THE CHARACTERISATION OF *MDM2* AND *CDK4* GENE AMPLIFICATION AND THEIR ASSOCIATION WITH RECURRENCE IN LIPOMATOUS TUMOURS

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by

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LIST OF SYMBOLS

%	Percentage
°C	Degree Celsius
≥	More than or equal to
n	Sample size
+	Amplified by FISH
-	Non-amplified by FISH
nm	Nanometre
mm	Millimetre
µL	Microlitre

LIST OF ABBREVIATIONS

ALT	Atypical lipomatous tumour
WDLS	Well-differentiated liposarcoma
DDLS	Dedifferentiated liposarcoma
MLS	Myxoid liposarcoma
PLS	Pleomorphic liposarcoma
MPLS	Myxoid pleomorphic liposarcoma
MDM2	Murine double minute-2
CDK4	Cyclin-dependent kinases-4
FISH	Fluorescence in situ hybridisation
FFPE	Formalin-fixed paraffin-embedded
NGS	Next-generation sequencing
P53	Protein 53
Rb	Retinoblastoma
DAPI	4′,6-diamidino-2-phenylindole
FITC	Fluorescein
TRITC	Tetramethyl rhodamine
CEP12	Centromeric reference probe
RT	Room temperature
STS	Soft tissue sarcoma
WHO	World Health Organisation
MLPA	Multiplex ligation-dependent probe amplification
RT-PCR	Reverse transcriptase-polymerase chain reaction
HPUSM	Hospital Pakar Universiti Sains Malaysia
RTU	Ready-to-use
MNCR	Malaysia National Cancer Registry
H&E	Haematoxylin and eosin
DISH	Dual-colour in situ hybridisation
HR	Hazard ratio
CI	Confidence interval
FDA	Food and Drug Administration
MPNST	Malignant peripheral nerve sheath tumour

HMGA2	High mobility group AT-hook 2
YEATS4	YEATS domain containing 4
UPS	Undifferentiated pleomorphic sarcoma
EMC	Extraskeletal myxoid chondrosarcoma

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PENCIRIAN PENGUATAN GEN *MDM2* DAN *CDK4* SERTA KAITANNYA DENGAN PERULANGAN DALAM TUMOR BERLEMAK

ABSTRAK

MDM2 dan *CDK4* merupakan gen yang kerap mengalami amplifikasi dalam liposarkoma, terutamanya dalam tumor tisu lemak atipikal/liposarkoma berbeza baik (ALT/WDLS) dan liposarkoma dediferensiasi (DDLS). Walaupun peranan onkogenik gen *MDM2* dan *CDK4* secara individu telah terbukti, kadar kejadian dan nisbah amplifikasi serentak, serta kepentingan prognostiknya dalam liposarkoma, masih belum jelas. Kajian ini bertujuan untuk menilai nisbah amplifikasi *MDM2* dan *CDK4* dalam tumor tisu lemak, menentukan status amplifikasi serentak, dan menilai hubungannya dengan prognosis. Data klinikal dan patologi bagi kes yang didiagnos secara histologi sebagai liposarkoma tanpa had saiz atau tumor tisu lemak benigna berukuran sekurang-kurangnya 10 cm (≥ 10 cm) di Hospital Pakar Universiti Sains Malaysia (HPUSM) dari Januari 2014 hingga Mei 2021, telah diperoleh secara retrospektif daripada Sistem Maklumat Makmal (LIS), Jabatan Patologi, HPUSM. Sampel tisu yang diawet formalin dan terbenam dalam parafin telah dianalisis menggunakan pendarfluor in situ hibridisasi (FISH) untuk mengesan amplifikasi gen *MDM2* dan *CDK4*. Nisbah amplifikasi ditentukan dengan membandingkan purata bilangan salingan gen *MDM2/CDK4* dengan sentromer 12, di mana nisbah melebihi 2.0 menunjukkan amplifikasi manakala nisbah kurang daripada 2.0 menunjukkan tiada amplifikasi. Kelangsungan hidup bebas kelakuan berulang dan bebas metastasis antara kumpulan amplifikasi telah dinilai menggunakan analisis Kaplan-Meier dan dibandingkan dengan statistik log-rank. Faktor prognostik untuk kelakuan berulang telah dianalisis menggunakan regresi bahaya Cox. Daripada 86 kes, 23 (27%) adalah

liposarkoma, manakala 63 (73%) adalah tumor tisu lemak benigna (≥10 cm) selepas pengelasan semula menggunakan FISH. Amplifikasi bersama *MDM2* dan *CDK4* (*MDM2*+/*CDK4*+; 13%) dikesan dalam semua (6/6) kes DDLS dan separuh (5/10) daripada kes ALT/WDLS. Lima kes menunjukkan amplifikasi *MDM2* tanpa amplifikasi *CDK4* (*MDM2*+/*CDK4*-; 6%), kesemuanya dikesan dalam ALT. Tiada amplifikasi gen *MDM2* atau *CDK4* (*MDM2*-/*CDK4*-; 81%) dikesan dalam liposarkoma myxoid, liposarkoma pleomorfik, atau tumor benigna. Nisbah amplifikasi *MDM2* dan *CDK4* lebih tinggi dalam DDLS (4.4 dan 2.8, masing-masing) berbanding ALT/WDLS (2.9 dan 2.6, masing-masing). Dalam kedua-dua subjenis, nisbah amplifikasi *MDM2* melebihi *CDK4*. Kumpulan *MDM2*+/*CDK4*+ menunjukkan kelangsungan hidup bebas kelakuan berulang (p=0.002; median 34 bulan) dan bebas metastasis (p=0.003; median 83 bulan) terendah berbanding dengan kumpulan lain. Analisis multivariat menunjukkan keberulangan laku berkait secara signifikan dengan pembedahan bersama kemoterapi (p=0.021), namun amplifikasi *MDM2* dan *CDK4* tidak muncul sebagai faktor prognostik bebas. Kesimpulannya, amplifikasi *MDM2* adalah lebih konsisten dan lebih tinggi secara kuantitatif berbanding *CDK4*, menyokong peranannya yang utama dalam perkembangan tumor. Walaupun amplifikasi bersama *MDM2* dan *CDK4* dikaitkan dengan prognosis yang lebih buruk, ia tidak terbukti sebagai faktor prognostik bebas dan menunjukkan kemungkinan pengaruh faktor klinikal lain. Namun, amplifikasi bersama ini berpotensi dalam pengenalpastian subkumpulan liposarkoma yang berisiko tinggi.

Kata Kunci: *MDM2*, *CDK4*, liposarkoma, FISH

THE CHARACTERISATION OF *MDM2* AND *CDK4* GENE AMPLIFICATION AND THEIR ASSOCIATION WITH RECURRENCE IN LIPOMATOUS TUMOURS

ABSTRACT

MDM2 and *CDK4* are frequently amplified genes in liposarcoma, particularly in atypical lipomatous tumour/well-differentiated liposarcoma (ALT/WDLS) and dedifferentiated liposarcoma (DDLS). Although the individual oncogenic role of *MDM2* and *CDK4* genes are well established, the prevalence and ratio of their concurrent amplification, as well as their prognostic significance in liposarcoma, remain unclear. The aim of this study was to evaluate *MDM2* and *CDK4* amplification ratios across lipomatous tumour subtypes, determine their concurrent amplification statuses, and assess associations with patients' prognosis. Clinicopathological data of cases histologically diagnosed as liposarcoma of any size or benign lipomatous tumours measuring at least 10 cm (≥10 cm), at Hospital Pakar Universiti Sains Malaysia (HPUSM) between January 2014 and May 2021, were retrospectively retrieved from Laboratory Information System of Pathology Department, HPUSM. Formalin-fixed paraffin-embedded tissue samples of eligible cases were subjected to fluorescence in situ hybridisation (FISH) for *MDM2* and *CDK4* gene amplification detection. Amplification ratio was determined by comparing *MDM2* or *CDK4* mean copy number with centromere 12 signals, where ratios more than 2.0 indicated amplification, and ratio less than 2.0 indicated no amplification. Recurrence-free and metastasis-free survival across amplification groups were evaluated using Kaplan-Meier survival analysis and compared with log-rank statistics. Prognostic factors of recurrence were analysed using Cox proportional hazard regression. Among 86 cases,

23 (27%) were liposarcoma and 63 (73%) were benign lipomatous tumours (≥10 cm) following reclassification by FISH. *MDM2* and *CDK4* co-amplification (*MDM2*+/*CDK4*+; 13%) was observed in all (6/6) DDLS and half (5/10) of ALT/WDLS cases. Five *MDM2*-amplified cases lacked *CDK4* amplification (*MDM2*+/*CDK4*-; 6%), all detected in ALT. No amplification of either gene (*MDM2*-/*CDK4*-; 81%) was detected in myxoid liposarcoma, pleomorphic liposarcoma, or benign tumours. DDLS showed higher *MDM2* and *CDK4* amplification ratios (4.4 and 2.8, respectively) than ALT/WDLS (2.9 and 2.6, respectively). In both subtypes, *MDM2* amplification ratio exceeded *CDK4*. *MDM2*+/*CDK4*+ group had the shortest recurrence-free (p=0.002; median 34 months) and metastasis-free survival (p=0.003; median 83 months) compared to other groups. Multivariate analysis showed recurrence was significantly associated with surgery combined with chemotherapy (p=0.021), but *MDM2* and *CDK4* amplification was not an independent prognostic factor. In conclusion, *MDM2* amplification was more consistent and quantitatively higher than *CDK4*, supporting its central role in tumourigenesis. While *MDM2/CDK4* co-amplification was associated with poorer outcomes, it lacked independent prognostic value, reflecting the potential influence of other clinical variables. Nevertheless, co-amplification may hold clinical relevance in identifying high-risk liposarcoma subgroups.

Keywords: *MDM2*, *CDK4*, liposarcoma, FISH

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Soft tissue sarcoma (STS) is a rare type of cancer that develops from soft tissues of the body. It accounts for only 1% of all cancers (Siegel et al., 2018; Yang et al., 2019), with an estimated global incidence of one to four cases per 100 000 people each year (Berwick and Wiggins, 2017). In Malaysia, the incidence of STS was not specified in the most recent (2017-2021) Malaysia National Cancer Registry (MNCR) report; however, prior MNCR reports had indicated a notable increase in STS cases, from 812 (0.78%) between 2007 and 2011 to 1066 (0.93%) between 2012 and 2016 (Azizah et al., 2016; Azizah et al., 2019). Moreover, the American Cancer Society has expected 13 590 new STS cases and 5200 deaths due to the disease in the United States by the end of 2024 (American Cancer Society, 2024).

STS comprises over 100 different histological subtypes despite its rarity. Liposarcoma, which develops from adipose tissue, is one of the most common subtypes as it accounts for about 20% of all adult STS (Codenotti et al., 2017). The World Health Organisation (WHO) has continually revised the classification of liposarcoma over the years to address its heterogeneity and improve diagnostic accuracy. In the most recent edition (WHO, 2020), five major subtypes of liposarcoma were recognised. This includes atypical lipomatous tumour/well-differentiated liposarcoma (ALT/WDLS), dedifferentiated liposarcoma (DDLS), myxoid liposarcoma (MLS), pleomorphic liposarcoma (PLS), and the newly introduced subtype, myxoid pleomorphic liposarcoma (MPLS).

Despite the classification, liposarcoma often exhibits overlapping histological features, making differentiation between subtypes, benign lipomatous tumour, and other STS difficult. A study on soft tissue tumours reported that liposarcoma was frequently misclassified as either benign or malignant (Jalaludin et al., 2017). Benign lipomatous tumours are more common than malignant ones, with an incidence rate of 2100 per 100 000 population. Due to their higher frequency and considerable histological overlap, malignant tumours are often presumed to be benign (Johnson et al., 2018). Moreover, there is an increased awareness of the potential for malignant transformation, particularly among large lipomas (Gungor et al., 2017). Prior studies reported that tumours measuring at least 10 cm in size is a significant predictive factor in distinguishing ALTs from lipomas (Bird et al., 2016). Although histologic examination and immunohistochemistry (IHC) are widely used, they may be insufficient in diagnosing challenging cases. Therefore, ancillary molecular tests are essential to confirm the diagnosis. Fluorescence in situ hybridisation (FISH) is particularly valued for its high sensitivity and specificity compared to IHC, and is considered the "gold standard" for detecting specific genetic aberrations in STS (Asif et al., 2018).

The standard treatment approach for liposarcoma is surgical resection, regardless of subtype (Demir et al., 2022). Adjuvant radiotherapy or chemotherapy in cases of metastatic tumours are also performed, although with the expense of limited efficacy and high-level toxicity. Therefore, molecular targeted therapies are being explored as promising treatment options for liposarcoma. It is well known that murine double minute-2 (*MDM2*) and cyclin-dependent kinases-4 (*CDK4*) genes serve essential roles in liposarcoma tumourigenesis, particularly in ALT/WDLS and DDLS subtypes. The amplification and overexpression of these genes inhibit two major

growth regulatory pathways mediated by the prominent tumour suppressors p53 and retinoblastoma (Rb), respectively. Their co-amplification is common in ALT/WDLS and DDLS, as well as in other STS, although being located in two discontinuous regions on chromosome 12q (Dembla et al., 2018, Martinez-Monleon et al., 2022). Researchers are actively testing numerous inhibitors targeting these two genes to improve outcomes for the disease.

Recent trials have highlighted brigimadlin as a promising MDM2-p53 antagonist, achieving stable disease in more than half of WDLS and DDLS patients (Gounder et al., 2022). Further trials are currently ongoing to compare its efficacy with standard first-line doxorubicin treatment in DDLS patients (Schöffski et al., 2023). Similarly, palbociclib has also shown effectiveness in stabilising the disease in phase 2 trials, although significant tumour shrinkage was lacking in most patients (Dickson et al., 2013; Dickson et al., 2016). This drug is one of the first *CDK4* inhibitors which was approved by Food and Drug Administration (FDA) for breast cancer treatment. However, for liposarcoma treatment, no MDM2 or CDK4 inhibitors have yet received FDA approval (Wang et al., 2024), as many inhibitors that produced positive initial results have failed to maintain efficacy in later-phase trials. Besides, the use of MDM2 and CDK4 inhibitors together is a debated strategy in liposarcoma, with some research indicating a synergistic effect, while other findings report antagonistic effects in terms of cytotoxicity (Laroche-Clary et al., 2017; Sriraman et al., 2018).

This highlights the critical need for a better understanding on the combined characteristics of *MDM2* and *CDK4* amplification to improve the development of targeted therapies for liposarcoma. Although extensive literature exists on *MDM2* amplification in liposarcoma, studies examining the concurrent amplification of both

MDM2 and *CDK4* is limited. Therefore, using the FISH technique, this study focused on evaluating both gene amplifications and their impact on patients diagnosed with liposarcoma of any size or large benign lipomatous tumours measuring at least 10 cm at HPUSM. In addition to evaluating the amplification ratios across subtypes, the association between clinicopathological variables and recurrence were also analysed in this study.

1.2 Problem Statement and Study Rationale

Although STS is rare, the gradual increase in the number of cases over the years in Malaysia and worldwide is concerning. As with STS, the incidence of liposarcoma is also on the rise by 19% in western population (Bock et al., 2020); however, data on its prevalence in Malaysia remains limited. The management of liposarcoma patients is complex and requires a multidisciplinary collaboration of pathologists, radiologists, surgeons, and oncologists. A recent study reported that the diagnostic accuracy is higher when a multidisciplinary approach is used (Pang et al., 2022). Despite this, uncertainties in diagnosing liposarcoma among soft tissue tumours is still an acknowledged issue, mainly because of their rarity and high heterogeneity.

Previous case reports have documented histologic similarities between large lipomas and ALT/WDLS (Widodo et al., 2020), DDLS and PLS mimicking other pleomorphic sarcomas (Le Guellec et al., 2014), and MLS resembling other myxoid STS (Suzuki et al., 2017). There are also several reports on potential overlap between the liposarcoma subtypes themselves, further complicating the diagnosis for pathologists (Thway, 2019; Iwasaki et al., 2015). Besides, imaging techniques are useful for soft tissue tumour evaluation, with magnetic resonance imaging (MRI) as one of the most advanced modalities for the disease (Church et al., 2017). However,

radiologists face difficulties in accurately differentiating between lipoma and liposarcoma using MRI, achieving only 73.5% accuracy (Ryan et al., 2018). This limitation was also highlighted in a recent study involving 240 lipomatous tumours, which found that 73.3% of the cases were correctly categorised with MRI, but 21.7% was over-diagnosed and 5% under-diagnosed compared to histological findings (Ballhause et al., 2022).

When clinical and imaging evaluations are inconclusive, a biopsy is performed to confirm the malignancy. However, the limited biopsy material may not demonstrate the distinctive histologic features of the tumour, which can lead to diagnostic misinterpretation. The overall diagnostic accuracy was only 62.8% for percutaneous biopsy of liposarcoma subtypes, with DDLS (36.5%) showing significantly lower accuracy compared to WDLS (85.1%) (Ikoma et al., 2015). Addressing these diagnostic challenges altogether are crucial to ensure that the growing incidence of liposarcoma is appropriately managed. MDM2 and CDK4 overexpression and amplification are both useful markers in distinguishing between different liposarcoma subtypes and benign lipomatous tumours. Overexpression is normally determined by IHC, but studies have shown IHC to be less specific than FISH when performed on soft tissue tumours (El Koubaiti et al., 2022). Therefore, this study utilised the 'gold standard' FISH technique for the accurate detection of *MDM2* and *CDK4* amplification, providing valuable data on their prevalence within the lipomatous tumour of local population.

Despite significant advances in treatment, prognosis of liposarcoma remains poor. The 5-year overall survival rate is 68%, but this varies depending on multiple factors including tumour subtype (Demir et al., 2022). ALT/WDLS and DDLS are known to be less sensitive to chemotherapy compared to other subtypes, resulting in

low response rates among patients (Crago and Dickson, 2016; Stacchiotti et al., 2022). Although MDM2 and CDK4 inhibitors as targeted therapy agents are already in clinical trials, many have failed to demonstrate a significant level of clinical benefit in liposarcoma patients. For instance, in a recent phase 3 trial of MDM2 inhibitor milademetan in DDLS patients, median progression-free survival did not significantly differ from chemotherapy agent trabectedin (Jones et al., 2023). Besides, it is also not clear whether targeting *MDM2* alone or in combination with *CDK4* will have a sufficient therapeutic advantage to patients. In this regard, an enhanced understanding on both genes is necessary for the improvement of targeted inhibitors.

To date, majority of studies investigating liposarcoma have largely focused on *MDM2* amplification alone. Despite the finding that *CDK4* frequently amplifies together with *MDM2* (Dembla et al., 2018), their concurrent amplification characteristics in liposarcoma and their impact on patient prognosis remains unclear. Previous research found that both amplifications associated with decreased disease-specific and disease-free survival among DDLS patients (Ricciotti et al., 2017). Another study on *MDM2*-amplified WDLS and DDLS found that *CDK4* amplification was associated with poor prognosis, however, *MDM2* did not show any prognostic significance (Lee et al., 2014). These studies have limited their cohort by subtypes like ALT/WDLS and DDLS, where both amplifications are characteristic. However, the comparative impact of both gene amplification statuses on prognosis was not explored in a cohort inclusive of all liposarcoma subtypes and large benign lipomatous tumours. Addressing this gap may enable better molecular stratification and provide insights into the development of more effective targeted therapies.

1.3 Research Questions

1. What is the frequency of *MDM2* and *CDK4* gene amplification in lipomatous tumours?
2. Is there any difference in *MDM2* and *CDK4* gene amplification in different types of lipomatous tumours?
3. Is there a statistically significant association between *MDM2* and *CDK4* gene amplification and median recurrence-free and metastasis-free survival time in lipomatous tumours?
4. Is there a statistically significant association between *MDM2* and *CDK4* gene amplification and the risk of recurrence following surgical resection in lipomatous tumours?

1.4 Research Objectives

1. General Objective:
    - To study the characterisation of *MDM2* and *CDK4* gene amplification and their association with recurrence in lipomatous tumours
2. Specific objectives:
    - To determine the frequency of *MDM2* and *CDK4* gene amplification in lipomatous tumours
    - To describe the characterisation of *MDM2* and *CDK4* gene amplification among the morphologic spectrum of lipomatous tumours
    - To determine the association of *MDM2* and *CDK4* gene amplification with median recurrence-free and metastasis-free survival time in lipomatous tumours

- To determine the association of *MDM2* and *CDK4* gene amplification as prognostic factor for recurrence in lipomatous tumours

CHAPTER 2

LITERATURE REVIEW

2.1 Liposarcoma Incidence

Liposarcoma is a rare malignancy originating from adipocytes. This tumour can occur in any part of the body, but extremities and retroperitoneum are the most common area involved (Crago and Brennan, 2015; Xiao et al., 2021). It represents about 16 – 20% of all STS cases, making it a significant entity of STS. Due to their rarity, available information relevant to liposarcoma incidence are limited. As shown in Table 2.1, several recent studies have reported the age-standardised annual incidence rate (ASR) of liposarcoma corresponding to the country population. Compared with the data from western countries, Asian countries (Thailand, Taiwan, and Iran) have a lower ASR ranging from 0.23 to 0.63 per 100 000 people per year. The variation between countries could be attributed to the use of different classification criteria, genetic, and socioeconomic factors (Kollár et al., 2019; Asef-Kabiri et al., 2021).

While the statistics of STS in Malaysia is available through the National Cancer Registry, the data are not robust enough to appreciate its subtypes, including liposarcoma. According to the information from MNCR report, a slight increase in the STS cases was observed from period 2007-2011 (0.78%) to 2012-2016 (0.93%) (Azizah et al., 2016; Azizah et al., 2019). Similar trend was noted in some literature reports from other parts of the world (Willburger et al., 2022; Adamkova et al., 2024). As with STS, liposarcoma also had a significantly increased trend over time. Bock et al. (2020) had reported a 19% increase from 2001 to 2016 in the United States. A more recently published study, which investigated sarcoma incidence in Canada over a period of two decades, had highlighted that liposarcoma had the largest increase in rates compared to other sarcomas (Alkazemi et al., 2023).

Table 2.1 Incidence rates of liposarcoma across countries

Country	Study Period	ASR (per 100 000 individuals – year)	Author
United States	2001 – 2016	1.01	Bock et al. (2020)
Germany	2012	0.97	Saltus et al. (2018)
Switzerland	1996 – 2015	0.92	Kollár et al. (2019)
Taiwan	2007 – 2013	0.63	Hung et al. (2019)
Thailand	2001 – 2015	0.23	Klangjorhor et al. (2022)
Iran	2009 – 2014	0.23	Asef-Kabiri et al. (2021)

2.2 Liposarcoma Subtypes

According to the new WHO classification of 2020, liposarcoma has been classified into five distinctive subtypes which are ALT/WDLS, DDLS, MLS, PLS, and MPLS (WHO, 2020). The first two histological types are among the commonest, comprising about 40% to 45% of all liposarcoma cases (Briski et al., 2018).

2.2.1 Atypical Lipomatous Tumour/Well-Differentiated Liposarcoma

Although ALT and WDLS are classified in the same entity, their prognosis differs depending on the tumour location. Tumours located in surgically accessible sites, such as the extremities, are termed ALT, while those in areas where achieving wide surgical margins is challenging, like the retroperitoneum, are classified as WDLS (Nagano et al., 2015). ALT has a better prognosis than WDLS since complete surgical removal is often unfeasible in the latter, leading to a higher likelihood of local recurrence and an increased mortality rate. Additionally, WDLS in these difficult-to-resect areas have a higher propensity to transform into DDLS.

Histologically, ALT/WDLS is often seen in the lipoma-like form, which is characterised by mature adipocytes of varying sizes and focal nuclear atypia within adipocytic and stromal component (Figure 2.1 A). This pattern closely mimics benign

lipomatous tumours, including lipomas and other variants, due to the presence of mature adipocytes. Other histological forms of ALT/WDLS also exist, such as the sclerosing, inflammatory, and spindle cell (Figure 2.1 B-D). In the sclerosing type, dense stromal fibrous areas are present, which may cause confusion with desmoid fibromatosis or other fibrotic soft tissue tumours (Noorily et al., 2024). The inflammatory variant shows chronic infiltration of lymphoplasmacytic cells to the extent that may obscure the adipocytic nature of the tumour. This variant is rarely seen, and such a case has the risk of being mistaken for lymphocyte predominate tumours including Hodgkin lymphoma, Castleman disease, or inflammatory pseudotumour (Kilpatrick, 2024). Spindle cell is another uncommon variant in which bland spindle cells are set in a fibrous or myxoid stroma, resembling spindle cell lipoma or malignant tumours such as MLS, myxofibrosarcoma, malignant peripheral nerve sheath tumour (MPNST), or low-grade fibromyxoid sarcoma (Peck et al., 2020; Liao et al., 2021). Nevertheless, the lipoma-like variant is considered the classic form of ALT/WDLS and is found in all types, although it can be present only focally (Kilpatrick, 2024). The presence of atypical hyperchromatic nuclei is an important diagnostic clue of this entity among other adipocytic tumours. However, when they are poorly represented or absent particularly in limited samples, the diagnosis of ALT/WDLS becomes challenging. Other features, such as hibernoma-like, angiolipoma-like, and intramuscular lipoma-like components, were also seen in this entity, causing further difficulty in reaching an accurate diagnosis (Burusapat et al., 2020; Saygin et al., 2020; Kojima et al., 2022).

Despite all the morphological resemblances, ALT/WDLS differ from its mimickers in terms of molecular aspect. ALT/WDLS is characterised by giant rod marker or supernumerary ring chromosomes derived from the amplified segments of 12q13-15 chromosomal region, which includes *MDM2*, *CDK4*, high mobility group

AT-hook 2 (*HMGA2*), and YEATS domain containing 4 (*YEATS4*) genes (Lee et al., 2018). *MDM2* and *CDK4* are the most prominent genes in the amplified sequences and their amplification are used as diagnostic markers for this entity. Benign lipomatous tumours and several mesenchymal malignancies lack *MDM2* and *CDK4* amplification. This would help to rule out their possibilities from the differential diagnosis of ALT/WDLS.

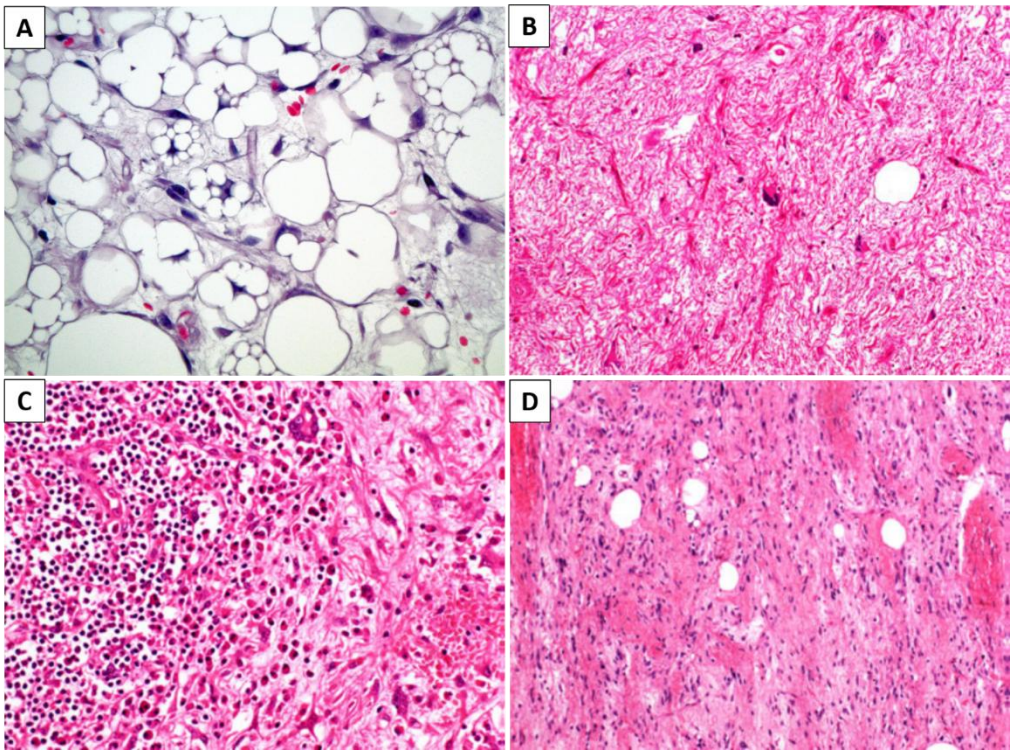

Figure 2.1. Histology of ALT/WDLS variants. (A) Lipoma-like (classic form of ALT/WDLS), with marked variation in mature adipocyte sizes and enlarged atypical hyperchromatic cells observed at intermediate magnification (Kilpatrick, 2024). (B) Sclerosing, with bizarre hyperchromatic cells in a background of dense fibrous tissue (C) Inflammatory, with scattered atypical stromal cells in a background obscured by chronic inflammation (D) Spindle cell, with bland spindle cell proliferation in a myxoid background (low magnification) (Dei Tos, 2014).

2.2.2 Dedifferentiated Liposarcoma

DDLS are aggressive lesions, associated with higher rates of local recurrences (57.2%) and metastasis (29.7%) (Tirumani et al., 2015). Majority of DDLS (90%) arises *de novo* as primary tumours, while the remaining (10%) is the result from the progression of pre-existing ALT/WDLS (WHO, 2020). The term "dedifferentiation" refers to the abrupt histological transition from a lipogenic ALT/WDLS component to a non-lipogenic, pleomorphic tumour component. The risk of dedifferentiation was reported to be higher in the retroperitoneal area, demonstrating worse survival than those from other sites (Gootee et al., 2019, Nguyen et al., 2021). Regardless of location, a recent study associated DDLS with the poorest 5-year survival rate (49.4%) compared to other liposarcoma subtypes (Amer et al., 2020).

Histologically, DDLS is characterised by the presence of ALT/WDLS sharply juxtaposed to a dedifferentiated component (Figure 2.2). Although the transition between the two components is mostly abrupt, it can also show a gradual or intermingling transition in rare cases (Lali et al., 2020). The DDLS area may gradually intermingle with the surrounding lipogenic area, simulating an ALT/WDLS tumour. In some cases, the tumour may be extensively dedifferentiated, showing minimal or no areas of its well-differentiated precursor, potentially misleading the diagnosis (Le Guellec et al., 2014). Due to its pleomorphism and varying dedifferentiation grade, DDLS has a broad differential diagnosis ranging from benign to malignant soft tissue tumours. This includes spindle cell lipoma, hibernoma, undifferentiated pleomorphic sarcoma (UPS), myxofibrosarcoma, gastrointestinal stromal tumour, MPNST, and many others (Kojima et al., 2022; Ohshima et al., 2023; Lali et al., 2020; Nishio et al., 2021; Chaudhary et al., 2022; Gajzer et al., 2020). The dedifferentiated component most frequently resembled UPS and myxofibrosarcoma (Lali et al., 2020; Nishio et al., 2021).

DDLS with extensive areas of myxoid stroma in combination with capillary vascular pattern, can also be confused with MLS particularly when the cells lack pleomorphism (Thway, 2019). In exceptional circumstances, DDLS can show homologous lipoblast differentiation, closely resembling PLS (Thway, 2019).

Heterologous differentiation is observed in about 5% to 10% of DDLS cases, and mostly towards rhabdomyosarcomatous, leiomyosarcomatous, or osteosarcomatous elements (Lokka et al., 2014; Thway, 2019; Yamashita et al., 2018). Less commonly, DDLS can show meningothelial-like whorls, as seen in neural tumours and follicular dendritic cell sarcoma. These whorls, which are associated with metaplastic bone formation, have been described to be an early sign of dedifferentiation in liposarcoma (Usman Tariq et al., 2020). The heterologous elements of this entity appear not to affect patient outcome; however, their prognostic relevance is still under debate. A recent study showed that DDLS with myogenic differentiation had a poor overall survival, in concordance with previous studies (Dorian Yarih et al., 2021; Kurzawa et al., 2020; Gronchi et al., 2015). Another study suggested that the risk of early local recurrences among DDLS patients with osteogenic differentiation did not always result in poor survival outcomes (Yamashita et al., 2018). Usman Tariq et al. (2020) mentioned that the aggressive behaviour of DDLS with meningothelial-like whorls were similar to those without the whorls.

Therefore, the ALT/WDLS component within the DDLS tumour is an important feature to recognise, as it is helpful in distinguishing DDLS from other soft tissue tumours. In the absence of its well-differentiated precursor on histological examination, molecular detection of *MDM2* and *CDK4* gene amplification within 12q13-15 region may facilitate the diagnosis. Although DDLS genetically overlaps with ALT/WDLS, it

is reported that the progression of DDLS tumour is further associated with additional genetic aberrations, including the amplification of chromosomal regions 1p23 and 6q23 (Chai et al., 2022).

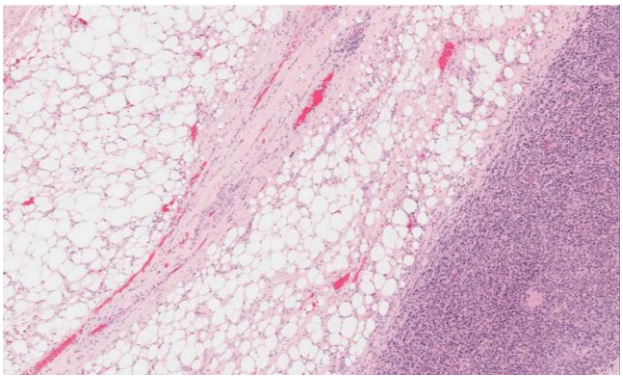

Figure 2.2. Histology of DDLS. The figure shows an abrupt transition from ALT/WDLS component to a more cellular, non-lipogenic component, 4x magnification (Dry, 2024).

2.2.3 Myxoid Liposarcoma

The next common subtype of liposarcoma after WDLS/DDLS is MLS, contributing one-third of all liposarcoma cases (Demir et al., 2022). Histologically, this subtype shows a mixture of round to ovoid mesenchymal cells with the presence of scattered signet ring lipoblast in an abundant myxoid stroma (Figure 2.3). One of its most defining microscopic features is the rich plexiform capillary vessel network that is often described as “chicken-wire” pattern. Round cell differentiation, which was formerly classified as a distinct entity, has been included in MLS category as high-grade lesions (Schaefer and Gronchi, 2022). This is because of its continuum of morphologic changes, ranging from the myxoid transition to round cell areas, are commonly observed in MLS. Similar genetic alterations exhibited by both morphologic patterns further supports the histologic continuum concept of a single entity MLS. The most commonly described chromosomal abnormality, which is found in almost all MLS cases, is the t(12;16)(q13;p11) translocation in which the CHOP/DDIT3 gene in 12q13 was fused with FUS/TLS gene in 16p11 (Lee et al., 2018). An alternative translocation

t(12;22)(q13;q12) was also detected in some MLS cases, resulting in the fusion of DDIT3 with EWSR1 instead of FUS (Lee et al., 2018). These rearrangements involving the DDIT3 gene aid in the differential diagnosis of MLS from other soft tissue tumours.

The diagnosis of MLS tumour is generally straightforward once its characteristic morphological features are recognised. However, similar to other liposarcoma subtypes, this entity exhibits a wide range of morphological variations, which can lead to confusion when encountering unusual histological forms. One type of tumour that often appears in the differential diagnosis alongside MLS is extraskeletal myxoid chondrosarcoma (EMC) (Nayel et al., 2020). In addition to the presence of eosinophilic chondroblast-like cells, EMC consists of a rich myxoid histology, which can resemble MLS under microscopic examination. Nevertheless, these tumours can be differentiated by the presence of specific gene translocation. MLS is characterised by t(12;16) translocation, while EMC is characterised by t(9;22) translocation (Nayel et al., 2020). Besides, it is also challenging to differentiate MLS with extensive lipoma-like changes from ALT/WDLS tumours that present with myxoid areas (Iwasaki et al., 2015). Amplifications of *MDM2* and *CDK4* in ALT/WDLS, and specific translocation in MLS at molecular level may help to render a definitive diagnosis. Other misleading morphology found in MLS includes hibernoma-like, chondroid lipoma-like, and spindle cell lipoma-like components (Kojima et al., 2022; Al-Malki and Al-Khamiss, 2015; Ohshima et al., 2023). It is crucial to accurately distinguish MLS from these tumours (Figure 2.6), as the prognosis and patient management for each entity is different. While lipoma and its variants are treated with simple surgical removal without the risk of recurrence or metastasis, MLS has a more complex clinical course. According to Durr et al. (2018), MLS patients who underwent limb-sparing surgical resection and radiation therapy had a 9% recurrence rate. Furthermore, it is reported that 18-24% of MLS

patients developed metastasis (Shinoda et al., 2020; Tuzzato et al., 2022). Their overall survival rates, particularly those with the presence of round cell areas exceeding 5%, are poor (80% for 5 years and 10 years, respectively) (Francesco et al., 2018).

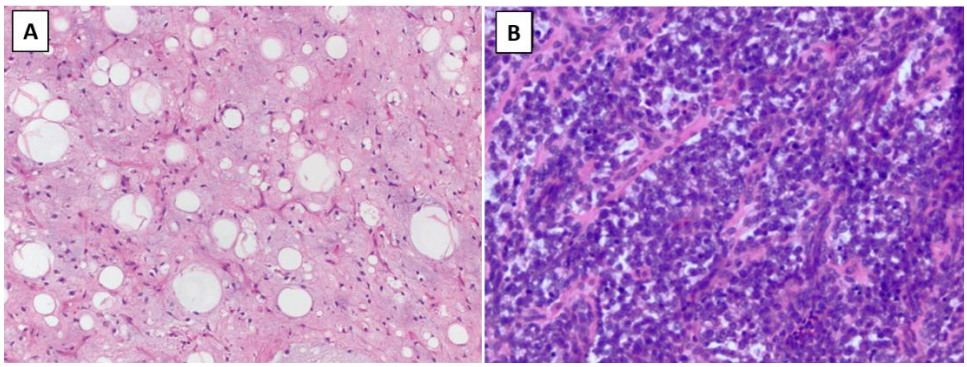

Figure 2.3. Histology of MLS. (A) Lipoblasts can be seen in a myxoid stroma with rich capillary vessel network (Doyle, 2014). (B) A focal hypercellular area with small round cells comprising 5% of the MLS tumour (low magnification) (Finlay et al., 2024).

2.2.4 Pleomorphic Liposarcoma

Pleomorphic variant is the rarest subtype of liposarcoma, making up to only 5% of all liposarcoma cases (Yu and Sokumbi, 2016). However, it is the most aggressive variant of liposarcoma with a 5-year survival rate of 66.2% (Gjorgova Gjeorgjievski et al., 2022). Histologically, this subtype is composed of pleomorphic lipoblasts which is an essential component to differentiate it from other high-grade sarcomas (Hadjimichael et al., 2023) (Figure 2.4). The use of IHC in diagnosing PLS is constrained due to its non-specific immunopanel, which exhibits variable positivity for a number of markers including SMA, desmin, and CD34 (Wakely et al., 2022). Genetically, PLS have no known targetable molecular alterations as it is found to have diverse complex chromosomal rearrangements and genomic changes. Several frequently observed genetic abnormalities in PLS involves common tumour suppressors such as p53, Rb, and neurofibromatosis type 1 (Wakely et al., 2022). Therefore, IHC and molecular

analyses have no more diagnostic value than the presence of pleomorphic lipoblasts in the differential diagnosis of PLS.

When pleomorphic lipoblasts are absent or minimal, PLS are most similar to UPS and myxofibrosarcoma (Gjorgova Gjeorgjievski et al., 2022). PLS with epithelioid-rich histology can be confused with non-mesenchymal neoplasms, including carcinoma and melanoma (Gjorgova Gjeorgjievski et al., 2022, Al-Attar et al., 2023). In such cases, PAX8 and SF1 expressions are useful markers to accurately differentiate carcinoma from PLS. On the other hand, positive staining for S-100 protein, SOX10, and other markers of melanocytes are useful for differentiating melanoma from PLS. Another differential diagnostic consideration of PLS is pleomorphic rhabdomyosarcoma, and the distinction between both are made with myogenin staining, a specific IHC marker for rhabdomyoblastic differentiation (Wakely et al., 2022). The diagnosis of PLS is further complicated by the presence of homologous lipoblastic differentiation in some DDLS cases (Thway, 2019). However, *MDM2* and *CDK4* gene amplification, which is the molecular hallmark of DDLS and ALT/WDLS tumours, are not exhibited in PLS. This may help to rule out tumours and establish a correct diagnosis. Additionally, PLS may also demonstrate extensive lipomatous differentiation, resembling ALT/WDLS. However, the lack of the characteristic gene amplifications, combined with the presence of lipoblast within the adipose tissue, provide useful diagnostic clues for identifying PLS (Wang et al., 2018). As patients diagnosed with PLS have distinct clinical outcomes, accurate diagnosis is therefore crucial. In the context of this entity, IHC and molecular analyses are primarily used to rule out other potential diagnoses, whereas the detection of pleomorphic lipoblasts remains the most important criterion in confirming the diagnosis of PLS.

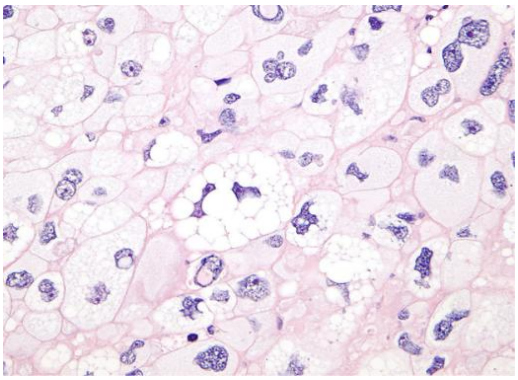

Figure 2.4. Histology of PLS. Lipoblasts is an essential component of PLS observed at intermediate magnification (Anderson and Jo, 2019).

2.2.5 Myxoid Pleomorphic Liposarcoma

In the year 2020, the WHO has introduced MPLS as a new distinct entity in the classification of liposarcoma subtypes. Initially discovered by Alaggio et al. (2009), this rare subtype has a predilection for mediastinum and exhibits a mixture of morphologic patterns from both MLS and PLS (Figure 2.5). However, this tumour has a poorer prognosis compared to the latter two subtypes, with studies reporting high rates of local recurrence and metastasis that often lead to death within a short time frame (Creytens et al., 2021; Dermawan et al., 2022). One of the authors proposed that increased recurrence rates in MPLS patients may be influenced by positive surgical margins and complex tumour location, such as the mediastinum (Dermawan et al., 2022).

Based on the limited studies and case reports available on MPLS, a notable molecular feature that frequently identified is the widespread loss of heterozygosity (LOH) (Dermawan et al., 2022; Tan et al., 2024). *TP53* mutations are also observed in these tumours; however, such mutations are not unique to MPLS and can also be found in PLS. This complicates the diagnosis, especially when PLS tumours exhibit myxoid areas (Dermawan et al., 2022). However, the absence of widespread LOH in PLS serves as a distinguishing factor from MPLS. Similarly, MLS also lacks widespread LOH, but it is instead characterised by specific mutations involving *DDIT3* translocation, which

are absent in MPLS (Creytens et al., 2021). In addition, DDLS with either homologous lipoblastic differentiation or myxoid areas may sometimes mimic MPLS histologically, but it can be distinguished by *MDM2* and *CDK4* amplifications (Choi et al., 2014; Dermawan, 2024). These amplifications are not found in MPLS, thereby allowing for their exclusion from the differential diagnosis (Creytens et al., 2021). These molecular differences altogether are useful to differentiate MPLS from other liposarcoma subtypes. Apart from liposarcoma, the morphologic appearance of benign tumours like spindle cell lipoma might also be similar to that of MPLS, but it does not show necrosis or the high level of mitotic activity that MPLS does (Creytens et al., 2021).

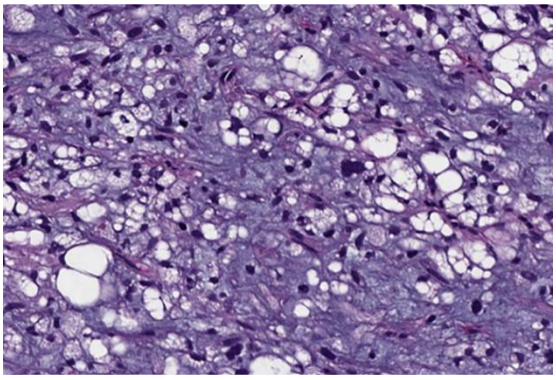

Figure 2.5. Histology of MPLS. An admixture of MLS and PLS are characteristic features of MPLS observed at low magnification (Dermawan et al., 2022).

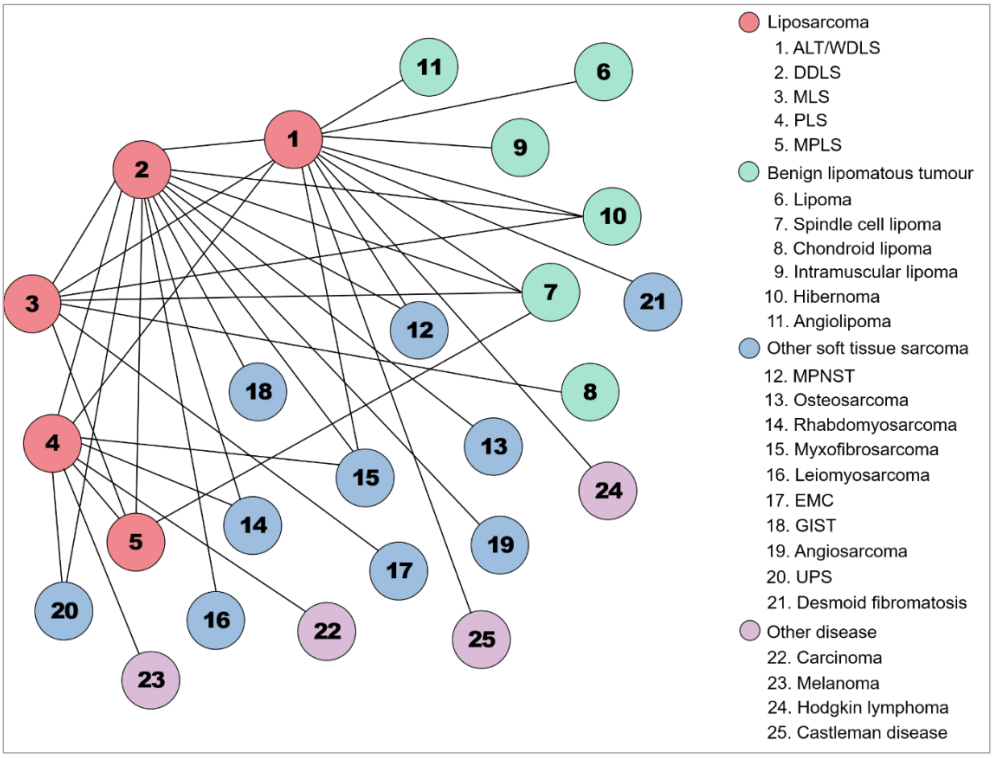

Figure 2.6. Histologic overlaps among liposarcoma and other tumours. The figure illustrates the documented overlaps between liposarcoma subtypes, benign lipomatous tumours, other STS, and carcinomas, highlighting diagnostic challenges.

2.3 Murine Double Minute-2 *(MDM2)* gene

The *MDM2* gene is located on the long arm of chromosome 12 (12q15) (Figure 2.7). It is known to possess oncogenic properties that is mostly associated with the inhibition of p53 tumour suppressor protein function. Upon exposure to DNA damage or any cellular stress, p53 is activated and often functions to prevent the growth of abnormal cells by promoting apoptosis and cell cycle arrest. It is widely understood that p53 act as a transcription factor and upregulates an array of target genes responsible for diverse cellular processes. Interestingly, *MDM2* is among the target genes that is transcriptionally regulated by p53, thus causing the production of MDM2 protein. The MDM2 protein, in turn, negatively regulates the p53 activity by degrading or suppressing its transcriptional activity. This results in the formation of negative feedback loop between MDM2 and p53 (Figure 2.8). This loop is essential in regulating

p53 expression in non-stressed tissues, given its strong ability to inhibit growth. In other words, p53 becomes harmful when MDM2 is not present. However, as a key negative regulator of p53, it is not surprising that *MDM2* are oncogenic when overexpressed and contributes to the accelerated malignant tumour development.

The *MDM2* gene is amplified in a small subset of cancers, occurring in only a few percent (3.5% to 4.4%) across various cancer types (Dembla et al., 2018; Kato et al., 2018). In a large study cohort analysing 523 patients of multiple cancer types, the occurrence of *MDM2* amplification was predominantly observed in sarcoma (57%) (Dembla et al., 2018). Research indicates that its amplification in sarcoma often manifest in the form of double minutes chromosomes, which are small and spherical chromatin bodies (Gambella et al., 2023). The *MDM2* amplification serves as a diagnostic marker for several sarcomas, specifically ALT/WDLS, DDLS, intimal sarcoma, and low-grade osteosarcoma (Sciot, 2021). Among these, it is most consistently detected in ALT/WDLS and DDLS tumours (Abeshouse et al., 2017). Therefore, its amplification status is utilised as a diagnostic aid to distinguish these malignant subtypes from benign tumours, like lipoma and its variants. While *MDM2* amplification is widely acknowledged for its diagnostic utility, its role as a prognostic indicator in liposarcoma remains limited. Previous studies reported that the level of *MDM2* amplification does not appear to be a useful prognostic factor in WDLS and DDLS patient outcomes, including recurrence-free, progression-free, and disease-specific survival (Jour et al., 2015; Lee et al., 2014). Alternatively, subsequent research finding revealed that the high *MDM2* amplification level in DDLS patients is significantly linked to a reduced time to relapse (Bill et al. 2019). The authors also noted that *MDM2* amplification in DDLS leads to a lack of sensitivity to standard chemotherapy regimens (Bill et al. 2019).

In other tumour types, *MDM2* amplification has been shown to have significant negative effect on prognosis. Several studies reported an association between *MDM2*-amplified intimal sarcoma and poor outcomes, showing median survival of less than a year in many cases (Frezza et al., 2020; Jimbo et al, 2019). Likewise, Wege et al. (2022) had linked elevated levels of *MDM2* in luminal breast cancer patients to unfavourable disease-free and overall survival rates. In addition to this, a recent pan-cancer analysis of large clinical datasets has in fact established that *MDM2* is an effective marker for assessing prognosis (Zheng et al., 2023).

2.3.1 *MDM2* Targeted Therapy

The frequent *MDM2* amplification in different cancer types, including liposarcoma provide rationale for targeting *MDM2* as a therapeutic approach. Various strategies were developed, most of which were to target the oncogenic MDM2-p53 pathway and restoring the p53 function (Somaiah and Tap, 2024). Nutlins are the first potent small-molecule inhibitors designed to disrupt the MDM2-p53 interaction, providing early evidence for their therapeutic potential in cancer. These molecules target the p53-binding region on MDM2, effectively blocking its ability to interact with p53 (Lu et al., 2021). This disruption enhances p53 stability, restoring its tumour-suppressive functions (Lu et al., 2021). RG7112, which is one of the derivatives of nultins, demonstrated notable preclinical efficacy against several cancers, including MDM2-amplified liposarcoma (Somaiah and Tap, 2024). However, RG7112-induced activation of p53 has been linked to the development of thrombocytopenia, an adverse effect related to reduced number of platelets in the circulation (Iancu-Rubin et al., 2014). This restricts its clinical use among patients.

Besides, SAR405838, which is also an MDM2 inhibitor, has shown potential in treating liposarcoma by accumulating p53 and suppressing tumour growth in preclinical models (Bill et al., 2016; Wang et al., 2014). In a phase 1 trial on advanced solid tumours, including liposarcoma, it was reported that 56% of patients achieved stable disease with manageable toxicity (de Jonge et al., 2017). Despite this, the trial did not demonstrate significant tumour shrinkage, indicating the need for further evaluation, particularly in combination with other therapies. Similarly, milademetan, another MDM2 inhibitor, showed encouraging results in DDLS patients in phase 1 clinical trial, with median progression-free survival of 7.4 months (Gounder et al., 2023). However, the phase 3 trial failed to show the desired effect on progression-free survival compared to trabectedin (Jones et al., 2023). Wang et al. (2024) indicated that the contrasting results between phase 1 and 3 might be due to the patients' prior treatment.

Recent preclinical studies evaluating brigimadlin, another potent MDM2 inhibitor, demonstrated tumour regression in DDLS models, outperforming doxorubicin. Subsequent clinical trials reported stable disease in a majority of WDLS (92.9%) and DDLS (88.9%) patients (Gounder et al., 2022). Currently, more trials are ongoing, exploring its use as a safer and more effective alternative to doxorubicin for advanced DDLS treatment (Schöffski et al., 2023). While there are numerous previous and current MDM2 inhibitors that achieved promising results in preclinical or clinical phase trials (Table 2.2), none have achieved FDA market approval as a therapeutic drug product.