S-PHASE FRACTION AS A PROGNOSTIC INDICATOR IN SIMILARLY STAGED TONGUE SQUAMOUS CELL CARCINOMA IN SOME EGYPTIAN PATIENTS

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ABSTRACT

To analyze whether the S-phase fraction (SPF) may contribute to prognosis of similarly staged tongue squamous cell carcinoma, DNA flow cytometric data from 30 cases of tongue squamous cell carcinoma were analyzed. The SPF values ranged between 2.30% and 34.32% (mean 10.92%). SPF was calculated and correlated with the disease free survival rate and the overall survival of the patients. By multivariate survival analysis, SPF was significantly associated with the disease free survival rate and the overall survival of patients ($P < 0.05$). The results indicate that SPF has a relevant prognostic power for tongue squamous cell carcinoma.

INTRODUCTION

Oral cancer still has a generally negative prognosis, with five-year survival figures of less than 50%, producing high rate of mortality and morbidity [1]. A report of the Middle-East Cancer Consortium (MECC) of the National Cancer Institute in Bethesda, USA, depicted that Egypt had one of the highest overall incidence rates of cancer of oral cavity and pharynx ($5.5/10^5$) among the MECC countries [2]. In Egypt, The National Cancer Institute (NCI) reported that in 2003-2004, oral cavity squamous cell carcinoma (SCC) constituted 17.06 % of the malignancies of all digestive organs and the tongue was the most common site (36.10%) [3]. The clinical course of patients with oral squamous cell carcinoma, specifically tongue, is variable. The clinician is often unable to predict which patients will develop local recurrence, much less who will die from the cancer.

Flow cytometry is playing an increasing role in the diagnosis and histologic classification of tumors. About 70% of human tumors contain cells with abnormal DNA content. The detection of a small number of malignant cells in an effusion or biopsy that contains mainly nonmalignant (inflammatory) cells is often difficult with conventional histology and light microscopy.[4] It now becomes apparent that flow cytometry and associated techniques can be used more successfully to provide information on cell constituents that allow one to evaluate the

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tumor cells embolic status and growth. These features can be of prognostic value and can bear clues with respect to tumor sensitivity toward particular treatment regiments [5].

Numerous studies have established that alterations in DNA ploidy and increase in the S phase fraction are associated with survival, patient's response to therapy as well as locoregional or systemic recurrences following surgery in many tumors. [6-9] Several investigators working on oral squamous cell carcinoma have found similar results [10,11]. Some studies strongly suggested that the cell proliferation has equal or even more importance than ploidy changes in predicting patient outcome [12,13].

To our knowledge, few studies looked solely at the S-phase fraction in tongue squamous cell carcinoma. The purpose of this study was to examine the relationship between S-phase fraction for similarly staged tongue SCC and disease free survival rate, and the overall survival of the patients, to verify if cell proliferation, as measured by flow cytometry, can really provide useful prognostic information in these tumors or not. This study focused on the tongue SCC because the tongue is the most frequently involved site in patients with oral cancer [14,15], the tongue is the site that demonstrates the lowest survival period [16] and also because such a focus would decrease the heterogeneity of the data.

MATERIALS AND METHODS

This retrospective study was carried out on thirty formalin-fixed and paraffin-embedded surgical specimens, previously histologically diagnosed as tongue squamous cell carcinoma. Each specimen was coded and patient's name was not shown for ethical reasons. They were selected from archives of Surgical Pathology unit, Pathology Department, National Cancer Institute (NCI), Cairo University, from January 2003 to December 2008. Charts were reviewed for Personal history, Clinical and histopathological examination. Age of the patients ranged from 29 to 75 yrs with a mean age of 48.7 yrs; 16 patients were males while 14 were females. All of the studied cases were of stage IV.

Survival was calculated from the first day of diagnosis until the last date of follow up or death. The follow up period ranged from 7 - 26 months with a median of 12 months. At the end of follow up, out of the 30 tongue SCC cases, 17 cases revealed no recurrence, 6 cases showed local recurrence, 6 cases showed distant metastasis and 1 case exhibited both local recurrence and distant metastasis. All patients were treated for their primary tumors by surgical excision with block neck dissection followed by palliative radiotherapy and chemotherapy. None of the patients had received chemo- or radiotherapy prior to the operation.

Ethical approval of this study was taken by our institution.

Histological Grading

After specimen selection, 5-μm-thick section was cut from each paraffin block, stained with haematoxylin and eosin (H&E) and re- examined under light microscopy for diagnostic confirmation. Of the 30 patients, four had grade 1, twenty-one presented with grade 2 and five with grade 3 tumor.

Flow Cytometric Analysis

For flow cytometric analysis, single cell suspensions were prepared by the method described by Hedley [17] . The sections were deparafinized in xylene for 10 minutes, rehydrated in ethanol at decreasing concentrations (100%, 95%, 70%, and 50%), washed with phosphate buffered saline, and then incubated in 2 ml of 0.5% pepsin solution (Sigma p6887) adjusted to pH 1.5 for 90 minutes at 37C^{o} in water bath, with intermittent tapping every 5 minute for mixing. Next, cells were filtered twice through a 50 μm pore size nylon mesh to remove debris and cell clumps that may give abnormal extra peaks or background noise during

analysis. After centrifugation (10 minutes at 2500 rpm), nuclear pellets were treated with 2 ml of a ribonuclease solution (Sigma). The cell suspension was incubated at 37c° for 1 hour. At the completion of incubation, the cell suspension was centrifuged and resuspended in a propodium iodide solution. The stained specimen was kept in the dark for 6-8 hours at 4 c° before flow cytometry.

Analysis was performed using the Mod fit software in the FAC scan flow cytometer, Becton & Dickinson (B-D) sunny vale, CA. The SPF was estimated as the percentage of cells occupying the region between the mean channel number for G0/G1 and that of G2/M, measured by the computer calculation program for DNA analysis. The cutoff for the SPF was set as the mean [18], and considered as either being low or high respectively.

Statistical Analysis

Chi-square test was used to examine the relation of clinico-pathological parameters and SPF and to determine whether there was a significant difference. In addition, the Kaplan-Meier analysis was used to assess the Survival analysis of recurrence free survival and the overall survival. p Values < 0.05 were regarded as significant. SPSS software (version 17.00; SPSS, Chicago, Illinois, USA) was used for statistical analysis.

RESULTS

DNA flow cytometry was successfully carried out in 30 stage IV tongue squamous cell carcinomas. The mean S-phase fraction (SPF) in the tumors studied was 10.92%, with maximum and minimum values of 2.30% and 34.32% respectively. Among the studied patients, 53.3% (16/30) had high SPF (number of cells in S-phase fraction was more than 10.92%), and 46.7% (14/30) had low SPF (number of cells in S-phase fraction was less than 10.92%). No significant differences were found between any of the clinico-pathological parameters and SPF.

Univariate analysis of recurrence free survival suggested a statistically significant correlation of SPF with DFS ($p = 0.001$). Cases with recurrent tongue SCCs, distant metastasis or both, showed higher SPF (>10.92%) in contrast to non-recurrent and non-metastasizing cases. At the end of the follow up period, DFS was 100% in cases with low SPF versus 18.75% in cases with high SPF (Table 1 & Fig. 1).

A statistically significant correlation of SPF was also found with the patient's overall survival (p=0.001). No death was reported in patients with low SPF, while half of the patients with high SPF died within about 2 years from the time of diagnosis. That is the OS was 100% in cases with low SPF versus 50% in cases with high SPF (Table 2 & Fig. 2)

TABLE (1): Survival Analysis for DFS_time (months)

	Total	Number Events	Number Censored	Percent Censored
S_phase up to 10	14	0	14	100.00
S_phase >10	16	13	3	18.75
Overall	30	13	17	56.67
		Statistic	df	Significance
	Log Rank	18.93	1	<0.001

TABLE (2): Survival Analysis for OS_time (months)

	Total	Number Events	Number Censored	Percent Censored
S_phase up to 10	14	0	14	100.00
S_phase>10	16	8	8	50.00
Overall	30	8	22	73.33
	Log Rank	Statistic 10.90	df 1	Significance .0010

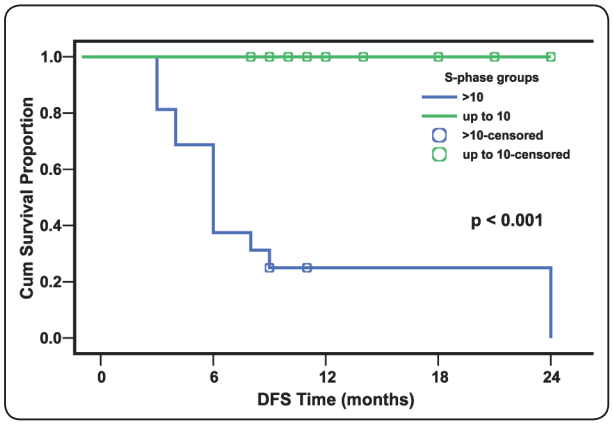

Fig. (1): Kaplan-Meier recurrence free survival curve of 30 patients according to SPF of tongue SCCs.

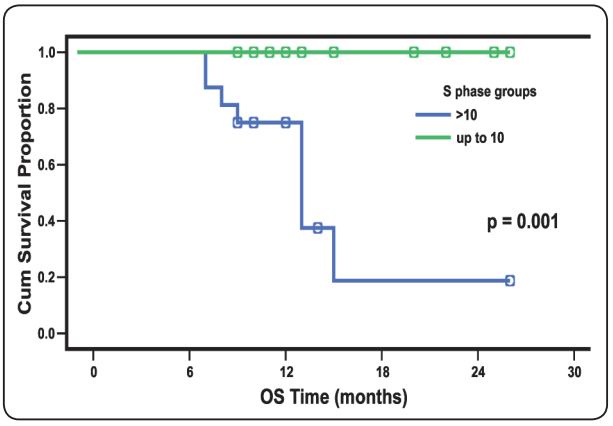

Fig. (2): Kaplan-Meier overall survival curve of 30 patients, according to SPF of tongue SCCs.

DISCUSSION

The study of prognostic factors in oncologic processes is of great interest because it allows us to know the natural history of the disease, makes it possible to form homogenous groups of patients in clinical trials, and facilitate the design of selective clinical trials. Furthermore, it explores possible interactions between the prognostic variables and the treatment, allows the prediction of the prognosis in patients, and can help to explain the variations detected in the survival between groups. In this way, it influences the therapeutic strategy, improves the stratification of patients in random studies and makes the comparison of results possible [19].

Intraoral squamous cell carcinoma is a biologically distinct group of tumors that are biologically aggressive [20] and require intensive chemotherapy in short duration to provide a better clinical response [21]. Recurrence of intraoral squamous cell carcinoma has been reported much earlier *i.e.*, within two years after operation in patients with advanced stage tumors and with lymph node metastasis[22]. While the TNM classification and pathologic features (e.g., histologic grade, nature of the invading margin) provide some useful prognostic information [23], they are semi-quantitative and susceptible to inter-observer variation. Moreover, they often fail to assist the clinician in deciding on the most appropriate treatment for an individual patient [24]. In contrast to the subjectivity of currently used prognostic criteria, analysis of solid tumors by flow cytometry (FCM) permits rapid, objective, quantitative evaluation of cellular DNA content and proliferative activity [25].

Assessment of proliferation has become popular in histopathology as a means of predicting the behavior of tumors-that is, their likelihood of local recurrence, their metastatic potential, and the growth of metastases, and thereby the disease-free survival and survival to death [26]. To assess tumor proliferation status, we evaluated the S phase fraction by flow cytometry.

The technical issues of validity and reproducibility related to prognostically useful methods of measuring tumor cell proliferation are a matter of controversy, but according to recent studies flow cytometric SPF is the most useful tumor cell proliferation marker to assess disease prognosis[24,25]. Studies suggested that the determination of SPF from one randomly chosen biopsy specimen is representative of the proliferative activity of the carcinoma, because intratumoral SPF variation is low [27].

Few studies up to now have examined only one anatomic site within the oral cavity, with even fewer looking specifically at the tongue. The purpose of this study was to analyze similarly staged squamous cell carcinoma of the tongue by flow cytometry with respect to trends between the % S-phase and the degree of local recurrence, distant metastasis or patient's survival.

Results of this research showed that, 53.3% of tongue SCCs had high SPF (>10.92%) while, low SPF (<10.92%) was present in 46.7% of cases. The mean SPF in this study is consistent with the reported results for flow cytometrically determined SPF of tongue SCC in a study by Monasebian and Ruskin [28].

The findings of this study showed that higher SPF in the tongue SCC was significantly related to early recurrence of the tumor, distant metastasis and death of the patients, while low SPF seemed to be associated with recurrence free survival and increase overall survival, despite of the similar clinical stage of the tumor and similar therapy administered to the patients. The results indicated that high growth potential of tumor cells allows them to develop into distant metastasis or local recurrence and increased the patient`s mortality. Those patients with higher SPF tumors may benefit from adjuvant chemotherapy[29] as rapidly proliferating tumors are more chemosensitive than others[30].

Tongue SCC tumors with high SPF were found to be more aggressive, and have been connected with poor patient outcomes and decreased 5-year survival rates, by many of the authors[28&31]. In some studies high SPF has been related to poor prognosis in oral cancer[32] , pharynx [33] , larynx [34] head and neck[35,36], gastric [37], and breast cancer [38,39,40], whereas others could not find such correlations [18, 19, 41].

Pinto et al.[38], stated that flow cytometric SPF is the most useful cell proliferation method in predicting the short term prognosis of patients with breast cancer, with the conventional median SPF category being the best indicator of disease outcome compared with other SPF variables and the Ki-67 index. Gasparini *et al*[39] compared SPF with other immunohistochemical indicators of cell proliferation, such as Ki-67 and proliferating cell nuclear antigen (PCNA), in a consecutive series of 195 patients with breast cancer, and also concluded that SPF is the best cell kinetics marker to assess disease prognosis. Similarly, Dettmar *et al*[40] determined SPF and MIB-1 indices in their retrospective study of 90 node negative breast carcinomas, and showed by multivariate analysis that SPF has the highest prognostic value. The number of sampled specimens, site and heterogeneity of tumors may account for the diversity of results.

As a conclusion, an estimation of the SPF may be useful for identification of patients with high risk for tumor recurrence or distant metastasis and pre-treatment can be performed by a single biopsy. SPF of tongue squamous cell carcinoma might be considered as an important discriminatory prognostic factor in patients with similarly staged tongue squamous cell carcinoma. These patients may benefit from more aggressive treatment, and chemotherapy.

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