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Abstract
Strategies that aim to limit the adaptive response to pathway inhibition in BRAF-mutated melanoma face the inherent limit of signaling redundancy and multiplicity of possible bypass mechanisms. Drug-induced expression of selected RNA-binding proteins, like the ubiquitously expressed HuR, has the potential to differentially stabilize the expression of many genes involved in the compensatory mechanisms of adaptive response. Here, we detect in BRAF-mutated melanoma cell lines having a higher propensity for adaptive response and in non-responding melanoma tumors, a larger proportion of HuRLow cells in the expression distribution of HuR. Using knockdown experiments, we demonstrate, through expression profiling and phenotypic assays, that increasing the proportion of HuRLow cells favors the adaptive response to BRAF inhibition, provided that the HuRLow state stays reversible. The MAPK dependency of melanoma cells appears to be diminished as the proportion of HuRLow cells increases. In single-cell assays, we demonstrate that the HuRLow cells display plasticity in their growth expression profile. Importantly, the adaptive […]

Reference

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Drug-induced expression of the RNA-binding protein HuR attenuates the adaptive response to BRAF inhibition in melanoma

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Strategies that aim to limit the adaptive response to pathway inhibition in BRAF-mutated melanoma face the inherent limit of signaling redundancy and multiplicity of possible bypass mechanisms. Drug-induced expression of selected RNA-binding proteins, like the ubiquitously expressed HuR, has the potential to differentially stabilize the expression of many genes involved in the compensatory mechanisms of adaptive response. Here, we detect in BRAF-mutated melanoma cell lines having a higher propensity for adaptive response in melanoma tumors, a larger proportion of HuR Low cells in the expression distribution of HuR. Using knockdown experiments, we demonstrate, through expression profiling and phenotypic assays, that increasing the proportion of HuR Low cells favors the adaptive response to BRAF inhibition, provided that the HuR Low state stays reversible. The MAPK dependency of melanoma cells appears to be diminished as the proportion of HuR Low cells increases. In single-cell assays, we demonstrate that the HuR Low cells display plasticity in their growth expression profile. Importantly, the adaptive over-proliferating cells emerge in the subpopulation containing the HuR Low cells. Therapeutic concentrations of lithium salts, although they moderately increase the global expression of HuR, are sufficient to suppress the HuR Low cells, induce an overall less resistant expression profile and attenuate in a HuR-dependent manner the adaptive response of melanoma cells in ex vivo assays. The therapeutic effectiveness of this approach is also demonstrated in vivo in mice xenografts. This study has immediate clinical relevance for melanoma therapy and opens a new avenue of strategies to prevent the adaptive response to targeted cancer therapy.
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1. Introduction

Numerous studies demonstrate that the adaptive response of BRAF-mutated melanoma cells to BRAF inhibition can potentially engage highly diverse bypass and alternative embryonic signaling pathways [1], among which Wnt5a, Notch1, Hedgehog, and Hippo signaling stand as examples. Many of these compensatory activations rely on variations at the post-transcriptional/translational steps of gene expression and are potentially modulated by RNA-binding proteins (RBPs) [2]. HuR (ELAVL1), which is a ubiquitously expressed stress-response nuclear RBP [3], has been implicated both in cell proliferation and differentiation and has likely a complex involvement in cancer in view of the available experimental data [4,5]. HuR activity, like most proteins, is controlled through post-translational modifications [6], however, and despite its own tight expression regulation [7], its abundance and compartmentalization are also involved in its trans-acting effects on its mRNA targets. Previously, we have shown that a transient overexpression of HuR suppresses the immediate paradoxical proliferation of melanoma subpopulations to suboptimal BRAF inhibition [8].

In this study, subsequent to ex and in vivo observations, we test the hypothesis that a relative low expression of HuR, contributes to

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the adaptive response to BRAF inhibition and that its moderate drug-induced expression sufficiently modifies the expression of important nodes within the signaling network, so as to reduce the adaptive response of melanoma cells and the quiescency of the cell subpopulations that gives rise to it.

2. Materials and methods

This section is described in Supplementary Materials and methods.

3. Results

3.1. HuR expression distribution in BRAF-inhibited melanoma

First, we analysed if the variability in the adaptive responses of BRAF-inhibited melanoma cells could be linked to differences in HuR expression distribution. Six well-characterized BRAF-mutated BRAF inhibitor-similarly-sensitive melanoma cell lines [9] were exposed to incremental increases in vemurafenib during four weeks using a standardized mild selection protocol (week 1: 50 nM, week 2: 100 nM, week 3: 200 nM, week 4: 300 nM). This protocol was systematically used in this study: (i) to avoid harsher pressure-induced mortality that will select minor mutated populations not reflecting the adaptive response; (ii) to be within the narrow window that catches differences in speed of acquired resistance; (iii) not to miss the early transition states of the subpopulations in single-cell experiments. Under this protocol, we did not observe any significant mortality even at the final dosage. Each derived cell line (TP2) was compared with its parental cell line (TP1) (Fig. 1A and B). At the end of the four weeks, acquired resistance occurred only in three cell lines (WM-88, Malme-3M, A375). Strikingly, FACS analysis of HuR expression distribution indicated that, compared with the three cell lines that remained sensitive, the three cell lines that had become resistant had at TP1, a relative larger proportion of HuRLow cells (Fig. 1D). These differences were not detectable in the cell lysates (Fig. 1C) and could not be attributed to the growth phenotype of the cell lines (Fig. 1E). For example, the fastest dividing Malme-3M and A375 cells had a large proportion of HuRLow cells. Additionally, we observed in TP2 cells a vemurafenib-

![Fig. 1. HuR expression distribution in BRAF-inhibited melanoma.](image-url)
induced immediate loose expression redistribution of HuR, suggesting an adaptive loss of its expression regulation (Fig. 1F). Again, cell cycle analysis demonstrated that these dynamic changes could not be linked to the change in the growth phenotype of the adapted cells (Fig. 1E and F).

We then did a preliminary analysis of biopsies performed on patients with BRAF-mutated metastatic melanoma before treatment. To evaluate the therapeutic response as much as possible on the same tissue in which HuR expression distribution was analysed, we had to ensure that, before therapy, patients had a residual tumor on the biopsied site or at least a similar tumor in the vicinity of the removed metastasis (most often an adjacent subcutaneous or lymph node metastasis). Considering that the number of patients we had access to, fulfilling this prerequisite and treated with vemurafenib, was low (n = 4), we included patients who received dabrafenib/trametinib combination therapy (n = 6) or both regimens (n = 1, total = 11) (Table S1). An immunohistochemistry-based automated quantification of HuR nuclear expression combined with S100 staining to identify malignant cells was blindly performed. Samples were then classified based on the therapeutic response and although their number was not large enough to reach statistical significance, consistent with our ex vivo results, we observed a strong trend toward an increased response to a two/three months BRAF inhibition in metastatic tumors containing a lower proportion of HuRLow cells (i.e. in samples with lower Z-scores < −2 SD) (Fig. 1G).

3.2. HuR knockdown consequences on adaptive response

Short-term knockdown experiments conducted in the Malme-3M cells demonstrated that an insufficient expression of HuR moderately affects the immediate adaptive response to BRAF inhibition. The reduced inhibitory activity of vemurafenib was nevertheless detected on both the MAPK and the PI3K/AKT pathways (Fig. 2A), and even on the downstream ribosomal protein S6 mainly at Ser240/244.

![Fig. 2. HuR knockdown in melanoma cells. (A) Western-blot of indicated markers in HuR (and Ctrl) siRNAi. (B) FACS analysis of HuR expression in SK-MEL28 sh HuR (and Ctrl) RNAi cells. The nascent HuRLow cells are shown emerging from the main population (left panel, arrow). The state designations and analysed time-points in the subsequent experiments are indicated. (C) Western-blot of indicated markers in parental (TP1), mildly (TP2) and harshly (TP3) adapted cells. The analysis was conducted in the low, mid and high-shift HuR knockdown states, see (B). The expression profile of the sh HuR high-shift cells at TP3 could not be compared with similarly exposed sh Ctrl cells as these cells underwent massive death under the same harsh adaptive regimen. (D) Cell cycle and viability analysis of the SK-MEL28 sh HuR low-shift and sh HuR high-shift cells at TP1 and TP3. Quantifications are shown below. (E) Clonogenic assays: resistance phenotype of SK-MEL28 sh HuR mid-shift and high-shift at TP1 and TP2. The sh HuR high-shift at TP1 became almost full-shifted after the corresponding three weeks clonogenic assay (designated as high-shift/full shift).]
Subsequent long-term knockdowns were conducted in the SK-MEL28 cells having a lower propensity for adaptive response and a relative higher expression of HuR. So as to explore with a dose effect the contribution of HuRLow cells to the adaptive response of the total cell population, we generated knockdown cells in which a HuRLow state could be distinguished (as opposed to having a shift of the whole cell population) and moreover occurred at varying proportion \( (\text{Fig. 2B}) \). Importantly, the progressive shift was due to an increasingly efficient knockdown occurring in each cell with two possible dominant states. Indeed, the nascent HuRLow cells emerged from the main population. Moreover, the slowness of the transition (three months) was presumably due to its reversibility in each cell, the HuRLow state ultimately becoming dominant.

In TP1 cells, despite the proportion of HuRLow cells still being weak (HuR low-shift), we observed a reduced inhibitory activity of vemurafenib on the MAPK pathway (Fig. 2C). Noticeably, the reduced inhibition of S6 phosphorylation at Ser\(^{235/236}\) was more pronounced than in the short-term knockdown, and combined, at the highest inhibitory concentrations of vemurafenib, with an increased S6 phosphorylation at Ser\(^{240/244}\). The phosphorylation of S6 being regulated by p70 S6 kinase 1, a TORC1 target or alternatively by p90 ribosomal S6 kinase, a downstream MAPK target \([10]\), the detectability of these changes in the cell lysates presumably reflects their respective strong compensatory activation and reactivation in the small proportion of cells being at any moment in a HuRLow state. Accordingly, before any pre-exposure to vemurafenib, these cells were more resistant to BRAF inhibition than their control counterpart were in long-term clonogenic assays (Fig. 2E). At TP2, these cells became even more resistant comparatively to their control, however FACS monitoring of similarly-exposed cells, indicated that by that time, the equilibrium had further shifted toward the HuRLow state (HuR mid-shift, detectable in the cell lysates) (Fig. 2B and C). At such mid-shift state in TP2 cells, we observed, compared with the control, rather than a further reduced inhibition of the MAPK and PI3K/AKT pathways, a reduced baseline activity of both pathways i.e. a reduced phosphorylation of ERK, S\(^{235/236}\)AKT, E\(^{451}\)BP1 and S\(^{240/244}\) and baseline expression of DUSP4 (Fig. 2C). Furthermore, at the highest inhibitory concentrations of vemurafenib, a slight increase in the expression (expected to be inhibited) of DUSP4 \([11]\), as well as BRAF, EGF and MET was detected.

A reduced baseline phosphorylation of MEK, S\(^{235/236}\), E\(^{451}\)BP1 and S\(^{240/244}\) in adapted cells became more obvious in the HuR high-shift knockdown cells (TP3) (Fig. 2C). In these cells, the sustained phosphorylation of ERK and reduced baseline expression and subsequent increase in vemurafenib-exposed cells of DUSP4, were additionally consistent with an overall reduction in the MAPK output and sensitivity to BRAF inhibition. The observed co-reduced activity of the PI3K/AKT pathway was also expected, as the two pathways mutually compensate each other \([12]\). Moreover, the increased expression of the resistance markers EGRF and MET \([1]\) was confirmed and associated with a decreased baseline expression of SOX10. Remarkably, contrary to the control knockdown cells, these cells could be exposed to an even harsher incremental increase vemurafenib regimen (same standard regimen followed by an increase in the dosage of 200 nM every three days up to 1.4 \(\mu\)M in two weeks, TP3) without suffering any mortality or any important decline in their growth rate (Fig. 2D), presumably due to their reduced dependency towards both MAPK and PI3K/AKT pathways. This timeframe of resistance and growth, however, ended as the cells acquired an almost full and irreversible knockdown of HuR. At this point, before exposure to the drug, their resistance to vemurafenib was similar to the HuR mid-shift knockdown cells (Fig. 2E). However, the almost full knockdown was disadvantageous for their adaptive resistance. When exposed to the same harsher regimen described above, although the cells did not suffer any mortality, they became growth-arrested (not shown). This phenotype did not occur in the untreated HuR almost full knockdown cells.

Collectively, these observations support the hypothesis that a moderate increase in the proportion of HuRLow cells favors the adaptive response of melanoma cells to BRAF inhibition. However, the reversibility of the HuRLow state seems necessary for adaptive proliferation, as opposed for the cells to become quiescent under BRAF inhibition.

### 3.3. HuR expression induction consequences on adaptive response

We hypothesized that the suppression of the HuRLow cells through inducing HuR expression could reduce the adaptive response to BRAF inhibition. We found in the literature that lithium salts can either increase the global expression of HuR \((\text{ex and in vivo}) [13]\) or its cytoplasmic translocation \((\text{ex vivo}) [14]\). We were not able to reproduce these observations to the extent reported, even upon cell fractionation in various melanoma cell lines (not specifically shown, visible in Fig. 3C). However, when using a sensitive immunocytochemistry (ICC)-based single-cell automated quantification of HuR total (nuclear and cytoplasmic) expression, we could repeatedly observe a positive shift in its expression distribution in lithium-treated \((\text{Li}2\text{CO}3)\) melanoma cells, including the A375 cells (Fig. 3A). The shift was observed at potentially in vivo nontoxic therapeutic concentrations \((0.5 \text{ mM or 1 mM, 48 h treatment})\) and was mainly due to an increase in the HuR nuclear fluorescence \((\text{see materials and methods})\). It did not occur in the SK-MEL28 HuR almost full knockdown cells nor in the A375 HuR non-reversible knockdown cells that we generated for this experiment as a valid negative control (Fig. 3E). The ICC quantification precisely reproduced the differences between the cell lines and their respective HuR knockdown in FACS analysis. The later was, however, less reproducibly detecting \((\text{not shown})\) the effect of the Li\(^2\text{CO}_3\) on HuR expression distribution.

Remarkably, long-term clonogenic assays demonstrated a significant reduction in the TP2 resistance phenotype of Li\(^2\text{CO}_3\)(1 mM)-treated A375 cells (Fig. 3B). A partial cell growth recovery was associated with this phenotype (Fig. 3D). Accordingly, in established A375 cells xenografts, following the initial response to vemurafenib, tumor relative regrowth was markedly attenuated in lithium-treated mice (Fig. 3F). Noticeably in vivo, before initiating the vemurafenib, tumor growth of lithium-exposed cells also decreased. Next, we compared in clonogenic assays, the resistance phenotype of Li\(^2\text{CO}_3\)-treated HuR\(^{\text{inducible}}\) A375 sh HuR cells, to the one of similarly treated HuR\(^{\text{inducible}}\) sh Ctrl cells, and could confirm that the lithium-induced reduced resistance phenotype occurred only in the latter but not the former and was therefore dependent on HuR induction (Fig. 3G). A secondary observation made here was that a monotonic knockdown of HuR (in A375 cells) had no impact on the adaptive response (Fig. 3G). This confirmed again that to favor the adaptive proliferative response to BRAF inhibition, the HuRLow state needs to be reversible.

Expression analysis of the same ex vivo experiment indicated that in the parental cells, Li\(^2\text{CO}_3\) (1 mM, during 24 h, TP1) reduced the baseline MAPK output as both ERK phosphorylation and the expression of DUSP4 were reduced (Fig. 3C). This effect was associated with a reduced expression of EGRF. However, in TP2Li\(^2\text{CO}_3\) cells, the sustained phosphorylation of ERK was also attenuated, the increased TP2 baseline expression of MEK was prevented, the baseline expression of resistant markers as EGF and CRAF \([15]\) was decreased and SOX10 baseline expression loss was slightly restored. This overall less resistant profile was associated with a slight increased baseline phosphorylation of AKT but its
dephosphorylation at the doses of vemurafenib reached in the adaptive regimen.

3.4. Single-cell analysis of the adaptive response

We conducted single-cell barcoded mass cytometry analysis in TP1, TP2 and TP2Li2CO3 (1 mM) A375 cells, of the expression distribution of HuR and two markers of proliferation (Ki67 and pRb). Additional markers (CASP3, p53, p21, EGFR, MET, PDGFRB, pERK1/2, pS6235/6, pAKT, p4EBP1) were used to define the subpopulations rather than for their own dynamic expression analysis, therefore the cells were harvested at steady state. The unsupervised clustering was mathematically defined among a multitude of computationally-run combinations of varying viSNE and SPADE parametrizations (see Materials and methods and Fig. S3).

The total population analysis confirmed that the emergence of a Ki67/pRbHigh over-proliferating subpopulation in TP2 cells was prevented in TP2Li2CO3 cells (Fig. 4C). Importantly, a HuRLow drag component in HuR expression distribution, in TP1 and TP2 cells, was not detected in the TP2Li2CO3 cells. Among the six defined clusters, the emerging TP2 over-proliferating cells were only detected in cluster 2 and 3 carrying a HuR Low component. Regardless of the clustering, we could visualize on the viSNE maps the two Ki67/pRbHigh over-proliferating subpopulations emerging at TP2 as corresponding to cluster 2 and an adjacent subcomponent of cluster 3. Their emergence was prevented in TP2Li2CO3 cells (Fig. 4A and B). Cluster 3 also included Ki67Low cells. This dual very low and high-proliferative state could be traced all the way down in the HuR lowest-expressing component of cluster 3, which was visualized in the upper part of cluster 3 and was almost nonexistent in the TP2Li2CO3 cells (Fig. S2). Despite its small size, (~500 cells, <10% of the total cell population), this subcomponent of cluster 3 carried at TP1 two equiproportionate number of the slowest-dividing cells of the whole cell population (pRbLow) and fast (pRbHigh) proliferating cells. Consistent with our previous data, these observations strongly suggest that the adaptive proliferative response to BRAF inhibition is emerging from the cell subpopulation having a sustained or intermittent lower expression of HuR, the size of which is reduced in lithium-treated cells.

Regarding the other markers (Fig. S1), the highest expression...
level of the well-defined targets of HuR, p21 and p53 [16], occurred in the HuR highest expressing cluster 4 that was stably across the three conditions (with no TP2 over-proliferation), the fastest proliferating cluster (Fig. 4C). Conversely, the expression of these two markers was low in a fraction of cells in the two over-proliferating cluster 2 and 3 in TP2 cells. This heterogeneous component was observed in cluster 3 for most markers as pERK1/2, pS6235/236, pAKT, p4EBP1 and PDGFRB in TP2 cells and suppressed in TP2Li2CO3 cells. Overall, clusters 2 and 3 were the most heterogeneous clusters for all markers. A striking heterogeneous “plastic” expression was even more noticeable, for many markers, in the HuR lowest-expressing subcomponent of cluster 3 carrying the “dual proliferative state” at TP1, and for some markers as pERK1/2 at TP2 (Fig. S2). Noticeably, cluster 5, which had the most homogeneous expression profile among all clusters, particularly for EGFR and MET compared with clusters 4 and 6, and for PDGFRB and pERK1/2 compared with cluster 1, had its size reduced in TP2 but not in TP2Li2CO3 cells (Fig. 4B). This overall relative synchronizing effect at steady state, is consistent with the lithium-induced partial cell growth recovery observed ex vivo in the adapted total cell population (Fig. 3D).

4. Discussion

A number of studies support that the adaptive response to pathway inhibition depends on the ability of malignant cells to switch to a state of slow-growth, dedifferentiation, and alternative use of embryonic signaling pathways [17]. This paradigm of phenotypic plasticity has been well explored in BRAF-mutated melanoma [18]. However, it is also in this disease that the reactivation of the targeted pathway has been best documented ex and in vivo, and recognized as the hallmark of the adaptive response to BRAF inhibition [19]. This apparent discrepancy is explainable by the heterogeneous constituency of malignant cell populations. Indeed, the stem-like slow-dividing dedifferentiated induced cells, can either represent a low proportion of cells, or have a minor
contribution to the MAPK output that emanates from the proliferative subpopulations. Currently, our understanding of the mechanisms of the equilibrium between these subpopulations is limited, the TGF-β/Smad signaling-induced epithelial-to-mesenchymal transition being a frequently transposed model to understand this equilibrium [17].

In this study, we describe a therapeutically actionable mechanism to reduce the proportion of a subpopulation of highly plastic and partly slowly dividing cells, in which the adaptive proliferation emerges under BRAF inhibition. At the whole cell population level, a moderate therapeutic induction of HuR expression is sufficient to modulate the expression and activation of multiple nodes within the adaptive signaling network. Complementary studies will clarify the molecular mechanisms that differentiate malignant cells that tightly regulate HuR expression, from those in which its expression is more fluctuating and will likely improve our therapeutic ability to stabilize HuR expression and attenuate the plasticity of malignant cells.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.06.154.

Author contributions

R.M. designed and supervised the project, performed experiments, analysed the data and wrote the manuscript. A.B.-M. performed most of the experiments. I.J.W. did the xenograft transplants. C.P. managed the clinical data. Y.D. ran the LC-ESI-MS/MS. C.S. ran the CyTOF. N.L. did the bioimaging analysis.

Conflicts of interest

R.M. is inventor on a patent application on the use of agents enhancing HuR/ELAV protein levels in the treatment of BRAF-mutated cancers.

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