

Expression of 14-3-3 σ in cervical squamous cell carcinomas: Relationship with clinical outcome

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Abstract. 14-3-3 sigma (σ) sequesters the cdc2-cyclin B1 complex in the cytoplasm resulting in G2 arrest. Inactivation and reduced expression of 14-3-3 σ have been reported in a variety of cancers. In the present study, we investigated the expression of 14-3-3 σ in a series of 297 cervical squamous cell carcinoma (SCC) to clarify the prognostic value. Using immunohistochemical methods we found high levels of 14-3-3 σ protein in cytoplasm of 143 (48.1%), in nucleus of 113 (38.0%) and in both cytoplasm and nucleus of 147 (49.5%) cases, whereas, low levels were present in cytoplasm of 154 (51.9%), in nucleus of 184 (62.0%) and in both cytoplasm and nucleus of 150 (50.5%) cases. Levels of 14-3-3 σ mRNA measured by reverse-transcription polymerase chain reaction (RT-PCR) and 14-3-3 σ protein were not significantly associated. 14-3-3 σ expression in cytoplasm, nuclear and cytoplasm/nuclear were not significantly correlated to disease-specific survival or disease-free survival. In conclusion, reduced expression of 14-3-3 σ protein in the cytoplasm and shuttle of 14-3-3 σ protein into the nucleus in a relatively high number of cases indicate that 14-3-3 σ may be important in the carcinogenesis of cervical SCCs by two different mechanisms; reduction and nuclear translocation of 14-3-3 σ protein. Furthermore, the non-significant correlation between expression levels of 14-3-3 σ mRNA and protein support a post-transcriptional regulation in cervical SCCs. The protein has no prognostic value in cervical cancers.

Introduction

Cervical carcinoma is the second most common cancer among women worldwide (1). Independent prognostic factors

include patient age, clinical stage, tumor size, lymph node metastasis and lymph-blood vessel invasion (2-7). These parameters are not sufficient to obtain an exact prediction of prognosis. Therefore, identification of new biological markers may be useful to determine patient outcome.

14-3-3 proteins represent a family of at least seven mammalian isoforms, which are highly conserved acidic proteins, involved in regulating signal transduction pathways, adhesion, apoptosis, differentiation, cellular proliferation and survival (8,9). Of these isoforms, 14-3-3 σ is restricted to be a human epithelial marker (10). Previous studies have shown that after DNA damage increased expression of 14-3-3 σ is mediated by p53 and p21. The increase of 14-3-3 σ protein expression results in G2/M arrest by binding to and sequestering the cdc2-cyclin B1 complexes in the cytoplasm (11). In addition, 14-3-3 σ binds to cdk2 and cdk4 and emerges as a new class of cdk inhibitors (12). These findings indicate that inactivation and low expression of 14-3-3 σ may be involved in malignant transformation.

Inactivation of 14-3-3 σ gene by CpG methylation and/or reduced expression of 14-3-3 σ have been reported in a variety of human cancers, including ovarian (13,14), cervix (15), vulvar (16,17), corpus (18), lung (19), oral (20), liver (21), skin (22), breast (23,24), gastric (25,26), renal (27), testis (27) and colorectal (28). Association of 14-3-3 σ with clinical outcome has been found in patients with endometrial carcinoma (18), ovarian carcinoma (14), breast carcinoma (24) and colorectal carcinoma (28). However, the clinical importance of 14-3-3 σ in patients with cervical carcinomas has not been studied. Therefore, in the present study, we investigated the expression of 14-3-3 σ in a series of 297 cervical SCC to clarify the prognostic value.

Materials and methods

Patients and clinical data. Tissue samples from 297 patients with cervical SCCs, FIGO stage I-IV were included in the present study (Table I). The patients were treated at The Norwegian Radium Hospital in the period 1995-2005. The ages of patients range from 26 to 92 years (median 55 years). One hundred and thirteen (38.0%) of the patients suffered a

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Table I. 14-3-3 σ in relation to clinical parameters.

Variables	Total no.	Cytoplasm			Nucleus			Cytoplasm/nucleus		
		Low	High (%)	P ^a	Low	High (%)	P ^a	Low	High (%)	P ^a
Age (years)				0.687			0.349			0.531
≤50	123	64	59 (48)		76	47 (38)		64	59 (92)	
50-70	104	51	53 (51)		60	44 (42)		48	56 (54)	
>70	70	39	31 (44)		48	22 (31)		38	32 (46)	
FIGO stage				0.638			0.227			0.569
I	65	37	28 (43)		38	27 (42)		36	29 (45)	
II	132	70	62 (47)		88	44 (33)		69	63 (48)	
III	74	35	39 (53)		46	28 (38)		33	41 (55)	
IV	26	12	14 (54)		12	14 (54)		12	14 (54)	

^aPearson χ^2 ; FIGO stage, International Federation of Gynecology and Obstetrics.

relapse and 105 (33.4%) died of cervical carcinoma. Follow-up for all patients range from 1.4 to 149 months (median 48 months), whereas, follow-up for patients still alive range from 16 to 139 months (median 107 months). Samples from 10 normal cervixes (patients who underwent amputation of the cervix for prolaps) were used as normal controls. The study was approved by The Regional Committee for Medical Research Ethics South Norway (S-06381a), The Social and Health Directorate (06/4509 and 06/4417) and The Data Inspectorate (06/01467-3).

Immunostaining method. Immunostaining was performed on sections from formalin-fixed, paraffin-embedded tissue, using the Dako EnVision™ + System, Peroxidase (DAB) (K4007, Dako Corp., CA, USA) and DakoAutostainer. After micro-waving in 10 mM citrate buffer pH 6.0 and treatment with 0.03% hydrogen peroxide (H₂O₂) for 5 min the sections were incubated with monoclonal 14-3-3 σ antibody (clone 1433S01, 1:75, 2.7 μ g IgG₁/ml, NeoMarkers, CA, USA) for 30 min at room temperature. The primary antibody is highly specific to 14-3-3 σ and shows no cross-reaction with other isoforms of 14-3-3 (information from NeoMarkers). The slides were then incubated with peroxidase labeled polymer conjugated to goat anti-mouse for 30 min, 3'-diaminobenzidine tetrahydrochloride (DAB) for 10 min and counterstained with hematoxylin, dehydrated, and mounted in Diatex. In all series normal skin has been included as positive controls, whereas, as negative controls mouse myeloma protein of the same subclass and concentration as mouse anti-14-3-3 σ were used. Both cytoplasmic and nuclear staining were evaluated as positive. Immunostaining was scored on a 3-tiered scale for both extent of staining (percentage of positive tumor cells 1, <10%; 2, 10-50%; 3, >50%) and intensity (1, absent/weak; 2, moderate; 3, strong). A composite score ranging from 1 to 9 for each section was obtained by multiplying the results of extent and intensity. Based on the staining pattern in normal cervical epithelium 14-3-3 σ expression in cytoplasm was defined as high when composite scores were 9, whereas in

Table II. 14-3-3 σ immunostaining.

Score	Cytoplasm n (%)	Nucleus n (%)	Cytoplasm/nucleus n (%)
0	1 (0.3)	184 (62.0)	1 (0.3)
1	1 (0.3)	0	1 (0.3)
2	5 (1.7)	7 (2.4)	5 (1.7)
3	24 (8.1)	98 (33.0)	24 (8.1)
4	7 (2.4)	0	7 (2.4)
6	116 (39.1)	6 (2.0)	112 (37.7)
9	143 (48.1)	2 (0.7)	147 (49.5)
Total	297 (100.0)	297 (100.0)	297 (100.0)

nucleus high expression group includes all the tumors with any staining.

RNA methods. 14-3-3 σ mRNA levels were assessed by quantitative real-time PCR (qRT PCR) analysis of 91 tumors as described previously (29). Total RNA was isolated from snap-frozen tumor specimens by use of TRIzol reagent (Invitrogen, Carlsbad, CA) followed by precipitation with isopropanol and 5 M lithium chloride. cDNA (10 ng), synthesized from the total RNA by use of Superscript III transcriptase (Invitrogen), was applied together with a pre-designed 14-3-3 σ (SFN) specific TaqMan probe and primer set (Applied Biosystems, Foster City, CA). Each reaction was carried out in triplicate, using the following amplification conditions: one cycle with of 50°C in 2 min, one cycle with 95°C in 10 min followed by 40 cycles of 95°C in 15 sec and 60°C in 1 min. The 14-3-3 σ mRNA level was calculated relative to the endogenous control B2M.

Statistical analyses. The correlation between 14-3-3 σ mRNA level and protein expression was investigated with Spearman

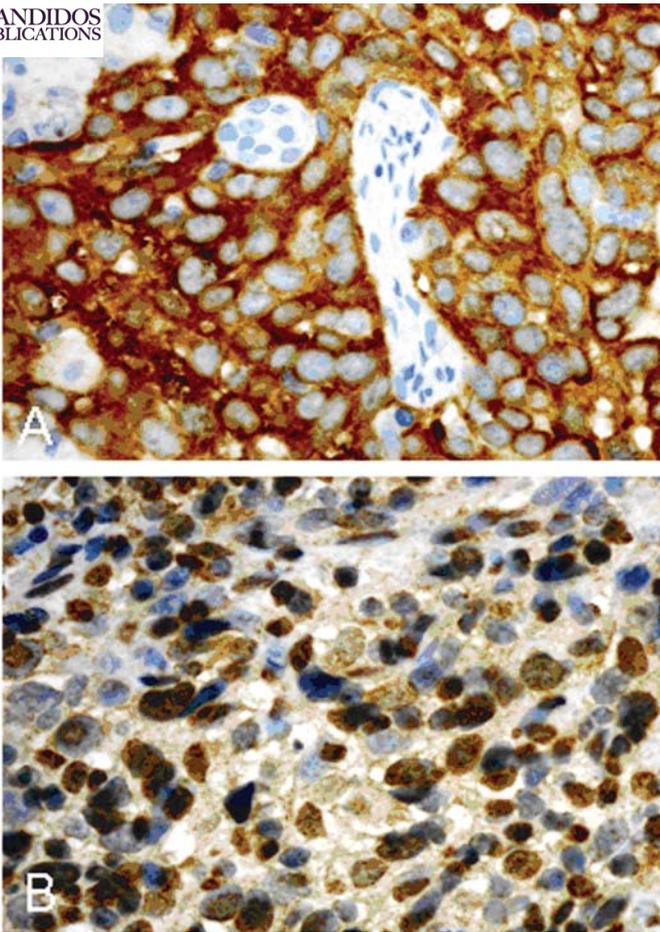


Figure 1. Immunohistochemical staining showing 14-3-3 σ protein in cytoplasm (A) and nucleus (B).

rank order analysis. The relation between 14-3-3 σ expression and age or FIGO stage was evaluated by the Person χ^2 test. Kaplan and Meier were used to calculate survival curves and

categories of 14-3-3 σ were compared by the log-rank test. Disease-specific and disease-free survival was defined as the time between diagnosis and death or relapse due to cervical cancer, respectively. For multivariate analysis of survival rate, the Cox proportional hazards regression model with a backward stepwise regression and a $P=0.05$ in univariate analysis as the inclusion criterion were used. The analysis was performed using the SPSS 15.0 statistical software package (SPSS, Chicago, IL). $P \leq 0.05$ was considered as statistical significance.

Results

In normal cervical epithelium, high expression of 14-3-3 σ protein in cytoplasm was found in 10/10 (100%) cases, whereas, no nuclear staining was seen. Positive staining for 14-3-3 σ was observed in basal, parabasal, middle and top layers of cervical epithelium. In cervical SCCs, high levels of 14-3-3 σ protein was observed in cytoplasm of 143 (48.1%), in nucleus of 113 (38.0%) and in both cytoplasm and nucleus of 147 (49.5%) cases, whereas, low levels were present in cytoplasm of 154 (51.9%), in nucleus of 184 (62.0%) and in both cytoplasm and nucleus of 150 (50.5%) cases (Table II, Fig. 1). There was no association between 14-3-3 σ mRNA level and protein expression, regardless of whether the protein level in the cytoplasm, nucleus or both was considered (Fig. 2).

The levels of 14-3-3 σ were not significantly associated to age and FIGO stage (Table I). In univariate analysis, 14-3-3 σ expression in cytoplasm, nuclear and cytoplasm/nuclear were not significantly associated to disease-specific survival ($P=0.705$, 0.193 and 0.793 , respectively) and disease-free survival ($P=0.519$, 0.200 and 0.573 , respectively). Increasing age and FIGO stage were significantly correlated to shorter disease-specific survival ($P<0.001$ and <0.001 , respectively) and disease-free survival ($P<0.006$ and <0.001 , respectively). In multivariate analysis, FIGO stage and age were of independent prognostic significance for disease-specific

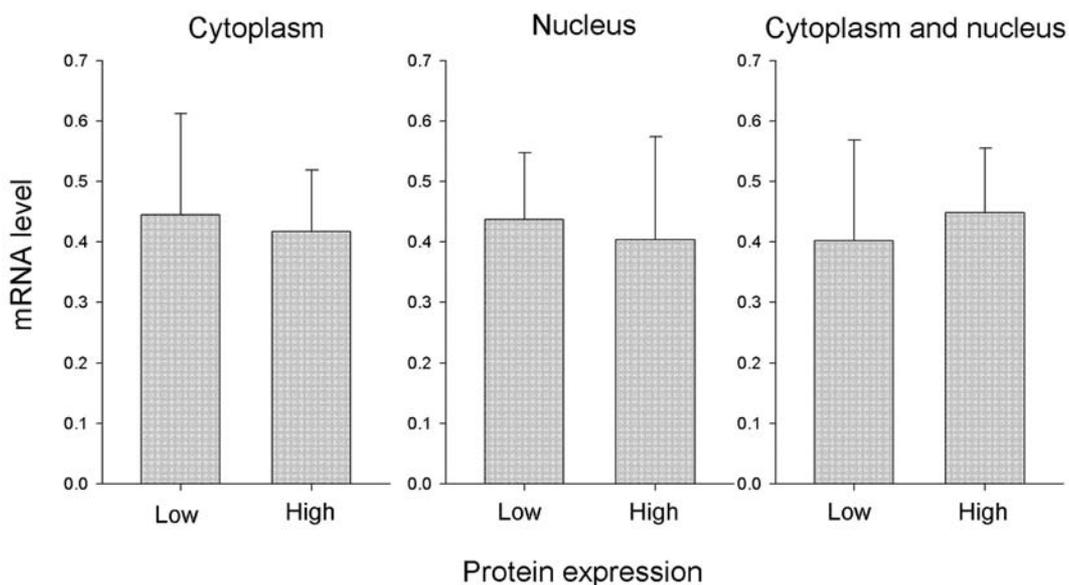


Figure 2. Gene expression in relation to protein expression of 14-3-3 σ .

Table III. Multivariate analysis.

Variables	Grouping	Hazard of risk (HR)	95% CI ^a	P-value ^b
Disease-specific survival				
FIGO stage	I vs. II vs. III vs. IV	2.08	1.67-2.59	<0.001
Age	<50 years vs. 50-70 years vs. >70 years	1.52	1.18-1.94	0.001
Disease-free survival				
FIGO stage	I vs. II vs. III vs. IV	2.03	1.64-2.51	<0.001

^a95% confidence interval of the HR. ^bTest for inclusion of variable.

survival, whereas only FIGO stage was of independent prognostic significance for disease-free survival (Table III).

Discussion

Reduced expression of 14-3-3 σ protein has been documented in ovarian (13,14), corpus (18), vulvar (17), lung (19), oral (20), liver (21), skin (22) and breast carcinomas (24). Furthermore, Mhaweche *et al* (15) identified low level of 14-3-3 σ protein in 3/9 (33%) cervical SSCs, whereas, Sano *et al* (30) did not find low level of 14-3-3 σ protein in any of the 29 cervical SSCs tested. In the present study we detected low level of 14-3-3 σ in about 50% of cervical SSCs when comparing with normal cervical squamous epithelium. These conflicting results may be explained by the small number of cases included in the previous works (15,30), the use of different 14-3-3 σ antibodies and immunohistochemical staining methods and the differences in scoring of the immunohistochemical staining. The low 14-3-3 σ protein level in a relative high number of cervical SSC and the finding of no association between 14-3-3 σ protein expression and FIGO stage, suggested that loss of 14-3-3 σ protein expression may occur as a relatively early event in cervical tumorigenesis.

In normal cervical epithelium high expression of 14-3-3 σ protein in cytoplasm was found in all 10 cases, whereas, no nuclear staining was seen. This is in accordance with a previous study of various mammalian cells localizing 14-3-3 σ mainly in the cytoplasm and only low levels in the nucleus (31). The high expression of cytoplasmic 14-3-3 σ in normal cervical epithelium may contribute to G2/M arrest by binding to and sequestering the cdc2-cyclin B1 in the cytoplasm (11). A different subcellular distribution of 14-3-3 σ protein was identified in cervical SCCs where high 14-3-3 σ expression was found in the cytoplasm and nucleus in 48 and 38% of the cases, respectively. The shuttle of 14-3-3 σ into the nucleus of cervical SSCs may reflect a mechanism by which 14-3-3 σ reduces the capacity to keep the cdc2-cyclin B1 complex in the cytoplasm resulting in failure of G2/M arrest and entering cells into mitosis. Therefore, 14-3-3 σ protein nuclear localization may be important in the carcinogenesis of cervical SCCs.

No previous studies are available on the prognostic significance of 14-3-3 σ protein expression in cervical SCC. We did not find a statistical association between 14-3-3 σ protein expression and disease-specific survival. This is in accordance with the data in breast carcinomas (24) and vulvar carcinomas (17). Previously, Akahira *et al* (14) have reported that in univariate analysis negative 14-3-3 σ immunoreactive ovarian carcinomas had a significantly poorer overall survival rates than positive cases. However, in multivariate analysis, 14-3-3 σ protein expression turned out not to be an independent prognostic marker. In contrast to these studies, Ito *et al* (18) showed that loss or absence of 14-3-3 σ expression was a statistically independent risk factor in overall survival in patients with early-stage endometrial cancer, whereas, Perathoner *et al* (28) identified that 14-3-3 σ overexpression was associated with significantly decreased survival time in multivariate analysis. Considering these studies together, it seems that 14-3-3 σ play different role in cancers from different human organs.

Silencing of the 14-3-3 σ gene, mainly by CpG methylation, occurs in numerous solid tumor types (9). However, 14-3-3 σ expression may also be regulated at the post-transcriptional level. The non-significant correlation between expression levels of 14-3-3 σ mRNA and protein in our study support a post-transcriptional regulation in cervical SCCs. This is in line with the results in cervical adeno/adeno-squamous carcinomas (30), vulvar squamous cell carcinomas (17) and colon cancer cell lines (28) where the expression of 14-3-3 σ mRNA and protein were not significantly associated. Different mechanisms have been indicated for the regulation at the post-transcriptional level. Audic and Hartley (32) have reported that there may be modifications of mRNA stability and/or translation efficiency. In addition, Urano *et al* (33) showed that Efp, a RING-finger-dependent ubiquitin ligase, targets proteolysis of 14-3-3 σ protein, indicating down-regulation of 14-3-3 σ protein through a proteasome-dependent mechanism.

In conclusion, reduced expression of 14-3-3 σ protein in the cytoplasm of 51.9% of the cases and shuttle of 14-3-3 σ protein into the nucleus of 38% of the cases indicate that 14-3-3 σ may be important in the carcinogenesis of cervical SCCs. Furthermore, the non-significant correlation between

SPANDIDOS levels of 14-3-3 σ mRNA and protein supports a transcriptional regulation in cervical SCCs. No significant correlation was seen between cytoplasmic and/or nuclear 14-3-3 σ protein and survival.

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