

Relationship Between RKIP Protein Expression and Clinical Staging in Thyroid Carcinoma

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Abstract: Raf-1 Kinase Inhibitory Protein (RKIP) belongs to a new class modulators of signalling cascades involved in maintaining of biological balance in many living organisms.

RKIP targets and inhibits different kinases (Mitogen Activated Protein (MAP) kinase, G- protein coupled receptor (GPCR) kinase and nuclear factor kappa B (NF-κB) signaling pathways), and its loss is associated with progression of many solid cancers and development of metastasis. In this study we analyzed RKIP expression in follicular, papillary and undifferentiated thyroid carcinoma tissues from 54 patients by western blotting.

We found that significantly reduced or lost RKIP expression was associated with lymph node (30 of 39 N1 tumors, 76.9%) and distant metastases (17 of 20 M1 tumors, 85%). No significant correlations were observed between RKIP expression and T-category, age, gender, histological type or tumor differentiation grade. Our results indicate that reduction of RKIP expression is a highly predictive factor for lymph node and distant metastasis in patients with thyroid carcinoma.

Keywords: RKIP, PEBP, thyroid carcinoma, MAP kinase.

INTRODUCTION

Thyroid carcinoma is the most common malignancy of the endocrine system and it includes follicular, papillary and undifferentiated thyroid tumors. Patients suffering from differentiated thyroid carcinomas such as papillary (PTC) and follicular (FTC) are generally well treatable and usually curable. Papillary thyroid carcinoma may occur at all ages, often with an optimistic prognosis and survival rates of more than 90%. Follicular thyroid carcinoma predicts more aggressive behaviour with recurrences or/and distant metastases to liver, lung and bones. Undifferentiated thyroid tumors (UTC), also termed, anaplastic, are much less common, metastasize early, and have a much poorer prognosis with a 5 years survival rates lower than 5% [1-4]. The majority of patients die due to metastatic disease. The list of tumor markers with prognostic or predictive values has been considerably increasing [5], however, only few of them correlate well with the presence of metastases in thyroid carcinoma [6-11].

RKIP was shown to be a critical down-regulator of MAP kinase signalling (Raf-MEK-ERK) and alteration of this pathway is involved in about 30% of all cancers [12]. Additionally to Raf-1 inhibition, RKIP is able to block the activation of NF-κB and GPCR kinase [13, 14]. Loss or reduction of RKIP has previously been shown to be associated with

metastasis of melanoma, prostate, colorectal, breast and ovarian cancers [15-20]. In this study we investigated the expression of RKIP in thyroid carcinomas.

MATERIALS AND METHODOLOGY

Thyroid tissues. A total of 54 primary thyroid tissues, including 23 follicular thyroid carcinomas (FTCs), 18 papillary thyroid carcinomas (PTCs) and 13 undifferentiated thyroid carcinomas (UTCs), were collected from patients operated on at the Department of Surgery, University of Halle. Tissues selected for RKIP analysis were primary tumors with complete clinicopathological data and TNM status (Table 1). Tumor tissues were staged according to the tumor-node-metastasis (TNM) staging classification (UICC-AJCC 1997). This study was approved by the ethical committee of the Martin Luther University, Faculty of Medicine, and all patients gave written consent.

Protein extraction. Resected thyroid tissues were immediately frozen in liquid nitrogen, homogenized and incubated in lysis buffer prepared by mixing 1.5 ml 5M NaCl, 1 ml 1M Tris/HCl pH 7.5, 50μl 0.5M EDTA pH 8.0, 50μl 0.5M pH 7.5 and 250μl Triton X-100 stock solutions, diluted with Aquabidest to 50ml. Protease inhibitors were added to obtain following end concentrations: 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml apoprotinin, and 1 μg/ml leupeptin. Protein lysates were incubated on ice for 15 min., followed by centrifugation twice at 16000 x g for 45 min. at 4°C. The supernatant was saved for protein analysis.

Western blot analysis. Protein extracts were separated on 12% polyacrylamide gels and blotted on PVDF membranes

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Table 1. Clinical Characteristic of Patients and the Protein Expression of RKIP

Histological Diagnosis	pTNM Status	Gender (F-Female, M-Male), Age (Years)	RKIP Signal Intensity (%)
FTC	pT4M1N1	F 61	0
FTC	pT4M1N1	F 72	18
FTC	pT4M1N1	M 45	61
FTC	pT3M1N1	M 72	46
FTC	pT4M1N1	M 65	0
FTC	pT3M0N1	F 52	87
FTC	pT3M0N1	M 49	113
FTC	pT4M0N1	M 54	118
FTC	pT2M1N1	F 67	0
FTC	pT3M1N1	M 58	37
FTC	pT4M0N0	F 43	124
FTC	pT4M0N0	F 36	120
FTC	pT4M0N0	F 52	138
FTC	pT3M0N0	M 64	132
FTC	pT4M0N1	F 41	9
FTC	pT2M1N1	M 63	127
FTC	pT3M1N1	M 52	22
FTC	pT3M0N0	F 77	36
FTC	pT3M0N0	M 61	67
FTC	pT4M1N0	F 82	29
FTC	pT3M1N0	F 74	5
FTC	pT2M1N0	F 68	8
FTC	pT4M1N1	F 71	0
PTC	pT2M0N1	F 39	62
PTC	pT3M0N0	M 57	130
PTC	pT4M1N1	F 26	65
PTC	pT1M0N0	M 55	130
PTC	pT4M0N1	M 66	116
PTC	pT2M0N0	F 15	122
PTC	pT2M0N1	M 53	16
PTC	pT4M0N1	F 15	33
PTC	pT4M0N1	F 15	119
PTC	pT2M0N1	M 33	0
PTC	pT4M0N1	F 75	113
PTC	pT4M0N1	M 56	53
PTC	pT4M0N1	M 16	41
PTC	pT4M0N1	M 14	61
PTC	pT4M0N1	F 82	46
PTC	pT4M0N1	F 16	38
PTC	pT4M0N1	F 17	37
PTC	pT4M0N1	F 26	0
UTC	pT4M1N1	M 68	9
UTC	pT3M1N0	M 28	53
UTC	pT3M1N1	M 63	0
UTC	pT4M0N0	F 71	126
UTC	pT3M0N1	M 63	22
UTC	pT4M1N1	M63	116
UTC	pT4M0N1	F 86	26
UTC	pT4M0N1	F 70	34
UTC	pT4M1N0	F 49	101
UTC	pT3M0N1	M 57	104
UTC	pT4M0N1	F 42	45
UTC	pT4M0N1	F 71	37
UTC	pT4M1N1	F 88	59

(Amersham Biosciences). Blocking was performed in 5% BSA in 1xTBS /Tween20 (0.1%) for 1h. After 3x washing with 1xTBS/Tween20, the membranes were incubated overnight with RKIP antiserum (#4742, 1:1000, Cell Signaling, Danvers, MA, USA), in blocking buffer. After washing steps, the secondary goat anti-rabbit serum (sc-2004, 1:20000, Santa Cruz Biotechnology, Heidelberg, Germany) was used. Specific protein bands were visualised by enhanced chemiluminescence on Amersham Hyperfilms (GE Healthcare, Buckinghamshire, UK) using the Pierce ECL Western Blotting Substrate (Pierce Biotechnology, Rockford, USA). Protein expression of RKIP was evaluated semi-quantitatively. Each western blot was scanned using desktop scanner HP 7400c (Hewlett Packard) and signals of RKIP intensity were measured using Quantity One software (Bio-Rad, Hercules, USA). Each band representing RKIP protein expression was evaluated in comparison to the positive control set as 100%. β -actin was used as an internal control. Patient tissues were classified into three groups according to the level of RKIP expression. One group included cases that showed no or very weak RKIP expression (signal intensity between 0-30%) and the other were composed of cases that showed reduced (signal intensity 30-70%) or normal RKIP (signal intensity over 70%).

Statistical analysis. Independent sample t and Kruskal-Wallis tests were used to compare the association between RKIP expression level and clinicopathological factors. All of the statistical tests were two-sided. Box plots were made employing SPSS software. The p values below 0.05 were considered to be statistically significant.

RESULTS

Expression of RKIP in thyroid carcinoma tissues. We analyzed the expression of RKIP in 54 thyroid carcinoma patients employing western blotting (Fig. 1). According to our criteria, RKIP was found to be very weak and reduced (signal intensity below 70%) in 36 of 54 cases analyzed (66.7%). We found no significant age and gender differences among patients with normal and reduced RKIP expression. Also no significant associations were found between down-regulation of RKIP and histological differentiation or tumor stage. Although the above-mentioned differences were not significant, patients with low or reduced RKIP expression bore more T4 tumors at the time of surgery than did patients without RKIP down-regulation (22 of 32 cases, 68.8%). Out of 35 patients with decreased RKIP expression; 22 of them bore T4 tumors (62.9%). Out of 19 patients with normal RKIP level, 10 suffered from T4 tumors (52.6%, Table 2).

RKIP expression is significantly reduced in metastatic thyroid carcinoma. As shown in Table 1, patients who developed lymph node or distant metastases, revealed reduced RKIP expression in 30 of 39 N1 cases (76.9%) and in 17 of 20 M1 cases (85%). Analysis of all RKIP signal intensities in various groups of patients with lymph node and/or distant metastases was evaluated employing box plot. We found that RKIP expression was highly reduced in all cases with either lymph node or distant metastases when compared to N0 or M0 cases, respectively (Fig. 2a, p<0.05; Fig. 2b, p<0.05). Comparison of lymph node and distant metastases negative (N0M0) patients with N1M0, N0M1 or N1M1 patients, also revealed significantly decreased RKIP expression in each

Table 2. Analysis of Patients with Thyroid Carcinoma According to the Expression Status of RKIP

	All Patients	Expression of RKIP			P Value
		Low (Signal Intensity 0-30%)	Reduced (Signal Intensity 30-70%)	Normal (Signal Intensity >70%)	
Age (mean ± SD, years)	53.2 ± 20.6	61.4 ± 16.1	43.2 ± 24.1	51.7 ± 17.0	NS
Gender					
Male	24	7	8	9	NS
Female	30	10	9	11	
Histological differentiation					
FTC	23	10	5	8	NS
PTC	18	3	9	6	
UTC	13	4	5	4	
T-classification					
T1	1	0	0	1	NS
T2	7	4	1	2	
T3	14	4	4	6	
T4	32	9	13	10	
Nodal involvement					
N0	15	3	3	9	<0.05
N1	39	14	16	10	
Metastatic status					
M0	34	6	14	14	<0.05
M1	20	11	6	3	

comparison group (Fig. 2c, $p < 0.05$). Comparisons within the metastatic groups (N1M0, N0M1, and N1M1) demonstrated no significant differences, however, the most reduced RKIP expression was observed in the metastatic group N1M1.

DISCUSSION

This is the first report demonstrating the association of RKIP with metastases of thyroid carcinoma. We show in this study that RKIP expression is significantly reduced in patients suffering from lymph node and/or distant metastases. RKIP has been identified as a growth suppressive gene and its expression was diminished or lost in metastases of melanoma, prostate, colorectal, breast and ovarian cancers [15-20]. Reduction of RKIP was also reported for insulinomas and hepatocellular carcinoma tissues [21, 22]. In patients with gastric adenocarcinoma, decreased expression of RKIP correlated positively with 5-year survival rates [23].

We demonstrated RKIP as a crucial inhibitor of MAP kinase pathway involved in thyroid pathogenesis. Previous studies reported several other MAP kinase effectors essential for progression of thyroid carcinoma, especially PTC. The most important are the tyrosine receptor kinase RET and the intracellular signalling effectors RAS and BRAF. It was shown that mutations in genes encoding these factors, lead to constitutive activation of MAP kinase signalling [24-26].

The specific actions involved in RKIP regulation of Raf signalling are not entirely clear. It has been shown that over-

expression of RKIP can inhibit MEK-Raf-1 or MEK-B-Raf interaction [27]. By using siRNA, it has been demonstrated that Raf-1 activation was blocked by preventing phosphorylation at serine 338 and tyrosine 341. Interestingly, activation of B-Raf was not regulated by RKIP [28]. Depletion of RKIP in embryonic fibroblasts from double knockout *Raf-1^{-/-}* mouse caused an increase of B-Raf mediated MEK activity. These results suggest that RKIP can regulate B-Raf independently of Raf-1 [29].

With regard to thyroid tissues, growth-suppressive function of RKIP was demonstrated for undifferentiated thyroid carcinoma only [30]. In that study RKIP expression in UTC tissues was reduced as compared to papillary carcinoma and normal thyroid tissues. However that study did not include follicular thyroid carcinoma tissues and no relation between RKIP expression and metastases of thyroid carcinoma was shown. Our results clearly demonstrated the correlation between the reduction of RKIP and the development of lymph node and distant metastases. The diminished expression of RKIP in both differentiated and undifferentiated thyroid tissues may serve as a risk marker for thyroid carcinoma.

CONCLUSION

Since RKIP was independent of other clinical parameters, such as histological type, tumor differentiation grade and size, this study fully supports our conclusion that RKIP acts as a tumor suppressor gene that influences metastasis in thyroid carcinoma.

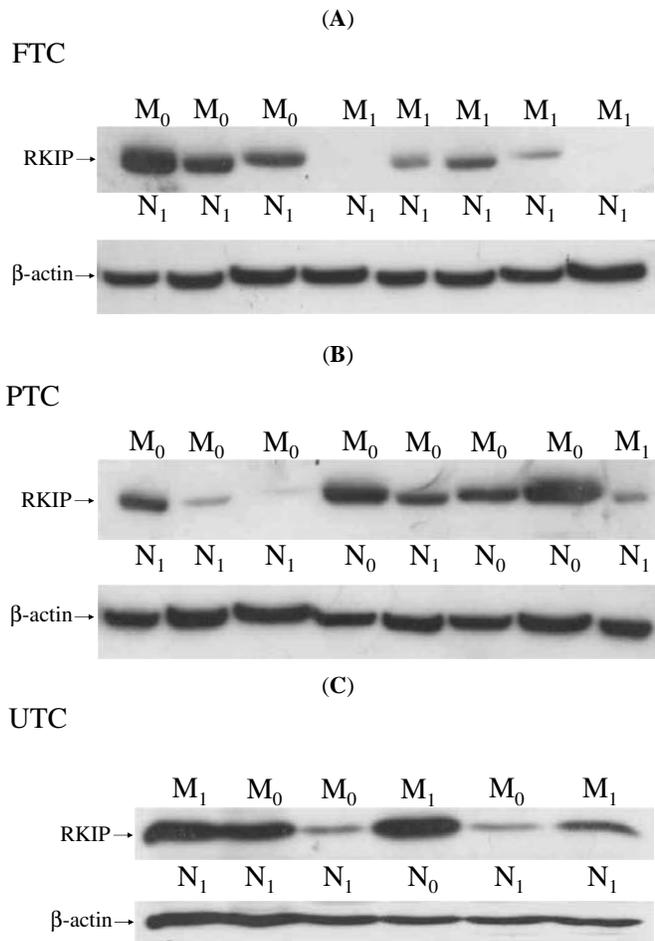


Fig. (1). Western blot analysis of RKIP expression in (A) follicular, (B) papillary and (C) undifferentiated thyroid carcinoma. RKIP is frequently down-regulated in patients who developed lymph node or/and distant metastases. N₀M₀-patients without lymph node and distant metastases; N₀M₁-patients without lymph node and with distant metastases; N₁M₀-patients with lymph node, but without distant metastases; N₁M₁-patients with lymph node and distant metastases; β-actin served as normalizing marker and internal control of protein loading.

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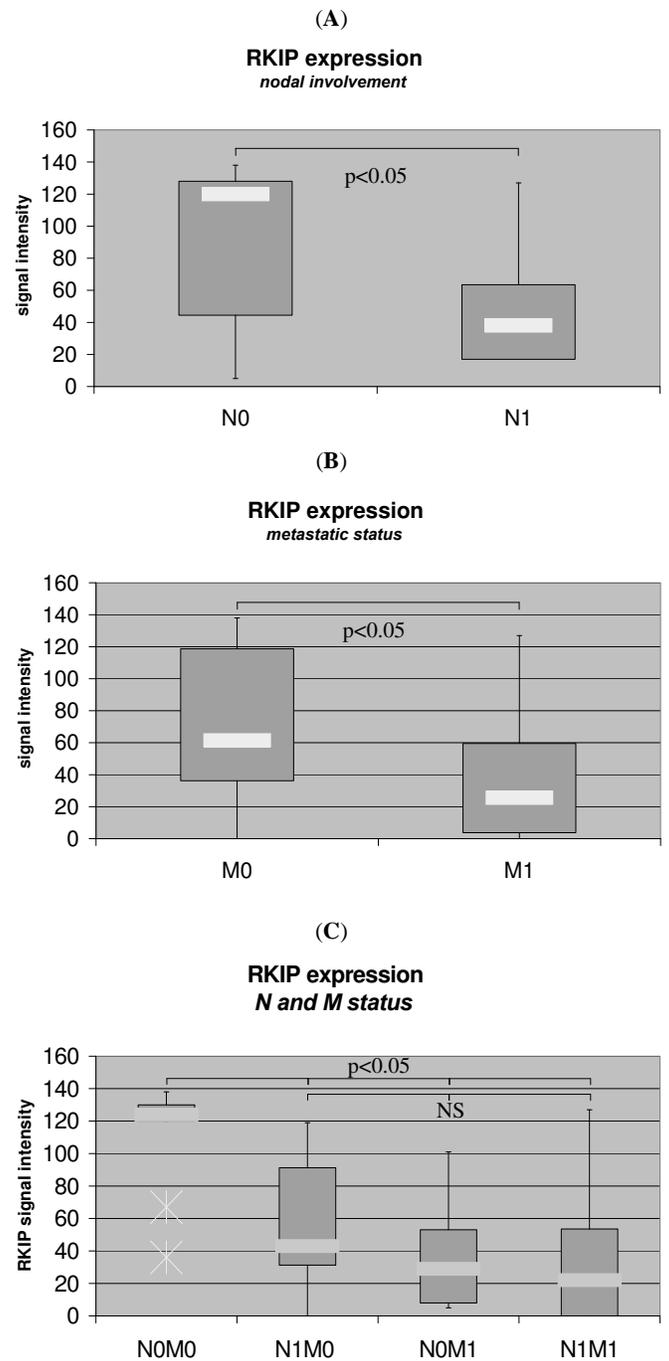


Fig. (2). Evaluation of RKIP protein expression according to lymph node and distant metastases status. Analysis of RKIP were performed according to (A) N status only (15 N₀ and 39 N₁ cases), (B) M status (34 M₀ and 20 M₁ cases) only and (C) both N and M parameters simultaneously (10 N₀M₀, 24 N₁M₀, 5 N₀M₁, and 15 N₁M₁ cases); p values <0.05 were considered as statistically significant.

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