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Modern Pathology (2005) 18, 340–348
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www.modernpathology.org

Downregulation of 14-3-3σ in ovary, prostate and endometrial carcinomas is associated with CpG island methylation

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The 14-3-3σ inhibitor of cell cycle progression has been shown to be target of epigenetic deregulation in many forms of human cancers; however, its role in urological and gynecological cancers has not been studied. Here, we have analyzed the expression of *14-3-3σ*, wild-type *p53* and mutated *p53* in over 300 cases of the most common cancers occurring in the urological and gynecological tracts and its normal counterpart tissue by immunohistochemistry using the multiple tumor tissue microarrays. 14-3-3σ expression was detected in normal epithelia from most organs with sporadic expression in renal tubules and absence in the testis. In contrast to normal tissue, 14-3-3σ expression was lost in 40–60% of adenocarcinomas of the breast, ovary, endometrium and prostate. There was no association between *14-3-3σ* and wild-type/mutated *p53* expression. By performing methylation-specific PCR, we showed a close association of 14-3-3σ CpG island methylation and low protein expression levels of 14-3-3σ. In addition, a direct link of 14-3-3σ mRNA expression levels to CpG island methylation is demonstrated in two human cancer cell lines. Loss of 14-3-3σ expression due to promoter hypermethylation may represent the most frequent molecular aberration in ovarian, endometrial and prostate adenocarcinomas.
Modern Pathology (2005) **18,** 340–348, advance online publication, 16 July 2004; doi:10.1038/modpathol.3800240

Keywords: urological and gynecological carcinomas; 14-3-3σ expression; CpG methylation

The 14-3-3σ protein belongs to a highly conserved family of acidic protein, the 14-3-3 family, which is constituted of at least seven mammalian isoforms.[1–3] From all seven isoforms, 14-3-3σ has been most directly linked to cancer. 14-3-3 proteins are defined as phosphoserine-binding proteins that can bind to the two consensus motifs RSXpSXP and RXY/FXpSXP, which are present in almost all of the 14-3-3-binding proteins. 14-3-3 proteins are involved in regulation of numerous cellular signaling pathways and one of their main activities is cell cycle regulation. A functional link between 14-3-3σ and the p53 protein has been proposed. Upon DNA damage, p53 induces 14-3-3σ expression.[4] By sequestration of CDC2 in the cytoplasm, *14-3-3σ* mediates a G2/M arrest. Genetic inactivation of *14-3-3σ* was used to demonstrate its requirement for a stable arrest after DNA damage. Furthermore, *14-3-3σ* may directly interact with and stabilize *p53* suggesting a positive feedback loop.[5] In addition to cell cycle regulation, 14-3-3σ proteins may play a role in controlling apoptotic cell death. Through interaction with proapoptotic proteins BAD and BAX, 14-3-3σ has been shown to inhibit apoptosis.

Compelling evidence suggest that *14-3-3σ* is a tumor suppressor gene that contributes to cancer development. First, DNA damage leads to upregulation of *14-3-3σ* in colon cancer cells in a p53-dependent manner. Second, gene inactivation of *14-3-3σ* in cells leads to a defect in G2/M arrest normally induced by DNA damage. Third, irradiated *14-3-3σ−/−* cells are not capable to stably arrest in G2 phase of the cycle to repair DNA damages leading to mitotic catastrophe and cell death.[4] In breast epithelial cells, 14-3-3σ expression has been

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Received 17 May 2004; revised 22 June 2004; accepted 24 June 2004; published online 16 July 2004

demonstrated to be post-translationally regulated through interaction with the estrogen responsive finger protein (EFP) leading to ubiquitinylation and rapid degradation. Furthermore, overexpression of EFP *in vivo* can induce tumors presumably by inducing genomic instability due to *14-3-3σ* loss.[6] Finally, in skin epithelia, expression of 14-3-3σ is upregulated upon differentiation in cells of the poststem cell compartment and immortalization of keratinocytes by experimental inactivation of 14-3-3σ, underlines furthermore its role as tumor suppressor gene.[7]

Significant progress has been made in understanding the molecular genetics of urological and gynecological malignancies. There is strong experimental and clinical evidence that in addition to distinct genetic mutations, epigenetic mechanisms may play an important role in the pathogenesis of these tumors.[8–10] Hypermethylation of CpG islands is a well-known epigenetic mechanism to inactivate a tumor suppressor gene. Silencing of gene expression by CpG hypermethylation has been shown to be an early event in cancer development, and in some cases may precede the neoplastic process.[11,12] Likewise to other malignancies, silencing of an increasing number of genes by CpG island methylation has been demonstrated in renal, urothelial, ovarian, prostate and endometrial cancers. Best known examples are silencing of the VHL tumor-suppressor gene in renal carcinoma or loss of hormone receptor genes (estrogen, progesterone, androgen) in endometrial and prostate cancers respectively.[13,14]

Following the hypothesis of *14-3-3σ* being a tumor suppressor gene, Ferguson *et al*[15] analyzed 14-3-3σ expression in breast cancer. Downregulation of *14-3-3σ* expression in tumor cell lines and primary tumor samples was associated with aberrant CpG island methylation. Following studies demonstrated epigenetic silencing of 14-3-3σ expression in gastric, liver, lung, cervical, oral squamous cell carcinoma, and basal cell carcinoma of the skin.[16–21]

The aims of this study were: first, to study the expression of 14-3-3σ by using immunohistochemistry on a multiple tumor tissue microarrays containing a large number of urological and gynecological cancers. Second, to explore the relation between 14-3-3σ expression and *p53* using the D07 and the PAB240 clone recognizing wild-type and mutated *p53* isoforms respectively. Third, by focusing on adenocarcinoma of three organs such as prostate, endometrium and ovary, we intended to evaluate the relation between downregulation of *14-3-3σ* and CpG island methylation in cell lines and primary tumor samples.

Materials and methods

Case Selection and Multitumor Tissue Microarray

A total of 350 cases of carcinomas arising in the urological and gynecological tracts has been selected from the surgical pathology archives. These malignancies include the following cases: urothelial bladder carcinoma ($n = 45$), squamous cell carcinoma of the uterine cervix ($n = 9$), endometrial carcinoma ($n = 46$), ovarian carcinoma ($n = 21$), breast carcinoma (ductal type $n = 36$, lobular type $n = 7$), renal carcinoma (clear cell type $n = 60$, chromophobe $n = 10$, papillary $n = 16$), adenocarcinoma of the prostate ($n = 40$) and testicular tumors (seminoma $n = 24$, embryonal $n = 36$).

Paraffin-embedded tissues were used. The tissue microarray was constructed as described previously by Kononen *et al.*[22] After carefully choosing the morphologically representative region on the individual paraffin-embedded chosen blocks (donor blocks), a core tissue biopsy of 1.6 mm was punched and transferred to the donor paraffin-embedded block (receiver block). To overcome tumor heterogeneity, two punch biopsies were performed from different areas of each tumor. In total, 42 normal tissue from each of cervix ($n = 2$), breast ($n = 10$), kidney ($n = 12$), prostate ($n = 10$), and testis ($n = 8$) were also included in the array. owing to the difficulty of taking punch biopsy of normal urothelium for the array, whole sections of five normal bladder mucosa were studied separately for the expression of *14-3-3σ*. Sections of 4 μm thickness were cut from the tumor microarray block and transferred to glass slides using the paraffin-sectioning aid system (adhesive-coated slides, adhesive tape, and UV lamp, Instrumedics Inc., Hockensack, NJ, USA). One section was stained by hematoxylin–eosin to evaluate the presence of the tumor.

Immunohistochemical Staining Procedure

Sections, of 4 μm thickness, from formalin-fixed, paraffin-embedded tissue microarray were processed for immunohistochemistry. Endogenous peroxidase was blocked with 0.3% hydrogen peroxidase for 5 min. Antigen retrieval was carried out in citrate buffer (0.01 M, pH 6.0) at 98°C for 3 min in a pressure cooker. Then, sections were incubated with the 14-3-3σ (rabbit polyclonal, 1:50, IBL, Japan), p53/PAB240 (mouse monoclonal, 1:50, DAKO, Copenhagen, Denmark), and p53/D07 (mouse monoclonal, 1:400, DAKO) antibodies at room temperature. A subsequent reaction was performed with biotin-free HRP enzyme labeled polymer of Envision plus detection system (DAKO). Diaminobenzidine complex was used as chromogen. Evaluation of the immunohistochemistry was evaluated semiquantitatively by two pathologists (PM and MA) using double-head microscope, who were not aware of the original histological diagnosis. For p53, the cutoff of 10% of cells with positive nuclei staining pattern was considered as positive. As for 14-3-3σ, staining was detected in the cytoplasm with sometimes nuclear staining. The cutoff of 50% positive cells was considered as positive.

Methylation-Specific PCR

Genomic DNA was isolated from 66 formaldehyde-fixed and paraffin-embedded cases (17 ovarian, 30 prostate, 19 endometrial tumors) by using standard techniques. For molecular analysis tumor regions were mechanically separated from normal tissue if necessary, to have over 80% tumor cells in a given sample. Bisulfite treatment and PCR amplification was performed as previously described with some modification.[15] In brief, 2 μg genomic DNA was denatured in NaOH (0.2 N) for 15 min at 37°C before adding 30 μl hydrochinone (20 μM, Sigma) and 520 μl of sodium bisulfite (3 M, pH 5.0). The samples were mixed and incubated at 50°C for 16 h. Modified DNA was purified with the QIAGEN PCR purification kit, following the manufacturer's protocol (QIAGEN, Hildesheim, Germany). To complete the conversion, 6.3 μl NaOH (5 N) was added to the eluted DNA (100 μl) and the samples incubated for 5 min at room temperature, followed by ethanol precipitation. DNA samples were then stored at −20°C until further use. High-performance *Taq* polymerase (Ampli*Taq*-GOLD, Applied Biosystems, Rotkreuz, Switzerland) was used for all PCR reactions. All samples were first analyzed for amplification of the wild-type (WT) *14-3-3σ* locus using the primers (WT-Forward: 5′-tat gag gac atg gca gcc ttc a-3′ and WT-Reverse: 5′-cc cct cca ggc agc cct ctg g-3′) recognizing a 138 bp genomic DNA fragment (GenBank Acc. Do. NM_006142, position 210–348). For methylation-specific PCR previously reported primer sets covering CpG dinucleotides numbers 3, 4, 8, and 9 of *14-3-3σ* were used.[15] For each PCR reaction, three controls were included: in addition to a water control (negative), two DNA samples of breast cancer cell lines (MDA-MB-435, MCF-7) were analyzed known to result in strong signals for the methylated and unmethylated alleles, respectively (MCF-7, MDA-MB-435).[15,20]

14-3-3σ Methylation and Messenger RNA Expression upon 5-Azacytidine Treatment

Nine urological and gynecological cancer cell lines (all from the American Tissue Culture Collection (ATCC) cultivated according to the supplier's recommendations) were analyzed for 14-3-3σ promoter methylation by PCR (as describe above). Two cancer cell lines (AN3CA, LNCAP) with strong 14-3-3σ CpG methylation were further analyzed for 14-3-3σ expression at the messenger RNA (mRNA) level before and after blocking cellular methylation by 5-azacytidine (5-Aza, SIGMA Biochemicals, Buchs, Switzerland) treatment as described previously.[15] Cells were treated for three consecutive days with 5-Aza at concentrations ranging from 0.1–10 μM. A fresh stock solution was prepared each day (0.5 mM in medium) immediately before adding to the cells. 14-3-3σ promoter methylation was checked by PCR before and after 5-Aza treatment. For quantitative mRNA expression analysis, total RNA was isolated using the Trizol™ reagent following the manufacturer's instruction (Invitrogen Life Technologies, Carlsbad, CA, USA). Obtained total RNA was quantified by optical densitometry and 0.5 μg were directly transcribed into first-strand complementary DNA (cDNA) using the first strand cDNA synthesis kit from Roche Molecular Biochemicals (Rotkreuz, Switzerland). To quantify the levels of *14-3-3σ* mRNA levels 1 μl of a 100 × dilution of the cDNA (20 μl) was used to proceed real-time PCR in iQTM SYBR Green Supermix (Biorad Laboratories Inc., Hercules, CA, USA). mRNA expression was evaluated by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis using an Icyler™ (Biorad). For each sample, PCR reactions were performed in triplicate. The following primers were used: sigma-G1 (forward primer) 5′-cca ggc tac ttc tcc cct c-3′ and sigma-G2 (reverse primer) 5′-ctg tcc agt tct cag cca ca-3′ resulting in a 99 bp fragment (GenBank Acc. Do. NM_006142; position 1165–1263). The specificity of the PCR reaction was further checked by sequence analysis of the product appearing as single band of expected size on a 2.5% agarose gel. Expression levels of 14-3-3σ mRNA were normalized to the endogenous mRNA levels of GAPDH gene (ΔCT method).

Statistical Analysis

The associations between protein expressions were determined using Fisher's exact test. Differences in the proportion of protein expression among organs and tumor types were assessed with Fisher's exact test. All analysis was performed using the Stata 8.2, Statistical Package (College Station, TX, USA).

Results

Distribution of 14-3-3σ Expression in Normal Urological and Gynecological Tissues

The distribution of 14-3-3σ protein expression in normal tissue was as follows: positive immunostaining was observed in the urothelial epithelium, periductal and periglandular cells of the prostate (Figure 1a, b) and breast and some glandular and ductal cells, and squamous epithelium of the exocervix of the uterus. On the other hand, no 14-3-3σ expression was seen in the germinal cells of the testis and in the ovary (follicules and stroma). There was very sporadic expression in renal tubules, but none in the glomeruli. There was very weak expression in the endometrial glands and endocervical glands.

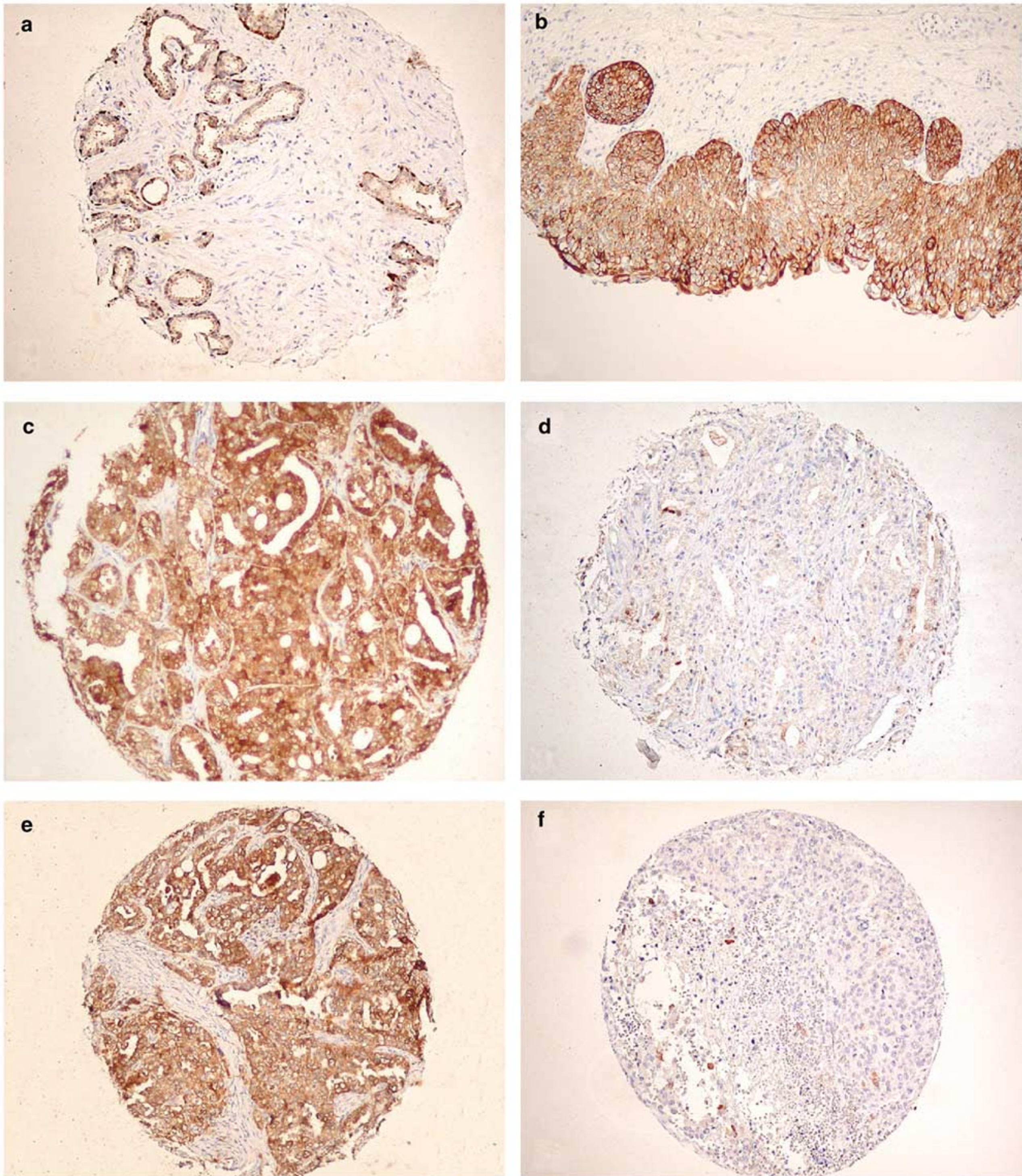

Figure 1 14-3-3σ protein expression in normal and malignant urological and gynecological tissues assessed by immunohistochemistry. (**a**) Normal prostate tissue stained with 14-3-3σ showed positivity of the basement membrane and some epithelial cells. (**b**) Normal bladder stained with 14-3-3σ showed positive staining of the cytoplasm of urothelial cells. (**c**) A case of prostatic adenocarcinoma sigma showing strong 14-3-3σ positivity in the cytoplasm and nuclei of tumor cells. (**d**) Prostatic adenocarcinoma negative for 14-3-3σ. (**e**) A case of endometrial adenocarcinoma showing strong positivity of 14-3-3σ. The expression by tumor cells was mainly cytoplasmic. (**f**) Endometrial adenocarcinoma negative for 14-3-3σ.

Expression of 14-3-3σ in Urological and Gynecological Tumor Tissues

As summarized in Table 1, 14-3-3σ protein expression was found in 141 (40.3%). of 350 overall tumors. The protein was expressed in 44 (98%) urothelial bladder cancer, 22 (55%) prostatic adenocarcinoma, six (66.7%) squamous cell carcinoma of the cervix, 26 (56.6%) endometrial adenocarcinoma, seven (33%) ovarian adenocarcinoma and 10 (23%) breast carcinoma. Similar to normal tissue, sporadic expression of 14-3-3σ was detected in 10 (11.7%) of renal tumors and 16 (26.7%) of testicular tumors (Figure 1 c–f).

Since 14-3-3σ has been shown to be regulated by *p53*, we next analyzed the tumors by using two antibodies recognizing *p53*, D07 recognizing an epitope in the amino (N)-terminus of the wild-type human p53 protein, and PAb240 recognizing only the mutant forms of *p53*. The *p53* D07 was expressed in 78 cases (22.4%) and negative in 270 cases (77.6%). Signals for mutated *p53* were seen in 57 cases (16.5%) and negative in 290 cases (83.6%). There was no significant association between 14-3-3σ and p53 expression as detected by the p240 or the DO7 antibody ($p=0.9$). Furthermore, and as expected, a significant association between p53 D07 and mutated *p53* ($p<0.001$) was observed.

Downregulation of 14-3-3σ Expression in Prostate, Endometrial and Ovarial Carcinomas is Associated with 14-3-3σ CpG Methylation

As summarized in Figure 2 and Table 2, methylation-specific PCR (MSP) analysis revealed signals for 14-3-3σ CpG island methylation in most of the cases analyzed. There was a significant association of CpG methylation with a weak or absent 14-3-3σ protein expression in the 66 cases chosen from the tissue microarray (Table 2). All cases without sign of methylation (weak or absent amplification of the

Table 1 Expression of 14-3-3σ, wild-type *p53* and mutated *p53* in different tumor tissues of the urological and gynecological tracts

	Total number	*14-3-3σ*		*p53 (DO7)*		*p53 (pAb240)*	
		Positive (n)	*%*	*Positive* (n)	*%*	*Positive* (n)	*%*
Urothelial bladder carcinoma	45	44	98	19	42.2	10	22.2
Prostatic adenocarcinoma	40	22	55	1	2.5	1	2.5
Squamous cell carcinoma of the cervix	9	6	66.7	1	11.1		0
Endometrial adenocarcinoma	46	26	56.6	9	19.6	3	6.5
Breast carcinoma	43	10	23	4	9.3	4	9.3
Ductal type	36						
Lobular type	7						
Ovarian adenocarcinoma	21	7	33	16	76.2	13	62
Renal tumors	86	10	11.7	6	7	0	0
Renal cell carcinoma	60						
Chromophobe carcinoma	10						
Papillary carcinoma	16						
Testicular tumors	60	16	26.7	22	38	26	45.6
Seminoma	24						
Embryonal carcinoma	36						

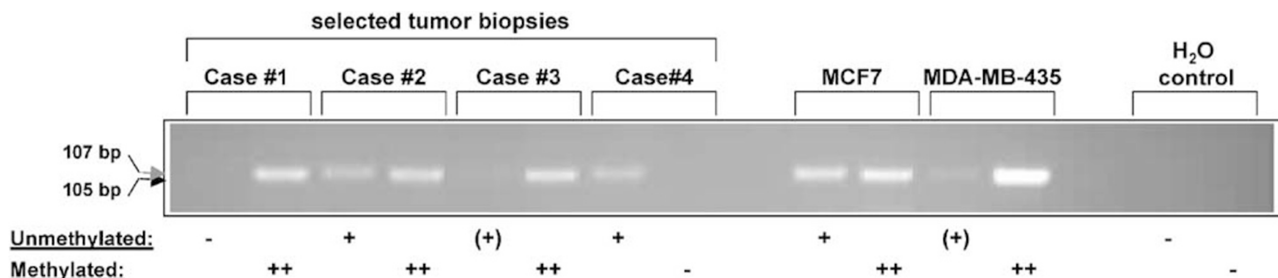

Figure 2 14-3-3σ methylation-specific PCR analysis in selected tumor biopsies. Methylation-specific PCR analysis showed cases with strong 14-3-3σ methylation and absence of unmethylated allele, cases with signals for unmethylated and methylated alleles of variable intensity [(+), +, ++] and cases with no signs of methylation. In each PCR reaction, in addition to a water negative control, DNA from breast carcinoma cells (MCF-7, MDA-MB-435) showing predominant signals for the unmethlylated and methylated alleles, respectively, were used. PCR products with close molecular weight (methylated allele, 105 bp; unmethylated allele, 107 bp) are marked with arrows.

Table 2 Association of 14-3-3σ CpG island methylation (by methyaltion-specific PCR) and protein expression in 66 tumors from the urological and gynecological tracts

	Methylation-specific PCR				
	–	(+)	+	++	
M (methylated)					
IHC-positive ($n=32$)[a]	2	15	14	1	
IHC-negative ($n=34$)[b]	0	6	19	9	$P=0.003$*
U (unmethylated)					
IHC-positive ($n=32$)	1	13	14	4	$P=0.006$**
IHC-negative ($n=34$)	9	16	9	0	

Association between IHC-negativity and methylation signal (M)* and IHC-positivity and unmethylation signal (U)**.
[a]IHC-positive cases (ovarian ($n=7$), prostate ($n=15$), endometrial ($n=10$)).
[b]IHC-negative (ovarian ($n=10$), prostate ($n=15$) and endometrial ($n=9$)).

Table 3 14-3-3σ CpG island methylation in urological and gynecological cancer cell lines

Cell line	*Origin*	*Unmethylated*[a]	*Methylated*[a]
DU-145	Prostate CA	++	–
PC-3	Prostate CA	++	–
LNcaP	Metastatic prostate CA	–	++
OVCA-429	Ovarian CA	++	–
OVCAR-433	Ovarian CA	++	–
OVCAR-3	Ovarian CA	–	++
HOC-1B	Ovarian CA	++	+
AN3CA	Endometrial CA	–	++
HEC-59	Endometrial CA	++	+

[a]Signal strength in methylation-specfic PCR (MSP) analysis (see Figure 2).

methylated allele) showed normal expression of 14-3-3σ immunohistochemical staining ($p=0.006$). In contrast, almost all cases showing evidence for strong methylation (weak or absent amplification of the unmethylated allele) showed only faint or absent 14-3-3σ protein expression ($p=0.003$). These results suggest that CpG methylation of 14-3-3σ may be responsible for downregulation of 14-3-3σ expression in urological and gynecological cancers.

To further support the hypothesis that CpG promoter methylation leads to down-regulation of 14-3-3σ expression in urological and gynecological cancers, we analyzed a panel of established cells lines from ovarian cancer (OVCA-429, OVCAR-3, OVCAR-433, HOC-1B), prostate cancer (LNCAP, DU-145, PC-3) and endometrial cancer (HEC-59, AN3CA) by methylation-specific PCR. As shown in Table 3, five out of nine cell lines showed some 14-3-3σ promoter methylation. In three of them, no signal for unmethylated alleles could be observed. We next ask whether demethylation by the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-Aza) may be able to trigger re-expression of 14-3-3σ in cells with strong CpG island methylation. As shown in Figure 3a an increased signal for the unmethylated allele is observed after 3 days of 5-Aza treatment of LNCAP (prostate cancer) and AN3CA (endometrial cancer) cells. However, it is interesting to note that the sensitivity of both cell lines differs by factor 10, with optimal signals after 1 and 10 μM 5-Aza treatment. Further increase of the 5-Aza dose results in nonspecific toxicity without any significant demethylation of 14-3-3σ (data not shown). To analyze the link of 14-3-3σ promoter methylation and gene expression, 14-3-3σ mRNA levels were further determined in both cell lines before and after 5-Aza treatment. As shown in Figure 3b, dose-dependent increase in 14-3-3σ mRNA can be observed in LNCAP and AN3CA cells upon demethylation by 5-Aza treatment. These findings show that downregulation of 14-3-3σ in urological and gynecological cancers is a consequence of promoter CpG island methylation and suggest that pharmacological redirection of 14-3-3σ using small-molecule DNA methyltransferase inhibitors may be of benefit in therapy of these cancers.

Discussion

In normal tissue, the 14-3-3σ protein was found to be expressed in the urothelial epithelium, periductal and periglandular cells of the prostate and breast and some glandular and ductal cells, the exocervix

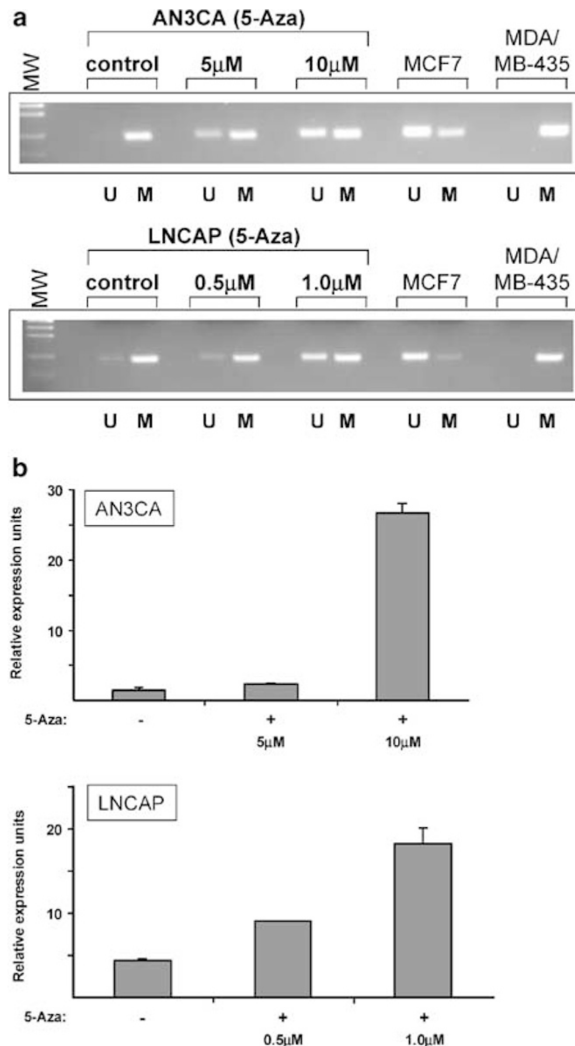

Figure 3 5-Aza treatment increases 14-3-3σ mRNA expression in selected cancer cell lines from prostate, ovary and endometrium. (**a**) AN3CA and LNCAP cells were treated for 3 days with 5-Aza, 0.1–10 μM) with renewal of the medium each day. Untreated AN3CA and LNCAP cells show an almost complete methylation of the 14-3-3σ promoter region. Upon 5-Aza treatment a dose-dependent signal increase for unmethylated 14-3-3σ alleles can be observed. (**b**) 14-3-3σ mRNA levels were compared upon treatment of AN3CA (top panel) and LNCAP (bottom panel) cells with 5-Aza (0.1–10 μM, 3 days) by real-time PCR. Relative levels were normalized to the GAPDH housekeeping gene mRNA expression levels. A dose-dependent increase in 14-3-3σ mRNA expression after 5-Aza treatment can be seen in both cell lines.

of the uterus, and its absence in the ovarian stroma, kidney, and testis. These results are very similar to those seen by Nakajima *et al*.[23]

In tumor tissues, 14-3-3σ was more frequently expressed in urothelial bladder cancer, squamous cell carcinoma of the exocervix, followed by prostatic and endometrial adenocarcinomas, and it is the least expressed in carcinomas of the breast and ovary and in renal and testicular tumors. By performing PCR analysis for CpG island methylation, we showed that decrease 14-3-3σ expression is associated with methylation-dependent transcriptional silencing. Although we have not analyzed our cases for 14-3-3σ mutations, previous works analyzing a large number of cases from various tissues have failed to detect any mutation that may be responsible to downregulate 14-3-3σ expression. Including our series, epigenetic transcriptional silencing of 14-3-3σ has been demonstrated in malignancies from prostate, endometrium, ovary, breast, lung, liver, skin, stomach and oral squamous cell carcinoma.[15–18,20,21,24–26] Another recent immunohistochemical study has shown loss of 14-3-3σ expression in prostate cancer.[27] By preselecting areas of highest Gleason score in their tumor biopsies and by using a different 14-3-3σ antibody, these authors find low or absent levels of 14-3-3σ in an even higher percentage of tumors.

Several studies have documented 14-3-3σ CpG methylation in dysplastic premalignant lesions or normal tissue adjacent to the tumor, suggesting that this process may represent an early event in carcinogenesis.[20,24] However, in contrast to most malignancies, gene expression profiling as well as tissue microarray screens have recently demonstrated overexpression of 14-3-3σ in pancreatic ductal adenocarcinomas and biliary cancers.[28,29] Interestingly, deregulated overexpression of 14-3-3σ was associated with CpG island hypomethylation. Close association of CpG island hypomethylation with overexpression of multiple genes indicates that gene hypomethylation is a frequent epigenetic event in pancreatic cancer.[30]

Normal regulation of 14-3-3σ seems to be cell-type dependent since in contrast to the tissues listed above, 14-3-3σ seems to be regulated by constitutive CpG island methylation in lymphoid cells. Using MSP, Bhatia *et al*[3] have shown that normal and malignant lymphoid cells constitutively express low levels of 14-3-3σ associated with CpG methylation.[31] This finding is of importance and has to be kept in mind when analyzing tumor samples for 14-3-3σ methylation. Strong inflammatory reactions within the tumor samples may lead to false-positive results. This phenomenon may also explain methylation-specific PCR signals in some cases of our series with normal 14-3-3σ as determined by immunohistochemistry.

Whether 14-3-3σ may play a role in renal or testicular malignancies is not completely understood. Sporadic and low levels of 14-3-3σ expression detected in our series of normal and malignant renal and testicular tissues suggest that 14-3-3σ may not play an important role in the pathogenesis of these lesions.

14-3-3σ has been identified as a p53-regulated inhibitor of G2/M cell cycle progression. DNA damage induced by irradiation or cytotoxic agents lead to a time and dose-dependent upregulation of 14-3-3σ expression.[4] Induced by DNA damage, stress and various oncogenes *p53* is the central regulator of the cells apoptotic machinery.[32]

Inactivation of *p53* in a large percentage of human cancers promotes checkpoint defects, cellular immortalization, genomic instability and inappropriate survival. Since *p53* inactivation by mutation of interaction with viral proteins is a frequent event in urological and gynecological tumors, we compared expression of p53 with 14-3-3σ expression in our large series.[33] Keeping in mind that p53/PAB240 detects about 80% of p53 mutations, in the present study the immunohistochemical status of p53 did not correlate with 14-3-3σ expression. In our tumors, we did not analyze the *p53* mutation but previous studies showed that no correlation between genomic alterations of p53 and 14-3-3σ expression was observed in various cancer cell lines, and in primary vulval squamous cancer and primary neuroendocrine tumors.[16,25,26] Like *p53*, its homologs *p63* and *p73* can trigger expression of 14-3-3σ. In addition, repression of 14-3-3σ expression was seen by a tumor-associated *p63* splice variant (Delta Np63) *in vitro*.[34,35] However, again no correlation was found between the *p63* status and 14-3-3σ expression in hyperproliferative skin disease.[20] These studies all suggest independence of 14-3-3σ expression of *p53* and its homologues *p63* and *p73*.

Interestingly, there is evidence that 14-3-3σ expression is also regulated at the post-transcriptional level. Cellular levels of 14-3-3 proteins including the sigma isoform have been recently shown to be regulated by tuberous sclerosis gene products hamartin and tuberin, suggesting that deregulation of 14-3-3 proteins might contribute to tumor formation in tuberous sclerosis patients.[36] In addition, in breast cancer cells 14-3-3σ has been shown to undergo proteolysis mediated by the *EFP*. The *EFP* is a target gene of the estrogen receptor acting as an ubiquitin ligase of 14-3-3σ.[6] Whether *EFP*, widely expressed in hormone-sensitive tissues like uterus, ovary and mammary glands may play a role in 14-3-3σ regulation of urological and gynecological cancers is subject of ongoing studies.

In summary, 14-3-3σ is downregulated in a significant fraction of urological and gynecological tumors. In addition, we showed an association between 14-3-3σ CpG methylation and low level of expression, indicating that epigenetic silencing of 14-3-3σ may present a frequent aberration in ovarian, endometrial and prostatic adenocarcinomas.

Acknowledgements

We thank Mr L Métral and the histopathology laboratory especially Mrs D Fontana for her help. This study was in part supported by grants from the Swiss National Science Foundation and ONCOSUISSE to JS.

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