

Suppression of Rho B Expression in Invasive Carcinoma from Head and Neck Cancer Patients¹

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ABSTRACT

Purpose: In contrast to *Ras* small GTPases, which contribute to human malignancy when overexpressed or constitutively activated, convincing evidence for the involvement of *Ras* homologous (Rho) GTPases in human cancer is still missing. In cell culture and animal models, RhoB antagonizes malignant transformation, but no data are available regarding the expression of RhoB in human tumors. In this study, we have analyzed the status of the RhoB protein and the closely related family member RhoA in human head and neck squamous cell carcinomas.

Experimental Design: Protein immunorexpression was quantitated by image analysis in the context of tumor invasion and differentiation. To account for possible individual variations, expression levels of RhoB and RhoA were evaluated in the tumor and its adjacent nonneoplastic tissue. Potential gene deletions or mutations were assessed by PCR and RT-PCR.

Results: RhoB expression is readily detected in normal epithelium, carcinomas *in situ*, and well-differentiated tumors, but it becomes weak to undetectable as tumors become deeply invasive and poorly differentiated. In contrast, Ki67 (proliferation marker) and RhoA protein levels increase with tumor progression. Furthermore, whereas in nonneoplastic keratinocytes RhoB is localized mainly in the nucleus, in carcinomas RhoB is predominantly located in the cytoplasm. *RhoB* gene deletions or mutations were not found.

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Conclusions: These results give additional support to the notion that RhoB may play a tumor suppressive role in squamous cell carcinomas of the head and neck. The lack of RhoB expression in deeply invasive carcinoma argues against inhibition of RhoB farnesylation as a mediator of farnesyltransferase inhibitors' antitumor activity.

INTRODUCTION

The Rho⁴ subfamily of low molecular weight GTP-binding proteins appears to control many aspects of cellular functions, including adhesion, morphology, motility, and cell cycle progression (1). Although 12 family members have been identified thus far, only 3 of these, RhoA, Rac1, and Cdc42, have been studied in detail (1). Whereas RhoA interferes with stress-fiber formation, and Rac1 and Cdc42 regulate formation of lamellipodia and filopodia, respectively, the function of RhoB remains unknown (2).

Despite the high homology of the different Rho isoforms, RhoA, RhoB, and RhoC, their physiological function appears to be distinct (3, 4). RhoB differs from other Rho proteins in its COOH-terminal isoprenylation and intracellular localization (5). Moreover, RhoB mRNA, but not that of RhoA, RhoC, Rac1, and Cdc42, is rapidly induced by UV irradiation and DNA damaging agents, indicating a role for RhoB in the early response of eukaryotic cells to genotoxic stress (6, 7). The induction of RhoB upon treatment of cells with DNA damaging agents either blocks DNA replication or causes apoptosis (8). Thus, it seems that RhoB plays a regulatory role sensing the level of cytotoxicity in the cell and quickly triggering subsequent protective responses, such as cell cycle arrest, through induction of the cell cycle kinase inhibitor p21^{WAF1/CIP1} or apoptosis (8–10).

Recent evidence suggests that RhoB may act as a tumor suppressor. First, as mentioned above, RhoB induction is an early response of eukaryotic cells to genotoxic stress (7). Second, overexpression of RhoB in human cancer cells results in inhibition of signal transduction pathways involved in oncogenesis and tumor survival, as well as induction of apoptosis (11). Third, when overexpressed in Ras-transformed cells, RhoB induces phenotypic reversion, cell growth inhibition, and activation of the cell cycle kinase inhibitor p21^{WAF1/CIP1} (12). Finally, overexpressing RhoB in human cancer cells antagonizes their anchorage-dependent and -independent cell growth and suppresses their growth in nude mice (11). Taken together, these results suggest that RhoB interferes with tumorigenesis, and thus it is of great interest to determine the genetic and functional status of RhoB in human primary tumors.

To address the question of whether RhoB expression is

⁴ The abbreviations used are: Rho, ras homologous; ROI, region of interest; RT-PCR, reverse transcription-PCR.

altered in human tumorigenesis, we analyzed RhoB levels in head and neck squamous cell carcinomas. Our results show that RhoB expression is prevalent in carcinomas *in situ* and well-differentiated tumors. This expression becomes weak to undetectable as the tumors become deeply invasive and more undifferentiated. Furthermore, whereas RhoB was localized mainly in the nucleus in nonneoplastic epithelial cells, in carcinomas RhoB was predominantly located in the cytoplasmic compartment.

MATERIALS AND METHODS

Selection of Cases. Human head and neck squamous cell carcinomas were selected from the archives of the Pathology Department at the H. Lee Moffitt Cancer Center and Research Institute (Tampa, FL) to evaluate protein expression in the context of the different histopathological stages in the progression from normal epithelium to invasive cancer. The selected cases had to fulfill all of the following criteria: (a) presence of areas of "nonneoplastic" squamous epithelium "adjacent" to the tumor and lacking histopathological features of dysplasia or malignancy; (b) presence of areas of dysplasia and/or *in situ* carcinoma; (c) presence of superficial and infiltrating components; (d) presence of at least two different degrees of differentiation; (e) specimens were properly processed (no delayed fixation or poor handling) and free of necrosis; (f) patients did not receive any previous treatment (with the exception of a diagnostic biopsy); and (g) patients were initially diagnosed, treated, and followed-up at the Moffitt Cancer Center to ensure collection of clinical data, adherence to Institutional Review Board requirements, and patient confidentiality. The diagnosis of squamous cell carcinoma was made by a head and neck pathologist using standard criteria that included intracellular bridges, diskeratinization, keratin pearl formation, and various degrees of cytological atypia. Degree of differentiation was assigned as follows: (a) well differentiated: large cells with obvious keratinization, intercellular bridges, or keratin pearl formation; (b) moderately differentiated: heterogeneous population of cells of moderate size with only focal keratinization, poorly preserved intercellular bridges, and a higher degree of cytological atypia; and (c) poorly differentiated: anaplastic population lacking keratinization and intercellular bridges and with minimal resemblance to squamous epithelium. Definitive stromal infiltration required desmoplastic reaction associated with ragged tumor borders. The superficial component of the invasive carcinoma was defined as that region of the tumor in continuity with the overlying squamous epithelium and that invaded the underlying stroma up to a distance of ~300 μm . Within this distance, there is a high probability of angiolymphatic invasion. Deep invasion was defined as those areas of the tumor invading to a distance >300 μm and/or with tumor tongues separated from the main mass. Clinicopathological staging was assigned following the recommendations of the American Joint Committee on Cancer (13).

Immunohistochemical Analysis of RhoB, RhoA, SP1, and Ki67. Five- μm sections from formalin-fixed, paraffin-embedded tissues were cut and placed on poly-L-lysine-coated slides. Slides were subjected to deparaffination in xylene and hydration through a series of decreasing alcohol concentrations,

following standard procedures. Endogenous peroxidase activity was quenched with a 3% solution of H_2O_2 for 20 min at 37°C, and the slides were washed in deionized water for 5 min. Antigen retrieval was performed by placing the slides in a clear plastic container with a vented top containing citrate buffer [0.1 M citric acid (4.5 ml), 0.1 M sodium citrate (21.5 ml), and deionized water (225 ml)] in a microwave oven set on high, twice for 5 min each. The slides were then allowed to cool for 10–20 min, rinsed in deionized water, placed in PBS for 5 min, and drained. Blocking serum was applied, and the slides were incubated in a humid chamber for 20 min at room temperature. After blotting, the following primary antibodies were applied at room temperature: polyclonal anti-RhoB (Santa Cruz Biotechnology); anti-RhoA (Santa Cruz Biotechnology); anti-Sp1 (Santa Cruz Biotechnology); or anti-Ki67 (Dako Corporation, Carpinteria CA). After 1 h, slides were rinsed with PBS and placed in PBS for 5 min. For detection, the Vectastain ABC Kit, Rabbit IgG, Elite series (Vector Laboratories, Inc., Burlingame, CA) was used, following the manufacturer's specifications. The biotinylated secondary antibody was applied for 20 min at room temperature in a humid chamber. At the end of this incubation, the slides were rinsed and placed in PBS for 5 min, followed by the addition of the avidin-biotin complex. The slides were incubated in a humid chamber for 30 min at room temperature and then rinsed and placed in PBS for 5 min. 3,3'-diaminobenzidine, prepared according to the manufacturer's specifications, was applied to the slides and color development monitored. When maximal intensity was reached (~5 min), the slides were rinsed in water and counterstained with modified Mayer's hematoxylin for 30 s. The slides were finally washed in running water for 10 min, dehydrated, cleared, and mounted with resinous mounting medium.

Image Analysis. The Optimas 6.5 (Media Cybernetics, Inc.) software was used to quantitate protein expression. Images were stored as TIFF images using identical magnifications ($\times 400$) and camera settings. The ROI (normal squamous epithelium, dysplasia, carcinoma *in situ*, superficial, and deep components of the tumor and different degrees of differentiation) were defined, and the total area and the number of nuclei in a given ROI were determined. Thresholds were set to discriminate between the brown color of RhoB-positive nuclei and the negative (blue) nuclei. A RhoB index was generated as the percentage of positive areas *versus* total area.

Statistical Analysis. The Wilcoxon-Rank Sum test was used to compare paired samples.

PCR and RT-PCR Analysis. DNA and RNA samples were obtained from the H. Lee Moffitt Cancer Center Tissue Procurement Laboratory. cDNA was synthesized from 1.0 μg of total cellular RNA by reverse transcription with Sensiscript reverse transcriptase (Qiagen, Valencia, CA) using random hexamers as primers. The histone H3.3A sense (5'-CCACTGAAGTTCTGATTCGC-3') and antisense (5'-GCGTGCTAGCTGGATGTCTT-3') primers correspond to sequences on two different exons. They were used as positive controls for the reverse transcription reaction and genomic DNA contamination (generation of an additional large PCR fragment). The following RhoB sense (5'-ATGGCGGC-CATCCGCAAGAAGC) and antisense (5'-TCATAGCACCTTG-CAGCAGTTG) primers were used. These primers amplify the entire *RhoB* gene from nucleotides 1 to 591. Of note, *RhoB* is a

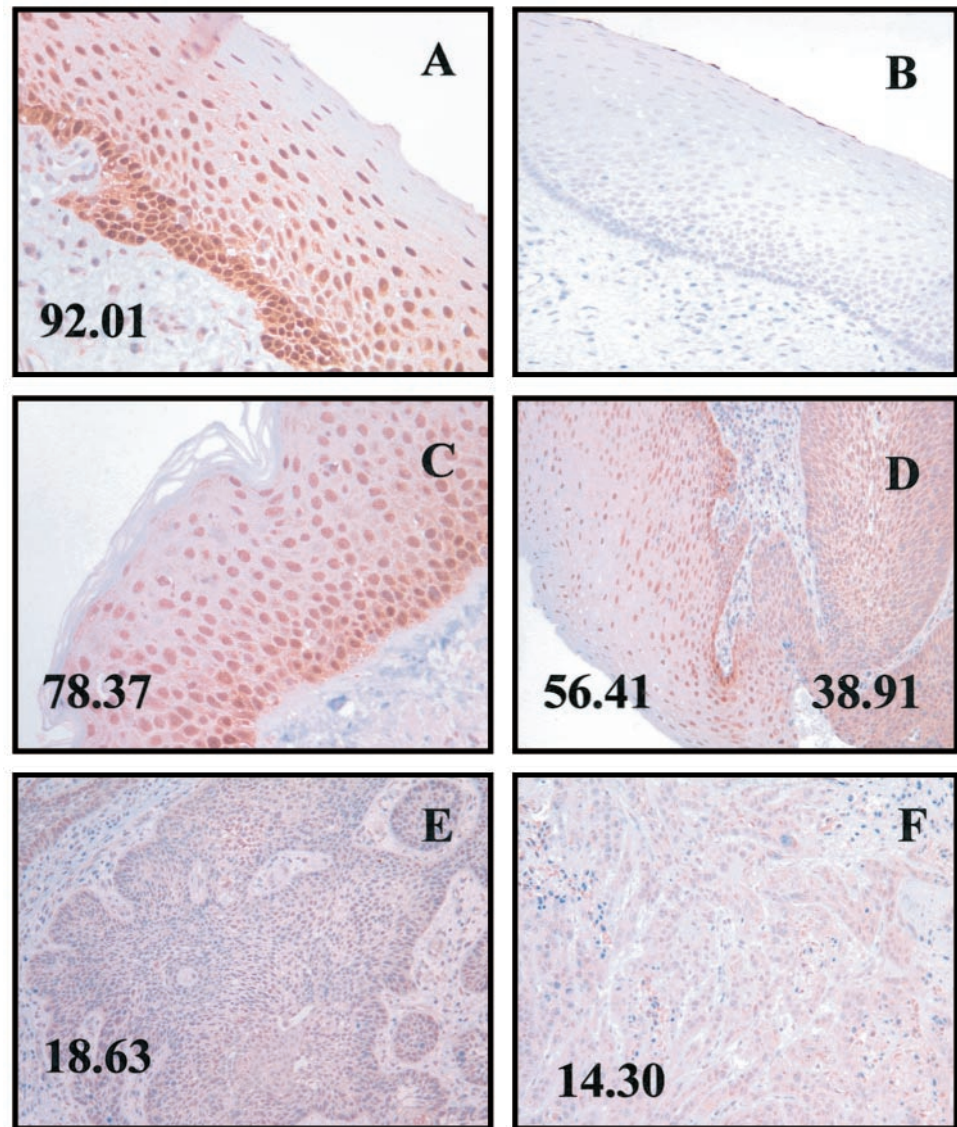


Fig. 1 RhoB expression in squamous cell carcinoma. Sections from formalin-fixed, paraffin-embedded tissues were processed and immunostained with anti-RhoB antibody as described in "Materials and Methods." The progressive loss of nuclear RhoB is observed in the transition from nonneoplastic epithelium (A) to carcinoma *in situ* (C), to superficially invasive carcinoma (D and E), to deeply infiltrating carcinoma (F). Staining is abolished by previous incubation of nonneoplastic epithelium with the specific RhoB peptide that was used to raise RhoB antibody (B).

591 bp intronless gene (3, 4), thus, RhoB primers will not allow the distinction of mRNA from genomic DNA. The PCR mixture contained 50 mM Tris (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 100 ng of each primer, 0.056 μM TaqStart Antibody (Clontech Laboratories, Palo Alto, CA), and 1 unit Taq polymerase (Life Technologies, Inc.). After an initial denaturation at 94°C for 5 min, amplification was carried out for 30 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min, followed by a final elongation step at 94°C for 2 min, 55°C for 2 min, and 72°C for 10 min. The PCR products were resolved on a 2% agarose gel and stained with ethidium bromide.

RESULTS

RhoB Expression Is Decreased in Invasive Carcinomas of the Head and Neck. To test whether RhoB expression is altered in tumorigenesis, we evaluated the status and subcellular

localization of RhoB protein in head and neck cancer patient specimens across the different histopathological stages from normal epithelium to invasive squamous cell carcinoma. Sections from formalin-fixed, paraffin-embedded tissues were immunostained with antibodies to RhoB and the expression index of RhoB, was determined using image analysis, selectively excluding nontumoral components such as stroma, vessels, and inflammation. The number of nuclei that were positive for RhoB staining was calculated by setting the software threshold to overlap the brown color of 3,3'-diaminobenzidine, whereas that of negative nuclei was calculated by setting the threshold over the blue color of the hematoxylin counterstain (14). To quantify cytoplasmic staining, the area showing cytoplasmic expression for a given marker was calculated as a function of the total cytoplasmic area of tumor cells contained within the region of interest. Images corresponding to the different tumor areas are

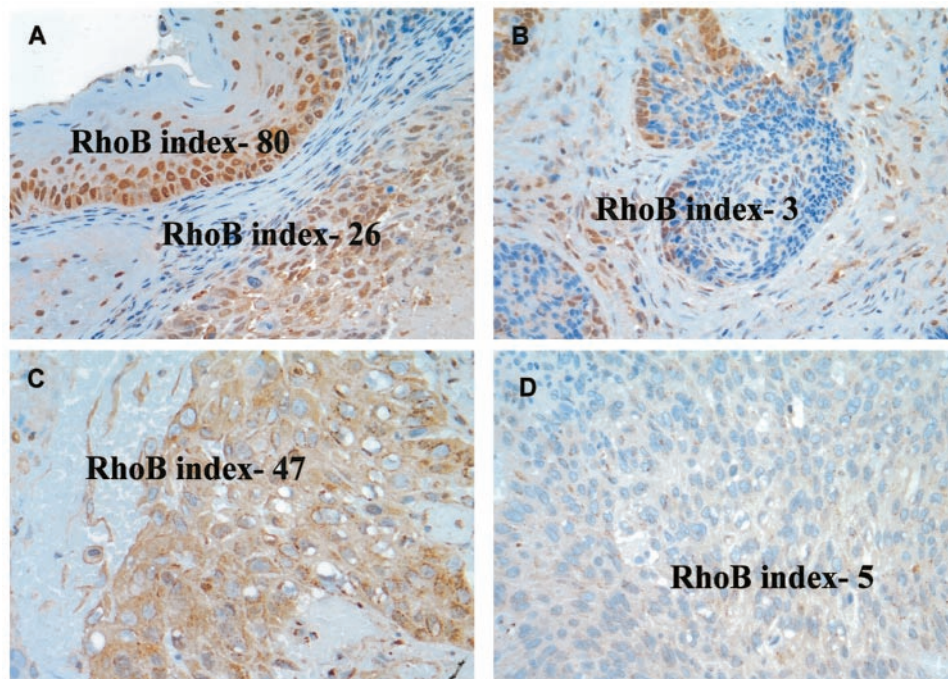


Fig. 2 Expression of RhoB in the context of differentiation. Tissue processing and immunostaining were performed as described under the legend to Fig. 1 and “Materials and Methods.” The figure shows two different head and neck squamous cell carcinomas (A and B) and (C and D). In A, areas of nonneoplastic (RhoB index: 80) squamous epithelium (top left) are shown next to a superficially invasive, well-differentiated (RhoB index: 26) region of the carcinoma (bottom right). RhoB, located in the nucleus of cells in the nonneoplastic epithelium, is predominantly cytoplasmic in the carcinoma. RhoB is markedly decreased in the poorly differentiated (RhoB index: 3) areas of the same tumor (B). A similar decrease in RhoB expression is observed in the poorly differentiated (RhoB index: 5) areas of the second tumor (D) compared with well-differentiated (RhoB index: 47) regions (C).

depicted in Fig. 1, showing the changes in protein levels and localization of RhoB along the successive oncogenic histological stages of head and neck squamous neoplasia from normal epithelium to carcinoma *in situ*, to superficially invasive carcinoma, to deeply infiltrating carcinoma. The specificity of the nuclear staining of nonneoplastic epithelium with the RhoB antibody (Fig. 1A) was ascertained by abolishing the staining in the presence of the immunizing specific peptide (Fig. 1B). RhoB nuclear expression was maintained in carcinoma *in situ* (Fig. 1C); however, in areas of transition of carcinoma *in situ* to invasive carcinoma, RhoB was progressively found in a cytoplasmic location in a higher number of tumor cells (Fig. 1D). Finally, in invasive carcinoma, RhoB could no longer be detected in the nucleus and its level in the cytoplasm was variable, and RhoB was often absent from the most deeply invasive areas of the tumor (Fig. 1, E and F).

Expression of RhoB in Squamous Cell Carcinoma Across Different Degrees of Differentiation. The results from Fig. 1 suggest that RhoB localizes in the nucleus in normal tissue and in the cytoplasm in tumors. To determine whether RhoB expression and localization is affected by the differentiation state of the tumors, RhoB expression was analyzed in tumors with different degrees of differentiation. Fig. 2 shows RhoB expression in two different tumors (Fig. 2, A and B from case 2 in Table 1; Fig. 2, C and D from case 18 in Table 1). Fig. 2A shows predominantly nuclear expression in overlying nonneoplastic epithelium and predominantly cytoplasmic RhoB expression in adjacent superficially invasive well-differentiated squamous cell carcinoma. The well-differentiated areas in both tumors (Fig. 2, A and C) show higher RhoB expression levels than the poorly differentiated areas in the same tumors (Fig. 2, B and D, respectively). The analysis in all 18 cases shows that

RhoB levels progressively decrease from well-differentiated areas (average RhoB index: 32), to moderately differentiated areas (average RhoB index: 12), to poorly differentiated areas (average RhoB index: 3.2). In well-differentiated areas, if decreased RhoB intensity is observed, it is restricted to deeply infiltrating areas. RhoB expression status was evaluated in 18 head and neck squamous cell carcinomas from different anatomical locations (Table 1). Cases were tabulated according to disease stage at presentation. Table 1 also lists the gender, age, status of the patient, RhoB indexes, and statistical analysis. A marked reduction in the staining intensity of RhoB was observed on all 18 cases studies. When compared with nonneoplastic tissue, carcinoma *in situ* shows a reduction of ~15%, the superficial component of the invasive carcinoma a reduction of 60%, and the deep component of the invasive carcinoma a reduction of 97%. Specifically, no significant differences in RhoB protein levels were found between nonneoplastic epithelium and carcinoma *in situ*. However, statistically significant differences ($P < 0.001$) were found when nonneoplastic epithelium ($78.5 + 11.5$) or carcinoma *in situ* ($66.5 + 9$) was compared with either superficial ($30.1 + 16$) or deeply invasive ($2.2 + 1.1$) carcinoma and across the three degrees of differentiation ($P < 0.001$). The levels of RhoB in superficial *versus* infiltrating areas are graphically represented in Fig. 3.

RhoB's amino acid sequence is 86% identical to that of the closely related family member RhoA. Therefore, we investigated whether the expression of RhoA was also altered in head and neck tumors. Sections from formalin-fixed, paraffin-embedded tissues were immunostained with antibodies to RhoB and to RhoA to evaluate the tissue expression of another Rho protein, Sp1, to evaluate the tissue status of a marker known to localize to the nucleus and Ki67 to evaluate cell proliferation. As shown

Table 1 Quantitation of RhoB expression in head and neck squamous cell carcinoma

The Optimas 6.5 Image Analysis software was used to quantitate nuclear RhoB expression in 18 squamous cell carcinomas containing superficial invasive component (SIC) and deeply invasive component (DIC) areas of the tumor, as well as in adjacent areas of carcinoma *in situ* (CIS) and nonneoplastic squamous epithelium. RhoB quantitation was also performed independently in well-differentiated (WD), moderately-differentiated (MD), and poorly-differentiated (PD) areas of the tumors. The intensity of RhoB staining is expressed as a RhoB index. The average and SD for each of the grouped areas are depicted at the bottom of the table. A Wilcoxon Rank-Sum test analysis showed significant differences ($P < 0.001$) between nonneoplastic epithelium, SIC, and DIC, between CIS, SIC, and DIC, and between SIC and DIC. No significant differences were found between nonneoplastic epithelium and CIS. Significant differences ($P < 0.0001$) were also observed among the three degrees of differentiation.

Case	Sex	Age (yrs)	Location	Size (cm)	TNM	Stage	Follow-up (mon)	Status	Nonneoplastic	CIS	SIC	DIC	WD	MD	PD
1	M	61	Pyriform sinus	2.0	T ₂ N ₀ M ₀	II	14	NED ^a	96	95	16	4	32	15	5
2	F	67	Oral cavity	2.6	T ₂ N ₀ M ₀	II	73	AED	80	87	7	2	26	4	3
3	F	76	Oral cavity	4.0	T ₂ N ₀ M ₀	II	18	AED	69	31	13	3	23	8	6
4	F	62	Larynx	3.1	T ₂ N ₀ M ₀	II	45	AED	70	42	50	4	46	6	5
5	M	70	Oral cavity	2.5	T ₂ N ₀ M ₀	II	35	NED	92	76	47	3	63	45	4
6	F	52	Oral cavity	3.5	T ₂ N ₁ M ₀	III	19	NED	72	30	27	0.5	29	3	1
7	M	77	Larynx	2.0	T ₃ N ₀ M ₀	III	21	NED	77	68	22	2	27	3	3
8	M	77	Larynx	3.7	T ₃ N ₀ M ₀	III	35	NED	87	52	19	1	23	4	1
9	M	42	Eyelid	4.2	T ₄ N ₀ M ₀	IV	14	NED	92	90	12	2	18	5	3
10	M	63	Larynx	4.1	T ₄ N ₀ M ₀	IV	16	NED	92	88	32	1	28	16	3
11	M	56	Tongue	6.6	T ₄ N ₁ M ₀	IV	36	DOD	73	62	43	1	64	8	2
12	F	82	Tongue	5.5	T ₄ N ₁ M ₀	IV	26	AED	82	69	17	1	34	7	1
13	M	54	Oropharynx	4.2	T ₄ N ₁ M ₀	IV	46	DOD	79	73	38	3	23	24	3
14	M	57	Larynx	3.1	T ₃ N _{2B} M ₀	IV	38	AED	77	70	43	1	13	18	4
15	M	69	Tongue	6.0	T ₃ N _{2B} M ₀	IV	12	NED	60	52	57	4	49	15	2
16	M	71	Pyriform sinus	8.0	T ₄ N _{2B} M ₀	IV	14	AED	66	67	18	1	10	9	0.5
17	F	69	Larynx	4.5	T ₄ N _{2C} M ₀	IV	10	NED	58	73	29	3	21	15	6
18	M	69	Lip	3.5	T ₄ N _{2C} M ₀	IV	12	NED	92	75	57	2	47	10	5
Average									78.5	66.5	30.1	2.2	32	12	3.2
SD									11.5	9.0	16.0	1.1	15.8	10.4	1.7

^a NED, no evidence of disease; AED, alive with evidence of disease; DOD, dead of disease.

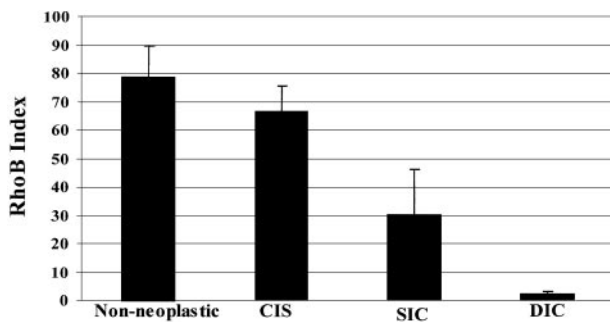


Fig. 3 Rho B expression in squamous cell carcinomas from 18 head and neck cancer patients. The average from Table 1 of RhoB index in nonneoplastic squamous epithelium (78.5 + 11.5), carcinoma *in situ* (CIS; 66.5 + 9), superficially invasive carcinoma (SIC; 30.1 + 16), and deeply invasive carcinoma (DIC; 2.2 + 1.1) is graphically represented.

before, in nonneoplastic epithelium, RhoB is present in the nucleus (Fig. 4A) and predominantly in the lower half corresponding to proliferating layers (Ki67 staining, Fig. 4G). While in invasive carcinoma, RhoB expression decreases (RhoB index 76 in Fig. 4A versus RhoB index 17 in Fig. 4B) and becomes predominantly cytoplasmic (Fig. 4B). In contrast to RhoB, RhoA localization is always cytoplasmic and its protein levels increased by 2-fold in invasive carcinoma as compared with nonneoplastic epithelium (index 87.16 versus index 43.23, respectively, Fig. 4, C and D). Expression of RhoA was further evaluated in 10 squamous cell carcinomas and adjacent nonneo-

plastic epithelium. RhoA was predominantly cytoplasmic and its levels were markedly increased in 80% of carcinomas compared with adjacent nonneoplastic epithelium. The expression status of the ubiquitous nuclear transcription factor Sp1 in carcinoma was similar to that in nonneoplastic epithelium (index 96 versus index 78, respectively, (Fig. 4, E and F), confirming that the lack of RhoB expression seen in the deeply invasive areas of tumors was not because of poor antigen preservation in these areas. In addition, levels of the proliferation nuclear antigen Ki67 were 5-fold higher in invasive carcinoma (index 71 in Fig. 4G) than in nonneoplastic epithelium (index 15 in Fig. 4H), again confirming that our immunohistochemical observations represent actual tumor status.

Analysis of RhoB Gene and mRNA in Head and Neck Squamous Cell Carcinoma. The low level or lack in RhoB protein levels in head and neck tumors could be attributable to either deletions or decrease expression of the *RhoB* gene. Therefore, we analyzed RhoB genomic DNA and RNA from 12 head and neck tumor samples that were previously examined for RhoB protein levels by immunostaining. Histone- and RhoB-specific primers were used to amplify genomic DNA. As shown in Fig. 5, only a 600-bp fragment corresponding to the full-length *RhoB* gene was present in the tissue samples examined, implying that there were no large *RhoB* gene deletions. However, this result does not exclude the possible occurrence of small deletions or point mutations, which cannot be detected by the PCR method used. Next, we analyzed RhoB RNA from these samples by RT-PCR using the same set of primers. Total RNA was reverse transcribed as described in "Materials and

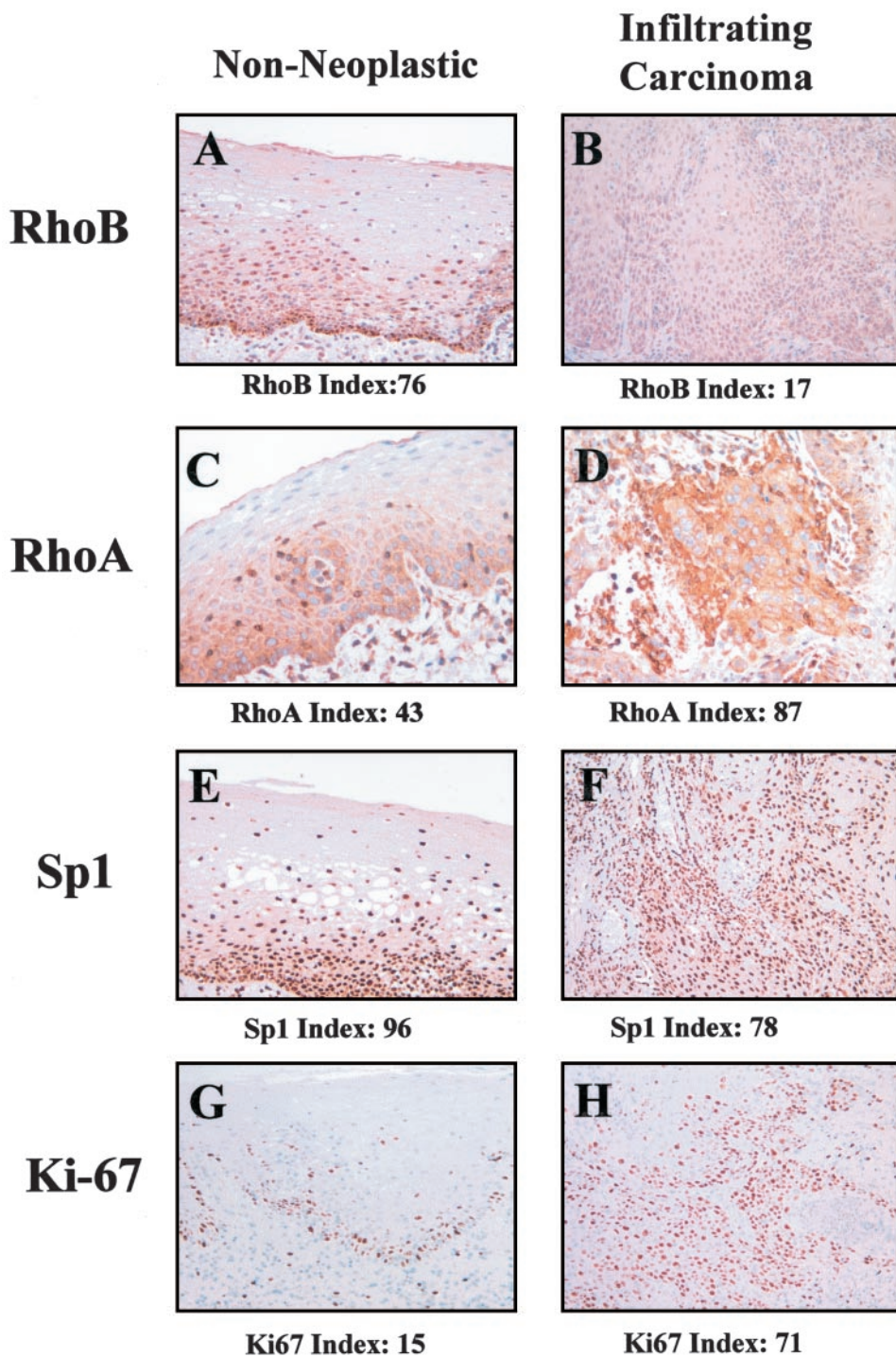


Fig. 4 Expression of RhoB (A and B), RhoA (C and D), Sp1 (E and F), and Ki67 (G and H) in nonneoplastic epithelium and deep regions of invasive carcinoma. Sections from formalin-fixed, paraffin-embedded tissues were processed and immunostained with anti-RhoB, RhoA, Sp1, and Ki67 antibodies as described in "Materials and Methods." RhoB is strongly expressed in the nuclei of cells from lower layers of squamous epithelium (index: 76.05) and with low intensity and only in rare cells in carcinoma (index: 17.22). RhoA is expressed in the cytoplasm of the lower two-thirds of the non-neoplastic epithelium (index: 43.23) and with increased intensity in the majority of carcinoma cells (index: 87.16). Sp1 is strongly expressed in the nuclei of cells from all layers in squamous epithelium (index: 96.75) and in the majority of carcinoma cells (index: 78.89). Ki67 is expressed in the nuclei of proliferating layers of non-neoplastic squamous epithelium and in the majority of the carcinoma cells (index: 71.03).

Methods," and the resulting cDNA was PCR amplified using primers specific for either the housekeeping gene, histone H3.3A, or RhoB. The histone H3.3A primers correspond to sequences in two different exons, thus, if genomic DNA contaminates a sample, an additional larger PCR fragment will be generated. However, RhoB primers will not allow the distinction of mRNA from genomic DNA because *RhoB* is an intron-

less gene. The RT-PCR experiments, using Sensiscript, for the reverse transcriptase reaction show that the PCR amplification with RhoB primers of tumor RNA without the addition of reverse transcriptase did not yield any bands (Fig. 5). In addition, RT-PCR reactions using histone primers yield similar levels of a single band in all of the tumor samples examined (data not shown). However, the level of RhoB RNA was low to

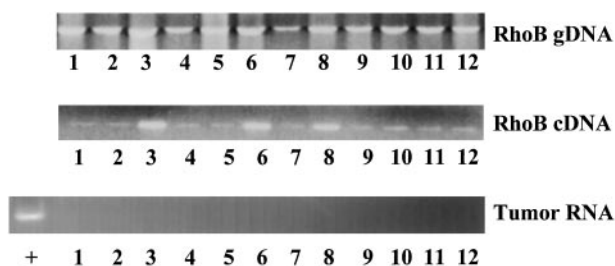


Fig. 5 Analysis of RhoB expression in genomic DNA and RNA from 12 patients with head and neck squamous cell carcinoma. Total RNA obtained from 12 head and neck squamous cell carcinomas were reverse transcribed as described in "Materials and Methods." The resulting cDNA along with genomic DNA were PCR amplified using primers specific for RhoB. Amplification of RhoB genomic DNA (*gDNA*) demonstrating that there are no deletions of the gene; RT-PCR reaction of tumor RNA, showing amplification of the *RhoB cDNA*; and PCR reaction using RhoB primers and *tumor RNA* (without the addition of reverse transcriptase) demonstrating that the RNA had no contaminating genomic DNA.

barely detectable in 9 of 12 (75%) of all tumors analyzed, suggesting that *RhoB* transcription may be down-regulated in the tumors. For the other 3 tumors, the absence of RhoB may be attributable to mechanisms other than decreased transcription such as decreased protein synthesis or increased protein degradation. The PCR products of all samples were sequenced using the RhoB PCR primers and an additional set of internal primers to the *RhoB* gene that allowed us to sequence the RhoB gene (bp 1–591) in both directions. Our sequence analysis did not reveal any mutations. However, it could be argued that in some tumors a mutation or deletion of the gene could be masked by contamination with normal tissue. The best way to avoid this problem would be to isolate our genetic material using laser capture microdissection of the areas of interest. However, for our analysis we used archived paraffin blocks, which unfortunately are not amenable to these procedures. Future prospective studies will include the collection of the paraffin blocks under conditions that would conserve the RNA. Taken together, our data suggest that the decrease in RhoB expression could be attributable to either transcriptional down-regulation or increased degradation of the mRNA or protein. The data also suggest that genetic alterations are most likely not involved.

DISCUSSION

The ability of RhoB to reverse the phenotype of Ras-transformed cells, inhibit human tumor growth in nude mice, and delay DNA replication or induce apoptosis in response to DNA damaging drugs suggests that RhoB may play a tumor suppressor role (7, 8, 11, 12, 15). However, the evidence in support of this hypothesis is limited to cell culture studies and animal models. Studies in cancer patient tumors are lacking. We reasoned that if RhoB contributes to tumor suppression, then its expression levels should be decreased in malignancy. To test this hypothesis, we evaluated the status and subcellular localization of RhoB protein in tumors from head and neck cancer patients showing the histopathological changes from normal epithelium to invasive squamous cell carcinoma. This analysis

of RhoB expression in 18 head and neck squamous cell carcinomas showed no significant differences in RhoB levels between nonneoplastic epithelium and carcinoma *in situ*. However, significant differences were found when nonneoplastic epithelium or carcinoma *in situ* was compared with either superficial or deeply invasive carcinoma ($P < 0.001$). Significant differences were also observed along the three degrees of differentiation. These results suggest that the absence of RhoB may have contributed to tumor progression and aggressiveness.

The absence of RhoB in advanced tumors was not because of deletion or mutation of *RhoB* gene, but rather it appears that RhoB may have been either transcriptionally down-regulated or its mRNA became unstable. In addition to its low abundance in advanced tumors, RhoB mislocalized to the cytoplasm. Indeed, in nonneoplastic epithelium, RhoB was present predominantly in the nucleus but, as the tumors progress to invasive carcinoma, RhoB was mostly present in the cytoplasm at levels varying from low to null in the most deeply invasive areas of the tumor. Consistent with our results, showing a nuclear presence of RhoB, is the association of RhoB with the transcription factor DB1 and the nuclear membrane, and its presence in an intranuclear laminar region (16). Thus, it seems that in normal cells, RhoB would translocate to the nucleus where it performs its function but, as cells progress toward a malignant phenotype, RhoB loses this ability to translocate to the nucleus and remains mainly in the cytoplasm. RhoB does not possess a nuclear localization signal, and it is unclear how RhoB is transported to the nucleus.

In contrast to RhoB, RhoA was predominantly cytoplasmic and its level increased by a 2-fold in 80% of carcinomas. This is in agreement with a previously reported study in which RhoA expression was shown to be up-regulated in colon and lung tumors and in breast tumors progressing from WHO grade I to grade III (17). Furthermore, the analysis of expression of RhoB and RhoA in 60 human cancer cell lines of the National Cancer Institute showed that RhoA was expressed in all cell lines, whereas RhoB protein was barely detectable.⁵ Thus, despite their high sequence homology, RhoA and RhoB appear to play distinct roles in the cell. In addition to differences in cellular localization, RhoB has several features that distinguish it from RhoA. Most notably, RhoB, but not RhoA, is inducible by a variety of stimuli, including growth factors, UV, and DNA-damaging agents (7, 18), and unlike RhoA, both RhoB mRNA and protein are labile with half-lives of ~20 min and 2 h, respectively. RhoB turnover occurs via ubiquitin-mediated destruction by the 26 S proteasome (19). The transcriptional induction and the short half-lives of RhoB, together with its posttranslational modifications (isoprenylation) and GTP/GDP binding allow the regulation of RhoB function at several levels and suggest that RhoB may be a component of a regulatory pathway(s).

Whereas in normal epithelium and in most of the well-differentiated areas, RhoB was predominantly nuclear, in moderately and poorly differentiated regions of the tumors, RhoB localized to the cytoplasm and its expression decreased dramati-

⁵ M. Blaskovich and S. Sebt, unpublished data.

ically. These results suggest that RhoB function may be involved in cellular differentiation. Interestingly, RhoB was previously cloned as a gene induced by Bone Morphogenetic Proteins during the early differentiation of neural crest cells, suggesting a role of RhoB in differentiation in response to Bone Morphogenetic Proteins signaling (20). Moreover, transforming growth factor β was shown to exert a stabilizing influence on RhoB, thereby resulting in its accumulation (19).

Mounting evidence points to a role of RhoB in tumor inhibition. Indeed, accumulation of geranylgeranylated RhoB, after treatment of cancer cells with farnesyltransferase inhibitors, has been suggested to be the event mediating the antitumor effect of farnesyltransferase inhibitors (10). In addition, under *in vitro* conditions, overexpression of RhoB results in inhibition of transformation, phenotypic reversion, and cell growth inhibition (11, 12). The data described in this study demonstrating a decrease of RhoB expression during tumor progression and the lack of expression of RhoB in deeply infiltrating carcinoma gives additional support for the tumor-suppressive activity of RhoB.

To summarize, our findings indicate that RhoB expression and localization become altered during tumor progression. On the basis of these studies, our results support the view that a decrease in RhoB expression is a critical event in malignant transformation and/or progression of human cancer cells. The molecular basis for this decrease in RhoB expression remains to be elucidated.

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