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Biological insights into BRAF^{V600} mutations in melanoma patient

Not mere therapeutic targets

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Some experimental evidence indicates that uncommon *BRAF* mutations consisting in the substitution of 2 adjacent nucleotides within codon 600 are in a *cis* configuration and associate with *BRAF* gene amplification. These findings suggest that *BRAF^{V600}* mutations are unlikely to occur as homozygous alterations in clinical melanoma samples, with gene amplification perhaps contributing to mask the heterozygous state.

Keywords: BRAF, homozygosis, immunotherapy, melanoma, MHC, vemurafenib

Abbreviations: BRAF, *v-raf* murine sarcoma viral oncogene homolog B1; BRAFi, BRAF inhibitor; ERK, extracellular signal-regulated kinase; FISH, fluorescence in situ hybridization; IFN, interferon; LOH, loss-of-heterozygosity; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; TAA, tumor-associated antigen; TIL, tumor-infiltrating lymphocyte

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The mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway is a highly conserved signal transduction cascade involved in the regulation of cell proliferation, differentiation, and survival in response to extracellular cues, and is frequently altered in human neoplasms. The most potent activator of MAPK/ERK kinases (MEKs) is the non-receptor kinase *v-raf* murine sarcoma viral oncogene homolog B1 (BRAF). The most predominant activating mutation of *BRAF* detected in human tumors involves a thymidine to adenosine transversion at nucleotide 1799 (exon 15), resulting in the substitution of valine at residue 600 with glutamic acid (V600E).[1] BRAF^{V600E} exhibits a 500-fold increase in kinase activity, relentlessly stimulating the activation of MEK/ERK signaling in the absence of extracellular stimuli. This corresponds to the emancipation of malignant cells from the need of external growth signals. The MEK/ERK pathway is frequently mutated in melanoma, with *BRAF* mutations being found in up to 70% of cases and *BRAF^{V600E}* being the most frequent alteration of all (> 90%).[1]

Because of its key role in the MEK/ERK signaling pathway, BRAF has been the subject of intense investigation, leading to the approval by regulatory agencies of 2 distinct BRAF inhibitors (BRAFis), vemurafenib and dabrafenib, for use in melanoma patients. Moreover, the assessment of *BRAF* mutations nowadays constitutes a fundamental diagnostic procedure.[2] However, many melanoma patients harboring the *BRAF^{V600E}* mutation frequently become resistant to BRAFis. This corresponds to a median duration of clinical responses that is significantly shorter than 1 y, in many cases followed by rapid disease progression.[2]

Novel therapeutic options for advanced melanoma rely on immune checkpoint inhibitors or the adoptive transfer of tumor-derived T cells (i.e., tumor-infiltrating lymphocytes, TILs) expanded and optionally activated in vitro. These approaches frequently result in the activation of robust antitumor T-cell responses and (at least in some cases) induce spectacular tumor regressions, reflecting the recognition of tumor-associated antigens (TAAs) presented on the surface of malignant cells in complex with MHC molecules

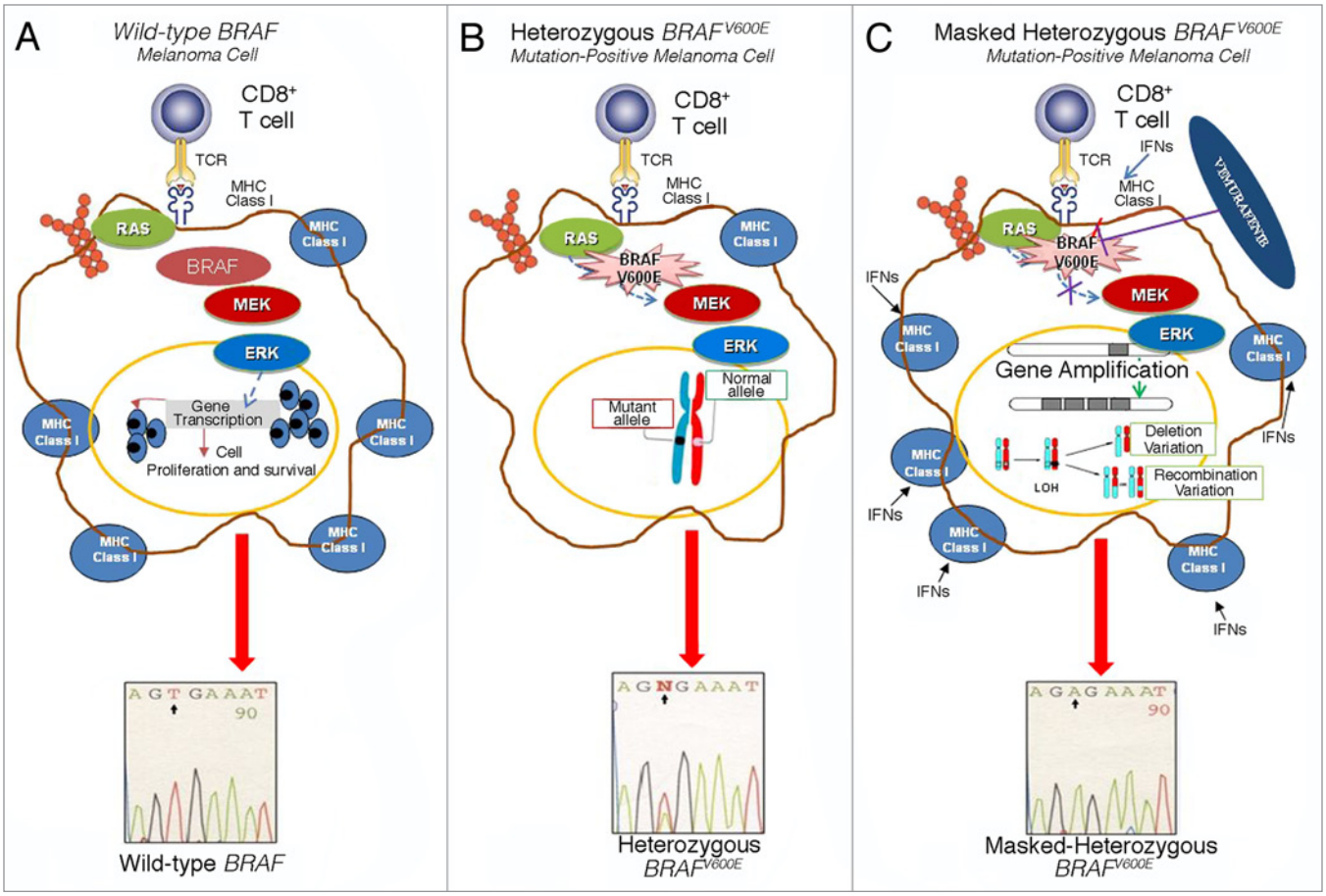

Figure 1. Vemurafenib increases interferon γ-induced MHC expression on melanoma cells harboring a masked heterozygous *BRAF*^{V600} mutation. (**A**) MHC expression levels are higher in wild-type melanoma cells that in cells bearing a *BRAF*^{V600} mutation. (**B**) Mutant *BRAF*^{V600} suppresses the expression of MHC molecules on the cell surface. (**C**) The administration of BRAF inhibitors (BRAFis) promotes the interferon γ (IFNγ)-induced expression of MHC molecules by melanoma cells that harbor a "masked" heterozygous *BRAF*^{V600} mutation in the context of *BRAF* amplification or loss-of-heterozygosity (LOH). ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; TCR, T-cell receptor.

by immune effector cells. As interferons (IFNs) are potent inducers of MHC class I and class II molecules,[3,4] they are generally expressed in the tumor microenvironment and can be used therapeutically; Sapkota et al. have recently investigated whether the *BRAF*^{V600E} mutation may affect the expression of MHC molecules by melanoma cells,[5] to optimally integrate strategies based on targeted kinase inhibitors and immunotherapeutic agents.

According to these authors, BRAF^{V600E} reduces the amount of MHC molecules on the cell surface and its inhibition significantly boost the ability of IFNγ and IFNα2b (the latter of which is approved for the therapy of melanoma) to upregulate MHC expression levels.[5] Thus, the inhibition of BRAF^{V600E}, either as a standalone therapeutic intervention or combined with the administration IFNα2b, may be a valid approach to enhance the expression of MHC class I molecules on the surface of melanoma cells, thus promoting their recognition by cytotoxic T cells (be they naturally present and active in the tumor microenvironment or unleashed by immunotherapy).[5]

One important aspect of this study is the impact of *BRAF*^{V600E} zygosity on the capacity of vemurafenib to boost the expression of MHC molecules induced by IFN. According to Sapkota et al., indeed, this effect occurred in melanoma cell lines harboring homozygous but not heterozygous *BRAF*^{V600E} mutations. These authors proposed that the percentage of homozygous *BRAF*^{V600E} mutations in melanoma is unclear but not a rare event.[5] In this regard, a study from Rubinstein et al. was cited, showing that roughly 50% of melanoma patients harboring the *BRAF*^{V600E} mutation, as assessed by a conventional sequencing procedure, are homozygous.[6] In further support of their findings, Sapkota et al. cite a study by Sigalotti et al. suggesting that the zygosity of *BRAF*^{V600E} can change over time from heterozygous to homozygous, as demonstrated using metachronous melanoma metastases from different anatomical locations, even though *BRAF*^{V600E} appears as a "stable" and early (present also in benign nevi) mutational event.[7] Sapkota et al. concluded by speculating that the assessment of *BRAF*^{V600E} zygosity may warrant further examination as a biomarker in melanoma patients bearing this mutation.[5]

At variance with these results, we believe that, perhaps with a few notable exceptions,[8] somatic (oncogene-activating?) mutations such as those found in most melanoma are unlikely to be stably

homozygous, as homozygosity is typical of germinal mutations and hereditary disorders. As a matter of fact, melanoma cells generally exhibit randomly acquired mutations that stochastically affect one of both alleles, as it normally occurs in cells replicating by mitosis (as opposed the meiotic formation of gametes). In this setting, mutations are very unlikely to simultaneously affect the same genetic locus on both alleles, rather resulting in a heterozygous somatic mutational pattern. Events that can "mask" heterozygous mutations and make them appear as homozygous are essentially two: the amplification of the mutated allele and the loss-of-heterozygosity (LOH) of one of the 2 alleles.

While gene amplification is not seen in normal cells, it occurs quite often in tumor (melanoma) cells. In the course of oncogenesis, indeed, (pre)malignant cells are under strict selective pressure and obtain a survival/growth advantage by expressing an inherently active protein, such as $BRAF^{V600E}$, that stimulates tumor growth, sustains chemoresistance (by delivering antiapoptotic signals), and facilitates the escape from immunosurveillance (by attenuating the baseline expression levels of MHC class I molecules). Indeed, amplifications of chromosome 7q (*BRAF* is located on 7q34) have repeatedly been reported in melanoma patients.[8,9] LOH, i.e., the loss of one allele affecting a heterozygous genetic locus, may result from different mechanisms, including chromosomal deletions, mitotic recombination, gene conversion, point mutations, or intragenic allele inactivation.[10] In particular, the locus may become homozygous following mitotic recombination, gene conversion, or chromosome loss with reduplication, hemizygous upon gene deletion or chromosome loss, complex heterozygous due to the introduction of an additional point mutation, or may remain heterozygous if one allele is inactivated intragenically.[10] A high incidence of LOH is regarded as an unfavorable prognostic factor for cancer patients, as it generally correlated with accelerated tumor progression and indicates the involvement of specific genomic regions in carcinogenesis.

Gene amplification and LOH, alone or in combination, may explain the phenomenon of apparent homozygosis in melanoma patients (**Fig. 1**). In this respect, thanks to our longstanding experience on the assessment of *BRAF* mutations in melanoma patients, we were recently able to observe *BRAF* mutations consisting in the substitution of 2 adjacent nucleotides within codon 600, namely GTG→GAA, GTG→AAG, or GTG→AGG, resulting in V600E, V600K, or V600R substitutions, respectively (unpublished data). To this aim, we employed melanoma specimens with a prevalence of malignant cells > 95% (to minimize the risk of contamination with wild-type DNA from stromal cells). Of note, all these double mutations were in a *cis* configuration, hence affecting the same allele (and not both) in individual cancer cells (and not distinct populations of malignant cells), as demonstrated by the sub-cloning of relevant amplification products in bacterial systems. Furthermore, tumor samples bearing double *BRAF* substitutions at codon 600 also showed an increased copy number of *BRAF*, as evaluated by fluorescence in situ hybridization (FISH). These findings suggest that melanoma patients are unlikely to be homozygous for $BRAF^{V600}$ and that gains in *BRAF* copy number may de facto mask an underlying panel of heterozygous mutations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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