RESEARCH PAPER

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Novel tumor suppressor candidates on chromosome 3 revealed by NotI-microarrays in cervical cancer

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Genetic and epigenetic alterations in cervical carcinomas were investigated using NotI-microarrays containing 180 cloned sequences flanking all NotI-sites associated with genes on chromosome 3. In total, 48 paired normal/tumor DNA samples, specifically enriched in NotI-sites, were hybridized to NotI-microarrays. Thirty genes, including tumor suppressors or candidates (for example, *VHL*, *RBSP3/CTDSPL*, *ITGA9*, *LRRC3B*, *ALDH1L1*, *EPHB1*) and genes previously unknown as cancer-associated (*ABHD5*, *C3orf77*, *PRL32*, *LOC285375*, *FGD5* and others), showed methylation/deletion in 21–44% of tumors. The genes were more frequently altered in squamous cell carcinomas (SCC) than in adenocarcinomas (ADC, $p < 0.01$). A set of seven potential markers (*LRRN1*, *PRICKLE2*, *VHL*, *BHLHE40*, *RBSP3*, *CGGBP1* and *SOX14*) is promising for discrimination of ADC and SCC. Alterations of more than 20 genes simultaneously were revealed in 23% of SCC. Bisulfite sequencing analysis confirmed methylation as a frequent event in SCC. High down-regulation frequency was shown for *RBSP3*, *ITGA9*, *VILL*, *APRG1/C3orf35* and *RASSF1 (isoform A)* genes (3p21.3 locus) in SCC. Both frequency and extent of *RASSF1A* and *RBSP3* mRNA level decrease were more pronounced in tumors with lymph node metastases compared with non-metastatic ones ($p \leq 0.05$). We confirmed by bisulfite sequencing that *RASSF1* promoter methylation was a rare event in SCC and, for the first time, demonstrated *RASSF1A* down-regulation at both the mRNA and protein levels without promoter methylation in tumors of this histological type. Thus, our data revealed novel tumor suppressor candidates located on chromosome 3 and a frequent loss of epigenetic stability of 3p21.3 locus in combination with down-regulation of genes in cervical cancer.

Introduction

Cervical cancer (CC) is the second most common cause of cancer deaths in women worldwide. Molecular as well as epidemiological studies demonstrate that high-risk human papillomaviruses (HPV) are the causative agents for cervical cancer. The recently introduced vaccines can prevent the initial infection by two of these high-risk types, HPV 16 and 18, which are responsible for about 70% of cervical cancers.[1] These vaccines provide very effective protection in previously non-exposed women, but they do not seem to possess a significant therapeutic effect in already infected individuals. Not all patients with HPV infection develop invasive lesions; thus, molecular tests for cervical cytology screening may help to identify women with increased risk for progression to cervical carcinoma. Based on this knowledge, a retrieval of new biomarkers for dysplastic cervical cells and additional signaling pathways targeted in malignant progression is necessary.

The important role of chromosome 3 in cancer is well known, its short arm (3p) harbors several regions that include many known tumor suppressor genes (TSGs) and TSG candidates.[2,3] Earlier, we performed a comprehensive deletion survey of 3p in more than 400 samples of major epithelial tumors, including CC and identified two most frequently affected regions ("hotspots") – LUCA at the centromeric and AP20 at the telomeric borders of

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the 3p21.3 locus.[4,5] It was shown that different genetic alterations of this locus are associated with cervical carcinogenesis.

At the present time methylation of CpG islands in promoter regions is known to be precisely regulated during cell differentiation, and plays a key role in the control of gene expression and in cancer (for a review see ref. 6). CpG islands are located in promoter regions of many genes associated with cancer and its hypermethylation has been observed as a frequent mechanism of TSGs inactivation, which contributes to malignant transformation. A comprehensive analysis of methylation status of chromosome 3 in CC was not still performed.

Recently, we developed a novel technology based on NotI-microarrays (NMA) and successfully used it for simultaneous analysis of deletions/methylations in lung,[7] ovary[8] and colorectal cancer.[9] NotI-microarrays can serve as a valued complement of the new techniques for advanced search of methylated cancer-associated genes, first of all TSGs. Moreover, NMA are the unique tools that permit to identify both structural (deletions) and epigenetic (methylation) changes simultaneously. This methodology has been described in detail earlier.[8,10] Briefly, the essence of this method consists of the ability of the NotI restriction enzyme to recognize and digest only the unmethylated CG-rich motif GCGGCCGC often found in CpG islands associated with regulatory region of many genes (**Fig. 1**). The technique involves a special procedure of isolation of genomic DNA fragments flanking by NotI-digested sites from total tumor/normal DNA and usage of them as a NotI-enriched probe (100–500 bp) for comparative hybridization on microarrays containing all 180 NotI-associated clones from a NotI-library of chromosome 3.[11,12] Lack or decrease of hybridization signals of tumor DNA compared with normal DNA indicates the deletion or methylation of NotI-associated DNA fragments.

The aim of our study was to identify new genes using NMA technology that can be potential markers for CC. For the first time, 30 genes have been revealed to be methylated/deleted on chromosome 3 in more than 20% of cervical primary tumors. For the majority of these genes, methylation and/or deletions (M/Ds) were not described in CC earlier. The NotI-microarray data were confirmed for a portion of revealed TGSs and candidate genes by bisulfite genomic sequencing and qPCR expression analysis. We also found a novel set of genes with epigenetic alterations that may be useful for discrimination between cervical adenocarcinoma (ADC) and squamous cell carcinoma (SCC) cells. In addition, for the first time we showed the frequent down-regulation of well-known TSG, *RASSF1 (isoform A),* without *RASSF1* promoter methylation that was specific for SCC of cervix. We showed that deletions and epigenetic inactivation of TSGs in chromosome 3 are frequent mechanisms in CC.

Results

Analysis of methylation/deletion frequency using NotI-microarrays. Chromosome 3 specific NMA containing 180 NotI linking clones associated with 188 genes were hybridized with NotI-enriched DNA probes from paired normal/tumor samples (**Fig. 2** and **Table 1**; **Table S1**). The statistical analysis showed 30 genes with M/D in more than 20% of the cervical cancer samples. The patterns of aberrations were different for two histological types - ADC and SCC. In general, genes were more frequently methylated/deleted in SCC than in ADC samples ($p < 0.01$) (**Fig. 2** and **Table 1**). We revealed that at least 9 out 39 (23%) tested cervical SCC samples had alterations of more than 20 genes simultaneously including genes from Ap20 and LUCA sub-regions (**Fig. 2B**, samples 11, 17, 20, 25, 27, 38, 42, 47 and 48). Among genes frequently methylated/deleted in CC, only a few were already known TSGs, for example, *RBSP3/CTDSPL*, *ITGA9* and *VHL*. The majority of found genes were previously not shown to be involved in cervical carcinogenesis, among them *LOC285205, FGD5, RPL32, ROPN1, CGGBP1, NBEAL2* and *LOC285375* (see **Table S2** for information about functions of the corresponding proteins and their association with carcinogenesis).

Confirmation of NotI-microarrays results by bisulfite genomic sequencing. To confirm the results of NMA hybridizations, methylation status of seven genes with frequency of M/D 27–44% according NMA were analyzed in 11 SCC samples by bisulfite sequencing (namely: *LRRN1*, *ITGA9*, *LRRC3B*, *NKIRAS1*, *VHL*, *RBSP3* and *FGF12*). The results of bisulfite sequencing are represented in **Table 2** and **Tables S3–S5**. Methylation of CpG promoter islands was confirmed in most of tested cases (19 out of 23). For 4 cases (*LRRN1* N°42, *NKIRAS1* N°17, *VHL* N°15 and *RBSP3* N°17), the reason of decreased hybridization signal at NotI-microarray was another than NotI-site methylation (number of samples in parentheses as **Fig. 2B**). According to our previous qPCR data, deletions are the main mechanism of *NKIRAS1* gene inactivation.[13] Probably, hemizygous deletions took place for three other unmethylated cases although a loss of NotI-sites due to point mutations cannot be excluded. So, among 23 randomly selected cases from NotI-panel with decreased signal of hybridization 19 cases (83%) showed methylation of gene CpG islands including NotI recognition sites. Thus, bisulfite sequence data are in good concordance with NotI-microarrays results and suggest that methylation of 5' regulator regions of genes is a frequent event in SCC.

Cervical SCC and ADC have different methylation/deletion patterns. We observed the difference of M/D patterns in SCC and ADC for 22 tested genes (**Fig. 2B**). However, only for two genes, *PPP2R3A* and *LRRN1,* this difference was statistically valid ($p = 0.03$ for both). But this difference was obvious for all 22 genes taken together (**Table 1**): mean frequency of methylation/deletion decreased from 37% for SCC to 5% for ADC samples ($p < 0.01$). The statistical analysis of our data suggested the most promising set of 7 genes (*LRRN1, PRICKLE2, VHL, BHLHE40, RBSP3, CGGBP1* and *SOX14*) that discriminate SCC and ADC. The revealing of alteration in two and more of these genes in a sample would indicate the presence of SCC cells but not ADC. The sensitivity of the set is equal to (67 ± 8)% and the specificity is 100%.

Simultaneous down-regulation of *RBSP3, ITGA9, VILL, APRG1/C3orf35* and *RASSF1A* genes in cervical SCC. Earlier, we demonstrated that expression loss of several genes from both LUCA and AP20 sub-regions within the 3p21.3 locus occurred frequently and simultaneously in the same tumor in lung cancer ($p << 0.01$).[14] Taking into account these data and a frequent

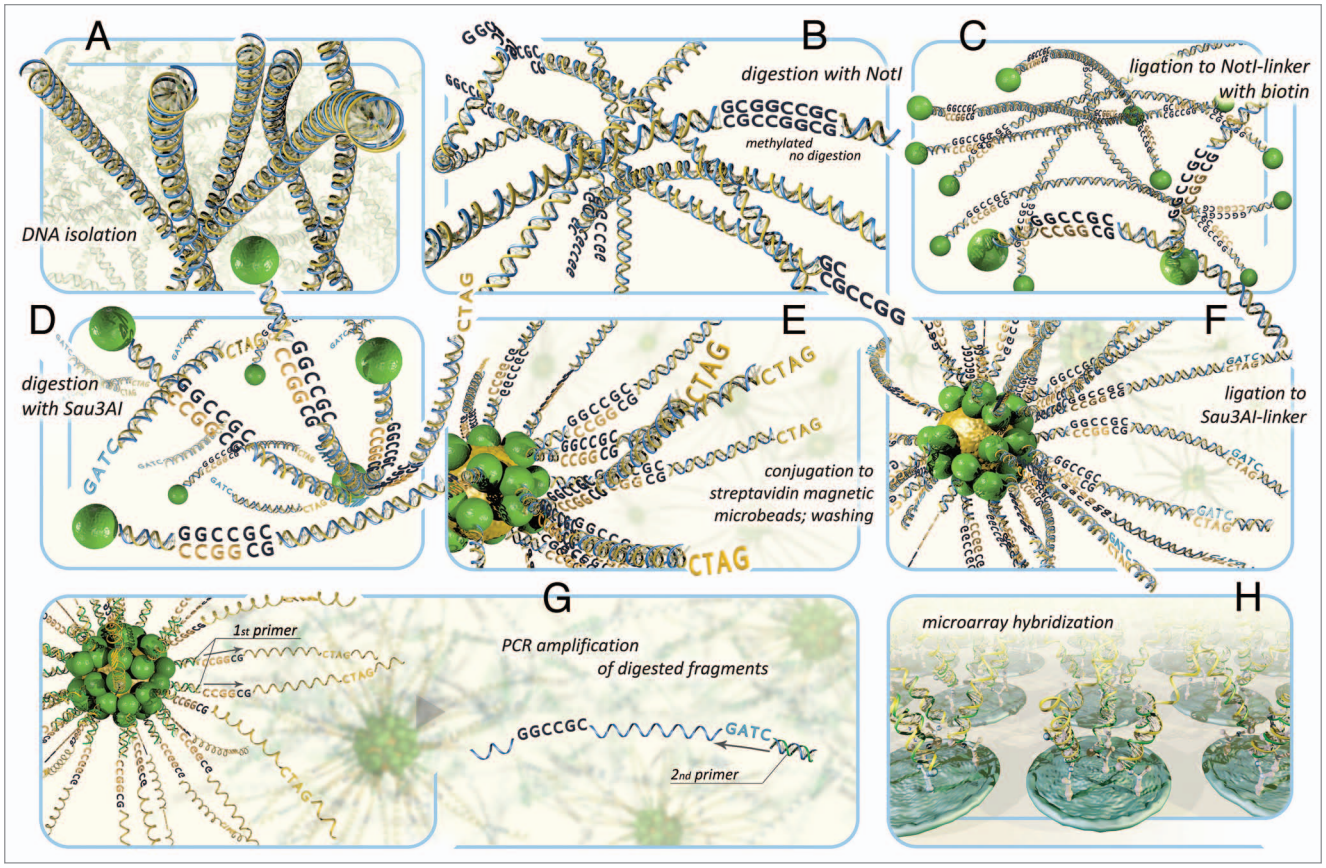

Figure 1. The scheme of DNA hybridization on NotI-microarrays. (**A**) Isolation of genomic DNA. (**B**) DNA digestion with methyl-specific rare-cutter enzyme NotI. (**C**) Ligation of the fragments to NotI-linkers containing biotin. (**D**) DNA digestion with 4-base restriction enzyme Sau3AI. (**E**) Fragments conjugation to microbeads containing streptavidin and washing. (**F**) Ligation of fragments to Sau3AI-linkers containing PCR primer-annealing site. (**G**) PCR amplification of DNA sequences that has been attached to the microbeads. Then the standard procedures can also be performed: subtraction hybridization in order to remove abundant DNA fragments and to enrich the probe with further sequencing. (**H**) NotI-enriched probe hybridization to microarrays.

simultaneous genetic/epigenetic destabilization of many genes at the 3p (**Fig. 2B**) we expected that analysis of NotI-sites will reveal a wide region of epigenetic destabilization where neighboring genes without NotI-sites in their CpG islands might also be down-regulated. To evaluate the rate of down-regulation of genes that may be simultaneously destabilized we have quantified and compared mRNA levels for several genes from 3p21.3 locus in SCC samples. We included in this analysis four genes from AP20 sub-region: two NotI–associated TSGs, *RBSP3* and *ITGA9,* and two candidate genes, *VILL* and *APRG1,* without NotI-sites in their regulatory regions. *RBSP3* and *ITGA9* are known to be involved in carcinogenesis and frequently methylated/deleted in many cancers, including cervical SCC according our NotI-microarray data (**Fig. 2B**). Though genes *VILL* and *APRG1* are localized in AP20, the data confirming their involvement in carcinogenesis are rather poor. We analyzed also well-known TSG *RASSF1A* from the LUCA sub-region. *RASSF1* has no NotI-sites in the promoter region, but we have selected it taking into account the following points: 1) its inactivation caused by promoter methylation has been demonstrated in many types of tumors; 2) published data about the *RASSF1* frequency of methylation in CC are contradictory (for a review see 15).

All five genes were down-regulated (2–78-fold) in the majority of cases of SCC (53–89%, **Figure 3** and **Table 3**). High frequencies of aberrations were inherent both for three known TSGs from two "hotspots" and too poorly studied genes. High frequency (60%) and extent of the *APRG1* mRNA decrease (up to 18-fold) indicates on the need for further research of this gene (**Table 3**).

Simultaneous decrease of mRNA levels of tested genes was observed in all SCC samples, except two ones (samples 1 and 2, **Figure 3**). Moreover, four genes (except, *APRG1*) revealed the tendency to increase frequency and extent of mRNA decrease in samples from patients with lymph nodes metastases compared with samples without metastases (**Table 3**). For *RASSF1A* and *RBSP3,* this difference was statistically significant ($p \leq 0.05$). Three TSGs, *RASSF1A, RBSP3* and *ITGA9,* showed concordant expression profiles: we observed a positive correlation between their mRNA levels. Spearmen rank coefficient values reflecting the correlation between mRNA levels were: 0.69 for *RASSF1A* and *RBSP3* ($p < 0.01$), 0.43 for *RBSP3* and *ITGA9* ($p = 0.07$), and 0.48 for *RASSF1A* and *ITGA9* ($p = 0.04$).

The frequencies of mRNA level decrease and NotI methylation/deletions were compared in the same samples for two genes

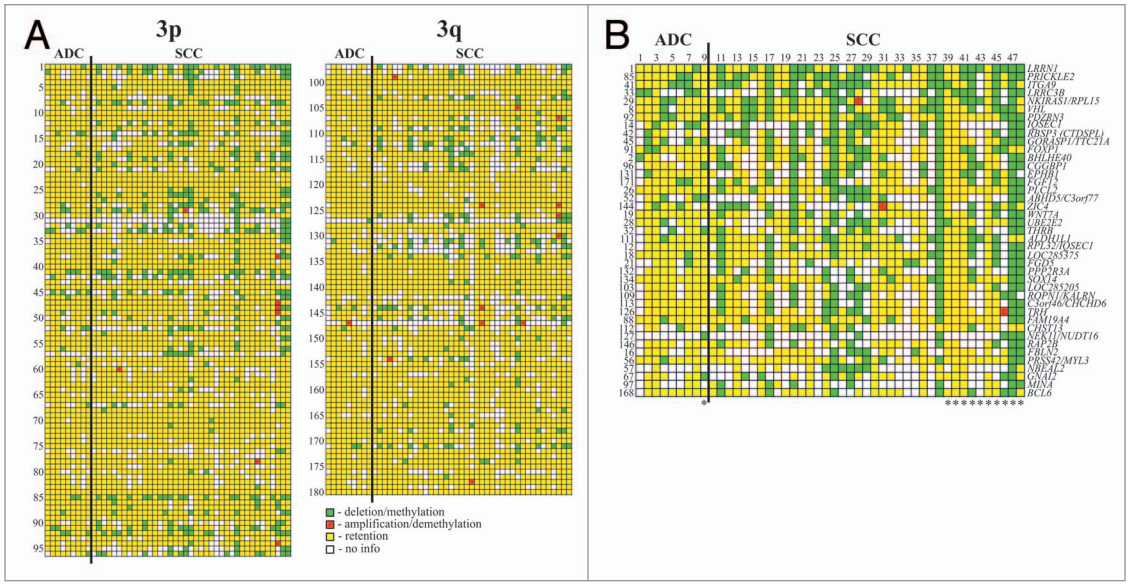

Figure 2. Hybridization pattern of DNA from cervical cancer samples on NotI-microarrays. (**A**) Vertically—180 NotI-sites arranged according their localization on chromosome 3 (from 3p26.2 to 3p11.1 and from 3q11.2 to 3q29). Horizontally—48 cervical cancer samples (9 ADC and 39 SCC). (**B**) Vertically—41 NotI-sites arranged by methylation/deletion frequency (from 44% to 15%). Numbers correspond to numbers from **Figure 2A**. *Samples from patients with lymph node metastases.

Table 1. List of chromosome 3 NotI-sites with methylation/deletion frequencies more than 20% in cervical cancer

n/n	Gene*	NotI-site**	Locus	Methylation/deletion frequency, %		
				ADC	SCC	ADC+SCC
1	*LRRN1*	NL6-FJ5R (C)	3p26.2	11 (1/9)	51 (20/39)	44 (21/48)
2	*PRICKLE2*	NR1-NJ9R (C)	3p14.1	22 (2/9)	49 (19/39)	44 (21/48)
3	*ITGA9*	NL1A401R (D)	3p21.3	33 (3/9)	44 (17/39)	42 (20/48)
4	*LRRC3B*	NL3-CA11RS	3p24	44 (4/9)	39 (15/39)	40 (19/48)
5	*NKIRAS1*	NL1-CJ4R (C)	3p24.2	22 (2/9)	41 (16/39)	38 (18/48)
6	*RPL15*	NL1-CJ4R (C)	3p24.2	22 (2/9)	41 (16/39)	38 (18/48)
7	*VHL*	NRLA404R (U)	3p25.3	11 (1/9)	41 (16/39)	35 (17/48)
8	*PDZRN3*	NL6-AF21R (C)	3p13	33 (3/9)	33 (13/39)	33 (16/48)
9	*IQSEC1*	NR1-XM13 (C) HSJ4-AB7R (C)	3p25.2	33 (3/9) 0 (0/9)	31 (12/39) 26 (10/39)	31 (15/48) 21 (10/48)
10	*RBSP3*	NLJ-003RD	3p21.3	11 (1/9)	36 (14/39)	31 (15/48)
11	*GORASP1*	NL3003R (U)	3p22-p21.33	44 (4/9)	26 (10/39)	29 (14/48)

*Four pairs of adjacent genes have one common NotI-site (5 and 6, 11 and 12, 19 and 20, 9 and 26), *IQSEC1* gene has two NotI-sites, **sequences available at www.ncbi.nlm.nih.gov/nuccore/.

Table 1. List of chromosome 3 NotI-sites with methylation/deletion frequencies more than 20% in cervical cancer (Continued)

n/n	Gene*	NotI-site**	Locus	Methylation/deletion frequency, %		
				ADC	SCC	ADC+SCC
13	*FOXP1*	NL1-BA6R	3p14.1	22 (2/9)	31 (12/39)	29 (14/48)
14	*BHLHE40*	NR5-IH18RS	3p26.1	11 (1/9)	31 (12/39)	27 (13/48)
15	*CGGBP1*	NR1-WE11RS	3p12-p11.1	11 (1/9)	31 (12/39)	27 (13/48)
16	*EPHB1*	NL1A079R (D)	3q21-q23	11 (1/9)	31 (12/39)	27 (13/48)
17	*FGF12*	NR1-NH1R (C)	3q28	22 (2/9)	28 (11/39)	27 (13/48)
18	*PLCL2*	NL4-AP18R (C)	3p24.3	0 (0/9)	31 (12/39)	25 (12/48)
19	*ABHD5*	NR1-AN24RS	3p21	33 (3/9)	23 (9/39)	25 (12/48)
20	*C3orf77*	NR1-AN24RS	3p21	33 (3/9)	23 (9/39)	25 (12/48)
21	*ZIC4*	NR1-PD1R	3q24	22 (2/9)	26 (10/39)	25 (12/48)
22	*WNT7A*	NL4-BK12R (C)	3p25	0 (0/9)	28 (11/39)	23 (11/48)
23	*UBE2E2*	NR1-WF18R (C)	3p24.2	11 (1/9)	26 (10/39)	23 (11/48)
24	*THRB*	NL4-BB6R (C)	3p24.2	22 (2/9)	23 (9/39)	23 (11/48)
25	*ALDH1L1*	NL4-BC8R (C)	3q21.3	11 (1/9)	26 (10/39)	23 (11/48)
26	*RPL32*	HSJ4-AB7R (C)	3p25.2	0 (0/9)	26 (10/39)	21 (10/48)
27	*LOC285375*	NR1-PL22R (C)	3p25.1	11 (1/9)	23 (9/39)	21 (10/48)
28	*FGD5*	NL4-DP2RS	3p25.1	11 (1/9)	23 (9/39)	21 (10/48)
29	*PPP2R3A*	NL1-FK10R (C)	3q22.1	0 (0/9)	26 (10/39)	21 (10/48)
30	*SOX14*	NR1-WJ2RS	3q22-q23	0 (0/9)	26 (10/39)	21 (10/48)

*Four pairs of adjacent genes have one common NotI-site (5 and 6, 11 and 12, 19 and 20, 9 and 26), *IQSEC1* gene has two NotI-sites, **sequences available at www.ncbi.nlm.nih.gov/nuccore/.

containing NotI-sites, namely, *RBSP3* and *ITGA9*. The *RBSP3* mRNA level was decreased in 68% of SCC samples and methylation/deletions were detected in 36%; for the *ITGA9* gene these values were 89% and 44%, respectively. Thus, these findings were in concordance with the observed frequent inactivation of the genes highlighted by NotI-microarray and suggest the existence of additional mechanisms of their inactivation besides of DNA methylation.

RASSF1A down-regulation of is not associated with hypermethylation of its promoter in cervical SCC. We revealed the decrease of the *RASSF1A* mRNA level in 58% (11 out 19) of cervical SCC (**Fig. 3** and **Table 3**). It was an unexpected result because according several reports *RASSF1* promoter methylation was rare event in SCC of cervix in opposition to ADC (for a review see 15). To understand the reasons of *RASSF1A* down-regulation, we estimated its mRNA level by qPCR and the methylation status

Table 2. Analysis of methylation status of 7 genes in cervical carcinomas from NMA panel by bisulfite genomic sequencing

Gene	Methylated samples*	Unmethylated samples*
LRRN1	17, 22, 37	42
ITGA9	13, 17, 20, 26, 27, 37	no
LRRC3B	17	no
NKIRAS1	no	17
VHL	17, 42	15
RBSP3	13, 20, 22, 36, 47	17
FGF12	17, 42	no

*Numerals indicate sample numbers from **Figure 2B**.

of the RASSF1 promoter by bisulfite sequencing in 17 primary SCC cervical tumors and 4 cervical carcinoma cell lines (**Fig. 4** and **Table 4**). Surprisingly the promoter methylation was detected only in the HPV-negative C33A cell line, that was used as positive control.[16] All primary tumors with the *RASSF1A* mRNA level decrease and 3 HPV-positive SCC cell lines demonstrated sporadic methylation of a few CpG sites of the *RASSF1* promoter. We did not revealed substantial methylation of the *RASSF1* promoter even in tumors with nearly complete absence of the *RASSF1A* mRNA (samples 16 and 17, **Table 4**). There is no difference in methylation patterns between healthy cervical tissue, SCC cell lines with more than 2-fold decrease of mRNA levels (C4–1, CaSki) and SiHa cells with unchanged mRNA level compared with healthy cervix (**Table 4** and **Figure 5B**).

Then the RASSF1A protein content was estimated in 4 cell lines with different *RASSF1A* mRNA levels by immunohistochemistry. Three cell lines demonstrated the decrease of cytoplasmic staining intensity compared with SiHa cells with unchanged mRNA level (**Fig. 5** and **Table 4**). The extent of fluorescence signal was correlated with the *RASSF1A* mRNA level. The strongest protein down-regulation was detected for cell line C33A, in which *RASSF1* promoter was methylated, but substantial decrease of protein levels were detected in cell lines without promoter methylation (CaSki, C4–1).

Thus using bisulfite sequencing we have confirmed that *RASSF1* promoter methylation is a rare event in cervical SCC and for the first time have demonstrated its down-regulation at both mRNA and protein levels without promoter methylation in tumors of this histological type. In addition these findings indicate that another mechanisms besides DNA methylation may be destabilized in the region of the *RASSF1* gene as well as in regions of *RBSP3* and *ITGA9* genes.

Discussion

In this study, using original technology of NotI-microarrays,[10] we found 30 genes on chromosome 3, including known TSGs and TSG-candidates (for example, *VHL*, *RBSP3, ITGA9*, *LRRC3B, ALDH1L1, EPHB1*) and also genes which have not been previously reported as involved in cancer development (*ABHD5, C3orf77, PRL32, LOC285375, FGD5, etc.*) with high frequencies (more than 20%) of methylation/deletion during cervical carcinogenesis (**Tables 1** and **S1**). Many of these genes (*LOC285205, FGD5, RPL32, ROPN1, CGGBP1, NBEAL2, LOC285375* and others) were not shown to be involved in cervical carcinogenesis previously, but according to our recent data many of them were involved in the development of non-small cell lung and ovarian cancer.[7,8] These findings suggest that genetic and epigenetic destabilization of genes at chromosome 3 is common mechanisms of epithelial tumors development. According literature data, the protein products of newly identified genes belong to pathways affected during development and progression of different cancer types (**Table S2**): in WNT signaling pathway (E3 ubiquitin-protein ligase, PDZRN3), in the regulation of cellular polarity and invasion (PRICKLE2), in regulation of the actin cytoskeleton (FGD5), in MAP kinase signaling pathway (FGF12) and others. Some of them are transcription factors involved in regulation of apoptosis (BHLHE40) and tissue-specific expression (FOXP1). Functions of some proteins are currently unknown, for example, LRRN1, LRRC3B and C3orf77. High frequencies of methylation/deletions, participation in signaling pathways affected in many tumors and inactivation at least in three types of epithelial tumors according our data argue in favor of tumor suppression functions of these genes.

Two out of 30 genes (*PDZRN3* and *SOX14*) had aberrations only in cervical cancer, but not lung and ovarian. PDZRN3 plays an important role in regulation of the surface level of MUSK (skeletal muscle-specific tyrosine-protein kinase receptor) on myotubules. SOX14 is involved in the regulation of embryonic development and in the determination of the cell fate (**Table S2**). These genes might be potentially used for discrimination between different cancer types.

We detected that the patterns of aberrations were different for two histological types of CC—ADC and SCC. In general, squamous cell tumors revealed higher rates of methylation/deletions than adenocarcinomas ($p < 0.01$). It is known that, in spite of general decline of CC incidence, the contribution of cervical ADC, absolute and relative to SCC, has been rising steadily in many countries, especially for younger women. This may be a result of the limitations of ADC detection during CC screening: adenocarcinomas are often diagnosed at an advanced disease stage (for a review see ref. 17). The statistical analysis of our data suggested the most predictive set of seven genes (*LRRN1, PRICKLE2, VHL, BHLHE40, RBSP3, CGGBP1* and *SOX14*) that may discriminate SCC and ADC with accuracy (73 ± 6)%. These genes might be useful for analysis by PCR-based technique on early stages of disease.

We have validated our microarray data for genes *LRRN1, ITGA9*, *LRRC3B*, *NKIRAS1*, *VHL*, *RBSP3* and *FGF12* by bisulfite genomic sequencing and have revealed that methylation of tested genes takes place in CC and is a prevalent event (83% of tested cases) compared with deletions that are also detected by NotI-microarrays. These results, and our recent data obtained for lung and ovarian cancers using NotI-microarrays,[7,8] suggest that methylation of promoter regions is the important mechanism leading to inactivation of TSG and TSG-candidates on chromosome 3.

We found out a group of cervical SCC (23% of cases) in which more than 20 NotI-sites were affected simultaneously (**Fig. 2B**).

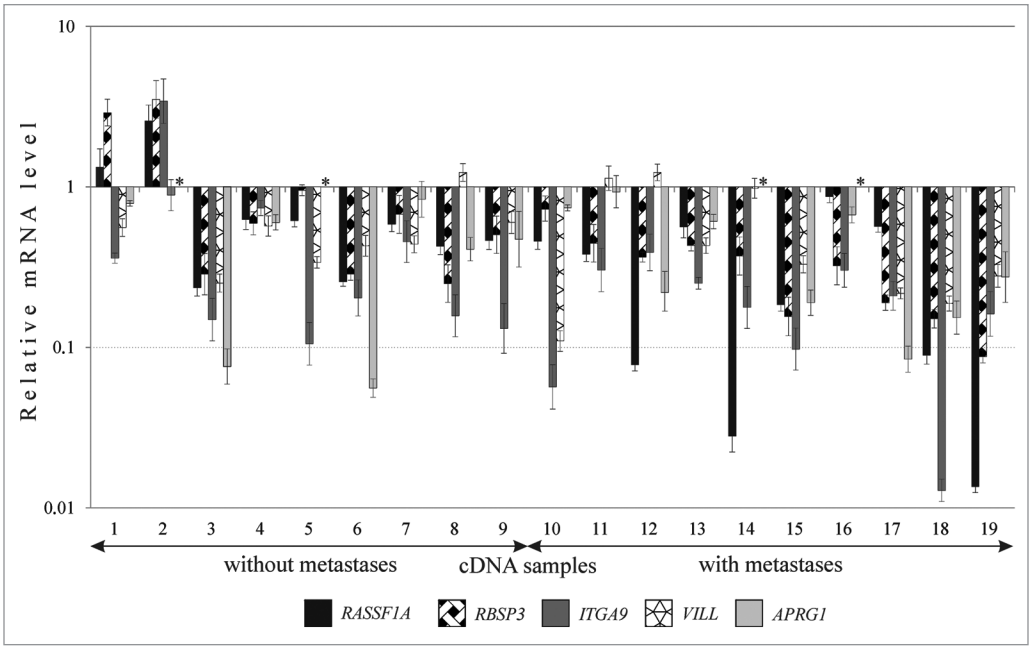

Figure 3. The relative expression level (R) of five genes (3p21.3) in SCC with different clinical characteristics. Results of qPCR. Samples without lymph node metastases (stages I and II), samples with lymph node metastases (stage III). **APRG1* mRNA was not detected in both tumor and adjacent normal tissues.

Table 3. QPCR analysis of five genes (3p21.3) in cervical SCC

Genes	Frequency of mRNA level decrease, %			Median of mRNA level decrease*, n-fold		
	Without metastasis	With metastasis	Total	Without metastasis	With metastasis	Total
RASSF1A	44 (4/9)	70 (7/10)	58 (11/19)	3 (2 – 4)	11 (2 – 74)	4 (2 – 74)
RBSP3	44 (4/9)	90 (9/10)	68 (13/19)	3 (2 – 4)	3 (2 – 11)	3 (2 – 11)
ITGA9	78 (7/9)	100 (10/10)	89 (17/19)	6 (2 – 9)	5 (3 – 78)	6 (2 – 78)
VILL	44 (4/9)	60 (6/10)	53 (10/19)	3 (2 – 4)	4 (2 – 9)	3 (2 – 9)
APRG1	57 (4/7)	62 (5/8)	60 (9/15)	4 (2 – 18)	5 (4 – 12)	5 (2 – 18)

*A range of mRNA levels decrease is shown in parentheses. $p < 0.01$ for each gene.

The majority of the corresponding genes is located on 3p, the short arm of chromosome 3. It is known that groups of tumors with high degrees of methylation (the CpG island methylator phenotype, or CIMP+) exist in colorectal and some other types of cancers.[18] CIMP+ tumors represent a clinically distinct group of cancers that is characterized by epigenetic instability. Our data suggest that CIMP+ and epigenetic instability on 3p are characteristic features of a portion of HPV-positive cervical SCC. Earlier hypermethylation of some genes have been identified not only in cervical cancer but also in its precursors, suggesting that assays for these molecular events might be clinically useful for cytological screening and diagnostics of CC at early stages.[19-22] Therefore, the usage of methylation markers (known and newly identified) in addition to conventional cytological screening and human papillomavirus testing may help to reveal cancer cells with CIMP+.

To confirm epigenetic deregulation of 3p21.3 locus, we evaluated mRNA levels of five genes from this sub-region, *RBSP3, ITGA9, APRG1, VILL* and *RASSF1A*, in cervical SCC. High frequencies (53–89%) of down-regulation of all these genes were revealed for the first time in cervical SCC excepting *RBSP3*.[23,24] Along with known TSGs, *RASSF1A, RBSP3* and *ITGA9* (see **Table S2**), two poorly studied genes, *APRG1* and *VILL,* were strongly down-regulated in 60% and 53% of cervical tumors respectively. There is only scarce information about their functions and involvement in cancer. *APRG1* is located proximal to the border of the homozygous deletion that takes place in a small cell lung cancer cell line ACC-LC5.[25] *APRG1* was suggested to have tumor suppression function in breast cancer.[26] This gene encodes a putative protein of 170 amino acids (isoform B), which has an N-terminal part conserved among members of the eukaryotic translation factor 6 gene family. The analysis by PROSITE, the database of protein domains, families and functional sites, had revealed peptide patterns corresponding to the cell attachment sequence Arg-Gly-Asp, which suggests

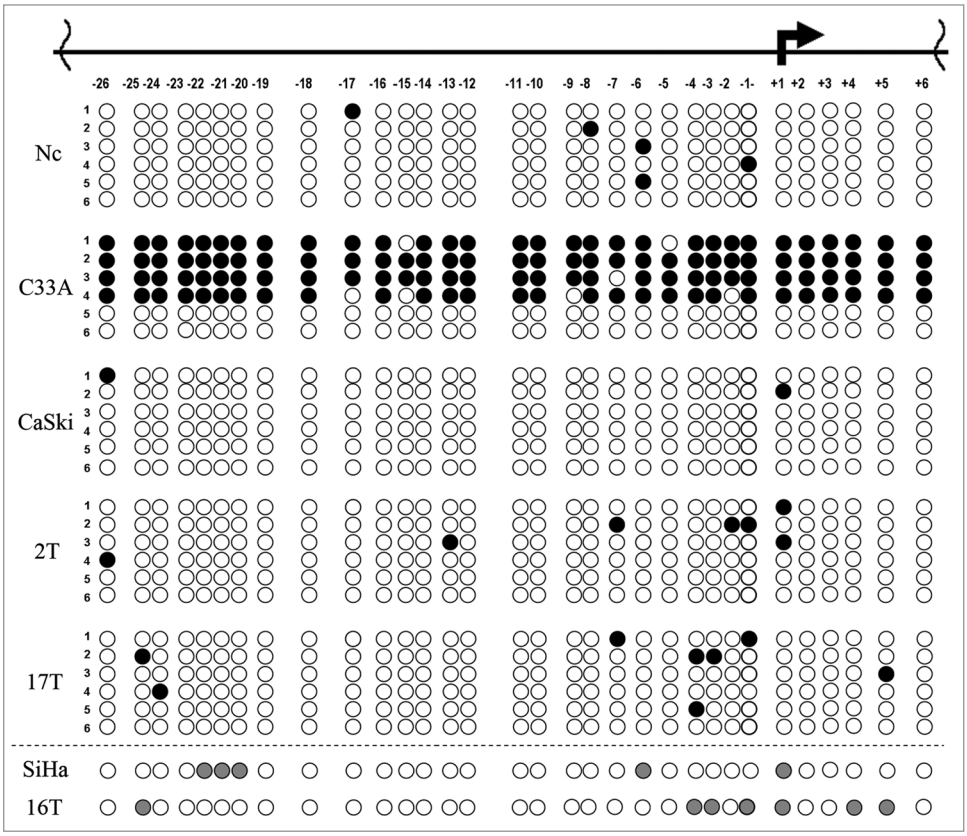

Figure 4. Analysis of methylation status of *RASSF1* promoter in cervical SCC samples and cell lines. Schematic representation of results of sodium bisulfite sequencing: numbers under the horizontal line indicate CpG dinucleotides within *RASSF1* promoter, the broken arrow – transcription initiation site; black circle,methylated CpG; white circle, unmethylated CpG; gray circle, partially methylated CpG; each row of circles represents a cloned DNA molecule for all samples excepting SiHa and number 16T, where the total PCR products are presented; T, tumor; Nc, normal cervix. Numbers of tumors correspond to **Table 4**.

the involvement of APRG1 in membrane interactions and cell adhesion.[25] The protein encoded by *VILL* belongs to the villin/gelsolin family. It is required for actin bundle assembly.[27] No association between *VILL* and tumorigenesis has been revealed, but villin 1, a member of this family, was reported to be either up or down-regulated in different kinds of cancer.[28,29] Our data suggest that these genes could be considered as TSGs candidates. Further research of their functions in carcinogenesis is necessary.

It was surprising that we detected inactivation of *RASSF1A* in 58% of cervical SCC. Previous published data concerning to *RASSF1* methylation status in CC were contradictory. A number of publications demonstrated that methylation of its promoter took place in cervical ADC but was a very rare event in cervical SCC. On the other hand, frequent *RASSF1* promoter methylation in cervical SCC and precancerous lesions was described in several publications (for a review see 15). Unfortunately, levels of *RASSF1A* expression in combination with *RASSF1* promoter methylation were not evaluated in these studies. In our experiments, we did not revealed pronounced promoter methylation in any tumor out of 10 SCC with decreased mRNA levels (**Table 4**). The *RASSF1A* inactivation at the mRNA and protein levels correlated with *RASSF1* promoter methylation only in one out of 4 cervical SCC cell lines (**Table 4**; **Figures 3** and 4). Thus, we have confirmed that the *RASSF1* promoter methylation is a rare event in cervical SCC and, for the first time, have demonstrated frequent *RASSF1A* down-regulation without *RASSF1* promoter methylation in tumors of this histological type.

Well-known TSGs, *RASSF1A, RBSP3* and *ITGA9,* showed coordinated down-regulation in the same SCC patients. Earlier, we demonstrated simultaneous inactivation of some genes from this 3p21.3 region, including *RASSF1A, RBSP3* and *ITGA9* in lung cancer.[14,30] These results point to the importance of this region for epithelium tumor development and allow to assume the existence of common mechanisms of gene down-regulation in this locus. Several groups of data indicate the existence of other epigenetic mechanisms, besides DNA methylation, inactivating these genes. We clearly demonstrated that at least one gene from the 3p21.3 locus, *RASSF1A*, was frequently silenced in the absence of promoter methylation in cervical SCC. We confirmed, using bisulfite genomic sequencing, that promoter methylation of *RBSP3* and *ITGA9* took place in a portion of CC; on the other hand, we demonstrated that the frequency of down-regulation revealed by qPCR was significantly higher than methylation/deletions rate showed by NotI-microarray (68 and 31% for *RBSP3;* 89 and 42% for *ITGA9*, respectively). The same results were obtained for these genes in lung cancer: the frequency of *RBSP3* and *ITGA9* mRNA level decreases (85%, for each gene) was also higher than the frequency of methylation/deletions (47.5 and 45%, respectively).[7] The lack of concordance between these results can be related to participation of chromatin modification and RNA interference in gene inactivation instead of, or in addition to, DNA methylation. To predict microRNA regulation of *RASSF1*, *RBSP3* and *ITGA9* genes, we used miRNA body map (www.mirnabodymap.org/index.php),[31] which includes data from five microRNA resources (TargetScan, DIANA, PITA, miRDB and MicroCosm) implementing different algorithms of microRNA target prediction. The analysis demonstrated that each of the three mRNAs (*RASSF1*, *RBSP3* and *ITGA9*) may be the target of three microRNAs (miR-346, miR-515 and miR-767–5p) simultaneously. All these miRNAs seem to play a role in tumors onset or progression. The miR-346 is overexpressed in follicular thyroid carcinoma[32] and in serum of

Table 4. *RASSF1* methylation status and relative mRNA levels of *RASSF1A* in cervical SCC and cell lines

n/n	TNM/Clinical stage	Methylation status of promoter region*	mRNA level**, n-fold	Comments
		Primary tumors		
1	$T_2N_0M_0$/II	U	2.6↑ (2.3↑ – 2.9↑)	Up-regulation
2	$T_2N_0M_0$/II	U	1.0↓ (1.4↑ – 1.4↓)	Retention
3	$T_1N_0M_0$/I	U	1.1↓ (1.4↑ – 1.5↓)	
4	$T_1N_0M_0$/I	U	1.2↓ (1.3↑ – 1.8↓)	
5	$T_2N_0M_0$/II	U	1.6↓ (1.4↓ – 1.8↓)	
6	$T_2N_1M_0$/III	U	1.7↓ (1.5↓ – 1.9↓)	
7	$T_1N_0M_0$/I	U	1.9↓ (1.4↓ – 2.6↓)	
8	$T_1N_0M_0$/I	U	2.2↓ (1.6↓ – 3.0↓)	
9	$T_2N_0M_0$/II	U	2.8↓ (2.0↓ – 3.8↓)	
10	$T_2N_0M_0$/II	U	2.8↓ (2.0↓ – 3.9↓)	
11	$T_2N_0M_0$/II	U	3.9↓ (3.4↓ – 4.4↓)	
12	$T_2N_0M_0$/II	U	3.9↓ (3.7↓ – 4.2↓)	Down-regulation
13	$T_1N_0M_0$/I	U	4.5↓ (3.2↓ – 6.2↓)	
14	$T_2N_1M_0$/III	U	11↓ (10↓ – 13↓)	
15	$T_1N_1M_0$/III	U	13↓ (12↓ – 14↓)	
16	$T_2N_1M_0$/III	U	36↓ (29↓ – 45↓)	
17	$T_2N_1M_0$/III	U	74↓ (68↓ – 80↓)	
		Cell lines		
18	SiHa	U	1.4↓ (1.1↓ - 1.7↓)	Retention
19	C4–1	U	4.5↓ (3.6↓ - 5.6↓)	Down-regulation
20	CaSki	U	8↓ (6↓ - 11↓)	
21	C33A	Met	105↓ (67↓ - 162↓)	

**qPCR data, standard deviations of relative mRNA levels are shown in parentheses. Samples arranged according to *RASSF1A* levels of down-regulation. ↑, mRNA level increase; ↓, mRNA level decrease. *bisulfite sequencing data: Met,methylated; U, unmethylated.

prostate cancer patients.[33] In vitro overexpression of miR-346 induced proliferation, whereas inhibition of its expression led to growth arrest.[32] miR-515, a precursor of miR-515–5p, was overexpressed in oral carcinomas.[34] The third microRNA, miR-767–5p, was found to play a major role in oncogenic processes in different tumors. Deregulation of any of these miRNAs might result in coordinate inactivation of the three genes. Thus, the relations of these miRNAs to the regulation of *RASSF1*, *RBSP3* and *ITGA9* need to be studied. Recently, it was demonstrated that epigenetic silencing can span large regions of the chromosome in prostate and colorectal cancer. Both methylated DNA and neighboring unmethylated genes can be coordinately suppressed by global changes in histone modification across an entire chromosome band within chromosomes 2 and 7 in these tumors.[35,36] These large regions were associated with regional histone deacetylation combined with subdomains of different epigenetic patterns, which include re-enforcement, gain or exchange of repressive histone, and DNA methylation marks. Authors believe that coordinate epigenetic control over larger regions may be a common phenomenon in cells during development and may be involved in cancer. Our data suggest that the 3p21.3 locus might be such long-range epigenetic silencing region in CC (**Fig. 2B**) and study of its chromatin structure in combination with DNA methylation should be interesting.

In general, our study revealed a frequent loss of epigenetic stability in combination with gene down-regulation in 3p21.3 locus in CC. This study reasserts that NotI-microarrays are powerful tools for disclosing TSG candidates and provides a basis for better understanding of mechanisms involved in development of CC.

Materials and Methods

Tissue specimens and cell lines. Paired specimens of tissues including 39 squamous cell carcinomas (SCC), 9 adenocarcinomas (ADC) of uterine cervix and adjacent morphologically normal tissues (conventional "normal" tissues) were obtained after surgical resection prior radiation or chemotherapy and stored in liquid nitrogen. An additional set of SCC (19 paired normal/tumor tissues) was used for further validation by qPCR. Tissues were obtained from patients with FIGO stages I, II and III of the disease at the N.N. Blokhin Cancer Research Center. The diagnosis was verified by histopathology and only samples containing 70–80% or more tumor cells were used in the study. All tumors were E7 HPV16/18-positive according to PCR analysis. Sample information is represented in **Table 5**. Tissues of healthy cervix were obtained from patients with uterine polyps or tumors and tested for HPV absence. All tissues were collected under the approval of the Institutional Review Board of

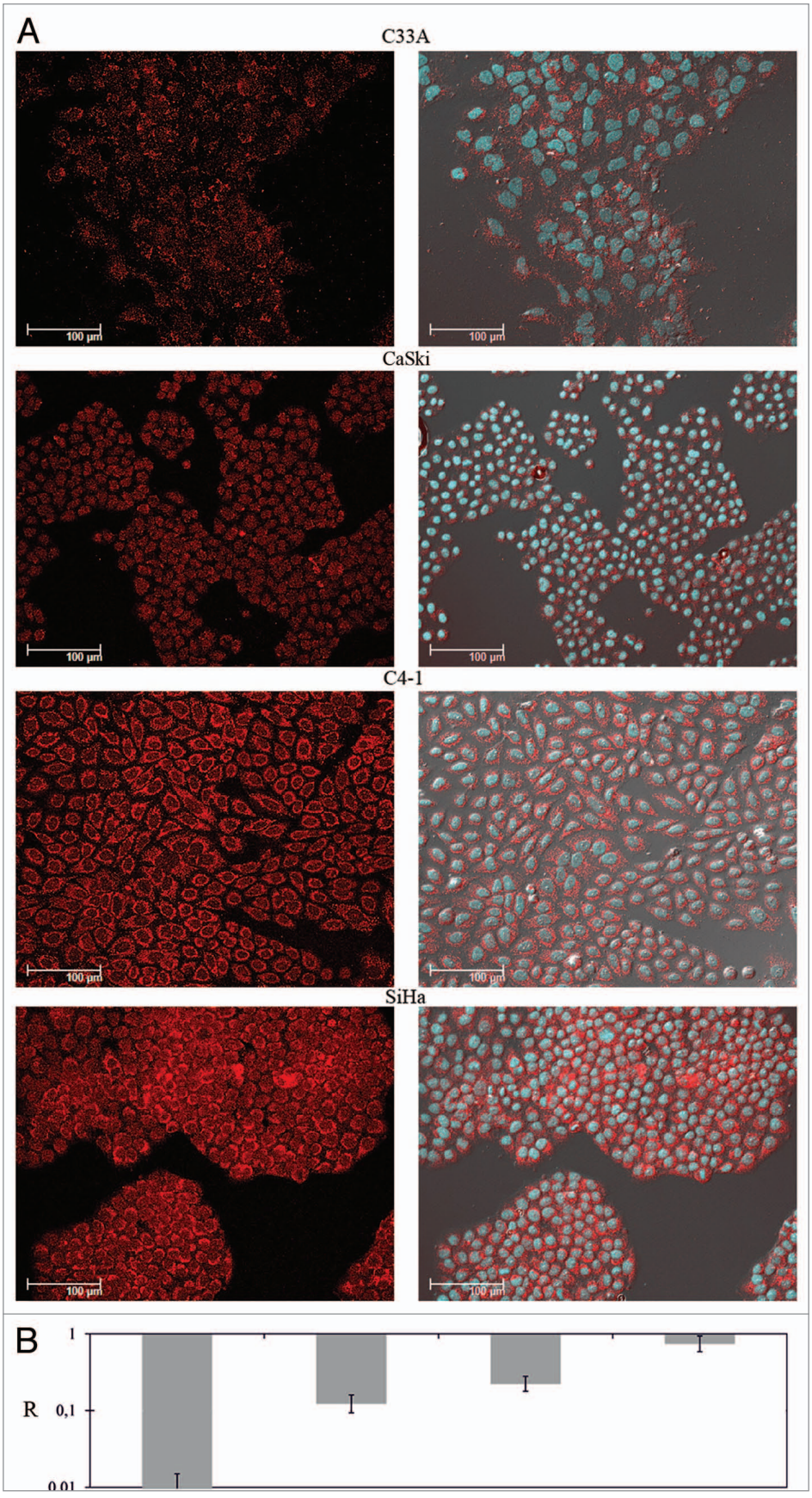

Figure 5. For figure legend, see page 11.

Figure 5. Analysis of RASSF1A protein and mRNA levels in cervical cancer cell lines. (**A**) Left column: immunochemical detection of RASSF1A; right column – the same as left column plus DAPI staining of nuclei (blue). (**B**) Results of qPCR. Columns mean averaged *RASSF1A* mRNA level of three different propagations of each cell line. All calculations were performed relatively to average value of three HPV-negative healthy cervical tissues.

the Blokhin Cancer Research Center. Informed consent was obtained from all patients. The study was done in accordance with the principles outlined in the Declaration of Helsinki. The HPV-positive human cervical SCC cell lines SiHa, CaSki, C4–1 and HPV-negative C-33A cell line (American Type Culture Collection) were maintained in DMEM supplemented with 10% FCS.

NotI-microarrays. One hundred eighty NotI linking clones from human chromosome 3 containing 188 genes with inserts up to 15 kb were immobilized on the glass slides in six replications.[10,12] Plasmid DNA for immobilization on the glasses was isolated with a HiPure Plasmid Midiprep kit (Invitrogen) and printed on the silanized glasses at a concentration of 0.25 μg/ml with a QarrayMini microarrayer (Genetix). DNA from *E. coli* was used as negative hybridization control. Preparation of NotI representations (NotI probes, NR) was done essentially as described previously.[37] Hybridization of NR was performed at 42°C for 15 h in a Lucidea Base device (Amersham Pharmacia Biotech) according to manufacturer's recommendations. Microarrays were scanned in a GenePix 4000A. The technique determines NotI-site methylation or deletion simultaneously. The results were processed with GenePix Pro 6.0 software (Amersham Pharmacia Biotech). Then data were analyzed using our program NIMAN, NotI-Microarray Analysis.[7]

Bisulfite genomic sequencing. The bisulfite conversion of DNAs was performed using an EZ DNA Methylation Kit (Zymo Research) according to the manufacturer's instructions. Primers for PCR are available upon request. After amplification of bisulfite treated DNAs PCR products were cloned and used for automated sequencing or were sequenced directly (ABI Prism 3100-Avant Genetic Analyzer, Applied Biosystems).

Quantitative PCR. Total RNA extraction and reverse transcription reaction was done as described earlier.[14] The sequences of primers and probes for *ITGA9*, *RBSP3* and *RASSF1A* genes were published previously.[14] For *VILL* gene we used the following sequences: forward primer—GCA CTG ACA GCC ACA ACA CCA, reverse primer—ATC ACC ATT ACA GCC CTT CCC A, probe—CCG TGC CTC ATC CCT CAA CTC CAG; and for *APRG1* gene: TTT GGA CCC AAG GTA AGA AAA CTG, CCA TCC AAT GCT GTG ATT CCA C and ACC AGC CTT CCA TTG CTC CAC ACA respectively. Reference genes *GAPDH* and *RPNI*[38] were used. All reactions were performed using 7500 Real-Time PCR System (Applied Biosystems). QPCR data were analyzed using the relative quantification or $\Delta\Delta C_t$-method as described.[39] Each reaction was repeated three times. At least 2-fold mRNA changes were considered as significant because of reference genes variability.

Immunohistochemistry. RASSF1A protein content in cervical cancer cell lines were estimated with immunohistochemical methodology using confocal microscope (SPES, Leica) with AOTF calibration before each measurement. We used specific primary monoclonal mice antibodies to RASSF1A (Acris) and secondary goat antibodies to mice conjugated with Atto 647 dye, which we chose because of their high photo stability. Fluorescence level in 3D images (reconstructions) was measured using Imaris software.

Table 5. Clinical and pathological characteristics of cervical cancer samples

TNM/Clinical stage	Number of samples	
	Histological type of cervical cancer	
	SCC*	ADC
$T_1N_0M_0$/I	21/24	6
$T_2N_0M_0$/II	8/17	2
$T_1N_1M_0$, $T_2N_1M_0$/III	10/17	1
Total	39/58	9

*Number of samples used for NotI-microarrays analysis/total number of samples used in all experiments.

Statistical analysis. Nonparametric Wilcoxon test was used to compare mRNA expression differences of target and reference genes in cervical cancer samples. Kruskal-Wallis and Mann-Whitney rank-sum tests, Fisher's exact test and χ^2 criteria were used for analysis of genomic DNA copy, methylation and mRNA level changes in cervical cancer groups with different histological characteristics. P-values < 0.05 were considered statistically significant. Spearmen's rank correlation coefficient was used for revealing correlations between mRNA level changes of different genes. All statistical procedures were performed using our NIMAN software created earlier[7] and BioStat software.[40] Sensitivity, specificity and accuracy were calculated according to.[41] Selection of groups of the genes, allowing potentially to distinguish two main histological types of CC - SCC and ADC was performed by often used statistical method SVM (support vector machine). The number of genes was chosen to create a set with the maximum distance between these two groups.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental materials may be found here:
http://www.landesbioscience.com/journals/epigenetics/article/24233/

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