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Circulating Melanoma Cells

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1. Introduction

Cutaneous Melanoma is an aggressive cancer which accounts for 80% of skin cancer deaths. Australia has the highest incidence world-wide and rates are increasing annually (10,000 new cases and 1,700 deaths per year). Notably it is the most common cancer in 15-39 year olds and the leading cancer related cause of death in young males (WA Cancer Registry 2007). In the USA, incidence is rising faster than other cancers, with 60,000 new cases and 8,000 deaths in 2007 ¹.

Mortality rates remain high due to metastasis of the tumour. Once melanoma has metastasised, survival is commonly 6 to 9 months, with 5-year survival rate less than 40% ²⁻⁴. Several prognostic factors have been identified, based upon pathological evaluation of the primary tumour ⁵ and lymph node metastases ⁶. However, metastatic disease particularly micrometastasis, is difficult to detect and occurs in over 30% of patients, including 8-30% of patients with *in situ* melanoma ⁷⁻⁹ and 15% of patients whose lymph nodes are negative at the time of surgical intervention ^{10,11}. In addition, metastasis can occur up to 35 years after diagnosis ¹². Effective therapies providing long term survival are very limited (<20%), often due to drug resistance associated with prevailing undetected mutations or acquisition of new mutations. Surprisingly few studies ¹³⁻¹⁵ have monitored circulating cells as a means of determining disease progression, and/or treatment efficacy or for design of personalised treatment strategies.

A great deal of information has been compiled in an attempt to identify prognostic factors that correlate with clinical outcomes. Current clinical staging is performed using measures of the pathological features of the primary tumour, lymph node and distant metastases ⁶, as well as LDH levels and now also, genetic changes in the primary tumour ¹⁶⁻¹⁹. The inability to accurately predict melanoma progression, may be related to the fact that the majority of studies use primary and less often, metastatic tumour tissue to stratify patients and delineate prognostic markers. Very few studies analyse the circulating tumour cell (CTC) phenotype that gives rise to the metastases. There is therefore an unmet need for detailed analyses of CTCs to provide early identification of metastatic risk and prognosis and evaluation of adjuvant therapies.

2. Circulating tumour cells

Millions of cells are shed from a tumour every day (approx 4×10^6 cells/g primary tumour) ²⁰, and these cells invade lymphatic and/or venous circulatory systems. Once they

have entered the blood stream they become a circulating melanoma cell population. The fact that metastasis can occur many years after surgery indicates that disseminated tumour cells may remain in the blood stream for decades, evading the immune system and apparently remaining quiescent. Interestingly, few establish clinically diagnosed metastasis^{21,22}. Those that do, particularly after many years, must acquire some activating change¹².

Since most cancer patients die as a result of metastatic spread rather than from their primary tumours^{22,23}, metastatic inefficiency of the primary tumour is likely overcome by the large number of tumour cells that enter the systemic circulation daily, estimated to be up to $\sim 4 \times 10^6$ tumour cells released per gram of primary tumour^{20,24}. Several studies have shown that the number of CTCs in patient peripheral blood increases with increasing stages of melanoma^{25,26-29, 30}. Yet the variety and phenotype of these CTCs, their ability to remain in circulation for many years thus evading the immune system, and their activation causing metastasis, remain largely unexplored, particularly for melanoma.

3. Melanoma metastasis

When cancer cells detach from the primary tumour and enter into blood vessels (or the lymphatic system), they can do so actively or passively⁷. Passive cell intravasation, where cells are simply dislodged from the primary tumour, occurs as a result of increased hemodynamic flow^{22,31,32}. By contrast, active migration occurs in cells which have separated from their neighbours and actively migrate. In addition, many circulating cells are apoptotic or necrotic and are unlikely to survive immune cell destruction³³⁻³⁷.

The question remains then, is there a phenotypic and/or genotypic difference between cells that are able to survive in the circulation and metastasise from those that cannot? Fundamentally, gene expression signatures that prompt a melanoma cell to proliferate *in situ* must be different from those that permit a cell to actively migrate, and survive as a circulating cell, and then establish a secondary tumour. Several studies have documented the differential gene expression associated with malignant progression of melanoma. Pathways associated with initiation of melanoma metastasis include the transition from radial to vertical growth phase, epithelial to mesenchymal transitions, alterations in cell adhesion properties and suppression of apoptosis^{38,39}. Included are several key steps; loss of adhesion, dermal invasion, migration from the primary site, intravasation followed by survival in the blood, migration into target tissues, and increased proliferation in the new tissue microenvironment followed by orchestration of angiogenesis at the new site⁴⁰.

Normal melanocyte cells in the epidermis are tethered tightly to other melanocyte cells and surrounding keratinocytes by cell surface molecules⁴¹. Once they become proliferative and malignant, melanoma cells lose many of the cell surface proteins that secure the tight epithelial cell-cell adhesive interactions³³. One of the key cell surface proteins, CDH1 (Cadherin 1, E-cadherin), is bound via its cytoplasmic tail to α -catenin and β -catenin, and thus to the actin cytoskeleton to maintain close cell junctions⁴². Once melanoma cells become invasive, they no longer express CDH1, but express rather CDH5 (V-cadherin) or CDH2 (N-cadherin), proteins synonymous with the start of an epithelial to- mesenchymal transition (EMT)⁴³. The EMT process, commonly utilised by migrating cells during embryonic development, involves switching of polarised epithelial cells to contractile, motile mesenchymal progenitor cells, and is triggered by secretion of growth factors (EGF (epithelial growth factor), FGF (fibroblast growth factor)) and chemotactic/pro-migratory factors SF/HGF (hepatocyte growth factor) and chemokines from stromal fibroblasts and

macrophages. This secretion induces intracellular transduction pathways (Wnt, Notch) which in turn activate transcription factors (Twist and SNAI1 and 2) ^{44,45} bringing about the invasion of melanoma cells from the epidermal, dermal border to invasion of the dermis and entry of the cells into the circulation and metastasis ^{27,46}. The invasive process is also the result of activated signalling cascades such as the NEDD9-DOCK3-Rac (Neural precursor cell expressed developmentally down-regulated protein 9- Dorsocross3 - Rac) pathway. The movement of these cells through the extracellular matrix and their migration towards blood vessels is assisted by integrin and matrix metalloproteinases ^{27,47-49}.

The next step in the active migration process is the attraction of tumour cells to lymph and blood vessels, a process mediated by ligand-receptor interactions between tumour cells and the stroma or endothelial cells. Tumour cells secrete CSF1 (colony stimulating factor 1) and growth factors such as EGF which activate the formation and proliferation of tumour-associated macrophages in the stroma. These cells in turn secrete chemokines including SDF-1 (stromal-cell-derived factor 1), SCL/CCL21 (chemokine C-C motif ligand 21) and I309/CCL1 (chemokine C-C motif ligand 1), which assist with chemotaxis of tumour cells expressing the appropriate receptors, CXCR4, CCR7 and CCR8, into blood vessels ^{27,50,51}.

Whether cells actively move toward and into nearby blood vessels or whether the process is passive and coincidental may be of some significance. Expression of specific genes that assist entry into the circulation, either passively or actively, may determine cell survival and metastatic ability. That is cells expressing genes associated with EMT, or cell migration, may be more prone to tumourigenesis and metastasis.

Model systems that quantify circulating cells leaving the tumour, show, in fact, that $3-4 \times 10^6$ malignant cells/day are shed per gram of tumour suggesting that millions of cells may be shed from a tumour every day ^{24,52,53}. Characterisation of these circulating tumour cells in patients with metastatic prostate and breast cancer indicates that they are predominantly apoptotic ³⁷ or necrotic and unlikely to survive ^{34,35,36}. Furthermore, circulating cells are often sheared and destroyed as they leave the tumour and enter the circulation. Moreover, immune cells in the circulation destroy the bulk of circulating cells and prevent all but the most active from producing metastases ^{33,54}.

Some cells do however survive for long periods of time in the vasculature ^{55,33} where they are usually found in clumps or clusters known as circulating tumour microemboli, surrounded by a "cloak" of platelets and leukocytes which assists tumour cell survival for some time by evasion of the immune system ⁵⁶⁻⁵⁸. Moreover, melanoma cell evasion of the immune system and thus survival in the blood stream is also due to the intracellular localisation of the ligand which typically activates NKD2D receptors on natural killer (NK) cells ⁵⁹. Thus evasion from attack by natural killer (NK) cells affords melanoma cells a powerful means of protection from NK cell mediated cytotoxicity. Metastatic melanoma cells also develop resistance to inhibitory cytokines through the modification of oncostatin M receptors ^{60,61}. With such an arsenal of survival mechanisms, melanoma cells may indeed survive in the circulation for long periods of time.

4. Detection of CTCs

Various methods have been used to quantify and characterise CTCs, including indirect methods, namely qRT-PCR ^{25,62-65}, and direct analyses such as immunomagnetic bead capture, or fibre-optic array scanning technology ^{28,29,66,67,68}. From these results it is obvious that; a) CTCs are present at relatively low concentrations; one tumour cell per 10^6 to 10^7

normal blood cells or on average, 1 cell per ml of blood ^{69,70,71}; b) the number of cells appears to be related to stage ^{2,25,72}; c) melanoma cell markers differ with respect to stage ⁷³; and c) CTC gene expression differs from that of the primary tumour ^{28,29}.

Quantitative RT-PCR has typically detected expression of melanocytic genes such as tyrosinase (TYR) ^{74, 75} since normal melanocytes are not thought to circulate in peripheral blood and therefore detection of transcripts from melanocytic genes should correlate to identification of CTCs ^{72,76}. The sensitivity and specificity of PCR for circulating melanoma cells is increased by analysis of multiple markers ⁷⁶, and these commonly include melan-A (MLANA), beta-1,4-N-acetyl-galactosaminyl transferase 1 (B4GALNT1), silver homolog (SILV), melanoma cell adhesion molecule (MCAM), melanoma associated antigen p97 (MFI2), melanoma antigen family A3 (MAGEA3) and microphthalmia-associated transcription factor 4 (MITF4) ^{63,64,77,78}. Several studies have shown that levels of gene expression associated with melanoma CTCs in patient blood correlate to AJCC stage, survival and disease recurrence ^{2,25,72}.

Alternately, CTCs can be positively selected from whole blood using immunomagnetic beads ⁶⁶. With this system, CTC numbers are shown to positively correlate with cell stage and be an independent prognostic indicator of progression-free and overall survival for breast cancer ⁷⁹, melanoma ²⁸ and many other cancers ⁷¹.

5. Differential gene expression profiles of circulating

The question remains then, how do we differentiate those cells that are able to survive in the circulation from those that cannot? Moreover, can we differentiate actively metastatic melanoma cells from those that are quiescent or apoptotic and is there a need to do so? It is thought that any cancer cell can acquire the ability to disseminate at any time, even early and prior to overt tumour formation ^{11,80}. For heterogeneous tumours such as melanoma, an unstable, genetically-variant, invasive cell may metastasise at any time ²⁷. Whether this is possible for all melanoma cells remains to be confirmed, but recent experiments suggest that all melanoma cells can initiate a new tumour when xenotransplanted into immunocompromised mice ⁸¹.

By contrast a plethora of information gathered over many decades indicates that melanoma cells express stage related markers that are associated with a more or less invasive tumour ⁷³. In Fig 1, for example, we have shows differential stage related expression of the melanoma cell adhesion molecule, MCAM. At early disease stages, MCAM is found in <50% cells of 4/10 primary tumours, as opposed to metastatic tissue, where all cells are MCAM+ (5/5, 93% cells).⁸². MCSP, on the other hand, is found on >80% of melanoma cells at all stages I-IV ^{14,83-85} so its expression is not stage related. CTCs are also likely to have differential gene expression signatures related to stage and these may differ relative to those of the primary tumour as previously demonstrated ^{28,29}. It is apparent that merely identifying the presence of CTCs using for example MCSP, does not necessarily provide evidence of disease progression. It may be necessary to analyse CTC phenotype or genotype to obtain more prognostic information.

In recent years a number of researchers have shown the existence of a sub-set of tumour initiating or melanoma stem cells within the primary tumour ⁸⁶⁻⁹⁰. These cells are believed to be responsible for relapse and metastasis by virtue of their ability to survive treatment and initiate new tumour formation. Rare cancer stem cells would therefore be capable of effectively managing the metastatic process ⁹¹ and would act as stem cells for metastasis

formation at a new site ⁶⁹. Melanoma stem cell markers include JARID1B (jumonji, AT rich interactive domain 1B) ⁹², ABCB5 (ATP-Binding Cassette Subfamily B (MDR/TAP) Member 5), ABCG2 (ATP-binding cassette sub-family G member 2), MDR1 (Multi-Drug Resistance 1) ^{87,93-99} and more recently CD271 ¹⁰⁰.

6. Markers of metastasis – Can they be identified in CTCs?

A plethora of studies have focused on identification of markers with sufficient specificity to accurately predict melanoma progression. Although many of these were identified using primary tissue or melanoma cell lines, they have been used for the multitude of CTC studies conducted thus far ^{72,101-103}. For qRT-PCR analysis of CTCs include SILV, MLANA, TYR, MAGEA3 and MAGE-A10 ^{25,62-64,72,104}, or more recently, ABCB5 ^{86,87} ¹⁰⁵. From high throughput analyses of melanoma gene expression, several key progression pathways have been identified ^{38,39,103,106,107} but remain to be tested as informative for CTC analysis. Key amongst these pathways are: tyrosine kinase receptors (TKRs) (e.g. VEGFR, ERBB2, TGF-betaR), the Ras / Raf / MEK / ERK pathway, the PI3K / Akt / PTEN / mTOR pathway, cell cycle regulation pathways (Rb / p53 / p16INKA / p14ARF / HDM2), epigenetic gene expression regulation and DNA repair pathways (DNA methylation, histone acetylation, RNA interference), apoptotic pathways (e.g. death receptors: FAS, TRAILR, TNFR; mitochondrial pathway: Bcl2 family), common apoptosis effectors, protein chaperoning, degradation (HSP, proteasome) ^{25,108,109} and epithelial to mesenchymal transition (reviewed in ¹¹⁰). A thorough screening of CTCs from metastatic melanoma patients for these activated pathways needs to be performed so as to establish their involvement in CTC survival, proliferation and intra- and extravasation. By detecting the presence and/or levels of genes associated with these activated / metastatic pathways, CTC analyses might be significantly enhanced.

Several reports also suggest that, in melanoma cells, altered regulation of melanocyte developmental pathways, are key to the acquisition of metastatic potential ¹¹¹⁻¹¹⁶. Indeed, melanoma metastasis reflects to some extent the migratory capacity of melanoblast developmental precursors, the neural crest cells. Moreover, genes that are critical for melanocyte development have been recognised as important factors of melanoma growth, for example MITF, DCT and SOX10 all function to maintain the stem or progenitor cell population of melanoblasts during migration from the neural crest and during melanoblast survival in the hair follicle niche ^{72,110,117-119} and may be equally important in melanoma cell maintenance and migration. It is important then to identify the expression of these developmental genes in CTCs and assess their association with metastasis.

7. Mutations in circulating melanoma cells

There is increasing evidence that melanoma metastasis is activated by mutations in multiple pathways including MAPK-ERK, PI3/AKT, PTEN and retinoblastoma pathways that regulate cyclin-dependent kinases (CDKs) (Table 1) ^{120,121}. MITF, a key transcription factor, is amplified in melanoma, and also regulated by c-KIT via MAPK/ERK and PI3/AKT pathways ¹²². Differing mutations in multiple pathways have also now been identified for different melanoma subtypes ^{120,123,124}. It remains for stage and subtype related mutations to be profiled in CTCs.

There are currently eight defined subtypes of melanoma, based on mutations in key melanoma genes, but not all melanoma cases fit into these subtypes ¹²⁵. The majority of

melanomas will have a mutation in either *BRAF* (57%) or *NRAS* (17%) but rarely have both ¹²⁴. Further details about known mutations are described below.

	Pathway(s) activated by mutations
1.	MAPK
2.	c-KIT
3.	GNAQ/GNA11
4.	NRAS
5.	MITF
6.	AKT/PI3K
7.	CDK
8.	P53/BCL
9.	Undetermined

Table 1. Melanoma subtypes - adapted from Vidwans et al. ¹²⁵

Many new treatments being developed for melanoma target these specific molecular pathways which are associated with tumour progression. Unfortunately the effectiveness of these potential treatments has so far been limited by drug resistance as a result of newly acquired mutations ¹²⁶ or the inadequate analysis of existing additional mutations. Since the presence or absence of certain mutations can drastically effect the success of targeted treatments it would be of benefit to develop a detailed profile of mutations that exist in an individual patient to maximise efficacy of treatment. One possibility is to use CTCs for stage and subtype related mutation analysis.

8. Conclusion

From our own (Fig. 1) and many other reported studies ^{33-36,54}, it is clear that positivity *per se* is not necessarily a prognostic indicator i.e. it is possible that not all circulating cells establish successful tissue metastases. Thus a more comprehensive set of experiments and additional markers are required to better understand the diagnostic and prognostic significance of circulating melanoma cells. These issues are best addressed by isolation, characterisation and quantification of circulating melanoma cells. Additionally, newly identified prognostic markers need to be measured in CTCs and assessed relative to patient outcome to delineate the metastatic potential of circulating melanoma cells and their usefulness as a prognostic indicator ^{72,127}.

We hypothesise that the ability of circulating melanoma cells to become activated, proliferative and migratory from a quiescent cell depends on several key genes. An alternate hypothesis is that malignant cells disseminate from the primary tumour early in tumourigenesis and remain in a clinically latent state until either the cells themselves or the host environment is receptive to the development of metastases. Quintana and colleagues ⁸¹ and more recently Roesch et al., ⁹² show that single human melanoma cells with no specifically identifiable gene signature can re-establish melanoma tumours when xenotransplanted into severely immunocompromised mice. It is of paramount importance therefore that we identify pathways associated with metastasis of circulating cells, ie those pathways that confer metastatic properties on quiescent melanoma stem cells capable of evading human anti-tumour immune responses. Furthermore, it is necessary to identify whether CTC numbers, gene expression profiles, or a combination of both, are key factors in

patient outcome. It also remains to be seen whether tumour related DNA mutations and the resultant activated proteins provide more accurate measures of melanoma progression. A combination of marker types is likely to be more accurate than measures that determine the presence and quantity of CTCs alone. Future studies in this field will need to be performed to address the multitude of issues alluded to in this chapter.

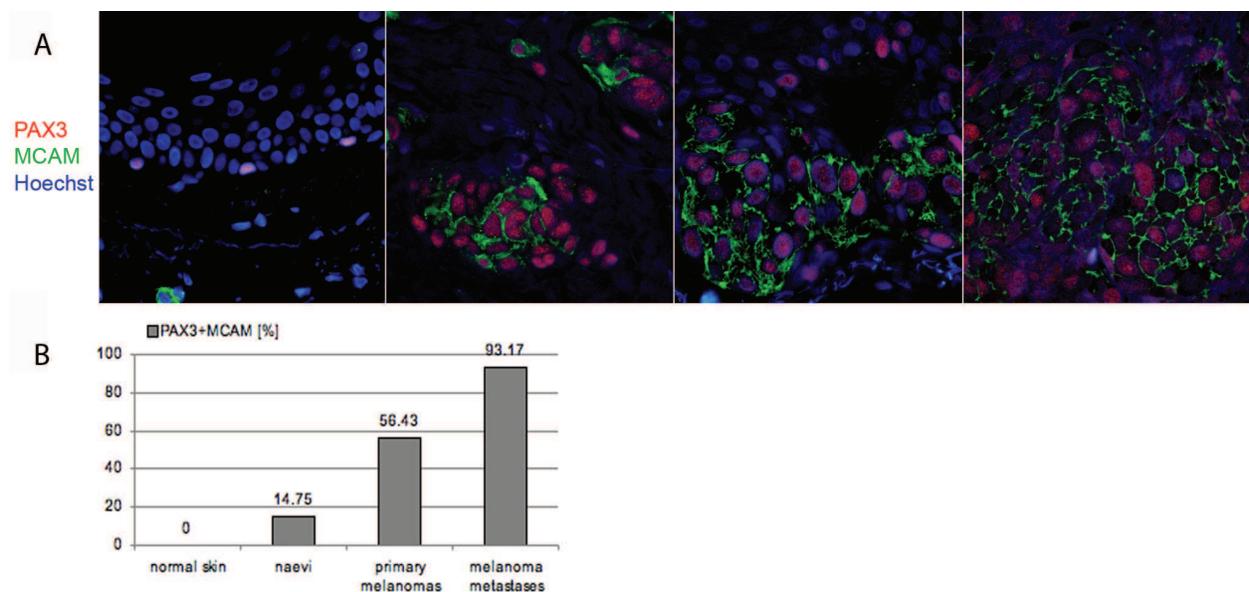


Fig. 1. A) Double immunofluorescent staining showing PAX3 (mouse monoclonal antibody, DSHB) and MCAM co-expression in normal skin (epidermal melanocytes), naevus, primary melanoma and melanoma metastasis respectively. Lines in (A) demarcate the epidermal-dermal border (EDB) or epidermal surface (ES). B) Graph showing the overall number of PAX3, MCAM double-labelled cells in normal skin, naevi, primary melanomas and melanoma metastases. Each column represents a percentage of PAX3-positive, MCAM-positive, averaged across all samples. Note: MCAM positive cells were all PAX3 positive (revised from 73)

9. References

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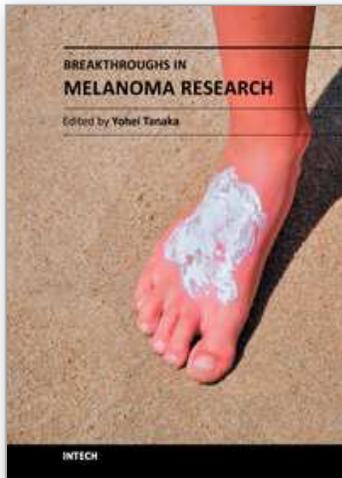
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Melanoma is considered to be one of the most aggressive forms of skin neoplasms. Despite aggressive researches towards finding treatments, no effective therapy exists to inhibit the metastatic spread of malignant melanoma. The 5-year survival rate of metastatic melanoma is still significantly low, and there has been an earnest need to develop more effective therapies with greater anti-melanoma activity. Through the accomplishment of over 100 distinguished and respected researchers from 19 different countries, this book covers a wide range of aspects from various standpoints and issues related to melanoma. These include the biology of melanoma, pigmentations, pathways, receptors and diagnosis, and the latest treatments and therapies to make potential new therapies. Not only will this be beneficial for readers, but it will also contribute to scientists making further breakthroughs in melanoma research.

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