Skin tumors induced by sorafenib; Paradoxical RAS-RAF pathway activation and oncogenic mutations of HRAS, TP53 and TGFBR1.

Running title: Molecular study of Sorafenib-induced skin tumors

Keywords: Sorafenib; Keratoacanthoma; Squamous cell carcinoma; BRAF inhibitor; Skin tumor, HRAS, TP53, TGFBR1 mutations

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Statement of Translational Relevance:

Skin tumors appearing during the course of RAF inhibitors-based therapy is an intriguing and potentially serious adverse effect of these new drugs. We performed a detailed clinical, pathological and molecular study of skin tumors in patients treated with sorafenib. In parallel, we studied the effect of sorafenib on normal keratinocytes in vivo in normal skin samples and in vitro. This translational study demonstrated that the RAF inhibitor sorafenib induces a time- and dose-dependent paradoxical activation of the MAP-kinase pathway in keratinocytes, leading to cell proliferation and associated with the emergence of benign (follicular cystic lesions), borderline (keratoacanthomas) and malignant (squamous cell carcinomas) tumors. Additional somatic events like oncogene mutations, possibly induced by UV rays exposure, might influence the evolution of benign lesions to malignant tumors. Understanding the mechanisms underlying this phenomenon is a first and mandatory step towards treatment and prevention of this worrisome side effect of our new targeted therapies.
Abstract:

Purpose: The emergence of skin tumors in patients treated with sorafenib or with more recent BRAF inhibitors is an intriguing and potentially serious event. We performed a clinical, pathological and molecular study of skin lesions occurring in patients receiving sorafenib.

Material and methods: 31 skin lesions from patients receiving sorafenib were characterized clinically and pathologically. DNA extracted from the lesions was screened for mutation hotspots of HRAS, NRAS, KiRAS, TP53, EGFR, BRAF, AKT1, PI3KCA, TGFBR1 and PTEN. Biological effect of sorafenib was studied in vivo in normal skin specimen and in vitro on cultured keratinocytes.

Results: We observed a continuous spectrum of lesions: from benign to more inflammatory and proliferative lesions, all seemingly initiated in the hair follicles. Eight oncogenic HRAS, TGFBR1 and TP53 mutations were found in 2 benign lesions, 3 keratoacanthomas (KA) and 3 KA-like squamous cell carcinoma (SCC). Six of them correspond to the typical UV-signature. Treatment with sorafenib led to an increased keratinocyte proliferation and a tendency towards increased MAPK pathway activation in normal skin.

Sorafenib induced BRAF-CRAF dimerization in cultured keratinocytes and activated CRAF with a dose-dependent effect on MAP-kinase pathway activation and on keratinocyte proliferation.

Conclusion: Sorafenib induces keratinocyte proliferation in vivo and a time and dose-dependent activation of the MAP-kinase pathway in vitro. It is associated with a spectrum of lesions ranging from benign follicular cystic lesions to KA-like SCC. Additional and potentially preexisting somatic genetic events, like UV-induced mutations, might influence the evolution of benign lesions to more proliferative and malignant tumors.
Introduction

BRAF is the most frequently mutated protein kinase in human cancer (1) and is the target of several anticancer drugs. The potency and the specificity of BRAF inhibitors available on the market or under clinical development are variable. Sorafenib (Nexavar®, Bayer/Onyx) is a Pan-RAF inhibitor which also blocks vascular endothelial growth factor receptors (VEGFR)-2, -3, platelet-derived growth factor receptor-β (PDGFR-β), fms-like tyrosine kinase 3 (FLT3), and KIT. Conversely, vemurafenib (Plexxikon/Roche) or GSK2118436 (GSK) are highly selective and very potent BRAF inhibitors. These latter drugs have a low IC₅₀ for mutated oncogenic BRAF, and are less active on wild type BRAF (wtBRAF) and other RAF kinases. The clinical activities of these inhibitors also differ from one another. Selective BRAF inhibitors like vemurafenib and GSK2118436 are efficient against tumors harboring BRAF mutations and dependent on the RAF/MEK/ERK pathway. Melanoma with V600E BRAF mutation is a case in point (2). Sorafenib activity is not linked to the presence of a BRAF mutation in the tumor and is more likely due to its antiangiogenic effect. Sorafenib is efficient and has been approved for the treatment of metastatic renal carcinoma (3)(4) and advanced hepatocellular carcinoma (4) independently of the mutational status of BRAF.

In spite of their variability in terms of BRAF selectivity and clinical activity, all three RAF inhibitors are associated with one and the same intriguing cutaneous side effect which is the emergence of borderline squamous cell neoplasms. Indeed, we and others have reported single or multiple cutaneous keratoacanthomas (KA) and squamous cell carcinomas (SCC) during treatment with sorafenib(5–9). These paradoxical keratinocyte proliferations arise in 6-7% of patients (5; 10). The induction of KA and SCC is even more frequent with vemurafenib
and GSK2118436, and has been described in 15 to 25% of the patients (2; 10).

We undertook a translational research in the aim of understanding the mechanisms of these skin cancer inductions. We first performed a detailed clinical, pathological and molecular study of the skin neoplasms arising in patients treated with sorafenib. We then looked at the biological effects of sorafenib in vivo on normal skin samples from patients treated with sorafenib or a placebo. In parallel, we analyzed the effect of sorafenib in vitro on cultured keratinocytes. We found that sorafenib exposure was associated with increased MAPK pathway activation and keratinocyte proliferation ex vivo and in vivo. Sorafenib induced dose-dependent BRAF/CRAF dimerization associated with strong activation of CRAF in cultured keratinocytes. Finally, oncogenic mutations in HRAS TGFBR1 and TP53 were detected in several benign or malignant skin tumors. Altogether, our results suggest that sorafenib induces a continuous spectrum of keratinocyte changes and proliferation via the paradoxical activation of the MAPK pathway in keratinocytes associated with BRAF/CRAF heterodimerization and subsequent CRAF activation. Additional events such as a HRAS mutation or EGFR activation giving rise to MAPK pathway co-activation might be required for full transformation of keratinocytes.

**Material and methods**

**Patients and skin samples:**

Thirty-one skin lesions diagnosed as follicular cystic skin lesions, perforating folliculitis, KA or SCC originating from 17 patients treated with sorafenib from 2005 to 2010 were studied.

For each patient, the tumor type, duration of sorafenib therapy before the emergence of the skin tumor and the clinical description of the skin lesions are reported. Four μm sections from formalin-fixed, paraffin-embedded biopsy samples of skin lesions were stained with hematoxylin and eosin and independently read by two pathologists.
Normal skin samples from patients included in the TARGET study, evaluating sorafenib versus a placebo in patients with renal cell cancer were also studied (3). Normal skin biopsy samples were collected from the forearms of patients who received sorafenib or a placebo for 6 weeks.

Each skin sample was cut into 4-μm sections, formalin-fixed, paraffin-embedded and stained with hematoxylin and eosin. A section from each case was analyzed immunochemically for Ki67 (monoclonal MIB-1, DAKO, Carpintera, CA) and phospho-ERK protein (monoclonal 20G11, Cell signaling technology, Boston, MA). Slides were deparaffinized and treated with an alcohol gradient to absolute ethanol. Endogenous peroxidase activity was blocked with the Peroxydase blocking solution (DAKO, S2001, Carpintera, CA). Antigen retrieval was achieved with Target Retrieval Solution (DAKO). Primary antibody was applied at 1:100 dilution and incubated at 4°C for 1 hour. Anti-mouse IgG with the DAKO Envision system (horseradish peroxidase) was employed according to the manufacturer’s instructions.
DNA isolation and mutation detection on biopsy specimens of skin lesions:

Genomic DNA was extracted from 10 to 20-μm-thick paraffin-embedded unstained slides of each skin lesion using the DNeasy Blood and tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. We analyzed full coding sequences of 70 exons including oncogenic mutational hotspots corresponding to AKT1 (NM_005163.2) exon 4, BRAF (NM_004333.4) exons 11 & 15, EGFR (NM_005228.3) exons 18 to 21, HRAS (NM_005343.2) exons 2 & 3, KRAS (NM_033360.2) exons 2 & 3, NRAS (NM_002524.3) exon 2 & 3, PIK3CA (NM_006218.2) exons 10 & 21, PTEN (NM_000314.4) exons 1 to 9, TP53 (NM_000546.4) exons 2 & 11, and TGFBR1 (NM_004612.2) exons 2, 4, 6 and 7.

Sequences were analyzed by Sanger direct sequencing performed after Polymerase Chain Reaction (PCR) amplification of target exons. Sequencing reactions were carried out using the BigDye® Terminator Cycle Sequencing Kit software according to the manufacturer’s recommendations (Applied Biosystems, Forster City, CA). The primer sequences are available upon request. Sequencing reactions were analyzed on a 48-capillary 3730 DNA Analyzer®. Sequence reading and alignment were performed with SeqScape® software (Applied Biosystems). All detected mutations were confirmed following at least one independent PCR reaction.

In vitro experiment using HaCat keratinocytes

Cell culture: HaCat keratinocytes were cultured in DMEM (Invitrogen) containing 10% fetal calf serum (FCS; Perbio) and antibiotics (penicillin/streptomycin; Invitrogen). Cellular proliferation was evaluated using the CellTiter assay (Promega). Sorafenib and PD153035 used for cell treatment was purchased from Alexis Biochemicals and dissolved in DMSO.

Protein expression: Cells were lysed in 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% NP40, 5 mM NaF, 2 mM Na3VO4, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. For immunoprecipitation, samples were incubated with 1 μg BRAF (clones F-7 or H145, Santa
Cruz) or CRAF (clone C12, Santa Cruz or clone 53/c-Raf-1, BD Transduction Laboratories) antibodies for one hour and immunoprecipitated by protein G-Sepharose (GE Healthcare) for one hour at 4°C. The immune complexes were washed three times with lysis buffer, proteins were separated by SDS-PAGE and Western blot analysis was performed according to standard protocols using the following antibodies: BRAF (F7 or H145); CRAF (C12 or 53/c-Raf-1); phospho-ERK (MAPK-YT; Sigma) and ERK (Upstate/Millipore); MCL1 (Abcam), EGFR and phospho-EGFR (Cell Signaling) and Actin (Abcam).

**Kinase Assay**: CRAF was immunoprecipitated from cellular extract and its activity toward MEK measured *in vitro*. Briefly, immunoprecipitated CRAF was incubated with 5 μg of purified GST-MEK in 30mM Tris (pH 7.5), 100μM EDTA, 10mM MgCl₂, 0.1% Triton 100, 5mM NaF, 1mM ATP, 0.3% β-mercaptoethanol for 30 min at 30°C. The reaction was stopped by adding Laemmli buffer and analyzed by SDS-PAGE with the phospho-MEK (Cell Signaling Technology) and CRAF antibody. Phosphorylated MEK and CRAF were visualized and quantified by fluorescence on an Odyssey imaging system.

**Proximity Ligation Assay (PLA)**: HaCat cells were grown on glass cover slips, treated with DMSO or sorafenib, fixed with methanol and permeabilized with acetone. The PLA was performed according to the manufacturer’s instructions (Olink) using B-Raf (F7) and C-Raf (C12) antibodies.

**RNA interference**: HaCat cells were transfected with a siRNA control (SCR) or targeting BRAF or CRAF as previously described (11).

**Statistics**: Simple univariate descriptive statistics were used to compare the population, using the t-test with a 5% significance level.
Results

Clinical and pathological characterization of skin lesions:

The gender, age, cancer type, daily dose of sorafenib, time interval between the start of sorafenib therapy and the first skin symptom, the number and types of lesions as well as the skin sites involved are detailed in table 1.

Various clinical aspects were observed but all patients developed small cystic follicular lesions that most frequently affected the face and also sometimes other parts of the skin such as the buttocks, axilla or inguinal areas (table 1). They occurred 3 to 5 months after the initiation of sorafenib. Cysts on the face were dominantly non inflammatory millimetric small and white lesions. Inflammatory cystic lesions exhibited erythematous hyperkeratotic papules, with a central, cone-shaped plug.

Lesions clinically suggestive of perforating folliculitis, meaning a more inflammatory infiltrate with a keratotic appearance occurred mostly on the buttocks and the upper part of the limbs (patients # 7, 11, 12, 13 and 14).

Patients 1 to 11 developed at least one lesion characteristic of KA or of KA-like SCC. Clinically, they were fast-growing firm round nodules with a central crateriform keratotic zone. Three of these patients had multiple skin lesions (patients # 4, 6, 7) occurring 1 to 9 months after the initiation of sorafenib.

A total of 22 lesions were excised and the pathological examination using H&E staining revealed a spectrum of lesions (Figure.1): i) non inflammatory cystic hair follicles, ii) cystic folliculitis, and perforating folliculitis (PF) with rupture of the hair follicle wall and perifollicular infiltrates, iii) KA defined as a crateriform epidermal neoplasm limited by sharp
beaks with no cytological atypia nor invasion, iii) crateriform KA-like SCC defined as crateriform epidermal neoplasms with irregular budding borders, cytological atypia and invasive nests (figure 1). Lymphatic metastatic embols were seen in one SCC (patient #8).

Patient #7 developed the entire spectrum of the skin lesions that were observed: non inflammatory facial cysts, cystic folliculitis, perforating folliculitis, multiple KA and SCC that emerged respectively 3 to 5 months after the initiation of sorafenib. Sorafenib was stopped due to cutaneous intolerance at the request of the patient after 24 months even though it was still effective against the progression of the renal cell carcinoma. All of her skin lesions, including skin tumors disappeared over a 4-month period following sorafenib interruption.

Oncogenic mutations

Eight oncogenic mutations were found in benign and malignant tumors. Three HRAS mutations on codons 12 or 13 were found in three lesions: one KA, one KA-like SCC and one case of benign perforating folliculitis (table 1). TP53 was also found to be mutated in 3 lesions: one KA, one in-situ SCC and 2(?) case of perforating folliculitis (exons 8 & 5). Moreover two Missense mutations of TGBR1 were observed in one KA (c.1120G>A ; p.Gly374Arg) and in one in-situ SCC (c.248C>T ; Pro83Leu) (table 1). Those TGBR1 mutations are somatic mutations because they were not present in other samples for the same patient. No additional mutation of the screened exons of BRAF, NRAS, KRAS, AKT1, PI3KCA, PTEN, nor EGFR genes was found in any of the other malignant or benign lesions. Six of these mutations are known to be typically UV-induced (Table 1). (13, 14)

Normal skin biopsy specimens :

The results of the immunostaining of normal skin samples with Ki67 and pERK for patients treated with sorafenib or a placebo are shown in figure 2.

Ki67 staining was found in the nucleus of a few scattered basal cells in non treated patients while it was found in the nucleus of 1 to 2 whole layers or multiple heaps of basal cells in
patients treated with sorafenib (figure 2 A). Ki-67 stained cells were counted by two independent pathologists in three representative fields of each specimen. (15) There was a significant difference between the numbers of Ki67-positive keratinocytes in the two populations of patients with more proliferating cells in the sorafenib treated group than in the placebo treated patients (Rank test , p= 0.03). (Figure2 B).

We also found a tendency for increased phosphorylation of ERK in normal skin of sorafenib-treated patients compared to normal skin of placebo-treated patients. A strong positive p-ERK staining was found in 7 out of 11 normal skin samples from sorafenib-treated patients (64%) whereas only 2 out of 8 samples from placebo-treated patients (25%) showed positive, albeit weak and focal p-ERK staining (figure 2 C & D) (p= 0.1 Fisher exact test). The lack of statistical significance might be due here to the small number of specimen.

**In vitro results on HaCat keratinocytes**

We first used sequencing to verify that HaCat cells did not harbor an oncogenic mutation in any of the RAS genes (HRAS, KRAS and NRAS) (data not shown).

HaCat cells were treated with increasing amounts of sorafenib and BRAF-CRAF heterodimerization was evaluated using co-immunoprecipitation experiments. We did not detect any heterodimers in DMSO-treated cells whereas sorafenib induced BRAF-CRAF heterodimers in a dose-dependent manner in keratinocytes (Figure 3 A). As BRAF-CRAF heterodimerization was previously shown to activate CRAF, we measured CRAF kinase activity in keratinocytes treated with increasing doses of sorafenib. Of note, sorafenib is a reversible inhibitor and therefore was no longer present during the *in vitro* kinase assay.

Treatment of HaCat cells with sorafenib stimulated CRAF activity in a dose-dependent manner (figure 3 B), which paralleled the dimerization of BRAF with CRAF (Figure 3 A), confirming that dimerization activates CRAF in keratinocytes. To analyze the biological effect of sorafenib-induced CRAF activation on the MAPK pathway, we measured ERK phosphorylation in response to sorafenib in a time course experiment. Sorafenib transiently
activated ERK at 1, 3 and 10 μM, attaining a maximum after 15 minutes (Figure 4 A). A more pronounced effect was observed with the lower doses of Sorafenib. To analyze the biological consequence of ERK activation, we measured the proliferation rate of HaCat cells treated with increasing doses of sorafenib. Cellular proliferation was increased with the low dose (1 μM) of sorafenib. Proliferation was inhibited with higher doses of sorafenib (3 and 10 μM). (Figure 4 B).

To determine whether this dual effect on cell proliferation could be explained by the action of sorafenib on the anti-apoptotic molecule MCL1, MCL1 expression was studied as a function of sorafenib concentration. We found that MCL1 expression decreased with higher sorafenib concentrations, (3 and 10 μM), whereas 1 μM of sorafenib did not influence MCL1 expression (figure 4 C). To test whether sorafenib-induced heterodimerization could be detected in situ, we used a Proximity Ligation Assay (PLA). In this assay, a pair of oligonucleotide-labeled secondary antibodies (PLA probes) generates a signal only when they have bound in close proximity, thus allowing the detection of protein-protein interaction in situ. HaCat cells were treated or not with sorafenib and fixed. BRAF-CRAF heterodimerization was visualized by PLA using a combination of a CRAF and a BRAF antibody. Figure 5 shows that sorafenib-induced BRAF-CRAF heterodimerization can be directly detected in keratinocytes.

RNA interference confirmed the specificity of the PLA signal as inhibition of BRAF or CRAF expression with siRNA strongly diminished the PLA signal induced by sorafenib (Supplementary Figure 1).
DISCUSSION

Targeted anticancer agents are associated with frequent and diverse cutaneous side effects. The description and study of these skin manifestations is of critical importance. Indeed, they frequently have an adverse impact on the patient’s quality of life and understanding their underlying mechanisms is certainly the best way to treat these skin manifestations in addition to offering new perspectives on skin biology (5; 6; 12–14).

Our earlier observations of skin manifestations induced by targeted agents had lead us to formulate the hypothesis that sorafenib-induced KA and skin SCC were due to its effect on RAF kinases. Indeed, these side effects were not seen after blockade of VEGFR, KIT, FLT3 or PDGFR, the other sorafenib targets (5; 12). Our hypothesis was further corroborated by the description of KA and SCC occurring with more specific BRAF inhibitors: vemurafenib and GSK2118436 (2; 15).

In the present work, we present a series of 31 skin lesions occurring in 17 patients treated with sorafenib 1 to 9 months after the beginning of the therapy. The lesions were benign and borderline or malignant skin tumors which might seem paradoxical since they occurred while under therapy with an anti-proliferative agent. The entity KA as a distinct tumor at the crossroad between benign and malignant tumors has been debated for decades. KA is a rapidly growing keratotic and crateriform tumor, very similar clinically and pathologically to well differentiated SCC (16). It is characterized by frequent spontaneous resolution and originates from hair follicles (17). For some authors, it is considered to be low grade SCC. (18) KAs can be observed sporadically, mostly on sun exposed areas, or in the context of genetic diseases, like for example the Multiple self-healing...
squamous epithelioma (MSSE) also called Ferguson Smith disease (19). Our pathological classification between KA and SCC depended on the existence of cytological atypia, invasive nests or lymphatic metastatic embols. When any of these sign were seen, we classified the crateriform epidermal neoplasms limited by sharp beaks as KA-like SCCs. When neither atypia, nor invasion was associated with the crateriform lesion, they were considered as KAs.

The first hypothesis that we put forward following our clinical and pathological studies of these tumors was that they seemed to belong to a common spectrum of lesions with the possibility of transition from benign lesions, i.e. from a cystic more or less inflammatory lesion to KA and SCC. This “continuity” hypothesis is based on several observations. First, several of our patients had associated benign and malignant tumors. All of them had several benign cystic lesions, in addition to the lesions that were biopsied and studied (table 1). One patient, in particular developed the complete spectrum of lesions, i.e. follicular cysts, perforating folliculitis, KAs and KA-like SCC (patient 7). Furthermore, all SCCs were crateriform in shape, resembling KA. We did not observe other clinical subtypes of SCC such as Bowen disease or classic sun-induced, ulcerated or nodular forms of SCC.

Second, all of these lesions seemed to have developed at the expense of or around hair follicles. Follicular dystrophic lesions and perforating folliculitis are obviously follicular lesions and relics of hair follicles were indeed observed in every biopsy sample. Follicular ontogeny for KA is also well documented (20). The microscopically lamellar structure as well as the type of cytokeratins found in KA suggest that they are hair follicle-derived tumors(21). Thus, our first hypothesis is that all these proliferative epidermal lesions could represent various steps of a same generic process initiated by RAF inhibition in the skin and particularly targeting follicular keratinocytes.
To explore the biological effect of sorafenib on normal skin in vivo, we studied normal skin samples of patients treated with sorafenib or a placebo. Our results showed a significant increase in keratinocyte proliferation, and no decrease of ERK phosphorylation, but, on the contrary, a clear tendency to an increase of ERK phosphorylation levels in the skin after treatment with sorafenib. We now have preliminary data on normal skin samples from patients treated with vemurafenib (before treatment and at day 21) showing similar results (data not shown).

These results, which at first glance might seem highly improbable, are in fact in line with recent published data showing that BRAF inhibitors induce activation instead of inhibition of the RAF/MEK/ERK pathway in wt BRAF cells (22–24). The proposed mechanisms underlying the activation of the pathway by a RAF inhibitor imply dimerization of RAF proteins, and homo- (BRAF-BRAF) or hetero- (BRAF-CRAF) dimerization. Binding of the RAF inhibitor enhances signaling via CRAF engaged in dimerization with BRAF (23). In a second model, binding of the inhibitor induces transactivation of the second element of the homo- or heterodimer (22). In both models, this activation requires wt BRAF together with an additional activation signal of the pathway, as is the case with RAS or EGFR activation. To understand whether this effect could explain keratinocyte proliferation in response to sorafenib, we used HaCat keratinocytes to see if we could reproduce these results in an in vitro system. We showed that sorafenib indeed regulates the activation of the MAPK pathway and cellular proliferation in keratinocytes in a dose- and time-dependent manner with an increase of both parameters when the dose of sorafenib was low (1 μM). For higher sorafenib concentrations (3 and 10 μM) the effect on ERK phosphorylation was transient and disappeared after one hour, and the effect on proliferation was rather inhibitory. This is in line with the transactivation model (22) where RAF inhibitors activate ERK signaling at low concentrations but inhibit it at higher concentrations in wt BRAF cells. The decreased effect
of higher concentrations of sorafenib on ERK phosphorylation could be explained by the direct binding and inhibiting activity of the drug on CRAF. As sorafenib has been previously shown to regulate the expression of the anti-apoptotic MCL1 molecule (25; 26), we explored the effect of various doses of sorafenib on MCL1 expression. We found that the regulation of MCL1 expression was dependent on sorafenib concentration with inhibited MCL1 expression at higher drug doses. This effect of high doses (3 and 10 μM) of sorafenib on MCL1 expression could explain that, although sorafenib activated ERK at all doses tested, it inhibits cell proliferation at higher doses. Thus, the complex action of sorafenib on the activation of the MAPK kinase pathway and on cell proliferation is dose-dependent and results from the combined effects of the drug on RAF kinase inhibition on the one hand and on the control of apoptosis via MCL1 regulation on the other hand. We were able to detect the formation of BRAF-CRAF dimers in vitro by co-immunoprecipitation and directly in cells by PLA for all sorafenib concentrations tested. This dimerization-induced CRAF activation in keratinocytes can explain MAPK activation at least at a low sorafenib concentration which might be the case in the skin. Indeed, the plasma sorafenib concentration in clinical therapeutic use ranges from 3 to 6 mg/l (6.5 to 13 μM) but the drug concentration in the skin is probably much lower than in plasma due to high protein binding(27). In most experimental cell lines models used, RAS activation seems required for RAF dimerization. We show here that RAS is not mutated in the HaCat keratinocytes and is rarely mutated in our biopsy specimen, however, it is possible that RAS is activated by upstream signaling like EGFR activation, which is very frequently observed in the skin. Indeed we showed that when EGFR activity was inhibited with PD153035 in HaCat cells, sorafenib could not induce RAF heterodimerization anymore (Supplementary Figure 2). This result shows that activation of the pathway upstream of RAF, presumably including RAS activation, is necessary for sorafenib-induced RAF dimerization in keratinocytes. Our data are therefore consistent with
models from the literature stating that drug-induced RAF dimerization requires RAS activation either through oncogenic mutation or through RTK activation. Thus, the model we propose is as follows: BRAF wild type skin keratinocytes submitted to low sorafenib concentrations activate the MAPkinase pathway via BRAF-CRAF dimerization sparking off CRAF activation which in turn leads to keratinocyte proliferation. Additional events could then precipitate the proliferation and transformation process. In accordance with this model, we found oncogenic mutations (3 HRAS and 3 TP53 mutations and 2 TGFBR1) in benign and malignant skin lesions. Sporadic KA and SC, beside the use of RAF inhibitors are known to harbor oncogenic RAS and TP53 mutations. (30–34).RAS mutations are reported in 30% of KA (HRAS) and in 12 to 46% of SCC (mostly HRAS) (30–32; 34). Furthermore, TGFBR1 mutations similar to the ones we found in our patients tumors have recently been shown to be germinal mutations directly involved in an autosomal genodermatosis characterized by the occurrence of multiple KAs, the Multiple self-healing squamous epithelioma (MSSE) also known as Ferguson Smith disease (19). Patient 10 had two lesions screened for mutations, one KA-like SCC with a TGFBR1 mutation (Pro83Leu) and benign cystic folliculitis that did not harbor any mutation, meaning that the TGFBR1 mutation was somatic and not germinal (Table 1).

The lesions presented by patients with MSSE are clinically and pathologically very similar to lesions presented in the context of RAF inhibitor treatment suggesting an effect of these drugs on the TGFB/TGFBR signaling pathway that warrants further exploration.

Six of the mutations found in our samples are known to be potentially UV-induced (patients #1, 3, 4, 6, 10 and 14), either located on typical UV sensitive dipyrimidine sites (patients #1, 3, 6, 10, 14) or on an ACA sequence known to give rise to a mutagenic photoproduct (patient #4) (35; 36). Oncogenic events such as TP53 mutations are found in normal skin keratinocytes on sun-exposed areas (37). Indeed, a recent paper demonstrated that intermittently sun-exposed skin contained an extensive number of intact cells with TP53...
mutations, with an estimated annual rate of 35,000 new persistent TP53 mutations per individual(38). In accordance with this hypothesis, the six lesions that were harboring mutations with UV-signature were located on sun-exposed areas, whereas, the two remaining mutations (patients #7 and 12) were found on non-exposed skin areas (palm and buttocks), (Table 1). Thus, sorafenib-induced skin tumor might result from the combined effect of paradoxical MAPkinase pathway activation and co-existing or even pre-existing oncogenic mutations that could have been generated by UV rays action on normal skin.

Altogether, the fact that we found oncogenic TP53, TGFBR1 and RAS mutations with an UV signature suggests that it is a probably wise move to advise patients receiving sorafenib or other RAF inhibitors to use efficient photoprotective measures.

The fact that we did not find oncogenic mutations in the other malignant lesions could be due to the existence of other mutations that we did not look for or by alternate ways of activating the MAPKinase pathway such as EGFR activation. Indeed, EGFR can also be activated in the skin by various stimuli such as physiologic sun exposure or wound healing.(28; 29)and its activation is sufficient to promote RAF dimerization in keratinocytes treated with sorafenib (Supplementary Figure 2).

It is possible that sun-induced RAF or TP53 mutation or EGFR activation were present in the keratinocyte prior to BRAF inhibition, and could have led to keratinocyte transformation in the presence of sorafenib. In some of our samples, a mutational event existing prior to malignant transformation is supported by the fact that we found oncogenic mutations of HRAS and TP53 in two benign cystic lesions.

Recently, oncogenic RAS mutations, (mostly HRAS) were found at a higher frequency in 71% of 21 KA and SCC induced by vemurafenib, suggesting that RAS involvement might be more preeminent than other oncogenes in the ontogeny of vemurafenib-induced tumors.(39). In
**conclusion,** we propose a model in which paradoxical ERK activation by RAF inhibitors in the skin induces changes in keratinocyte biology with activation of the MAK pathway and cell proliferation associated with a spectrum of skin lesions ranging from more or less inflammatory benign follicular cystic lesions to KA-like SCC. Additional and maybe preexisting somatic genetic events might influence the evolution of the benign lesions to more proliferative and malignant tumors. The fact that these skin lesions occur more frequently with more potent and specific BRAF inhibitors than with the pan-RAF inhibitor sorafenib could result from the differential effect of these drugs on CRAF or from various bioavailabilities of the drugs in the skin. These findings have other important clinical implications. Indeed, they also suggest that the use of RAF inhibitors might be pointless and even deleterious in normal tissues especially in cells harboring a RAS mutation, as it can be found in lung and colon tissue for example (40; 41). Indeed, we cannot exclude the hypothesis that RAF inhibitors could promote cell transformation and/or proliferation in these wt BRAF cells with RAS activation and lead to internal tumors. Fortunately, such events have not been reported so far.

Altogether, the effect of RAF inhibitors in the skin illustrates the complexity and the variety of the consequences of the systemic blockade of an universal pathway like the MAPK pathway and reminds us that efficient targeted therapies always have off-target effects that need to be very carefully evaluated and monitored.
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References


Figure Legends: Table 1 : Patients and cutaneous lesions characteristics

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Abbreviations : KA, keratoacanthoma ; SCC, Squamous cell carcinoma ; PF, perforating folliculitis ; WT, wild type ; NT, not tested.

Delay (month): Interval in months between the initiation of sorafenib and the emergence of the first skin lesion

Details of the mutated sequences:

- **TP53-R181H** for mutation in exon 5 of TP53 (NM_000546.6) encoded c.542G>A; p.Arg181His.
- **TP53-C277F** for mutation in exon 8 of TP53 (NM_000546.6) encoded c.830G>T; p.Cys277Phe.
- **TP53-G293R** for mutation in exon 8 of TP53 (NM_000546.6) encoded c.877G>A; p.Gly293Arg.
- **HRAS-G13V** for mutation in exon 2 of HRAS (NM_005343.2) encoded c.38G>T; p.Gly13Val.
- **HRAS-G13R** for mutation in exon 2 of HRAS (NM_005343.2) encoded c.37G>C; p.Gly13Arg.
- **HRAS-G12S+G12D** for double mutation in exon 2 of HRAS (NM_005343.2) encoded c.34G>A and c.35G>A; p.Gly12Ser and p.Gly12ASP.
- **TGFBR1-P83L** for mutation in exon 2 of TGFBR1 (NM_004612.2) encoded c.248C>T; p.Pro83Leu.
- **TGFBR1-G374R** for a mutation in exon 6 of TGFBR1 (NM_004612.2) encoded c.1120G>A; p.Gly374Arg.

Figure 1:
Clinical and pathological spectrum of skin lesions.

Non-inflammatory cystic follicular lesions (A and E); inflamed follicular cysts: folliculitis with onset of perforation (B and F); perforating folliculitis (C and G), keratoacanthoma (D and H).

Figure 2: Normal skin biopsies of patients treated with sorafenib or placebo

A: Ki67 Immunostaining on normal skin specimen from a placebo treated patient (left) and a sorafenib treated patient(right)

B: Dot graph showing the mean numbers of Ki67 positive keratinocyte nuclei in 3 representative fields of normal skin samples in placebo and sorafenib-treated patients

C: Numbers and ratios of normal skin specimen expressing p-ERK in the epidermis among the patients treated with placebo or sorafenib for renal cell cancer

D: Example of immunostaining results in a placebo treated patient (left) and a sorafenib treated patient (right)

Figure 3: Sorafenib induces BRAF/CRAF dimerization and CRAF activation in HaCat keratinocytes.

HaCat were treated with DMSO (0) and increasing doses of sorafenib (1, 3 or 10 μM) for 4 hrs and lysed. (A) CRAF was immunoprecipitated and probed for BRAF and CRAF. Quantification is shown below the blot. (B) CRAF was immunoprecipitated and its kinase activity against MEK measured in vitro. Similar results were obtained in three independent experiments.

Figure 4: Effect of sorafenib on ERK phosphorylation, cell proliferation and MCL1 expression in HaCat keratinocytes

(A) HaCat were treated with DMSO (0) or with increasing doses of sorafenib (1, 3 or 10 μM) for the indicated time and lysed. Lysates were probed for phosphorylated ERK (ppERK) and total ERK. The ratio ppERK/ERK is shown on the graph which represents the mean +/- SD of three independent experiments. (B) Proliferation of HaCat treated with DMSO (0) an increasing dose of sorafenib for 3 days was measured using the CellTiter assay. The graph represents the mean of two experiments in
triplicate. (C) Lysates from A were probed for MCL1 and actin. The ratio MCL1/actin was calculated for the indicated dose of sorafenib after 6h or 24h of treatment. Graphs represent the mean +/- SD of two experiments.

**Figure 5 : Proximity ligation assay showing BRAF/CRAF dimerization induced by sorafenib in keratinocytes in vitro.**

Sorafenib induces HaCat grown on coverslip where treated with DMSO, 1 μM or 10 μM of sorafenib for 4 hrs and fixed. BRAF-CRAF heterodimerization was visualized as red dots using a Proximity Ligation Assay and detected with a fluorescent microscope, cell nuclei were stained with DAPI. Each dot represent one BRAF-CRAF complex.
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**Table 1**: Patients and cutaneous lesions characteristics

UV signature

...non UV-induced

PF: Perforating folliculitis; KA: Keratoacanthoma; SCC: Squamous cell carcinoma
Figure 1:

Clinical and pathological spectrum of skin lesions.

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D: Example of immunostaining results in a placebo treated patient (left) and a sorafenib treated patient (right)
A

ppERK/ERK ratio (arbitrary units)

Time (h)

1 μM Sorafenib
3 μM Sorafenib
10 μM Sorafenib

B

Cellular proliferation (arbitrary units)

0 1μM 3μM 10μM

C

Mcl1/Actin ratio (arbitrary units)

0 1μM 3μM 10μM

6 hrs 24 hrs
# Clinical Cancer Research

## Skin tumors induced by sorafenib; Paradoxical RAS-RAF pathway activation and oncogenic mutations of HRAS, TP53 and TGFBR1

Jean-Philippe Arnault, Christine Mateus, Bernard Escudier, et al.

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