ORIGINAL ARTICLE

Prognostic significance of ploidy and S-phase fraction in primary intraoral squamous cell carcinoma and their corresponding metastatic lymph nodes

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Abstract *Background:* Despite improvements in diagnosis and therapy of oral and oro-pharyngeal carcinomas during the past 30 years the 5-year disease-free survival is still poor. Patient's prognosis is affected by cervical lymph node metastasis rather than primary tumors. The DNA ploidy and S-phase fraction (SPF) are associated with tumor aggressiveness and patient outcome in many solid tumors.

Purpose: Analysis of DNA ploidy and SPF in primary oral squamous cell carcinoma (OSCC) and corresponding node metastasis as prognostic markers in relation to conventional prognostic factors and disease-free survival (DFS).

Methods: Ploidy status and SPF (mean value) of 37 formalin-fixed paraffin embedded (FFPE) primary OSCC tumors and their corresponding lymph node metastasis were assessed by flow cytometry (FCM) and correlated with clinicopathologic prognostic parameters and DFS.

Results: Most of OSCC tumors (86.5%) were Grade II. Among primary OSCC the incidence of aneuploidy was 19%, 51.4% showed high SPF (>10.62%) and 48.6% had low SPF (<10.62%).Border line significance ($P = 0.10$) was detected between ploidy status and SPF in primary tumors. In lymph node metastases all tumors were diploid, 78.4% of metastatic tumors

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revealed low SPF and only 21.6% showed high SPF. There was a statistically significant correlation ($p = 0.02$) between site of tumors and DFS and a highly statistically significant correlation ($p = 0.01$) between SPF of primary tumors and DFS.

Conclusions: High SPF of primary OSCC tumors assessed by FCM was significantly associated with decreased disease free survival rates. DNA ploidy showed no relationship to bad prognostic indicators in either primary OSCC or their metastatic tumors.

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Introduction

The incidence of oral squamous cell carcinoma remains high [1]. Oral and oro-pharyngeal carcinomas are the sixth most common cancer in the world [2]. Despite of all improvements of diagnosis and therapy the overall survival of patients did not improve significantly with the 5-years survival rates between 45% and 50% [1].

In Egypt according to National Cancer Institute registry in 2003–2004 oral cavity squamous cell carcinoma constituted 2.45% of total malignancies and 17.06% of all digestive organs cancer. The tongue was the most common site (36.10%) [3].

The patient's prognosis varies with tumor size, stage of disease (TNM), tumor primary site, and the status of surgical margins. Also the cumulative effects of tobacco, betel nut, and alcohol decrease the survival rate [4].

Flow cytometry is a laser based technology used to quantify DNA content of cells and measure the percentage of cells in each of the different phases of cell cycle. Several studies have reported that alterations in the DNA content (ploidy) and/or increase in SPF (as a measure of proliferative activity) were associated with poor patient's outcome in a variety of neoplasms [5,6].

In most of the researches flow cytometric data of intraoral squamous cell carcinoma (OSCC) have been lumped with tumors of the head and neck [7–10] due to its lower incidence rate. However, these results do not necessarily reflect the clinical importance of DNA ploidy in site specific tumors [11]. Moreover, the studies that compared the ploidy status and SPF of primary OSCC tumors and their lymph node metastasis are very few and no conclusive reports are available on the DNA pattern of metastatic lymph nodes in this group of patients.

Objective: To analyze the DNA content and SPF of primary OSCC tumors and corresponding metastatic nodal lesions using flow cytometry and to assess its relationship to prognosis in comparison to clinicopathological findings and DFS.

Patients and methods

The paraffin blocks of 37 cases of OSCC surgical specimens were retrieved from the archives of surgical pathology unit, Pathology Department, National Cancer Institute, Cairo University between years 2000 and 2007. Only patients with hospital files showing full clinical data and complete follow up findings as well as sufficient tumor tissue in paraffin blocks from primary tumors and their nodal secondaries were enrolled in this study. None of the patients received pre-operative radiation or chemotherapy. Clinical staging of the tumors was done according to criteria set by WHO [12].

Histopathological examination

Hematoxylin and eosin (H&E) stained sections from the primary tumors and metastatic lymph nodes were examined to confirm histopathological diagnosis, assess tumor grade and to assure that tumor tissue constitutes > 70% of the section with minimal hemorrhagic and necrotic foci. Averages of 10–15 cervical lymph nodes were examined in each case.

DNA flow cytometry

For DNA analysis single cell suspension from FFPE tumors was prepared according to a modified method described by Hedley [13]. Two to three sections (50 μm thick) were cut from each block. Sections were deparaffinized with xylene and rehydrated in graded ethanol followed by two washes in phosphate buffer saline (PBS 0.1 M, pH 7.4). Each sample was digested by incubation with pepsin 0.5%, pH 1.5 (Sigma, P6887) at 37 °C in water bath for 90 min with intermittent tapping every 5 min for mixing. The disaggregated tissues were filtered through 50 μm pore size nylon mesh in order to remove debris and cell clumps that may give abnormal extra peaks during analysis. After two washes in PBS, trypsin inhibitor, and ribonuclease A (CycleTest PLUS DNA Reagent kit, Cat. No. 340242, Becton–Dickinson, USA) were added to the sample to stop the action of pepsin and digest RNA. After washing using PBS the cell count was adjusted to $2–3 \times 10^6$ cells/ml. Nuclear staining for DNA content was done using 50 μg/ml propidium iodide (PI) (CycleTest PLUS DNA Reagent kit, Cat. No. 340242, Becton–Dickinson, USA) at room temperature for 10 min in the dark. The stained samples were acquired by flow cytometer (Facscan, Becton–Dickinson (B–D) Sunnyvale, CA). Calibration of the flow cytometer was done using PI labeled chicken RBC's. Normal human peripheral blood lymphocytes were used to identify the normal diploid peak that served as reference peak for subsequent analysis. From each sample at least 10,000 cells were acquired by flow cytometer at a rate of 100–200 cells/s and data were presented as DNA distribution histograms. Data were analyzed using Modfit software (Becton–Dickinson (B–D) Sunnyvale, CA). Interpretation of DNA histograms was done according to Das et al. [14] and Baretton et al. [15]. Histograms showing a single G0/G1 peak were defined as diploid. Tumors that exhibit two distinct histogram G0/G1 peaks (second peak is > 10% away from diploid one) were classified as aneuploid. The DNA index (DI) was obtained by calculating the ratio of mean channel number of abnormal aneuploid G0/G1 peak to the mean channel number of the normal (diploid) G0/G1 peak. Table 1 shows the five arbitrary DI classes that were used for further classification of aneuploid tumors [16]. The mean co-efficient of variation (CV) of the measurement recorded

by flow cytometer ranged from 5% to 8%. Cut-off of the SPF was determined by calculating mean SPF values.

Statistical analysis

The study data were summarized as frequency and percentage (qualitative data) or as mean ± standard deviation (SD) (numerical data). Data were analyzed using SPSS win statistical package version 12. Chi-square test (Fisher's test) was used to examine relationship between qualitative variables while for quantitative data Mann–Whitney test was used for variables not normally distributed. Survival analysis was done using Kaplan–Meier method. Comparison between two survival curves was performed using log rank test. P value $\leqslant 0.05$ was considered as statistically significant.

Results

Clinical and histopathological findings

The patient population comprised 21 males (56.8%) and 16 females (43.2%). Their mean age at first diagnosis was 52.5 years (min. 29 years and max. 75). Primary sites were mainly identified in the tongue (51.4%), buccal mucosa (21.6%), retromolar gingiva (18.9%), and floor of mouth (8.1%). According to TNM classification, 37.8% of OSCC patients had Stage III tumors while 62.2% were Stage IV. Most of patients (86.5%) had moderately differentiated tumors (Grade II) while Grade III and Grade I comprised 8.1% and 5.4%, respectively.

Disease free survival (DFS) status

The follow up period ranged from 3 to 24 months with a median of 6 ± 0.2 months. At the end of follow up, 21.6% of SCC cases revealed no recurrence, 54.1% of cases showed local recurrence, 18.9% showed distant metastasis. Two cases (5.4%) exhibited both local recurrence and distant metastasis. Five patients died from disease after local recurrence or distant metastasis (Fig. 1).

Flow cytometric findings

Primary OSCC tumors
Among 37 OSCC tumors analyzed, 30 tumors (81%) were diploid. Seven primary OSCC tumors were aneuploid (19%) and were further classified into six hyperdiploid tumors (DI = 1.32–1.87, mean of 1.71) and one tetraploid tumor (DI = 1.93) (Fig. 2). Nineteen out of 37 (51.4%) tumors had high SPF (number of cells in SPF > 10.63%) while 18 out of 37 cases (48.6%) had low SPF (number of cells < 10.63%).

Table 1 DNA index (DI) intervals used for analysis of OSCC.

Ploidy	DNA index (DI)
Hypodiploid	DI < 0.95
Diploid	0.95 ≤ DI < 1.05
Hyperdiploid	1.05 ≤ DI < 1.90
Tetraploid[a]	1.90 ≤ DI < 2.10
Hypertetraploid	DI ≥ 2.10

[a] Tetraploidy: proportion of the G2M cells of diploid population exceeds 15%.

The SPF ranged from 1.24% to 43.66% with a mean of 10.63% ± 3.96. Border line significant relationship ($P = 0.10$) was detected between ploidy status and SPF. Among the 30 diploid tumors, 14 tumors (46.7%) had high SPF (mean 22.3%) while 16 (53.3%) of cases revealed low SPF (mean 9.12%). On the other hand most of aneuploid tumors (71.4%) showed high SPF (mean 22.65%) and only two tumors revealed low SPF (mean 5.58%) (Table 2).

Metastatic lymph nodes
In lymph node metastases all tumors were diploid. Regarding SPF 78.4% (29/37) of metastatic tumors revealed low SPF and 21.6% showed high SPF. The SPF ranged from 1.24% to 23.58% (Fig. 3).

Correlation between flow cytometric parameters and clinicopathological findings

None of the clinical variables (patient age, gender, tumor site, histopathological grade, and stage) compared to ploidy status or SPF was of statistical significance in either primary tumors or metastatic LN tumors. Borderline significance ($P = 0.10$) was found on relating patient sex with SPF of primary tumors. Most of the females had low SPF (62.5%) while most of the males (61.9%) have high SPF (Table 3).

Disease free survival analysis

Disease free survival of OSCC patients reached 27.03% at 6 months. Female patients exhibited higher DFS (31.3%) at 6 month than males (23.8%) but relationship was not statistically significant ($p = 0.51$). Regarding the site, patients were classified into those with tongue tumors and those with tumors in all other sites. A statistically significant correlation ($p = 0.02$) was noticed between site of tumors and DFS. At 6 months DFS was 10.5% in tongue tumors while it was 44.4% in tumors of other sites (Fig. 4). No statistically significant relationship was observed between histopathological grade and DFS. Border line significance ($p = 0.10$) was noticed on relating DFS with tumor stage. DFS at 6 months for Stage III tumors was 38.9% while for Stage IV cases it was 15.8% (Fig. 5). When the SPF was divided into high (>10.63%) and low (<10.63%) groups, a highly statistically significant correlation was found between SPF of primary tumors and DFS ($p = 0.01$). DFS at 6 months was 44.4% in cases with
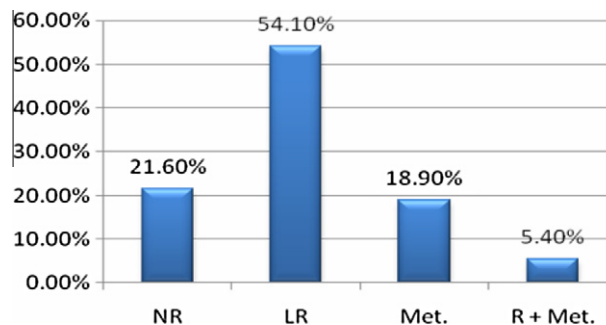

Figure 1 Disease free survival status of OSCC patients (NR, no recurrence; LR, local recurrence; Met., metastasis; R + Met., recurrence and metastasis).
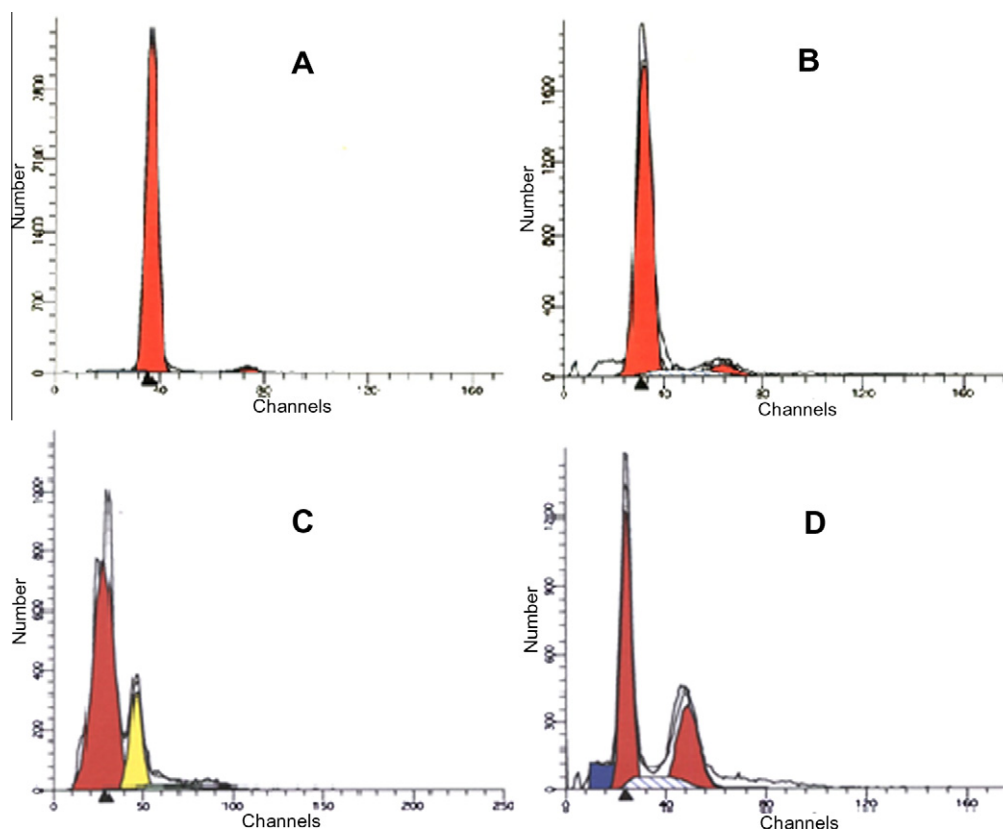
Figure 2 DNA ploidy pattern of primary OSCC tumors, (A) diploid tumor with low SPF (3.01%), (B) diploid tumor with high SPF (11.70%), (C) aneuploid (near diploid) pattern with low SPF (4.93%), (D) tetraploid (G2M = 32.47%) aneuploid tumor with high SPF (12.39%).

Table 2 Relationship between ploidy and SPF in primary OSCC tumors.

Tumor	Ploidy		P value
	Diploid	Aneuploid	
Primary OSCC	30 (81%)	7 (19%)	
SPF* (10.63% ± 3.96%)			
High (> 10.63)	14 (46.7%)	5 (71.4%)	0.10**
Low (< 10.63)	16 (53.3%)	2 (28.6%)	

* SPF (mean ± SD).
** Border line significance.

low SPF versus 10.5% of tumors with high SPF (Fig. 6). Although patients with aneuploid primary tumors fared worse than diploid ones as regard DFS (14.2% versus 30%) however the correlation was not of statistical significance.

Discussion

Oral cancer is often discovered when cancer has metastasized to another location most likely lymph nodes of the neck. Prognosis at this stage of discovery is significantly worse than localized intraoral disease [17].

There are several types of oral cancers but about 90% are squamous cell carcinoma. In addition to poor histologic differentiation, presence of metastatic lymph nodes has often been reported as the single most important prognostic factor in pa-

tients with OSCC [18]. Association of DNA pattern of primary tumors as well as their metastatic nodes with disease free survival is still controversial.

In the present research around half of OSCC tumors were located in the tongue (51.4%), followed by buccal mucosa (21.6%). Sargeran et al. [19] reported that the most common oral cancer site was the tongue accounting for 50% of oral cavity cancer. Out of 19 tongue squamous cell carcinoma 14 patients were less than 60 years. This was emphasized by other authors who stated that tongue squamous cell carcinoma constitute a large percent of head and neck cancers, and the incidence among young patients is increasing [20].

Nineteen percent of the analyzed primary OSCC tumors were aneuploid. A higher incidence of aneuploidy among OSCC tumors was reported by western researchers (30–58%) [14,21,22]. The discrepancy can be explained by dominance of Grade II OSCC tumors in our study (86.5%) while Grade III and Grade I comprised only 8.1% and 5.4%, respectively. In agreement with our results, some researchers showed that the incidence of aneuploidy among their series of OSCC Grade II tumors was 20% [22] and 15.43% [23].

It was generally observed that the researchers who used OSCC fresh tissues reported a higher incidence of aneuploidy; 58% [24] than those who used FFPE OSCC samples; 30% [21], 39% [22], and 41% [23]. Furthermore, authors observed that DNA ploidy is heterogeneous within squamous cell carcinoma of the oral cavity and the incidence of aneuploidy was very high when multiple tissue samples from each tumor were analyzed [15].
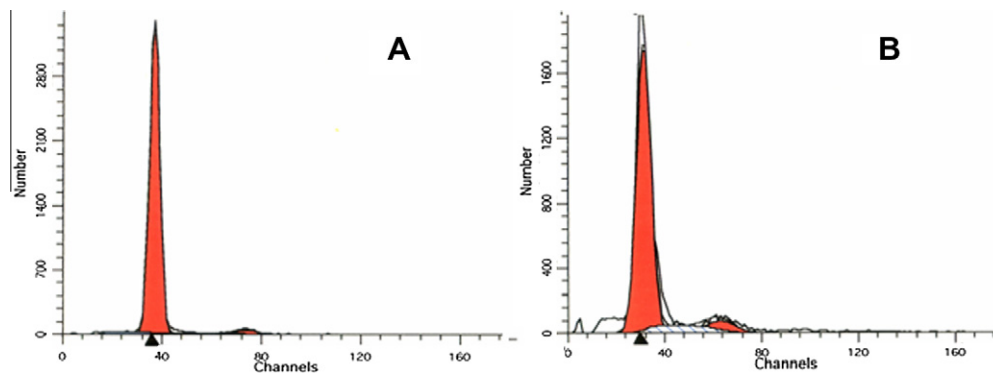
Figure 3 DNA ploidy profile of lymph node metastatic deposits of OSCC tumors, (A) diploid tumor with low SPF (3.58%), (B) diploid tumor with high SPF (12.14%).

In the present study none of the metastatic lymph node OSCC tumors was aneuploid. Discrepancy between ploidy status in primary OSCC tumors and their corresponding lymph node metastasis was reported by other investigators who analyzed ploidy status and SPF of 96 FFPE primary OSCC tumors and their 85 metastatic nodal tumors [22]. They mentioned that shift down to diploidy was observed in 25 out of 39 metastatic lymph nodes associated with aneuploid primary

tumors. One possible explanation is that the diploid cells rather than aneuploid ones are responsible for causing lymph node metastasis; however they gradually change into aneuploid cells during the process of progression in the metastatic site as they do at the primary sites.

After revising the H&E sections of metastatic LN again we noticed that even if metastatic tumor cells heavily affected most of the lymph node(s) area yet the existence of some

Table 3 Clinicopathological parameters and FCM findings of primary OSCC tumors.

Variables		SPF		p-Value	Ploidy state		p-Value
		Low	High		Diploid	Aneuploid	
Age	< 40	2	5		6	1	
		28.6%	71.4%		85.7%	14.3%	
	40–60	6	17	0.30	19	4	0.35
		26%	74%	NS[*]	82.6%	17.4%	NS
	> 60	3	4		5	2	
		43%	57%		71.5%	28.5%	
Sex	Male	8	13		16	5	
		38.1%	61.9%	0.10	76.2%	23.8%	0.89
	Female	10	6	BS[**]	14	2	NS
		62.5%	37.5%		87.5%	12.5%	
Site	Tongue	6	13		16	3	
		31.6%	68.4%		84.2%	15.8%	
	Retromolar area and gingiva	2	5	0.87	6	1	0.85
		28.6%	71.4%	NS	83.3%	16.7%	NS
	Buccal mucosa	2	6		6	2	
		25%	75%		75%	25%	
	Mouth floor	0	3		2	1	
		0%	100%		66.7%	33.3%	
Grade	I	0	2		2	0	
		0%	100%	0.18	100%	0%	0.78
	II	11	21	NS	26	6	NS
		44.4%	65.6%		81.3%	18.8%	
	III	0	3		2	1	
		0%	100%		66.7%	33.3%	
Stage	III	5	12		14	4	
		43.4%	66.6%	0.86	77.8%	22.2%	0.58
	IV	6	13	NS	16	3	NS
		31.6%	68.4%		84.2%	15.8%	

[*] NS, not significant.
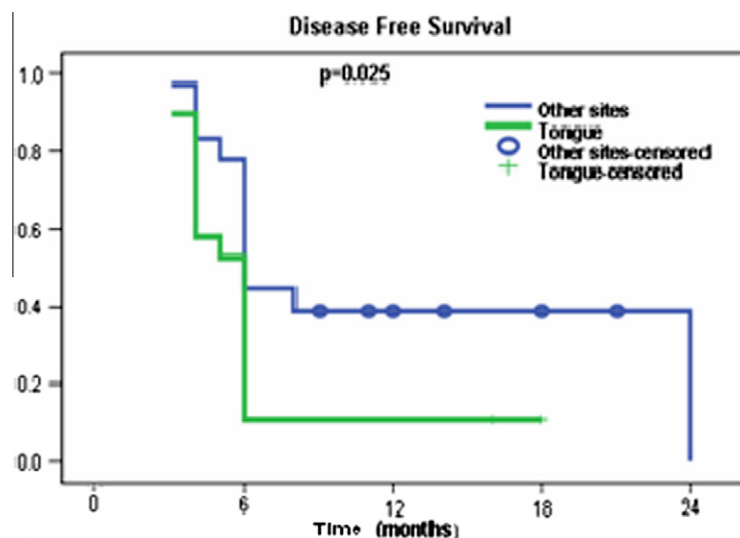[**] BS, borderline significance.

Figure 4 Disease free-survival in relation to OSCC site ($p = 0.025$).
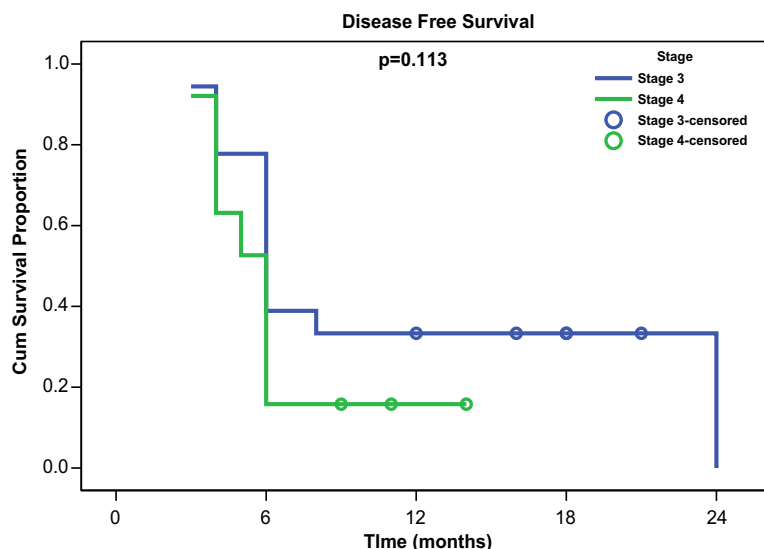

Figure 5 Disease free-survival in relation to tumor stage among 37 OSCC patients ($p = 0.118$).

reactive lymphoid elements or even one or two reactive lymph nodes on the same section was inevitable. From the technical point the presence of significant number of non-tumorous cells in the sample may hide small aneuploid populations of cells especially in FFPE with wide CV. Microdissection of metastatic tumor cells from the lymph nodes may overcome this problem by enriching the analyzed samples with tumor cells and minimizing the fraction of normal and/or reactive lymphocytes during flow cytometric analysis especially if tumor cells are widely separated.

In contrast to DNA ploidy analysis, investigators suggested that determination of SPF from one randomly chosen biopsy specimen is representative of the proliferative activity of the carcinoma, because intratumoral SPF variation is low [25].

Our study showed that the mean SPF was lower in metastatic lymph nodes ($6.58 \pm 1.32\%$) than that in primary tumors ($10.63 \pm 3.96\%$). Concordant results were shown by other investigators who reported a lower mean SPF of lymph node metastasis (6.96%) than that of primary OSCC tumors (11.24%) [14].

Border line significant relationship ($p = 0.10$) was detected between DNA ploidy and SPF of the primary OSCC tumors. The results revealed that 71.4% of the aneuploid tumors versus 46.7% of the diploid tumors had high SPF. Several authors reported, that the SPF was significantly higher in aneuploid OSCC tumors than in diploid carcinomas [14,16,24].

No significant association was found between DNA ploidy of either primary or metastatic tumors with age, sex, tumor site, histopathologic grade, and tumor stage.

The relationship between clinicopathological variables and DNA ploidy in OSCC is frequently debated. Some authors in accordance to our results did not identify an association
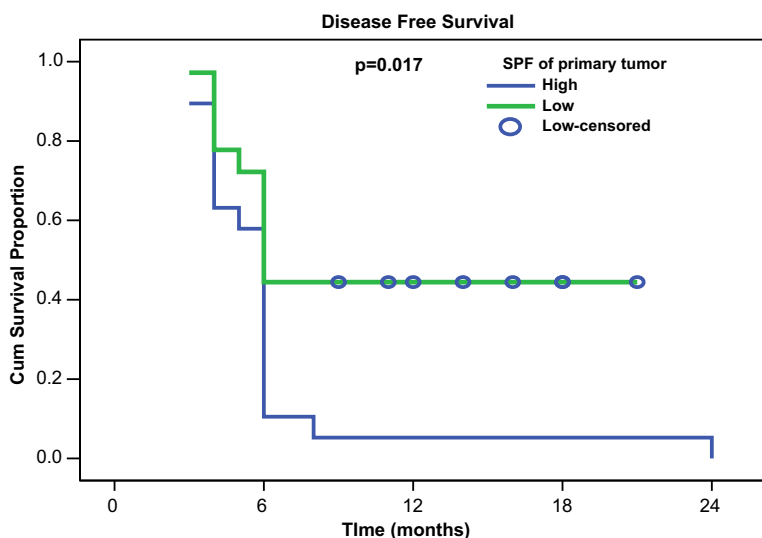
Figure 6   Disease free-survival in relation to SPF of primary OSCC tumors ($p = 0.017$).

between them [14,26] while others did. Investigators reported that aneuploid tumors were significantly more likely to be found in men and to have poor differentiation and advanced stage (III, IV) [27].

A significant correlation was found between the DFS and tumor site ($p = 0.02$) as local recurrence and distant metastasis were detected in 61% (13/21) and 55.5% (5/9), respectively, of tongue tumors rather than lesions in other sites in oral cavity. These findings were in agreement with the studies of many researchers who concluded that tongue SCC was associated with poor survival compared with other oral cavity and head and neck sites [28–30]. The clinical stage revealed a border line significance ($p = 0.10$) in relation to DFS.

A highly statistically significant correlation ($p = 0.01$) was detected between SPF of primary tumors and DFS. While the correlation did not reach the level of statistical significance ($p = 0.35$) in metastatic lymph node tumors.

Russo et al. [31] revealed that among 36 patients with locally advanced squamous cell carcinoma, the major significant predictor of both disease relapse and death was high SPF and TP53 mutation. Hass et al. [32] performed flow cytometric analysis of 48 primary and recurrent head and neck squamous cell carcinoma tumors. Their findings indicated that proliferative activity was significantly associated with malignant progression and recurrence.

Although patients with aneuploid primary tumors fared worse than diploid ones as regard DFS (14.2% versus 30%) however their relationship was not statistically significant. Comparable results were reported by other authors [32,26].

Conclusion

High SPF of primary OSCC tumors assessed by FCM was significantly associated with decrease disease free survival rates. DNA ploidy showed no relationship to bad prognostic indicators in either primary OSCC or their metastatic tumors.

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