



## Flow Cytometry Analysis of DNA Ploidy and S-Phase Fraction in Salivary Gland Tumors of Egyptian Patients

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### Abstract

**Aim:** The aim of this study is to investigate the DNA ploidy and S-Phase Fraction (SPF) of some Salivary Gland Tumors (SGTs) in Egyptian patients and to investigate the correlation between these two biological parameters and the presumptive behavior of these neoplasms.

**Methods:** Flow cytometric analysis of DNA ploidy and S-Phase Fraction (SPF) was done in 50 fresh tumor tissue sections of SGTs which diagnosed as 15 benign and 35 malignant tumors.

**Results:** 93.3% of the benign SGTs tumors were diploid, while only 34% of malignant tumors were diploid and 66% were aneuploid. The malignant SGTs had higher SPF than the benign tumors but with no significant difference. There was no significant correlation of ploidy status or SPF with the tumor grades of mucoepidermoid carcinomas.

**Conclusion:** DNA aneuploidy may be a key indicator for tumors activity and malignancy in SGTs, while the SPF has a minor role in the evaluation of SGTs activity.

**Keywords:** Flow cytometry; Salivary gland neoplasm; Aneuploidy; Mitotic Index; Diploidy; Mucoepidermoid carcinoma

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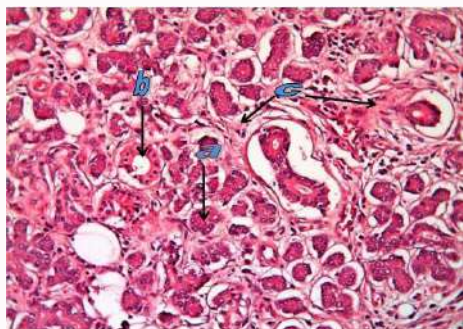
### Introduction

Salivary Glands Tumors (SGTs) are rare and represent 2-4% of head and neck neoplasm. The global annual incidence for all SGTs as quoted by the World Health Organization (WHO), varied from 0.4-13.5 cases per 100,000 populations [1]. They categorized into benign neoplasm, tumor-like conditions, and malignant neoplasm [2]. Benign and malignant SGTs may resemble each other grossly if seen early in their clinical course [3].

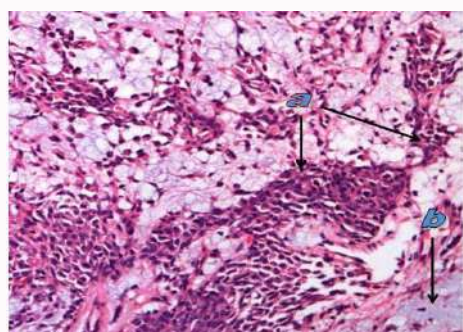
DNA analysis is an area of interest because chromosomal aberration is a characteristic feature of neoplasia [4,5]. The clinical behavior of tumors may be assisted by flow cytometric analysis of DNA content that allows rapid and reliable identification of cell with chromosomal aberrations that result in a measurable deviation from the DNA content of the normal diploid cells [6]. The flow cytometry allows a fast estimation of ploidy status and cell proliferation of the neoplasm by the determination of nuclear DNA contents and provide 2 functional factors related to neoplastic progression, DNA ploidy and the Synthesis Phase Fraction (SPF) [7,8].

DNA ploidy is a term used to describe the nuclear DNA contents. The deviation of DNA content from the normal diploid value referred to as aneuploid, and it is widely accepted as an indicator of malignancy in human tumors [9]. The detection of aneuploidy by flow cytometry may provide practical informations on clinical behavior for SGTs [10]. DNA content analysis in SGTs had been studied but with conflicting results. Some studies showed an overall low proportion of cases with DNA aneuploidy [8,11]. Others, however, reported a high frequency of DNA aneuploidy in this type of tumors [12]. Abnormal DNA content has been related to aggressive behavior in Mucoepidermoid Carcinomas (MEC), Adenoid Cystic Carcinomas (AdCC), Acinic Cell Carcinomas (ACC) and oncocytomas (Onc) [8]. S-phase value is correlated with the behavior of the tumors [13,14].

The aim of this study is to investigate the DNA ploidy and S-Phase Fraction (SPF) of some



**Figure 1:** Normal submandibular salivary gland tissue showing serous acini (a) and duct (b) in connective tissue stroma (c). (H&E. stain X200).



**Figure 2:** Pleomorphic adenoma in submandibular salivary gland showing tumor cells (a) and myxoid component (b). (H&E. stain X200).

Salivary Gland Tumors (SGTs) in Egyptian patients and to investigate the correlation between these two biological parameters and the presumptive behavior of these neoplasms.

## Materials and Methods

### Patients' selection

The current study included fresh tissue of 50 patients presented with SGTs which had been encountered in the last 4 years. Clinical data and patients characteristics were retrieved from the clinical charts. The diagnosis was 15 benign and 35 malignant SGTs. The benign cases included 7 Pleomorphic Adenomas (PA), 5 Warthin Tumors (WT) and 3 oncocytomas (Onc). The malignant cases included 5 AdCC, 3 Polymorphous Low Grade Adenocarcinoma (PLGA), 3 ACC, 14 MEC, 3 Myoepithelial Carcinomas (MC) and 7 carcinoma ex-pleomorphic adenomas (CexPA). MEC included 7 high grade cases (grade III), 3 intermediate (grade II) and 4 low grade (grade I). The tissue sections were collected after surgical resection at the Department of Surgical Oncology, South Egypt Cancer Institute, Assiut University and Oral and Maxillofacial Department, Faculty of Dentistry, University of Alexandria.

The study was approved by Ethical Committee of Faculty of Dental Medicine, Al-Azhar University, Assiut.

### Surgery

For parotid glands tumors, we performed parotidectomy (superficial or total) with an adequate margin of normal tissue with preservation of the facial nerve unless it is directly infiltrated by tumor. Regarding submandibular gland tumors, benign neoplasms of the submandibular gland required complete excision of the gland while, malignant neoplasms required submandibular triangle excision without resection lingual and hypoglossal nerves unless it was directly

infiltrated by tumor to obtain clear margins. For minor salivary glands tumors, we performed complete excision with adequate safety margin. Selective or modified radical neck dissection was performed in case of clinically or pathologically positive neck lymph nodes with malignant SGTs.

### Flow cytometry study

Three sections of 50  $\mu$ m thickness from each tumor tissue were cut and transferred to flow cytometry Unit, Clinical Pathology Department, South Egypt Cancer Institute for flow cytometric analysis of DNA ploidy and SPF. Single nuclear suspensions were prepared by filtering through a 50- $\mu$ m nylon mesh. The DNA contents were measured in the suspensions using FACS Calibur flow cytometer (Becton Dickinson Biosciences, San Jose, California USA). DNA histograms of at least 50000 nuclei were analysed. The DNA-diploid cell population of normal submandibular salivary gland with retention cyst is used as a reference standard for the identification of DNA-aneuploid clones. The percentages of the cell cycle phases as well as the DNA indices of the aneuploid clones were calculated using the Modfit software package. DNA histograms were classified as diploid if there was a single G0-G1 peak and aneuploid if additional G0-G1 peaks were present. The ratio of aneuploid G0-G1 peak values to diploid G0-G1 peak values was expressed as a DNA index. All specimens had a G0-G1 peak coefficient of variation of no more than 4%. The following were taken as cytometric variables: DNA ploidy, DNA Index (DI), and SPF. The cases with DI between 0.95 and 1.05 were considered as DNA diploids, and those less than 0.95 (hypodiploid) or greater than 1.05 (hyperdiploid) as DNA aneuploids. The SPF was estimated as 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 cut off for the SPF was set as the mean  $\pm$  2 standard deviation (SD) and considered as either being low or high. One section from each block was cut at 5  $\mu$ m thickness and stained with Hematoxylin and Eosin (H&E) for confirmation of histopathological diagnosis, classification, and assures that the tumor tissue constitutes >70% of the paraffin section with minimal necrotic and hemorrhagic foci. (Figure 1, 2)

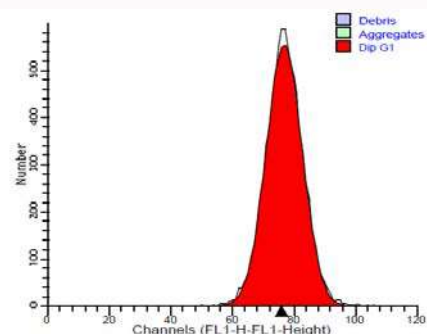
### Statistical analysis

Data was statistically described in terms of mean  $\pm$  SD, median and range, or frequencies (number of cases) and percentages when appropriate. Comparison of numerical variables between the study groups was done using Mann Whitney U test for independent samples when comparing benign and malignant lesions and Kruskal Wallis test when comparing MEC grades. For comparing categorical data, Chi square ( $\chi^2$ ) test was performed.

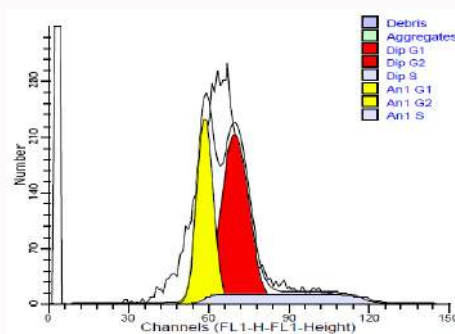
## Results

The average age of patients in the current study was 45.8 years. 16 of 35 (47.7%) of patients with malignant tumors were between 30 and 60 years, while 60% of cases with benign tumors were above 60 years. The male to female ratio was 1.5:1 for benign and malignant tumors. 28 tumors (56%) were originated in the parotid gland while 9 were originated in the submandibular gland (18%) and the remaining (26%) were originated in the minor salivary glands.

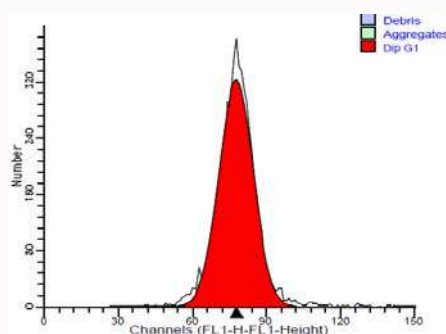
The normal standard of submandibular gland tissue showed a single diploid G0/G1 peak. They showed no cells in the S-phase or at G2/M peak (Figure 3). 14 cases (93.3%) of the benign tumors were diploid and only one case was aneuploid, its diagnosis was WT. While



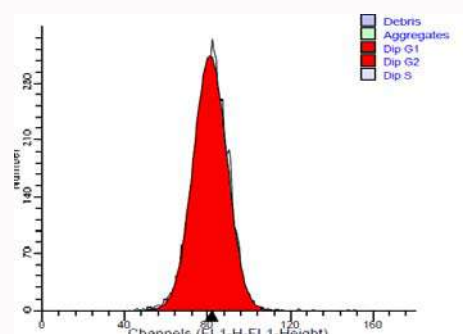
**Figure 3:** DNA frequency histogram of diploid standard (normal salivary gland tissue), showing single G0/G1 peak and no SPF cells.



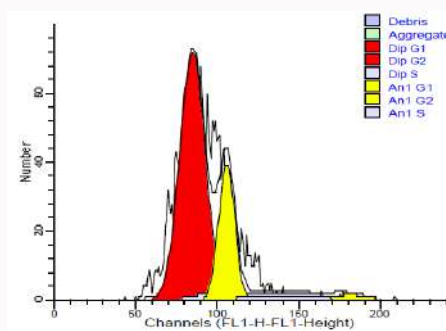
**Figure 6:** DNA frequency histogram of aneuploid malignant salivary gland tumors showing hypodiploidy (DI= 0.81).



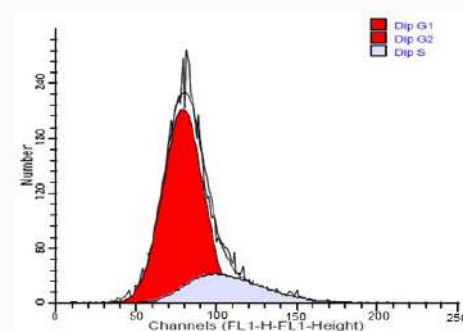
**Figure 4:** DNA frequency histogram of diploid salivary gland tumors, showing single G0/G1 peak.



**Figure 7:** DNA frequency histogram of salivary gland tumors showing low SPF (1.7%).



**Figure 5:** DNA frequency histogram of aneuploid salivary gland tumors showing hyperdiploidy (DI= 1.2).



**Figure 8:** DNA frequency histogram of salivary gland tumors showing high SPF (21.6%).

24 cases (66%) of malignant SGTs were aneuploid and 11 (34%) were diploid. The diploid tumors showed a single diploid G0/G1 peak similar to that of the reference peak (Figure 4). In some cases G2/M peak were also identified as a small peak that contained less than 15% of whole cells.

The aneuploid tumors showed an additional peak with its main channel. Aneuploid tumors were further classified into 23 hyperdiploid cases (22 malignant and 1 benign) and 2 hypodiploid. In the hyperdiploid, the additional peak was to the right of G0/G1 diploid peak with DI ranged from 1.08 to 1.4 with a mean of 1.25 (Figure 5). The hypodiploid peak was to the left of G0/G1 diploid peak with DI ranged from 0.81 to 0.85 with a mean of 0.83 (Figure 6). The difference in the ploidy state (diploid and aneuploid DNA pattern) between benign and malignant SGTs was statistically highly significant ( $P = 0.001$ ).

The SPF values of the diploid benign SGTs ranged between 1.7%

and 17.8% with a mean of 7.3%. For DNA diploid malignant SGTs, SPF values ranged between 1.3% and 21.6% with a mean of 10.86% and the SPF of the aneuploid malignant tumors ranged between 6.1% and 53.7% with a mean of 15.89%. 9 cases (64.28%) of the benign tumors had low SPF (all had diploid DNA) (Figure 7), and 6 cases (35.72%) had high SPF (2 diploid and 1 aneuploid). Among the diploid malignant tumors 54.55% had high SPF and 45.45% had low SPF. In the aneuploid malignant SGTs, about 79.16% had low SPF, and 20.84% had high SPF (Figure 8). There is no significant difference in the SPF between benign and malignant tumors ( $p = 0.528$ ) and between diploid and aneuploid tumors ( $p = 0.319$ ). There was no significant difference in the SPF between benign and malignant tumors ( $p = 0.528$ ) or between diploid and aneuploid tumors ( $p = 0.319$ ).

Five cases of the 7 grade III MEC (71.4%), 2 cases of grade II MEC and 2 cases of grade I had aneuploid DNA pattern. Four cases of grade III and 2 of grade II MEC had high SPF while the remaining



had low SPF. The differences between grades in the ploidy states and SPF were not statistically significant ( $P=0.772$ ). Also, none of the clinical variables, patient's age, sex and tumor site had no correlation with the ploidy status or SPF.

## Discussion

Aneuploidy in the present Egyptian study was higher than that reported by other researchers in SGTs [4,7,13]. This may be due to late diagnosis of these tumors in our patients as most cases diagnosed at advanced stage and the sections were taken from deep tumor tissue to increase the number of tumor cells in our samples. The deep samples demonstrated aneuploidy in the DNA content than the superficial sample of the same tumor and correlated with atypical histological and immunohistochemical features [15]. Agreement with our results, Takashima [16] found that among the studied SGTs, 69% of malignant tumors were aneuploid. Pinto [8] performed flow cytometric analysis on 97 SGTs, and found that all benign tumors exhibited a DNA diploid pattern and 46.7% of high grade carcinomas showed DNA aneuploidy. Also, Vargas [11] reported that all the PAs were diploid and 44% of the high grade malignant lesions analyzed were aneuploid. Monteiro [17] reported that 55% of malignant SGTs had diploid DNA and 45% had aneuploidy DNA histograms. In the present study, we had 5 cases of WT; one of them had aneuploid DNA pattern which may be due to the aggressive behavior of WT or the presence of malignant clones in the tumors section that was analyzed by flow cytometry. This result was comparable with that of Atula [18] who reported that 2 of 33 WTs showed clear aneuploid DNA and seven cases were near diploid. The aneuploid WT in the present study was a recurrent tumor with long duration. Martins [19] mentioned that 4 of the 16 benign SGTs have aneuploidy pattern with 3 of them being recurrent lesions. This indicates that the DNA aneuploidy may contribute to the occurrence of recurrence and gains further importance to the flow cytometric DNA analysis in predicting the recurrence of SGTs.

The SPF represented a continuous variable related to any proliferating cell population so it represented a biomarker of malignancy. This study showed that malignant SGTs had higher SPF than the benign SGTs but there was no significant difference. Comparable results were obtained by a study of Kelsch [20] who investigated 10 samples of PLGA that contain adequate tumor tissue and found that all diploid tumors had S-phase percentage less than those of the lowest aneuploid cell line but with no significant difference. Nordgard [4] revealed that flow-cytometric analysis on fresh and paraffin-embedded material of SGTs correlated well concerning DNA ploidy, but not for the S-phase. Lin [21] noticed in their research on ACC of the trachea and bronchus that aneuploidy tumors associated with high SPF than diploid one but no variable marker could be present as a prognostic indicator. Other studies found a statistically significant difference in the SPF of benign versus malignant tumors, diploid versus aneuploid tumors [7,8,22]. We found that there was no significant correlation of ploidy status or SPF with the tumor grades of MEC. This may be due to the few numbers of cases involved in this study. This result was also observed by other investigators [8,13], who reported that there was no significant relationship between DNA ploidy status and histopathological grading. Other studies in oral cancer [23,24] reported that there is no relationship between the histopathological grade of oral epithelial dysplasia or oral squamous cell carcinoma and the ploidy status. In contrast, two studies [25,26] on ACCs reported an association between tumor grade and DNA

ploidy. In the present study, the clinical data of patients (age, sex and tumor site) didn't have any correlation with flow cytometric parameters (ploidy status or SPF). These results are in agreement with the previous study [11,17,27].

## Conclusion

DNA aneuploidy may be a key indicator for tumor activity and malignancy in SGTs, while the SPF has a minor role in the evaluation of SGTs activity.

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