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Report

BRAF and RKIP are significantly decreased in cutaneous squamous cell carcinoma

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Abbreviations: AK, actinic keratosis; SCC, squamous cell carcinoma; BCC, basal cell carcinoma; NMSC, non-melanoma skin cancer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NTC, non-template control

Key words: actinic keratosis, squamous cell carcinoma, BRAF, RKIP, mRNA expression, RT-qPCR, AS-qPCR, V600E mutation

Background: **Actinic keratosis (AK) is a well-established pre-cancerous skin lesion that has the potential to progress to squamous cell carcinoma (SCC). However, little is known about the implication of BRAF and RKIP expression, or about the incidence of BRAF mutations in the formation of these cutaneous diseases. The RAS oncogene has been proposed to significantly contribute to skin cancer development. Moreover, numerous BRAF mutations have been detected in melanoma biopsy specimens and cell lines.**

Objectives: **This study aimed to measure the mRNA levels of the genes BRAF and RKIP in AK, as well as their possible implication in the progress of AK to SCC. All biopsy specimens were also screened for BRAF mutations within exons 11 and 15.**

Results: **Significant downregulation was noted for both genes in SCC, compared to normal tissue (for *BRAF*, p = 0.002; for *RKIP*, p < 0.001). *RKIP* expression levels were significantly higher than the corresponding levels of *BRAF* (p < 0.001), whereas the two genes showed a negative correlation not only in AK and SCC, but in the adjacent phenotypically normal skin tissue, as well. No mutation was detected, either in AK or SCC, within exons 11 and 15 of the BRAF gene.**

Patients and methods: **Expression levels of the genes BRAF and RKIP were examined in 16 AKs and 12 SCCs by RT-qPCR. A novel allele-specific qPCR method, in combination with direct DNA sequencing, was performed in order to inspect the frequency of the V600E mutation in exon 15, as well as to examine the mutation status of the gene within exon 11.**

Conclusion: **Both *BRAF* and *RKIP* expression levels exhibit a decrease from normal skin tissue and AK, going to SCC. The decrease of *RKIP* mRNA levels in SCC, suggests one novel mechanism in the deregulation of the *BRAF* signaling pathway. Our results further indicate that *BRAF* does not appear to be frequently mutated either in pre-cancerous skin lesions (AK) or in non-melanoma skin tumors (SCC).**

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Introduction

Actinic keratosis (AK) is a well-established pre-cancerous skin lesion that has the potential to progress to squamous cell carcinoma (SCC). Cutaneous SCCs are the most prevalent keratinocyte-derived tumors, second to basal cell carcinomas (BCCs).[1] Clinically AKs appear primarily on chronically sun-exposed areas,[2] and sites adjacent to AKs may contain significant histological alterations, suggesting extensive pre-neoplastic alterations in sun-damaged skin.[3] It has been found that 82.4% of SCCs arise either within (26.7%) or in close proximity to AKs (55.7%),[4] and the risk of AK progression to SCC is reported to be between 0.025–16%.[5] Additionally, 72% of SCCs are associated with contiguous AKs.[6]

The RAS gene has been found to carry mutations in SCC, at a frequency of 10–20%.[7-13] Moreover, BRAF gene mutations have been proposed to contribute to cancer development.[14] BRAF is a kinase that activates the RAF/MEK/ERK signal transduction cascade. Increased activity of the RAF/MEK/ERK pathway prevents apoptosis and induces cell cycle progression.[15,16] The most commonly reported mutation, was a T to A missense transversion at nucleotide 1799 (leading to a V600E amino acid change in the BRAF protein) observed in 80% of the malignant melanoma tumors and cell lines. Functional analysis revealed that this transversion was the only detected mutation that caused constitutive activation of the BRAF kinase activity, independently of RAS activation, by converting BRAF into a dominant transforming protein.[14] Besides melanoma,[17,18] BRAF mutations have also been investigated in BCC.[19] However, no previous studies have investigated whether BRAF is mutated in pre-cancerous skin lesions (AK) or non-melanoma skin tumors, such as SCC.

RKIP, or Phosphatidyl-ethanolamine-binding protein 1 (PEBP-1), had initially been characterized to be involved in many different physiological activities, including reproduction and

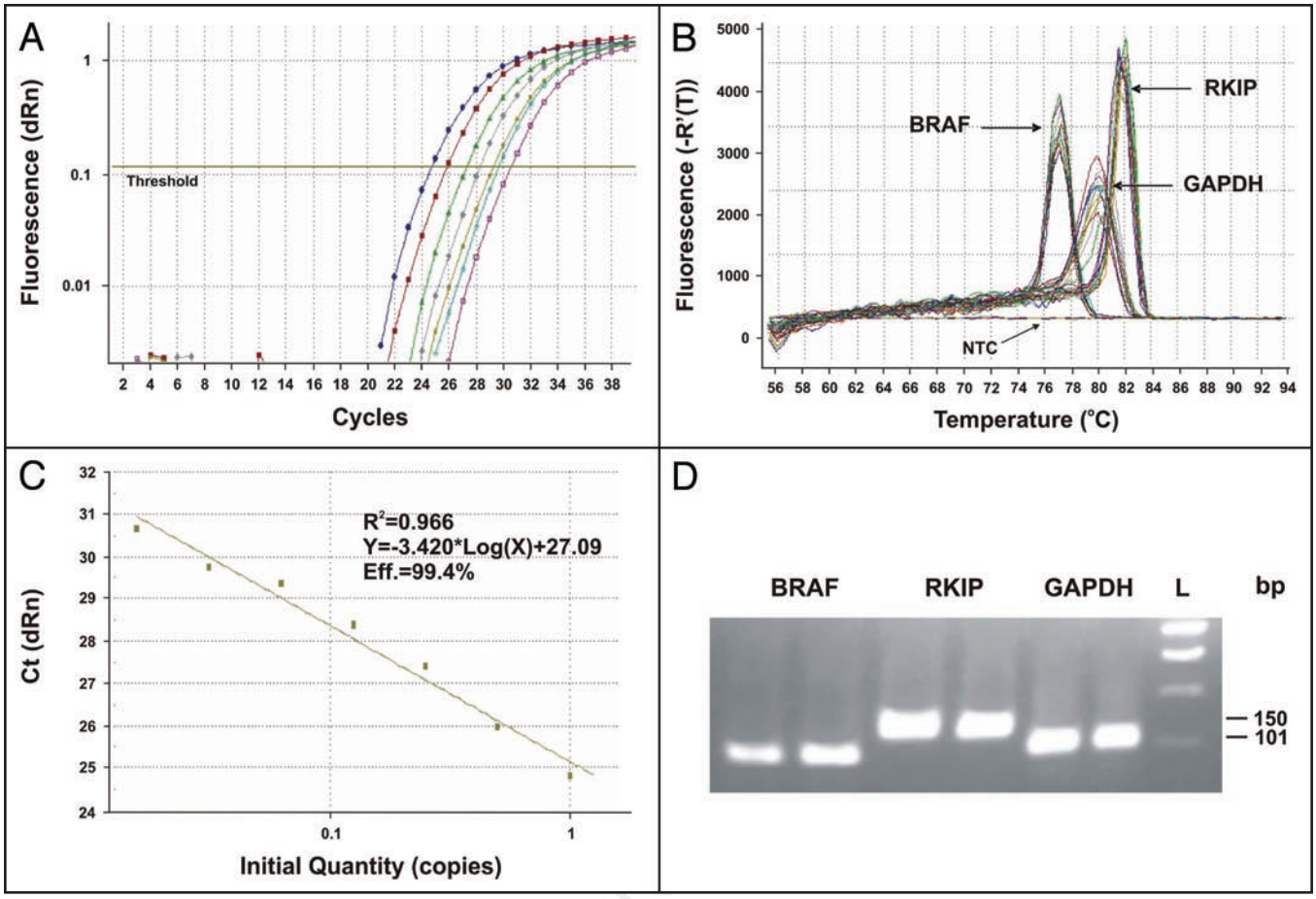

Figure 1. Selective Amplification Curves (A) and Melting Curves (B) used for expression analysis and product specificity of the genes BRAF and RKIP. GAPDH was used as a normalising gene. (C) A Standard Curve was constructed from samples used in a series of consecutive dilutions for the mentioned genes. All characteristics necessary for a proper standard curve are shown. (D) Representative examples of PCR products after analysis in 2% agarose gel of the genes studied. L, 100-bp DNA molecular marker.

neurophysiology.[20] Previous findings, however, identified RKIP as a modulator of apoptosis and metastasis through the regulation of important signaling cascades, i.e., the RAF-MEK-ERK kinase cascade, G protein-coupled receptors and the NFκB pathway.[21-23] RKIP blocks the RAF-induced phosphorylation of MEK, via direct interaction with RAF-1 kinase, and consequently, the activation of ERK.[22] RKIP also has a weak binding affinity to MEK-1 and ERK-2, interfering with downstream phosphorylation events. In addition to its modulation of RAF signaling, RKIP inhibits NFκB activity by interacting with upstream NFκB activators such as the NFκB-inducing kinase (NIK) and TGF-B-activated kinase 1 (TAK1).[24] Another study showed a decrease in RKIP expression in malignant melanoma and the absence of RKIP expression in melanoma metastases.[25] However, RKIP expression levels in pre-cancerous skin lesions (AK) and SCC have yet to be investigated.

We hypothesized that pre-neoplastic AK and SCC may exhibit either or both alterations, namely, *BRAF* mutation and decreased expression of *RKIP*. These two alterations may induce the constitutive activation of the RAF-1/MEK/ERK cell survival pathway. In the present study, the following were examined using samples derived from patients with AK and SCC and compared to adjacent normal tissues: (1) Examination of the presence of V600E mutation (*BRAF* exon 15) using a novel allele specific qPCR method, as well screening of the entire *BRAF* exon 11 by direct DNA sequencing, (2) Expression levels of *BRAF* by RT-qPCR and (3) Expression levels of *RKIP* by RT-qPCR. The findings demonstrate that both *BRAF* and *RKIP* levels were decreased in SCC and suggest one novel mechanism in the deregulation of the BRAF signaling pathway.

Results

This study was conducted in order to screen AK and SCC samples for the presence of *BRAF* mutations within exons 11 and 15. Moreover, we wanted to detect the expression levels of the genes BRAF and RKIP in AK and SCC, and compare the genes with the corresponding levels from the adjacent normal skin tissue.

In all AK and SCC samples, both sequence analysis of the PCR products and AS-qPCR failed to reveal mutations within exons 11 and 15 of the BRAF gene (Figs. 2 and 3).

Regarding the second part of this study, *BRAF* and *RKIP* were expressed in the pathological skin tissue, in both AK and SCC, as well as in the adjacent normal skin tissues studied. According to the ratio of the level of expression of each gene in AK and SCC, compared to the adjacent normal tissues, we defined three groups

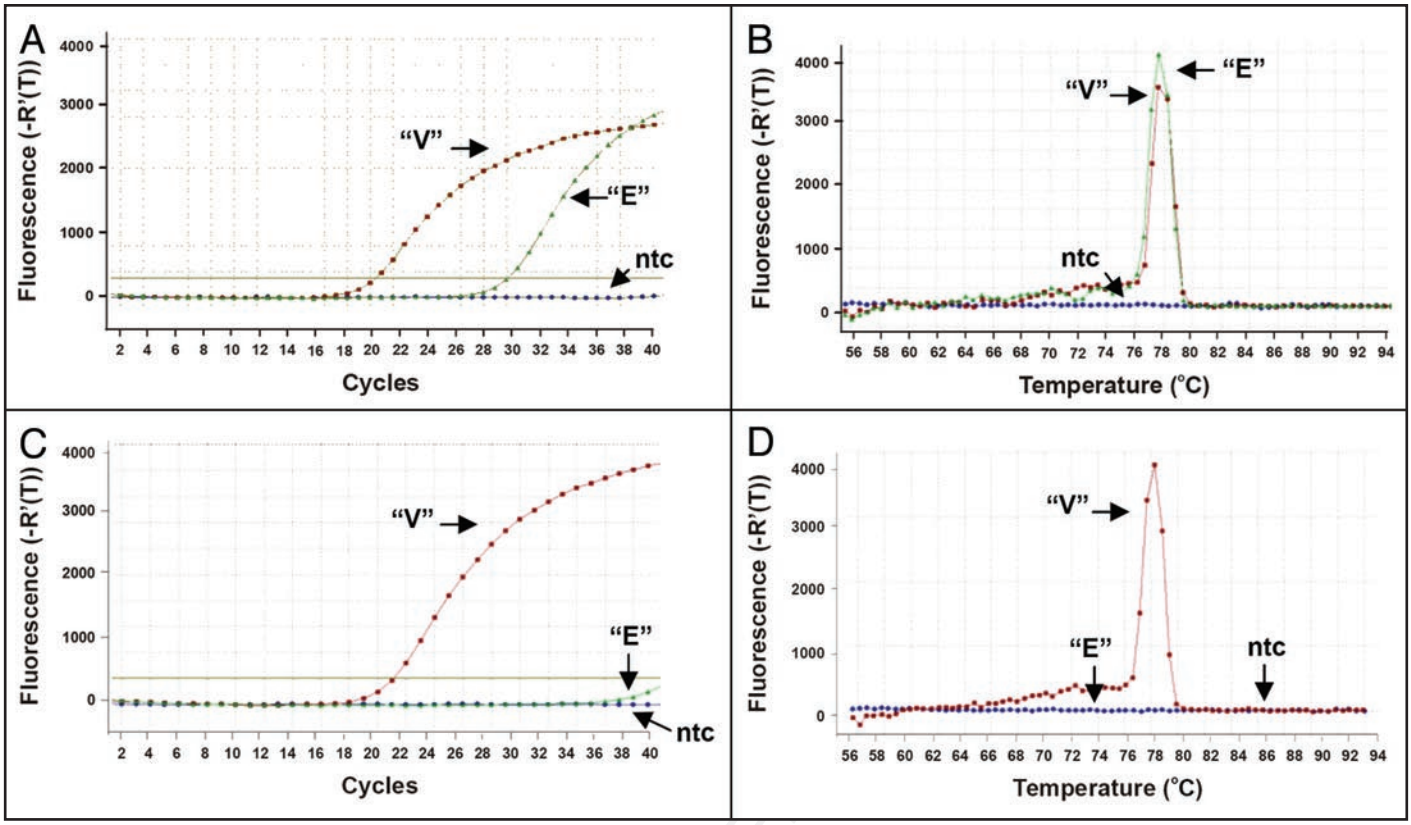

Figure 2. Allele-Specific real-time PCR (AS-qPCR) was used in order to detect the BRAF V600E mutation, in AK and SCC samples. All AK and SCC samples were wt at this locus. Upper panels (A and B) show the Amplification and Dissociation Curves from a melanoma DNA sample bearing the V600E mutation, which served as a positive control. Lower panels (C and D), exhibit the Amplification and Dissociation Curves of a wt SCC sample.

of expression (<0.5, underexpression; 0.5–2.0, equal expression and >2.0, overexpression), as shown in Table 1. Moreover, Table 2 depicts the expression levels of the genes studied in AK and SCC, compared to the adjacent normal skin tissues. All *BRAF* and *RKIP* normalized mRNA expression levels were significantly different among AK, SCC and the adjacent normal tissue (*BRAF*, p = 0.001; *RKIP*, p = 0.030; Kruskal-Wallis test). In SCC, both BRAF and RKIP genes presented significant levels of under-expression (58.3% and 75%, respectively), and these differences were statistically significant (p = 0.002 and p = 0.011, respectively; Mann-Whitney U test); whereas in the majority of the AK samples, the two genes exhibited equal mRNA levels with the adjacent normal skin tissue (43.7% and 48.7%, respectively). *BRAF* mRNA levels were also higher in AK compared to SCC (p < 0.01; Mann-Whitney U test). Furthermore, five AK samples (31.2%), showed higher *BRAF* expression levels compared to the normal skin tissue, but the difference was not statistically significant (Fig. 4).

Regarding the most significant finding of our study, *BRAF* and *RKIP* under-expression values in SCC, were 0.23 ± 0.12 and 0.34 ± 0.11, respectively, when compared to the normal skin tissue (mean-fold difference ± SD, range) (Table 1).

Moreover, *RKIP* mRNA levels were significantly higher than the corresponding *BRAF* levels, both in AK and SCC, as well as in the adjacent normal skin tissue (p < 0.001, in all three types of skin tissue; Mann-Whitney U test).

Finally, using the Spearman rank correlation, we tested the co-expression patterns of the genes in a pair-wise manner in AK, SCC and the adjacent control tissue. This test examines whether the genes studied, are up or downregulated concomitantly (positive correlation), or whether one gene has a reduced expression when the other is overexpressed (negative correlation). Interestingly, *BRAF* and *RKIP* were negatively correlated in SCC and the normal tissue (p = 0.004, Correlation Coefficient = -0.760** and p = 0.005, Correlation Coefficient = -0.512**, respectively). No statistically significant correlation between the two genes could be deduced in AK.

Discussion

Previous studies have suggested that the RAS family genes contribute to skin cancer development.[7-13] Many tumors exhibited alterations in the RAS-RAF-MEK-ERK-MAP kinase signaling pathways resulting in an increased signaling intensity via this cascade: more than 50% of colon tumors bear active mutants of *KRAS* and almost 70% of melanomas have a constitutively active BRAF protein.[29,30] Mutation of *BRAF* has been proposed to play a role in cancer growth, including primary and metastatic melanoma.[14-16,18] Moreover, Libra et al. recently attempted to determine *BRAF* mutations within exons 11 and 15 in BCC, but their study failed to give any positive results.[19] However, the incidence of *BRAF* mutations has yet to be investigated in pre-

cancerous lesions such as actinic keratosis, or non-melanoma skin tumors apart from BCC, such as squamous cell carcinoma. The present study is the first to do so. AK and SCC biopsy samples from a total of 28 patients were screened for *BRAF* mutations. In the pre-cancerous and tumor biopsy specimens, both the sequence analysis of the PCR products and AS-qPCR of the genomic DNA, failed to reveal mutations within exons 11 and 15 of the BRAF gene. Our results are in accordance with the negative outcome that Libra et al. mentioned in their study, which focused on BCC.[19] To the best of our knowledge, our research and the one mentioned above, are the only two studies to have screened *BRAF* for mutations, in non-melanoma skin tumors, and it appears that this gene is mutated exclusively in melanoma, but not in non-melanoma skin tumors, or pre-cancerous lesions. Since novel interactions between the MAPK and PTEN pathways have recently been found in cutaneous melanoma, other gene alterations such as *PI3K* mutations have been suggested to be able to cause the development of BCC.[19] Should that be the case, the same may apply for SCC and/or AK.

RKIP expression has been reported to be significantly decreased in malignant melanoma and to be completely absent in melanoma metastases.[25] This is the first study to investigate the mRNA levels of RKIP, in combination with BRAF, in non-melanoma skin tumors, as well as in pre-cancerous skin lesions. Although we found no significant difference in the expression of the two genes, nor between AK and adjacent normal skin tissue, the corresponding expression levels of the two genes significantly diminished in SCC, when compared to the normal skin tissue. In prostate cancer, RKIP has been described to be decreased and totally absent in metastases. We suggest that in the case of cutaneous lesions, RKIP follows a similar pattern, where the normal skin tissue and pre-cancerous skin lesions such as AK, express the highest levels of RKIP. Moreover, the cancerous tissue (SCC and/or BCC) expresses significantly reduced mRNA levels. Finally, its expression is diminished in metastatic melanoma, as previously reported.[25]

Recently, RKIP was found to downregulate BRAF kinase activity in melanoma cancer cells.[23] In our study, *BRAF* and *RKIP* were negatively correlated in SCC as well as in the adjacent normal skin tissue reflecting the inhibitory action that the latter exerted upon the former. Interestingly, this inhibitory action appears to occur in normal skin tissue, as well. Supporting this, *RKIP* expression levels were significantly higher than the corresponding *BRAF* levels, in all pathologic skin tissues, as well as in normal skin. Therefore, the inhibitory action of RKIP upon BRAF may not be confined to cancer tissue only, but also occurs in the phenotypically adjacent normal skin tissue.

Novel drugs are currently under study, the most promising of which appear to be rituximab, a chimeric mouse antihuman CD20 monoclonal Ab (mAb). This drug was recently reported to induce the expression of RKIP, which has been declared to be a prognostic marker of the pathogenesis of human cancer cells and tumors, following treatment with clinically relevant chemotherapeutic drugs.[31] Moreover, Baritaki et al. showed that the overexpression of RKIP sensitizes tumor cells to TRAIL-induced apoptosis via the inhibition of NFκB and YY1, and the upregulation of DR5 expression.[31] Therefore, rituximab may prove to be a hopeful

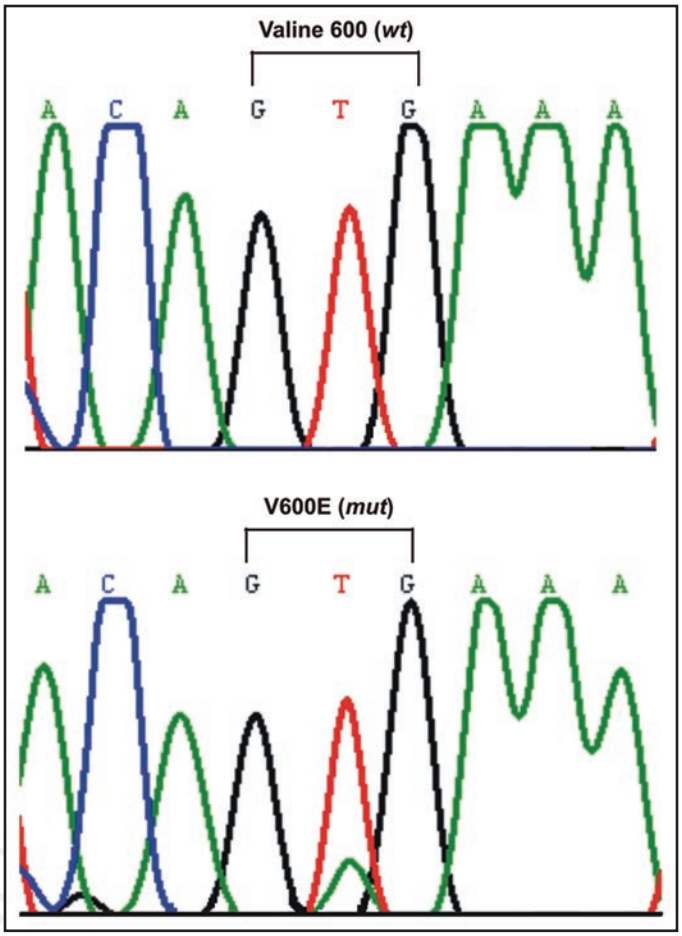

Figure 3. Direct sequencing of the PCR products verified the absence of mutations in exons 11 and 15 in the AK and SCC samples. Upper panel depicts a representative wt BRAF exon 15 sequence, which codes for the amino acid Valine at position 600. The sequencing electrophoregram in the lower panel shows a positive control for the V600E mutation, derived from a melanoma cell line bearing this transversion.

Table 1 Expression status of the genes studied in AK and SCC compared to adjacent normal tissues

Gene	lesions	Relative mRNA expression		
		↑ (%) mean ± SD	— (%) mean ± SD	↓ (%) mean ± SD
BRAF	AK	5/16 (31.2) 2.57 ± 0.53	**7/16 (43.7) 1.55 ± 0.33**	4/16 (25) 0.21 ± 0.15
	SCC	2/12 (16.6) 12.26 ± 4.07	3/12 (25) 1.03 ± 0.38	**7/12 (58.3) 0.23 ± 0.12**
RKIP	AK	2/16 (12.5) 3.79 ± 2.43	**11/16 (68.7) 1.22 ± 0.55**	3/16 (18.7) 0.16 ± 0.14
	SCC	1/12 (8.3) 16.56 ± 0.00	2/12 (16.6) 1.42 ± 0.54	**9/12 (75) 0.34 ± 0.11**

Note: The majority of the SCC samples exhibited a significant underexpression for both genes, whereas most of the AK samples had an equal to the normal tissue expression. ↑: overexpression; —: equally expressed samples; ↓: underexpression; SD, standard deviation.

Table 2 The expression status of the genes BRAF and RKIP in AK (A) and SCC (B), compared to the adjacent normal tissue (N), stated as x-fold difference

A

Patient No	x-fold difference (AK/N) BRAF	RKIP
1	2.04	1.25
2	1.91	0.20
3	1.75	1.51
4	2.90	0.27
5	3.23	1.96
6	0.26	0.66
7	2.04	1.95
8	2.62	1.87
9	0.18	2.07
10	1.79	0.00
11	0.39	0.65
12	1.51	0.64
13	1.51	1.34
14	1.51	0.88
15	0.91	0.66
16	0.02	5.50

B

Patient No	x-fold difference (SCC/N) BRAF	RKIP
1	0.74	0.19
2	0.90	0.30
3	1.46	0.16
4	9.38	0.33
5	0.00	16.56
6	0.39	0.43
7	0.21	1.03
8	0.20	1.80
9	15.14	0.34
10	0.29	0.42
12	0.28	0.47
13	0.23	0.41

Note: Red color denotes overexpression, green denotes equal expression and blue denotes underexpression between the corresponding pathologic tissue and the adjacent normal one.

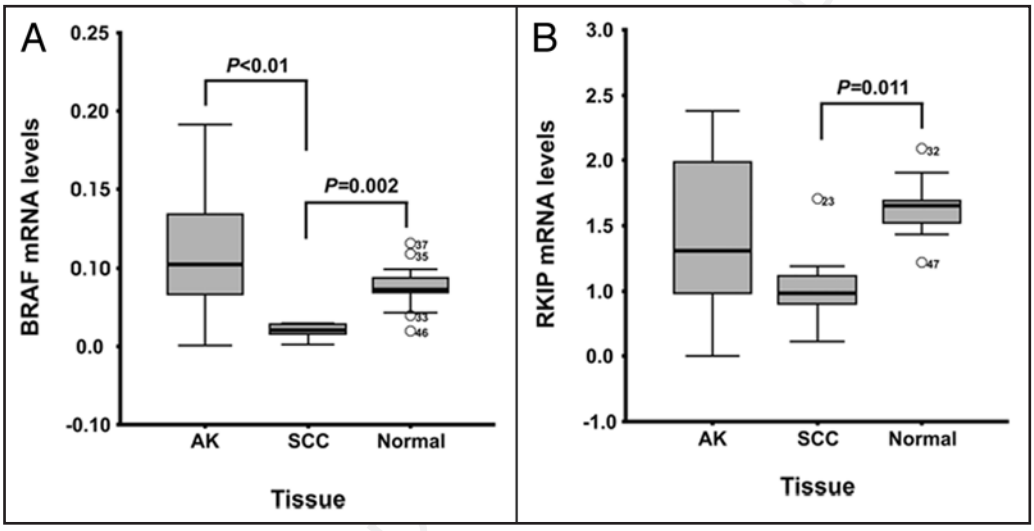

Figure 4. All BRAF and RKIP normalized mRNA expression levels were significantly different among AK, SCC and adjacent normal tissue (BRAF, P=0.001; RKIP P=0.030; Kruskal-Wallis test). The adjacent normal skin tissue presented higher BRAF (A) and RKIP (B) mRNA levels, compared to SCC (for BRAF, P=0.002; for RKIP, P=0.011, Mann-Whitney U test). BRAF mRNA levels were also significantly elevated in AK when compared to SCC (P<0.01; Mann-Whitney U test). Boxplots show the 25th, 50th (median), and 75th percentile values. Whiskers show the minimum and maximum values. AK, actinic keratosis; SCC, squamous cell carcinoma; Normal, adjacent normal skin tissue.

treatment not only against SCC, but probably against BCC and/or melanoma as well, in the future.

In summary, our results are the first to indicate that *BRAF* does not appear to be frequently mutated in pre-cancerous lesions such as AK and non-melanoma skin tumors such as SCC. Moreover, *BRAF* and *RKIP* exhibit decreased mRNA expression levels in SCC, compared to the adjacent normal skin tissue. *RKIP* mRNA levels are reduced in tumors and completely diminish in metastasis. Therefore, this gene may be a useful prognostic marker and potential target for therapeutic treatment against SCC.

Materials and Methods

Tumor specimens and DNA extraction. Sixteen AK with adjacent normal skin tissues and 12 SCC samples with 6 adjacent normal tissues were obtained from patients treated at 'A. Sygros' Hospital (Athens, Greece) with the approval of the donors and the institute's ethical committee. Patients with AK lesions were between the ages of 63 and 92 years (average 76; SD ± 7.2; 16 males and 10 females). Patients with SCC lesions were between the ages of 63 and 88 years (average 75; SD ± 7.5; 7 males and 5 females). A total of 8 AK patients presented contiguous SCC. All examined lesions (adjacent normal tissues included) were located on sun-exposed parts of the body, such as the head and neck in the case of both sexes, as well as the chest and shoulders for males and the legs for females.[26] Sample diagnosis was histologically confirmed. Immediately following dissection, the specimens were stored at -80°C until DNA/RNA extraction. Genomic DNA was extracted using proteinase K (Promega, Madison, WI), followed by phenol extraction and ethanol precipitation according to standard procedures. All specimens were examined for the presence of amplifiable DNA using a set of primers for the β-globin gene.

PCR and direct DNA sequencing. We amplified *BRAF* exons 11and 15 by polymerase chain reaction (PCR) using the primers: *BRAF* exon 11 forward, 5'-TCT CTT CCT GTA TCC CTC TC-3' and *BRAF* exon 11 reverse, 5'-ACT TGT CAC AAT GTC ACC AC-3', (PCR product: 257 bp); *BRAF* exon 15 forward, 5'-ATC TAC TGT TTT CCT TTA CTT-3' and *BRAF* exon 15 reverse, 5'-TAG TAA CTC AGC AGC ATC T-3', (PCR product: 216 bp). All primers were located in the introns flanking the coding exons of the gene to include sequences from the intron/exon boundaries in the amplified products and the final sequencing traces. PCR conditions were: initial denaturation at 95°C for 5 min, followed by 37 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for exon 11, at 52°C for exon 14 and at 51°C for exon 15, for 40 sec, and elongation at 72°C for 30 sec. The reaction ended with a final extension at 72°C for 10 min.

Direct DNA sequencing was used to identify and/or verify mutations within exons 11 and 15 of the BRAF gene. The primers used for the forward reading of the reactions were: BRAF exon

11, 5'-TGT TTG GCT TGA CTT GAC-3' and BRAF exon 15, 5'-CCC TGA GAT GCT GCT GAG TT-3'. The sequencing reactions were carried out using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) in a 10-μl volume containing purified PCR product and the sequencing primer. The temperature conditions set for the sequencing reactions were 96°C for 2 min followed by 25 cycles at 96°C for 30 sec, 54°C for 10 sec and 60°C for 4 min. The reaction products were precipitated with 2-propanol, washed with 75% ethanol, re-suspended in 25 μl water and loaded onto an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Forward and reverse strands were sequenced separately. Sequencing data were analyzed using sequence analysis software (Sequence Analysis 3.7; Applied Biosystems). The sequence of each PCR product was compared with that of the GenBank accession number M95712.

Allele-specific real-time PCR (AS-qPCR) for the detection of the BRAF V600E mutation. For verification of the absence of the BRAF V600E mutation in the genomic DNA of the AK and SCC biopsies, we used an allele-specific real-time PCR assay. Specifically, two forward primers with variations in their 3' nucleotides were used in order that each was specific for the wild-type (V; AGG TGA TTT TGG TCT AGC TAC AG*T*) or the mutated variant (E; AGG TGA TTT TGG TCT AGC TAC AG*A*) and one reverse primer (AS; TAG TAA CTC AGC AGC ATC TCA GGG C). Genomic DNA (100 ng) was amplified with primer mix V and E. The fluorescence produced was measured at the end of each cycle. The samples were analyzed in duplicate. The amplification conditions used were as previously described, on an Mx3000P thermal cycler (Stratagene).[27]

RNA extraction and reverse transcription. Tissue specimens were homogenized in TRIzol® reagent (Invitrogen, Carlsbad, CA) using a power homogenizer followed by chloroform addition and centrifugation. Total RNA was precipitated from the supernatant with isopropanol, washed with 75% ethanol and resuspended in 50 μl of DEPC-treated water. RNA concentration was calculated using the NanoDrop™ 1000 Spectrophotometer.

Reverse transcription reactions for the preparation of first-strand cDNA from 1 μg of total RNA were performed using the RETROscript® Kit, according to the manufacturer's protocol (Ambion, USA). Random hexamers were used as amplification primers.

qPCR analysis. The samples were treated collectively from the mRNA extraction and its conversion to cDNA, to the qPCR analysis. Moreover, all the qPCR primer sets were optimized for their concentration and optimal annealing temperature, so as to give the smallest Cts with the highest DRns. Transcribed products were subjected to qPCR assay with the SYBR Green qPCR Mastermix (2X), according to the manufacturer's instructions (Stratagene, USA) in an Mx3000P programmable thermal controller apparatus (Stratagene). The two genes used as internal controls were: GAPDH and b-actin. However, on comparing the expression stability measure *M* for each gene with the geNorm software, GAPDH was determined to be the most stable housekeeping gene with which we normalized the BRAF and RKIP mRNA expression levels. The primer pairs were designed to span at least one intron in order to avoid amplification of contaminating genomic DNA along with cDNA (Fig. 1). The primer sequences used and their annealing conditions were as previously described.[27] All reactions were performed in duplicate. PCR products were further confirmed by analysis on 2% ethidium bromide-stained agarose gels. The gene transcription levels were calculated using the ΔΔCt Method, as previously reported.[28] A 2-fold increased (≥2) or decreased (≤0.5) value was considered mRNA overexpression or downregulation, respectively, in each skin sample.

Statistical analysis. RNA levels were initially evaluated by the one-sample Kolmogorov-Smirnov goodness of fit test in order to determine whether they followed a normal distribution pattern. Depending on the results, Pearson's correlation or the non-parametric Spearman rank correlations were used to examine their relationship pair-wise and their association with continuous variables. The Mann-Whitney U and Kruskal-Wallis H tests were used to examine the *BRAF* and *RKIP* expression status with various clinicopathological parameters after stratification. Statistical analyses were performed with SPSS 11.5 (SPSS, Chicago, IL). Statistical significance was set at the 95% level (p-value <0.05).

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