Letter to the Editor

Raf kinase inhibitor protein expression correlates with differentiation but not with ERK phosphorylation in cutaneous squamous cell carcinoma

The classical mitogen-activated protein kinase (MAPK) signaling pathway is integral to a number of essential cellular functions and hyperactivation of this pathway is associated with cellular transformation and metastasis in a variety of cancers [1,2]. The best-characterized constituents of the MAPK pathway are Ras, Raf-1, MEK and ERK. Raf kinase inhibitor protein (RKIP) is a key regulator of MAPK activity. RKIP inhibits MAPK signaling by physically binding either Raf-1, BRAF or MEK preventing the Raf-1 or BRAF mediated activation of MEK [1,2]. Several studies recently demonstrated reduced RKIP expression in malignant tumors and tumor cell lines compared to normal tissue [3–6]. Additionally, there appears to be an inverse correlation between RKIP expression and metastatic potential [3–6]. A recent study demonstrated homogenous expression of RKIP in malignant melanoma in both primary and metastatic lesions, but tumors with strong RKIP staining had a higher proportion of phospho-ERK (p-ERK) positive cells [7]. These findings suggest that the normal inhibitory function of RKIP may be altered under some cellular conditions. Cutaneous squamous cell carcinoma (SCC) is the second most common skin cancer, accounting for 10–20% of all malignant skin tumors. Histologically, SCC is subclassified into three groups based on the degree of keratinization: well-, moderately- and poorly differentiated SCC. A subset of SCCs, especially poorly differentiated SCCs, are more likely to progress, recur, and metastasize, leading to poor patient prognosis. No studies have examined the relationship between RKIP expression levels and MAPK pathway activation in cutaneous SCCs with regard to their differentiation status.

We investigated the relationship between differentiation grade and RKIP expression in 53 cutaneous SCC samples. RKIP immunostaining was seen in tumor nests in well- and moderately differentiated SCC with relatively less staining towards the periphery (Fig. 1a, asterisks). All 24 of the well-differentiated SCC demonstrated RKIP staining at the staining grade 4 level (Fig. 1a and Table 1). In contrast, the nine poorly differentiated SCC examined demonstrated weak or negative RKIP staining (Fig. 1a and Table 1). There was a significant correlation between the differentiation status and staining grade of RKIP expression ($p < 0.001$, Table 1). We hypothesized that there should be a reciprocal localization of RKIP expression and activated ERK in SCC nests. Thus, we next examined the relationship between RKIP expression and ERK phosphorylation in SCC lesions. In the majority of tissue sections, the tumor cells exhibited nuclear immunoreactivity for p-ERK irrespective of differentiation status, but p-ERK positive cells were singly distributed randomly throughout the tumor nests with no apparent preference for subcellular localization or focus formation (Fig. 1a). Weak cytoplasmic staining was also seen. Overall, there was no reciprocal relationship appreciated between the histological localization of RKIP and activated ERK in the 53 SCC specimens examined. Additionally, there was no significant correlation between the tumor differentiation status and the proportion of p-ERK-positive cells (Table 1).

A role for RKIP in inhibiting tumor metastasis has been proposed for a variety of malignant tumors [3–6]. Therefore, we performed an immunohistochemical analysis for RKIP and p-ERK expression in lymph node metastases and primary lesions in 11 patients with SCC. The grade of RKIP staining did not differ between the metastatic lesions compared with their primary lesions ($p = 0.257$, Fig. 1b). Likewise, levels of ERK phosphorylation in the metastatic lesions were not increased compared to those seen in the primary tumors ($p = 0.527$, Fig. 1c). However, detailed evaluation of individual cases revealed that in three out of 11 metastatic cases (one well- and two moderately differentiated SCC) the RKIP staining intensity in the metastatic lesions was markedly reduced, and the corresponding staining of p-ERK was increased (cases 2, 3 and 8).

We demonstrated a statistically significant correlation between the differentiation grade of SCC and RKIP staining. This is consistent with the RKIP staining seen in normal skin, and shows that RKIP staining intensity is associated with the degree of terminal keratinocyte differentiation; namely, RKIP was not expressed by cells of the undifferentiated basal layer and was weakly expressed in the lower spinous layer, while the well-differentiated upper spinous layer was strongly RKIP positive [8]. However, the RKIP staining did not correlate with levels of ERK phosphorylation in the tumors analyzed. P-ERK positive keratinocytes predominately localize to the basal layer of normal epidermis [9], indicating that RKIP and p-ERK positive keratinocytes are reciprocally localized in normal epidermis. Taken together, these findings suggest RKIP mediated inhibition of ERK phosphorylation is lost in many cases of cutaneous SCC despite the retention of differentiation-dependent RKIP expression seen following malignant transformation. Recently, Zaravinos et al. showed that the RKIP mRNA expression levels were reduced in 12 SCC samples. However, they did not determine levels of RKIP protein and the activity status of ERK [10].

RKIP expression is reduced in metastatic lesions compared to the corresponding primary tumors in several malignancies [3,4,6]. When considered together with *in vitro* data examining the function of RKIP in cell proliferation, a possible role for RKIP as a tumor metastasis suppressor has been hypothesized. However, our current data demonstrate no statistically significant correlation between the RKIP staining in primary SCCs and their metastatic lesions, implying that RKIP-mediated inhibition of tumor metastasis may not function in all cancer types. Similar to our results in patients with SCC, uniformly strong RKIP expression was seen in the most of 180 melanoma samples examined, irrespective of whether it was a primary tumor or a metastatic lesion [7]. Notably, 3 of 11 SCCs with corresponding metastatic lesions examined in this study demonstrated decreased RKIP staining in the metastatic lesions compared to the primary tumors, and there

Table 1
Relationship between RKIP staining grade, p-ERK and differentiation status in SCC.

Differentiation	Cases (%)			
	Staining grade			
	1	2	3	4
RKIP				
Well	0 (0)	0 (0)	0 (0)	24 (100)
Moderate	0 (0)	5 (25)	8 (40)	7 (35)
Poor	6 (67)	3 (33)	0 (0)	0 (0)
Phospho-ERK				
Well	2 (8)	7 (29)	4 (17)	11 (46)
Moderate	4 (20)	9 (45)	5 (25)	2 (10)
Poor	1 (11)	4 (44)	2 (22)	2 (22)
Total ERK				
Well	0 (0)	0 (0)	7 (29)	17 (71)
Moderate	0 (0)	0 (0)	5 (25)	15 (75)
Poor	0 (0)	0 (0)	2 (22)	7 (78)

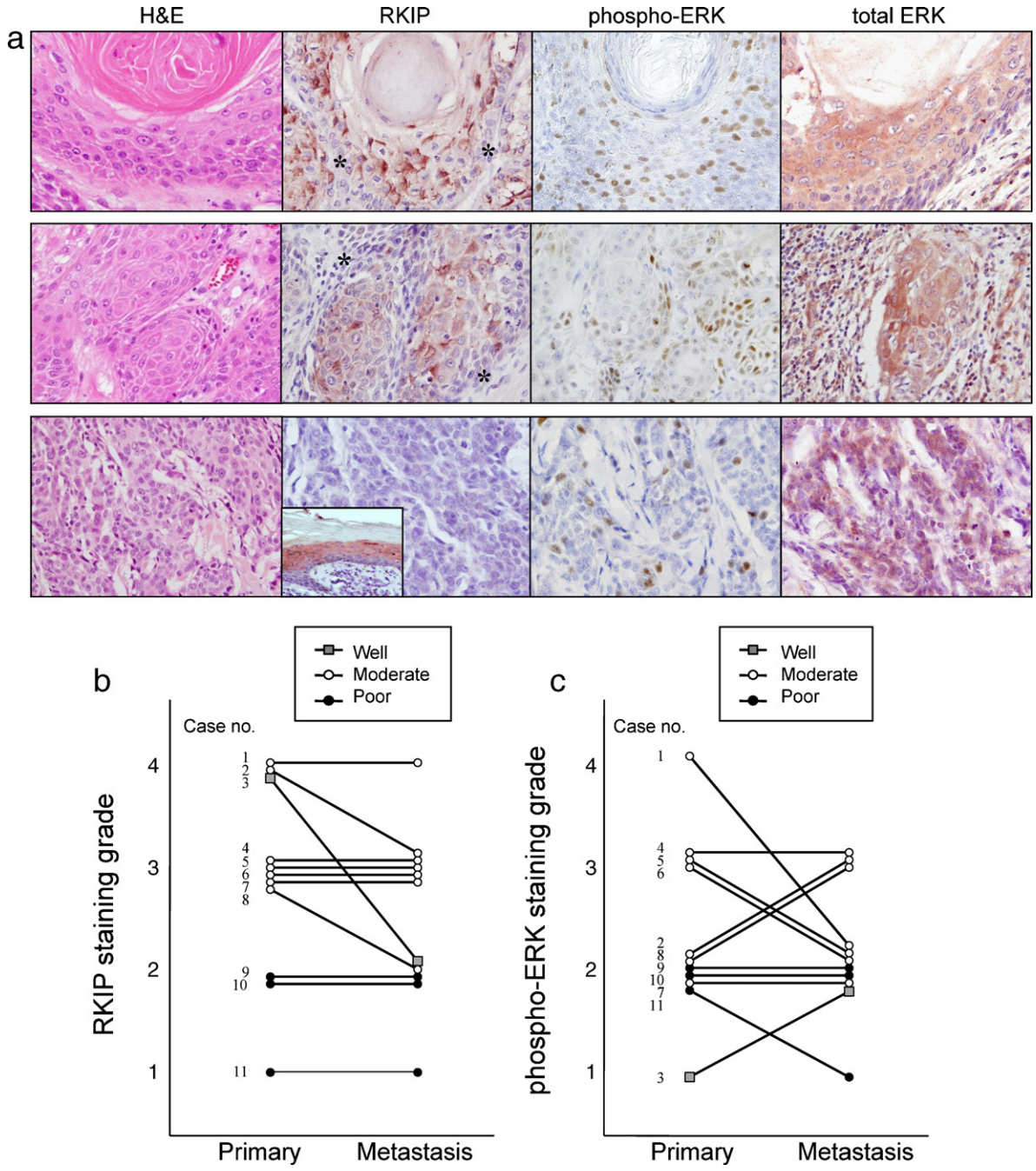

Fig. 1. (a) Immunohistochemical staining for RKIP and p-ERK in well-differentiated (upper panels), moderately-differentiated (middle panels), and poorly-differentiated (lower panels) SCC. The anti-RKIP antibody was previously published [8]. Anti-p-ERK (Thr202/Tyr204) antibody was purchased from Cell Signaling Technology (Danvers, USA), anti-ERK1/2 antibody was from Santa Cruz Biotechnology (Santa Cruz, USA). All the tumor samples were serially sectioned. Inset demonstrates positive staining of the epidermis in the same specimen as an internal control. Original magnification: ×200. (b and c) Comparison of immunohistochemical grading of RKIP and p-ERK levels between premetastatic primary tumors and metastases in 11 patients. All samples used in this study were obtained from patients who underwent surgery in the Department of Dermatology, Hirosaki University Hospital between 1998 and 2008. A total of 53 patient primary cutaneous SCC samples, including 24 well-differentiated, 20 moderately-differentiated, and 9 poorly-differentiated neoplasms, were used. Samples of metastatic lymph node lesions were obtained from 11 of the SCC patients. All samples were used after obtaining written informed consent from each patient. The staining intensity was graded as negative, weak, standard, and strong, and staining comparable to or greater than standard was defined as *positive*. For evaluation of immunohistochemical staining, the expression levels were graded on a scale from 1 to 4 as follows: 1, no positive staining; 2, positively stained tumor cells less than 30%; 3, positively stained cells less than 60%; 4, positively stained cells more than 60%. For statistical analyses of immunohistochemical results, significance among the groups was calculated using the Kruskal–Wallis test. Two-sided Wilcoxon's signed rank test was used for comparison of RKIP and p-ERK staining levels between the two groups of primary lesions and metastatic lesions.

was a reciprocal increase in p-ERK staining in these lesions. Therefore, in a limited number of SCC samples, reduced intracellular RKIP expression may represent one of the many mechanisms contributing to ERK activation and facilitate tumor metastasis.

When considered together with previous findings, there appear to be conflicting roles for RKIP in promoting tumor proliferation and metastasis, and further studies are needed to better define the role of RKIP and MAPK signaling in the growth and spread of cancer.

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Letter to the Editor

Fluctuations of BMP signaling pathway during hair cycles in skin of mice with mutant genes *we*, *wal* and *Fgf5^{go}*

In contrast to other mammalian organs that grow constantly, hair growth undergoes cyclic changes, from growth phase (anagen) through apoptosis and regression stage (catagen) to relative quiescence phase (telogen) [1]. Interactions of mice mutant genes *wellhaarig* (*we*), *waved alopecia* (*wal*) and *angora* (*Fgf5^{go}*) cause hair cycle alterations and hair loss. In double homozygous mice *we*/*we wal*/*wal*, the structure of hair coat is altered, and almost total alopecia is developed [2]. *Fgf5$^{go\text{-}Y}$* gene weakened alopecia development in *Fgf5$^{go\text{-}Y}$*/*Fgf5$^{go\text{-}Y}$ we*/*we wal*/*wal* mice, in which first signs of alopecia were seen just at 21st day after birth, 7 days later as compared to double homozygotes [3]. It is known that in case of insufficient BMP signaling pathway activity in hair follicles, hair loss is observed [4]. Our aim was to analyze the impact of interactions between mutant genes *Fgf5$^{go\text{-}Y}$*, *we* and *wal* on the features of expression of *Bmp2* and *Id1* genes mRNA as the markers of BMP signaling pathway in skin.

Double homozygous mice *a*/*a we*/*we wal*/*wal* and triple homozygous black mice *Fgf5$^{go\text{-}Y}$*/*Fgf5$^{go\text{-}Y}$ we*/*we wal*/*wal* were obtained at Vavilov Institute of General Genetics RAS. Heterozygous mice *a*/*a* +/*we* +/*wal* and mice of normal genotype (C57BL/6 strain) were used as the control. All surgical operations with the animals were performed using anesthesia. 10× 5 mm back skin fragments of C57BL/6 mice were taken at the day 7, 16, 20, 24, 30, 40, 45, 50 after birth. In mutant mice, skin fragments were gathered every two days during the interval between 7th and 40th day after birth and at 45th, 50th, 60th and 90th day of life. Skin samples were used for RNA extraction and to prepare paraffin sections. Phases of hair cycle were determined according to Müller-Röver and co-authors [1]. Real-time PCR was performed with SYBR Green I/ROX staining for amplification of *Bmp2* (primers Bmp2F (5′-AAGCAACAGAAGCCCAGTTG-3′) and Bmp2R (5′-GACCTTTAGGAGACCGCAGT-3′)) and *Id1* [5] genes in samples of cDNA from mouse skin. We used 2×, 3× and 4× dilutions of purified PCR products previously generated by PCR with specific primers for *Bmp2* and *Id1* genes as the standards. For relative quantification of real-time PCR data, C_t values for both samples in a pair were normalized to C_t value in reaction with *Gapdh* probes.

Histological analysis reveals that the duration of anagen of first generation (G1) of gurad hair in C57BL/6 mice is 16 days, catagen – 4 days, and telogen – 8 days (Table 1). Assessment of *Bmp2* and *Id1* genes' expression dynamics in skin of C57BL/6 mice during native G1 and second generation (G2) guard hair cycles suggests defined correlation between mRNA levels of these genes and actual hair cycle phases. The obtained data confirm results of other authors about high activity of BMP pathway during anagen phase [5]. Our present study demonstrates that level of *Bmp2* gene mRNA decreased not only at the end of telogen phase, but also at the