A blood test to identify when melanoma metastasizes: a reality for melanoma management?

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Background: why do we need a blood test for metastatic melanoma?

Malignant melanoma is an aggressive skin cancer arising from the melanocytes. For many patients, the appearance of metastatic disease occurs (sometimes years) after apparently curative surgical excision of primary lesions. In others, presentation occurs with metastatic disease. In either case, once the disease becomes unresectable and management becomes entirely medical, long-term survival is unusual. For many years, the medical management of metastatic melanoma was largely ineffective and the prognosis of advanced disease was accordingly poor, with survival after diagnosis of visceral and/or CNS metastases typically being only a few months. The reasons for this dismal situation were several-fold:

- The frequent presentation of metastatic disease with multifocal, large-volume, rapidly growing lesions;
- The strong propensity of the disease to metastasize to visceral and CNS sites;

- The very limited efficacy in melanoma of conventional cytotoxic drugs that are active in epithelial cancers;
- The absence of effective adjuvant (post-operative) therapy, which, in other tumor types, can favorably modulate the prognosis and outcomes.

The limitations of traditional systemic therapy with the alkylating agent dacarbazine (the most frequently used cytotoxic drug) are illustrated by the reported response rates of 7–15% in stage 4 melanoma. Moreover, responses are often differential and almost never result in long-term disease control. Against this historical background, the therapeutic landscape of melanoma management is now undergoing a phase of unprecedented (and rapid) evolution. Advances in medical treatment have come on two fronts. First, the genomic era has identified at least three ‘druggable’ driver mutations in melanoma (with more certain to follow). The most frequent of these is in the *BRAF* gene, with mutations at codon 600 occurring in 40–60%

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of melanomas. Inhibitors of the mutant BRAF protein have shown remarkable (albeit frequently ephemeral) efficacy. Examples include (the first-in-class) vemurafenib, dabrafenib and others currently in development [1]. A second frequent molecular genetic event is mutation in the *NRAS* gene, reported in 15–20% of metastatic melanomas and frequently associated with clinically aggressive disease. Preclinical data suggest that the presence of *NRAS* mutations results in sensitivity to pharmacological inhibition of MEK, and trials of MEK inhibitors in *NRAS*-mutated stage 3C and 4 melanoma are now in progress. In addition to *BRAF* and *NRAS*, a further subset of melanomas, particularly those arising from mucosal surfaces, contain mutations in the *C-KIT* gene, which confer sensitivity to imatinib. Taken together, such genetically stratified melanomas comprise a significant majority of melanoma cases. Second, there is now a convincing and rapidly growing volume of evidence suggesting that activation of host immunity by specific monoclonal antibodies directed at inhibitory cell surface molecules on host T lymphocytes can result in immune activation and immune-mediated tumor cytoreduction. Evolving experience in the use of these new immunotherapeutic agents strongly suggests that long-term disease control and potentially cure could be achieved in some patients [2].

Tumor markers

Blood-borne tumor markers have long been used in oncology in order to facilitate diagnosis and, more commonly, to monitor response to therapy. These include CA125 (ovarian cancer), CA153 (breast cancer), CEA (colorectal cancer) and CA19.9 (pancreatic cancer). In melanoma, S100 is sometimes detectable in peripheral blood, but has had little use in directing management. Although an attractive concept in principle, the use of these established tumor markers in order to identify early relapse and/or metastatic disease and thus to inform the expeditious use of systemic therapy has not resulted in major improvements in overall survival. For example, in ovarian cancer, the use of chemotherapy determined solely by a rising serum CA125 does not confer any survival benefit [3]. However, the very different approaches to systemic therapy in melanoma, particularly the confirmed efficacy of immunomodulatory therapies with antibodies such as ipilimumab and anti-PD1, imply that deployment in early, low-volume metastasis is

likely to result in better outcomes (including, in some patients, the potential for cure), and the evidence from clinical trials clearly supports this hypothesis. For example, in the seminal paper by Hodi *et al.*, in which the efficacy of ipilimumab was first reported, subgroup analysis revealed that patients with stage 4A and 4B disease had significantly better outcomes than those with stage 4C (disseminated) disease [4]. This suggests that, unlike diseases such as ovarian cancer, in which cytotoxic therapy remains the basis of medical management and cure is rarely achievable in recurrent disease, immunomodulatory agents may achieve cure in a subset of advanced melanoma patients, and the probability of this is significantly greater when these drugs are deployed in early metastatic disease. Therefore, the implication is clearly that the identification of patients with either residual melanoma postresection or subclinical relapsed metastatic disease could inform the early use of agents whose efficacy is highly likely to be optimal in low-volume disease, a situation analogous in some respects to conventional adjuvant chemotherapy in epithelial malignancies, such as breast and colorectal carcinomas. The ability to identify patients with subclinical relapsed/metastatic disease is therefore a priority in melanoma research.

Epigenetic biomarkers

Whereas cancer genetics involves the study of structural or numerical changes in the genes that occur in cancer, epigenetics refers to heritable, nonstructural changes that affect gene expression. A major modification that affects gene transcription is methylation at CpG dinucleotides [5]. In cancer, regions of the genome in which CpGs occur at high density (CpG islands) are typically unmethylated in normal cells, but may become methylated in neoplasia, with concomitant transcriptional silencing. Detection of methylated genomic DNA in tissue or serum has several properties that make it an attractive potential source of biomarkers:

- Methylation is frequently (but not invariably) specific for neoplasia;
- Methylated genomic DNA is relatively stable in biofluids;

The methodology for analysis of methylated DNA is readily amenable to automation, allowing large numbers of samples to be processed.

How can novel epigenetic biomarkers be identified?

A number of strategies have been described for the identification of genes whose methylation might have clinical utility as biomarkers. The most frequently used is the candidate gene approach. This usually entails analysis of a gene in which methylation-dependent transcriptional silencing makes biological and mechanistic sense.

A second approach utilizes genome-wide analysis of the RNA expression of cell lines for which a primary (non-metastatic) line is available, together with derivative cell lines that have acquired metastatic properties. This can be effectively combined with genome-wide methylation array technology in order to correlate aberrant CpG island methylation with down-regulated expression in the metastatic cell lines. Candidates identified by this approach can then be tested in clinical series. A number of genes have been reported whose methylation in tissue is associated with poor prognosis in melanoma, and a subset of these have also been examined in serum, including *RASSF1A* [6], *AIM1* [7] and *TFPI2* [8].

What do we need to do to make this a reality?

The principle that methylated genomic DNA can be detected in the peripheral blood of melanoma patients is now clearly established. However, several caveats exist that must be addressed before the routine use of this technology in clinical practice can be envisaged:

Optimal analytical methodologies combining sensitivity, specificity and cost-effectiveness need to be established. Some laboratories have used quantitative methylation-specific PCR (e.g., with systems such as MethyLight®) in order to successfully detect methylated DNA in serum. The advantages of quantitative methylation-specific PCR are: technical simplicity; compatibility with existing 'real-time' quantitative PCR infrastructure; and the PCR reaction is specific for methylated DNA and hence the presence of background levels of 'normal' genomic DNA in blood do not dilute the positive methylation signal, thereby reducing the possibility of false-negative samples. In other studies, pyrosequencing has been employed for the detection of methylated DNA. Pyrosequencing allows for the quantification of methylation at each individual CG dinucleotide within an amplified fragment

of DNA. As such, the technique enables high-resolution analysis of methylation, which is not possible with methylation-specific PCR. Furthermore, small changes in methylation at each CG site can be easily detected. The disadvantages of pyrosequencing are that it requires a dedicated pyrosequencer and software and that the presence of large amounts of normal DNA in blood may dilute the percentage of methylation, masking methylated dinucleotides and so resulting in the possibility of false-negative results.

The sensitivity and specificity of individual candidate genes for the identification of subclinical metastasis and relapse must be prospectively validated in large, independent, well-annotated clinical series. It remains to be determined whether analysis of a single gene such as *TFPI2* [8] will afford sufficient sensitivity, or whether a panel of marker genes will be required.

We need to determine exactly how the detection of methylated genomic DNA in serum is optimally employed as a clinical biomarker. For example, is it sufficient simply for levels of methylated DNA to be above predesignated cut-off levels (established in large-scale analyses of appropriate populations), or does a 'positive' test require evidence of rising levels of methylated DNA in serial samples?

Resolution of the above issues will then lead to the ultimate validation of blood tests: namely, whether their use improves patient outcomes. A potential trial to test this would involve comparing outcomes in patients whose systemic therapy was initiated as a result of positive blood testing versus those treated according to the conventional criteria of metastatic relapse (i.e., radiological evidence of metastases with or without tissue rebiopsy).

Clinical use of biomarkers

Now that sensitive and specific biomarkers are being identified and undergoing validation, it is highly likely that, in the near future, the utility of one or more genes will be confirmed as sensitive and specific. How, then, might this promising progress be translated into clinically useful technology in order to inform the optimal management of metastatic melanoma?

The first scenario is the use of serum testing in order to detect the presence of subclinical disease in asymptomatic, apparently disease-free patients. Given the emerging evidence (discussed earlier) that new treatment options for melanoma are likely to be most effective when

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deployed in low-volume disease, a robust blood test for the identification of such patients would be invaluable in informing medical intervention. A second clinical situation in which blood testing might be valuable is in the population of patients being considered for sentinel lymph node biopsy. Although the clinical benefits of sentinel lymph node biopsy remain uncertain (and controversial), patients may suffer significant morbidity for as-yet unproven diagnostic and therapeutic benefits. The availability of a blood test that is indicative of the presence of metastatic disease would be of obvious benefit in sparing disease-free patients from these morbidities. Third, a positive blood test performed after apparently curative surgical excision could be useful in the identification of patients for whom adjuvant therapy would be appropriate. As noted earlier, no agent has yet demonstrated unequivocal evidence of efficacy in the adjuvant setting in melanoma. However, there is every possibility that one or more of the anti-CTLA4 and/or anti-PD1 antibodies will be active as adjuvant therapies. Given the considerable toxicities associated with these drugs, the ability to identify patients who would benefit from treatment would be extremely helpful in clinical decision-making.

Conclusion

There is little doubt that we now have the technology to detect the presence of methylated genomic DNA in blood, and there is a growing evidence base suggesting that the presence of methylated DNA from specific genes may indicate the presence of metastatic melanoma. We are now entering a key phase in which known candidates are undergoing rigorous validation and new candidates are emerging from *de novo* gene-finding strategies. Combining this evolving diagnostic approach with the emerging new therapeutic agents is highly likely to change the current treatment algorithms in melanoma from watchful waiting to active hunting for metastasis.

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References

- Sullivan RJ, Lorusso PM, Flaherty KT. The intersection of immune-directed and molecularly targeted therapy in advanced melanoma: where we have been, are and will be. *Clin. Cancer Res.* 19, 5283–5291 (2013).
- Ott PA, Hodi FS, Robert C. CTLA-4 and PD-1/PD-L1 blockade: new immunotherapeutic modalities with durable clinical benefit in melanoma patients. *Clin. Cancer Res.* 19, 5300–5309 (2013).
- Rustin GJ. Follow-up with CA125 after primary therapy of advanced ovarian cancer has major implications for treatment outcome and trial performances and should not be routinely performed. *Ann. Oncol.* 22(Suppl. 8), viii45–viii48 (2011).
- Hodi FS, O'Day SJ, McDermott DF *et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *N. Engl. J. Med.* 363, 711–723 (2010).
- Lopez J, Percharde M, Coley HM *et al.* The context and potential of epigenetics in oncology. *Br. J. Cancer* 100(4), 571–577 (2009).
- Mori T, O'Day SJ, Umetani N *et al.* Predictive utility of circulating methylated DNA in serum of melanoma patients receiving biochemotherapy. *J. Clin. Oncol.* 23, 9351–9358 (2005).
- Hoshimoto S, Kuo CT, Chong KK *et al.* *AIM1* and *LINE-1* epigenetic aberrations in tumor and serum relate to melanoma progression and disease outcome. *J. Invest. Dermatol.* 132, 1689–1697 (2012).
- Lo Nigro C, Wang H, McHugh A *et al.* Methylated tissue factor pathway inhibitor 2 (*TFPI2*) in serum is a novel circulating DNA biomarker of metastatic melanoma. *J. Invest. Dermatol.* 133, 1278–1285 (2013).