Prognostic Value Of Expression Of Survivin And Ki67 In Head And Neck Squamous Cell Carcinoma Treated By Chemoradiotherapy

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Abstract: Aim of the work: to evaluate prognostic value of expression of survivin and ki67 in head and neck squamous cell carcinoma treated by chemoradiotherapy. **Patients and Methods:** Between Jan. 2005 and Dec. 2009, all patients who were treated with primary chemoradiotherapy with curative intent for squamous cell carcinoma of the head and neck (SCCHN) at our department of clinical oncology. All initial pretreatment specimens were examined for expression of survivin and ki67 using immunohistochemical staining. **Results:** One hundred and fifty four patients were eligible for this study. Survivin expression was low in 107 (69.5%) and high in 47cases (30.5%). According to immunoreactive score (IRS), the staining was negative in 78 (50.7%), weak in 28 (18.2%), moderate in 22 (14.3%) and strong in 26 (16.9%) cases. Nuclear staining of Ki-67 was positive in 72 tumors (46.8%) and negative in 82 tumors (53.2%). The median follow-up was 19.5 months (range: 3 - 55 months). There were 114 local treatment failures (74%) and 86 deaths (55.8%), of which 72deaths (83.7%) were caused by disease. High survivin expression was correlated significantly with higher disease free survival and overall survival. Patients with high survivin expression in their tumors had a median disease free survival of 32 months compared with 16 months for patients with low expression tumors (P = 0.007). The median overall survival of patients with high survivin expression was 36 months versus 24 months for those with low survivin expression, (P = 0.04). The expression of Ki-67 significantly correlated with tumor grade but it was not significantly correlated with either disease free survival, (P = 0.5) or overall survival, (P = 0.7). **Conclusion:** the present study demonstrated that high survivin expression predicts better local control and superior overall survival in advanced HNSCC treated with radiochemotherapy. Survivin might be used as a stratification marker to define HNSCC patients, who would potentially benefit from radiochemotherapy. .Further investigation is necessary to clarify and understand the roles of survivin in patients with HNSCC.

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the fifth most common cancer in men.[1] Over 70% of head and neck cancer patients present with advanced stage III and IV disease. Concomitant chemoradiation (CCRT) leads to improved local control (LC) and overall survival (OS) in advanced head and neck cancer compared with conventional radiotherapy,[1-4] making this modality the most suitable curative treatment option in these patients currently. However, CCRT is not effective in all patients, and when unsuccessful, patients suffer its potential side effects and toxicities. Therefore, the identification of new prognostic factors is so crucial that the appropriate therapy can be tailored better for individual patients,[5].

Studies in the literature have examined the prognostic significance of various biomarkers, including cell cycle regulators, members of the proapoptotic family, angiogenesis markers, and proliferation markers in head and neck squamous cell cancer with mixed results ,[6-9].

Survivin is one of the most cancer-specific biomarkers identified to date. It belongs to the apoptosis inhibitor gene family. It inhibits apoptosis either by directly or indirectly interfering with caspase-3 and caspase-7 function and caspase-9 processing [10]. Furthermore, survivin enhances cell proliferation and promotes angiogenesis. Survivin is expressed during embryonic and fetal development but is undetectable in terminally differentiated normal adult tissue. However, it is re-expressed in transformed cell lines and several human cancer cells at a frequency of 34-100% [11]. Although high expression of survivin in cancer cells is a common phenomenon, which is supposed to be critically involved in tumor progression by inhibition of apoptosis, the reason for this abundant protein expression remained unclear and the relevance of survivin for the clinical course of HNSCC still has to be defined.

Ki-67 is a nuclear antigen expressed mainly in the S and M phases of the cell cycle, and it has been used for estimating the growth fraction in many studies investigating various tumor types [12].

The aim of his study is to evaluate prognostic value of expression of survivin and ki67 in head and neck squamous cell carcinoma treated by chemoradiotherapy.

2. Materials and Methods

This is a retrospective study that enrolled all patients who were treated with primary chemoradiotherapy with curative intent for squamous cell carcinoma of the head and neck (SCCHN) at our department of clinical oncology, Assiut University, between Jan. 2005 and Dec. 2009. Eligible patients have to have pathologically proven SCCHN without evidence of distant metastases, ≥18 years old and ECOG P.S. of <2. All patients should undergo a full endoscopic examination and CT scan of the head and neck before initial treatment, 1-2 months after completion of chemoradiotherapy & on recurrence. Patients treated with chemotherapy alone, brachytherapy or surgeries for the primary tumor were excluded.

Clinical data retrieved from patient charts included gender, age, tobacco use, tumor site, and clinical T and N classifications according to the TNM system by AJCC (AJCC 2002), [13], reevaluation after finishing treatment course, any recurrence or distant metastases during the period of follow up.

Chemotherapy: The chemotherapy regimen consisting of cisplatin 20 mg/m2 once weekly during radiotherapy. All patients received adequate hydration and serotonin antagonist against emesis during cisplatin administration. Full blood count examination was performed weekly. If the white blood cell count was lower than 3.0×10^9/l, the platelet count below 100×10^9 /l, or hemoglobin less than 10 g/dl, the subsequent chemotherapy dose was delayed for one week, without interruption of radiotherapy.

Radiation Therapy*:*

Pre-treatment CT of the head and neck was done to assess the extent of the primary tumor, as well as the neck nodes. The treatment volume included the primary tumor site and the neck nodes above the clavicle. The patients were treated with 6 MV photons. The fractional daily dose was 2 Gray (Gy) with a planned total dose of 60 Gy. This was delivered using a 6-MV linear accelerator or Cobalt-60 at a dose fraction of 2 Gy per day five times a week, without any intended gaps, up to a dose of 60–66 Gy (depending on TNM classification; T1 and T2 tumors and negative nodes were treated with a dose of 60 Gy, while patients with T3, T4 and/or positive nodes were treated with a dose of 66 Gy). Reproducibility of head and neck positioning was achieved by using a fixation device (Orfit mask; MEDTEC Inc Orange City, Iowa, USA). The treatment volume included the primary tumour site plus adequate margins and the neck nodes at risk. Usually, parallel-opposed fields were used to irradiate the primary tumour and the upper neck. A separate, anterior supraclavicular field was used to irradiate the lower neck and supraclavicular fossa. The spinal cord was protected after 40 Gy. The prescribed dose was 50 Gy to the clinically negative neck and 60–66 Gy to the gross target volume and positive neck nodes.

Histopathology

All histology slides from initial pretreatment biopsies were reviewed by two authors (Refaiy A M. and Elosaily GM.) to confirm the diagnoses. Tumors were graded as well differentiated (Grade 1), moderately differentiated (Grade 2), or poorly differentiated (Grade 3).

A representative block was selected for each patient. Unstained sections were cut for routine staining with hematoxylin and eosin and for immunohistochemistry.

Immunohistochemistry

Four micron Paraffin sections were cut and mounted on coated slides with polylysine and stained for survivin and Ki immunohistochemistry according to manufacturer's protocol. Tissue sections were deparaffinized, rehydrated in graded alcohol, and transferred to phosphate buffered saline (PBS; PH 7.6). The slides were rinsed twice with PBS, and then endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in methanol for 5 minutes.

After three times wash with PBS, antigen retrieval was done by using microwave at 700W for 15 min in citrate buffer. After cooling the slides were washed three times with PBS. The slides were incubated for 18 h (overnight) at 4 C with primary antibody for survivin (mouse monoclonal antibody Ab1) and Ki 67 (rabbit polyclonal antibody Ab4) ,Thermo scientific CA, USA, at dilution of 1:100 and 1: 200 respectively. The slides were then rinsed three times with PBS and incubated for 10 min. with the biotinylated goat antipolyvalent (Thermo Scientific, CA,USA) at room temperature. After further rinsing with PBS, the slides were incubated for 10 min. with Streptavidin peroxidase (Thermo Scientific, Ca, USA) at room temperature. The slides were again washed three times with PBS, and diaminobenzidine was applied for 5 min at room temperature. Finally, the slides were rinsed in D.W., counterstained with Mayer's hematoxylin, dehydrated and mounted. Positive control sections for survivin were from human placenta and for Ki 67

were from lymph node with reactive hyperplasia. Specificity of staining was checked on negative control slides by omitting the primary antibody.

Expression of survivin was determined in the nucleus and in the cytoplasm by assessing semi-quantitatively the percentage of marked tumor cells and the staining intensity. The percentage of positive cells was rated as follows: 1, 1-10% positive cells; 2, 11-50%; 3, 51-80%; and 4, > 80% positive cells. Staining intensity was scored as 1, weak; 2, moderate, and 3, intensive. Scores for percentage of positive cells and scores for expression intensities were multiplied to calculate an immunoreactivety score (IRS) [14]; 0-2 = no staining; 3-4 = weak staining; 6-8 = moderate staining; 9-12 = strong staining. For statistical analyses, ‘‘none’’ and ‘‘weak’’ staining were combined and counted as ‘‘low expression’’ whereas ‘‘moderate’’ and ‘‘strong’’ staining were grouped together and scored as ‘‘high expression.’’

Ki 67 evaluation was done by counting number of positive nuclei in 1000 tumor cells and the percentage was calculated as ki 67 labelling index.

The endpoints were disease free survival and overall survival. Local treatment failure was defined as either presence of neoplasm after radiotherapy or the appearance of local recurrence at the irradiated site confirmed by histology.

Statistical analysis

Data were recorded on specialized forms and all statistical tests were performed using SPSS version 16 for windows (SPSS Inc, Chicago, IL, USA) and Microsoft Excell (Realmond, W.A, USA) software. Descriptive analysis (e.g., mean , standard deviation, frequencies, percentage) were calculated and analysis was performed using the student’s t-test and Fisher ExactT- Test. A multivariate analysis using the Cox regression model14 was then performed on all variables with significant prognostic influence in univariate analysis (P _ 0.05). The survival curves were made using the Kaplan-Meier method and comparison was with the log rank test.

3. Results:

One hundred and fifty four patients were eligible for this study. They were composed of 93 men (60.4%) and 31 women (39.6%) with a mean age at the time of diagnosis of 54 years (range, 21–80 years). The site of the primary tumor was the the larynx in 64 patients (41.6%), oropharynx and oral cavity in 27 patients (17.5%), the hypopharynx in 52 patients (33.8%), and the nasopharynx 11 (7.1%) (Table 1). The majority of patients had advanced T-stage tumors (71% T3–T4) and enlarged L.N. (63.3% N1-N3).

Immunostaining

The immunostaining for survivin was prevalently cytoplasmic with sporadic prominent nuclear staining. So, we recorded the specimens as positive without considering the intracellular localization of the signal (cytoplasmic or nuclear). No survivin expression was detected in adjacent normal tissues. Survivin expression in the cytoplasm of tumor cells and / or nucleus by immunohistochemistry was low in 107 (69.5%) and high in 47cases (30.5%); (Table.1). According to immunoreactive score (IRS), the staining was negative in 78 (50.7%), weak in 28 (18.2%), moderate in 22 (14.3%) and strong in 26 (16.9%) cases (Fig 1, 2).

Based on a 20% threshold value, nuclear staining of Ki-67 was positive in 72 tumors (46.8%) i.e. having high proliferation rate (Fig 3, 4) and negative in 82 tumors (53.2%) i.e. with low proliferation rate.

Clinical outcome and survivin expression

The follow-up ranged from 3 months to 55 months (median, 20.5 months).There were 114 local treatment failures (74%) and 86 deaths (55.8%), of which 72 deaths (83.7%) were caused by disease.

High survivin expression was correlated significantly with higher disease free survival and overall survival (Fig.5). Patients with high survivin expression in their tumors had a median disease free survival of 32± 3.62 (95% CI, 18.927-43.073) compared with 16 ±1.1 (95% CI, 13.912-18.088) for patients with low expression tumors (P = 0.007). The median overall survival of patients with high survivin expression was 36± 1.81months (95% CI, 32.45-39.55) months, versus 24 ±3.47 months (95% CI, 18.18-31.82) for those with low survivin expression. This difference of survival rates was statistically significant (P = 0.04). The expression of Ki-67 was not significantly correlated with either disease free survival 19 ±5.3 months (95% CI, 8.618-29.382) for patients with negative ki67 vs. 17± 1.7 months (95% CI, 14.712 - 19.288) for positive ki67 (P = 0.5) or overall survival 26 ±4.4 months (95% CI, 13.285 - 40.715) for patients with negative ki67 vs. 20 ±5.3 months (95% CI, 11.615 - 42.366) for positive ki67, (P = 0.729), (Fig.6).

Analysis of Subgroups

Both univariate and multivariate analyses showed no significant correlation between survivin expression and age, sex, histological grade, tumour stage, or the presence of lymph node metastasis (Table 2). Ki67 correlated significantly with age and tumor grade but did not correlate with other factors (Table 3).

Table 1: Patients characteristics

		Frequency (N=154)	Percent
Sex	Male	93	60.4
	Female	61	39.6
Age (years)	Mean ± SD	53.99 ± 14.07	
	Median	54	
	Min - Max	21-80	
Site	Larynx	64	41.6
	Nasopharynx	11	7.1
	oral cavity	23	14.9
	Oropharynx	4	2.6
	Hypopharynx	52	33.8
T	1	6	3.9
	2	38	24.7
	3	69	44.8
	4	41	26.2
L.N	0	56	36.4
	1	47	30.5
	2	45	29.2
	3	6	3.9
Grade	1	51	33.1
	2	73	47.4
	3	30	19.5
Response	CR	83	53.9
	PR	38	24.7
	NR	28	18.2
	PD	5	3.2
Survivin	Low	107	69.5
	High	47	30.5
Ki67	Negative	82	53.2
	Positive	72	46.8

Table 2: correlation of survivin with patients characteristics

		Survivin1				P-value
		Low		High		
		N	%	N	%	
sex	Male	65	60.7	28	59.6	0.5
	Female	42	39.3	19	40.4	
Age (years)		53.57 ± 14.3		56.35 ± 12.68		0.3
Site	Larynx	36	33.6	28	59.6	0.06
	Nasopharynx	7	6.5	4	8.5	
	Oro-pharynx/oral cavity	21	19.6	6	12.8	

		Survivin1				P-value
		Low		High		
		N	%	N	%	
	Male	65	60.7	28	59.6	
	Hypo-pharynx	43	40.2	9	19.1	
T	1	2	1.9	0	0.0	0.34
	2	14	13.1	8	17.0	
	3	51	47.7	28	59.6	
	4	40	37.4	11	23.4	
L.N	0	50	46.7	31	66.0	0.06
	1	20	18.7	2	4.3	
	2	35	32.7	10	21.3	
	3	2	1.9	4	8.5	
Grade	1	25	23.4	6	12.8	0.06
	2	68	63.6	25	53.2	
	3	14	13.1	16	34.0	

Table 3: correlation of ki67 with patients characteristics

		Ki67				P-value
		Negative		positive		
		N	%	N	%	
sex	Male	54	65.9	39	54.2	0.13
	Female	28	34.1	33	45.8	
Age (years)		59.38 ± 13.789		49.49 ± 12.902		0.002
	Larynx	36	43.9	28	38.9	0.3
	Nasopharynx	3	3.7	8	11.1	
	Oro-pharynx/oral cavity	13	15.9	14	19.4	
	Hypo-pharynx	30	36.6	22	30.6	
T	1	2	2.4	0	0.0	0.08
	2	14	17.1	8	11.1	
	3	33	40.2	46	63.9	
	4	33	40.2	18	25.0	
L.N	0	32	39.0	49	68.1	0.06
	1	14	17.1	8	11.1	
	2	30	36.6	15	20.8	
	3	6	7.3	0	0.0	
Grade	1	28	34.1	3	4.2	0.000
	2	50	61.0	43	59.7	
	3	4	4.9	26	36.1	

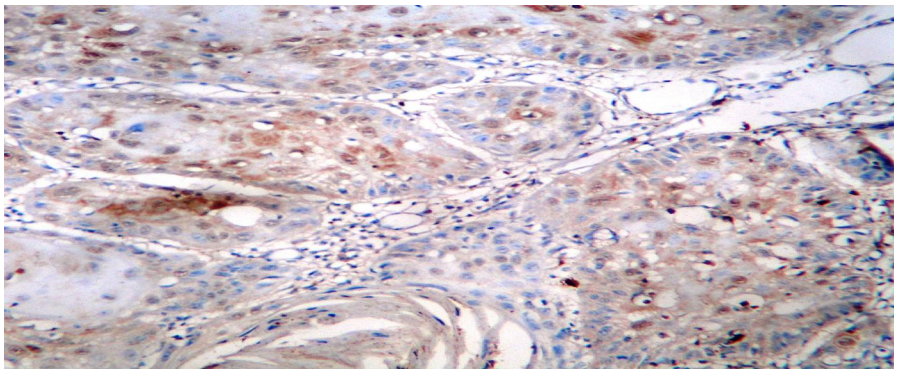

Fig 1: Moderate survivin expression in moderately differentiated SCC (x200)

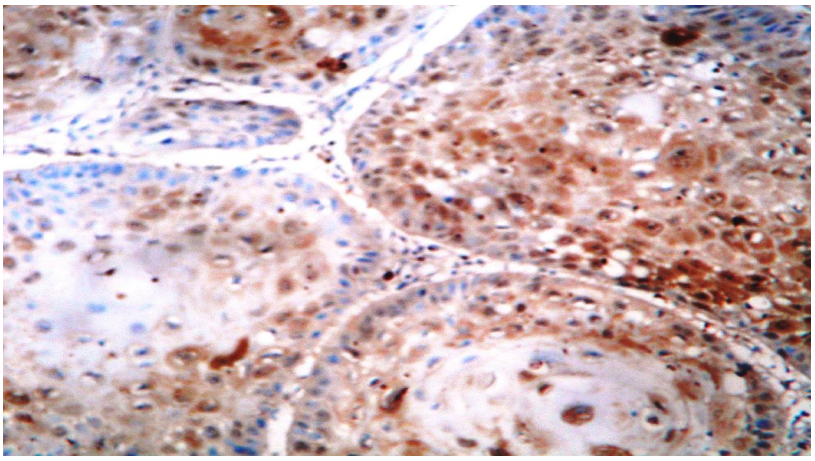

Fig 2: Strong survivin expression in well differentiated SCC (x200)

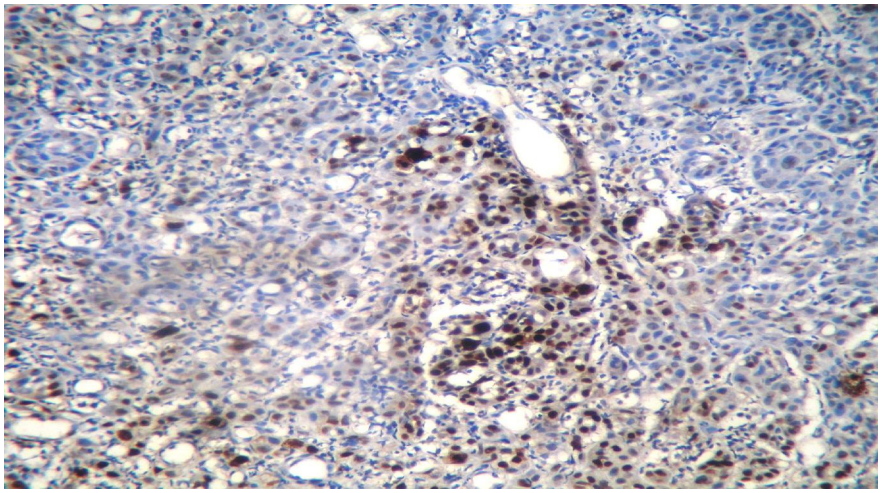

Fig 3: Ki67 immunohistochemical expression in moderately differentiated squamous cell carcinoma showing brown nuclear staining (x100)

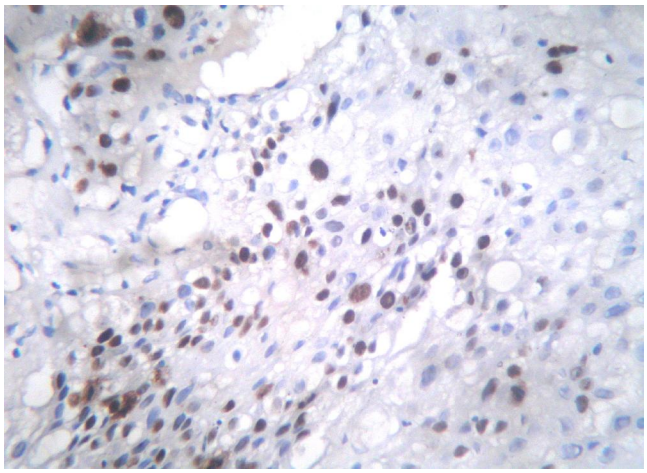

Fig 4: Ki67 expression in moderately differentiated SCC higher power view (x200)

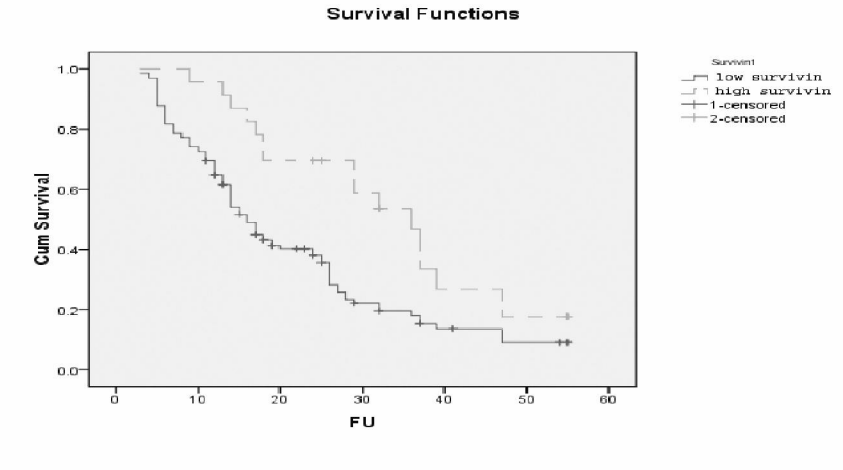

Fig.5A. correlation of survivin with disease free survival.

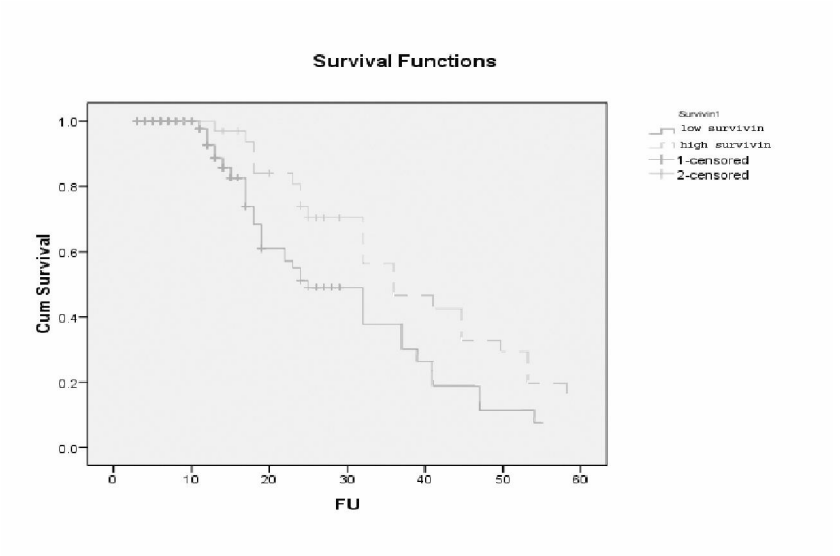

Fig.5B: correlation of survivin with overall survival

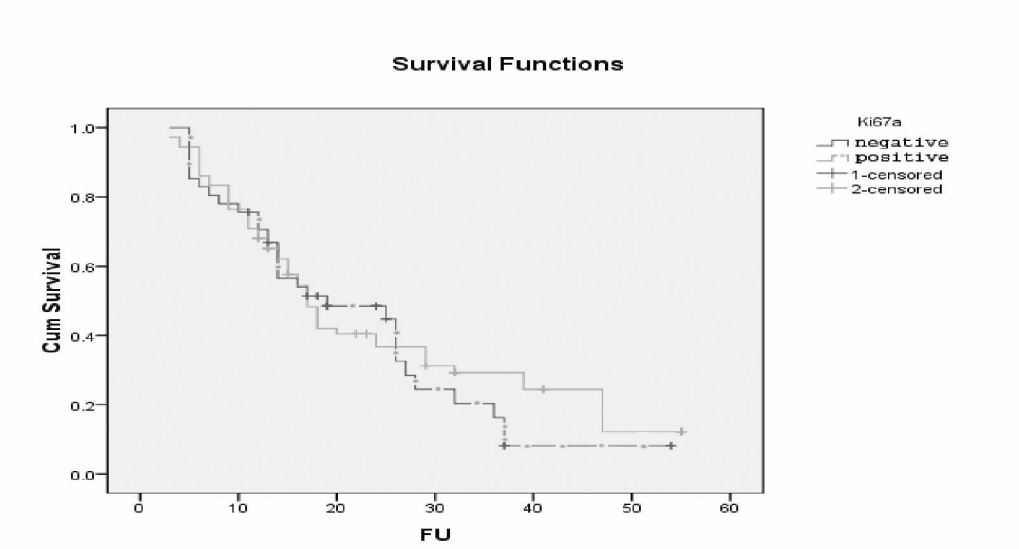

Fig.6 A: correlation of ki67 with disease free survival

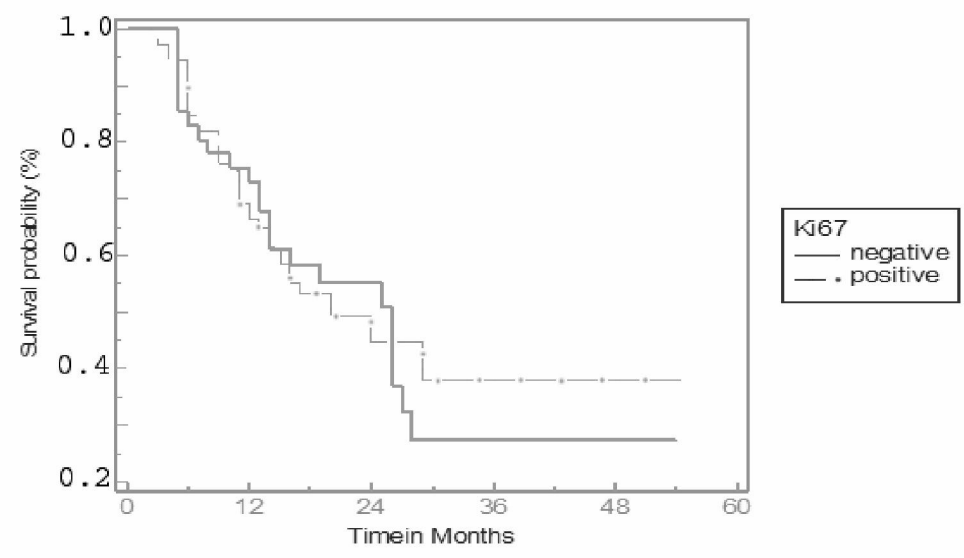

Fig.6 B: correlation of ki67 with overall survival.

4. Discussion

The aim of the present study was to delineate prognostic value of expression of survivin and ki67 in head and neck squamous cell carcinoma treated by chemoradiotherapy.

Khan et al. [15] reported that Survivin is expressed in a varying proportion of cells, and in majority of patients it was localized in cytoplasm in oral squamous cell carcinoma. Others [16,17] demonstrated nuclear subcellular survivin localization. However Engels et al. [18] suggested that the difference between cytoplasmic and nuclear survivin is an indicator for survivin activity in tumor cells.

The clinical implications for subcellular localization of survivin expression remains controversial. Among the 19 publications relevant to survivin localization in nuclei or cytoplasm in various cancer tissues reviewed by Li et al. [19], 9 publications showed that survivin expression in cancer cell nuclei was an unfavorable prognostic marker, whereas 5 publications proposed the opposing notion that nuclear survivin expression represented a favorable prognostic marker. Similarly, overall survivin expression, its discrete intracellular localization, and its implication as a prognostic marker were also analyzed in several HNSCC studies, albeit with opposing results [16,20,15].

In the present study, high survivin expression in HNSCC was associated with favorable patients' outcome. Consistent with our data are those reported by Freier et al [19] who found that high survivin expression was an independent predictor of increased 5- and 10-year overall survival of patients with oral squamous cell carcinoma (OSCC). Within a subgroup of patients, who received radiation therapy, they also found that high survivin expression was the only predictor of favorable 3-, 5- and 10-year overall survival in a multivariate cox regression analysis including UICC stage and age as covariables[21]. Also, recent studies in several different tumor entities like breast carcinoma, [22] colon carcinoma, [23] osteosarcoma[24] and transitional bladder cancer[25] showed similar results of high survivin expression predicting increased overall survival of the patients.

However, our data are in contrast to recent studies in oral squamous cell carcinoma, [26,27], laryngeal basaloid squamous cell carcinoma[16], and adenoid cystic carcinoma of the head and neck [28] in which high survivin expression was associated with adverse patients' outcome. Similar results of correlating survivin with an unfavorable clinical outcome were reported by previous studies in a variety of cancers, including colorectal cancer [29], breast cancer [30], lung cancer [31], and esophageal cancer [32].

However, in esophageal carcinoma, a tumor entity, which derives from the epithelium of the upper aerodigestive tract just like HNSCC, an association of high mRNA survivin expression and favorable outcome after neoadjuvant radiochemotherapy was recently found [33]. This was corroborated by a previous study, which showed that survivin protein expression correlates with the proliferative index but not the apoptotic index in esophageal carcinoma, which might enhance the responsiveness to induction radiochemotherapy eventually resulting in survivin-dependent superior overall survival [34]. According to our data, one might speculate that increased proliferation activity of the tumor cells induced by high survivin expression makes the tumor cells more liable for radiation-induced cell damage. This could explain the observed increased overall survival of patients with high survivin expression treated by radiochemotherapy. If the assumption of high survivin expression being a predictor of radiation response could be verified in further functional analysis, survivin might be a promising stratification, which should be evaluated in prospective, controlled clinical studies to marker to preselect patients, who would benefit from radiochemotherapy in the clinical management of HNSCC.

In this study, we found a statistically significant correlation between Ki67 expression and tumor grade but not correlated with other factors. Our results are in agreement with several reports of evidence that Ki67 expression increases with the severity of dysplastic changes in the head and neck squamous cell carcinoma, [35, 36].

In conclusion, the present study demonstrated that high survivin expression predicts better local control and superior overall survival in HNSCC patients treated with radiochemotherapy for an advanced tumor. Survivin might be used as a stratification marker to define HNSCC patients, who would potentially benefit from radiochemotherapy. .Further investigation is necessary to clarify and understand the roles of survivin in patients with HNSCC.

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