Simultaneous down-regulation of tumor suppressor genes \textit{RBSP3/CTDSPL}, \textit{NPRL2/G21} and \textit{RASSF1A} in primary non-small cell lung cancer

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Abstract

\textbf{Background:} The short arm of human chromosome 3 is involved in the development of many cancers including lung cancer. Three bona fide lung cancer tumor suppressor genes namely \textit{RBSP3} (AP20 region),\textit{NPRL2} and \textit{RASSF1A} (LUCA region) were identified in the 3p21.3 region. We have shown previously that homozygous deletions in AP20 and LUCA sub-regions often occurred in the same tumor (P < \(10^{-6}\)).

\textbf{Methods:} We estimated the quantity of \textit{RBSP3}, \textit{NPRL2}, \textit{RASSF1A}, \textit{GAPDH}, \textit{RPN1} mRNA and \textit{RBSP3} DNA copy number in 59 primary non-small cell lung cancers, including 41 squamous cell and 18 adenocarcinomas by real-time reverse transcription-polymerase chain reaction based on TaqMan technology and relative quantification.

\textbf{Results:} We evaluated the relationship between mRNA level and clinicopathologic characteristics in non-small cell lung cancer. A significant expression decrease (\(\geq 2\)) was found for all three genes early in tumor development: in 85% of cases for \textit{RBSP3}; 73% for \textit{NPRL2} and 67% for \textit{RASSF1A} (P < 0.001), more strongly pronounced in squamous cell than in adenocarcinomas. Strong suppression of both, \textit{NPRL2} and \textit{RBSP3} was seen in 100% of cases already at Stage I of squamous cell carcinomas. Deregulation of \textit{RASSF1A} correlated with tumor progression of squamous cell (P = 0.196) and adenocarcinomas (P < 0.05). Most likely, genetic and epigenetic mechanisms might be responsible for transcriptional inactivation of \textit{RBSP3} in non-small cell lung cancers as promoter methylation of \textit{RBSP3} according to NotI microarrays data was detected in 80% of squamous cell and in 38% of adenocarcinomas. With NotI microarrays we tested how often LUCA (\textit{NPRL2}, \textit{RASSF1A}) and AP20 (\textit{RBSP3}) regions were deleted or methylated in the same tumor sample and found that this occurred in 39% of all studied samples (P < 0.05).

\textbf{Conclusion:} Our data support the hypothesis that these TSG are involved in tumorigenesis of NSCLC. Both genetic and epigenetic mechanisms contribute to down-regulation of these three genes representing two tumor suppressor clusters in 3p21.3. Most importantly expression of \textit{RBSP3}, \textit{NPRL2} and \textit{RASSF1A} was simultaneously decreased in the same sample of primary NSCLC: in 39% of cases all these three genes showed reduced expression (P < 0.05).
(RB protein serine phosphatase from chromosome 3 gene or HYA22 or CTDSPL; CTD small phosphatase family), NPRL2 (nitrogen permease regulator-like 2 gene or G21 or TUSC4; NPR family) and RASSF1A (Ras association domain family member 1 gene). RBSP3 was mapped to AP20 and the others two to the LUCA region [1,11-13].

The RBSP3 gene occupies more than 120 kb and contains at least 8 exons coding for a 4.8 kb mRNA that is ubiquitously expressed in normal tissues including lung. By sequence analysis RBSP3 belongs to a gene family of small C-terminal domain phosphatases that may control the RNA polymerase II transcription machinery [14]. Two sequence splice variants of RBSP3 (A and B) were identified and an initial analysis of RBSP3 was performed in lung and other cancers [12]. The expression of the gene was greatly decreased in several small cell lung cancer (SCLC) and NSCLC cell lines. RBSP3 showed growth suppression with regulated transgenes in cell culture and suppression of tumor formation in SCID mice. It was demonstrated that transient expression of variant A and B resulted in drastic reduction of the phosphorylated form of RB protein presumably leading to a block of the cell cycle at the G1/S boundary. In addition, frameshift, nonsense and missense mutations in RBSP3 have been reported [15]. All these features are consistent with classical characteristics of a TSG.

The NPRL2/G21 gene covers 3.3 kb and contains 11 exons coding for the main 1.8 kb transcript with multiple splice isoforms that are expressed in all tested normal tissues including lung. By sequence analysis, the main product of NPRL2/G21 encodes a soluble protein that has a bipartite nuclear localization signal, a protein-binding domain, similarity to MutS core domain, and a newly identified nitrogen permease regulator domain 2 domain with unknown function. This information suggests that the nuclear protein NPRL2/G21 may be involved in DNA mismatch repair, cell cycle checkpoint signaling, and regulation of the apoptotic pathway. NPRL2 plays an important role in cisplatin-induced resistance in human non-small-cell lung cancer cells [16,17]. Previously obtained results indicated that NPRL2/G21 is a multiple tumor suppressor gene [16,18,19].

The RASSF1 gene occupies 7.6 kb and contains 5 exons coding for 2 kb alternatively spliced mRNAs [6,11,20]. One of the major splicing forms is RASSF1A. Several studies have shown that loss of RASSF1A expression occurs in many different cancers because of tumor acquired promoter DNA methylation and the gene is able to suppress growth of lung cancer cells in culture and tumor formation in mice [13,21-24]. For example, RASSF1A is silenced by promoter hypermethylation in 100% of SCLCs and in 63% of NSCLCs cell lines and in 50-100% SCLC and 21-58% NSCLC primary tumors [25-28]. As in the case of RBSP3, frameshift, nonsense and missense mutations in RASSF1A have been discovered [15,29]. The amino acid sequence of RASSF1A (340 amino acids) contains a predicted diacylglycerol (DAG) binding domain and a Ras association domain. Association of human proteins RASSF1C and RASSF1A with Ras protein was demonstrated [30,31]. RASSF1A can induce cell-cycle arrest by engaging the Rb-family cell cycle checkpoint [32]. RASSF1A is involved in several growth regulating and apoptotic pathways and regulates cell proliferation, cellular integrity and cell death [24,27]. These and other results strongly suggest that RASSF1A is an important human TSG involved in the development or progression of many epithelial tumors.

Previously only few studies were performed to compare expression of several 3p TSG in the same tumor sample [33,34]. To investigate this further we chose RBSP3, NPRL2 and RASSF1A and analyzed their expression by qPCR in primary tumors: non-small cell lung cancer (NSCLC) – adenocarcinoma (AC) and squamous cell lung cancer (SCC).

For the first time we found that expression of all three genes was significantly decreased in 67-85% of tested NSCLC cases. Moreover, the simultaneous down-regulation of RBSP3, NPRL2 and RASSF1A in the same tumor sample was observed in 39% of all cases. Both genetic and epigenetic mechanisms contributed to deregulation of these three genes representing two TSG clusters in 3p21.3.

Methods
Tissue specimens
Paired specimens of non-small cell lung cancer (NSCLC) tissues including 41 squamous cell carcinomas (SCC), 18 adenocarcinomas AC) and adjacent morphologically normal tissues (conventional “normal” matched control samples) were obtained after surgical resection of primary lung cancer prior radiation or chemotherapy and stored in liquid nitrogen. “Normal” matched controls were obtained minimum at 2 cm distance from the tumor and confirmed histologically as normal lung epithelial cells. The diagnosis was verified by histopathology and only samples containing 70% or more tumor cells were used in the study. The samples were collected in accordance to the guidelines issued by the Ethics Committee of Blokhin Cancer Research Center, Russian Academy of Medical Sciences (Moscow). All patients gave written informed consent that is available upon request. The study was done in accordance with the principles outlined in the Declaration of Helsinki. All tumor specimens were characterized according to the International System of Clinico-Morphological Classification of Tumors (TNM), based on the tumor-node-metastasis and staging
classification of 1999 [35] and WHO criteria classification of 1999 [36]. Relevant clinical and pathological characteristics of the patients with NSCLC included in this study are summarized in Table 1. Normal lung tissues (autopsy material) were obtained post mortally from ten healthy individuals (age 23-49 lacking cancer history with absence of chronic diseases).

**DNA and total RNA extraction and reverse transcription reaction**

DNA was extracted using the Dneasy Tissue kit (Qiagen, USA) and total RNA was isolated with Rneasy mini kit (Qiagen, USA) according to the manufacturer’s recommendation. RNA quality was assessed with spectrophotometer Nano Drop ND-1000 (NanoDrop Technologies Inc. USA) and by gel electrophoresis. All RNA samples were treated with RNase free DNase I (Fermentas, Lithuania) and cDNA was synthesized using MMLV reverse transcriptase and random hexamers according to standard manufacturer’s protocol (Fermentas, Lithuania).

**Analysis of mRNA and DNA copy number by qPCR**

The sequences of primers and probes are shown in Table 2. All reactions were performed using ABI 7000 PRISM™ SDS (Applied Biosystems) with RQ software (PCR program: 10 min at 95°C, then 40 two-step cycles 15 s at 95°C and 60 s at 60°C) in total volume 25 μl in triplicate. All probes contained the dye FAM at 5′-end. Final concentrations of primers and probes for target and reference genes were: RBSP3 cDNA primers - 350 nM, probe - 150 nM; RBSP3 DNA primers - 150 nM, probe - 100 nM; ACTB DNA primers- 200 nM, probe - 100 nM, NPRL2 cDNA primers - 500 nM, probe 300 nM; RASSFIA cDNA primers - 300 nM, probe 300 nM; GAPDH cDNA primers - 300 nM, probe - 150 nM; RPNI cDNA primers - 350 nM, probe - 200 nM; RPN1 DNA primers - 200 nM, probe - 100 nM; GUSB cDNA primers - 350 nM, probe - 250 nM; GUSB DNA primers - 200 nM, probe - 200 nM.

PCR products were analyzed in 1.8% agarose gels and nucleotide sequences of the amplicons were verified by sequencing with 3730 DNA Analyzer automated sequencer (Applied Biosystems). qPCR data were analyzed using the relative quantification or ΔΔCt-method [37,38] based on mRNA (or DNA) copy number ratio (R) of a target gene versus reference gene in a given tumor sample relative to matched normal control sample (see above Tissue specimens section) according to the formula:

\[
R = \left( \frac{2^{\Delta \Delta CT}}{2^{\Delta \Delta CT}} \right)^{1/E}
\]

where \(E\) - efficiency of reaction, \(C_T\) - threshold cycle, ref - reference gene, tar - target gene.

All preliminary validation steps have been done: standardization of all assays, reproducibility of the qPCRs in parallel and in independent runs, selection of reference samples and testing of reference genes at http://www.gene-quantification.info/.

**NotI-microarray analysis**

Microarrays were constructed essentially as previously described [39,40]. In brief, two oligonucleotides: NotX: 5′-AAAAAGAATGTCAGTGTGTCACGTATGGACGAATTGC-3′

and NotY: 5′-GGCCCGCAATTCGTGCTCAGTGCACGTGACATCC-3′

were used to create the NotI linker. Annealing was carried out in a final volume of 100 μl containing 20 μl of 100 μM NotX, 20 μl of 100 μM NotY, 10 μl of 10×M buffer (Roche Molecular Biochemicals) and 50 μl of H₂O. Two micrograms of tumor and normal control DNA (50 μg/ml) were digested with 20 U of Sau3A (Roche Molecular Biochemicals) at 37°C for 5 h and then 0.4 μg of the digested DNAs were circularized overnight with the T4 DNA ligase (Roche Molecular Biochemicals) in the appropriate buffer in 1 ml reaction mixture. Then DNA was concentrated with ethanol, partially filled in and digested with 10 U of NotI at 37°C for 3 h. Following digestion, NotI was heat inactivated and DNAs were ligated overnight in the presence of a 50 M excess of NotI linker at room temperature. NotI-representation (NR) probes were labeled in a PCR

**Table 1 Clinical and pathological characteristics of patients with NSCLC**

| Variables                  | Patients |
|---------------------------|----------|
| Gender/n                  | Female/7, Male/52 |
| Age                       | Mean 60, Range 31-76 |
| TNM/Stage                 | Histological type of NSCLC |
| T1N0M0/Stage IA           | SCC       |
| T1N1M0/Stage IB           | 6         |
| T1N2M0/Stage IIA          | 5         |
| T2N0M0, T2N1M0/Stage IIIB | 19        |
| T2N1M0, T2N2M0/Stage IIIA | 11        |
| T2N2M0/Stage IIIB         | –         |
| N0 Stage (no metastases)  | 24        |
| N1 Stage +N2 Stage (with metastases) | 17 |
| Central cancer            | 27        |
| Peripheral cancer         | 9         |
| ND                        | 5         |
| Total                     | 41        |

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reaction with NotX primer. The majority of products of the DNA amplification step were in the 0.2-1.0 kb range. Repeated PCR was conducted for labeling NR with fluorophores.

Hybridization of coupled normal/tumor NotI samples was carried out at 42°C for 15 h in a Lucidea Base device (Amersham Pharmacia Biotech) according to manufacturer’s recommendations. Automatic washing of the microarrays was performed in the same device using manufacturer’s protocol. The following solutions were sequentially used for the washing: 1) 0.2% SDS+1 SSC; 2) 0.2% SDS+0.1 SSC; 3) 0.1 SSC; 4) de-ionized water; 5) isopropyl alcohol. Then microarrays were scanned in the GenePix 4000 A and results were processed with GenePix Pro 6.0 software (Amersham Pharmacia Biotech).

### Statistical analysis

Nonparametric Wilcoxon test was used to compare mRNA expression differences of target and reference genes for the same NSCLC sample. Then groups of samples were compared in respect to average level of mRNA decrease (LDav) and the frequency of decrease (FD). The LD was calculated as 1/R and reflects the n-fold factor by which the mRNA content decreased in the tumor compared to normal tissue. Nonparametric Kruskal-Wallis and Mann-Whitney rank-sum tests were used to test mRNA differences (both LDav and FD) for each target gene in NSCLC (AC, SCC) and with and without metastases. Nonparametric Spearman’s criterion was used to calculate the coefficient of correlation between the levels of mRNA decrease (LDav) for each

### Table 2 Primers and probes for target and reference genes for expression levels and copy number studies

| Gene Name | Primers (F, R) and probe (Z) sequences 5’ → 3’ | Amplicon length |
|-----------|-----------------------------------------------|----------------|
| **Target genes** | | |
| RBSP3/CTDSPL | cDNA | 154 |
| NM_00108892 | F: GAGAGAAAGCTCTCCATG | R: CCACCTCTCCCTCCACG | Z: CCACATGTAATCACGAGC | |
| NPRL2/G2I | cDNA | 126 |
| NM_006545 | F: GCACACTACTACAACAACATCG | R: GTCAAGACTGCTAGTAGCAC | Z: ACACCCGAAGTTTCAGCCAGGACGAT | |
| RASSF1A | cDNA | 120 |
| NM_007182 | F: CGCGATAGGAAATCC | R: AGGTCGTCAGGTGTCG | Z: CGCTGCTGCTGCTGCTGCTGCTG | |
| **Reference genes** | | |
| RPN1 | cDNA | 125 |
| NM_002950 | F: CACCCCTAAAGGCAAGAAG | R: TGAGTTCGTGTTCTGCTG | Z: CCCTCTGCTGCTGCTGCTGCTG | |
| GUSB | cDNA | 171 |
| NM_000181 | F: GATGGAAGAAGTGGTGCGT | R: TTAGAGTTGCTCACAAAGTCC | Z: GCTCATACCTGAATCTGTGGCTACT | |
| ACTB | cDNA | 160 |
| NM_001101 | F: GTGCTCAGGGCTTCTGCTCTG | R: CCTACGCACCACTTCCATC | Z: GAGCCCTCGTCTCCTTCCTTCT | |
| GAPDH | cDNA | 139 |
| NM_002046 | F: GGAGTGAAGGGCTTCTGCTCTT | R: TTTCTCCCTCCCTCCCTTCCCTT | Z: CTCTGGTCTCCTCCCTTCTT | |
set of pairs of target genes. P-values < 0.05 were considered statistically significant. All statistical procedures were performed using the BioStat software [41].

Results

Expression of RBSP3, NPRL2 and RASSF1A genes in lung tissues of healthy donors

Genomic structure and location of primers and probes is shown in Figure 1.

It is known that normally looking cells surrounding tumors can already harbor genetic and epigenetic changes compared to the cells isolated from the same tissue from healthy individuals. Therefore we decided to check expression of these 3 genes in samples obtained from healthy donors and in biopsies of "normal" (matched) control samples obtained from patients with NSCLC. The mRNA levels were measured in lung tissues from healthy donors using different reference samples. RNA pool isolated from lung tissues of 10 healthy donors was used in one set of experiments and in another test the same samples were examined against RNA pool isolated from adjacent to tumor "normal" tissues of 30 NSCLC patients. The GAPDH was used as a reference gene and its expression did not vary more than 2-fold in all samples examined. Very similar results were obtained for target genes in both experiments. We concluded that expression of all three genes did not significantly vary (R = 1.5 ± 0.5) in normal lung tissues from healthy donors and patients with NSCLC (‘normal’ matched controls).

Expression of three TSGs in primary NSCLC - AC and SCC

The results of mRNA level quantification for three genes and statistical analysis are shown in Figures 2 and 3 and Table 3. The variability of GAPDH and RPN1 mRNA was less than 2-fold in studied samples, therefore in this study we considered significant greater than or equal to 2-fold decrease of target genes expression.

RBSP3

Significant (from 2 to 94-fold) decrease of the mRNA level (LD) was observed in 85% (50 of 59, P < 0.001) of all NSCLC cases. Frequency of mRNA decrease (FD) was high in both AC (78%) and SCC (88%). Interestingly at Stage I of the SCC cases expression of RBSP3 was down-regulated already in 100% cases and only in 71% of AC samples (Table 3). On the other hand the tendency of more severe deregulation of RBSP3 transcription during tumor progression was more evident for AC cases: in average a 3-fold declined expression in 70% of cases without metastases and a 6-fold in 88% of samples with metastases. The same was correct for cases at Stage I (LD_{av} = 3, FD = 71%) and Stage III (LD_{av} = 6, 26%)}
Figure 2 The relative expression levels of RBSP3, NPRL2/G21 and RASSF1A genes in AC and SCC. The mRNA level of each target gene was normalized by that of the reference gene GAPDH. Data for ADC and SCC were presented according to cancer progression stages and to the presence of metastases.
FD = 100%). Spearman’s coefficient of reverse correlation ($r_s$) between tumor Stages (I, II, III) and expression R values was 0.36 ($P = 0.2$, i.e. non-significant).

**NPRL2**

Significant decrease of LD (from 2 to 100-fold) was seen in 73% (43 of 59, $P < 0.001$) of all NSCLC cases. Down-regulation of NPRL2 was more significant in SCC compared to AC: 9-fold and 85% vs. 4-fold and 44%, respectively, $P = 0.002$ (see Table 3). No expression was found in 12% (5 of 59) of SCC cases. The FD was comparable in SCC cases with metastases and without (82% and 88%, respectively). However FD in AC samples without metastases was less than with metastases (20% against 75%; $P = 0.08$). Again at Stage I of the SCC cases expression of NPRL2 was down-regulated already in 100% cases and in AC only 14% samples showed decreased expression. The tendency of more frequent decrease of NPRL2 mRNA level with tumor progression was seen only for AC cases, where Spearman’s coefficient of reverse correlation ($r_s$) between tumor Stages (I, II, III) and expression R values was 0.47 ($P = 0.06$). Eighty three percent of AC cases had decreased expression at Stage III as compared to 14% at Stage I.

**RASSF1A**

Significant decrease of LD (from 2-fold to 57-fold) was detected in 67% (24 of 36, $P < 0.01$) of all NSCLC cases, including 64% of AC and 68% of SCC samples (Figure 2, Table 3). More severe deregulation of RASSF1A expression was observed during tumor progression both in AC and SCC. Spearman’s coefficient of reverse correlation ($r_s$) between tumor Stages (I, II, III) and R values was 0.54 ($P = 0.05$) for AC. Statistically significant decrease of LD$_{av}$ and increase of FD was observed in AC for patients with metastases and without (7-fold and 100% vs. 4-fold and 29%, $P < 0.05$). The same tendency was seen for LD$_{av}$ and FD in SCC cases with and without metastases: namely, 6-fold and 81% vs. 4-fold and 33%, respectively; $P = 0.196$. Moreover an average decrease of RASSF1A mRNA (LD$_{av}$) in SCC at Stage I was 4-fold and at Stage III it was already 7-fold. FD increased for both SCC (33% vs. 82%) and AC (29% vs. 100%) during tumor progression from Stage I to Stage III.

Surprisingly a 3-to-4 fold increase of mRNA level of two genes - NPRL2 and RASSF1A was found for several AC samples at Stage I (Figure 2, samples 1, 3, 5). These
tumor samples were highly differentiated in contrast to samples 2, 4, 9, 10 and others that displayed low degree of differentiation and significant decrease of NPRL2 and RASSF1A. Some of them showed so high degree of anaplasia that the epithelial phenotype was almost undetectable. These undifferentiated cells were highly malignant and showed the strongest decrease of NPRL2 and RASSF1A expression.

Simultaneous down-regulated expression of three genes in the same tumor samples
As expression of all three target genes was frequently decreased in the same NSCLC samples we tested the probability that this decrease was not random. To do this we used 36 NSCLC samples and two control reference genes GAPDH and RPNI (Figure 3, Table 4). Results demonstrated that this simultaneous down-regulation was very frequent and statistically valid: all three genes had reduced expression (≥2) in 39% (P < 0.001) of NSCLC specimens (36% in AC and 41% in SCC). Simultaneous decreased expression of RBSP3 and NPRL2 was observed in 61% of cases and for RBSP3 and RASSF1A this occurred in 50%. In the case of NPRL2 and RASSF1A simultaneous down-regulation was detected in 44% of tumors (Table 4). Spearman’s coefficient values rs for RBSP3, NPRL2/G2I, RASSF1A gene pairs were high (0.63 - 1.0, P < 0.001) in different groups especially in AC and SCC with metastases.

Decreased expression of RBSP3, NPRL2 and RASSF1A in primary NSCLC can be caused by genetic and epigenetic factors
To understand the mechanism underlying the observed down-regulation of RBSP3 in NSCLC samples we used qPCR to test DNA copy number changes, i.e. genetic factors [9,10] and Not1 microarrays to examine methylation i.e. epigenetic factors [39]. Methylation was detected in 38% of AC (3 of 8) and in 80% of SCC (8 of 10) cases with decreased expression of RBSP3. Deletions were detected in 25% of AC samples and in 30% of SCC tumors (Table 5). Both methylation and hemizygous deletions were observed in two SCC specimens. Methylation or/and deletions of RBSP3 were detected in 63% of AC and in 90% of SCC cases. The data suggested that both genetic and epigenetic mechanisms are important for transcriptional inactivation of RBSP3 in NSCLC.

Using Not1 microarrays we cannot test methylation of NPRL2 and RASSF1A promoters because they don’t contain Not1 sites. However we can check genes SEMA3F and GNAI2 that are located in the same LUCA sub-region as NPRL2 and RASSF1A. We tested how often deletions and methylations occurred in the same NSCLC sample at both LUCA and AP20 sub-regions and found that this occurred in 58% of all studied cases (15 of 26). Thus, most likely both genetic and epigenetic mechanisms are responsible for simultaneous down-regulation of expression of these three genes.
were reported [10,12,15]. In some leukemia cell lines and lung cancer. However, frequent deletions and mutations of RBSP3 [6,16] in NSCLC and SCLC cell lines [6,16]. There are no methylation groups of NPRL2 [10,42,43]. The test is based on aberrations in the loci NLJ-003 (AP20) and NL3-001 (LUCA) as different events was carried out using a permutation test for four types of cancers: lung, renal, breast, and ovarian. This test also revealed a significant correlation between different aberrations in these two loci (P < 10^-6). The same results were obtained using Pearson correlation for numeric values of copy number changes of these loci. Indeed, homozygous deletions in both regions often occur in the same tumor (P < 3 × 10^-7).

Table 4 The frequencies of simultaneous mRNA level decreases (FD) for the combination of genes RBSP3, NPRL2/G21 and RASSF1A in different groups

| Groups     | RBSP3/NPRL2/G21 | RBSP3/RASSF1A | RASSF1A/NPRL2/G21 | RBSP3/NPRL2/G21/RASSF1A |
|------------|-----------------|---------------|-------------------|-------------------------|
| AC         | 14 (1/7)        | 14 (1/7)      | 0 (0/7)           | 0 (0/7)                 |
| I          | 14 (1/7)        | 14 (1/7)      | 0 (0/7)           | 0 (0/7)                 |
| II-III     | 71 (5/7)        | 100 (7/7)     | 71 (5/7)          | 71 (5/7)                |
|             | rs = 0.88       | rs = 0.88     | rs = 0.74         |                         |
| Total      | 43 (6/14)       | 57 (8/14)     | 36 (5/14)         | 36 (5/14)               |
| SCC I      | 67 (4/6)        | 17 (1/6)      | 33 (2/6)          | 17 (1/6)                |
| II-III     | 75 (12/16)      | 56 (9/16)     | 56 (9/16)         | 50 (8/16)               |
|             | rs = 1.00       | rs = 0.88     | rs = 0.88         |                         |
| Total      | 73 (16/22)      | 45 (10/22)    | 50 (11/22)        | 41 (9/22)               |
| NSCLC I    | 38 (5/13)       | 15 (2/3)      | 15 (2/3)          | 8 (1/13)                |
| II-III     | 74 (17/23)      | 70 (16/23)    | 61 (14/23)        | 57 (13/23)              |
|             | rs = 0.68       | rs = 0.94     | rs = 0.74         | rs = 0.69               |
| Total      | 61 (22/36)      | 50 (18/36)    | 44 (16/36)        | 39 (14/36)              |

# Discussion

Several candidate TSG from the 3p21.3 AP20 and LUCA sub-regions were examined in the gene inactivation test, GIT [2,12,13,16,34,42,43]. The test is based on the functional inactivation of analyzed genes that can be achieved in different ways: by mutation, deletion, methylation etc. According to these results, at least three genes can now be considered as bona fide lung TSG: NPRL2 and RASSF1A from LUCA and RBSP3 from AP20 sub-regions [12,13,16].

Earlier the decrease of RBSP3 expression was shown in SCLC, NSCLC, cervical, renal, breast, and ovarian. In NSCLC, cervical cell lines and primary tumors by Northern blot analysis, RT-PCR and qPCR [12,44,45]. The decrease or absence of NPRL2/G21 expression was detected in some SCLC, NSCLC and renal cancer cell lines using Northern blot analysis, RT-PCR and qPCR [12,44-46]. The decrease or absence of expression was detected in some SCLC, NSCLC and many other tumors and cancer cell lines [see 24,27,28].

It was reported that promoter methylation was the main mechanism of RASSF1A loss of expression in lung cancer (see Introduction). Homozygous deletion of 3'-part of NPRL2 gene and rare mutations were found in NSCLC and SCLC cell lines [6,16]. There are no methylation data explaining the loss of RBSP3 expression in lung cancer. However, frequent deletions and mutations were reported [10,12,15]. In some leukemia cell lines (up to 98%) and acute leukemia lymphoma blood samples (24%) methylation of the promoter region of RBSP3 was reported [44]. Methylation (up to 26%), deletions and decreased expression of RBSP3 were significantly associated with poor prognosis of cervical cancer [45]. Thus, inactivation of RBSP3 might be one of the early events in cervical carcinogenesis.

Loss of heterozygosity and quantitative real-time PCR demonstrated that aberrations in both LUCA and AP20 sub-regions occurred simultaneously in the same tumor with high probability. Thus, it was suggested that aberrations in both LUCA and AP20 sub-regions could be linked [9,10]. Indeed, homozygous deletions in both regions often occur in the same tumor (P < 3 × 10^-7). The estimation of possible interdependency between all aberrations in the loci NLJ-003 (AP20) and NL3-001 (LUCA) as different events was carried out using a permutation test for four types of cancers: lung, renal, breast, and ovarian. This test also revealed a significant correlation between different aberrations in these two loci (P < 10^-6). The same results were obtained using Pearson correlation for numeric values of copy number changes of these loci. Indeed, proteins RBSP3 and RASSF1A could collaborate in cell cycle arrest: RASSF1A by inhibiting cyclin D1 [32] and RBSP3 by dephosphorylating pRB [12]. Thus functional collaboration of these two genes could result in activation of the RB1 gene.

In this study we tested the hypothesis that TSG in AP20 and LUCA regions were not only deleted but their expression could also be simultaneously down-regulated in NSCLC. This suggestion was indirectly supported by other studies that showed that genes over large chromosomal regions could be regulated in a coordinated fashion [33,47-49].

First we found that expression of all three genes is rather uniform in lung samples isolated from healthy donors and from normally looking lung samples obtained from NSCLC patients ("normal" matched control samples). Thus adjacent morphologically normal tissues from the patients can be used as paired reference controls to tumor samples. In the study two parameters were analyzed - the level of mRNA decrease and frequency of mRNA decrease in two major NSCLC histological subtypes (AC and SCC) and their subgroups with different characteristics such as clinical Stage, grade, tumor localization, presence of metastases and others. Although both parameters reflect deregulation of gene expression, they are not randomly but rather functionally related.

Expression analysis of the three genes revealed the following main features.

1. Expression of the three studied TSG was significantly decreased in NSCLC: 85% for RBSP3, 67% for RASSF1A and 73% for NPRL2 (P < 0.001). It was statistically valid both for SCC and AC.
2. Down-regulation of the three genes was already evident at Stage I of NSCLC samples. Statistically significant down-regulation of both NPRL2 and RBSP3 was seen in 100% cases at Stage I of SCC.

3. The degree and frequency of the expression decrease for all three genes was more strongly pronounced in SCC than in AC samples (see Table 3). This difference was statistically valid in the case of NPRL2 ($P = 0.002$).

4. All studied genes were involved in progression of AC. The tendency of more severe expression down-regulation of the RBSP3 was evident during tumor progression of AC with respect to FD and LD (70% and 3-fold in cases without metastases in contrast to 88% and 6-fold decrease in cases with metastases, $P = 0.13$). For NPRL2, this tendency was also seen only for AC - 83% of cases had decreased expression at Stage III compared to 14% at Stage I and in 75% of AC cases with metastases vs. 20% of cases with metastases, $P = 0.08$, see Table 3). Expression of RASSF1A revealed the most strongly pronounced correlation between decrease of expression (FD and LD) and tumor progression both in SCC and AC. For example, difference in FD values was obvious between cases with and without metastases. For SCC cases this difference was 33% vs. 81% ($P = 0.196$) and for AC it was even more sharp, 29% vs. 100% ($P < 0.05$).

5. Expression of RBSP3 and RASSF1A was most seriously affected in respect to FD. For RBPS3 it was detected in 85% of all NSCLC cases and for RASSF1A in 67%. However, regarding LD, the expression of NPRL2 was most strongly inhibited (LD$_{av} = 8$), while RBSP3 showed weaker inhibition (LD$_{av} = 5$).

6. Preliminary data suggested that no statistically significant difference was observed in cases with relation to age, smoking history and other cytological and pathological characteristics.

7. NotI microarrays and qPCR on genomic DNA we tested for possible mechanisms of the declined expression of RBSP3 in NSCLC. The data suggested that both genetic and epigenetic mechanisms were important for transcriptional inactivation of RBSP3 in NSCLC. Altogether deletions were detected in 25% of AC samples and in 30% of SCC patients. Methylation of RBSP3 was detected in 38% of AC and in 80% of SCC cases. With NotI microarrays we also tested how often LUCA (NPRL2 and RASSF1A) and AP20 (RBSP3) regions were deleted or methylated in the same tumor and found that this occurred in 58% of all studied cases (18 of 26). Thus, most likely both genetic and epigenetic mechanisms are responsible for simultaneous down-regulation of expression of these three TSG.

**Conclusion**

The detailed analysis of mRNA expression levels of three 3p TSG was performed in two histological

### Table 5 Comparison of qPCR data and NotI-microarrays for RBSP3 gene

| Sample number | qPCR mRNA decrease | aDNA copy number | bNot I-microarrays | Possible reason of mRNA decrease |
|---------------|--------------------|------------------|-------------------|----------------------------------|
| AC            |                    |                  |                   |                                  |
| 1             | 0.39               | +                | +                 | deletion                         |
| 2             | 0.15               | -                | +                 | methylation                      |
| 4             | 0.29               | -                | -                 | another mechanism                |
| 5             | 0.49               | +                | +                 | deletion                         |
| 7             | 0.37               | -                | -                 | another mechanism                |
| 11            | 0.12               | -                | -                 | another mechanism                |
| 16            | 0.29               | -                | +                 | methylation                      |
| 17            | 0.12               | -                | +                 | methylation                      |
| SCC           |                    |                  |                   |                                  |
| 23            | 0.16               | -                | +                 | methylation                      |
| 25            | 0.13               | +                | +                 | deletion and methylation         |
| 26            | 0.21               | -                | +                 | methylation                      |
| 27            | 0.39               | +                | +                 | deletion and methylation         |
| 43            | 0.48               | -                | +                 | deletion                         |
| 46            | 0.14               | -                | +                 | methylation                      |
| 52            | 0.06               | -                | +                 | methylation                      |
| 53            | 0.46               | -                | +                 | methylation                      |
| 54            | 0.08               | -                | +                 | methylation                      |
| 55            | 0.26               | -                | -                 | another mechanism                |

*a* + deletion, - retention  
*b* + deletion and/or methylation, - no changes
subtypes of NSCLC - AC and SCC respectively. The most important finding was that expression of RBSP3, NPLR2 and RASSF1A decreased in the same samples of primary NSCLC: all 3 genes have reduced expression in 39% of cases (P < 0.05). Declined expression of primary NSCLC: all 3 genes have reduced expression in 3 Blokhin Cancer Research Center, Russian Academy of Medical Sciences, 2 Microbiology and Tumor Biology Center, Department of Clinical Science Cooperation in Research and Higher Education (STINT), the Swedish Institute, Swedish Research Council, the Swedish Foundation for International 2008/07/07) and by research grants from the Swedish Cancer Society, the 15457; 15656; 15656-49408; The Agency for Strategic Technologies (Agreement 1-444, 2005), The Agency for Strategic Technologies (Agreement 1-444, 2006) and the Ministry of Science and Innovations (State Contracts no 02.512.11.2241, Agreement 1-1112, 2007). The authors declare that they have no competing interests. Received: 21 July 2009 Accepted: 1 March 2010 Published: 1 March 2010

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