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 mRNA 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-III A. Our results indicate that RECK could be classified as a tumor suppressor gene and is an interesting target for further investigation of MMP inhibitors.
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 – IIB 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.

Assessment 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’-AATAAGAAGTITGATACGGGTAC-3’; M-antisense, 5’-AAAAACGCGAAATACCTCGAA-3’ and for the unmethylated sequence: U-sense 5’-TAAAGGATTITGGTATGGGATGT-3’; U-antisense 5’-CTCCAAAACCCACAAATACCTCAA-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’-ATCATTTCCCGATCTACTAC-3’; reverse primer 5’-ATATGGGCTAGGACAATGCGAG-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.

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 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.
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.

### Discussion

The MMPs are enzymes which are involved in many processes associated with tumor growth and metastasis, e.g. angiogenesis, degradation of the extracellular matrix and basal membrane during tumor enlargement 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.: Expression of RECK in NSCLC.

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

<table>
<thead>
<tr>
<th>N</th>
<th>Tissue</th>
<th>Median expression (RECK/GAPDH)</th>
<th>Wilcoxon p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>NSCLC</td>
<td>0.0397797</td>
<td>0.0032</td>
</tr>
<tr>
<td>49</td>
<td>Control</td>
<td>0.1102843</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>SCC</td>
<td>0.0297737</td>
<td>0.0003</td>
</tr>
<tr>
<td>24</td>
<td>Control</td>
<td>0.1043990</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Adenocarcinoma</td>
<td>0.1138185</td>
<td>0.3208</td>
</tr>
<tr>
<td>19</td>
<td>Control</td>
<td>0.1359807</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>SCC</td>
<td>0.0297737</td>
<td>0.0051</td>
</tr>
<tr>
<td>19</td>
<td>Adenocarcinoma</td>
<td>0.1138185</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>N</th>
<th>Stage</th>
<th>Median expression (RECK/GAPDH)</th>
<th>Two-sample test (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>IA</td>
<td>0.0543656</td>
<td>p=0.0455</td>
</tr>
<tr>
<td>39</td>
<td>IB-IIIA</td>
<td>0.0338051</td>
<td></td>
</tr>
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</table>

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

<table>
<thead>
<tr>
<th>Studied groups</th>
<th>DFI</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methylation status</td>
<td>Log-rank p-value</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>NSCLC</td>
<td>23</td>
<td>22</td>
</tr>
<tr>
<td>SCC</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>11</td>
<td>5</td>
</tr>
</tbody>
</table>

(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.
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 adenocarcinoma 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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References


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