

ORIGINAL ARTICLE

Cyclin D1 expression in acral melanoma: a case control study in Sarawak

Zainal Abidin IBRAHIM *MPath*, M Zulkarnaen A NARIHAN *MPath*, Dk Norlida A OJEP *MPath*, Ashley Edward Roy SOOSAY *DPhil** and Kok Long PAN *FRCS***

*Departments of Pathology, *Para Clinical Sciences and **Orthopaedics, Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak, Kuching, Malaysia*

Abstract

Acral melanoma has been reported to have distinctive clinical presentation and ethnic distribution compared to other histological types of malignant melanoma. Acral melanoma also exhibits distinctive focused gene amplifications, including cyclin D1 overexpression. We reviewed archived histological material of malignant melanoma in the Sarawak General Hospital from year 2004 to 2010. 43 tumours, comprising 28 acral melanoma and 15 non-acral melanoma, had sufficient material to be included in the study. The majority (36%) of acral melanoma tumours occurred in the heel. The tumours were analyzed for cyclin D1 expression by immunohistochemistry. 68% of acral melanoma were cyclin D1 positive compared to a positivity of 33% in non-acral tumours. This difference was statistically significant ($p < 0.05$). This finding may improve the histological diagnosis of acral melanoma and detection of positive resection margins.

Keywords: cyclin D1, acral melanoma

INTRODUCTION

Traditionally, morphological and histopathological features of malignant melanoma (MM) have been the mainstay for classification and prognostic evaluation in MM. The availability and the increasing number of molecular markers for MM have potentiated improvement in diagnosis, prognostic factor determination and therapeutics options. They have also enabled the disease to be further categorized in a more detailed and objective manner.

There are established ethnic variations in the incidence and anatomical distribution of MM. Generally, non-Caucasian populations including Malaysians face a lower risk of MM compared to Caucasians.^{1,2} Within the Asian population, MM exhibits unique characteristics whereby it is regularly found at non-pigmented areas of the skin i.e. on the palm, soles and under the nail. This distinct variant of MM is known as acral melanoma (AM). AM is defined by the World Health Organization as MM located on the non-hair bearing skin of the palms and soles or the nail bed (Figure 1).³



FIG. 1 A subungual acral melanoma of the big toe affecting the nail bed. The arrow points to the dark pigmented tumour mass which occupied and destroyed the nail bed.

Address for correspondence and reprint requests: Zainal Abidin Ibrahim, Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak, Lot 77 Section 22 KTL D, Jalan Tun Ahmad Zaidi Adruce, 93150 Kuching, Sarawak, Malaysia. Tel: 082 292143 / 012 3258504. Fax: 082 422564. Email: rzabidin@fmhs.unimas.my

Another unique distinction between AM compared to other MM variants is the associated risk factor. Generally, exposure to ultraviolet (UV) radiation is the major risk factor for MM. On the contrary, AM arises in relatively or completely sun-protected sites. The causative role of UV radiation in AM is considered to be negligible due to its protected anatomic location involving the extremities and the presence of thick epidermal stratum corneum of the palm and sole. In addressing these differences, Bastian *et al* have demonstrated that there are clear differences in the genetic make-up among subtypes of MM particularly AM.⁴

Due to its unique site of involvement, AM patients usually show an advanced clinical stage at presentation compared to other variants of MM. This could be due to AM lesions at these obscured sites not been noticed by the patient. The lack of the protective effect of skin pigmentation at the acral areas is also said to be a contributing factor. AM has been documented to clinically mimic several common benign lesions as the affected areas are covered by thick stratum corneum.⁵ Consequently, the invasive nature of the disease at the early stage may be clinically obscured.

Takata *et al* introduced a different approach in the classification of MM.⁶ They classified MM based on the anatomical sites and the presence of chronic UV-induced skin damage. This classification is substantiated by molecular analysis, in contrast to the currently used histological pattern based categorization which has demonstrated limited contribution to the clinical management of patients. Takata's classification includes four categories: MM on skin with chronic sun damage, MM on skin without chronic sun damage, AM and mucosal MM.⁶

Bastian *et al* in their study using comparative genomic hybridization of malignant melanoma have shown a distinct difference between AM and non-AM.⁴ All of their cases of AM exhibited gene amplification compared to only 13 percent in non-AM. The most common amplified region is chromosome 11q13. This amplification was detected in 50 percent of their acral melanoma cases. Sauter *et al* had demonstrated that cyclin D1 is one of several candidate genes for this region.⁷ It was also observed that cyclin D1 gene was always accompanied by overexpression of the cyclin D1 protein. Inhibition of cyclin D1 expression had led to apoptosis or tumour shrinkage in *in vitro* and xenograft models.

The mitogen-activated protein kinase (MAPK) signaling pathway engages an important role in regulating cellular growth and survival of various human neoplasms including MM.² MAPK pathway mediates signal transduction from cell surface receptors to downstream transcription factors that lead to cellular responses such as cell proliferation, growth, motility, survival and apoptosis. The cyclin D1 promoter is a sensor for growth signals relayed through the MAPK cascade and links this pathway to the cell cycle machinery.⁸ Cell cycle progression regulation from G₁ to S phase involves complex interaction between several groups of proteins. In cellular proliferative phase, the complex of cyclin D1 and cyclin dependent kinases (cdk) phosphorylates the retinoblastoma (Rb) protein and releases its inhibition on E2F transcriptional factor 1 (E2F1). E2F1 is believed to be the ultimate mediator of G₁ to S progression by promoting wide range of genes transcription vital for S phases (Figure 2). Cyclin D1 is a converging point for MAPK and Rb pathways.

The orderly progression of cells through various phases of cell cycles is orchestrated by cyclins and cyclin-dependent kinases (cdk) and their inhibitors. Cyclin D is the first cyclin to be increased during the cell cycle. It appears in the middle of the G₁ phase and not detectable in the S phase. There are three forms of cyclin D namely D1, D2 and D3. G1/S-specific cyclin-D1 is a protein that is encoded by the cyclin D1 genes (CCND1). Cyclin D1 interacts with four cdks namely cdk2, 4, 5 and 6. Cyclin D1-cdk4/6 complex accumulation plays a pivotal role in cell cycle progression. Cyclin D1-cdk4/6 complex partially phosphorylates Rb. The partially phosphorylated Rb is able to induce expression of genes such as cyclin E which is vital for S phase progression (Figure 2).

Deregulation of G₁ cell cycle regulatory proteins has been demonstrated in a variety of human neoplasms including AM. The cyclin D1 gene (CCND1) is a putative oncogene. Evidence shows that cyclin D1 was overexpressed in 50 percent of breast carcinomas.⁹ The overexpression phenomenon occurs through mechanism such as translocation and gene amplification. Translocation is the *modus operandi* for cyclin D1 activation in parathyroid adenoma and mantle cell lymphoma whereas in many cancers including colon, breast, lung, bladder and head and neck cancer, cyclin D1 is activated by gene amplification.^{10, 11, 12, 13}

NRAS and BRAF mutations are known to

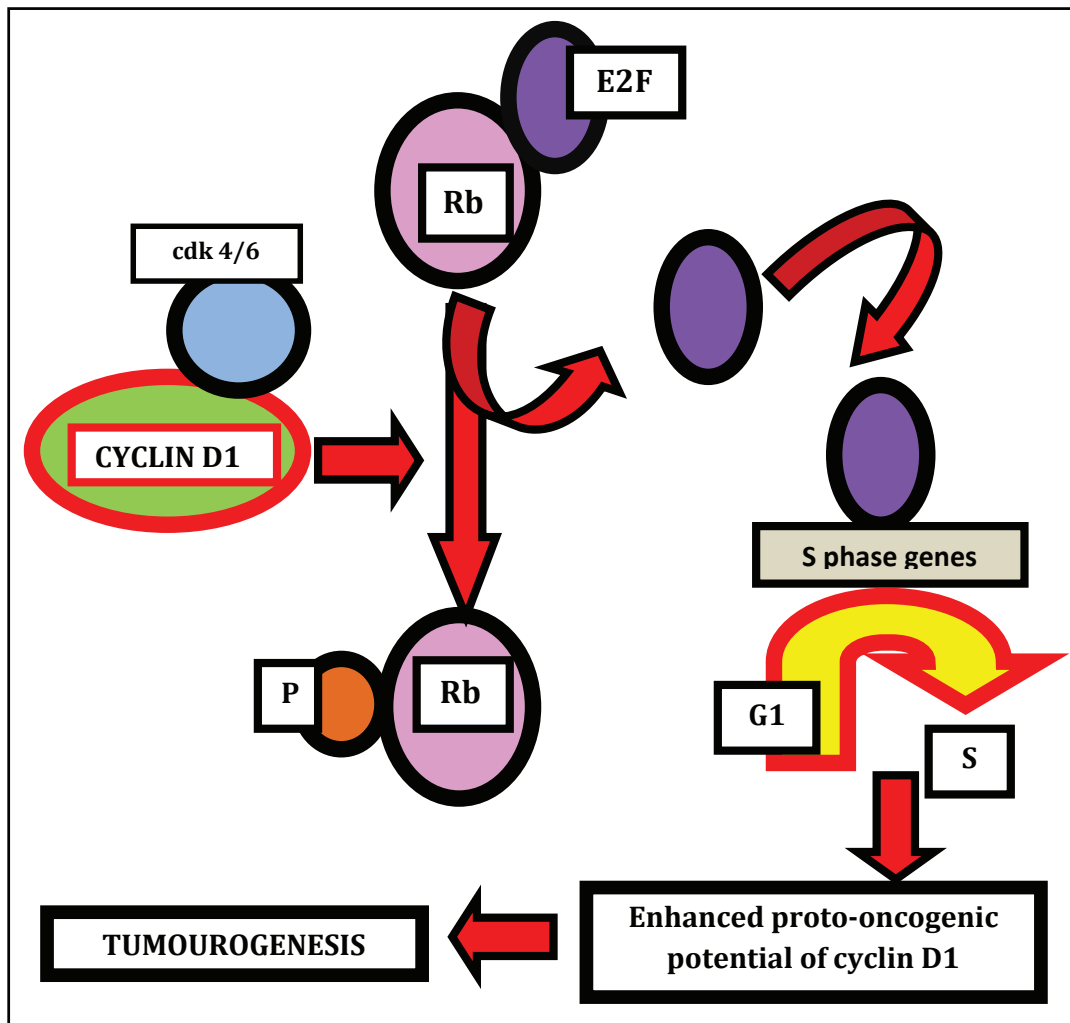


FIG. 2 Cyclin D1 overexpression and tumourogenesis.

Cyclin D1 interacts with enzymes cdk4 or cdk6 initiating phosphorylation of retinoblastoma (Rb) family of transcriptional repressor. E2F transcriptional factors are liberated allowing the synthesis of S-phase genes. The activity of cyclin D1–cdk4/6 peaks in the late G1 phase. It is among the main determinants of the initiation of DNA replication and autonomous completion of the cell cycle. The cyclin D1 cell cycle function contributes to the potential of cyclin D1 becoming oncogenic when it is expressed at high level in neoplastic process.

be rare in AM and mucosal MM in which UV exposure is not a risk factor for the pathogenesis.² Takata *et al* in their study highlighted that the MAPK signaling pathways is constitutively activated in a majority of AM despite low frequency of NRAS/BRAF mutation.² They also noted that most of AM cases that lack evidence of constitutive MAPK pathway activation, harbour CCND1 amplification.² Takata *et al* concluded that increase gene dosage of CCND1 may produce similar effects to phosphorylated ERK protein in cell growth.² Cyclin D1 protein is an important down-stream effector of the

MAPK pathway. Overexpression of cyclin D1 in neoplastic cells either contributes to neoplastic cellular progression past the G1-S phase of the cell cycle or acts as a pro-survival factor.

In our opinion, the understanding of MM heterogeneity with respect to its anatomical location, degree of UV exposure and histological characteristics mediated by distinct molecular variation is of great clinical importance. It is likely to result in novel targeted therapeutic approaches and more objective prevention strategies in AM. To our knowledge, up to date, cyclin D1 overexpression in AM has not been

assessed among Malaysian subjects.

The purpose of this study was to highlight the potential of cyclin D1 expression detection in facilitating histopathological diagnosis of AM cases. Cyclin D1 immunohistochemistry also has the prospect to be utilized as a marker to determine the adequacy of resection in AM cases and assessment of metastatic MM cases. This study aimed to demonstrate cyclin D1 expression in our local cases of AM.

MATERIAL AND METHODS

Archival paraffin-fixed tissue of MM cases was retrieved from the Department of Pathology, Sarawak General Hospital, Malaysia from the year 2004 until 2010. This laboratory is the histopathology referral center for the state of Sarawak. For the purpose of this study, MM cases that were found at anatomical sites other than the acral region were classified as non-acral melanoma (NAM). The retrieved cases were reevaluated and further confirmed by three accredited histopathologists. Cases with inadequate remaining tissue for immunohistochemical studies were excluded from the study.

Immunohistochemistry

Immunohistochemical staining by using cyclin D1 antibody was performed on the paraffin-fixed tissue sections from these cases. Expression of cyclin D1 was determined using anti-cyclin D1 antibody (monoclonal SP4)(Biocare Medical) at 1:50 dilution according to standard procedure using 3-amino-9-ethylcarbazole as the chromogen. Cyclin D1 positive breast cancer tissue served as positive control. The immunoreactivity of the tumours were assessed using X20 objective. Only cells that demonstrated definitive nuclear staining were assessed. Scores were assigned semi-quantitatively as follows (a) 0 if less than 10% of nuclei were stained (Figure 3A); (b) 1+, 10% to 25% of nuclei were stained; (c) 2+, >25% to 50% of nuclei stained; (d) 3+, >50% to 75% of nuclei stained; and (e) 4+, >75% of nuclei stained (Figure 3B). For the purpose of statistical analysis, scores 1+ and above are regarded as positive.³ Statistical analysis was performed by using Statistical Package for the Social Sciences (SPSS) version 17.

RESULTS

There were 35 acral melanoma and 20 non-acral melanoma cases retrieved for this study. 7 acral

melanoma and 5 non-acral melanoma cases were excluded due to insufficient tissue for further studies. Of the 43 cases of MM included in the study, 28 cases AM while 15 were non-acral MM. Table 1 shows the anatomical locations of the acral melanoma cases. The majority (36%) occurred in the heel.

Table 2 compares the cyclin D1 immunostaining scores between AM and non-AM cases. Taking scores of 1+ and above as positive, 68% of AM were cyclin D1 positive compared to a positivity of 33% in non-AM tumours (Table 3). This difference was statistically significant ($p < 0.05$).

DISCUSSION

Curtin *et al* in their study reiterated that AM has a higher degree of chromosomal aberration and it was uniquely characterized by a much

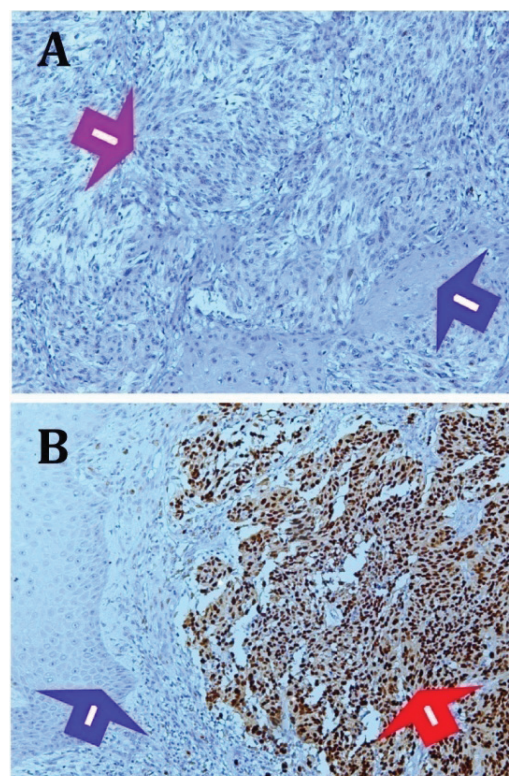


FIG. 3 **A:** Immunonegativity for cyclin D1. The blue arrow points to the overlying epidermis; the purple arrow indicates clusters of MM cells with negative cyclin D1 staining (score 0). **B:** Positive immunoreactivity towards cyclin D1. The blue arrow points to the overlying epidermis; the red arrow indicates clusters of malignant melanoma cells which are strongly immunoreactive towards cyclin D1 (4+ score).

TABLE 1. Anatomical distribution of the 28 acral melanoma cases

Anatomical site	No. (%)
Heel	10 (35.7%)
Sole (other than heel)	9 (32.1%)
Nail bed	7 (25.0%)
Web spaces	2 (7.1%)

TABLE 2. Cyclin D1 immunoreactivity score distribution of acral melanoma and non-acral melanoma

Score	Acral melanoma n=28	Non-acral melanoma n=15
0	9 (32.1%)	10 (66.7%)
1+	3 (10.7%)	1 (6.7%)
2+	1 (3.6%)	1 (6.7%)
3+	4 (14.3%)	0 (0.0%)
4+	11 (39.3%)	3 (20.0%)

Score 1+ and above is taken as positive.

TABLE 3. Comparison of Cyclin D1 expression in acral melanoma and non acral melanoma cases

Melanoma type	Cyclin D1 status		χ^2 value (df)	<i>p</i> value
	No. (%)			
	Positive	Negative		
Acral melanoma	19 (67.9%)	9 (32.1%)	4.721 (1)	<0.05 (0.032)
Non-acral melanoma	5 (33.3%)	10 (66.7%)		

higher frequency of focal gene amplification.¹⁴ In their study, gene amplifications were found in 89 percent of AM cases.¹⁴ The commonest amplified region is chromosome 11q13.⁴ It is correlated with amplification of the cyclin D1 locus which has been confirmed by fluorescence in situ hybridization (FISH) and immunohistochemistry.¹⁵ Cyclin D1 gene amplification occurs early in the AM tumorigenesis. This amplification has been found to occur even before the formation of the invasive malignant melanoma appears (in situ phase). It is also reported to be detectable on neoplastic melanocytes that were found up to 3 mm beyond the histologically recognizable tumour.¹⁶ These melanocytes play an important role in the recurrence of the tumour after inadequate surgical resection. Surprisingly, these particular melanocytes were reported to morphologically resemble normal non-neoplastic melanocytes.⁴ This further emphasizes the

importance of detecting these cells in AM. With the detection, the adequacy of surgical resection margins for AM cases can therefore be more definitively determined.⁴

Curtin *et al* also claimed that AM exhibited similar genetic aberrations regardless of whether the histological subtypes were acral lentiginous, superficial spreading or nodular melanoma.¹⁴ This observation led us to group our MM cases based on the anatomical location and disregard the histological subtypes. We used IHC-demonstrated cyclin D1 protein expression as a surrogate for cyclin D1 gene amplification. In our study we have identified a distinct cyclin D1 IHC immunoreactivity pattern among the AM cases in comparison to non-AM cases. This result is consistent with findings in other studies of different ethnicity.^{2,14,15}

With regards to the immunoreactivity pattern detection, Ewanowich *et al* highlighted distinctive pattern of cyclin D1 immunoreactivity in MM

compared to benign melanocytic lesions.¹⁷ They emphasized that only sporadic or zonal pattern of cyclin D1 overexpression was seen in dysplastic naevi and Spitz naevus. In malignant cells, the cyclin D1-positive cells were diffusely scattered throughout the lesion. They concluded that the percentage of positivity was more prominent in severely dysplastic cells.¹⁷ Therefore the distribution pattern and the intensity of the reaction need to be considered in interpreting the IHC findings. In relation to this finding, only score 1+ and above were taken as positive.

Looking at a similar study at this region, Shandika *et al* in their MM IHC study among neighbouring Indonesian subjects also observed significant cyclin D1 expression in MM cases. The majority of their MM subjects were AM.¹⁸ They found that none of the benign melanocytic naevus cases expressed cyclin D1.

Characterization of AM by means of cyclin D1 expression will enable pathologists to identify possible primary sites of a confirmed metastatic MM particularly affecting the lymph nodes. This is in view of frequent occurrences of occult AM primary sites and its presentation that may mimic several benign lesions as discussed earlier. In addition, tumour regression in MM can add difficulty in identification of the principal lesion. We propose that cyclin D1 IHC may serve as a useful adjunct to distinguish AM and non-AM and assessment of adequacy of surgical resection margins. The expression of cyclin D1 in metastatic lymphadenopathy was not performed in this study.

Cyclin D1 gene amplification represents a crucial event in the progression of MM. The increase in the number of copies of cyclin D1 genes were inversely correlated with mutation in BRAF genes.¹⁴ BRAF mutations are commonly detected in MM arising in the areas intermittently exposed to the sun. It is however rare in chronically sun exposed skin, acral skin or mucosal membrane.¹⁴ Curtin *et al* emphasized that BRAF and N-RAS mutations in MM do not show typical effects of ultraviolet "fingerprint" mutation.¹⁴ They concluded that genetic alterations at different MM sites and the levels of UV exposure indicate distinct genetic pathways. Cyclin D1 had been implicated as independent oncogenes in MM without mutation of BRAF or N-RAS. Because of its major role in cell division and proliferation, cyclin D1 overexpression is known to be associated with increase tumour cell proliferation and increases sensitivity to chemotherapy.¹⁹

With detection of cyclin D1 overexpression, there is a potential for targeted immunotherapy in AM. Ishii *et al* noted in their study on breast cancer cells that cyclin D1 protein can be stabilized by treatment with proteasome inhibitor bortezomib. They also found that overexpression of cyclin D1 is associated with improved patients' survival.¹⁹ Stabilization of cyclin D1 further amplifies the cyclin D1-dependent repression of anti-apoptotic transcription factor signal transducer and activator of transcription 3 (STAT3) *in vitro*. This eventually promotes apoptosis and slows down tumour growth *in vivo*.¹⁹

Bortezomib was also found to amplify the pro-apoptotic function of cyclin D1 raising the possibility that cyclin D1 can be a marker to predict the clinical response to this drug.¹⁹ Bortezomib promotes apoptosis by inducing stress in the endoplasmic reticulum leading to calcium release. Uptake of calcium by mitochondria causes activation of several caspases.¹⁹ Ishii *et al* found that treatment with bortezomib slowed tumour growth specifically in the cyclin D1-overexpressing breast tumour cells compared to non-overexpressing group.¹⁹ Overexpression of cyclin D1 therefore can be the candidate molecular marker for the selection of this treatment. To our knowledge, no study has been performed to assess the effects of bortezomib in AM cases.

CONCLUSION

This finding would affect the design of future studies in the diagnosis and management of AM. Cyclin D1 expression is significantly more prominent in our AM cases compared to the non-AM cases. This finding can improve the histological diagnosis of AM and has the potential to enhance the management and treatment efficacy in these cases.

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