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In vivo profiling reveals immunomodulatory effects of sorafenib and dacarbazine on melanoma

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Abbreviations: AE, adverse events CMR, complete metabolic response; ISG, IFN-stimulated gene; PMD, progressive metabolic disease; PMR, partial metabolic response; SAE, serious adverse events SMD; stable metabolic disease.

Sorafenib is a multi-kinase inhibitor used alone or in combination with dacarbazine to treat metastasized melanoma. Our study investigated the relationship between metabolic response assessed by PET-CT and global transcriptome changes during sorafenib and dacarbazine therapy in patients with advanced melanoma. We conducted an open-label, investigator-initiated study that enrolled 13 sorafenib-naïve Stage IV melanoma patients, whose metastases were accessible for repeated biopsies. Treatment regimen included orally administered sorafenib and intravenous dacarbazine. Biopsies of skin or superficial lymph node metastases were taken before treatment (baseline), during sorafenib and after dacarbazine therapy and used for transcriptional profiling and validation experiments. Serum samples were evaluated for cytokine production. Metabolic response to therapy was observed in 45.5% of patients. The study drugs were well tolerated. We observed a clear upregulation of interferon (IFN)-stimulated immune response genes in profiled metastases. The IFN γ -induced gene signature seemed to be enhanced after addition of dacarbazine to sorafenib. Serum IFN γ also increased during therapy, particularly after addition of dacarbazine. Induction of IFN γ stimulated genes correlating with increased serum IFN γ was predictive of better clinical outcome and responders who had significantly higher serum IFN γ levels lived longer. Our data reveal *in situ* changes in melanoma metastases during treatment with sorafenib and dacarbazine and suggest an additional mechanism of action through immunomodulation.

Introduction

Over the last 4 decades, the rate of melanoma incidence has been constantly increasing.¹ Despite regional variability, European countries have not yet reached the incidence rates of Australia and the United States (46.7 and 22.5 per 100,000 people, respectively).¹⁻⁴ Treatment of metastatic melanoma remains challenging,^{1,3,4} even though prolonged survival has been achieved by the use of the recently FDA-approved molecules, such as the anti-CTLA-4 blocking antibody ipilimumab and the BRAF kinase inhibitors vemurafenib and dabrafenib.⁵ With registration of ipilimumab, which will surely be followed by other compounds intervening at immunological checkpoints, immunotherapy of melanoma has been implemented into standard therapeutic regimens worldwide.

Sorafenib is an orally available multi-kinase inhibitor that targets tumor cell proliferation and angiogenesis. It intervenes in the protein kinase RAF/MEK/ERK pathway by inhibiting several Raf kinase isoforms, including RAF1, wild-type BRAF and mutant BRAF (for a review see ref. ⁶) Furthermore, sorafenib acts against several other receptor tyrosine kinases including vascular endothelial growth factor receptor (VEGFR)2/3, platelet-derived growth factor receptor β PDGFR β , fms-related tyrosine kinase 3 (FLT-3) and c-KIT. Sorafenib is currently registered for the treatment of metastasized renal cell carcinoma, hepatocellular carcinoma and differentiated thyroid cancer. In melanoma, sorafenib has been tested as monotherapy or in combination with different compounds, such as dacarbazine, temozolamide, carboplatin, paclitaxel and others.⁶ Dacarbazine is an alkylating agent, first introduced some 30 years ago and still considered as a

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reference single agent for the management of advanced melanoma.⁷ Phase I and II trials using sorafenib combined with dacarbazine in advanced melanoma demonstrated acceptable toxicity profiles and limited anti-tumor activity with disease stabilization in up to 20%.⁸⁻¹¹ However, patients in Phase III trials of sorafenib added to other chemotherapeutics such as carboplatin and paclitaxel did not have better outcome compared to chemotherapy alone in the treatment of advanced melanoma.^{12,13} Despite the long history of dacarbazine use in melanoma therapy, little is known about mechanistic effects of the both sorafenib and dacarbazine, as well as their combination on antitumor responses in melanoma.

We initiated an open-label, investigator-initiated study for sorafenib-naïve patients with advanced melanoma to evaluate the *in situ* effects of dacarbazine and sorafenib on metastases that were accessible for repeated biopsies. Herein, we report that dacarbazine and sorafenib have additional immunomodulatory properties in addition to their cytotoxic and kinase inhibitory activities.

Results

Response and toxicity

Out of 14 enrolled patients with advanced melanoma, 13 patients (5 females, 9 males, mean age: 61.7 years \pm 11.93) were actually treated with the study medication. Of these, 2 patients did not complete the study, with patient number 6 deceased on day 54 due to tumor progression and patient number 12 discontinued the medication at day 37 due to side effects (fatigue and loss of appetite). The remaining 11 patients were evaluable for the analysis at the end of the study (day 60). Metabolic response of target lesions evaluated using PERCIST 1.0 criteria was seen in 5 out of 11 patients (45.5%) (Table 1). At the end of the study (day 60), 5 responders (all complete metabolic response [CMR]) and 6 non-responders (2 stable metabolic disease [SMD] and 4 progressive metabolic disease [PMD]) could be defined. The

median progression-free survival (PFS) was 63 days (range 24-307 days). Responders defined by PERCIST criteria showed a significantly longer median PFS (307 days) than non-responders (58.5 days; log rank test $p = 0.001$). Only one out of 5 responders had mutated BRAF (V600E mutation).

The treatment was well tolerated. Most of the adverse events (AEs) were mild or moderate and included constitutional symptoms, skin changes, impairment of bone marrow function and gastrointestinal side effects. A summary of AEs is shown in Table S1. Likewise, most of the serious adverse events (SAEs) were due to the impairment of bone marrow function, constitutional symptoms or toxicity to the gastrointestinal tract and liver. The death events on study were due to underlying disease (Table S1).

In vivo sequential transcriptome response to therapy

In-depth pathway analysis during therapy with sorafenib and its combination with dacarbazine

To evaluate transcriptional changes in melanoma metastases during therapy, we performed gene expression profiling using exon array analysis on melanoma metastases specimens obtained from 9 patients before treatment (baseline), during treatment with sorafenib (day 10) and during treatment with sorafenib and dacarbazine (day 16). Using our filtering criteria, we obtained 603 differentially expressed probes during sorafenib monotherapy (day 10) compared to baseline, accounting for 367 genes (169 upregulated, 198 downregulated). For the duration of sorafenib and dacarbazine therapy (day 16), we identified 786 differentially expressed probes compared to baseline, accounting for 455 genes (269 upregulated and 186 downregulated). A part of these genes, exactly 144, were shared between 2 time points (Fig. 1A). Genes from both treatment points were then used in pathway analysis. For this purpose ClueGO, a Cytoscape plug-in was employed using GO biological process descriptions, in order to assess the functional grouping of genes and enable visualization of interactions. Furthermore, a comparison analysis of genes differentially expressed during sorafenib monotherapy vs. sorafenib

Table 1 Patients' characteristics

Patient No	Sex	Age	Stage at baseline	B-raf status	Previous therapies	PERCIST at day 60*	Status
1	M	74	IV	Wild-type	Surgery	CMR	Responder
2	M	67	IV	Wild-type	Surgery	SMD	Non-responder
3	M	73	IV	Wild-type	Surgery, chemotherapy	CMR	Responder
4	M	63	IV	Mutated (V600E)	Surgery, topical and experimental immunotherapy	CMR	Responder
5	F	61	IV	Mutated (V600E)	Surgery, interferon- α , experimental immunotherapy	PMD	Non-responder
6	M	59	IV	Wild-type	Surgery	n.e.	Non-responder
7	F	48	IV	Wild-type	Surgery, interferon- α	PMD	Non-responder
9	M	60	IV	Wild-type	Surgery	SMD	Non-responder
10	M	75	IV	Wild-type	Surgery	CMR	Responder
11	M	42	IV	Wild-type	Surgery	PMD	Non-responder
12	F	76	IV	Wild-type	Surgery	n.e.	Non-responder
13	F	63	IV	Wild-type	None	CMR	Responder
14	F	41	IV	Mutated (V600E)	Surgery, topical immunotherapy, interferon- α , radiotherapy	PMD	Non-responder

*CMR (complete metabolic response), PMR (partial metabolic response), SMD (stable metabolic disease), PMD (progressive metabolic disease) according to PERCIST 1.0 criteria (see Material and Methods).

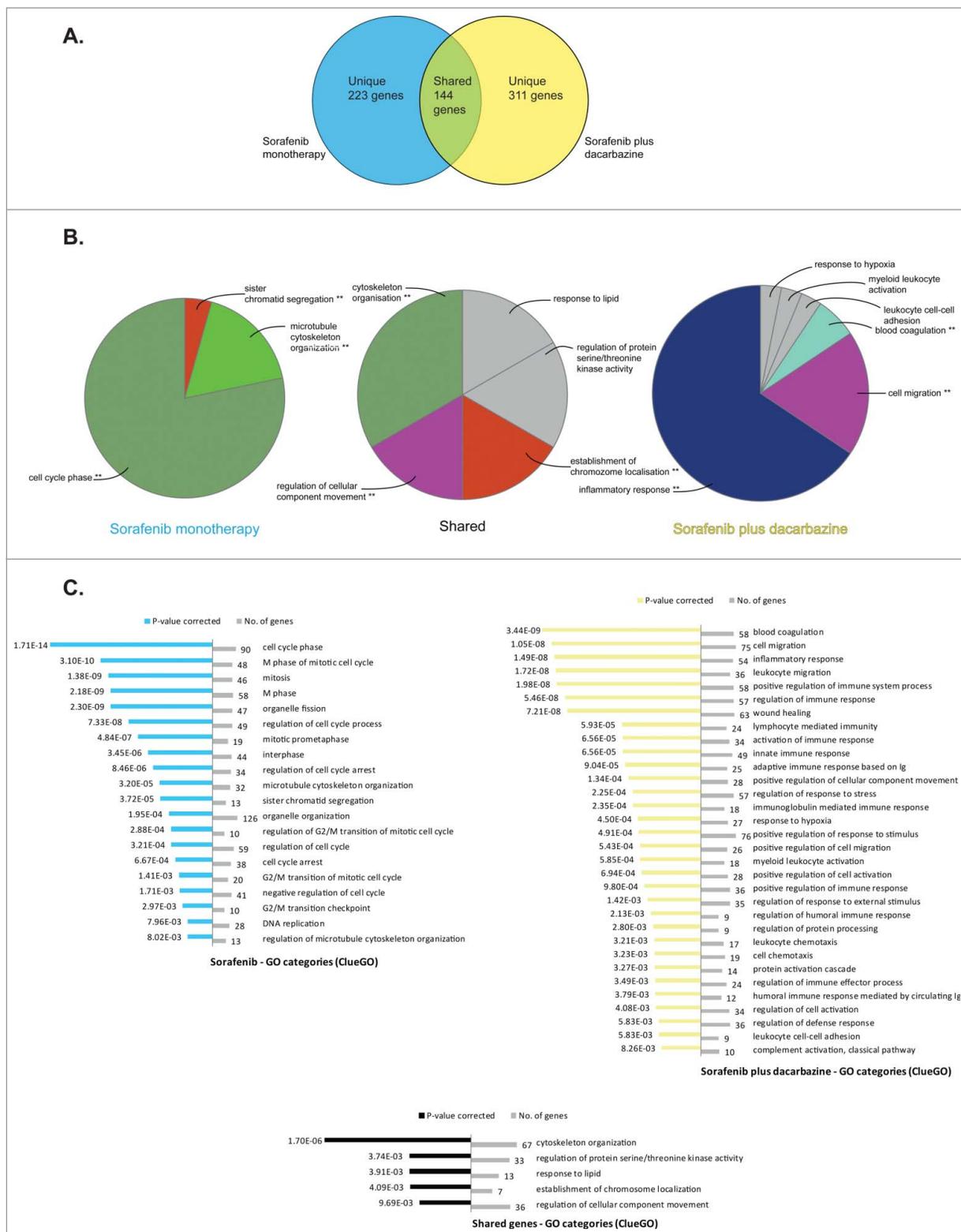


Figure 1. Melanoma metastases differentially express genes during therapy with sorafenib and/or dacarbazine. Exon array analysis on melanoma metastases specimens obtained from patients (n=9) before treatment (baseline), during treatment with sorafenib (day 10) and during treatment with sorafenib and dacarbazine (day 16). **(A)** Venn diagram of over/under differentially expressed genes in response to therapy and representing genes specific for or shared by different therapies. **(B-C)** Mining of functional gene associations was performed using Cytoscape, an open source application for visualization of gene interactions and pathway analysis (<http://www.cytoscape.org>). **(B)** Overview charts are specifying leading functional terms specific for or shared by sorafenib monotherapy or sorafenib plus dacarbazine. The size of the group indicates the number of the terms included in group. **(C)** Bar graphs representing gene ontology (GO) categories of genes specific for with sorafenib and/or dacarbazine combination, shared genes, their P-values and number of genes per GO category.

plus dacarbazine was also performed, in order to define biological processes specific for a particular treatment. The analysis criteria for ClueGO are described in Material & Methods and the results are shown in **Figure 1**. Function-based network of differentially expressed genes is shown in **Figure S2**.

The majority of differentially expressed genes were allocated to functional groups implicated in the regulation of the cell cycle, cellular component organization and inflammatory / immune response (**Fig. 1B, C**). Control of cell cycle together with spindle apparatus / cytoskeleton organization appeared to be specific for the sorafenib monotherapy (**Fig. 1B**), with highest *P*-values for GO categories containing cell cycle and mitosis control (blue bars diagram, **Fig. 1C**). On the other hand, genes involved in inflammatory / immune response, cell and leukocyte migration were represented in the groups specific for sorafenib and dacarbazine combination (**Fig. 1B**), with highest *P*-values for GO categories comprising inflammatory response and cell migration (yellow bars diagram, **Fig. 1C**). Common to both treatment points were functional groups implicated in cytoskeleton and cellular compartment organization, the regulation of kinase activity and response to lipid (**Fig. 1B, C**). Functional signatures pertinent to sorafenib action on cell cycle processes could be therefore detected at both time points, whereas upregulation of genes involved in inflammatory response was observed particularly at day 16, *i.e.*, following addition of dacarbazine to sorafenib therapy.

Delineation of genes predicting clinical response to therapy

After having underlined the most important gene function categories expressed during sorafenib (day 10) and sorafenib plus dacarbazine therapy (day 16), we next sought to identify genes that could predict clinical response. For this purpose, we analyzed genes at these time points using SVM algorithm and their correlation to clinical outcome evaluated by PET-CT (see Methods) at the end of the study (day 60). In this way, we identified 114 genes for sorafenib and 94 genes for sorafenib plus dacarbazine therapy, which were predictive of clinical outcome with the lowest error rate. Fourteen genes were shared between the 2 treatment points (**Fig. 2A**). These predictor genes were then queried using Genemania and MCODE plug-ins in Cytoscape. Genemania data mining is performed using a databank of functional interaction data in combination with GO biological process descriptions, whereas MCODE determines the most densely connected regions (so-called modules). To our surprise, the majority of predictor genes assessed by Genemania plug-in represented genes involved in different aspects of immune response. The highest ranked functional categories during sorafenib monotherapy encompassed activation of immune response ($P < 0.016 \times 10^{-14}$) and its subcategories. At the point of sorafenib plus dacarbazine therapy, response to interferon (IFN) γ ($P < 0.014 \times 10^{-16}$), IFN γ signaling pathway and cellular response to IFN γ ($P < 0.05 \times 10^{-15}$) were the most significant functional categories, followed by antigen processing / presentation and their subcategories. Likewise, in the group of shared genes between 2 time points, response to IFN γ ($P < 0.02 \times 10^{-13}$) dominated over other functional themes. Using MCODE plug-in, we further

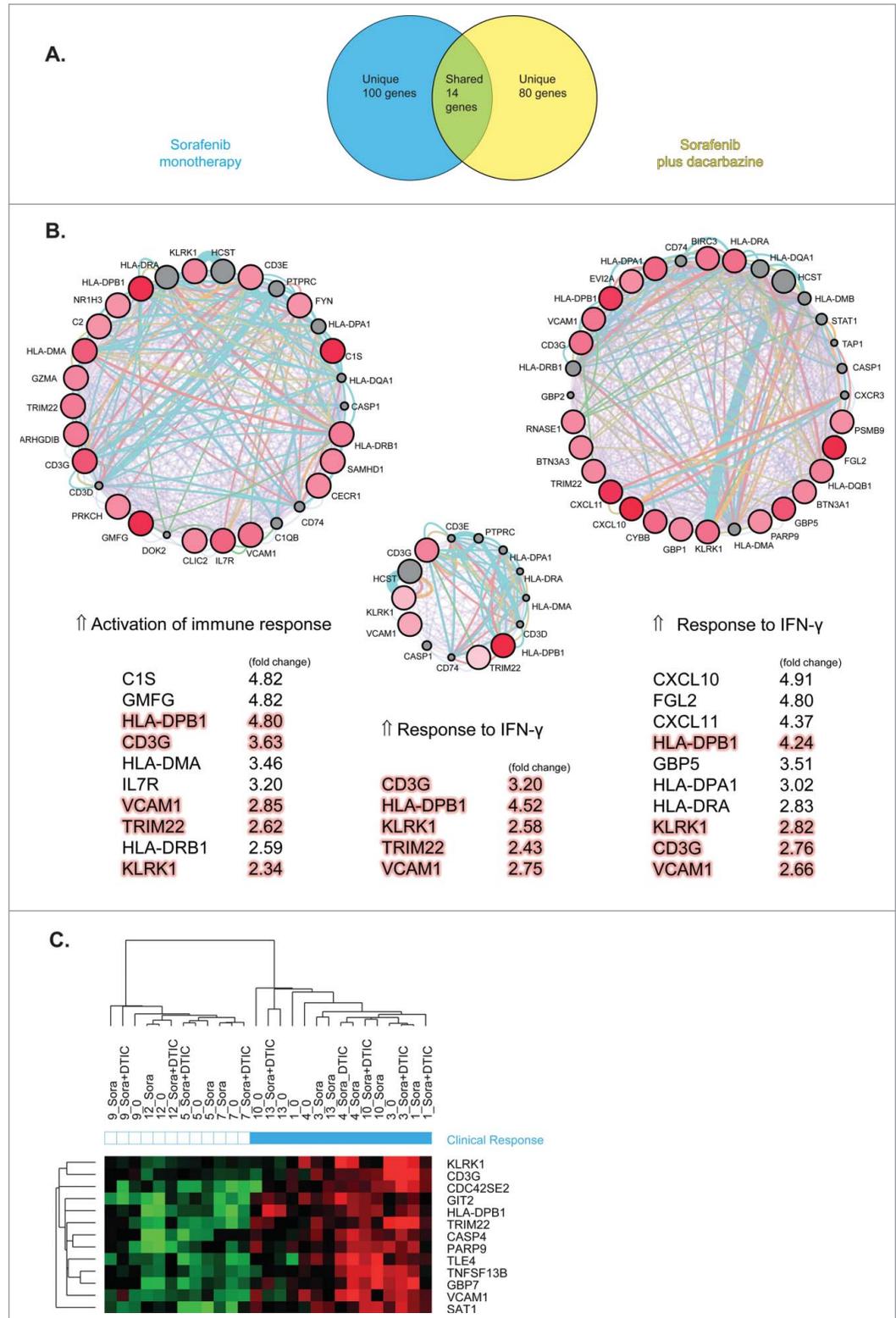
dissected the most densely connected modules within the differentially expressed genes in the treatment groups and determined their overlapping genes. The highest ranked modules as circular networks and top 5-10 genes per module are shown in **Figure 2B**. In line with the above observation, we observed upregulation of IFN γ -stimulated genes, like *CXCL10*, *CXCL11*, *HLA-DP/R*, *CD3G*, *VCAM1* and several encoding guanylate binding proteins (GBPs) to be more prominent during combined sorafenib and dacarbazine therapy. IFN α -stimulated genes, like *OAS1*, *IFI44*, *IFIH1*, several poly [ADP-ribose] polymerase (PARP) family members, and others, were upregulated at both time points but to a lower extent when compared to IFN γ -induced genes. Unsupervised 2-way clustering analysis using 14 shared predictor genes between treatment groups confirmed upregulated expression of these genes in responder patients (**Fig. 2C**). Although most of the treated patients clustered near each other in the responder arm, it was interesting to observe that some of the baseline samples were intermixed, suggesting the presence of an activated antitumor microenvironment prior to the initiation of therapy, which, in turn, may be permissive for positive responses in the course of the therapy.

Validation of clinical response predictors by PCR and immunohistochemistry

We next focused on verifying the expression of predictor genes and their proteins in our samples by real-time PCR and immunohistochemistry. Samples from 10 patients (4 responders and 6 non-responders) were tested using real-time PCR. IFN γ (type II IFN) inducible transcripts (*CD3G*, *CXCL11*, *VCAM1* and those encoding HLA-DR/HLA class II) and IFN α / β (type I IFN)-inducible MXA genes products were evaluated. The chemokine (C-X-C motif) ligand 11 (CXCL11, also known by the synonyms I-TAC and IP9) is a chemokine primarily induced by IFN γ with its best known role in leukocyte trafficking.^{14,15} CD3 and HLA-DR are cellular receptors present on T lymphocytes and antigen presenting cells, respectively.¹⁶ The *VCAM1* gene encodes vascular cell adhesion molecule 1, a molecule involved in leukocyte migration to site of inflammation and is also IFN γ inducible.¹⁷ The mRNA expression of all tested IFN γ -inducible genes was significantly higher in responders (2-way ANOVA; *CXCL11* $P = 0.002$, *VCAM1* $P = 0.026$, *CD3G* $P = 0.010$ and *HLA-DRA* $P < 0.0001$, **Fig. 3**). Moreover, the expression levels of most of these genes at either of the 2 time points (*i.e.*, day 10 or day 16) was able to discriminate between responders and non-responders in PET-CT as determined by receiver operating characteristic (ROC) curves (true predictors are shown in **Fig. S3**).

Although the *MXI* gene was not present on the predictor genes list, we have chosen this gene and its product MX dynamin-like GTPase 1 (MX1, better known as MxA) protein as an additional molecular marker for type I IFN responses in the skin.¹⁸ The *MXI* gene product (*i.e.*, the MxA protein) is a large GTPase that is involved in cellular antiviral response.^{19,20} The expression of *MXI* mRNA did not significantly differ between responders and non-responders (2-way ANOVA $P = 0.352$), implying that IFN γ -induced gene products are more relevant than those stimulated by IFN α in predicting clinical response. Nevertheless, *MXI* gene expression in

Figure 2. Gene predictors of clinical response of melanoma patients to therapy with sorafenib and/or dacarbazine. Differentially expressed genes from melanoma biopsy samples from patients (n = 9) treated with sorafenib (day 10) and sorafenib plus dacarbazine therapy (day 16) were analyzed for a correlation to clinical outcome. **(A)** Venn diagram representing gene predictors specific for or shared by sorafenib monotherapy or sorafenib plus dacarbazine therapy group. **(B)** Circle networks of most densely connected modules within treatment groups obtained by Genemania and MCODE plug-ins in Cytoscape. Shared genes by both groups are shown in the middle. The top upregulated genes are listed for each group and fold-change values to baseline are indicated. Orange highlights represent shared genes between the groups. Genes are identified by their gene symbols. **(C)** Two-way clustering analysis of the baseline, sorafenib (day 10) and sorafenib plus dacarbazine therapy (day 16) using 14 shared predictor genes. The similarity of gene expression profiles between examined samples is summarized in a dendrogram above the heatmap, in which the pattern and the length of the branches reflect the relatedness of the samples. The red color in the heatmap indicates a high relative level of expression, whereas the green color indicates a low relative level of expression. Clinical response is represented by blue squares, whereas non-responders are represented by white squares. Genes are represented by their gene symbols.



obtained biopsies could discriminate between responders and non-responders according to ROC analysis (Fig. S3).

Similar results were obtained by immunohistochemistry, even though a reduced number and partially unpaired tissue samples

were available. Total of 6 baseline and 7 samples from day 16 (sorafenib plus dacarbazine) were used for immunohistochemical stains for CD3 (T cells), HLA-DR (MHC class expressing inflammatory cells), MxA (IFN α activity) and VCAM-1

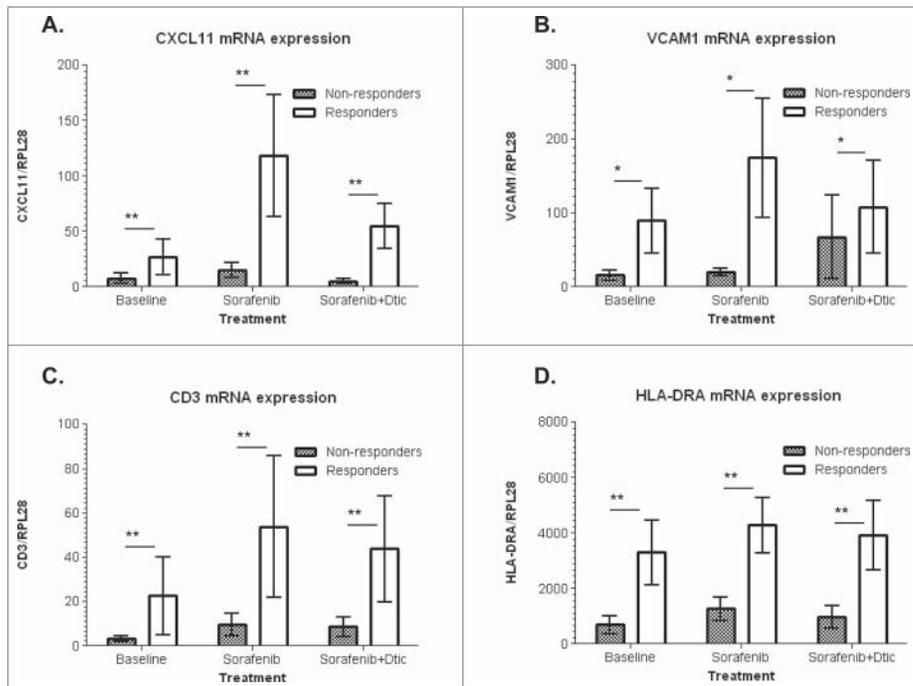


Figure 3. Validation of gene predictor of clinical response to therapy. Expression of response predictor genes in biopsies ($n = 10$ patients), including 4 responders and 6 non-responders, were tested using real-time PCR. (A–D) Gene expression levels were assessed by quantitative PCR and tested for significance using 2-way ANOVA. The bars show mean expression of the respective gene normalized to RPL28 housekeeping genes at baseline, day 10 (sorafenib) and day 16 (sorafenib plus dacarbazine) as well as their grouping according to response, while whiskers represent standard error of the mean (SEM); * $P < 0.05$; ** $P < 0.01$.

revealed analogous results, with only CD3 and HLA-DR discriminating response (Fig. 4C and D).

Systemic IFN γ induction during therapy with sorafenib and its combination with dacarbazine

(adhesion molecule). CD3 expression revealing T-cell presence, albeit on the level of significance, was more prominent in the responder group (Mann-Whitney U-test $P = 0.055$, Fig. 4A). HLA-DR expression was also stronger in responder biopsies (Mann-Whitney U-test $P = 0.008$, Fig. 4B). MxA and VCAM-1 (data not shown) protein expression, on the other hand, did not show any statistical difference between responders and non-responders. ROC analysis of immunohistochemistry markers

Since therapy with sorafenib, either monotherapy or in combination with dacarbazine, seems to induce expression of IFN-stimulated genes, we measured serum levels of IFN α and IFN γ in our patients. For this purpose, we analyzed sera of 8 patients (4 responders and 4 non-responders) at baseline, day 10 and day 16. Although detectable, serum IFN α levels were only marginally different between responders and non-responders (2-way ANOVA $P = 0.055$, Fig. 5A). In contrast and in conjunction with the PCR data presented above,

it came as no surprise that serum IFN γ were only detectable in responders ($P < 0.0001$, Fig. 5B). Furthermore, serum IFN γ continuously increased during the treatment, showing the highest levels after addition of dacarbazine (0 to

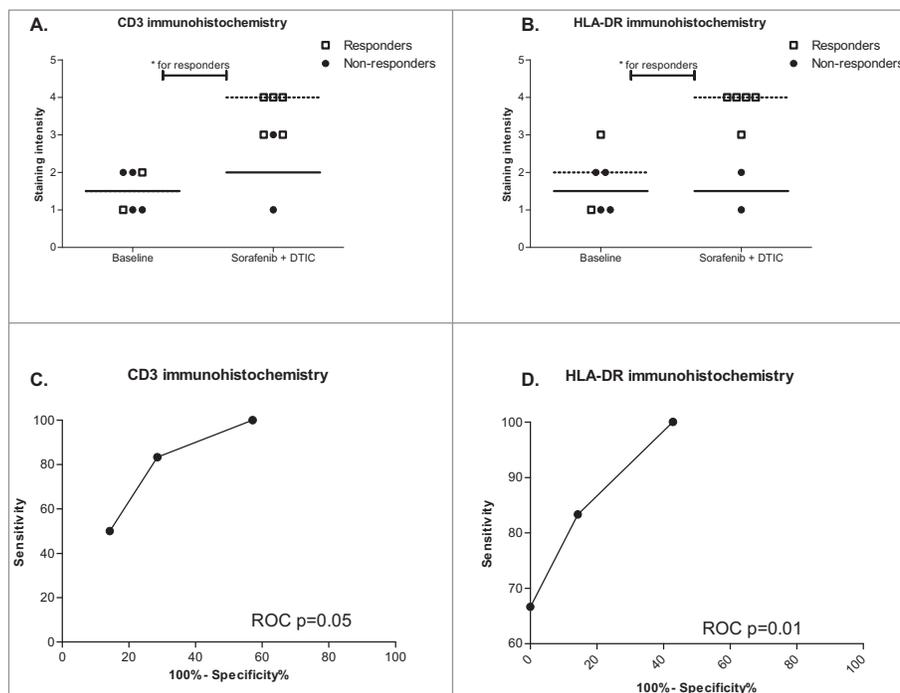


Figure 4. Expression of response predictor gene products in melanoma biopsies. (A and B) CD3 and HLA-DR protein expression was evaluated by immunohistochemistry and difference between responders and non-responders tested using Mann-Whitney U-test. P values for each test are shown in the graph, $P < 0.05$ values are highlighted with one asterisk (*), $P < 0.01$ values with 2 asterisks (**). The scatter plots show individual staining intensity at baseline and day 16 (sorafenib plus dacarbazine), different labeling according to response, with horizontal line representing median value for each group. (C and D) ROC diagrams depict discrimination between responders and non-responders using CD3 and HLA-DR expression.

66.89 pg/mL vs. 120.8 pg/mL; 2-way ANOVA $P = 0.0095$). Serum IFN γ levels were good discriminators between responders and non-responders in PET-CT using ROC analysis ($P = 0.0009$; Fig. 5C). Furthermore, patients who had detectable serum IFN γ levels had significantly longer progression-free survival than the ones without (307 days vs. 56.5 days; log-rank test $P = 0.006$; Fig. 5D). These data reveal, to our knowledge for the first time, detectable systemic IFN γ production *in vivo* during sorafenib and dacarbazine therapy and its positive relation to clinical response and survival in melanoma patients.

Discussion

Our pilot study revealed a crucial aspect of the response to kinase inhibitors and chemotherapy in melanoma, an immunologic effect that has so far been considered important primarily in immunotherapies. We evaluated the efficacy of sorafenib and dacarbazine combination in patients with advanced melanoma of Stage IV. Overall metabolic response rates of 45.5% observed in our study are better than the 12% reportedly observed by others^{9,11} but may be due to a low patient number and different response evaluation parameters (PERCIST) utilized in our study. Comparison studies of PERCIST and RECIST evaluations in solid tumors reveal higher sensitivity of PERCIST in detecting complete remission and stable disease,^{21,22} which may partly account for the high response rate observed in our patient population. The study drugs were well tolerated by our patients. Only one out of 5 responders had a mutated *BRAF* gene, suggesting that MAPK pathway inhibition by sorafenib was not the main mechanism governing the response in these patients. Similar data were published recently by Wilson et al., showing that *BRAF* mutation status was not predictive of response or survival in melanoma patients treated with sorafenib and chemotherapy.²³

Subsequently, we focused on elucidating genome-wide transcriptional changes during sorafenib monotherapy followed by its combination with dacarbazine in accessible skin and lymph node metastases. Despite their potential influence on the Raf/MEK/ERK pathway,⁶ the molecular mechanisms by which sorafenib exerts its antitumor activity have not been fully elucidated. Taking into account that modulation of signaling through the Raf/MEK/ERK pathway can interfere with the cell cycle, cytoskeleton organization and cellular movement,²⁴⁻²⁶ our finding that these biological categories were highlighted in our analysis

was anticipated. Similar results were obtained by others who performed gene expression profiling of tumors responding to sorafenib, including hepatocellular carcinoma²⁷ and acute myeloid leukemia.²⁸ We detected no significant changes related to inhibition of angiogenesis pathways by sorafenib. Dacarbazine, on the other hand, is classically considered to be a multimodal cytotoxic agent interfering with cell cycle and DNA organization, which could also be seen in our current study and by others.²⁹

There is increasing evidence that both conventional and targeted therapies have broader and immunomodulatory activities.^{30,31} Sorafenib appears to enhance antitumor immunological responses by modulating macrophage and natural killer (NK) activity, thus rendering tumor cells susceptible to NK cell-mediated killing.³² Similarly, sorafenib selectively influences T-cell subsets, as it promotes activation of effector CD4⁺ T cells while reducing immunosuppressive regulatory T cells.^{33,34} Moreover, a recent study by Romero et al. showed that sorafenib promotes T helper (Th) type 1 polarization and accumulation of peripheral CD4⁺ T cells expressing NK group 2D (NKG2D) ligands in melanoma patients treated with sorafenib and temozolomide, an alkylating agent related to dacarbazine.³⁵ The frequencies of NKG2D⁺ cell subtypes, however, failed to show an association with clinical response in this study. In our patient collective, the expression of NKG2D was one of the predictors of clinical

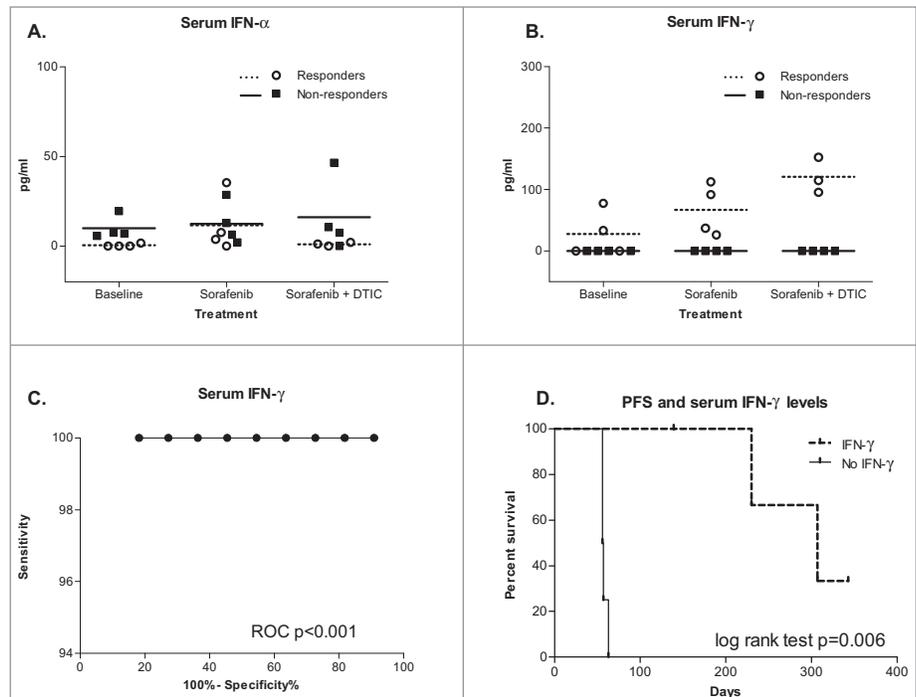


Figure 5. Serum IFN α and IFN γ levels during therapy with sorafenib and/or dacarbazine. (A and B) Serum cytokine levels were measured by ELISA and tested by 2-way ANOVA. The scatter plots show individual interferon α (IFN α) and interferon γ (IFN γ) levels at baseline, day 10 (sorafenib) and day 16 (sorafenib plus dacarbazine) as well as their grouping according to response, the horizontal lines represent mean values of each response group. (B) ROC diagram depicts discrimination between responders and non-responders using IFN γ serum levels. (C) Kaplan-Meier curve depicts a difference in progression-free survival in study patients with detectable vs. undetectable serum IFN γ , as determined by log-rank test.

response. Dacarbazine seems also to exert not only cytotoxic but also immunomodulatory effects. Hervieu et al. reported that dacarbazine upregulates the expression of NKG2D ligand on tumor cells leading to NK-cell activation.³⁶ This, in turn, induces IFN γ secretion, which upregulates the expression of MHC class I molecules on the surface of tumor cells, making them susceptible to killing by cytotoxic CD8⁺ T cells in a mouse melanoma model. Interestingly, melanomas in T-cell deficient mice do not respond to dacarbazine, implying that the activation of NK cells is mandatory for the therapeutic *in vivo* effect of dacarbazine.³⁶ This is in line with our data showing increased expression of NKG2D encoding transcripts (*KLRK1*) throughout the therapy with sorafenib and later with dacarbazine. More importantly, global gene expression profiling analysis of cutaneous melanoma metastases from patients treated with dacarbazine revealed gene expression signatures consistent with T-cell infiltration, immune activation and response to wounding.²⁹ Therapy with dacarbazine induces expression of various chemokines, cytokines, lymphocytic markers and MHC class II genes, which we could also see in our analysis. Particular to our patient collective is the induction of multiple IFN γ stimulated genes, emphasizing the importance of adaptive antitumor immune response.

The importance of antitumor immunity in host control of tumor development is one of the cornerstones of the “cancer immunoediting” theory.³⁷ Accordingly, the immune system is not only capable of destroying tumor cells through the activation of adaptive and innate immune IFN-dependent pathways, it can also facilitate the selection of tumor cells capable of escaping immune pressure, a 3-phase process composed of Elimination, Equilibrium and Escape.^{37,38} In the context of protective antitumor immunity and elimination, the role of intact lymphocyte compartment, type I (IFN- α/β) and type II (IFN γ) interferons is well recognized (reviewed in).³⁹ Antineoplastic agents such as conventional chemotherapeutics may thus stimulate immunosurveillance by increasing tumor antigenicity (e.g., via upregulation of MHC class I immunopeptidome), immunogenicity (via emitting danger signals) and susceptibility to immune effector cell killing (e.g., via upregulation of co-stimulatory molecules, death receptors, NK cell activating receptor ligands [mentioned above] or via downregulation of inhibitory molecules),³¹ most of which we could identify in our analysis (see Fig. 2). It has become increasingly appreciated that specific immune activation signatures can be associated with better prognosis and/or response to therapy in various types of human cancers including melanoma.³⁹⁻⁴² The majority of these signatures falls in to functional categories encompassing Th1 activation (e.g., STAT1, IRF1/ IFN γ pathways), chemokines and their ligands (CXCR3/ CXCL9-11 and CCR5/ CCL3-5 pathways), cytotoxic factors (granzyme, perforin, granulysin/ TIA1/ caspase pathways) and adhesion molecules (VCAM1, ICAM1 and others).⁴¹ It is, therefore, not surprising that our response prediction analysis delivered some of the same genes as those previously described in other similar melanoma studies.⁴³⁻⁴⁷ This observation also refers to transcripts encoding CD3,⁴³⁻⁴⁶ HLA class II,⁴³⁻⁴⁶ CXCL11^{44,47} and VCAM1^{43,45}, markers

which we used in our validation experiments. It is noteworthy that these immune activation signatures were typically described in patients receiving immunotherapy^{43,44,46,47} and rarely in chemotherapy.^{29,43} Our study clearly underlines the fact that tumor regression is, for the most part, immunologically mediated, not only in immune modulation-based but also in chemotherapy and targeted therapies.

The molecular pathways represented in signatures predictive of responsiveness to anticancer therapy largely involve coordinated expression of IFN-stimulated genes (ISGs). Both type I (IFN α/β) and type II (IFN γ) interferons induce a unique and partially overlapping set of ISGs, some of which were investigated here in the context of predicting responsiveness to therapy. Our results are consistent with downstream effects of IFN γ , which we could also detect in the serum of treated patients. Not only did we find higher IFN γ levels in patients who responded to sorafenib and dacarbazine therapy, patients who did not clinically respond to therapy presented no measurable serum IFN γ at all. Prior cytokine studies by others indicate that insufficient production of Th1 cytokines, including IFN γ , in patients with metastasized melanoma can be improved by immunotherapeutic interventions.⁴⁸⁻⁵⁰ In spite of the fact that our patients did not receive immunotherapy, we could still clearly detect an increase in IFN γ serum levels in patients after receiving dacarbazine in combination with sorafenib. This is to our knowledge the first such report showing differential modulation of IFN γ serum levels by tumor-specific therapies.

Lastly, responders presented with an activated antitumor microenvironment prior to the initiation of therapy (as shown by clustering analysis), which obviously favored positive response to therapy. Applied therapy probably resulted in the release of tumor antigens and an IFN γ -polarized immune response, which was detectable in the remaining tumors and serum. It is unlikely that serial biopsies might have accounted for the signatures that we observed, since wound healing after skin injury does not induce an IFN γ -dominated immune response.⁵¹⁻⁵³

In conclusion, we show that combined therapy using sorafenib and dacarbazine can induce tumor regressions in patients with metastasized melanoma, which are associated with an unexpected immune effector mechanism. The limitation of our study is the small number of patients from which the results were drawn. Nevertheless, we demonstrated an induction of a proinflammatory environment dominated by IFN γ and its positive influence on clinical response. As IFN γ is considered to be one of the key players determining the clinical outcome to anticancer therapy,³⁹⁻⁴² further investigations of other chemotherapeutics and targeted therapies are necessary to fully understand and exploit their immunomodulatory potential. There is evidence that sorafenib potentiates responses to regional chemotherapy with temozolomide,⁵⁴ whereas its combination with alkylating agents like dacarbazine and temozolomide leads to more superior stimulation of antitumor immunity.^{32,35} Thus, unique combinations of different therapeutic agents may be more advantageous than monotherapy and such contexts should be taken into consideration.

Methods

Patients

The study protocol was approved by the institutional and regional ethical committee (reference number 799) and Swissmedic (notification number 2008DR1336). The trial was registered in www.clinicaltrials.gov (reference number NCT00794235). The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and good clinical practice.

Patients with advanced melanoma in Stage IV⁵⁵ with histologically-confirmed skin or lymph node metastases, larger than 1 cm in diameter and measurable by PET/CT-scan were eligible for the study. Patients had to be sorafenib-naïve, not received any systemic chemotherapy for at least 3 months prior to study inclusion, show an elevated level of serum LDH (>1.1 ULN) and adequate bone marrow, liver and renal function. Patients with ocular melanoma were excluded as were individuals with significant comorbidities (e.g., severe cardiovascular, renal, hepatic or psychiatric conditions), severe infections, metastatic neoplasm other than melanoma or symptomatic metastatic brain or meningeal tumors. All patients gave written informed consent prior to study inclusion. Patients' characteristics are shown in **Table 1**.

Study design and response evaluation

The study was an investigator-initiated, open-label, pilot study performed at the Department of Dermatology, University Hospital Zurich, Switzerland performed between January and September 2009. Treatment regimen included orally given sorafenib (Nexavar[®], Bayer, Switzerland) 400 mg bid from day 1 to 56 and intravenous dacarbazine (DTIC, Dacin[®], Lipomed, Switzerland) 1000 mg/m² on days 14 and 42 (**Fig. S1**). In patients showing disease stabilization (i.e., tumor regression or stable disease) by the end of the study, the study medication was continued after the end of the study. In the event of disease progression, participation on the study was terminated. Adverse events were documented according to the National Cancer Institute Common Terminology for Criteria Adverse Events (NCI CTCAE) version 3.0 (www.cancer.gov). PET-CT scans with 2-deoxy-2-[fluorine-18]fluoro- D-glucose (FDG) were performed at baseline and day 60. Response to therapy was assessed by PET-CT using PERCIST 1.0 criteria as described by Wahl et al.²². Complete (CMR) and partial metabolic response (PMR) were considered as metabolic response; stable disease (SMD) and progressive metabolic disease (PMD) were evaluated as metabolic non-response.

Sequential response profiling

Biopsies of lymph node or superficial skin metastases were performed before treatment (baseline), during treatment with sorafenib (day 10) and after treatment with dacarbazine (day 16). The biopsies within one patient were taken from the same tumor and were divided and either preserved in RNAlater (Ambion, Life Technologies Corp., Carlsbad, CA, USA) for transcriptional profiling or in formalin for immunohistochemistry.

Transcriptional response profiling and data analysis

Total RNA was extracted from tissue biopsies using Qiagen Mini-Tissue kit (Qiagen, Venlo, Netherlands). RNA quality and integrity was assessed using Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA, USA). SPIA whole-genome cDNA amplification and labeling was performed using Applause kit (NuGen, San Carlos, CA, USA). The amplified, fragmented and labeled cDNA was purified from the reaction mix with Qiagen's MinElute[®] Reaction Cleanup Kit. Five µg of labeled cDNA were used for hybridization onto Human GeneChip[®] Exon 1.0 ST microarray (Affymetrix, Santa Clara, CA, USA) at the Functional Genomics Center Zurich.

Microarray data were analyzed using Partek software as described by Mojica and Hawthorn.⁵⁶ Baseline, sorafenib (day 10) and sorafenib plus dacarbazine (day 16) treated samples were used for analysis. Briefly, after RMA summarization and normalization using core annotations, principle component analysis and analysis of variance is performed followed by the development of mixed ANOVA model, Benjamini-Hochberg multiple testing correction (P -value cut-off 7lt;0.005) and fold change filtering (cut-off $\geq \pm 2.5$). Analysis on exon level was performed as described previously.^{57,58} Subsequent response prediction analysis was done using support vector machine (SVM) module from Partek software.

Mining of functional gene associations was performed using Cytoscape, an open source application for visualization of gene interactions and pathway analysis (<http://www.cytoscape.org>).⁵⁹ For this purpose, ClueGO,⁶⁰ GeneMANIA⁶¹ and MCODE⁶² Cytoscape plug-ins were employed. Clustering analysis was performed with the Cluster program, and the display of the rearranged data was generated with the Tree View program (both programs are available through <http://rana.lbl.gov>).

Real-time quantitative PCR

Total RNA was isolated from biopsies using TRIzol reagent according to manufacturer's instructions (Invitrogen/Life technologies, Carlsbad, USA). One µg aliquots of RNA were reverse transcribed with Reverse Transcription System (Promega, Madison, USA) according to the manufacturer's instructions. Data collection and analysis were performed by ABI Viiia7 Fast Real-Time PCR Systems (Applied Biosystems/ Life technologies, Carlsbad, USA). The primers were purchased from Qiagen (Venlo, Netherlands): *OAS1* (Hs_OAS1_1_SG), *MX1* (Hs_MX1_1_SG), *HLA-DRA* (Hs_HLA-DRA_1_SG), *CXCL11* (Hs_CXCL11_2_SG), *CD3g* (Hs_CD3G_1_SG), and *VCAM1* (Hs_VCAM_1_SG). Gene expression values of averaged triplicate reactions were normalized to *RPL28* expression levels. The sequence of our *RPL28* primers was: 5'-GCAATTGGTTCCGCTACAAC-3' and 5'-TGTTCTTGCGGATCATGTGT-3'.

Immunohistochemistry

Paraffin-embedded blocks from biopsies were collected and used for validation. Immunohistochemical staining using alkaline-phosphatase-anti-alkaline-phosphatase technique (APAAP) was performed as previously described.¹⁸ The following

antibodies were used: anti-CD3 (Dako, Baar, Switzerland), anti-HLA II (Novocastra, Leica Microsystems GmbH, Wetzlar, Germany), anti-VCAM1 (Abcam, Cambridge, United Kingdom), anti-MxA (clone CL143, kind gift of Dr. Jovan Pavlovic, Institute of Medical Virology, University of Zurich, Zurich, Switzerland). Staining intensity was graded from 0 (no staining) to 4 (maximal staining).

Measurement of serum IFN α and IFN γ levels

Serum levels of IFN α and IFN γ in patient sera were analyzed by human IFN α and IFN γ ELISA sets (eBioscience, San Diego, USA) according to manufacturer's protocol.

Statistical analysis

Statistical analysis of the data other than exon arrays was performed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego California USA). Statistical analysis of PCR and ELISA data was done using 2-way ANOVA, for immunohistochemistry data using Mann-Whitney U Test. Comparison of Kaplan-Meier survival curves was performed using log-rank test. *P* values of less than 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

Statement of Significance

To our knowledge this is the first report to show differential modulation of systemic interferon production by tumor-specific and non-immunomodulatory therapies, such as sorafenib and dacarbazine.

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