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14-3-3 sigma and p53 expression in gastric cancer and its clinical applications

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Abstract. 14-3-3 sigma (σ) induces G2 arrest enabling the repair of damaged DNA. The function of 14-3-3 σ is frequently lost in tumor cells, indicating a potential tumor suppressor function. The purpose of this study was to evaluate the prognostic value of 14-3-3 σ expression in human gastric cancer. 14-3-3 σ expression was analyzed by immunohistochemistry in 157 tumor samples of patients, who underwent resection for gastric cancer. Since 14-3-3 σ is involved in the p53 network, p53 expression was detected in parallel and correlated with 14-3-3 σ. 14-3-3 σ was found to be overexpressed in 75 (47.8%) of 157 cases, the overexpression rate of p53 protein was 27.4%. 14-3-3 σ overexpression was statistically significantly associated with pT-stage ($p = 0.041$) pN-stage ($p = 0.015$) and UICC-stage ($p = 0.019$) and showed a borderline significance with Lauren classification ($p = 0.057$). Univariate survival calculations revealed a coexistent 14-3-3 σ and p53 overexpression as a significant predictor of disease-free survival. Multivariate analysis did not unfold 14-3-3 as an independent prognostic factor for disease-free survival and overall survival. Concomitant 14-3-3 σ and p53 overexpression in tumor cells of patients with gastric cancer identifies a population of patients with relatively unfavorable prognosis.

Keywords: Gastric cancer, 14-3-3 sigma, tumorigenesis

1. Introduction

14-3-3 proteins have a critical role in a wide variety of cellular responses including signal transduction, cell cycle regulation, apoptosis, cytoskeleton organisation and malignant transformation [1–3]. The 14-3-3 protein family comprises 7 isoforms whereas 14-3-3 σ is the only isoform induced by p53 protein in response to γ irradiation and other damaging agents [4]. 14-3-3 σ sequesters the mitotic initiation complex cdc2-cyclin B1 from entering the nucleus, thus preventing initiation of mitosis. In this manner, 14-3-3 σ induces G2 arrest enabling the repair of damaged DNA [1]. As a target gene of p53, 14-3-3 σ has reciprocally influence on the p53 network. It specifically increase p53 stability and enhance p53 transcriptional activity [5].

The function of 14-3-3 σ is frequently lost not only in tumor cells but also in surrounding pre-dysplastic tissue, indicating an important tumor suppressor function that becomes lost at an early stage in the progression to invasive cancer [6,7]. Besides, Wilker et al. indicate that 14-3-3 σ is important in the physiological down-regulation of new protein synthesis during and immediately after mitosis and is required for the translation of a protein kinase that is essential to the proper completion of cytokinesis [3]. The loss of function is either due

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to hypermethylation of the 14-3-3 σ promoter or to induction of an oestrogen-responsive ubiquitin ligase that specifically targets 14-3-3 σ for proteosomal degradation [6–12]. CpG islands hypermethylation and loss of 14-3-3 σ expression was frequently found in different tumor entities such as hepatocellular carcinoma, lung cancer, prostate cancer, oral cancer, ovarian cancer or gastric cancer whereas in colorectal cancer CpG islands were unmethylated [10–16]. In the present study we aimed to explore the expression of 14-3-3 σ in the tissue of patients with resected gastric cancer and to elucidate the relationship to biological behaviour and prognosis.

2. Material and methods

2.1. Patients and specimens

The study was conducted according to the regulations of the local ethics committee and Austrian law. Tumor tissues of 157 consecutive cases of adenocarcinomas of the stomach {97 men (62%) and 60 women (38%) at a mean age of 67.2 years (95% CI: 65.8–69.2)} were investigated in this study. All patients had been operated between 1993 and 2004 at the Department of Surgery, Innsbruck Medical University, Austria with curative intent ($n = 157$). Tumor tissues were routinely processed (formalin-fixed and paraffin-embedded) and were classified according to the guidelines of the Union Internationale Contre le Cancer (UICC) and were graded according to WHO classification [17,18]. Adjuvant or additive chemotherapy was recommended to patients with UICC stages III and IV. Adjuvant chemoradiation as propagated by Macdonald et al. was not used routinely but carried out in selected patients [19]. Demographic data of all patients were recruited from our computerised surgical documentation system (ChiBASE). All data regarding date and cause of death were confirmed by the "Tumorregister Tirol", a cancer-register maintained by the Tyrolean government. Demographic data and tumor characteristics are summarised in Table 1.

2.2. Immunohistochemistry

Sections of a tissue microarray were deparaffinized by prolonged incubation in xylene (3–4 min), followed by prolonged washing and rehydration in ethanol (96% ethanol 2–3 min, 80% ethanol 3 min, and 70% ethanol 3 min). Endogenous peroxidase activity was blocked by 0.5% hydrogen peroxide (H2O2) in methanol for 20 min. Antigen retrieval was achieved by heating the slides to 86°C in citrate buffer (0.01 M) for 15 min. Immunohistochemistry was performed as described earlier [20]. Briefly, slides were incubated with anti-14-3-3 σ antibody (2 lg/ml, Neomarkers, Fremont, CA, USA) or anti-p53 antibody (clone DO7, DakoCytomation, Glostrup, Denmark, 1:50) in a humidified chamber for 60 min at room temperature. After washing, the slides were incubated with biotinylated rabbit anti-mouse IgG (Dako, Copenhagen, Denmark) at a dilution of 1:500, and detected with an ABC-peroxidase- Kit (Vector Laboratories, Burlingame, CA) with diaminobenzidine as the substrate, according to the manufacturer 's protocol. Sections were counterstained with Mayer's hemalaun (Merck, Darmstadt, Germany), washed in tap water, and mounted using Aquatex (Merck). The specificity of 14-3-3σ antibody was assessed by blocking controls. Native 14-3-3σ protein (ProSci Incorporated, Poway, CA) was added to the primary antibody solution in tenfold excess 1 h before application onto tissue sections. Blocking of 14-3-3σ antibody resulted in no 14-3-3σ staining on 14-3-3σ positive tissue sections (not shown) demonstrating no cross reaction of this antibody with other members of the 14-3-3 protein family. Negative controls without primary antibody were included in each run.

Slices were evaluated by two independent pathologists (P.M. and K.S.) using light microscopy who had no prior knowledge of the clinical data. Antigen expression was defined as the presence of specific staining of cytoplasm of tumour cells for 14-3-3 σ and nuclear staining for p53. 14-3-3 σ and p53 overexpression was evaluated by calculating a total immunostaining score as the product of a proportion and intensity score. The proportion score described the estimated fraction of positive stained tumour cells (0 = none; 1 =< 10%; 2 = 10–50%; 3 = 51–80%; 4 => 80%). The intensity score represented the estimated staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong). The total score ranged from 0–12. Regarding the total score the tissue samples were bimodally distributed with the nadir at a total score of 3–4. Therefore, 14-3-3 σ "overexpression" was arbitrarily defined as a total score > 4 as described previously [20].

2.3. Statistical analysis

Statistical analysis was performed using the Statistical Package of Social Science (SPSS, Version 16.0, Chicago IL). Descriptive statistics for continuous measures are given as the mean with the respective 95% confidence interval (CI) in parenthesis. For discrete da-

Table 1
Patients ($n = 157$) characteristics and clinicopathological features

		n / mean	% / min-max
Age (years)		67.2	27–94
Gender	female	60	38.0
	male	97	62.0
Operative Procedure	subtotal gastrectomy	53	33.8
	total gastrectomy	82	52.2
	resection of the gastric remnant	13	8.3
	limited resection	9	5.7
Lymph node dissection	D1	41	26.1
	D2+D3	116	73.9
pT-category	pT1	28	17.8
	2	87	55.4
	3	37	23.6
	4	5	3.2
pN-category	pN0	71	45.2
	1	46	29.3
	2	26	16.6
	3	14	8.9
M-category	M0	146	93.0
	1	11	7.0
UICC-stage	IA	25	15.9
	IB	34	21.7
	II	43	27.4
	IIIA + IIIB	33	21.0
	IV	22	14.0
R-Stage	R0	150	95.5
	R1	7	4.5
Tumor Differentiation	G1-2	53	33.8
	G3-4	104	66.2
Lauren Classification	intestinal	92	58.6
	diffuse	65	41.4
Chemotherapy	neoadjuvant	9	5.2
	adjuvant	14	9.1

min-max. . . lowest and highest value.

ta (e.g. 14-3-3 σ expression) frequency counts and percentages were tabulated. Results of the various estimates were compared using either the χ^2 test or One-way ANOVA. Survival curves were calculated using the Kaplan and Meier method and compared by the log-rank test. Follow-up time was censored if the patient was lost to follow-up. Patients who died without documented disease recurrence were considered censored for disease-free survival but were included as deaths for overall survival analysis. Factors shown to be of prognostic significance in univariate models were evaluated in a multivariate Cox regression model. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of 14-3-3 σ and p53

Out of all cancer samples, 14-3-3 σ was immunohistochemically detected in the cytoplasm of tumor cells in 122 (77.7%) specimens whereas 35 (22.3%) patients showed a negative staining reaction (Fig. 1). With regard to 14-3-3 σ positivity ($n = 122$), 65 (53.3%) patients were with strong staining fraction (score 4), 50 (41.0%) with moderate (score 3), and 7 (5.7%) with weak (score 2) staining contingent. According to the above mentioned criteria, 14-3-3 σ was found to be overexpressed in 75 (47.8%) of 157 cases. Adjacent normal epithelial cells were stained negatively or very weakly. Contrary, p53 immunostaining was detectable only in the nucleus of tumor cells (Fig. 2). Out of all cancer samples 67 (42.7%) were immunopositive for p53, whereas 90 (57.3%) lacked a positive staining reaction. Referring to immunopositivity, 49 (73.1%) patients were with strong staining fraction (score 4), 7 (10.4%) with moderate (score 3), and 11 (16.4%) with weak (score 2) staining rate. The overexpression rate of p53 protein was 27.4%. Adjacent normal epithelial cells were stained negatively for p53.

3.2. Relationship between 14-3-3 σ and p53 overexpression and clinicopathologic features

14-3-3 σ overexpression in carcinoma cells was statistically significantly associated with pT-stage ($\chi^2 =$

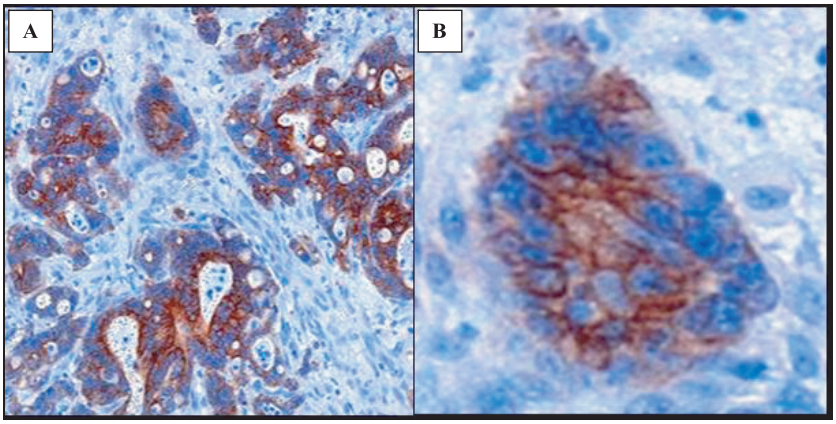

Fig. 1. Immunohistochemical staining for 14-3-3 σ expression in human gastric cancer tissues with anti-14-3-3 σ antibody, (A original magnification x 100; B original magnification x 400). The examples show a strong specific cytoplasmic staining for 14-3-3 σ and no staining in the nucleus and plasma membrane.

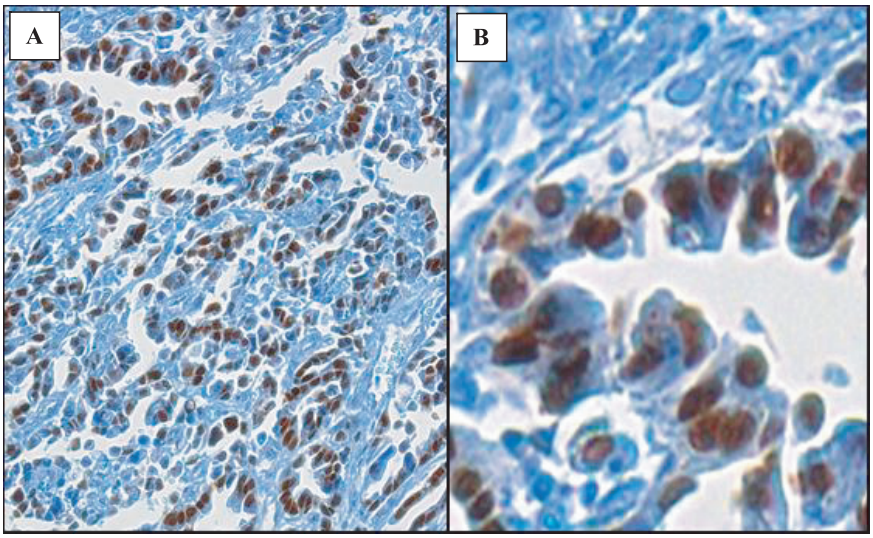

Fig. 2. Immunohistochemical staining for p53 expression in human gastric cancer tissues with anti-p53 antibody, (A original magnification x 100; B original magnification x 400). The examples show a strong nuclear p53 staining.

8.24; df = 3; $p = 0.041$) pN-stage ($\chi^2 = 5.38$; df = 1; $p = 0.015$) and UICC-stage ($\chi^2 = 13.53$; df = 5; $p = 0.019$) and showed a borderline significance with Lauren classification ($\chi^2 = 5.73$; df = 2; $p = 0.057$) (Table 2). No statistically significant association was found between p53 and common clinicopathologic features.

3.3. Relationship between 14-3-3 σ expression and p53 status

Due to the fact that 14-3-3 σ is induced by p53 protein in response to γ irradiation and other damaging agents and that 14-3-3 σ has reciprocally influence on the p53 network we examined the relationship between 14-3-3 σ and p53. Statistical analysis did not show significant association between the two investigated parameters.

3.4. Survival analysis

Regarding disease-free survival and overall survival, 14-3-3 σ overexpression did not reveal statistical significance (Fig. 3). Univariate survival calculations for the total cohort revealed Lauren classification, tumor differentiation, depth (T-stage) of wall invasion, nodal status, presence of distant metastasis, UICC-stage, tu-

Table 2
Relationship between 14-3-3 σ overexpression and p53 overexpression and various clinicopathological parameters in 157 gastric cancer specimens

		All cases ($n = 75$; 47.8%)	14-3-3 σ overexpression	p* ($n = 43$; 27.4%)	p53 overexpression	p*
Gender						
	male	97	44 (58.7)	n.s.	29 (67.4)	n.s.
	female	57	31 (41.3)		14 (32.6)	
Lauren classification						
	intestinal	92	51 (68.0)	0.057	16 (37.2)	n.s.
	diffuse	65	24 (32.0)		27 (62.8)	
Tumor differentiation						
	Grade 1/2	53	29 (38.7)	n.s.	15 (34.9)	n.s.
	Grade 3/4	104	46 (61.3)		28 (65.1)	
pT-category						
	pT1	28	7 (9.3)	0.041	9 (20.9)	n.s.
	pT2	87	43 (57.3)		22 (51.2)	
	pT3	37	22 (29.3)		10 (23.3)	
	pT4	5	3 (4.1)		2 (4.7)	
pN-category						
	N-pos	79	45 (60.0)	0.015	25 (58.1)	n.s.
	N-neg	78	30 (40.0)		18 (41.9)	
M-category						
	M0	146	69 (92.0)	n.s.	38 (88.4)	n.s.
	M1	11	6 (8.0)		5 (11.6)	
UICC stage						
	IA	25	5 (6.7)	0.019	8 (18.6)	n.s.
	IB	34	20 (26.7)		10 (23.3)	
	II	43	19 (25.3)		12 (27.9)	
	IIIA + B	33	18 (24.0)		2 (4.7)	
	IV	22	13 (17.3)		11 (25.6)	

**Probability, P, from χ^2 test.*

Table 3
Prognostic factors examined in 157 adenocarcinomas of the stomach: an univariate approach to tumor relapse and cancer-specific mortality

	DFS		OS	
	X^2	P*	X^2	P*
Age ($\leqslant 70$ vs. > 70))	0.001	n.s.	4.83	0.028
Lauren classification (intestinal vs. diffuse)	6.01	0.049	2.58	n.s.
Operative procedure (subt. vs. total gastr. vs. gr vs. limited)	2.35	n.s.	6.9	n.s.
Lymph node dissection (D1 vs. D2/3)	1.76	n.s.	6.06	0.014
Tumor differentiation (G1/G2 vs. G3/G4)	8.10	0.004	3.27	n.s.
pT-category (pT1 vs. pT2 vs. pT3 vs. pT4)	31.80	0.001	22.36	0.001
pN-category (pN− vs. pN+)	11.15	0.001	12.23	0.007
M-category (M0 vs. M1)	18.03	0.001	21.54	0.001
UICC-stage (IA + IB vs. II + IIIA/B + IV)	46.28	0.001	25.66	0.001
Residual disease (R0 vs. R1+R2)	3.00	n.s.	0.59	n.s.
Anemia ($\leqslant 11$ g% vs. > 11 g%))	2.25	n.s.	1.89	n.s.
Tumor size ($\leqslant 4$ cm vs. > 4 cm))	12.43	0.001	1.67	n.s.
14-3-3 σ overexpression (yes vs. no)	1.57	n.s.	0.55	n.s.
p53 overexpression (yes vs. no)	2.50	n.s.	0.88	n.s.
14-3-3 σ / p53 overexpression (yes vs. no)	4.91	0.027	0.23	n.s.

DFS. . . disease- free survival; OS. . . overall survival;
**Probability, P, from χ^2 test.*

mor size and concomitant 14-3-3 σ and p53 overexpression as significant predictors of disease-free survival (Fig. 4). Regarding overall survival, age, lymph node dissection, T stage, nodal status, presence of distant metastasis and UICC-stage were of prognostic significance, whereas gender and 14-3-3 σ overexpression were not (Table 3).

In multivariate analysis, UICC stage was an independent prognostic factor for disease-free survival and overall survival (Table 4).

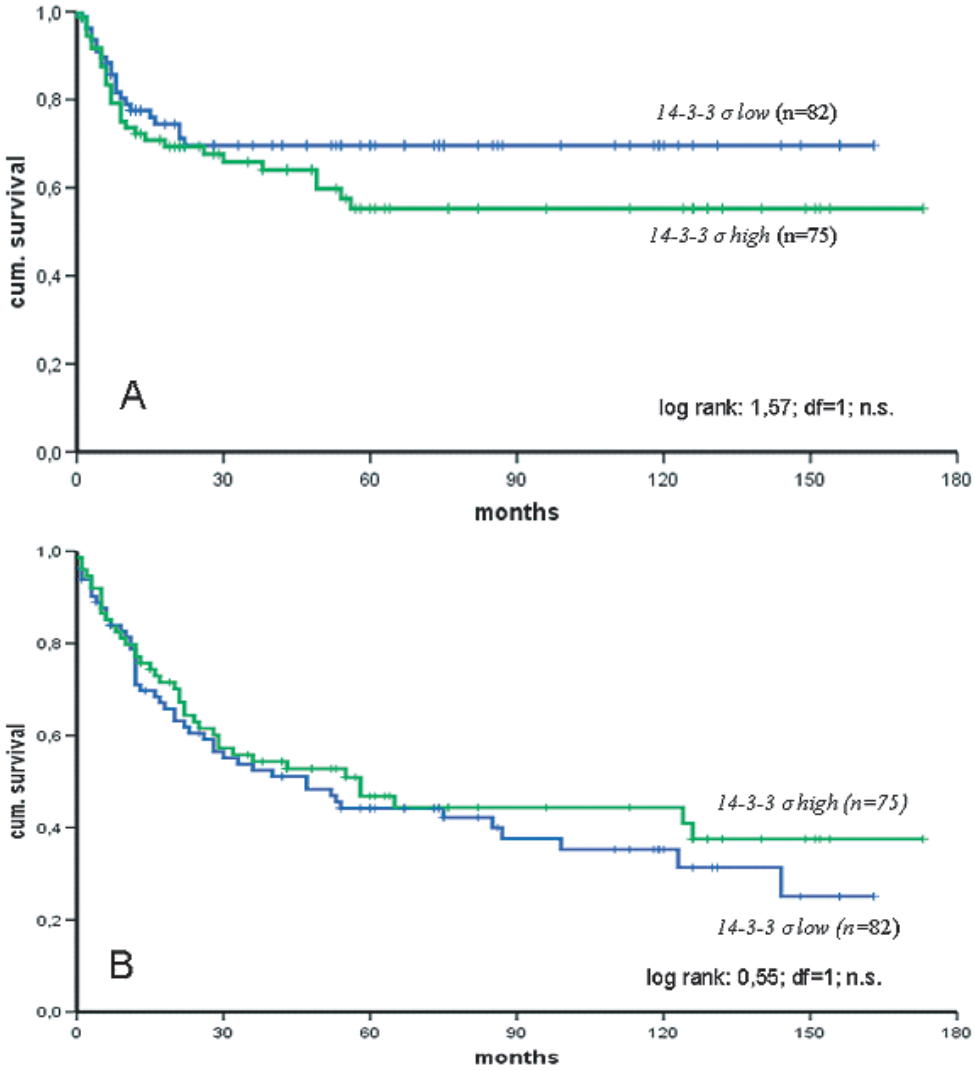

Fig. 3. Kaplan Meier survival curves with regard to disease-free-survival (A) and overall survival (B) of 157 patients with gastric carcinomas grouped according to their 14-3-3 σ expression. *high*, patients with 14-3-3 σ overexpressing tumor ($n = 75$). *low*, patients with tumor without 14-3-3 σ overexpression ($n = 82$).

4. Discussion

14-3-3 proteins are a family of highly conserved cellular proteins that play key roles in the regulation of central physiological pathways. *14-3-3* σ, one of the seven $14-3-3$ genes, is said to hold an important role in human tumorigensis [21]. Several lines of evidence suggests that 14-3-3 σ expression contributes to cancer development via the conference of apoptosis resistance, chemoresistance, and/or growth factor advantage to cancer cells, under certain growth conditions [22,23]. The present study revealed the expression of 14-3-3 σ and its prognostic value in gastric cancer.

Recent studies have demonstrated that the loss of function of 14-3-3 σ is either due to hypermethylation of the promoter or to induction of an oestrogen-responsive ubiquitin ligase that specifically targets 14-3-3 σ for proteosomal degradation [6–12]. CpG islands hypermethylation and loss of 14-3-3 σ expression was frequently found in different tumor entities such as hepatocellular carcinoma, lung cancer, prostate cancer, oral cancer, ovarian cancer or gastric cancer, whereas in colorectal cancer CpG islands were unmethylated [10–16].

Investigations by Suzuki et al. revealed that 14-3-3 σ methylation was frequently observed in poorly dif-

Table 4
Multivariate analysis regarding disease-free and overall survival

	DFS		OS	
	RHR (95% CI)	P*	RHR (95% CI)	P*
UICC-stage (II + IIIA/B + IV vs. IA + IB)	5.91 (1.99–16.80)	0.000	2.59 (2.26–11.95)	0.000
14-3-3 σ overexpression (yes vs. no)	0.59 (0.33–1.86)	n.s.	0.26 (0.36–1.31)	n.s.

*DFS. . . disease- free survival; OS. . . overall survival; RHR. . . relative hazard ratio; CI. . . confidence interval; *Probability, P, from χ^2 test.*

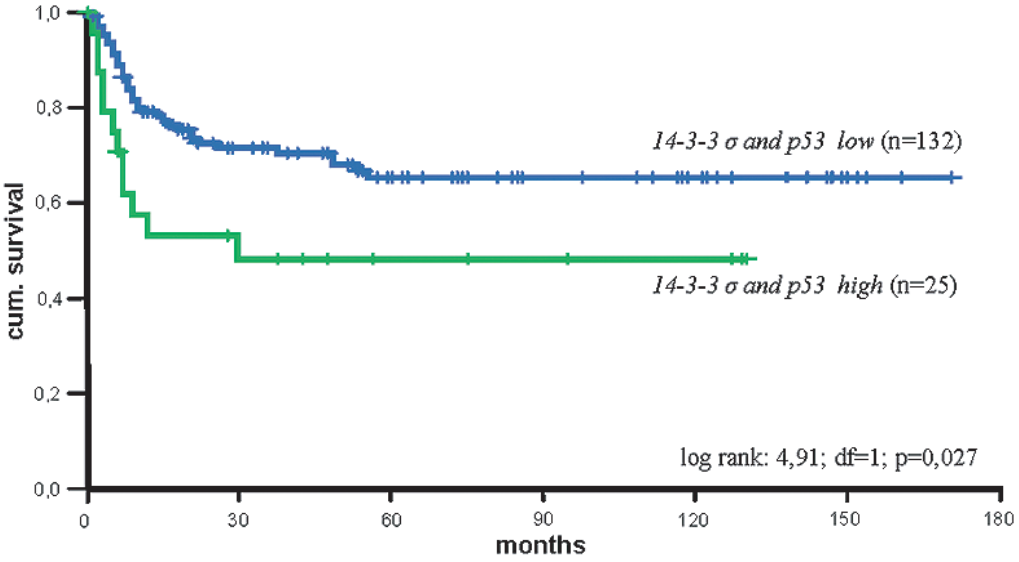

Fig. 4. Kaplan Meier survival curves with regard to disease-free-survival of 157 patients with gastric carcinomas with concomitant 14-3-3 σ and p53 overexpression ($n = 25$). *high*, patients with concomitant 14-3-3 σ and p53 overexpression ($n = 25$). *low*, patients without concomitant 14-3-3 σ and p53 overexpression ($n = 82$).

ferentiated adenocarcinoma of the stomach suggesting that decreased expression of 14-3-3 σ is associated with the developement of undifferentiated gastric cancer. In a series of 60 gastric cancer tissue samples they observed 14-3-3 σ hypermethylation in 43%, interpreting that the expression of 14-3-3 sigma is lost [11]. In contrast, other reports could show that 14-3-3 σ mRNA and protein expression was strongly up-regulated in more than two thirds of gastrointestinal cancer patients [24]. This goes along with our findings where 77.7% of the study patients showed positivity for 14-3-3 σ with an overexpression rate of 47.8%. Tanaka et al. allready published data on 14-3-3 σ expression in primary gastrointestinal malignancy. The analysis of the relationship between 14-3-3 σ expression and common clinicopathologic factors in 34 gastric cancer patients revealed only the gross appearance as a significant parameter. Gastric cancer with Borrmann types 1 or 2 showed significantly higher 14-3-3 σ expression in cancer cells than those with Borrmann types 3 or 4 [24]. The study population in this research is very small and a limiting factor for a clear conclusion. So we conducted an analysis with a larger study group to be able to make an improved statement. We found immunopositivity in 77.7% of all patients and out of these a high expression pattern in 74.2% of the cases. 14-3-3 σ overexpression was statistically significantly associated with pT-stage, pN-stage, and UICC-stage, and showed a borderline significance with Lauren classification.

Contrary to other reported tumor entities, Kaplan-Meier survival analysis in this study showed that 14-3-3 σ overexpression does not predict survival in gastric cancer patients [20,26]. p53 mutation and thereby the loss of the suppressive function is a key event in half of all human cancers [27]. Since 14-3-3 σ is integrated in the p53 network, dysregulation of 14-3-3 σ may be jointly responsible for tumor development in p53 wild-type cancers. Nevertheless, most examinations on gastric cancer have not shown predictive or prognostic impact of p53 [28,29]. In the present study, we can confirm these findings. No correlation was found between p53 expression and prognosis. Interestingly,

concomitant 14-3-3 σ and p53 overexpression was extracted as a significant predictor of disease-free survival in univariate analysis.

Since the bigger part of the patients in this study did not receive perioperative chemotherapy or radiation treatment, the 14-3-3 σ expression might not be influenced by therapy-induced DNA damage *in vivo*.

In conclusion, we demonstrated on a large study population with gastric cancer that 14-3-3 σ is immunopositive in 77.7% of the patients. Concomitant 14-3-3 σ and p53 overexpression in tumor cells of patients with gastric cancer identifies a population of patients with relatively unfavorable prognosis, however 14-3-3 σ does not predict survival in gastric cancer patients like it does in other tumor entities.

5. Conflict of interest

No potential conflict of interest relevant to this article was reported.

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