoblasts with an alveolar growth pattern, or packed tumor cells separated by fibrous septa without any alveolar growth pattern representing alveolar RMS solid type (Figure 2A and 2B). Spindle cell RMS was seen in 13.9% of cases. These were tumors with majority of cells exhibiting spindle or elongated nuclei with a moderate eosinophilic cytoplasm (Figure 2C). Among the spindle cell types, sclerosing RMS (3 cases) was characterized by proliferation of spindle cells in a dense sclerosing fibrous stroma and little pleomorphism (Figure 2D). Three cases were sub classified as embryonal RMS anaplastic variant and other three cases were sub classified as alveolar RMS solid variant.

The myogenin positivity was expressed as dark brown intranuclear color ranging from strong (Figure 3C), moderate (Figure 3B) and weak (Figure 3A). On the other hand, among the 41 cases suspected to be RMS by authors during sampling but had other diagnoses, 11 cases (27%) were positive for myogenin, making a total of 36 cases of confirmed RMS. Myogenin immunoreactivity was strong in 18 cases (50%), moderate in 6 cases (16.7%) and weak in 12 cases (33.3%). Among different histological subtypes, all six cases of alveolar RMS (100%) showed strong nuclear immunostain positivity. Majority (71.4%, n=10) of embryonal RMS exhibited strong immunostain positive reaction. None of the eight pleomorphic RMS showed strong nuclear immunostain positivity. Figure 3D corresponds to a known case of RMS which was used as a control for myogenin stain.

Among the 36 cases which were positive for myogenin, only 8 cases (22.2%) were positive for PTAH stain. The positivity was seen as dark blue to pale brown intracytoplasmic striations (Figure 4A) or intracytoplasmic fibrillar material (Figure 4B). Only areas of the tumor with well differentiated rhabdomyoblasts were able to visualize cross striations on PTAH stain.

Some variables were compared between childhood RMS and adult RMS: head and neck
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In one study conducted in 1995 by Pappo in USA, 35% of cases were found in head and neck region. However, when the sites were subdivided in specific anatomical sites of head and neck, they found that head and neck was comprised of 10%, orbit, 9% and parameningeal 16% [4]. When comparing children (<18 years) with adult patients (≥18 years), this study found head and neck tumors being predominant in children at 68% while they were 17% in adults (p value=0.006), a finding similar to what Sultan has reported in a retrospective study involving 2600 cases [14].

Only 35% (25/71) of cases initially diagnosed as RMS were positive for myogenin. This low proportion is highlighting challenges in the diagnosis of RMS with H&E alone. This high rate of misdiagnosed cases of RMS has also been found in a yet unpublished study conducted in Mulago hospital (Uganda) by Eva Mbwilo in a retrospective study involving 200 cases (10 years period) initially diagnosed as RMS on H&E, only 53 cases (26%) were positive for myogenin as confirmed cases of RMS. H&E alone is not accurate for diagnosing RMS as this study has revealed a very low specificity, low sensitivity, and low accuracy. In this study, pleomorphic RMS was the second most common histology type followed by alveolar RMS and spindle cell RMS. Weiss [15] has found that tumors were more predominant in children with 64.8% (13/19) compared to adult where they were 17.6% (3/17) and the difference was statistically significant (p value=0.006, Fisher's Exact Test). The most predominant histology type in younger patients was embryonal rhabdomyosarcoma with 73.7% (14/19) while it was 17.8% (3/17) in adult patients (p value=0.000; Fisher's Exact Test). These results are summarized in Table 2.

Discussion

Immunohistochemistry with myogenin stain was initially used in this study as a gold standard method to confirm the diagnosis of RMS before performing PTAH stain. Several studies have revealed the high sensitivity and specificity of myogenin immunohistochemistry stain [9, 11-13] in the diagnosis of RMS but there is scanty information in the literature about the usefulness of PTAH stain histochemistry in the diagnosis of RMS. This study aimed to elucidate the usefulness of PTAH stain in the diagnosis of RMS in a setting where more reliable methods are not readily available, as well as establishing the clinical-pathological features of RMS in Tanzania.

The median age among myogenin positive group was 16 years with 61% being ≤18 years of age, a finding that tallied with some published data. Sultan did a retrospective study combining children and adults with RMS from 1973 to 2005 in USA and found almost similar data with the age range from birth to 96 years, and the median age of 16 years [14]. The age distribution in myogenin positive group in this study was skewed to the right; with decreasing number of cases as the age were increasing and a slight rise after the age of 40 years corresponding with pleomorphic RMS cases in adults (Figure 1). This age distribution corresponds to the one described by Weiss [15]. In regard to anatomic site of RMS, majority of tumors were located at head and neck site (44%). Some authors have found a similar predominance of head and neck tumors especially in children, where in one study conducted in 1995 by Pappo in USA, 35% of cases were found in head and neck region. However, when the sites were subdivided in specific anatomical sites of head and neck, they found that, head and neck was composed of 10%, orbit, 9% and parameningeal 16% [4]. When comparing children (<18 years) with adult patients (≥18 years), this study found head and neck tumors being predominant in children at 68% while they were 17% in adults (p value=0.006), a finding similar to what Sultan has reported in a retrospective study involving 2600 cases [14].

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the most predominant subtype was embryonal RMS followed by alveolar RMS and the least one being pleomorphic RMS. It is likely that pleomorphic RMS comes in second position in this study because there were a considerable number of adult patients where this subtype is commonly found. Other studies are mainly studying RMS in the context of pediatric malignancies. When children and adults are separated, Embryonal RMS was the most predominant in children (74%) while pleomorphic RMS was exclusively (100%) found in adults with 87% (7/8) of patients being above 40 years. This finding is similar with what Furlong [16] published in Washington DC in 2001, regarding pleomorphic RMS where majority of patients were old with a median age of 54 years. Eleven cases were found to be RMS among 41 cases suspected to possibly be rhabdomyosarcoma. These were previously diagnosed as follow: three fibrosarcoma, two cases as neuroblastoma, two soft tissue sarcoma, one undifferentiated carcinoma, one Ewing’s sarcoma, one pleomorphic MFH and one case as synovial sarcoma or a MPNST. All the above mentioned initial diagnoses are included in differential diagnoses of RMS. Poorly differentiated neuroblastoma and Ewing’s sarcoma can mimic an alveo-
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Of cases. Majority of biopsies were incisional biopsies at 69.4% (25/36). Excision biopsies were counting for 30.6% (11/36). The absence of rhabdomyoblasts was strongly associated with incisional biopsy (P=0.009, Fisher’s exact test). The predominance of incisional biopsies of cases. Majority of biopsies were incisional biopsies at 69.4% (25/36). Excision biopsies were counting for 30.6% (11/36). The absence of rhabdomyoblasts was strongly associated with incisional biopsy (P=0.009, Fisher’s exact test). The predominance of incisional biopsies

Figure 3. Myogenin stain photomicrographs. A. Weak nuclear stain intensity. B. Moderate nuclear stain intensity. C. Strong nuclear stain intensity. D. Positive tissue control.

Figure 4. PTAH stain photomicrographs. A. Dark blue intracytoplasmic striations (arrow). B. Intracytoplasmic fibrillar material (arrows).
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Table 2. Compared variables between childhood RMS and adult RMS

<table>
<thead>
<tr>
<th>Variables</th>
<th>&lt;18 years (n=19)</th>
<th>≥18 years (n=17)</th>
<th>Total</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Frequency</td>
<td>%</td>
<td>Frequency</td>
<td>%</td>
</tr>
<tr>
<td>Male</td>
<td>11</td>
<td>57.9%</td>
<td>9</td>
<td>52.9%</td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>42.1%</td>
<td>8</td>
<td>47.1%</td>
</tr>
<tr>
<td>TOTAL</td>
<td>19</td>
<td></td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Site</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head and neck</td>
<td>13</td>
<td>68.4%</td>
<td>3</td>
<td>17.6%</td>
</tr>
<tr>
<td>Extremities</td>
<td>5</td>
<td>26.3%</td>
<td>8</td>
<td>47.2%</td>
</tr>
<tr>
<td>Genito-urinary</td>
<td>1</td>
<td>5.3%</td>
<td>3</td>
<td>17.6%</td>
</tr>
<tr>
<td>Trunk</td>
<td>0</td>
<td>0.0%</td>
<td>3</td>
<td>17.6%</td>
</tr>
<tr>
<td>TOTAL</td>
<td>19</td>
<td></td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Histology type</td>
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<td></td>
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<tr>
<td>Embryonal RMS</td>
<td>14</td>
<td>73.3%</td>
<td>3</td>
<td>17.8%</td>
</tr>
<tr>
<td>Alveolar RMS</td>
<td>2</td>
<td>10.5%</td>
<td>4</td>
<td>23.5%</td>
</tr>
<tr>
<td>Pleomorphic RMS</td>
<td>0</td>
<td>0.0%</td>
<td>8</td>
<td>47%</td>
</tr>
<tr>
<td>Spindle cell RMS</td>
<td>3</td>
<td>15.8%</td>
<td>2</td>
<td>11.7%</td>
</tr>
<tr>
<td>TOTAL</td>
<td>19</td>
<td></td>
<td>17</td>
<td></td>
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</tbody>
</table>

*Fisher’s Exact Test.

can be explained by the predominance of head and neck tumors in our patients, a site which does not easily allow wide excision. The more predominance of rhabdomyoblasts in excision biopsies may be explained by the relatively sufficient amount of tissue which allowed pathologists to take several sections from the tumor or larger tissue therefore increasing the chances to see rhabdomyoblasts. The absence of rhabdomyoblasts in majority of our cases was also explained by the poor differentiation in many tumors which were only made of small round monomorphic primitive cells with scanty cytoplasm.

Among 36 cases which were positive for myogenin, only 8 cases (22%) were positive for PTAH stain with visualization of cross-striations in the cytoplasm. This finding is showing that PTAH alone is not efficient in diagnosing RMS. Although embryonal RMS subtype was showing higher number of PTAH positive cases (36%) compared to other RMS subtypes, the difference in proportions was not statistically significant. The literature on the use of PTAH stain in the diagnosis of RMS is scanty and therefore comparable findings are limited.

In summary, this study is the first to address the challenges in diagnosis of RMS in Tanzania, and among the first studies to elucidate the usefulness of PTAH in RMS diagnosis. Age range, predominance of head and neck tumors, predominance of embryonal RMS in children and high rate of misdiagnosis are all comparable with other studies. This study has also revealed that there is high probability of misdiagnosing RMS by H&E staining and that PTAH stain is inferior and substandard test for RMS diagnosis.

We recommend that all suspected RMS should be subjected to a myogenin stain for confirmation of the diagnosis and it should readily be available for routine use.

Disclosure of conflict of interest

None.

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