Immunohistochemistry for an improved clinical workflow in the era of personalized melanoma therapy?

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In recent years, we have gained a significant amount of insight into the molecular pathways involved in melanoma. Activating mutations in the oncogenes *BRAF* or *NRAS* occur in roughly 50 and 20% of melanomas, respectively, and result in the constitutive activation of the MAPK cell signaling pathway [1]. Recently, small molecule inhibitors of mutant BRAF and the downstream kinase MEK (MAPK inhibitors) have changed the management of *BRAF*-mutant metastatic melanoma and improved overall survival compared with standard chemotherapy in *BRAF* V600-mutated metastatic melanoma patients [2–4]. *NRAS* has proven to be undruggable so far; however, single-agent MEK inhibition has shown clinical activity in *NRAS*-mutated metastatic melanoma [5] and several Phase II and III studies are ongoing, most of them including *NRAS*-mutated metastatic melanoma.

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histopathologic evaluation of melanoma specimens and provides additional precious information to predict outcome and select optimal therapeutic approaches. For this purpose, tumor tissues may need to be sent as rapidly as possible to specialized laboratories for molecular testing. Several methods have been used to detect *BRAF* mutations, including Sanger sequencing, SNaPshot, high-resolution melting, mutation-specific real-time PCR, pyrosequencing and mass spectrometry. Recently developed low-cost sequencing platforms allow concurrent sequencing of multiple targets. Each method has its own cost, turnaround time, sensitivity and specificity, the latter depending on the percentage of mutated tumor cells and on the quantity and quality of the extracted DNA from formalin fixed paraffin embedded (FFPE) specimens. Molecular testing requires extra time since it is always performed after conventional histopathological evaluation (upon mandatory assessment of the percentage of tumor

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cells, and most often, tumor cells enrichment by macro/microdissection). In addition, molecular biology techniques may not be accessible to many pathology laboratories; some detection methods are time-consuming and expensive to perform and may require different confirmatory methodological approaches resulting in unacceptably delay in response in clinical practice.

In contrast to molecular methods, conventional immunohistochemistry (IHC) is extensively and routinely used in pathology laboratories and mutation testing by IHC may offer several potential advantages: it is associated with a faster turnaround time, it is cheaper, requires minimal tissue and is particularly suitable in cases of low tumoral cellularity, it allows recognition of individual antigen-bearing tumor cells, and it carries a low risk of potential contamination.

The *BRAF*V600E mutant-specific monoclonal antibody (clone VE1) was introduced to identify *BRAF*-mutant protein in FFPE tissue specimens from different malignancies, including melanoma [6]. Because V600E represents the large majority of *BRAF* mutations in melanoma, IHC of the V600E mutant protein has been proposed as an alternative mutation detection method to molecular genetic assays [6–12]. The VE1 antibody has originally shown very good specificity (98%) and sensitivity (97%) for the recognition of the *BRAF* p.V600E mutation [6] and this high diagnostic accuracy was validated in other independent cohorts [7–10,13]. Interestingly, previous immunohistochemical analyses by the use of *BRAF*V600E antibody have shown that intratumoral heterogeneity of *BRAF* mutation is not common [7,11,12] and up to 100% concordance between matched primary and metastatic melanomas has been reported [11,13]. The interpretation can simply be based on the visual inspection of the percentage of immunostained cells and staining intensity, with high reproducibility [14] and a negative IHC result should prompt immediate screening by molecular methods to identify *BRAF* mutations other than V600E.

Besides *BRAF*, testing for *NRAS* is now integral part in the assessment of metastatic melanoma patients for at least three reasons: there is evidence that *NRAS*-mutated patients may obtain clinical benefit through MEK inhibitors; the combinations of inhibitors to target *NRAS*-activated signaling through MEK and PI3K, MEK and AKT, MEK and PI3K/mTOR, MEK and CDK4, as well as MEK and VEGF receptor inhibition, are now in early-phase clinical trials; *RAS* mutation is a relatively common mechanism

of acquired resistance during treatment with *BRAF* inhibitors [15].

With regards to *NRAS*, the most common oncogenic change (80% of all *NRAS* mutations) reported is a point mutation at position 61, with mutations at positions 12 and 13 occurring less frequently [15]. In a very large, retrospective study investigating the prognostic role of *BRAF* and *NRAS* in metastatic melanoma patients, the most common *NRAS* mutation was represented by the glutamine to arginine substitution at position 61 (*NRAS* Q61R) [16].

By interrogating a relative large series of patients evaluated both with IHC and molecular analysis, our group recently demonstrated that the N-Ras (Q61R) antibody is highly sensitive and specific for the detection of mutant *NRAS* Q61R melanoma in FFPE samples [17]. This is the first formal, clinical demonstration that IHC is a valuable and reliable tool to detect the most frequent *NRAS* mutation in melanoma.

Considering that in the first above-mentioned Phase II prospective trials investigating MEK162 in *NRAS*-mutated melanoma patients, most of them actually harbored the *NRAS* Q61R mutation [5], our data suggest there is room for IHC in the design of clinical trials as well as, potentially, in the clinical practice as soon as single agents or combination therapies will be introduced for *NRAS* melanoma patients.

As a general consideration, a number of limitations can arise when using IHC for mutational testing in routine practice [18]. First, the different parameters of the preanalytical phase need to be well controlled. Some false-negative results can occur in the case of hypo- or hyper-fixation with formaldehyde. Acid treatment for decalcifying bone melanoma metastases could hamper molecular methods, but also IHC. In this context it is mandatory to record the critical preanalytical and analytical parameters and, as for genetic molecular testing, it is required to actively participate in external quality assurance programs to improve IHC protocols and limit false-positive or -negative IHC results.

The dynamic and complex interactions between melanoma cells and the microenvironment cannot be recapitulated by the mere mutational status of tumor cells and analysis of the tumor microenvironment in a systematic fashion with the goal of identifying predictive biomarkers being a major priority and challenge. Collectively, a number of observations point toward downstream resistance mechanisms at the level of the tumor

microenvironment as a potential explanation for tumor escape [19]. Tumor cell PD-L1 expression and other microenvironmental features, such as PD-1 expression in tumor-infiltrating immune cells and the intensity of T-cell and B-cell infiltrates, have been recently investigated in correlation to response to anti-PD-1 pathway blockade [20]. Thus, a multiple analytical approach considering both tumor cells as well as qualitative and/or quantitative features of the immune cells in the microenvironment is emerging as a major indicator for efficacy, and potentially can be required to reach the correct patient and disease classification as well as the optimal therapeutic strategy, beyond BRAF inhibitors.

In this perspective, it is expected that follow-up studies focusing on clinical correlations will further extend the use of IHC for an improved clinical workflow in predicting response to therapy in melanoma patients. Ultimately, best practice recommendations on the use of IHC markers in melanoma for predictive purposes are strongly needed and this optimal approach will likely reshape the clinical management of melanoma patients in the near future.

5-year perspective

With the advent of personalized medicine, a multiple analytical approach considering both malignant cells as well as qualitative and/or quantitative features of the immune cells in the microenvironment is required to reach the correct patient and disease classification as well as the optimal therapeutic strategy. In the next 5 years it is expected that translational studies focusing on clinical correlations will further extend the use of IHC, and, ultimately, an integrative approach, which includes IHC, will reshape the clinical management of melanoma patients.

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