Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Su F, Viros A, Milagre C, et al. RAS mutations in cutaneous squamous-cell carcinomas in patients treated with BRAF inhibitors. N Engl J Med 2012;366:207-15.

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Supplemental Information for: Su, Viros, Alegre *et al*. RAS mutations in cutaneous squamous cell carcinomas with BRAF inhibitors

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Supplemental Figure Legends

Supplemental Figure 1. Additional examples of cutaneous squamous cell carcinomas or keratoacanthomas (cuSCC/KA) in patients treated with vemurafenib. A and B) Clinical and pathologic presentations and corresponding RAS status (additional details in Supplemental Table 3b; magnification, 1.25X; bar, 100 μM) from two cases in the validation set (A: patient 3, B: patient 10). C and D) Examples of Sanger sequencing of mutations in *HRAS* and *KRAS*: C) Sequencing tracings of a left temple SCC/KA from patient 4 from the initial set demonstrating a $HRAS^{Q61L}$ mutation, but wild type *KRAS*; D) Sequencing tracings of a right cheek SCC/KA from the same patient 4 demonstrating a $KRAS^{G12C}$ mutation, but wild type *HRAS*.

Supplemental Figure 2. Examples of pERK staining and sequencing for *RAS* mutations in cuSCC/KAs and surrounding unaffected skin. A) Lesion from patient 1 of the initial series, a KA from the arm with a $HRAS^{Q61K}$ mutation in a patient without a prior history of cuSCC/KAs. The basal cell layer shows strong pERK staining in cuSCC, with pERK staining present in normal epithelia, but to a lower extent. B) Lesion from patient 7 from the initial series, a cuSCC from the arm in a patient with prior history of other cuSCCs. The lesion demonstrated a $TP53^{P278S}$ mutation and no mutation in *RAS*. This case demonstrates low pERK staining, but it is present in the more differentiated tumor cells. ERK phosphorylation is present in normal epithelia, but to a lower extent, and there is pigmentation in the basal cell layer. C and D) Sequencing traces at the $KRAS^{G12}$ codon demonstrating the presence of a mutation in the cuSCC/KA lesions but not in the normal skin surrounding the lesions.

Supplemental Figure 3. Differential gene expression profiling of murine SCC cells exposed to BRAF inhibitors. A) Comparison of differentially expressed genes in murine B9 cells following overnight exposure to PLX4720 and vemurafenib (in replicates, labeled A and B respectively). Red indicates higher and green indicates lower expression compared to

untreated cells. B) PCR confirmation of differentially expressed genes identified in the gene microarray studies. B9 cells and $BRAF^{V600E}$–expressing melanoma cell lines, A375 and SK-MEL-28 were treated with 0.5 μM of vemurafenib or 1 μM of PLX4720 respectively for 6 or 16 hours, total RNA was extracted then RT-PCR was performed (Applied Biosystems). Each time point and concentration has triplicate samples. Blue bars represent fold changes of genes in the B9 mouse cuSCC cells and red bars represent average fold changes of genes in A375 and SK-MEL-28 human melanoma cell lines.

Supplemental Figure 4. Studies of vemurafenib-induced proliferation and signal transduction in A431 human SCC cells with or without oncogenic $HRAS^{Q61L}$. A and B) A431 cells transfected with $HRAS^{Q61L}$ or a control plasmid were analyzed using the MTT assay (Sigma) for proliferation (A) or by plotting the number of viable cells (B). C) A431 cells stably transduced with a lentiviral vector expressing $HRAS^{Q61L}$ or a control empty lentiviral vector (wt) were treated with increasing doses of vemurafenib for 3 days and analyzed by an MTS-based cell viability assay. Values on the y-axis are the relative proliferation of cells in comparison to vehicle (DMSO) treated cells. Comparisons of replicate samples were statistically significant (all $p < 0.05$) between the vemurafenib concentrations of 0.0005, 0.05 and 2.5 μM (two-way ANOVA with Bonferroni posttest analysis).

Supplemental Figure 5. Additional effects of vemurafenib on signaling and colony growth of $HRAS^{Q61L}$ mutant cells. A) A431 cells stably transduced with a lentiviral vector expressing $HRAS^{Q61L}$ or a control empty lentiviral vector (wt) were treated with vehicle (DMSO) control, 1 or 5 μM vemurafenib for 1 hour or 24 hours. Cells were lysed in cold RIPA buffer with a cocktail of protease/phosphatase inhibitors, and protein concentration was measured by BCA assay (Thermo Scientific, Rockford, IL). 8 μg of total protein was loaded in each well for SDS-PAGE. Total and phosphorylated kinases were assessed by immunoblotting. B) NIH3T3 cells transfected with a control vector or with mutated $HRAS^{Q61L}$ were cultured in increasing concentrations of vemurafenib for soft agar proliferation assay.

Supplemental Figure 6. BRAF inhibition accelerates growth of non-melanoma skin tumors in mice. A) Representative photomicrographs of exophytic papillomas and sharply demarcated exophytic cuSCC/KAs (left and centre panels; scale bars 1mm), with their corresponding dermal invasive fronts (right panels; scale bars 50 um). Arrows indicate deep dermal mitoses. B) Sanger sequencing profiles of representative cuSCC/KAs lesions from mice treated with DMBA/TPA and DMBA/TPA/PLX4720. The position of the CTA codon encoding the *HRAS*^{Q61L} mutation is indicated by the arrow.

Supplemental Figure 7. PLX4720 induces paradoxical activation of the MEK/ERK pathway and stimulates proliferation of *HRAS*^{Q61L}-transformed keratinocytes. A) Western blots for BRAF, CRAF, phosphorylated MEK (pMEK), phosphorylated ERK (pERK) and total ERK2 (loading control) in whole cell lysates from PDV cells treated with DMSO, PD184352 (PD; 2 μM, 60 min) and/or PLX4720 (PLX; 300 nM, 60 min). B) Graph showing proliferation in PDV cells treated with DMSO (control) or PLX4720 (PLX; 300 nM, 96 hours). Error bars represent standard deviation from the mean. C) Graph showing the induction of palpable lesions on the skin of DMBA, TPA and PLX4720-treated mice. Mice in one of the cohorts (represented by the green line) were additionally treated with PD184352 (+PD) after 47 days (indicated by the arrow).

Supplemental Online Methods

cuSCC/KA initial sample collection, processing and analysis

Tissues included in the original and validation sets were recruited independently. Surplus material was obtained following pathological analysis of suspicious lesions excised for clinical purposes. Available samples at the time of analysis were included in both series, with the centrally analyzed series using the first set of samples, and the validation set including samples provided by academic investigators. Samples were sent for centralized dermatopathology and molecular analyses (initial set). Suspicious skin lesions in formalin-fixed paraffin embedded (FFPE) tumor blocks or slides were submitted to a central dermatopatholgy laboratory (Dermpath, Palm Beach Gardens, FL) for histopathological review. Confirmed cuSCC/KA lesions were then forwarded to a central molecular pathology laboratory (HistoGeneX, Berchem, Belgium) for further molecular analyses. A validation set of 14 cases was assembled at three study sites (UCLA, Vanderbilt University and Peter McCallum Cancer Center) from cuSCC/KA biopsies in 12 patients. Samples in the validation set were assessed by the pathology departments of the hospitals of origins and by Dr Jorge Reis-Filho and Dr Amaya Viros to confirm diagnosis. Samples were included if there was a common diagnosis of atypical squamous cell proliferations with a keratoacanthoma-like differentiation or as atypical squamous cell proliferations with a predominantly invasive compartment.

Oncogenic analyses of tumor specimens

DNA was extracted from cuSCC/KA specimens with tumor cell proportion of at least 50% using the QIAamp DNA Mini Kit for FFPE Tissue (Qiagen, Hilden, Germany). *HRAS* (exons 1 & 2), *NRAS* (exons 1 & 2), *KRAS* (exons 1 & 2) and *CDKN2A* (exon 2) gene sequences were analyzed using direct DNA sequencing according to the Sanger technology[22]. For each

normalized sample, 5 ng template DNA was amplified in duplicate in independent polymerase chain reactions (PCR) for the amplicon of interest (Supplemental Table 1). Uracil-DNA glycosylase (UNG) was added to avoid generation of artifacts. The DNA products of the nested PCR reactions were purified and subjected to double strand (forward and reverse) Big Dye Terminator (BDT) cycle sequencing using ABI3730XL DNA Analyzers (Applied Biosystems, Carlsbad, CA). Data were analyzed using Phred, Phrap and Polyphred (University of Washington, Seattle, WA). Optimization studies demonstrated that the sensitivity of the Sanger sequencing procedure is approximately 20% for the detection of somatic mutations in the background of non-mutated DNA (data not shown). Sequence traces were manually reviewed by two qualified persons. Sequence variation was detected in the forward and corresponding reverse reactions and confirmed in either the forward or reverse reaction of the replicate. Single-base substitution or deletion mutations in *TP53* exons 2 through 11 were analyzed in DNA isolated from tumor specimens using the investigational AmpliChip® p53 Test (Roche Molecular Systems, Inc, Pleasanton, CA), which is accomplished through PCR amplification, fragmentation and labeling, then microarray hybridization and scanning.

For the validation set, DNA was extracted following microdissection of tumour samples to ensure neoplastic cell poportion >70%. PCR amplification using the primers described in Supplemental Table 2 was performed to allow direct DNA sequencing. PCR reactions consisted of 40 cycles of 95 degrees C 30'', 55 degrees C 1', 72 degrees C 1', after initial denaturation at 95 degrees C for 5'. PCR products were purified using Quiagen PCR Purification kit and then used as templates for forward and reverse sequencing reactions using Big Dye v3.1 (Applied Biosystems).

Mouse *HRAS* exon 3 was PCR amplified and sequenced from microdissected tumor samples containing >70% neoplastic cells as described[33,34]. PCRs were performed as described above.

For the RT-PCR confirmation of the findings of Affymetric gene microarray data, pre-designed gene expression assays targeting genes were obtained from Applied Biosystems (Foster City, CA). Primers and probes are listed in Supplementary Table 4. All gene expression assays were performed on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Briefly, 1 ug total RNA was used for ss cDNA Synthesis using Quanta qScript cDNA SuperMix (Quanta BioSciences Cat# 95048). PCR mix consisted of 10 µl PerfeCTa® qPCR FastMix, ROX™ (Quanta, Gaithersburg, MD), 1 µl Taqman or custom assay, and 2 µl DEPC-treated water (Ambion, Applied Biosystems, Foster City, CA) for each reaction. cDNA samples were diluted to 10 ng/µl in RNase-free water (Ambion, Applied Biosystems, Foster City, CA), and 7 µl added to a 384-well optical plate (Applied Biosystems, Foster City, CA) containing 13 µl pre-distributed assay PCR mix. Thermocycling conditions consisted of 45 °C for 2 min, 95 °C for 3 min, followed by 40 cycles of 95°C for 15 sec then 60°C for 45 sec using Quanta PerfeCTa qPCR Fast Mix (Quanta BioSciences Cat #95077). Each measurement was performed in triplicate. The expression levels of target genes were normalized to reference gene levels and represented as relative expression (E), $E = 2^{(\Delta Ct)}$, where ΔCt is the difference between reference and target gene cycles at which the amplification exceeds an arbitrary threshold. The level of expression of the target genes were normalized to 4 genes of reference (GOR: 18S, Beta-Actin, GAPDH, and GusB) and the geometric mean* of the 4 GORs.

Additional methods for the tissue culture experiments

The B9 murine cuSCC cell line was cultured in RPMI-1640 medium (Mediatech Inc., Manassas, VA) containing compounds added and changed once a week. After three weeks of exposure, colonies greater than 100 µm in diameter were scored by AxioVision Rel 4.8 software (Carl Zeiss, Wake Forest, NC).

For studies in the mouse cuSCC/KAs cell line PDV, antibodies for CRAF, BRAF, and ERK2 were from Santa Cruz Biotechnology. Phospho-ERK1/2 and tubulin antibodies were from Sigma. PD184352 and PLX4720 were synthesized in-house. PDV cells were maintained in DMEM supplemented with 10% FBS. Cell viability was measured by Cell Titer Glo (Promega) according to the manufacturer's instructions. To prepare cell lysates, cells were washed once with ice-cold PBS and harvested into 500 µl lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1%NP-40, 10 mM NaF, 1 mM Na_3VO_4, 1 mg/ml leupeptin, 1 mg/ml aprotinin) per 10 cm dish. The lysates were cleared of insoluble material by high-speed centrifugation, and protein concentrations were determined by the bicinchoninic acid (BCA) protocol from Pierce (Thermosicentific, UK). For Immunoprecipitations 1 mg of protein lysate was immunoprecipitated for 2 hours with 5 µg CRAF antibody and captured on protein G sepharose beads (Thermosicentific, UK). Western blots were performed by standard techniques and analyzed on an Odyssey Infrared Scanner (Li-COR Biosciences).

Supplemental Table 1. List of Primers used for PCR reactions in the initial series.

Primer name	Gene/Exon	Primer Sequence	Amplicon Length
KRAS2-ex02-F	KRAS exon 2	5'-GTGTGACATGTTCTAATATAGTCA-3'	214
KRAS2-ex02-R		5'-GAATGGTCCTGCACCAGTAA-3'	
KRAS2-ex02N-F	KRAS exon 2	5'-ATGTTCTAATATAGTCACATTTTC-3'	202
KRAS2-ex02N-R		5'-GTCCTGCACCAGTAATATGC-3'	
KRAS2-ex03-F	KRAS exon 3	5'-TAAAAGGTGCACTGTAATAATCC-3'	279
KRAS2-ex03-R		5'-TAAAAACTATAATTACTCCTTAATG-3'	
KRAS2-ex03N-F	KRAS exon 3	5'-CACTGTAATAATCCAGACTGTG-3'	264
KRAS2-ex03N-R		5'-CTATAATTACTCCTTAATGTCAGC-3'	
HRAS-ex02-F	HRAS exon 2	5'-AGGGACCGCTGTGGGTTTGC-3'	379
HRAS-ex02-R		5'-ACAGGGCCACAGCACCATGC-3'	
HRAS-ex02N-F	HRAS exon 2	5'-CTGGCTGAGCAGGGCCCTC-3'	292
HRAS-ex02N-R		5'-CACCATGCAGGGGACCAGG-3'	
HRAS-ex03-F	HRAS exon 3	5'-GAGAGGTACCAGGGAGAGGC-3'	331
HRAS-ex03-R		5'-TGCCTGGACGCAGCCGGCC-3'	
HRAS-ex03N-F	HRAS exon 3	5'-AGGCTGGCTGTGTGAACTCC-3'	312
HRAS-ex03N-R		5'-CTGGACGCAGCCGGCCTGG-3'	
NRAS-ex02-F	NRAS exon 2	5'-CAAATGGAAGGTCACACTAGG-3'	333
NRAS- ex02-R		5'-TTACTTTCTCTCCTCTTATTCC-3'	
NRAS- ex02N-F	NRAS exon 2	5'-ATAGAAAGCTTTAAAGTACTG-3'	275
NRAS- ex02N-R		5'-TTCCTTTAATACAGAATATGG-3'	
NRAS- ex03-F	NRAS exon 3	5'-CCTCCACACCCCCAGGATTC-3'	205
NRAS- ex03-R		5'-GCTCCTAGTACCTGTAGAGG-3'	
NRAS- ex03N-F	NRAS exon 3	5'-CACACCCCCAGGATTCTTAC-3'	190
NRAS- ex03N-R		5'-CTGTAGAGGTTAATATCCGC-3'	
CDKN2A-ex02-I-F	CDKN2A exon 2 -I	5'-AAGCTTCCTTTCCGTCATGC-3'	243
CDKN2A-ex02-I -R		5'-CCCAGGCATCGCGCACGTC-3'	
CDKN2A-ex02-II-F	CDKN2A exon 2 -II	5'-ACTCTCACCCGACCCGTGC-3'	294
CDKN2A-ex02-II -R		5'-GGAAAATGAATGCTCTGAG-3'	
CDKN2A-ex02N-II -F	CDKN2A exon 2 -II	5'-ACCCGACCCGTGCACGACGC-3'	279
CDKN2A-ex02N-II -R		5'-ATGCTCTGAGCTTTGGAAGC-3'	

Supplemental Table 2. List of Primers used for PCR reactions in the validation set.

Primer name	Gene/Exon	Primer Sequence	Amplicon Length
HRAS- codon 12/13-F	HRAS codon 12&13	5'-CAGGAGACCCTGTAGGAGGA-3'	139
HRAS- codon 12/13-R		5'-TCGTCCACAAAATGGTTCTG-3'	
HRAS- codon 61-F	HRAS codon 61	5'-GTGGTCATTGATGGGGAGAC-3'	133
HRAS- codon 61-R		5'TGGTGTTGTTGATGGCAAAC-3'	
KRAS- codon 12/13-F	KRAS codon 12&13	5'TCATTATTTTTATTATAAGGCCTGCTG-3'	185
KRAS- codon 12/13-R		5'AGAATGGTCCTGCACCAGTAA-3'	
KRAS- codon 61-F	KRAS codon 61	5'CCAGACTGTGTTTCTCCCTTC-3'	152
KRAS- codon 61-R		5'AAAGAAAGCCCTCCCCAGT-3'	
NRAS- codon 12/13-F	NRAS códon 12&13	5'GGTTTCCAACAGGTTCTTGC-3'	153
NRAS- codon 12/13-R		5'CACTGGGCCTCACCTCTATG-3'	
NRAS- codon 61-F	NRAS códon 61	5'CACCCCCAGGATTCTTACAG-3'	148
NRAS- codon 61-R		5'TGGCAAATACACAGAGGAAGC-3'	

Supplemental Table 3a. Clinical presentation and oncogenic events in cuSCC/KAs from the initial series of patients treated with vemurafenib

ID	Gender	Age	Chronic sun damaged skin	History of prior SCC/KA	Time from start of therapy until 1st SCC/KA (wks)*	SCC/KA skin location	Pathological characterization	Oncogenic events in cuSCC/KA
1	M	62	Yes	No	16	Arm	SCC-KA subtype	$HRAS^{Q61K}$
2	F	67	Yes	No	11	Head and neck	SCC	None detected
						Torso	SCC-KA subtype	$HRAS^{G12D}$ $NRAS^{G12D}$
3	M	52	No	No	8	Head and neck	SCC-KA subtype	None detected
						Torso	SCC	$HRAS^{Q61R}$
						Leg	SCC-KA subtype	$HRAS^{G12D}$
						Leg	SCC-KA subtype	None detected
						Torso	SCC-KA subtype	$HRAS^{Q61K}$
						Torso	SCC-KA subtype	$HRAS^{Q61L}$
4	M	83	Yes	Yes	6	Head and neck	SCC-KA subtype	$HRAS^{Q61L}$ Intron TP53 mutation
						Arm	SCC-KA subtype	None detected
						Arm	SCC-KA subtype	$HRAS^{G13D}$
						Torso	SCC-KA subtype	$HRAS^{G13V}$

						Head and neck	SCC-KA subtype	$KRAS^{G12C}$ $TP53^{R196X}$
5	M	56	Yes	No	5	Head and neck	SCC	$HRAS^{Q61L}$
6	M	44	Yes	No	8	Leg	SCC-KA subtype	None detected
7	M	60	Yes	Yes	8	Arm	SCC	$TP53^{P278S}$
8	F	53	Yes	No	6	Leg	SCC (with features of KA)	None detected
9	F	54	Yes	No	9	Leg	SCC-KA subtype	$HRAS^{Q61L}$
10	M	71	Yes	No	15	Torso	SCC-KA subtype	None detected
11	M	61	Yes	Yes	15	Arm	SCC-KA subtype	$HRAS^{Q61L}$

*At the time of the first cuSCC/KA all patients were taking vemurafenib 960 mg orally twice daily, except patient #4 who received 720 mg orally twice daily.

Legend: SCC: squamous cell carcinoma; KA: keratoacanthoma.

Supplemental Table 3b. Clinical presentation and oncogenic events in cuSCC/KAs from the validation series of patients treated with vemurafenib

ID	Gender	Age	Chronic sun damaged skin	History of prior SCC/KA	Time from start of therapy until 1st SCC/KA (wks)*	SCC/KA skin location	Pathological characterization	Oncogenic events in cuSCC/KA	RAS mutations in surrounding skin
1	M	80	Yes	No	9	Head and neck	SCC	None detected	ND
2	M	67	No	No	5	Head and neck	SCC	$HRAS^{Q61L}$	Wild type
3	M	69	No	No	9	Torso	SCC-KA subtype	$KRAS^{G12D}$	Wild type
4	F	66	Yes	No	12	Arm	SCC-KA subtype	None detected	ND
5	M	66	Yes	No	12	Torso	SCC-KA subtype	$HRAS^{Q61L}$	ND
6	M	51	No	No	15	Head and neck	SCC-KA subtype	$HRAS^{Q61R}$	ND
7	M	84	Yes	No	18	Arm	SCC	$KRAS^{G12C}$	Wild type
						Leg	SCC	None detected	ND
8	M	62	Yes	No	4	Arm	SCC-KA subtype	None detected	ND
9	F	72	Yes	Yes	6	Leg	SCC	None detected	ND
10	M	46	No	No	22	Head and neck	SCC-KA subtype	$HRAS^{Q61L}$	ND
11	M	55	Yes	No	18	Head and neck	SCC	None detected	ND

						Head and neck	SCC	$KRAS^{G12C}$	Wild type
12	M	84	Yes	No	3	Head and neck	SCC	$KRAS^{G12D}$	ND

Legend: SCC: squamous cell carcinoma; KA: keratoacanthoma; ND: Not done.

Supplemental Table 4. Baseline characteristics of patients with cuSCC/KA lesions.

		Initial series	Validation set	Total
Clinical Trial	Vemurafenib phase 1	7	2	9
	Vemurafenib phase 2	4	7	11
	Vemurafenib phase 3	0	1	1
	Drug-drug interaction study	0	2	2
Gender	Female	3	2	5
	Male	8	10	18
Age	Mean	60	66	60
	Range	44-83	46-84	44-84
Country	Australia	4	1	5
	USA	7	11	18
Number of Reported cuSCC/KA Events	Mean	2	4	3
	Range	1-6	1-10	1-10
Average Time to First Occurrence of cuSCC/KA (weeks)	Mean	9	11	10
	Range	5-16	3-22	3-22
Dose at Day of 1st cuSCC/KA Excision	Vemurafenib 720 mg	1	1	2
	Vemurafenib 920 mg	10	11	21