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Running Head: Circulating Cells and Metastatic Progression of Cutaneous Melanoma

Title: Genetic Factors in Metastatic Progression of Cutaneous Melanoma; The Future Role of Circulating Melanoma Cells in Prognosis and Management.

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Abstract

The greatest potential for improvement of outcome for patients with Cutaneous Malignant Melanoma lies in the prevention of systemic metastasis. Despite extensive investigation, current prognostic indicators either alone or in combination, although related to melanoma progression, are not sufficient to accurately predict the pattern of progression and outcome for any individual patient. Metastasis related death has been recorded in patients initially diagnosed with early stage tumour and in other patients many years after initial tumour removal. The trouble finding a predictable pattern in the puzzle of melanoma progression may possibly be linked to the fact that most of the material studied for prognosis is either, cutaneous primaries or metastatic deposits rather than the melanoma cells in the circulatory system which are responsible for disease progression. In this review article we discuss the potential use of circulating tumour cell (CTC) detection and quantification for identifying patients at risk of metastatic deposits. We also discuss current therapies for the treatment of metastatic melanoma and analyse how the use of CTCs may be used to evaluate the effectiveness of current therapies and to pinpoint patients who require further treatment.

Introduction

Cutaneous Malignant Melanoma (CMM) is the most severe of all skin cancers, accounting for 80% of skin cancer related deaths.Lewis [1]. CMM is the most common cancer in Australia for persons aged 15-39 and the deadliest cancer affecting young Australian males. In 2006 in Australia there were 10,000 new cases, 1,700 deaths and rates are continuing to rise [2]. In the United States CMM is an emerging problem as the incidence has increased more than that of any other cancer over the past two decades. More specifically, in 2005 the American Cancer Society recorded approximately 59,580 new cases and over 7,910 new deaths [3, 4].

CMM is often aggressive, unpredictable and difficult to treat. Mortality rates continue to remain high due mainly to progression of multiple drug resistant metastases for which the cure rate is currently less than 20% [5, 6]. Much effort has been put into identifying prognostic factors that correlate with clinical outcomes to enable The American Joint Committee on Cancer (AJCC) to devise an accurate TNM clinical staging system. Histopathologists determine the tumour thickness (T) component of this system, which most accurately characterises the CMM once excised [7]. Other features including ulceration, mitotic rate, clinical or surgical detection of lymph node (N) or distant metastasis (M) make up the information needed to stage and thus prognosticate and treat appropriately [8]. Other details including sex, age, LDH levels and anatomical location of the melanoma and metastases, residual disease following initial resection and genomic changes in the primary tumour have been linked with prognostic changes but so far have little material influence on treatment plans [9-11].

Despite extensive investigation, the above factors either alone or in combination, although related to melanoma progression are not sufficient to accurately predict the pattern of progression and outcome for any individual patient. Metastasis related death has been recorded as long as 35 years following initial removal of the primary tumour (uveal melanoma) [12]. The trouble finding a predictable pattern in the puzzle of melanoma progression may possibly be linked to the fact that most of the material studied was based on either cutaneous primaries or metastatic deposits rather than the quantity and phenotype of melanoma cells in the circulatory system. Circulating tumour cell (CTC) detection and quantification may hold the key to identifying patients at risk of metastatic deposits. Analysing CTCs has the potential not only to pinpoint patients who may require further treatment but evaluate the effectiveness of current therapies.

Current Methods of Treatment of Melanoma

CMM, generally referred to as melanoma, arises from melanocytes, cells of neural crest origin which migrate into the skin [13]. CMM can grow laterally in the epidermis or vertically through to the dermis ultimately leading to vascular invasion and metastatic disease. Stage 1 or 2 melanoma can be cured by wide surgical excision resulting in a 50-100% 5 year survival rate, decreasing with increasing tumour thickness [14]. Following surgery, patients are examined and depending on the histological features of the primary they may have further investigations including sentinel node biopsy, CT or PET for staging purposes [14]. Patients with tumours under 2mm do not require any investigations for staging as at this level imaging is not specific enough to identify metastases accurately, even though up to 44% of these people eventually die from metastatic disease despite having the primary removed

[14, 15]. Patients diagnosed with lymph node or systemic metastasis have a 5 year survival rate that plummets to between 5-60% [8, 16]. So far however, there is no specific gene within the tumour cells in the primary that can accurately predict the aggressiveness of the tumour [17].

There is no doubt that a cancer's mortality rate is determined by the speed of metastasis [18, 19]. In all tumours, millions of tumour cells (reported to be around $4x10^6$ per gram of tumour over a 24 hour period [20, 21]) invade the venous or lymphatic circulation very early in tumour development and result in disease progression [22-25]. Moreover a meta-analysis has shown that the number of CTCs in a patient's peripheral blood increases with increasing stages of melanoma [26]. Despite these recent advances there is still much to discover, including the phenotype of the melanoma CTCs that are able to stay dormant in the circulation for many years, evading the immune system, and the events leading to their activation, seeding and proliferation in distant bodily organs.

Once the tumour metastasises, and thus needs systemic treatment, there is a considerable lack of investigations available to give information to measure the current disease load and response of treatments. The AJCC currently uses metastatic site and serum LDH to put a number to the 5 year survival rate for prognostication purposes [8]. Treatment is measured in terms of the Objective Response Rate (ORR) defined as the percentage of patients with identifiable tumour shrinkage plus the percentage of patients with complete tumour disappearance following treatment [27].

Analysis of volume and phenotype of CTCs could have a place in monitoring current treatment responses. Additionally, development of markers that can reliably detect CTCs could potentially identify which tumours may respond to current treatments and highlight possible avenues for future treatment development. Current treatments are purely palliative as they have a response rate under 20% and do not have a significant survival benefit [28, 29]. CTCs as a marker could be used to determine whether the gold standard treatment DTIC is effective in treating disease as to date there is no placebo controlled trial to confirm its efficacy [28, 29]. Interleukin 2 (IL2) immunotherapy, a treatment developed in the 1990's has shown an overall response rate of 16% and these responders had a progression free period of over 5 years[28]. As IL2 is known to have severe side effects it is restricted to those with an excellent performance status and organ function, but ideally it would be desirable to have a way to identify who will respond. There is a possibility that analysis of CTCs would provide a way of identifying responders so that this treatment can be given to those most likely to benefit.

Vaccine treatment has been used in the effort to find a treatment that improves survival. Due to the heterogeneity of melanoma cells there has been a limited response rate of 12% with a mean increased overall survival (OS) of 21.3 months, triple that of DTIC [28, 30]. With further analysis of CTCs the genetic identity of the responders to IL2 or vaccines could be identified and vaccines could be developed to target the markers found in patient subsets.

B-RAF inhibitors are an exciting new mode of treatment targeting a mutation in B-RAF, an oncogene that promotes growth and angiogenesis when activated [31]. A

selective inhibitor of active B-RAF, PLX4032 has been shown to have a 78% response rate in phase 1 trials and phase 2 and phase 3 trials began in September 2009 and January 2010. Despite their initial success however, reports indicate that there is rapid acquisition of drug resistance resulting in a restriction of therapeutic response to an average 8 months [32-34]. There is, therefore, an urgent need for early detection of resistance to kinase inhibitors. The method currently used to evaluate the response of this treatment is an overall Response Evaluation Criteria in Solid Tumours (RECIST) which uses current imaging methods to find measurable lesions and monitor size before and after treatment. CTCs have the potential to enhance this process by using tumour cell load to monitor microscopic response to treatment and early acquisition of drug resistance, while current methods can only measure macroscopic metastasis presence/shrinkage.

Knowledge to date on the process of melanoma cell metastasis – not as simple as originally thought.

Understanding the processes that lead to metastasis are important for choosing significant molecular markers to characterise CTCs for future treatment strategies. The process of metastasis in melanoma requires loss of intracellular adhesion, dermal invasion, migration from the primary site, intravasation, intravascular survival, attachment to the vasculature of target tissues, migration into target organs, proliferation and angiogenesis at the target site [35]. Of particular relevance in this instance is the process of tumour cell dissemination to seed metastasis.

Melanoma cells arise in the epidermis, where initially, like melanocytes, they are tethered tightly to the surrounding melanoma cells, melanocytes and keratinocytes by a complex network of desmosomes, the adhesion molecules and gap junctions which allow transfer of melanin [22, 36, 37]. Once melanoma cells enter the invasive stage they progressively loose these adhesion surface interactions [38-40]. One of the key surface proteins, Epithelial Cadherin (E-Cadherin/CDH1), is bound via its cytoplasmic tail to α- catenin and β-catenin which links it directly to the actin cytoskeleton of the cell [41]. In fact the switching of E-Cadherin/CDH1 with Non-epithelial Cadherin (N-Cadherin/ CDH2) indicates the initiation of an epithelial to mesenchymal transition for the cell (EMT) [37]. The EMT process, initially utilized by migrating cells during embryonic development, allows the cell to transform from a polarised epithelial cell to a contractile, motile mesenchymal cell and this process is triggered by secretion of growth factors from fibroblasts and macrophages (e.g. hepatocyte growth factor (HGF), and/or fibroblast growth factor (FGF)). This secretion induces intracellular transduction pathways (Transforming growth factor-β (TGF-β), Platelet Derived Growth Factor (PDGF), Wnt and Notch) which in turn activate transcription factors (Snail and E47) [37, 42]. This cascade leads to the aforementioned switching of E-Cadherin to N-Cadherin allowing the cell to detach from the epithelium and migrate into the circulation leading to tumour progression [22, 43].

When melanoma cells detach from the primary tumour and enter the bloodstream or lymphatics they can do so actively or passively [18]. Passive entry occurs when cells are simply dislodged from the primary tumour due to increased blood flow and low CDH1 levels [18]. By contrast, active migration occurs in cells when NEDD9, a

melanoma metastasis protein, forms a complex with DOCK-3, a guanine nucleotide exchange factor, to activate Rac, a GTPase which acts on actin assembly, actomysin contractility and microtubules [44]. The result of Rac activation is that the cell becomes mobile and elongated so that it can pass between other cells [44]. The movement of these cells is assisted by matrix metalloproteinases that destroy integrins (cell receptors that mediate attachment to surrounding cells) and other extracellular materials [45].

The next step in the active migration process is the attraction of the tumour cells to lymph and/or blood vessels, a process mediated by ligand-receptor interactions between tumour cells and the stroma or endothelial cells. The tumour cells secrete Colony Stimulating Factor 1 (CSF-1) and growth factors such as Epithelial Growth Factor (EGF) which activate the formation and proliferation of tumour associated macrophages in the stroma. These macrophages secrete growth factors, cytokines, chemokines and enzymes that regulate tumour growth, angiogenesis, invasion and/or metastasis [22, 46].

Whether cells actively move toward and into surrounding blood vessels or whether this process is passive and coincidental may be significant. Expression of the genes and production of the complexes mentioned above, that result in active entry, may enhance survival of the cell once it is in the blood and increase metastatic ability. Thus markers for the above factors may provide a better measurement of metastatic potential than sheer number of circulating cells.

In order to investigate circulating tumour cells in more detail, models have been developed in which a tumour and its vascular system are isolated and blood vessels are monitored so as to quantify and characterise the cells entering and exiting the tumour [18, 20, 47]. Notably, a large volume ($3\text{-}4x10^6$) of cells are shed every day, with few of these developing into clinically detectable metastases. Characterisation of these circulating cells in patients with advanced breast and prostate cancer indicates that they are predominantly apoptotic and necrotic and are unlikely to survive [48-51]. Furthermore, it is believed that the bulk of circulating cells are destroyed by sheer stress; moreover circulating immune cells prevent all but the most proficient from producing secondary colonies [38, 52].

Nevertheless, some cells do survive for long times in the vasculature, though not independently, with the exception of hematopoietic cancers [38, 53]. Tumour cells are usually found in clumps, also known as circulating tumour microemboli, which are hidden beneath a "cloak" of platelets and leukocytes which may enhance survival [38, 54, 55]. Another factor promoting melanoma cell survival in the vasculature is evasion of natural killer (NK) cells, whose primary role is to pinpoint and destroy cancer cells. One mechanism melanoma cells have is intracellular retention of the NKG2D ligand which is usually expressed on the surface of infected or mutated calls and targets the cell for NK cell mediated destruction [56]. Another way it evades the immune system is by not expressing the oncostatic M receptor, which, when activated by IL-6 prevents cell division [6, 57].

It is well established that a melanoma cell may invade the circulation and survive without clinical evidence of a primary tumour, and so, not infrequently a patient may

have metastatic melanoma without a known cutaneous primary site [58, 59]. In fact, melanoma can still be lethal even when tumours cannot form due to the patient having an inbuilt inability for the tumours to form a vascular supply [4]. In this case the patient developed high levels of cells in the blood and CSF and eventually succumbed to renal failure from tumour lysis syndrome. Taken together with the fact that 9-45% of surgical excisions result in recurrences and up to 8% of melanoma in situ diagnoses result in recurrence, it is clear that circulating cells have a significant prognostic value and a more detailed investigation of the phenotype of actively metastasizing CTCs would have major implications for future clinical diagnoses and treatments [60-65].

Current knowledge of the genetic identity of melanoma cells

The question remains then, how do we identify the few CTCs that are capable of surviving in the circulation and metastasizing? The genetic factors needed to survive in the circulation must be different to those needed to proliferate in situ and also separate from those needed to establish a secondary tumour, so the cells surviving in the circulation for an extended period of time are the ones needed to identify this phenotype and survival advantage. Analysis of the genes expressed in circulating cells compared to primary and secondary tumour cells is one way of highlighting some of the changes in phenotype and adaptability necessary for their survival. This approach has been supported by observations of the different genes expressed in primary versus circulating cells from the same patient with melanoma [23, 24]. It has also been shown that there are subpopulations of cells within a primary tumour that resemble metastatic deposits; the cells are changed enough that they will survive to metastasis [66]. Alternately, there is no reason that any cancer cell cannot develop the ability to

metastasise, even prior to clinically detectable tumour formation [67]. Therefore, there is a possibility that for heterogeneic tumours like melanoma, an unstable, genetically variant and invasive cell could survive in the circulation but not exist in any volume in the primary tumour [22, 68]. Genetic changes that provide an edge in metastasis and proliferation at a secondary site may not be advantageous in the primary tumour and thus too rare to be identified in its population-averaged gene expression profile [22]. The preponderance of patients with in situ or occult melanomas (discussed in previous sections in more detail) supports these theories.

In the last few years a number of researchers have shown the existence of a subset of tumour initiating melanoma stem cells within the primary tumour that possess two important features. One of these features is the ability to self renew as well as differentiation into cancer progenitor cells. The other of these features is the innate resistance to chemotherapy and radiation [69-72]. These rare cancer stem cells are present early on (rather than immediately prior to metastasis as originally thought) and effectively manage the metastatic process when the detectable bulk of the tumour visible by current techniques has been successfully treated [73]. They evade therapy due to their stem cell properties of slow turnover and chemotherapy resistance, and upon reaching their destination would act as a seed for metastasis formation [74]. Melanoma stem cells have been identified in primary tissue and cell lines by expression of recently identified melanoma stem cell markers, namely, Multi-Drug Resistance gene product 1 (MDR-1) [75], CD20 [70, 76-78], CD44/CDH5/VE-Cadherin [78, 79], CD133 [70, 77, 78, 80], ABCB5 [75, 77, 78, 80], ABCC2 [75], ABCG2 [70, 80], human telomerase reverse transcriptase (hTERT) [75], nanog [75] and JARID1B [81]. However unlike normal stem cells, cancer stem cells may be

termed stem-cell like since they have mutations in key signalling pathways that may lead to 'phenotype instability' enabling cancer cells to switch their phenotype in response to microenvironmental cues [82].

Evidence of melanoma stem cells in the circulation of melanoma patients remains to be demonstrated but similar cells have been found in the bone marrow in breast cancer patients [83].

Melanoma markers identified

A large volume of studies have been dedicated to identification of markers with sufficient sensitivity and specificity to accurately predict melanoma progression. Even though many of these markers were identified from primary lesion tissues, these markers have been tested for in some CTC studies [84-87]. Mentioned previously, melanocytic and melanoma markers commonly used for qRT-PCR of CTCs include MAGE-A3 [64, 87-89], Mitf [87, 90], MART-1/Melan-A [64, 87-89], Tyr [26, 87, 89] and most recently ABCB5 [71, 77]. As a result of high throughput analyses of melanoma progression pathways several key pathways have been identified including epithelial-mesenchymal transition [91] but many of these are yet to be tested as informative for CTC analysis using either gene expression or whole cell detection techniques [17, 86, 92, 93]. Key amongst these pathways are: tyrosine kinase receptor (TKR) pathways (e.g. VEGFR, ERBB2 and the TGFβ receptor) [37], the Ras/Raf/MEK/ERK pathway [37], the PI3K/Akt/PTEN/mTOR pathway [37], cell cycle regulation pathways (Rb/p53/p16INKA/p14ARF/HDM2/CXCR4/CDC42) [94], epigenetic gene expression regulation and DNA repair (DNA methylation, histone

mitochondrial pathway and RNA interference) [95, 96], apoptotic pathways (e.g. death receptors: FAS, TRAILR, TNFR; mitochondrial pathway: Bcl2 family) [97], and epithelial to mesenchymal transition (CDH2, SPP1 and SPARC) [98]. Epithelial to mesenchymal transfer (EMT) has been thought to be the key to metastasis by giving malignant melanoma cells stem like capabilities to migrate within and through tissues. In 2010 Roesch [81] has added a further marker to this bank, JARID1B, which identifies slow cycling melanoma stem cells, first identified by Adams and Strasser [99] in 2008. This marker is expressed on melanoma cells which are slow to divide and so also resistant to current treatments but was not linked with the classic EMT signature or any other stem cell markers suggested to date.

It is possible that if these pathways are analysed further in CTCs that they may play a role in CTC survival, proliferation and intra- and extravasation. Furthermore, with continuing discovery of markers specific to melanoma stem cells and metastatic pathways a marker may be found that reliably detects metastatic melanoma which would be useful especially in detecting amelanocytic melanoma where there are only small levels of melanocytic gene expression and tracking down CTCs in patients with no primary melanoma. Moreover melanoma stem cell markers may be able to be used to identify residual melanoma in patients with complete regression according to the current radiological methods of monitoring treatment effectiveness.

In summary, conventional staging and treatment of melanoma is not particularly precise and effective. This has the potential to be dramatically improved by investigating molecular and cellular markers/pathways which lead to tumour progression. Such markers could include proteinases (proteinases of the plasmin

system, serine proteinases and matrix metalloproteinases) which degrade the extracellular matrix on the path to the circulation or create a basement membrane at the target site. Another possibility is the analysis of the superficial glycoproteins, factors responsible for cell adhesion (integrins) and intercellular communication (cadherins). Furthermore neoangiogenesis markers such as vascular endothelial growth factor (VEGF), endoglin (CD105), a transmembrane glycoprotein which is a component of the receptor for activating TGFβ, as well as neuropilin (NRP1), the co-receptor for VEGF could be included [37]. As previously described, markers of EMT transition (Twist, PI3K/Atk and CDH1) and melanoma stem cell markers (e.g. ABCB5) may also be of value in this analysis.

It has been reported that alteration of the pathways involved in melanocyte development may be the key to acquiring metastatic potential in melanoma [13, 100-103]. In fact, melanoma metastasis mimics the migratory capacity of neural crest cells, which are the embryonic precursors of melanocytes. Furthermore, some of the genes responsible for melanocyte development have been implicated in the process of melanoma formation, for example, PAX3 [87, 98, 104-106], Mitf [82, 87, 98], dopachrome tautomerase (DCT) [98, 106] and SOX10 [98]. These genes maintain a population of melanoblasts during migration from the neural crest during embryogenesis and from the hair follicle niche in mature skin. It is possible that these cells may also be the key to understanding melanoma stem cell maintenance and migration. The developmental genes and the signalling pathways required for maintenance of stem cell niches may help in the understanding of melanoma heterogeneity and the biological properties of melanoma cell subpopulations [82].

The use of CTCs as a prognostic indicator – valuable or not?

From past studies it has been noted that positivity for CTCs is not a prognostic indicator in itself i.e. not all circulating cells establish successful metastases. Circulating breast cancer cells have been found many years after mastectomy where only 1% develops recurrence though patients with circulating cells shortly after mastectomy are more likely to develop recurrence [107]. In uveal melanoma the quantity and genetic profile was more predictive than mere presence of CTCs in the blood, which was relatively inaccurate [108]. Moreover there is not yet any evidence that tumour progression is linked to increased genetic marker levels in the circulating CTCs. A more thorough analysis of the phenotype of CTCs associated with progression is still needed to confirm whether this may lead to a reliable marker. It is possible that increased marker level is due to change in phenotype rather than increase in number of CTCs.

A more comprehensive set of analyses of CTCs are required to better understand the diagnostic and prognostic significance of CTCs. We propose that these issues be addressed by isolation, characterisation and quantification of circulating melanoma cells. Additionally, application of newly identified markers to the long term follow up of patients will delineate the metastatic potential of circulating melanoma cells and their usefulness as a prognostic indicator.

Conclusion

This article addresses the transformation of cells from clinically latent to metastatic proliferating cells. We hypothesise that the ability of circulating melanoma cells to become activated, proliferate and migratory from a clinically latent cell depends on several key genes. An alternate hypothesis is that malignant cells disseminate from the primary tumour early in tumour development and remain in a clinically latent state until either the cells themselves or the host environment is receptive to metastasis. Quintana [109] showed that at least 25% of randomly harvested human melanoma cells from 12 different patients will re-establish a melanoma when xenotransplanted as single cells into severely immuno-compromised mice. Since this is unlikely in humans with an immune response and these cells did not form metastases, it is important to identify the pathways responsible for transforming melanoma stem cells, which are normally clinically latent, into cells capable of metastasis. Microarray analysis of tumour cells from different stages of melanoma has demonstrated that the genetic signature of the cell changes as the tumour progresses [24]. This adds to the evidence that analysing the phenotype of circulating cells may be beneficial in prognosis.

Current methods use only histological analysis and imaging to determine patient prognosis. While novel therapies are continuing to be trialled for the treatment of melanoma, mortality rates remain high [110]. Measurement of circulating melanoma cells has not yet been refined sufficiently so as to make it an effective diagnostic or prognostic tool but there is still potential for further discoveries to make this leap. Future studies involving isolation and gene expression analysis of circulating cells may provide crucial information about the changes in genes required for metastatic success and the numbers of cells expressing these genes needed to establish successful

metastasis. As well as the benefit identification of these genes may make to prognostic information, they may also be harnessed as targets for future treatment regimes.

Markers that highlight active, metastatic cells in the circulation would potentially enable understanding of melanoma metastasis like that already identified for breast cancer. Detection of CTCs is already a significant prognostic indicator in breast cancer patients as it relates directly to disease status and treatment efficacy [111, 112]. Indeed it more reliably correlates with overall survival than current imaging methods as they are relatively insensitive, especially in early metastasis [113, 114]. Finally, it is important to note that most cells that enter the circulation are apoptotic or necrotic yet current, conventional qRT-PCR is unable to exclude these cells. Preferentially, a paradigm embracing the isolation, quantification and characterisation of melanoma stem cells could potentially act as a more reliable and sensitive measure of progression and clearance of malignant melanoma which has already undergone existing or newly developed therapies.

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