Significance of Methylation Status and the Expression of *RECK* mRNA in Lung Tissue of Patients with NSCLC

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Abstract. *Objectives: RECK (reversion-inducing cysteine-rich protein with Kazal motifs) is a glycoprotein which negatively regulates the activity of matrix metalloproteinases (MMPs). We analyzed differences in RECK mRNA expression in histological types of non-small cell lung cancer (NSCLC) and the relationship between promoter methylation status of RECK gene, level of RECK mRNA expression and clinicopathological values of patients with NSCLC. Patients and Methods: Methylation status of the promoter and the expression of RECK mRNA were analyzed in paired tissue samples (tumor and control) of 50 patients with NSCLC. The methylation status of the RECK promoter was assessed using methylation-specific PCR. The level of RECK mRNA expression was measured using an RT real-time PCR method. Results: Lower expression of RECK mRNA in NSCLC tissue was recorded compared to normal tissue (p=0.0032). Significantly lower expression of RECK in squamous cell carcinoma (SCC) tissue was observed in comparison with adenocarcinoma tissue (p=0.0051). Significant differences in expression of RECK in stages IB-IIIA were found in comparison with stage IA (p=0.0455). There was a significantly lower expression of RECK mRNA in NSCLC tissue in samples with positive RECK promoter methylation status in comparison with samples with negative promoter methylation status (p=0.0400). Conclusion: We showed that there were differences in expression between histological types of NSCLC (SCC, adenocarcinoma). There was a higher expression of RECK in stage IA in comparison with stages IB-IIIA. Our results indicate that RECK could be classified as a tumor suppressor gene and is an interesting target for further investigation of MMP inhibitors.*

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RECK (reversion-inducing cysteine-rich protein with Kazal motifs) is a membrane anchored glycoprotein which regulates matrix metalloproteinases (MMPs) and inhibits angiogenesis. Recent analyses indicate that RECK expression is frequently down-regulated in tumor tissues in comparison with the surrounding non-tumorous tissues in several common types of cancer including colon, mammary, and pancreatic carcinoma (1). We focused on RECK expression in lung cancer, which is the leading cause of cancer-related mortality, not only in the Czech Republic, but also around the world (2, 3).

RECK protein is able to inhibit MMP-2, MMP-9 and MMP-14. Reduction of active MMP-2 is probably due to direct inhibition of its processing enzymes, MMP-14 and also MMP-2, by RECK: purified recombinant RECK was found to inhibit the proteolytic activities of MMP-2 and MMP-14 *in vitro*. The mechanism for the reduction of pro-MMP-9 is less clear. Pro-MMP-9 production is probably halted at some point between transcription and secretion (1). The effect of RECK in tumorigenesis is realized mainly through the relationship with MMPs (1). MMPs not only degradate the extracellular matrix (ECM) components and basal membranes, but they also influence changes in the growth, apoptosis, and migration of healthy cells. Through remodelling or destruction of the ECM, MMPs contribute to processes of tumor cells migration (4).

The decreased inhibition of active MMPs is trigged by the down-regulation of RECK expression by K-ras (5). Two mechanisms involving K-ras were described. The first is mediated by the methylation of CpG islands in the *RECK* promoter. Oncogenic RAS increases the binding of DNA methyltransferase DNMT3b to the promoter of *RECK* and this binding induces promoter methylation, which could be reversed by DNMT3b small interfering RNA (siRNA) (6). The second mechanism of this RECK down-regulation is *via* the target SP1 site on the *RECK* promoter sequence and appears to be multifactorial and also tumor specific. This theory supposes that Ras facilitates the phosporylation or other modification of Sp1/Sp3 factors which increases binding to the SP1 site in the promoter region of *RECK* gene

and thus decreases the transcription activity of *RECK*. This theory was confirmed by the results of Chang *et al*. (7). The next possible regulatory mechanism of the expression of RECK was published by Zhang *et al*., which showed that RECK was a *bona fide* target of miR-21 (8).

We investigated differences in *RECK* mRNA expression in histological types of non-small cell lung cancer (NSCLC). We further analyzed the relationship between promoter methylation status of *RECK* gene, level of *RECK* mRNA expression and clinicopathological values of patients with NSCLC.

Patients and Methods

Patients. We studied a group of 50 patients with NSCLC (median age of 62.4 years, range 47.5-77.8, stage IA 12 (24%) and stages IB – IIIB 38 (76%)), who had undergone lung surgery at the Department of Surgery, University Hospital Pilsen, between 2005-2007.

Tissue samples. Fifty paired (tumor and control) lung tissue samples were taken directly from tumor tissue and from the adjacent, histologically cancer-free lung tissue (normal lung tissue) in the same patient during surgery. These resected tissue samples were immediately frozen to –70˚C and stored at this temperature until use. All the samples were histologically verified. The distribution according to histology is shown in the Figure 1.

Assesment of the methylation status of the RECK promoter. DNA was isolated from approximately 20 mg of 50 tumor lung tissue samples using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). Genomic DNA conversion was performed using EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA). DNA after conversion was used for analyses of the methylation status of the *RECK* promoter using methylation-specific PCR. The sequence of primers taken from the publication of Chang *et al*. was modified (9). The following primers were used for the methylated sequence: M-sense 5'-AATAAAGAGTTTTGGTACGGGGTAC-3'; M-antisense, 5'-AAAACCGCGAAATACTCGAA-3' and for the unmethylated sequence: U-sense 5'-TAAAGAGTTTTGGTAT GGGGTATGT-3'; U-antisense 5'-CTCCAAAACCACAAAATA CTCAAA-3', synthesized by GeneriBiotech (Hradec Kralove, Czech Republic).

Quantitative estimation of mRNA using RT real-time PCR. Total RNA was isolated from 100 mg of 50 paired control and tumor lung tissue. We used the fast RNA Pro Green Kit (Q-BIOgene, Irvine, CA, USA). Reverse transcription (RT) was performed from 3 μg of total RNA with Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) and oligo $d(T)_{21}$ as a primer. The sequence of primers used for *RECK* was as follows: forward primer 5'-ATCATTCCCGTCGATCACTATC-3'; reverse primer 5'-ATATGTCC AGAGCAAGTGCAAG-3' synthesized by GeneriBiotech (Hradec Kralove, Czech Republic).

In all the samples, we also assessed the expression of mRNA of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The real-time PCR procedure and the sequence of *GAPDH* primers were described in our previous publication (10). The results are presented as normalized values, the ratio of the number of copies of *RECK* to the housekeeping gene *GAPDH*. Statistical analysis was performed using software SAS 8.02 (SAS Institute Inc., Cary, NC, USA). The statistical results were calculated by a Wilcoxon two-sample test. For the maximum hazard ratio (disease-free interval, DFI; overall survival, OS) the Cox regression hazard model was used. After finding the optimal cut-off for the examined markers, the Kaplan-Meier survival distribution functions of this optimal cut-off in given groups were computed.

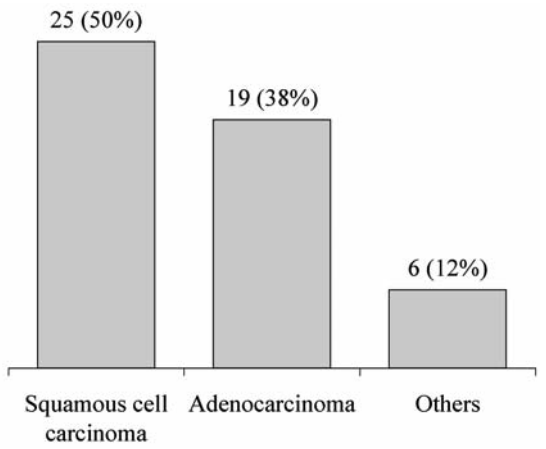

Figure 1. *The distribution of carcinoma tissues according to histological type*.

Results

We investigated differences in the expression of *RECK* mRNA in NSCLC and normal lung tissue. We found a statistically significant lower expression of *RECK* in tumor tissue in comparison with normal lung tissue (p=0.0032). We recorded a statistically significant lower expression of *RECK* in squamous cell carcinoma (SCC) tumor tissue in comparison with normal lung tissue (p=0.0003), but we did not observe differences between adenocarcinoma tissue and normal lung tissue (p=0.3208) (Table I). Futhermore we recorded a significantly lower expression of *RECK* in the SCC tumor tissue in comparison with the adenocarcinoma tissue (p=0.0051) (Figure 2).

We also observed a significantly higher expression of *RECK* in stage IA in comparison with IB-IIIA (p=0.0455) using the median two-sample test (Table II).

The samples of tumor tissue tested positively on promoter methylation status expressed a statistically significant lower level of *RECK* mRNA in comparison with unmethylated *RECK* promoter tumor tissue samples (p=0.0400) (Figure 3).

We found no statistically significant relation between the expression of *RECK* and clinicopathological values (DFI, OS) in all studied groups (NSCLC, SCC and adenocarcinoma), hence the p-values are not presented here. We recorded no statistical significance in the relation between RECK promoter methylation status and DFI and OS in all compared groups

Table I. *Differences in expression of RECK mRNA.*

N	Tissue	Median expression *RECK/GAPDH*	Wilcoxon *p*-Vaue
50	NSCLC	0.0397797	0.0032
49	Control	0.1102843	
25	SCC	0.0297737	0.0003
24	Control	0.1043990	
19	Adenocarcinoma	0.1138185	0.3208
19	Control	0.1359807	
25	SCC	0.0297737	0.0051
19	Adenocarcinoma	0.1138185	

Table II. *Difference between expression of RECK in stage IA and IB-IIIA.*

N	Stage	Median expression *RECK/GAPDH*	Two-sample test
11	IA	0.0543656	*p*=0.0455
39	IB-IIIA	0.0338051	

Table III. *Relation of DFI and OS to RECK promoter methylation status (only the patients with obtained DFI and OS data were included).*

Studied groups	DFI			OS		
	Methylation status		Log-rank *p*-value	Methylation status		Log-rank *p*-value
	–	+		–	+	
NSCLC	23	22	0.0869	24	24	0.4275
SCC	11	12	0.2878	11	13	0.7679
Adenocarcinoma	11	5	0.5682	12	6	0.6575

(NSCLC, SCC and adenocarcinoma) (Table III). It is interesting to note the *p*-value of 0.0869 for the relation between the *RECK* promoter methylation status and DFI in NSCLC group.

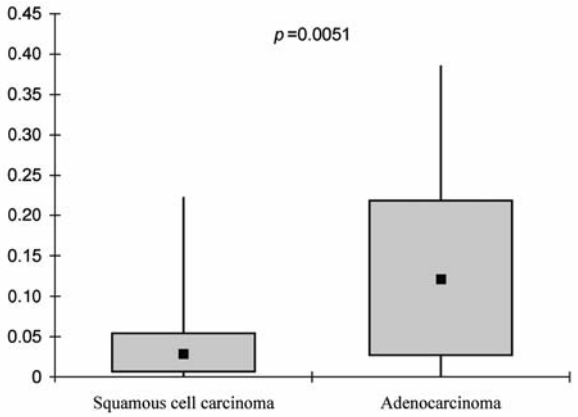

Figure 2. *Difference between expression of RECK in SCC and adenocarcinoma tumor tissue. There was a significantly lower expression of RECK in the SCC tumor tissue in comparison with the adenocarcinoma tissue. Minimum and maximum (line), lower and upper quartile (rectangle) and median (small square) values are shown.*

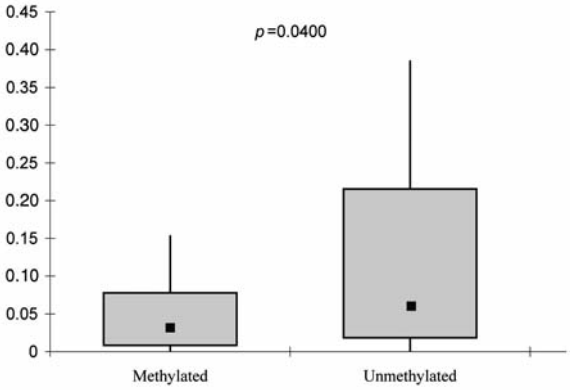

Figure 3. *Relation between methylation status and expression of RECK in NSCLC tissue. The samples with positive promoter methylation status (24 patients) expressed a statistically significant lower level of RECK mRNA (median of expression 0.0283) in comparison with tumor tissue samples unmethylated RECK promoter (24 patients; median of expression 0.0560). Minimum and maximum (line), lower and upper quartile (rectangle) and median (small square) values are shown.*

Discussion

The MMPs are enzymes which are involved in many processes associated with tumor growth and metastasis, *e.g.* angiogenesis, degradation of the extracelular matrix and basal membrane during tumor enlargment and invasion (11, 12). Therefore molecules regulating their expression and function attract the attention of investigators, for instance as a potential target of anticancer therapy (11, 13). Previous investigation of tissue inhibitors of MMPs has identified the molecule metallopeptidase inhibitor 1 (TIMP-1) as a promising prognostic marker and the study of other molecules with a strong effect on the function on MMPs continues (14-17).

Another molecule which inhibits MMPs (MMP-2, MMP-9, MMP-14) is RECK. We investigated expression of *RECK* mRNA and the methylation status of *RECK* gene promoter in NSCLC. The expression of *RECK* mRNA is decreased in NSCLC tissue in comparison with adjacent cancer-free lung tissue. In contrast, to results of Chang *et*

al. (9), we recorded a lower expression of *RECK* in SCC tissue in comparison with normal lung tissue, but we did not register differences between adenocarcinoma tissue and normal lung tissue. Futhermore, we observed lower expression of *RECK* in the SCC tumor tissue in comparison with the adenocarcinoma tissue. This shows that these histological types are different in *RECK* expression. Expression of RECK in NSCLC was also investigated by Takemoto *et al*., but these investigators did not find differences in expression in histological types (SCC, adenocarcinoma). The same authors did describe a much higher expression of MMP-9 in SCC than in adenocarcinoma (18). Taken together with the results of Takagi *et al*. that RECK negatively regulates MMP-9 transcription (19), this corresponds with our finding that *RECK* expression is lower in SCC tissue.

Next we investigated whether the expression of *RECK* in NSCLC depends on the stage of the disease. We compared stage IA (better prognosis) and stage IB-IIIA (worse prognosis). We observed higher expression of *RECK* in stage IA in comparison with stages IB-IIIA. Takemoto *et al*. observed similar results, but only in adenocarconoma of the lung (18). According to these results, we supposed that patients having higher expression of *RECK* in tumor tissue would achieve longer DFI and OS, but we did not confirm this idea, neither in NSCLC nor in subgroups (SCC, adenocarcinoma). Nevertheless Takemoto *et al*. recorded a relationship between lower *RECK* expression and shorter survival in patients with adenocarcinoma (18). Assessing the RECK protein (20, 21), Takenaka *et al*. observed a higher 5-year survival rate for patients with tumors with strong RECK expression.

We investigated the relation between the methylation status of *RECK* promoter and DFI and OS in NSCLC, but we did not observe any statistically significant differences (DFI, p=0.0869; OS, p=0.4275). We only found, that in solid tumor tissue of NSCLC, the expression of *RECK* is down-regulated by *RECK* promoter methylation. This result agrees with data published by Chang *et al*., who showed a correlation between *RECK* expression down-regulation and promoter methylation (9). More correctly, however, these observations do not exclude other mechanisms which could contribute to *RECK* down-regulation.

In conclusion, we show that the expression of *RECK* mRNA in SCC is lower in comparison with normal tissue and there are also differences in expression between histological types of NSCLC (SCC, adenocarcinoma). There is a higher expression of *RECK* in stage IA in comparison with stages IB-IIIA. These results show that *RECK* could be classified as a tumor suppressor gene with deregulated expression in the SCC tissue. *RECK* is an interesting target for further investigation of MMP inhibitors, in relation to tumors, at least as TIMPs.

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