Diagnostic Accuracy of Immunohistochemical Expression of p16, MDM2, and CDK4 in Well-Differentiated and De-Differentiated Liposarcoma in MDM2 Fluorescent *in situ* Hybridisation Confirmed Cases

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ABSTRACT

Objective: To establish the diagnostic utility of immunohistochemistry markers p16 along with MDM2 and CDK-4 in confirming the diagnosis of well-differentiated and de-differentiated liposarcoma while taking Fluorescent *in situ* Hybridisation (FISH) as a gold standard.

Study Design: A cross-sectional study.

Place and Duration of the Study: Department of Histopathology, Armed Forces Institute of Pathology, Rawalpindi, Pakistan, from 30th January 2022 to 30th June 2023.

Methodology: A standard panel of three immunohistochemistry markers p16, MDM2, and CDK4 were applied to 36 cases of atypical lipomatous tumours, well-differentiated liposarcoma (WDLPS), and de-differentiated liposarcoma (DDLPS), on which the gold standard FISH was already performed. The sample size was calculated with the help of a WHO calculator taking prevalence 1-2% in Pakistani population. Qualitative variables such as gender and site of tumour were presented by calculating frequency and percentages and comparison of Immunohistochemistry results with FISH was done by using a 2x2 table.

Results: The sensitivity and specificity of this triple marker panel for detecting WDLPS/DDLPS were 43.47% and 15.38%, respectively. The sensitivity and specificity of CDK4 for detecting WDLPS / DDLPS were 82.6% and 15.4%, those of MDM2 were 73.9% and 61.5 %, and those of p16 were 60.9% and 53.8%, respectively.

Conclusion: Among all three markers, CDK4 was the most sensitive and MDM2 was the most specific marker for detecting WDLPS-DDLPS. It also showed that a combination of these three markers improves the diagnostic credibility of the immunohistochemistry in diagnosing DDLPS and WDLPS but FISH is the most reliable and confirmatory method.

Key Words: *De-differentiated liposarcoma, Well-differentiated liposarcoma, P16, CDK4, MDM2.*

How to cite this article: Aslam A, Din HU, Qadir A, Aslam U, Humayoun S, Ahmed W. Diagnostic Accuracy of Immunohistochemical Expression of p16, MDM2, and CDK4 in Well-Differentiated and De-Differentiated Liposarcoma in MDM2 Fluorescent *in situ* Hybridisation Confirmed Cases. *J Coll Physicians Surg Pak* 2024; **34(09)**:1051-1055.

INTRODUCTION

Adipocytic neoplasm is a common soft tissue neoplasm. Their incidence varies from <6% cases per year per 100,000 population in Europe to <15 cases per year per 100,000 population in the United States.[1] No significant risk factor has been discovered yet. However, these are associated with family history and prior radiation exposure. Liposarcoma (LS) is a rare mesenchymal tumour characterised by the presence of lipoblasts. It is broadly classified into three groups: Atypical lipomatous tumours (ALT) with or without differentiation, the cellular myxoid spectrum, and the pleomorphic LS.[2]

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Received: November 15, 2023; Revised: August 30, 2023; Accepted: August 31, 2024
DOI: https://doi.org/10.29271/jcpsp.2024.09.1051

These tumours show distinct clinical, behavioural, molecular, and treatment sensitivity patterns. ALT / well-differentiated LS (WDLPS) showing non-lipogenic areas are called as de-differentiated LS (DDLPS). They constitute the most common type of LS, and both are radiosensitive and chemosensitive. They share morphological and molecular similarities, indicating that DDLPS arise mostly within precursor WDLPS lesions, with 8-10% within areas of locally recurrent WDLPS and 80-90% of DDLPS are found within primary WDLPS.[3]

Histologically, WDLPSs are characterised by mature adipocytes and variable-sized fat cells showing a pleomorphic indented nucleus. The adipocytes are intersected by fibrous septae containing atypical and pleomorphic cells. DDLPSs are characterised by more cellular and non-lipogenic sarcomatous areas.[4] Pathophysiology of LS is complex, however, both WDLPS and DDLPS are characterised by an amplified segment of chromosome 12q13-15 that contains a number of oncogenic genes. It is also associated with additional amplifications of the MDM2 and CDK4 with cell cycle oncogene protein overexpression. MDM2 is

an ubiquitin-protein ligase that is a key negative regulator of P53 and is nearly expressed in 100% of the patients.[5,6] These LS also overexpress the cell cycle regulator p16. The p16 is a tumour suppressor protein encoded by the *CDKN2A* gene. It inhibits cyclin D-dependent protein kinases (CDK4 and CDK6), thereby, maintaining *Rb* in its hypophosphorylated state. Positivity of p16 is seen in lesions with inactivation of the Rb gene in many other carcinomas. However, the immunohistochemical (IHC) expression of p16 is important in diagnosing DDLPS and WDLPS. If it is used in conjugation with MDM2 and CDK4, the diagnostic yield of the panel is increased. These three IHC markers (p16, MDM2, and CDK4) are used for the diagnosis of liposarcoma.

Limited data is available on the combined utility of p16, MDM2, and CDK4 panel for the confirmation of diagnosis but the purpose of this study was to devise a standardised IHC panel with more accurate and helpful results to diagnose the well-differentiated and de-differentiated liposarcomas, using the above markers.

METHODOLOGY

This is a descriptive cross-sectional study carried out in the Department of Histopathology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan, from 30th January 2022 to 30th July 2023, after obtaining approval from the Institutional Review Board. Sample size was calculated with the help of the WHO calculator. With a confidence interval of 95% and a margin of error of 4% and taking into consideration the prevalence of LS in the population as 1-2%,[7] the optimal sample size for this project was 36 patients with a diagnosis of atypical lipomatous tumours including both WDLPS and DDLPS. Fluorescent *in situ* hybridisation (FISH) was performed on these cases and definite confirmed cases of WDLPS and DDLPS were obtained.[8] Informed consent was taken from the patients, giving them the right to withdraw from the study at any time. In a prospective way, a non-probability convenient sampling technique was employed for these patients.

Inclusion criteria were all MDM2 FISH confirmed cases of WDLPS and DDLPS (true positive, TP), FISH-negative cases of ALT (true negative, TN), and all patients of both genders between 20-80 years of age.

Exclusion criteria were pleomorphic LS and myxoid LS, and all those patients already taking chemotherapy and radiotherapy.

Other demographic features, such as patients' age, gender, and site and size of the tumour were also recorded. Dual-color FISH was performed *via* using MDM2-specific probe together with a specific probe for 12 chromosomes. A formalin-fixed paraffin-embedded (FFPE) tissue containing sufficient neoplastic cells was preferred. Three microns sections were mounted over adhesive slides and with the use of appropriate filter sets, hybridisation signals of the probe appearing green for MDM2 and orange for CEN 12 were evaluated. A MDM2 / CEN12 ratio >2 was considered amplified for the MDM2 gene. Each test was repeated twice with a good positive control to label it negative.

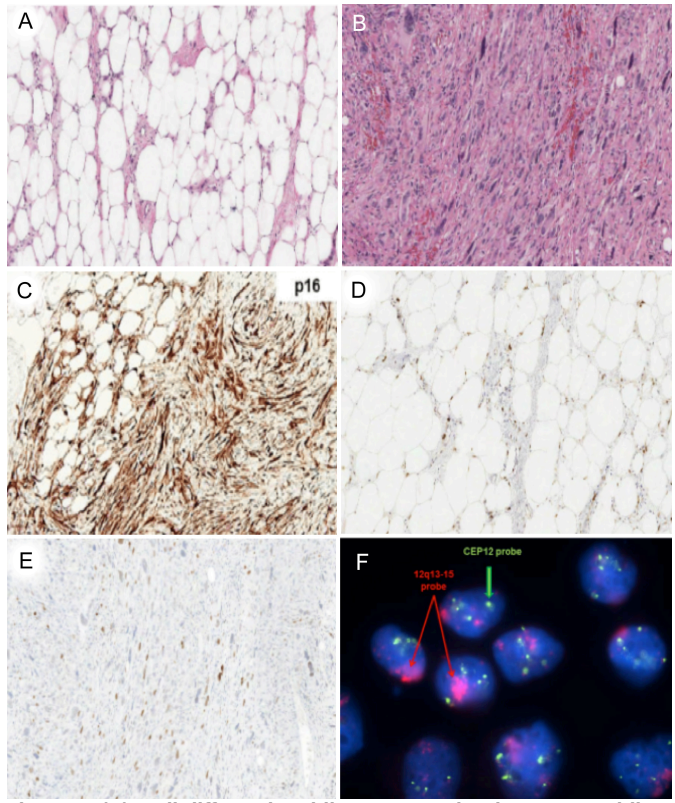

Figure 1: (A) Well-differentiated liposarcoma showing scattered lipoblasts. (B) De-differentiated liposarcoma. (C) P16 showing both cytoplasmic and nuclear positivity. (D) MDM 2 showing positivity in WDLPS. (E) CDK4 showing positivity in (DDLPS). (F) MDM2 amplification detected by FISH dual probe.

IHC was performed on five microns thick FFPE sections. P16 (mouse monoclonal antibody), CDK4 (Rabbit polyclonal antibody), and MDM2 (Mouse monoclonal antibody), were performed as a primary antibody. Subsequently, staining was performed with an auto stainer. All the slides were counterstained using haematoxylin. The presence of brown nuclear precipitate indicated positive staining for CDK4 and MDM2 and the presence of both cytoplasmic and nuclear staining for p16 indicates positive staining (Figure 1 A-F).[9] Appropriate positive controls were applied throughout and results of both FISH and IHC markers were analysed by two senior histopathologists, independently.[10,11] To prevent errors in diagnosis, a second opinion was ensured.[12] Each tumour was assessed into four-tier system; absent, focal weak positive, moderate positive, and strong positive. The tumour staining was also semi-quantitively evaluated as negative (0% staining of the cells), focal positive (1-10% positive cells), moderately positive (11-50% positive cells), and strong positive (>50% positive cells). For each antibody, only well-defined positivity (moderate and strong) was considered positive; faint staining (focal <10% positivity) despite good control was considered negative.

All the statistical data variables were analysed *via* using SPSS version 20. Qualitative variables such as gender and site were presented by frequency and percentages. For the comparison of IHC results with FISH, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV)

were calculated by using a 2×2 table. False positive (FP) is defined as a result that indicates the presence when it is not actually present. On the other hand, false negative (FN) states wrong negative results that does not hold true.

RESULTS

Out of 36 cases, male 64% (n = 23) to female 36% (n=13) ratio for this tumour was 1.7:1. Common site of involvement was the retroperitoneum (Table I). FISH was applied to 36 cases of WDLPS and DDLPS, 63.89% (n = 23) cases turned out positive and 36.11% (n = 13) cases were negative. The panel of three IHC markers were applied and the sensitivity, specificity, PPV, and NPV were evaluated for each individual marker and also for the trio of this combination. Sensitivity was calculated with the help of the following analyser positive WDLP-DDLP / total WDLP-S-DDLPS, specificity was calculated as negative WDLPS-DDPLS / total WDLPS-DDLPS, PPV was calculated TP / TP+FN, and NPV was estimated *via* TN / TN + FN (Table II).

Table I: Main demographic and tumour-related variables including gender, tumour size, and location (n = 36).

Characteristics		Results
Age (years), Mean ± SD		56.67 ± 12.53
Age	21-40 years	2 (%)
	41-60 years	19 (%)
	61-80 years	14 (%)
	>80 years	1 (%)
Gender	Males	23 (%)
	Females	13 (%)
Tumour size (cm), Mean ± SD		14.47 ± 4.6
Tumour size	≤10 cm	8 (%)
	>10 cm	28 (%)
Tumour location	Retroperitoneum	20 (%)
	Extremities	8 (%)
	Others	8 (%)

Table II: Sensitivity, specificity, positive predictive value, and negative predictive value of three IHC markers, in combined trio and in isolated forms.

IHC antibodies	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Trio	43.47%	15.38%	76.92%	66.66%
p16	60.9%	53.8%	68.42%	40.0%
MDM2	73.9%	61.55	76.19%	20.0%
CDK4	82.6%	15.4%	62.06%	53.84%

DISCUSSION

In this study, the use of IHC in the diagnosis of liposarcoma was evaluated to rule out myxoid and pleomorphic liposarcoma. This combination of three markers helped to distinguish the WDLPS-DDLPS from other liposarcomas. P16 is the least used antibody in the diagnosis of LS but the current study showed that it has good sensitivity and specificity and its use with CDK4 and MDM2 improves the yield of IHC panel to diagnose the WDLP-S-DDLPS.

Atypical lipomatous tumours constitute a major group of lipomatous tumours, being one of the most common sarcomas of adults. Tumours which are placed in WDLPS/DDLPS group are morphologically and genetically distinct from other LS subtypes, i.e, pleomorphic and myxoid liposarcomas.[6,13] WDLPS grossly and microscopically, closely resemble the lipomas. But on keen inspection, it shows lipoblasts, large univacoulated cells showing hyperchromatic nuclei. These liposarcomas show three distinct patterns, inflammatory LS, lipoma-like LS, and sclerosing LS. DDLS are diagnosed when non-lipogenic components arise in LS. It shows heterologous elements including high-grade rhabdomyosarcomas, angiosarcomas, and chondrosarcomas. Despite showing these heterologous elements and their confirmation on IHC, both WDLPS and DDLPS show the same genetic mutations.[14] Benign mimickers of WDLPS are lipoblastomas, spindle cell and pleomorphic lipomas, lipomas showing lipoatrophy, necrosis, and myxoid change. Therefore, it is important to always exclude the possibility of liposarcomas in lipomas showing large size (>10cm), old age, deep location, and showing numerous lipoblasts. For the diagnosis of LS, it is always necessary to have an excisional tissue biopsy as the needle core biopsy is not optimal for diagnosis.[15] Radiological details of soft tissue mass showing thick septae and fat tissue density mass favours the diagnosis of LS. The presence of fat necrosis in lipomas favours the lipoblast-like morphology and sometimes p16, MDM2, and CDK4 stain these giant and multinucleated foamy macrophages in lipomas. This makes a big problem for a histopathologist to satisfactorily report it. It is always necessary to rule out LS as it is more aggressive than lipomas and dedifferentiated components that arise in it.

A study conducted in the UK showed the sensitivity and specificity of this trio for detecting WDLPS / DDLPS as 71% and 98%, respectively. The sensitivity and specificity of p16 in the diagnosis of WDLPS was 89.4% and 68.2%, CDK4 was 68.4% and 97.7%, and MDM2 was 89.5% and 97.7%, respectively. The sensitivity and specificity of p16 for the diagnosis of DDLPS to be 94.0% and 70.0%, of CDK4 to be 83.0% and 95.2%, and of MDM2 to be 100% and 30.7%, respectively.[16] Similarly, a study conducted in France showed that p16 had a sensitivity of 94.4% but a specificity of 70% in detecting ALT. However, MDM2 and CDK4 showed sensitivity and specificity of 100% and 30%, and 83.3% and 95.5%, respectively.[17] Another study from Japan compared the diagnostic utility of p16, CDK4, and MDM2 in the diagnosis of WDLPS and DDLPS. The sensitivity and specificity of p16 was 100% and 69.0%, CDK4 was 100% and 53.3%, and MDM2 was 100% and 93.3%, respectively.[18] A study from Paris compared the sensitivity and specificity of p16, MDM2, and CDK4 immunostaining in identifying WDLPS / DDLPS were 87% and 69%, 97% and 92%, and 83% and 95%, respectively.[19] A study conducted in Belgium comparing the diagnostic utility of MDM2 and CDK4 in MDM2 FISH-confirmed cases of atypical lipomatous sarcomas and found similar results.[20] Another study conducted in India showed that positive expression of p16 and CDK4 was seen in 51.4% and 10.0%, respectively.[21]

In all of the studies, the sensitivity and specificity of each marker was more than 60%, which was comparable to the present study.[22] Specificity of MDM2 and p16 were also comparable; however, the specificity of CDK4 is not comparable to this study.

This low specificity of CDK4 could be due to a small sample size in the present population, the antibody clone used in CDK4 immunostaining or defect in the enzyme blockage site. The combined use of p16, MDM2, and CDK4 showed sensitivity and specificity of 43.47 % and 15.38%, respectively. So one can use this triple marker panel for initial screening and diagnosis of LS but for definite diagnosis and in problematic cases where IHC results and morphological findings are not clear, FISH should be used as it is the gold standard.[3,22]

CONCLUSION

In the diagnosis of WDLPS and DDLPS, this panel of three IHC markers (p16, MDM2, and CDK4) showed good sensitivity and can be used for screening soft tissue sarcoma. But the specificity of this trio is less.

ETHICAL APPROVAL:

This cross-sectional study received approval from the Ethics Committee of Armed Forces Institute of Pathology, Rawalpindi, Pakistan. (Approval No: FC-FSP-5/READ-IRB/22/1288; Dated: 24 March 2022).

PATIENTS' CONSENT:

Written informed consent was taken from all the patients.

COMPETING INTEREST:

The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:

AA: Data collection, introduction and discussion write-up, literature search, and performed reference setting.
HUD: Conception and study design, critical evaluation, and statistical analysis.
UA, AQ: Material and methods section, discussion section, and interpretation of the data.
SH, WA: Graphics and pictures.
All authors approved the final version of the manuscript to be published.

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