High Mutability of the Tumor Suppressor Genes **RASSF1** and **RBSP3 (CTDSPL)** in Cancer

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**Abstract**

**Background:** Many different genetic alterations are observed in cancer cells. Individual cancer genes display point mutations such as base changes, insertions and deletions that initiate and promote cancer growth and spread. Somatic hypermutation is a powerful mechanism for generation of different mutations. It was shown previously that somatic hypermutability of proto-oncogenes can induce development of lymphomas.

**Methodology/Principal Findings:** We found an exceptionally high incidence of single-base mutations in the tumor suppressor genes **RASSF1** and **RBSP3 (CTDSPL)** both located in 3p21.3 regions, LUCA and AP20 respectively. These regions contain clusters of tumor suppressor genes involved in multiple cancer types such as lung, kidney, breast, cervical, head and neck, nasopharyngeal, prostate and other carcinomas. Altogether in 144 sequenced **RASSF1A** clones (exons 1–2), 129 mutations were detected (mutation frequency, MF = 0.23 per 100 bp) and in 98 clones of exons 3–5 we found 146 mutations (MF = 0.29). In 85 sequenced **RBSP3** clones, 89 mutations were found (MF = 0.10). The mutations were not cytidine-specific, as would be expected from alterations generated by AID/APOBEC family enzymes, and appeared *de novo* during cell proliferation. They diminished the ability of corresponding transgenes to suppress cell and tumor growth implying a loss of function. These high levels of somatic mutations were found both in cancer biopsies and cancer cell lines.

**Conclusions/Significance:** This is the first report of high frequencies of somatic mutations in **RASSF1** and **RBSP3** in different cancers suggesting it may underlay the mutator phenotype of cancer. Somatic hypermutations in tumor suppressor genes involved in major human malignancies offer a novel insight in cancer development, progression and spread.

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**Introduction**

We have performed a comprehensive deletion survey of 3p on more than 400 of lung, renal, breast, cervical and ovarian carcinomas (major epithelial cancers) using a defined set of markers, combining conventional LOH with quantitative real-time PCR (QPCR), comparative genomic and NotI microarrays hybridisations [1,2,3,4,5]. We identified two most frequently affected 3p21.3 regions, LUCA (Lung Cancer) at the centromeric and AP20 at the telomeric border of 3p21.3. Aberrations of either region were detected in more than 90% of the studied tumors suggesting they harbor multiple tumor suppressor genes (TSG) [5,6,7].

One of them is **RASSF1** gene (from LUCA region) that can exist in different alternative splicing forms (at least 7 different isoforms). In this work we studied the most important **RASSF1A**, the largest splicing form [8]. Several studies have shown that loss of **RASSF1A** expression occurs because of tumor acquired promoter DNA methylation in many different cancers. For example, **RASSF1A** is silenced by promoter hypermethylation in over 90% of small cell lung carcinomas (SCLC) and clear cell renal cell carcinomas
(RCC) and in about 40% of non-small cell lung carcinomas (NSCLC). The gene is able to suppress growth of lung and renal cancer cells in culture and tumor formation in mice [6]. In addition, occasional missense mutations in RASSF1A have been reported. RASSF1A codes for 340 amino acids. The amino acid sequence of RASSF1A contains a predicted diacylglycerol (DAG) binding domain and a Ras association domain. RASSF1A can induce cell-cycle arrest by engaging the Rb-family cell cycle checkpoint [9]. These and other results strongly suggest that RASSF1A is an important human tumor suppressor protein acting at different levels of tumor progression [6].

Another gene is RBSP3 also called HYA22 and CTDSPL. It exists in two splice forms (A, 265 amino acids and B, 276 amino acids) that map to AP20 region and belongs to a gene family of small C-terminal domain phosphatases that may control the RNA polymerase II transcription machinery [10]. Expression of the gene was greatly decreased in several SCLC and NSCLC cell lines. RBSP3 showed growth suppression with regulated transgenes in culture and suppression of tumor formation in SCID mice. It was demonstrated that transient expression of both A and B forms resulted in drastic reduction of phosphorylated form of RB protein presumably leading to a block of the cell cycle at the G1/S boundary. After this finding the gene was renamed (RB protein serine phosphatase from chromosome 3). All these features are consistent with classical characteristics of a TSG.

Interestingly, both RASSF1 and RBSP3 could collaborate in cell cycle arrest: the former by inhibiting cyclin D1 [9] and the latter by dephosphorylating RB [10]. This supports the hypothesis that TSGs in these two regions could act synergistically [4,5]. Moreover two other TSGs from these regions could cause increasing mutation frequencies in tumors (MLH1 from AP20 and G21/NPRL2 from LUCA) [11,12,13].

It is well known that cancer is the result of genetic and epigenetic changes and point mutations is one of the most important mechanisms for the development of cancer [14,15]. Previously, others and we detected numerous single-base changes/mutations in RASSF1A that were believed to be SNPs [8,16,17]. Moreover, RBSP3 mutations were detected in all 14 tumors of different origins expressing the gene [10].

To study the apparently high mutation frequencies of TSG(s) in these regions of 3p21.3, we performed a comprehensive mutation analysis of RASSF1A [18,19] and RBSP3/HYA22 [10] in several cancers. Here we show that exceptionally frequent single-base mutations occur in these genes in multiple cancer types. The mutations were not cytidine-specific as would be expected if generated by AID [20] or other APOBEC family [21,22] enzymes. These mutations were not due to RNA editing and appeared de novo during cell divisions.

**Results**

**Bioinformatics analysis of EST cDNA clones reveals high mutation frequency of RASSF1 and RBSP3**

First we examined publicly available EST sequence data for RASSF1A and RBSP3 (for RASSF1A Accession No. NM_007182; RBSP3A, Accession No. AJ575644, and for RBSP3B, Accession No. AJ575645). Sequences with homology below the threshold (see Materials and Methods) i.e. containing multiple distinct mismatches to the annotated genes and unknown nucleotides (N) were not considered. Sequences close to the end of reads were also not considered. The data presented in Table 1 show that the RASSF1A and RBSP3 genes were mutated at extremely high rates. For the RASSF1A we considered only 17 clones (mutation frequency per 100 bp, MF = 0.22). Six of them were obtained from cancer tissues

![Table 1](image-url)
and all of them contained mutations (MF = 0.42). Eleven sequences were from normal tissues (four clones with one mutation) and MF = 0.1, i.e. mutation frequencies were statistically significantly different (P = 0.025).

Eighty one per cent of the RBSP3 sequences (63 out of 79) contained mutations/mismatches. MF for RBSP3 ESTs was 0.63. Again it was much higher in clones isolated from cancer (MF = 1.03) than from normal tissues (MF = 0.43). This difference was also significant (P < 0.001). The difference was even more pronounced for mutations changing amino acid sequences (MF 0.72 versus 0.24) and similar for RASSF1A clones (MF 0.53 versus 0.1).

The number of available (and mutated) EST sequences was significantly higher for both RASSF1A and RBSP3, but due to the very stringent criteria many were excluded from analysis. Importantly, we have also detected hypermutations in other exons of RASSF1A, shared with RASSF1C (recently shown to be a TSG with a different tissue specificity than RASSF1A, see [23]). MF for the exons 3–6 was 0.43 and for the mutations changing amino acids MF = 0.25 and therefore RASSF1C is also hypermutated.

A similar bioinformatic analysis was done for the insulin gene (333 bp, complete ORF). No mutations were detected in more than 1000 sequenced clones isolated from cell lines and somatic tissues. In 20 available p16/INK4a (exons 1–3, 447 bp) clones sequenced from cancer and normal cells we found no mutations and in 6 clones for GPR14 (1170 bp) only 1 mutation was found in cancer cells (MF = 0.01). In our experiments described below (see next Section and Section “Different mutations frequencies in other genes”) in 31 sequenced GPR14 clones no mutations were found indicating that this mutation is rather rare. The mutation frequency for GPR14 was statistically significantly different as compared to both the RASSF1A and RBSP3 (P = 0.01).

Frequent mutations in RASSF1A in human carcinomas, cancer and haematopoietic cell lines

During analysis of RASSF1A we have isolated several mutant clones including one double mutant [16]. This high frequency of mutations was surprising since for RASSF1A and other candidate genes in the AP2O and LUCA regions the mutation frequencies were reported to be low to none [6,19]. At the same time many polymorphisms were recorded for RASSF1A and in many cases it was not clear whether it was a real single nucleotide polymorphism or somatic mutation in cancer cells because control normal cells were not available [8]. Importantly, in all these studies single-strand conformation polymorphism (SSCP) and direct sequencing from PCR products was used. The admixture of stroma, blood vessels, lymphocytes and other normal cells would hamper detection of mutations using these methods (see M/M). Tumor heterogeneity creates additional problems for recognizing mutations. Therefore we decided to re-investigate the mutational status of RASSF1A in multiple tumor types including primary tumors and cancer cell lines. First, RASSF1A cDNA was isolated from an RCC biopsy (T356) and the surrounding normally looking kidney parenchyma (N356). Several cDNA clones were sequenced. In six clones derived from normal kidney parenchyma, no mutations were found. However of seven clones from the tumor tissue, mutations were detected in three (P = 0.14). All were A to G substitutions. To exclude RNA editing, genomic DNA from the same patient was isolated and the first two exons (DAG domain) of RASSF1A were amplified by PCR. Several clones derived from normal and tumor tissue were then sequenced: all six clones from the tumor biopsy showed mutations while of the fourteen analyzed clones from normal tissue only one was mutated (P < 0.001). The observed mutations in the cDNA from tumor cells were not created by RNA editing because the mutations were detected also on genomic DNA level. Normal cell contamination and high expression of RASSF1A in normal cells, compared to cancer cells, can explain the different ratios between mutated and normal RASSF1A clones from cDNA and genomic DNA. Most surprising was the fact that with the exception of two genomic clones from the tumor biopsy with deletion of C at position 234 (Accession No. NM_007182), all other detected mutations were in different positions.

As a control we amplified GPR14 from the same patient and sequenced 10 clones from cancer and from the surrounding normal tissue. No mutations were found proving that high mutation rate is specific for the RASSF1A gene.

To check whether different mutations in the same tumor occurred due to the tumor heterogeneity or some other mechanism(s), we isolated and sequenced RASSF1A clones (only exon 1 and 2; 391 bp) from genomic DNA of four RCC cell lines. In TK164 all three and in KRC/Y (2+2) all four sequenced clones contained mutations. In TK10, among 22 clones, 9 were mutated. Importantly, the majority of clones contained different mutations. Only one clone was sequenced from Caki1, and it was mutated.

We also sequenced this gene from genomic DNA of four lymphoid cell lines (BL2 and RAMOS are Burkitt cell lines, and IARC171 and MutuIII are lymphoblastoid cell lines) and the results were very similar to the RCC cell lines (Table 2). Altogether, among 84 sequenced clones 55 contained mutations that in most cases differed. MF in RASSF1A in these experiments was between 0.14 and 0.70.

In all further experiments, we analyzed genomic DNA (exon 1 and 2 for RASSF1A and the whole RBSP3 transgene in pETE vector) if not specially mentioned.

Mutations in RASSF1A can be generated de novo

To distinguish between the possibility that different mutated RASSF1A genes were mutated at once (“burst of mutations”) or were constantly generated over time, we performed experiments with single cells. In this experiment BL2 cells, (which previously showed the highest rate of mutation: 10 clones with 25 mutations), were diluted and plated into wells with an expected frequency of 0.3 cells per well. Three randomly selected wells (designated as BL2-cl.1, 2 and 3) containing single cells were expanded and further analyzed. DNA was isolated from these clones after 10 days (approximately 10 divisions, 10^3 cells).

The results were as follows: for BL2-cl.1, five of 10 sequenced clones were mutated (mutation frequency per 100 bp (MF), was 0.14), for BL2-cl.2, five of 13 clones (MF = 0.15; two clones contained T43T mutations with codon changed from ACA to ACC) and for BL2-cl.3, three of 17 clones were mutated (MF = 0.07; two clones contained N70G mutations). Altogether 16 single base pair mutations were detected, all were transitions and only five of them showed mutated G or C. This experiment clearly shows that mutations in the RASSF1A locus could be generated de novo during cell proliferation.

The complete list of 129 mutations (111 mutations were different) found in exons 1 and 2 of RASSF1A in all experiments is shown in Table S1A. See also Table 2 and 3 and Figure 1A. Altogether 144 clones were sequenced (56.3 KB) and the average frequency of mutations was 0.23/100 bp for transcribed sequences and 0.17/100 bp for coding sequences. Among them, there were four nucleotide changes that occurred in non-coding 5’, three stop (nonsense) and five frameshift (deletions) mutations. Of the remaining 127 mutations, 82 were missense and 35 synonymous.

RBSP3 is also hypermutated in various cancers

During previous analyses of small cell lung carcinoma (SCLC) cell line N417, two RCC, one breast carcinoma (BC) and two
ovarian carcinoma (OC) biopsies that all expressed RBSP3, we detected mutations in the RBSP3 cDNA in all six cases [10; see Table S2A].

To test whether the hypermutation feature is a characteristic only of the RASSF1A gene or a more general phenomenon, we similarly analyzed the recently identified multiple tumor suppressor gene RBSP3 located in AP20, 3p21.3 telomeric region [10].

Using RT-PCR, cDNA was isolated from two of each RCC, BC and OC biopsies and the SCLC cell line N417. Multiple clones were sequenced. Results, presented in Table S2A, Table 3 and Figure 1B, showed that almost all isolated clones suffered mutations. As reverse transcriptase used in RT-PCR has a significantly higher error rate than other polymerases used in PCR, we attempted to reproduce the observed high mutation rate at the genomic DNA level, as in the case with RASSF1A. Unfortunately, it was difficult to perform this experiment on the genomic RBSP3 due to the large size of the gene (more than 120 kb), numerous small exons (at least 9), and high GC content (reaching 100% in some regions). However this problem was solved using cloned RBSP3 in SCID mice.

RBSP3 revealed high mutability in SCID mice on genomic level

SCLC cell line ACC-LC5 and RCC cell line KRC/Y were transected with RBSP3A and RBSP3B splicing isoforms in the pETE vector and stable cell clones were isolated. Four of these clones (AHA1 and AHB1 for ACC-LC5 and KHA4 and KHB9 for KRC/Y) were inoculated into SCID mice (see M/M). Cell clones KHA4 and KHB9, containing RBSP3A or RBSP3B were grown in vitro in parallel with tumors in SCID mice. After 8 weeks DNA was isolated from grown tumors and cell lines, and the RBSP3A and B genes were amplified by PCR from pETE vector and cloned. Again multiple clones were sequenced and results of the experiment are shown in Table 4 and Table S2B. Only 30% of RBSP3A KHA4 and KHB9 plasmid clones were mutated in vitro, as compared to 85% mutated clones after growth in SCID mice. This difference according to Fischer test is statistically significant (P<0.001).

In summary, in RBSP3 experiments we identified 89 mutations among which 79 were individually distinct (see Tables S2A and S2B). The average frequency of mutations was 0.10/100 bp for transcribed sequences. This frequency is more than 0.11/100 bp for coding sequences (see Table 3 and Figure 1B). Among them, seven nucleotide changes occurred in non-coding regions and five were frameshift (deletions) mutations. Of the remaining 77 mutations, 68 were missense and 9 synonymous.

Thus, the mutation frequency was 2.5 fold less than for the first two exons of the RASSF1A (see above). The significant difference in mutation frequencies could be accounted for by differences in nucleotide composition of the genes, or it could reflect intrinsic differences in the hypermutation rates of the genes. It could also be important that for the RBSP3 the whole gene was sequenced while for the RASSF1A only its 5’ end.

**RASSF1A and RBSP3 amplified by PCR from E.coli DNA don’t show high frequency of mutations**

We have performed PCR amplification of E. coli DNA containing plasmids (i.e. total DNA isolated from E.coli containing mixture of genomic and plasmid DNA) with these two genes. For each gene ten and four ng of DNA was used. Unfortunately lower

| Locus | RASSF1A/Cell line | Description | Number of clones, mutated+nonmutated | Mutation frequency, per 100 bp | Total number of mutations | Deletions | Transitions over transversions | Mutations of G/C nucleotides |
|-------|------------------|-------------|--------------------------------------|-------------------------------|---------------------------|-----------|-------------------------------|-------------------------------|
| IARC171 | Burkitt’s lymphoma derived cell line | 11+7 | 0.23 | 15 | no | 2 | 9 |
| BL2 | Burkitt’s lymphoma derived cell line | 10+0 | 0.70 | 25 | 3 | 1 | 16 |
| RAMOS | Burkitt’s lymphoma derived cell line | 11+2 | 0.56 | 26 | 1 | 1.2 | 15 |
| mutulli | Burkitt’s lymphoma derived cell line | 7+0 | 0.56 | 14 | 1 | 1.6 | 9 |
| TK10 | renal cell carcinoma derived cell line | 9+13 | 0.15 | 12 | no | 0 | 8 |
| TK164 | renal cell carcinoma derived cell line | 3+0 | 0.28 | 3 | no | 2 | 2 |
| KRC/Y | renal cell carcinoma derived cell line | 2+2 | 0.14 | 2 | no | 0 | no |
| T356(RCC) | renal cell carcinoma biopsy | 6+0 | 0.51 | 11 | 3 | 0.6 | 5 |
| N356(RCC) | normal renal cell biopsy | 1+13 | 0.02 | 1 | no | 0 | no |
| Caki1 | renal cell carcinoma derived cell line | 1+0 | 0.28 | 1 | no | 0 | 1 |

**Table 2. Mutations in RASSF1A exon 1and 2 in different cell types.**

| Gene, length | Number of sequenced clones | Total length/coding sequences, Kbp | Number of mutations | Mutation frequency, per 100 bp |
|-------------|----------------------------|-----------------------------------|---------------------|------------------------------|
| RASSF1, exons 1–2 | 144 | 56.3/51.4 | 129 | 0.23 | 0.17 |
| RASSF1, exons 3–5 | 98 | 50.6 | 146 | 0.29 | 0.29 |
| RBSP3, exons 1–8 | 85 | 85.3/70.6 | 89 | 0.10 | 0.11 |

**Table 3. Experimental mutations frequency in the RASSF1A and RBSP3 genes.**

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amount of E. coli DNA didn’t produce sufficient amount of PCR products for further cloning. Ten clones in each experiment were sequenced and no mutations were detected. These results indicate that the observed hypermutation rate of RASSF1A and RBSP3 cannot be explained by PCR polymerase errors.

Search for founder mutations in RASSF1A in single cell clones

The main idea of this experiment was the following. If a mutation originates in the cell and not in the tube in vitro then in the cell population grown from one cell some fraction (depending on the number of alleles present in the single cell) of plasmid clones should contain the same (i.e. a founder) mutation. To perform this experiment we isolated 15 single cell KRC/Y clones as described in the section “Mutation generated de novo”. In this case we grew the cells for three weeks to obtain more DNA and generate more mutated clones. KRC/Y cells were used instead of BL2 cells as it was easier to detect that we have one cell in the well. However, we of course cannot exclude that in some of the 15 selected wells there were more than one cell. RASSF1A exons 3–5 were tested in this experiment (see M/M) as they were more easily isolated than exons 1 and 2 and contained more sequence information (516 nt vs. 391 nt). Moreover, according to EST sequence data this part of RASSF1A has higher MF. From each PCR reaction 10 plasmid clones were selected and DNA was isolated. However, due to different technical problems (no or rearranged insert, bad quality DNA or sequencing, etc.) usually only six-seven plasmid clones were further analysed. Totally 98 plasmid clones were sequenced (Table 5). One founder mutation was detected in all cell clones and in 46 (47%) of plasmid clones were further analysed. Totally 98 plasmid clones were sequenced (Table 5). One founder mutation was detected in all cell clones and in 46 (47%) of plasmid clones. It was a change of A to G (nt26735, Accession No. AC002481) just at the border of intron 2 and exon 3. This mutation destroyed the splice acceptor site AG/G and thus inactivated the gene. As this founder mutation appeared in all single cell clones most probably it originated before we started to do this experiment. Forty other founder mutations specific for each cell clone were also detected (see Table 5 and Table S1B). Interestingly in one case it was possible to construct a tree showing how founder mutations were accumulated. First it was only one mutation than two and then additional independent mutations (Figure 2).

Table 4. Mutation frequency of the RBSP3A and RBSP3B genes in vitro and in vivo in the gene inactivation test.

| Gene/cell clone | In vitro | In vivo |
|-----------------|----------|---------|
|                 | tested  | mutated | tested | mutated |
| RBSP3A/KHA4     | 11      | 3       | 13     | 10      |
| RBSP3B/KHB9     | 12      | 4       | 15     | 14      |
| Total           | 23      | 7       | 28     | 24      |

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Table 5. Founder mutations in single cell KRC/Y clones.

| Cell clone | Mutation | Number of sequenced plasmid clones with founder mutation |
|------------|----------|----------------------------------------------------------|
| All 15 clones | m26.735(A→G) | 46 |
| 1 | T196T | 3 |
| 2 | R240R, R241R | 2 |
| 3 | D157N | 4 |
| 4 | I139T | 5 |
| 4 | V225A | 4 |
| 5 | N155S | 3 |
| 8 | K232R | 3 |
| 9 | I139N | 3 |
| 9 | I146N | 2 |
| 10 | L256W, P274P | 2 |
| 12 | E126G | 2 |
| 14 | L260S | 4 |
| 15 | D262G | 3 |
| Total founder mutations | | 86 |

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Different mutation frequencies in other genes

Similar sequencing experiments were performed with insulin and albumin genes isolated from KRC/Y cell line (see M/M). In contrast to RASSF1A and RBSP3 results, only one of 21 sequenced insulin genomic clones (999 bp including complete ORF) and one of 19 albumin cDNA clones (700 bp, exons 12–15) were mutated (MF for both genes was less than 0.01). However in both cases, we could not exclude the possibility of polymorphisms. Additionally, two more genes were tested for mutations in genomic DNA. GPR14 (G protein-coupled receptor 14, 1018 bp) and transcription elongation factor A (SII) TCEA1 (1066 bp) were PCR amplified (see M/M) from DNA of KRC/Y cells and 11 clones for each gene were sequenced. All clones contained normal copies of the gene. No mutations were found in other 3p21.3 candidate genes: mut in the list of mutations [http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=11186]. No SNP were found in RBSP3 sequences.

Figure 2. Flow chart showing accumulation of mutations (including two founder mutations) in RASSF1A exons 3–5 in the single cell clone #9. Synonymous mutation Pro122Pro was caused by nucleotide change ATC→AAC. Mutation GTC→GTA also didn’t result in any amino acid change (Val174Val).
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Mutations in RASSF1A, RBSP3A and RBSP3B are not generated by AID or APOBEC related mechanisms

It has been recently shown that the activation-induced cytidine deaminase (AID) is responsible for somatic hypermutations in activated B cells. Moreover hypermutations generated by this enzyme in oncoproteins can cause malignancies in haematopoietic cells [24]. Although much remains to be learned concerning AID, several target sequence motifs for the mutations have been identified, namely WRC, RGYW and DGYW causing C/G mutations. The large family of APOBEC genes, also shown recently to mutate genes on DNA level [21,22], mostly targeted the RCW motifs causing mutations in C/G. Therefore, we checked whether these motifs were targeted or more frequent in RASSF1 and RBSP3 sequences when compared to the stable insulin gene. The frequency of the WRC motif per 100 bp varies from 12.5 to 14.3 for RASSF1 and RBSP3 sequences, and the insulin gene contains 16.5 such motifs per 100 bp. Other motifs showed the same distribution (also higher in the insulin gene), arguing against the involvement of these enzymes in hypermutating the RASSF1 and RBSP3 genes described here. Indeed, the APOBEC and AID enzymes cause mutations almost exclusively in C/G nucleotides, while we observed mutations of all 4 nucleotides (Figure 3). The results actually showed that mutations in A/T were even more frequent than in C/G. We tried to find a recognition motif. We studied all mutations (Figure 3A) or a subset of mutations (Figures 3B and 3C), but no obvious motifs have been yet identified.

More studies are needed to resolve this question as this pattern can be different in normal and cancer cells and could be dependent on nucleotide composition of a gene. These small differences in patterns could mask the recognition motif.

RASSF1A and RBSP3 mutants from RCC biopsy and lung cancer cell line have significantly reduced growth-inhibiting activity

We tested one RASSF1A gene, isolated from an RCC biopsy that contained two mutations (Cys65Arg and Val211Ala), for growth inhibition under cell culture conditions following transfection into the KRC/Y and prostate cancer LNCaP cells. In KRC/Y cells the mutated gene had a significantly reduced growth inhibition under cell culture conditions following transfection that contained two mutations (Cys65Arg and Val211Ala), for both genes was less than 0.01). However in both cases, we could not exclude the possibility of polymorphisms. Additionally, the APOBEC and AID enzymes cause mutations almost exclusively in C/G nucleotides, while we observed mutations of all 4 nucleotides (Figure 3). The results actually showed that mutations in A/T were even more frequent than in C/G. We tried to find a recognition motif. We studied all mutations (Figure 3A) or a subset of mutations (Figures 3B and 3C), but no obvious motifs have been yet identified.

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Conclusions

By sequencing 327 RASSF1A and RBSP3 clones, we detected 364 mutations with frequencies reaching 0.70 per 100 bp. Interestingly many clones contained more than 1 mutations (see Table S3A, B, C). Only one SNP was detected in RASSF1A ten clones (exon1 A→G) and it was excluded from the list of mutations [http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=11186]. No SNP were found in RBSP3 sequences.

The frequency of mutations was similar to other reported cases of somatic hypermutations found in Rho/TTF, MYC and BCL6 in large-cell lymphomas (MF was from 0.12 for MYC to 0.69 for
Figure 3. Distribution of mutations in \textit{RASSF1A} (A) and \textit{RBSP3} (B and C). For \textit{RASSF1A} all mutations were analyzed. For \textit{RBSP3} mutations found in GIT (B) and in human cancer (C) were analyzed separately. Bubble graphs depict the proportion of substitutions occurring at each of the four bases in the \textit{RASSF1A} and \textit{RBSP3}, depending on the distance from the mutated nucleotide (No. 0). N, any nucleotide; B = C, G or T; D = A, G or T; S = G or C; V = A,C, or G; W = A or T.

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However it was significantly lower than for immunoglobulin genes (12.7 mutations per 100 bp, see [25]). However, for the first time we found high frequency of somatic mutations in different tissues including non-haematopoietic and in tumor suppressor genes contrary to the previous reports where oncogenes were studied.

As AccuPrime™ Pfx DNA polymerase creates maximally one error in $3 \times 10^9$ bp, our results proved that the observed

Figure 4. Reduced growth inhibiting activity of RASSF1A (A) and RBSP3A (B) mutants. A. Growth of stably transformed KRC/Y RCC cells with wild type and mutant RASSF1A (Cys65Arg and Val211Ala) without doxycycline (the gene is on) is presented in A. On day 6, the number of cells with wt RASSF1A was $3 \times 10^5$ and the number of cells with mutant RASSF1A was $5 \times 10^5$ (1.7 times more than wt). On day 10, the number of cells with wt RASSF1A was $6 \times 10^5$ and the number of cells with mutant RASSF1A was $1.8 \times 10^6$ (3 times more than wt). The effect of expression of wild type and mutant RBSP3A and RBSP3B on colony formation efficiency in KRC/Y cells is shown in B. Mutants were isolated from the N417 SCLC cell line (His139Tyr), the ovarian tumor biopsy T4 (three mutations: Asn31Asp, Pro79Ser and Glu87Lys) and the KRC/Y cell line (three mutations: Lys35Met, Asp103Gly and Leu181Pro). The number of blasticidin-resistant colonies compared to the empty pETE were: 90% for mutant from N417, 15% for mutant from T4 biopsy and 25% for mutant from KRC/Y. The number of colonies for wtRBSP3A were 5–10% compared to the pETE colonies.

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hypermutation frequencies in the experiments could not be explained by erroneous performance of polymerases. In our experiment with SCID mice when AccuPrime™M™ DNA polymerase and 25 cycles were used, 85% of RBSP3 clones contained mutations.

During the growth of the same cell lines in vitro, 30% of RBSP3 clones (also 25 cycles and AccuPrime™M™ DNA polymerase) were mutant.

In experiments with RASSF1A (391 bp of the first and second exons), 65% of clones contained mutations (experiments with normal cells are not included). Moreover, in our experiments, we used different polymerases with different error rate (see M/M) and no significant differences in mutation frequency were observed, arguing against the generation of the mutations during PCR amplification. Different mutation frequencies between in vivo and in vitro experiments and in ESTs isolated from normal and cancer cells is an additional argument against the artificial nature of the hypermutation rate observed in RBSP3 and RASSF1 genes.

Mutations were detected with similar frequency both in cDNA and genomic DNA for RBSP3 and RASSF1, however, no high mutability either on genomic or cDNA level were found for albumin, insulin, GPR14, TSG p16/INK4a or transcription elongation factor A (SII) TCEA1. Moreover, no mutations were found in experiments with SCID mice for 5 genes: BLU, 101F6, PL6, mutFUS1 and mutP53. Expression of RBSP3 [10] in six tested samples differed almost 50-fold and on genomic level RBSP3 was present usually in 3–8 copies [26]. Our previous experiments using marker NL3-001 located 90 kb apart from the RASSF1A demonstrated that in tumor cells this region in most cases is present in 1–5 copies [4,5]. Still the frequency of mutations was almost the same. Thus number of template molecules didn’t influence significantly mutability level. Moreover, repeated sequencing of the same plasmid and isolated by different persons and at different time gave identical results (6 RASSF1A and 6 RBSP3 plasmid clones were sequenced) excluding frequent sequencing errors.

Both genes are CG rich however it seems that although high CG content can induce additional mutations it cannot explain the fact that two genes with significantly different CG content (RASSF1A, exons 1–2, 72.3%; RASSF1A, exons 1–6, 59.8% and RBSP3, exons 1–8, 54.3%) both possess high mutability while other genes with similar CG content (e.g. GPR14, 72.5%; p16/INK4a, 71.6%; insulin 61.6%) didn’t show any high frequency of mutations.

Using the same PCR conditions plasmids containing RBSP3 and RASSF1A were amplified from E.coli and no mutations were discovered arguing against generation of mutations during PCR amplification.

Experiments to find founder mutations with single-cell clones additionally confirm that mutations originate in the cell. Interestingly that from the single cell clone No. 9 we isolated plasmids with one, two or three mutations. This fact clearly showed how these mutations originate from one parental cell clone (Figure 2). Importantly after sequencing exons 3–5 of RASSF1A gene from KRC/Y we discovered founder mutation (destroying splice acceptor site) that was present in approximately 50% of 98 sequenced plasmid clones. This founder mutation appeared in all single cell clones and thus most likely it originated before we started this experiment.

For identification of tumor suppressor genes, we use the gene inactivation test, GIT [26,27]. This test is based on the functional inactivation of the analyzed genes during tumor growth in SCID mice. Our hypothesis was that under selective pressure in vivo the introduced TSG must be inactivated in growing experimental (xenografted) tumors (by deletion, mutation, promoter methylation) as in the naturally growing tumors. The expression of the tested gene in the GIT was regulated by tetracycline and the level of expression was under physiological conditions. In our published papers [10,23] wild type RBSP3 and wild type and mutated RASSF1A genes were tested in GIT. The genes were PCR amplified from tumors and sequenced. In contrast to the wild type RBSP3 and RASSF1A genes, that were inactivated (i.e. deleted, non-expressed, mutated) in all 32 grown tumors, the mutant RASSF1A was not additionally mutated in any of four analyzed tumors. Importantly, in these GIT experiments we used direct sequencing of PCR products. These experiments showed that “founding mutations” really do exist.

Analysis of public EST databases confirmed our experimental data. It should be noted that the frequency of mutations in RASSF1A and RBSP3 found in EST databases even using very stringent criteria was significantly higher than found in our experiments. MF for all mutations for RBSP3 was 0.63 and for RASSF1 it was 0.22. Probably, this discrepancy could account for the differences between the cell types analyzed in our experiments and in the EST database.

Unfortunately only 17 RASSF1A clones could be analysed because other EST sequences were either not sufficiently good or could be other isoforms of the RASSF1 gene.

Interestingly, mutations of RASSF1A and RBSP3 changing amino acids were found even in clones isolated from normal cell RNA, however, at a lower frequency than in cancer cells (MF ratios for cancer/normal sequences were 3.3 and 3, respectively). This difference for both genes was statistically significant (<0.001) This probably reflects the selection for and the advantage of coding mutations during cancer progression. Important to mention that “normal” sequences include also non-annotated sequences so we cannot exclude that some of the “normal” sequences actually represent cancer cells.

In fact, these results correlate with the data from the mouse in vivo experiments that showed a higher frequency of mutations in SCID tumors than in the same cells grown in vitro. Interestingly, the same mutations were observed in cells grown in vitro and in vivo, in SCID mice (see Table S2).

We have also experimentally tested whether RASSF1A genomic DNA, exons 1 and 2 harvested mutations in normal tissues and found one mutated clone out of 14 in normal kidney (normal control to T356, see section “Frequent mutations in RASSF1A in human carcinomas”). Important to note that so called “normal” kidney could be already partially transformed despite of normal phenotype because it was obtained from tissues adjacent to the tumor. We also sequenced complete RASSF1A cDNA from normal heart and detected six mutated clones out of 15 tested. All six heart mutated clones contained the same two mutations: L214L with codon changed from CTA to CTG and V236V with codon changed from GGT to GAC. Furthermore, in all 32 grown tumors, the mutant RASSF1A was not additionally mutated in any of four analyzed tumors.

As we found mutations in all 3 coding exons of RASSF1A (the last 6th exon contains only 48 amino acids = 144 bp). It means that other six known isoforms of RASSF1A are also frequently mutated.

Exceptionally high level of germ line SNP mutations in RASSF1A found in several studies [8] support our data that the two genes we studied have rather frequent mutations even in normal cells. The pattern of mutations was very different compared to those reported for AID and APOBEC enzymes and cannot be explained
by polymerase errors. This is the first report of high mutation frequencies of \textit{RASSF1} and \textit{RBSP3} genes in different epithelial malignancies. In our preliminary paper [20] we analyzed mutations in \textit{RASSF1A} gene in NPC samples and the results supported the present observations. In the NPC experiments 35 mutations were detected in 23 patients and mutations were considered real if at least two clones from the same patient contained the same mutation. Ten clones for each sample were sequenced in these experiments. Both DNA strands were sequenced.

At present, we don’t know the nature of the mechanism responsible for this hypermutability, and only speculations could be done for its physiological function(s) in normal cells. There are several DNA polymerases in vertebrate cells that inaccurately copy templates and could be involved in generating hypermutations [29]. One of them, \textit{POLH} (error rate 3 \times 10^{-5}), has a mutation target motif WA and may contribute to hypermutagenesis of immunoglobulin genes at A-T bases [30]. \textit{POLH} is expressed in all tissues and, in principle, could cause hypermutations in non-haematopoietic cells. We found that 50\% of all observed mutations in \textit{RBSP3} happened in A or T surrounded by G or C. That means that the mutation target motif for 50\% of mutations in \textit{RBSP3} is SWS and is different from the \textit{POLH} motif. It is reasonable to suggest that other(s) yet unknown DNA polymerase(s) may be responsible for the high mutability rates we report here and more than one polymerase contributes to hypermutations [29,31].

Our results also argue that mutations are not completely random. They are not correlated with predicted numbers (Tables 1, 2, 3). For example according to statistical calculations our sequences of \textit{RASSF1A} exons 1 and 2 should contain 0.026 nonsense mutations but in reality we detected 3 nonsense mutations, P<0.001 (see Table S1A). For \textit{RASSF1A} exons 3–5 the predicted number of nonsense mutations is 0.037 and we found 3 such mutations, P<0.001 (see Table S1B). This fact may reflect the nature of cancers and normal tissues studied here. We cannot also exclude that these mutations still have some preferable motif(s).

We mentioned in the text that clonal selection for more aggressive growth of cancer cells could add to changing proportion of different mutations. In our previous paper [28] we also observed an unusual distribution of mutations. Among 35 detected mutations we found 30 transitions, 3 transversions, 2 deletions (frameshift), 3 nonsense (stop), 26 missense and only 4 were synonymous.

High frequency of mutations in different cancers and normal cells was reported earlier for P53 [32]. However, at present it is difficult to compare these results with our study as different methodologies were used and most likely different mechanisms of mutagenesis were involved.

When this manuscript was completed two new publications appeared in PNAS that support our observations and concept [33,34]. Interestingly, in the paper of Yang et al. [35] hypermutability was demonstrated in damaged single-strand DNA formed at double–strand breaks in yeast \textit{S. cerevisiae}. Although yeast data may not apply to human cells, it is worthwhile to note that AP20 and LUCA regions where \textit{RASSF1} and \textit{RBSP3} are located were found extensively damaged (deletions, amplifications) in 90\% of studied major epithelial cancers [2,4,5, see Introduction].

Materials and Methods

Ethics Statement

All work with mice was performed in special “Animal House” in MTC according to the standard rules. The study was done in accordance to the guidelines (incl. husbandry) issued by the STOCKHOLMS Norra Djurforsoksetiskt Namnd (Animal Ethic Committee of North Stockholm).

Paired tumor/normal samples were obtained from the Blokhin Cancer Research Center, Russian Academy of Medical Sciences after surgical resection of primary tumors and stored in liquid nitrogen. Top and bottom sections (3–5 \textmu m thick) cut from frozen tumor tissues were examined histologically 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 Ethic Committee of the 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.

Cell lines and experiments with SCID mice

Cell lines were obtained from the MTC-KI (Stockholm, Sweden) cell lines collection. Cell and tumor growth assays were done as described previously [13,16,23,36]. GIT was performed as described previous [16,26,27].

In brief, plasmid DNAs were purified using R.E.A.L. Prep kit (Qiagen, Valencia, CA). Transfections were performed using LipofectAMINE PLUS Reagent (Life Technologies, Rockville, MD) according to the manufacturer’s protocol. After transfection, cells were selected with 5 \textmu g/ml Blasticidin and 200 ng/ml doxycycline for two-four weeks. For colony formation assay cells were selected for 2 weeks, fixed, stained with Giemsa and counted for transfection efficiency. For isolation of stably transfected cell clones, selection was done for four weeks. PCR positive clones from each recombinant were tested for expression using Northern hybridization and selected clones, 5 \times 10^6 cells/mouse, were inoculated subcutaneously with or without Matrigel (BD, Franklin Lakes, NJ) into six-week-old female SCID mice. Each mouse received only 1 injection. SCID mice were observed for tumor formation twice a week for up to seven weeks, if tumor formation was observed, tumors were measured using calipers. The tumors were explanted for DNA preparations.

General methods

All molecular biology and microbiology procedures were performed as described previously [10,13,36]. DNA and RNA were isolated from total tumor samples containing less than 30\% of non-tumor cells according to histopathology examination.

Construction of pETE vector and KRC/Y and LNCaP cell lines producing tetracycline trans-activator tTA were described in ref. [26].

Polymersases used for PCR

In experiments with cell lines and biopsies we used natural Taq polymerase (New England Biolabs, Ipswich, MA, USA) and JumpStartTM AccuTaqLA DNA Polymerase (Sigma-Aldrich, St. Louis, MO, USA). In some experiments (for comparison) we used AccuPrimeTM PfX DNA polymerase (Invitrogen, Carlsbad, CA, USA). No significant difference was observed between these three polymerases. Usually 30 cycles were used.

In experiments with single cell clones and SCID mice AccuPrimeTM PfX DNA polymerase and 25 cycles were employed.

Natural Taq polymerase has an error rate 4.5–5 \times 10^{-1} (i.e. maximally 1 mistake per 200,000 bp) and the JumpStartTM AccuTaqLA DNA Polymerase exhibits 6.5 fold higher fidelity. In many experiments, to exclude the possibility of generating mutations during the polymerization, we used the most error free polymerase
available (15-26 fold better fidelity than ordinary Taq polymerase) AccuPrime™Pf DNA polymerase and only 25 cycles.

The size of the RBSP3B is 1003 bp and the accuracy of ordinary Taq polymerase is approximately one error in 2 × 10^3 bp. This means that after 30 cycles 15% of clones would be expected to contain mutation(s) in the RBSP3 and after 25 cycles 12.5%. In the case of AccuPrime™Pf DNA polymerase, after 30 cycles 1% of clones would be mutant and after 25 cycles only 0.34%. In our experiment with SCID mice, 85% of RBSP3 clones contained mutations (AccuPrime™Pf DNA polymerase, 25 cycles).

During the growth of the same cell lines in vitro, 30% of RBSP3 clones (also 25 cycles and AccuPrime™Pf DNA polymerase) were mutant.

PCR and Sequencing

PCR primers were purchased from Invitrogen (Carlsbad, CA, USA). PCR was done as described earlier [2]. Initial denaturation was done for 2 min at 95°C following 25–30 cycles; 95°C for 15 sec, 64°C for 30 sec and extension at 68°C for 1 min per 1 kb.

RBSP3A and RBSP3B gene fragments (ORF) have been obtained by PCR from cDNA isolated from different cell lines using the following primer sets, according to the manufacturer’s manual.

**RBSP3B:** 120C: 5’-GCCGCGCGCCGCGGCGGACC-CATGAGCCCGCCGACACATC-3’ (nucleotides 1-40) and HYA22C: 5’-AAAAACAAAAAGGATAGCATTGGGCACTTCATT-3’ (nucleotides 1003-973). See GenBank Accession No. AJ575645

**RASSF1A:** genomic fragments (GenBank Accession No. AC002481).

- Ex1-Ex2: F2A: 5’-GCCAAAGCCGGCCGAGAC-3’ (nucleotides 1003-1807) and Ex2F2: 5’-ACCCAGG-CAGCCCTGGAGA-3’ (nucleotides 21066-21047).
- Ex3-Ex5: RassF1-2intrF: 5’-GTGCCAATTCGCCAGCAAGAC-3’ (nucleotides 25730-25754).

**RASSF1A:** cDNA fragment cDNA (ORF):

- F2A: 5’-GCCCAAGCCGAGCCGACAC-3’ (nucleotides 97-116) and
- F2B: 5’-AGCAGTACTGCTGCTGAGAGGACCAG-3’ (nucleotides 1343-1319), see GenBank Accession No. NM_007182

**GPR14:**

- GPR14F: 5’-GCCATCTGAGGAGCTGCTCTC ATA-3’ (nucleotides 26713-26734) and RassF1-5exR: 5’-CACCTGCAATTCGGTCCTGCTC-3’ (nucleotides 27530-27554).

**Bioinformatics**

For RASSF1A only exons 1 and 2 with a total length of 357 bp (GenBank Accession No. NM_007182) were analyzed. For RBSP3 the longest isoform B (GenBank Accession No. AJ575645; the total length is 831 bp) was analyzed. The gene sequences were searched against GenBank EST division, a collection of expressed sequence tags, or short, single-pass sequence reads from mRNA (cDNA). The statistically significant thresholds for the alignment (score) that provided elimination of alien mRNA sequences was set for RASSF1A at 462 and for RBSP3 at 404. These thresholds were obtained from expertise estimation to cut off clusters of short and non-significant homologies to the query sequences. An additional manual refinement against low quality sequences was performed. Nucleotide similarity searches were performed with BLAST 2.2.

In all experiments we always compared a given sequence with the annotated sequences as shown in previous paragraph.

Probabilities of mutation frequency differences were calculated using Poisson distribution.

**Supporting Information**

**Table S1**

| Found at: | doi:10.1371/journal.pone.0005231.s001 (0.39 MB DOC) |

**Table S2**

| Found at: | doi:10.1371/journal.pone.0005231.s002 (0.16 MB DOC) |

**Table S3**

| Found at: | doi:10.1371/journal.pone.0005231.s003 (0.15 MB DOC) |

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**Author Contributions**

Conceived and designed the experiments: VIK VMB LK YSZ ITE MIL GKR ERZ. Performed the experiments: VIK TVP EVG AK SPY JL FW EP ARP MZ VS VIZ TE.

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References

1. Alimov A, Kost-Alimova M, Liu J, Li C, Bergerheim U, Imreh S, et al. (2000) Combined LOH/CGH analysis proves the existence of interstitial 3p deletions in renal cell carcinoma. Oncogene 19: 1392–1399.

2. Braga E, Senchenko V, Bazov I, Loginow W, Liu J, Ermilova V, et al. (2002) Critical tumor-suppressor gene regions on chromosome 3p in major human epithelial malignancies: allelotyping and quantitative real-time PCR. Int J Cancer 100: 534–541.

3. Li J, Protopopov A, Wang F, Senchenko V, Petrushik V, Voronova O, et al. (2002) Nod subtraction and Nod-specific microarrays to detect copy number and methylation changes in whole genomes. Proc Natl Acad Sci U S A 99: 10724–10729.

4. Senchenko V, Liu J, Braga E, Mazurenko N, Loginow W, Seryogin Y, et al. (2003) Deletion mapping using quantitative real-time PCR identifies two distinct 3p:21.3 regions affected in most cervical carcinomas. Oncogene 22: 2904–2909.

5. Senchenko VN, Liu J, Loginow W, Bazov I, Angeloni D, Seryogin Y, et al. (2004) Discovery of frequent homozygous deletions in chromosome 3p:21.3 LUCa and AP20 regions in renal, lung and breast carcinomas. Oncogene 23: 5719–5728.

6. Zabarovsky ER, Lerman MI, Minna JD (2002) Tumor suppressor genes on chromosome 3p involved in the pathogenesis of lung and other cancers. Oncogene 21: 6915–6935.

7. Imreh S, Klein G, Zabarovsky ER (2003) Search for unknown tumor-antagonizing genes. Genes Chromosomes Cancer 36: 307–321.

8. Dammann R, Schagdarsurengin U, Strunnikova M, Rastetter M, Seidel C, Imreh S, Klein G, et al. (2003) Search for unknown tumor-antagonizing genes. Genes Chromosomes Cancer 36: 307–321.

9. Shivakumar L, Minna J, Sakamaki T, Pestell R, White MA (2002) The region on human chromosome 3p21.3: identification and evaluation of the tumor suppressor locus 3p21.3. Cancer Res 62: 3498–3502.

10. Li J, Wang F, Protopopov A, Malyukova A, Kashuba V, Minna JD, et al. (2002) The RASSF1A tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation. Mol Cell Biol 22: 4309–4318.

11. Kashuba VI, Li J, Wang F, Senchenko VN, Protopopov A, Malyukova A, et al. (2004) RBSP3 (HYA22) is a tumor suppressor gene implicated in major epithelial malignancies. Proc Natl Acad Sci U S A 101: 4906–4911.

12. Buermeier AB, Deschesnes SM, Baker SM, Liskay RM (1999) Mammalian DNA mismatch repair. Annu Rev Genet 33: 333–569.

13. Protopopov AI, Kashuba VI, Rahvaldion K, Protopopov A, Duhl FM, Geil L, et al. (2004) Functional characterization of the candidate tumor suppressor gene NPR2/G21 located in 3p21.3C. Cancer Res 64: 6438–6443.

14. Spandidos DA (1980) A unified theory for the development of cancer. Biochii Rep 68: 691–708.

15. Bischof JM (1987) The molecular genetics of cancer. Science 235(4786): 305–311.

16. Dreijerink K, Braga E, Kuzmin I, Geil L, Duhl FM, Angeloni D, et al. (2001) The candidate tumor suppressor gene, RASSF1A, from human chromosome 3p:21.3 is involved in kidney tumorigenesis. Proc Natl Acad Sci U S A 98: 7504–7509.

17. Kuzmin I, Gillespie JW, Protopopov A, Geil L, Dreijerink K, Yang Y, et al. (2002) The RASSF1A tumor suppressor gene is inactivated in prostate tumors and suppresses growth of prostate carcinoma cells. Cancer Res 62: 3498–3502.

18. Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP (2000) Epigenetic inactivation of a RAS association domain protein from the lung tumor suppressor locus 3p21.3. Nat Genet 25: 315–319.

19. Lerman MI, Minna JD (2000) The 630-kb lung cancer homozygous deletion region on human chromosome 3p:21.3: identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium. Cancer Res 60: 6139–6143.

20. Muto T, Muramatsu M, Tanakawa M, Kinoshita K, Honjo T (2000) Isolation, tissue distribution, and chromosomal localization of the human activation-induced cytokine deaminase (AID) gene. Genomics 68: 85–90.

21. Beale RC, Petersen-Mahrt SK, Watt IN, Harris RS, Rada C, Neuberger MS (2004) Comparison of the differential context-dependence of DNA deamination by APOBEC enzymes: correlation with mutation spectra in vivo. J Mol Biol 337: 585–596.

22. Contecllo SG, Thomas CF, Petersen-Mahrt S, Neuberger MS (2005) Evolution of the AID/APOBEC family of polynucleotide (deoxy)cytidine deaminases. Mol Biol Evol 22: 367–77.

23. Li J, Wang F, Protopopov A, Malyukova A, Kashuba V, Minna JD, et al. (2004b) Inactivation of RASSF1C during in vivo tumor growth identifies it as a tumor suppressor gene. Oncogene 23: 5941–5949.

24. Okazaki IM, Hiei H, Kakou N, Yamada S, Muramatsu M, Kinoshita K, et al. (2003) Constitutive expression of AID leads to tumorigenesis. J Exp Med 197: 1173–1181.

25. Pasqualucci L, Neumister P, Goosens T, Nanjangud G, Chaganti RS, Kipper R, et al. (2001) Hypermutation of multiple proto-oncogenes in B cell diffuse large-cell lymphomas. Nature 412: 341–346.

26. Protopopov AI, Li J, Winberg G, Giatroullin RZ, Kashuba VI, Klein G, et al. (2002) Human cell lines engineered for tetracycline-regulated expression of tumor suppressor candidate genes from a frequently affected chromosomal region, 3p:21.3. J Gene Med 4: 397–406.

27. Li J, Protopopov AI, Giatroullin RZ, Kiss C, Kashuba VI, Winberg G, et al. (1999) Identification of new tumor suppressor genes based on in vivo functional inactivation of a candidate gene. FEBS Lett 451: 289–294.

28. Pan ZG, Kashuba VI, Liu XQ, Shao JY, Zhang RH, Jiang JH, et al. (2005) High frequency somatic mutations in RASSF1A in nanopharogyal carcinoma. Cancer Biol Ther 4: 1116–1122.

29. Gearhart PJ, Wood RD (2001) Emerging links between hypermutation of antibody genes and DNA polymerases. Nat Rev Immunol 1: 187–192.

30. Rogozin IB, Pavlov YI, Bebeuuk K, Manuda T, Kunkel TA (2001) Somatic mutation hotspots correlate with DNA polymerase eta error spectrum. Nat Immunol 2: 530–536.

31. Zeng X, Winter DB, Kasmer C, Kraemer KH, Lehmann AR, Gearhart PJ, et al. (2001) Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. Nature 412: 341–346.

32. Strauss BS (2000) Role in tumorigenesis of silent mutations in the TP53 gene. Mutation Research 457: 93–104.

33. Bruldard M, Lorphelin D, Collignon O, Lorphelin W, Thouvenot B, Godiee E, et al. (2007) Nonrandom variations in human cancer ESTs indicate that mRNA heterogeneity increases during carcinogenesis. Proc Natl Acad Sci U S A 104: 7522–7527.

34. Yang J, Gonzalez KD, Scaringe WA, Tais K, Liu N, Gu D, et al. (2007) Evidence for mutation showers. Proc Natl Acad Sci U S A 104: 8403–8408.

35. Yang Y, Sterling J, Storici F, Resnick MA, Gordenin DA (2008) Hypermutability of damaged single-strand DNA formed at double-strand breaks and unapped idenocytes in Yeast Saccharomyces cerevisiae. PLoS Genet 4(11): e1000264.

36. Chumakov IM, Zabarovsky ER, Prassolov VS, Mett VL, Kisselov LL (1982) Human nucleotide sequences related to the transforming gene of a murine sarcoma virus: studies with cloned viral and cellular DNAs. Gene 17: 19–26.