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SPECIAL ARTICLE

Guidelines for biomarker testing in metastatic melanoma: a National Consensus of the Spanish Society of Pathology and the Spanish Society of Medical Oncology

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Abstract This consensus statement, conceived as a joint initiative of the Spanish Society of Pathology (SEAP) and the Spanish Society of Medical Oncology (SEOM), makes diagnostic and treatment recommendations for the management of patients with advanced or metastatic melanoma based on the current scientific evidence on biomarker use. This document thus provides an opportunity to improve healthcare efficiency and resource use, which will benefit these patients. Based on the data available so far, this expert group recommends routinely testing patients with metastatic melanoma for *BRAF* mutation status, as the result affects the subsequent therapeutic management of these patients. The analysis of genetic alterations in *KIT* may be reasonable in patients with primary tumours in acral or mucosal sites or on chronically sun-exposed skin, in an advanced condition, but not in patients with other types of melanomas. This panel believes that testing for other genetic alterations, such as *NRAS* mutation status in patients not carrying *BRAF* mutations, *GNAQ/GNA11* mutational analysis or genetic alterations in *PTEN*, is not currently indicated as routine clinical practice, because the results do not influence treatment planning in these patients at the present time. Other important issues addressed in this document are the organisational requirements and quality controls needed for proper testing of these biomarkers, and the legal implications to be borne in mind.

Keywords Biomarkers · *BRAF* · *KIT* · *MAPK* · Dabrafenib · Vemurafenib

Introduction

The aggressiveness and resistance to chemotherapy of metastatic melanoma are a clear reflection of the complex

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mechanisms of tumorigenesis or oncogenesis involved in melanoma. Only in the last few years, however, it has proved possible to identify some of the most important genetic aberrations underlying this malignancy. In 2005, Bastian's group demonstrated the existence of several molecular pathways of malignant transformation in melanoma, each bearing a different relationship to ultraviolet light exposure and, hence, to the type of sun damage. This also opened up a new diagnostic and pharmacological paradigm, which has produced one of the most remarkable and badly needed changes in therapy in recent years [1]. This elegant study provided the conceptual framework for identifying a range of potential targets, and allowed the development of molecules that possess absolutely extraordinary activity against melanoma, given the scant progress made with conventional chemotherapy in the preceding years [2].

At the same time, following the definition of key mechanisms and molecules in anti-tumour immune regulation in experimental models, monoclonal antibodies have been developed that show anti-tumour activity and disease control rates superior to those obtained with chemotherapy [3]. This progress means that the diagnostic and treatment protocols for metastatic melanoma need updating, leading the Spanish Society of Medical Oncology (SEOM) and the Spanish Society of Pathology (SEAP) to propose a new consensus statement on biomarker use in this disease.

Molecular classification of melanoma

The most important molecular finding in the pathogenesis of melanoma was the discovery of the key role played by the mitogen-activated protein kinase (MAPK) pathway and microphthalmia-associated transcription factor (MITF) in the development of this tumour. This has enabled targeted therapies to be developed, some of which have demonstrated an improvement in the care of patients with advanced melanoma. The MAPK pathway is activated in almost all melanomas [4], which assists their growth and survival. Members of the *RAS* gene family, which belong to this pathway, activate proteins that act as critical signal-transduction mediators.

The gene *NRAS* has been found to be mutated in 10–15 % of melanomas, and this is considered an important instigator of oncogenesis [5–7]. Thus, a mutation in the *NRAS* gene produces sustained constitutive activation of the NRAS protein, which promotes cell cycle progression, malignant transformation of cells and increased cell survival. This cascade of events may be mediated by overexpression or overactivation of various growth factor receptors, such as c-Met, epidermal growth factor receptor (EGFR) and KIT [8–10].

However, the most important mediators of activation of this pathway are BRAF and CRAF [11]. *BRAF* mutation is enough on its own to activate the pathway, whereas *CRAF* mutation requires additional steps. This explains why *BRAF* mutations are present in 40–50 % of melanomas, whereas activating *CRAF* mutations have not been described [12, 13]. Activation of RAF, in its homodimer or heterodimer form, sets off the phosphorylation of MAPK kinase (MEK), which in turn induces phosphorylation of extracellular signal-regulated kinase (ERK), its sole substrate [11, 14]. ERK activation promotes growth and signal transduction through its interaction with a number of molecules critical to tumour pathogenesis. *BRAF* mutation seems to be an acquired event, which occurs initially in invasive melanomas and induces clonal expansion and tumour progression. However, *BRAF* mutation is not a decisive event in cancer development, but assists malignant transformation by the acquisition of successive oncogenic stimuli. This explains why *BRAF* mutations are so common in melanocytic naevi (70–80 %), melanomas in the vertical growth phase or metastatic melanomas (40–50 %), but are uncommon in melanomas in the radial growth phase or in situ (6–10 %) [12, 15, 16]. However, the precise relationship between naevi and melanomagenesis has not yet been elucidated. What is clear is that when *BRAF* mutations develop in invasive melanomas, they induce activation of MEK and then ERK, giving rise to oncogenesis through the promotion of cell growth and inactivation of apoptosis [17]. The mutations most often seen in the *BRAF* gene are V600E (40–60 %) and V600K (20 %), while V600D, V600E2, V600R, V600A, V600G and K601E are much rarer.

Most melanomas contain mutations or genomic aberrations [1, 4], which can lead to activation of the MAPK pathway, creating an oncogenic addiction to the overactivated protein. *BRAF* and *NRAS* mutations are more common in melanomas that develop in intermittently sun-exposed skin without chronic sun-induced damage, such as on the trunk (60 %). In uveal melanoma, the MAPK pathway is upregulated by an activating mutation in *GNAQ* or *GNA11*, seen in 80 % of cases. However, *BRAF*, *NRAS* and *KIT* mutations are uncommon in these melanomas [18, 19]. Lastly, in acral and mucosal melanomas, mutation is the most common in the *KIT* gene (between 15 and 40 %), whereas *BRAF* and *NRAS* mutations occur in fewer than 10 % of cases. The prognosis for advanced melanomas is influenced by the specific mutations they harbour. Thus, melanomas with somatic *BRAF* or *NRAS* mutations and acral or mucosal melanomas with *KIT* mutations have a worse prognosis, although they can respond to BRAF, MEK or KIT inhibitors, respectively, which increase the overall survival (OS) of patients. As regards *GNAQ* or *GNA11*

mutations in uveal melanomas, these also seem to be associated with a worse prognosis [20].

MITF is crucial to melanin production and plays a vital role in the cell cycle during melanocyte differentiation, and invasion during the physiological migration period and melanocyte survival. However, its deregulation contributes to melanoma pathogenesis. Twenty percent of melanomas have mutations in the *MITF* gene, which also gives them a worse prognosis [21, 22]. MITF and the MAPK pathway are intimately related. MITF serves as a direct substrate in the phosphorylation of serine 73 by MAPK. BRAF antagonists block the phosphorylation, ubiquitination and degradation of MITF. It is postulated that BRAF antagonists might enhance the antigenicity of melanomas by increasing the stability of MITF and hence boosting the expression of melanocyte antigens [23].

Melanomas with *BRAF* (V600E) mutations can be treated with drugs that block this protein. Paradoxically, however, its inhibitors can overstimulate RAF kinases in cells with wild-type *BRAF* when the RAS pathway is activated [24–26]. Over time, patients treated with *BRAF* inhibitor drugs end up progressing due to the development of resistance.

Several resistance mechanisms have been identified:

- Activation of an alternative pathway that ends up reactivating ERK. Signals are restored through activation of receptor tyrosine kinases, such as platelet-derived growth factor receptor β (PDGFRB) and human epidermal growth factor receptor 2 (ERBB2) [27, 28], *NRAS* mutation [28, 29], *CRAF* activation [27, 30], MEK mutation [31, 32] or carnitine octanoyl transferase (COT) activation [27].
- Formation of short forms of BRAF protein [33], generated by "alternative splicing" of the gene, that can activate the MAPK pathway.
- Parallel activation of the phosphatidylinositol 3-kinase (PI3K) pathway, initiated by insulin-like growth factor 1 receptor (IGF-1R) or by genetic alterations in components of the pathway itself (e.g., those that lead to PTEN loss of function) [34].

Clinical importance of biomarkers in melanoma

Markers of genetic risk

It is estimated that between 10 and 18 % melanomas have a hereditary basis [35, 36]. For this reason, identifying markers of genetic risk is of great interest. A genetic predisposition should be suspected in the event of multiple cases of the disease in the same family, multiple melanomas in the same patient, or onset of melanoma at a very early age [37, 38].

Although the genetic risk factors are unknown in most cases, the factor most often implicated is the presence of mutations in the cyclin-dependent kinase inhibitor 2A gene (*CDKN2A*). *CDKN2A* is located on chromosome 9p21 and is mutated in up to 40 % of cases of familial melanoma [39]. The probability of this gene being mutated increases with the number of melanoma cases found in the family, being 1, 4, 8 and 38 % for families with one, two, three or more than three cases, respectively [40, 41]. Onset of the disease at an early age or the presence of multiple melanomas in a single individual is less associated with the presence of this mutation, so 2 % of patients with two melanomas or 1 % of patients under 40 years old at presentation harbour the mutation [41, 42]. The presence of atypical mole/pancreatic cancer syndrome in the family increases the likelihood of detecting a mutation in *CDKN2A* [41]. Due to the low frequency of these mutations, genetic testing is currently recommended for cases in which three or more melanomas exist in the same individual or two or more cases of melanoma or pancreatic cancer occur in the family.

BRAF mutation

Approximately half of melanomas contain an activating mutation in *BRAF*. In three quarters of cases, the mutation is V600E, with V600K and other variants being less common [43]. This mutation seems to be an early event in tumour development and has allowed the development of drugs that specifically inhibit the mutated protein. BRAF inhibitors are more effective than conventional chemotherapy in the treatment of disseminated melanoma. Until the advent of these drugs, objective response rates of less than 10 % and median overall survivals of 6–9 months were achieved with conventional chemotherapy such as dacarbazine. Combination chemotherapy or biochemotherapy failed to improve the outcome [44].

Vemurafenib has been the first oral inhibitor of BRAF approved by regulatory agencies. A phase II trial showed a response rate of 52 %, a median time to progression of 6.8 months and a median OS of 15.9 months [45]. In a phase III trial, vemurafenib was found to be superior to dacarbazine, with a response rate of 48 versus 5 %, a median disease-free survival of 5.3 versus 1.6 months, and an OS of 12.5 versus 9.5 months [46]. The advantage in OS may actually be underestimated, because nearly half the patients assigned to the dacarbazine arm received a BRAF inhibitor in second-line.

Efficacy results of another orally administered BRAF inhibitor, dabrafenib, have been recently reported. When dabrafenib was compared with dacarbazine, median progression-free survival was 6.7 versus 2.9 months (median overall survival not reached at the time of report) [47].

BRAF inhibitors induce not only major but also minor responses, so 90 % of patients experience some degree of tumour shrinkage, with the resultant symptomatic relief. Considering the clinical benefit and prolongation of OS obtained in these patients, BRAF inhibitors have become the treatment of choice for advanced BRAF-mutant melanoma. At the time of writing, however, access to vemurafenib or dabrafenib treatment is still limited in Spain, so referral for participation in clinical trials is strongly encouraged.

Despite the high activity of the new BRAF inhibitors, most patients eventually suffer tumour progression. The mechanisms involved in resistance to these drugs are being actively investigated. Although BRAF usually remains mutated, new mutations appear or are selected for, leading to reactivation of the MAPK pathway [48]. This evidence supports research into drug combinations.

BRAF inhibitors cause skin toxicity, normally mild or moderate, in most patients. This may consist of photosensitivity, rash, folliculitis, pruritus and the development of keratoacanthomas or squamous cell carcinomas. Other common forms of toxicity are fatigue, arthralgia and elevated liver enzymes.

Genetic alterations in *KIT*

Melanomas that arise in mucous membranes, acral regions and chronically sun-exposed skin seldom have *BRAF* mutations. However, they may harbour mutations (10 %) and/or amplifications (25 %) of the *KIT* gene [49].

KIT is a transmembrane receptor with tyrosine kinase activity [50]. Binding of its ligand, stem cell factor (SCF) induces dimerisation and autophosphorylation of the receptor, which results in activation of the MAPK and PI3K-protein kinase B (AKT) signalling pathways involved in cell proliferation and survival. Mutations in *KIT* are detected with differing frequencies in the following exons: exon 9 (2 %), exon 11 (60–70 %), exon 13 (15–20 %) and exon 17 or 18 (10–15 %) [51–53]. Given the clinical success of treating gastrointestinal stromal tumours (GISTs) with inhibitors of KIT tyrosine kinase activity, such as imatinib and sunitinib, a similar strategy has been developed in melanoma. However, the types of mutations are different, as is the frequency with which they are detected in the various exons. Also, the increased *KIT* copy number seen in these melanomas is a rare event in GISTs, which suggests that the clinical activity of these agents in patients with *KIT*-mutant melanoma may be lower.

Studies evaluating the prognostic role of *KIT* mutations in melanoma are inconsistent. Thus, Kong et al. examined 502 Chinese patients with melanoma, of whom 38.4 and 33.3 % had acral and mucosal melanomas, respectively. In this study, the presence of a *KIT* mutation was found to be associated with a shorter OS than was seen in patients with wild-type melanoma [54]. However, in a Swedish study involving 71 patients with mucosal melanomas mutated in *KIT* (35 %), *NRAS* (10 %) and *BRAF* (6 %), *KIT* mutation status was not associated with survival [55]. Two other phase II prospective studies have evaluated the clinical activity of imatinib in patients with melanoma and genetic alterations in *KIT* [52, 53]. The observed response rates of approximately 22 % were lower than expected from published clinical experience. Both studies suggest that imatinib is more active against tumours with mutations in exon 11 or 13, and has more variable activity in patients with other alterations.

These data suggest that some patients with *KIT*-mutant melanomas may respond to KIT inhibitors. It remains to be determined whether this limited efficacy of KIT inhibitors reflects the tumour's dependence on certain mutations or the drug's ability to interact with certain mutated residues in KIT. Preliminary data suggest that a *KIT*-mutant to wild-type allelic ratio of more than 1 can predict the response [52]. However, the mechanisms potentially involved in treatment resistance, such as activation of alternative signalling mechanisms or amplification, are unknown. Clinical trials are in progress to evaluate other KIT inhibitor drugs.

Genetic alterations in NRAS

The RAS protein family includes NRAS, KRAS and HRAS. RAS proteins can activate the MAPK and PI3K signalling pathways through the interaction of extracellular growth factors with transmembrane receptors or through the acquisition of activating mutations [56]. Among *RAS* genes, the mutations most common in melanoma are in *NRAS* (10–20 %), whereas mutations in *HRAS* and *KRAS* are detected in approximately 1 and 2 % of melanomas, respectively [57]. Most *NRAS* mutations are found in codon 60 or 61 of exon 2 (80 %), and in codon 12 or 13 of exon 1 (20 %). Also, mutations in *NRAS* and *BRAF* appear to be mutually exclusive, as they are seen together in fewer than 1 % of patients [58]. The simultaneous presence of mutations in *BRAF* and *NRAS* has been described recently after treatment with selective BRAF inhibitors, such as vemurafenib, although only the *BRAF* mutation was detectable before treatment began [28].

Selective pharmacological inhibition of NRAS has met with little success to date, because of the difficulty of designing NRAS antagonist drugs that inhibit its guanosinetriphosphatase (GTPase) activity. Tipifarnib is an inhibitor of the enzyme farnesyltransferase involved in the post-translational modification of RAS. Tipifarnib has been evaluated as monotherapy in a phase II study in patients

with metastatic melanoma, but no objective responses were seen in the patients involved, so the study was closed prematurely [59]. The patients included were not selected on the basis of the presence or absence of *NRAS* mutations, but this drug has also failed to show any efficacy in other known *RAS*-mutant tumours. Given the unavailability of specific RAS inhibitors, investigation has turned to the blockade of other effector molecules in the RAS pathway. In this respect, promising new evidence exists concerning the benefit that may be obtained by patients with metastatic melanoma harbouring an *NRAS* mutation when treated with MEK kinase inhibitors [60].

Genetic alterations in GNAQ/GNA11

The genes *GNAQ* and *GNA11* encode part of the guanosine triphosphate (GTP) binding protein that permits signalling from the cell surface to the protein kinase pathway, and is therefore an important signalling regulator [61]. Up to 83 % of uveal melanomas contain mutations in one of these two genes, located in codons 209 and 183 [19]. *GNAQ* mutations are found in 45 % of primary uveal melanomas and 22 % of their metastases. On the other hand, *GNA11* mutations occur in 32 % of primary tumours and 57 % of their metastases [18, 19]. The presence of these mutations does not seem to be associated with disease prognosis [62], but their value may lie in the possibility of developing new targeted therapies [63], like MEK inhibitor therapy. Currently, however, given the lack of therapeutic or prognostic implications of these genes, it is not considered necessary to test for them in the healthcare setting.

Genetic alterations in PTEN

The PI3K/AKT/phosphatase and tensin homologue (PTEN) system plays a critical role in the modulation of cell functions such as proliferation, growth, survival and metabolism in response to extracellular stimuli mediated by cytoplasmic membrane receptors and G proteins. In the absence of such a stimulus, PTEN phosphatase generates the phospholipid messenger phosphatidylinositol 4,5-bisphosphate (PIP2) by phosphorylating phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP2 cannot stimulate the phosphorylation of PI3K and this maintains suppression of the cell cycle and growth. The stimulation of membrane receptors or G proteins produces activation of PI3K, which leads to a rise in levels of phospholipid PIP3. This in turn causes AKT to translocate to the plasma membrane, where it is activated by phosphorylation and in turn phosphorylates its substrates. These include the serine/threonine kinase mammalian target of rapamycin (mTOR). Activation of mTOR phosphorylates S6 Kinases (S6K) and inhibits eIF4E binding protein (4E-BP), which causes increased synthesis of proteins and other targets that regulate cell division and apoptosis [64].

The inactivation of PTEN is associated with various types of cancer, including glioblastoma, melanoma and carcinoma of the prostate, breast and endometrium. In 20–40 % of melanomas, immunohistochemistry can detect a loss of or significant reduction in PTEN expression in tumour specimens [65]. Both somatic point mutations in the *PTEN* gene and homozygous deletions are rare. Because of its function, 82 % of melanomas with *PTEN* loss have increased expression of phosphorylated protein kinase B (pAKT) [66]. Three isoforms of AKT exist. AKT1 is involved in apoptosis and protein synthesis, AKT2 in glucose metabolism and AKT3 in multiple processes. An association has been reported between AKT alterations and various types of human cancer, including melanoma. Over 70 % of primary and metastatic melanomas display increased AKT activity as detected by immunohistochemistry [65, 67]. Amplification of the 1q43–44 genomic region, which contains AKT3, is also often detected. Lastly, AKT may be deregulated in patients with melanoma associated with *BRAF* mutation.

The PI3K protein family is divided into three classes and several subclasses, depending on primary structure, regulation and in vitro specificity for the lipid substrate. Class Ia is the best understood of them all, partly because of its role in cancer. These proteins possess a catalytic unit (p110) and a regulatory unit (p85). Higher expression of PI3K has been described in melanomas than in blue naevi, and association with a worse prognosis has been reported [68]. However, activating point mutations of PI3K are very rare (1 % of primary melanomas) and comparative genomic hybridisation studies have not shown gene amplification [66].

Both PTEN loss of function and AKT activation have been associated with *BRAF* mutation [58, 69]. In murine models involving *BRAF*-mutant melanocytes, gene silencing of *PTEN* is required for malignant transformation [70]. There are also preclinical models that implicate the activation of this system in the acquisition of resistance to BRAF inhibition [71, 72]. For all the above reasons, and as the PI3K/AKT/PTEN pathway can be modulated pharmacologically, its main components are likely to become biomarkers of predictive potential in the near future.

Other genetic alterations

MITF regulates the development, differentiation and maintenance of melanocytes. MITF is activated by the MAPK system or the cyclic adenosine monophosphate (cAMP) pathway, leading to the transcription of genes associated with pigmentation, cell cycle progression and survival. It may also contribute to a rise in Bcl-2 activity

[73] and transcription of cyclin-dependent kinase 2 (CDK2). It has been found that the *MITF* locus is often amplified in melanoma. Amplification of *MITF* is correlated with shorter OS and increased resistance to chemotherapy [21].

Deletion of *p16INK4* is described in approximately 50 % of melanomas, and 10 % of them contain point mutations in this gene [74]. It may also be silenced by methylation of its promoter. A reduction in p16INK4 levels is correlated with a worse prognosis [75]. Table 1 lists some of the most relevant genetic abnormalities described in melanoma [2].

Pathology tests for biomarkers in melanoma

Histological diagnosis as the first biomarker

Melanocytes are cells derived from the neural crest that are located in the skin, mucous membranes, leptomeninges and uvea, so melanomas may be of different origins. The diagnosis of this disease relies on conventional histological examination, sometimes backed up by immunohistochemical techniques like those employing protein S-100, HMB-45, melanoma antigen recognised by T-cells 1 (MART-1), etc. In the case of cutaneous melanoma, histological examination provides the main prognostic factors, such as thickness or depth in mm (Breslow thickness), the presence or absence of ulceration and the number of mitoses per mm^2. The seventh edition of the American Joint Committee on Cancer (AJCC) publication includes these three pieces of information in the T staging category [76]. The mitotic count has been shown to be an independent prognostic factor, second in importance after thickness [77]. Other data that may be included in a melanoma pathology report, but of disputed prognostic importance, are the presence of inflammatory infiltrate, regression, vascular or lymphatic invasion and perineural infiltration.

The classification of melanomas into classical clinicopathological types is of no prognostic relevance [78]. Finding microsatellitosis (a tumour nest exceeding 0.5 mm separated from the main tumour by at least 0.3 mm of normal dermis) classifies a melanoma as stage N2C, and the same is true of clinical satellitosis or in-transit metastases [79]. Melanomas with mutant *BRAF* are more often well delimited, with pagetoid growth, a pattern of intraepidermal nests and large pigmented cells, whereas those with mutant *NRAS* seem not to have any distinctive features [80].

Establishing the N category begins with an examination of the sentinel lymph node. There are two protocols for embedding it: section the node along its longest axis and embed both halves, which is the method proposed by the European Organisation for Research and Treatment of Cancer (EORTC), or cut multiple 1–3 mm sections and embed them all. The second method appears to offer greater advantages [81]. Also, in the new TNM classification, micrometastases are defined as metastases not detected by eye. Although inclusion of the size and site of the metastasis in the report is not specified, it is important to reflect the size and location within the node

Table 1 Genetic markers considered more relevant in melanoma

	Gene alterations	%	Pathways	Histologic subtypes
BRAF	Point mutation	~50	MAPK	Superficial spreading melanoma
				Nodular melanoma
				Others
KIT	Point mutation	~1	MAPK and PI3K	Mucosal (10 %)
				Acral lentiginous (10 %)
				Lentigo maligna melanoma (<10 %)
NRAS	Point mutation	~20	MAPK, PI3K and RALGDS	Superficial spreading melanoma
				Nodular melanoma
				Others
GNAQ	Point mutation	<1	PKC pathway	Uveal melanoma (40 %)
GNA11	Point mutation	<1	PKC pathway	Uveal melanoma (40 %)
PTEN	50–60 point mutation or heterozygous deletion; 10 homozygous deletion		PI3K	All types
MITF	Amplification	~20	Melanocyte lineage and cell cycle	All types
CDKN2A^{p16}	Point mutation or deletion	~30	Cell cycle	All types

Table 2 Histological data to be included in a melanoma pathology report

Primary melanoma
Must be included[a]
Thickness or depth of invasion (mm) [98]
Presence or absence of ulceration
Mitotic index per mm^2
Microsatellitosis
Recommended
Associated inflammatory infiltrate[b]
Histological regression[c]
Vascular and/or lymphatic invasion
Perineural infiltration
Status of surgical margins
Other data[d]
Histological type
Cellularity
Associated naevus
Sentinel lymph node
Presence or absence of metastasis
Site[e]
Size of largest metastasis[f]
Extracapsular spread

[a] In order to determine T and N categories

[b] State whether inflammatory infiltrate is absent, scant or prominent

[c] State whether regression involves more or less than 75 % of the tumour

[d] Not essential

[e] Subcapsular, parenchymal

[f] Largest diameter in mm

of the largest one. Some authors regard cases with metastases of less than 0.1 mm and subcapsular location as N0 [82]. Table 2 summarises the histological data that should be included in a melanoma pathology report.

Pre-test phase

In order to achieve optimum performance of molecular techniques it is essential to comply with the highest quality standards at all stages, and obtaining the right histopathological diagnosis is undoubtedly the first step. Therefore, whenever there is clinical or radiological suspicion of metastasis it is advisable to confirm the diagnosis by performing a cytological or biopsical study. This approach also provides representative material from the metastasising component of the tumour, whereas if only the primary melanoma is examined molecular tests might be biased because of the possibility of tumour heterogeneity. Although it is preferable to have as much of the tumour as possible, good results can also be obtained with very small specimens, such as alcohol-fixed, Papanicolaou-stained cytological preparations from tumour imprints or fine needle aspiration [83]. Also, when examination takes place at a referral centre, it is advisable for the diagnosis to be confirmed by expert pathologists or dermatopathologists.

Most melanoma-related specimens come from formalin-fixed paraffin-embedded tissue. The specimen should be fixed immediately after it is obtained, using 10 % buffered formaldehyde for approximately 24 h, aiming not to exceed 48 h. The most suitable paraffin block for subsequent examination should be chosen by looking at a haematoxylin–eosin-stained section representative of the tissue contained in the block at the time of examination. The original section used to make the initial diagnosis can be very different from the remaining tissue, especially if, after it was obtained, further sections were cut to perform special techniques. The integrity of nucleic acids in paraffin-embedded material is lost over time, so the most recent specimen should be chosen. Also, it should be borne in mind that the best results are obtained when fixing time has been optimal and when the specimen contains a lot of tumour and not much necrosis [84]. Lastly, blocks containing less melanin pigment should be chosen, because this can itself inhibit deoxyribonucleic acid (DNA) polymerase enzymes, invalidating the results [84].

Once the block has been chosen, 5 μm sections are cut, placed in tubes or on slides and fixed in an oven at 37 ± 2 °C overnight or 56 ± 2 °C for 30 min. If the size of the tumour tissue is 1 cm^2 or more a single section is enough, but if the specimen is smaller more sections should be used. After the sections have been obtained, the nucleic acid extraction process should be carried out quickly, within 48 h. If the extraction cannot be done quickly, the specimen should be stored in a refrigerator at 2–8 °C, preferably in the dark. To prevent cross-contamination, worktops and the microtome can be cleaned with a nuclease decontamination solution (e.g., 10 % bleach and 70 % ethanol). Nuclease-free water can then be applied and dried with a paper towel. It is advisable to use a new disposable blade in the microtome for each case and to wear disposable gloves when handling blocks [85].

The higher the proportion of tumour in the specimen, the more accurate the results will be. Therefore, if the viable tumour represents less than 80 % of the tissue in the paraffin block, macro-dissection should be performed to select as much viable tumour as possible, discarding normal tissue and areas of necrosis or melanin hyperpigmentation. The area of interest is marked with a permanent marker pen on a haematoxylin–eosin-stained histological preparation and compared with the surface of the block or an unstained section placed on a slide. The macro-dissection can be done on this section, by scraping the tumour with a scalpel tip, or softening the paraffin block in a 65 °C oven for an

hour, and using a scalpel to separate the area to be tested from the rest of the specimen [85].

Test phase

Testing for BRAF mutations

The molecular methods currently used to test for *BRAF* mutations are the same as those used for other molecular pathology tests. Many published protocols exist for identifying *BRAF* mutations in either tumour tissue or peripheral blood, using DNA and ribonucleic acid (RNA). However, we shall focus on the mutation identification systems routinely used in pathology laboratories, which can basically be divided into polymerase chain reaction (PCR) and sequencing methods, and methods based on real-time PCR (RT-qPCR).

Sequencing methods include direct sequencing by the Sanger method, which involves directly analysing a DNA sequence previously amplified by PCR. This is a method of high specificity that can detect all possible mutations. However, the sensitivity of this technique is suboptimal (25 %), which can lead to false negatives. It requires specific equipment not generally found in pathology laboratories, and experienced technical staff are needed to read the result. Another sequencing method consists of pyrosequencing. This is based on a PCR reaction in which, for every new base added to the DNA strand, the pyrophosphate released results in the emission of ultraviolet light. The advantages of this technique are that it takes place in real time, has high analytical sensitivity, and is simple and cost-effective. It can identify all possible mutations within a region of interest. Its main disadvantages are that the reagents can be expensive and it requires a pyrosequencer, which is not commonly available in conventional pathology laboratories. One of the most widely used commercial kits is Qiagen's Therascreen BRAF Pyro® kit, which complies with the European in vitro diagnostics directive.

The technique based on RT-qPCR is currently the method most widely used in pathology departments for identifying molecular biomarkers in tumours. This technique uses a thermal cycler incorporating a fluorescence reader, enabling the DNA synthesised moment by moment to be quantified. The main advantage of the RT-qPCR method is that it is highly sensitive, more than bidirectional Sanger sequencing. It is a simple, quick method that can make use of platforms already in existence within pathology departments for testing for other mutations, such as *KRAS* in colorectal carcinoma and *EGFR* in non-small cell carcinoma of the lung. Its biggest disadvantage is that it only detects known mutations. However, bearing in mind that the most common *BRAF* mutations at the moment are V600E (40–60 %) and V600K (20 %) [86–88], and that both are candidates for BRAF inhibitor treatments, this technique is ideal for identifying mutations of this type. There are two sorts of probes for *BRAF* mutation testing, called TaqMan® and Scorpions®.

The prototype TaqMan® probe for testing for *BRAF* mutations uses the cobas® 4800 BRAF V600 diagnostic test, which is approved by the US Food and Drug Administration (FDA) and bears the European CE mark of conformity (CE). Although this test was originally designed to test for V600E mutations, it can also detect 70 % of V600K mutations [89], as well as V600D and V600E2. The main advantages of this technique are that it is very quick (can be done in less than 8 h), allows batches of 3 samples to be analysed with no reduction in reagent yield, and automatically reports objective results without any need for amplification curves to be read by the operator. For the RT-qPCR technique with Scorpions® probes, the test currently used is Qiagen's BRAF Rotor-Gene Q® (RGQ) PCR kit. This kit is designed to test for the somatic *BRAF* V600E mutation, and also detects the V600K, V600E complex (*GAA*) and V600D mutations. It now possesses the CE mark for diagnostics. Identification of the mutations relies on knowing how to read the amplification curves, which requires experience and the use of positive and negative controls.

High-resolution melting curve analysis is a RT-qPCR-based technique capable of distinguishing between a fully complementary double-stranded DNA molecule and another containing a mismatched base or mutation, based on the temperature difference in DNA melting point. The main advantages of this technique are its speed, its high analytical sensitivity and its high specificity. It detects both known and unknown mutations. One of its disadvantages, however, is that although it confirms whether or not a mutation is present, it cannot determine what that mutation is, so sequencing must then be performed.

Other PCR-based methods exist, including laboratory-specific and commercially available diagnostic tests based on conventional PCR that detect the presence of point mutations in *BRAF*. Detection involves specifically amplifying the mutation contained in the sample. The PCR amplification product can then be detected using various systems, ranging from agarose gels to capillary electrophoresis. In this respect, the recently developed CLART® CMA KRAS-BRAF-PI3K kit from Genómica can detect point mutations in *BRAF* by conventional PCR. Mutations are identified by hybridising the amplification product to specific probes in low-density microarrays, with automatic reading. The advantages of these methods are that they are suitable for clinical diagnosis, do not require real-time PCR systems (so can be done using a conventional thermal cycler), are fast

and sensitive, and can identify and distinguish between V600E and V600K mutations. The same microarray can also be used to test for mutations in *KRAS* and *PI3K*. Lastly, there are techniques based on multiplex PCR, such as the Applied Biosystems® BRAF Mutation Analysis Reagents system, which can detect V600E, V600A and V600G mutations. This is a highly sensitive method developed for research purposes, but is not valid for diagnostic use at the present time. Its main disadvantage is that reading the results requires a capillary electrophoresis system, which is not easy to find in conventional pathology departments.

Testing for other biomarkers

A small subset of melanomas displays alterations in *KIT* [49]. *KIT* abnormalities occur selectively in 17 % of melanomas with chronic sun-induced damage. Lentiginous melanoma and mucosal melanoma are mutated in *KIT* more often than in *BRAF* [90]. Although the clinical importance of *KIT* mutations in melanoma is not clear, 20 % of cases with these mutations are sensitive to imatinib and other KIT inhibitors, although this indication is not currently licensed. Mutations occur more often in exon 11 and less often in exons 9, 13, 17 and 18 [51–53]. In exon 11, most mutations (34 %) cause the substitution of leucine by proline in codon 576. In any case, mutational analysis should be performed for all five exons of the gene. If DNA quantity or quality is a limiting factor, exons 9 and 11 should be tested preferentially. The two most widely used test methods for *KIT* mutations are direct sequencing and RT-qPCR. The technique's limit of detection, defined as the minimum percentage of tumour cells required in a specimen, varies depending on the method used and the laboratory. In any case, regardless of the technique used, it is important to select a sample with an appropriate percentage of tumour cells. In some cases, it is advisable to perform a microdissection to limit the presence of wild-type alleles from non-tumour cells. Cytological samples are also useful, and as they are not formalin-fixed the DNA is generally of better quality [83]. It is important to stress that immunohistochemical detection of KIT protein (CD117) is unreliable for predicting mutations, so molecular tests must be done [91].

Post-test phase

Table 3 lists the information that should be included in a pathology report in the post-test phase.

External quality controls

Laboratories that conduct tests for molecular biomarkers serving as therapeutic targets for the treatment of patients with melanoma should use technologies validated in terms of their specificity, sensitivity and predictive value. It is also advisable for these laboratories to take part in external quality control programs, such as the one set up by SEAP for the mutational analysis of *EGFR* in patients with pulmonary adenocarcinoma and *KRAS* in those with colorectal adenocarcinoma. This program assesses the pre-test, test and post-test quality of each laboratory. For this purpose, participating laboratories are sent mutant and wild-type cases chosen in advance by the site coordinating the program, to be tested following routine operating procedures. The results obtained by each laboratory are evaluated and discussed by the SEAP expert committee. If referral centres for biomarker testing exist, these should possess the necessary accreditation and certification according to the standards set by the health authority in each self-governing region.

Table 3 Melanoma pathology report in the post-test phase

Identification of the patient and the doctor who ordered the test
Anatomical origin of the specimen
How the specimen was obtained[a]
Outcome of immunohistochemical tests[b]
Molecular tests:
Type of specimen[c]
Specimen quality[d]
Identification of technique used to test for biomarker(s)
Mutations and/or other alterations detected
Identification of the laboratory responsible for molecular testing
Further comments of interest to the person placing the order[e]
Information about accreditation in quality programs
Final pathological diagnosis

[a] By surgery, biopsy, needle biopsy, etc.

[b] If done

[c] Fresh, frozen, paraffin-embedded tissue, smears, etc.

[d] Tumour percentage, microdissection, etc.

[e] If deemed necessary

Practical issues

Work flow and where to do the tests

Testing for *BRAF* gene mutations in patients with metastatic melanoma is urgent, because the result will influence pharmacological treatment. The test should therefore be done without delay. In order to achieve this, it is vitally important in these cases to work together in a multidisciplinary fashion in specific cancer committees made up of the medical oncologist, pathologist, medical radiologist, dermatologist, surgeon, molecular biologist, pathology

technician and other staff involved. It is essential that the people responsible for conducting the molecular analyses are represented on these cancer committees, where the approach to be taken with each patient will be discussed. In any case, molecular studies should be supervised by specialist pathologists who can make sure that the best part of the tumour is chosen, containing the least necrosis or pigmentation and as many cancer cells as possible.

Recommended and acceptable timescales

As stated above, time is of the essence for these patients. Therefore, once it has been decided that molecular tests must be done, the pathologist should choose the best histological tissue available. When doing so, it should be borne in mind that, although in principle *BRAF* mutations tend to be fairly stable, whenever possible it is preferable to use the most recent tumour material. With regard to the recommended optimal timescale, it should take 4–5 days (never more than 7 days) from the order being received by the pathology department to the issuing of the report containing the result of molecular analysis of *BRAF* gene mutation.

Which patients need biomarker tests?

The results of recent clinical trials with BRAF and MEK inhibitors have been so statistically and clinically remarkable that the inclusion of *BRAF* mutation analysis in metastatic melanoma should today be considered routine practice [92, 93]. Therefore, when pathology departments lack the necessary experience or infrastructure to conduct these tests, they should contact reference laboratories to have them done. In Spain, there are many accredited laboratories that are able to perform molecular analysis of *BRAF* mutations, and recently the BRIGHT platform (http://www.biomarkerpoint.com) has been launched as referral centre to perform this analysis. Apart from the molecular analysis, the detection of the mutation BRAF V600E by immunohistochemistry (VE1) has just been reported; however, new confirmatory studies are needed before incorporating this analysis into the routine clinical practice.

Testing for *NRAS* mutations in patients with *BRAF* wild-type metastatic melanoma is of interest in the clinical trial context [94]. The same can be said of GNAQ/GNA11 mutation analysis in ocular melanomas, as the role of these tests in the choice of treatment is currently being assessed in prospective clinical studies, with no evidence as yet to warrant them being done routinely [94]. Testing for *KIT* mutations can be considered in patients with unresectable disease and primary tumours in acral or mucosal sites, but cannot be recommended for other melanomas [94]. Immunohistochemical detection of the ligand of programmed death 1 protein (PD-L1) might be of interest, if the activity of anti-PD-1 and anti-PD-L1 monoclonal antibodies in metastatic melanoma is confirmed [95].

Legal implications

The approval of vemurafenib in August 2011 and February 2012 by the FDA and the European Medicines Agency (EMA), respectively, for the treatment of patients with unresectable or metastatic melanoma carrying the *BRAF* V600E mutation, as well as the cobas® 4800 BRAF V600 diagnostic mutation test, has meant a radical change in the diagnostic methods employed in this disease, because licensing clinical use of a new drug the activity of which depends on the presence of a specific biomarker means that tests for this have to be done [96, 97].

Tests designed to detect mutant *BRAF* have therefore been added to the resources used in the accredited diagnostic protocols for this disease, and should form part of routine healthcare in patients with advanced or metastatic melanoma eligible for systemic treatment. Based on the information currently available, the routine use of other biomarkers of possible therapeutic interest, such as KIT, PD-L1 or NRAS, cannot be considered warranted for healthcare purposes, despite the evidence reported to date.

Conclusions

Based on the data available so far, this expert panel considers that the main diagnostic method for melanoma should be conventional histopathological examination with haematoxylin/eosin, and in certain cases with immunohistochemistry techniques. Likewise, this committee believes that Breslow thickness, the presence of ulceration, mitotic index and the presence of metastasis should be regarded as the most important prognostic factors for patients with metastatic melanoma.

When planning the treatment of any patient with metastatic melanoma, *BRAF* V600E mutation status must first be known. This should be tested within 7 days in accredited laboratories with sufficient expertise. Testing for other biomarkers such as *KIT* or PD-L1 might be useful in specific cases of advanced melanoma, or if the activity of anti-PD-1 and anti-PD-L1 monoclonal antibodies is confirmed, but for the time being it is not thought that this should form part of routine clinical practice.

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