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Letter to the editor

Intraoperative flow cytometry for head and neck lesions. Assessment of malignancy and tumour-free resection margins

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ABSTRACT

Head and neck cancer poses a significant health problem worldwide. We set out to investigate the value of rapid intraoperative cell cycle analysis by flow cytometry for the intraoperative characterization of head and neck lesions and surgical margins. Seventy patients with head and neck lesions suspicious of malignancy were included in the study. There were 31 neoplastic and 39 benign lesions. Flow cytometry permitted the intraoperative detection of neoplastic lesions within 6 min with high sensitivity and specificity based on cell cycle fractions. In the cases in which surgical margins were assessed, intraoperative flow cytometry had complete concordance with pathology. Intreoperative flow cytometry is a novel promising technique for rapid intraoperative characterization of malignancy and tumour free resection margins in head and neck lesions.

Introduction

Head and neck cancer poses a significant health problem worldwide [1]. Although significant advances have been made in several surgical fields, head and neck cancer treatment needs further refinement. Conventional methods that use clinical inspection and microscopic examination to assess the extent of disease have not been so effective. Thus, novel technologies should be explored for assessment of margin status [2]. Intraoperative DNA content analysis by flow cytometry in solid lesions has been recently introduced and can be performed within 6 min [3,4]. In brain tumour surgery, intraoperative flow cytometry permitted the assessment of grade of malignancy, histology and tumour margins [3,5,6]. Additionally our ongoing work on breast cancer samples has generated very encouraging results [7]. The aim of this study was to investigate the value of rapid intraoperative flow cytometry for characterization of head and neck lesions and tumour-free resection margins.

Material and methods

We prospectively studied patients with head and neck lesions, suspicious of malignancy, who underwent surgery over a 1.5 year period. A tumour sample (about 3–5 mm^2) was collected during surgery (from tumour and excision margins when available). Half of the sample was sent for flow cytometry analysis, by Ioannina protocol as described previously [3], and the other half for standard pathology evaluation. The pathologist and flow cytometry operator were blinded from each other's results. Permanent section diagnosis by an expert pathologist (A.B.) was used as the “gold standard”. Diagnosed tumours were graded according to the World Health Organization 2017 classification scheme. Written informed consent was obtained from all participants before tissue collection as per institutional review board protocol.

Results

Between May 2016, and December 2017, 70 patients (54 men, 16 women, mean age 59, 5 years, range 6–92) met the inclusion criteria for the study. There were 39 benign lesions and 31 neoplastic lesions. The pathological diagnoses of the neoplastic lesions were twenty-one squamous cell carcinoma, five lymphomas of B-cell origin, 3 basal cell carcinoma, one neuroendocrine tumour and one tumour of mesenchymal origin. There were 58 diploid and 12 aneuploid lesions. All aneuploid lesions were neoplastic. Neoplastic lesions had lower G0/G1 and higher S-phase and G2/M phase fractions than non-neoplastic lesions (78.5% ± 11.5 vs 92.4% ± 2.1, $p < 0.0001$, 9.7 ± 5 vs 4 ± 1.4, $p < 0.0001$ and 11.8 ± 9.9 vs 3.75 ± 1.8, $p < 0.0001$ respectively). A cut-off value of 88% for G0/G1 phase had a 97.4% sensitivity and 90% specificity for the diagnosis of neoplastic lesions. For the S-phase fraction the cut-off value for the diagnosis of neoplastic lesions was higher or equal to 6% (97.4% sensitivity and 73.3% specificity) and for the G2/M phase fraction the cut-off value was higher or equal to 5% (80% sensitivity and 86.7% specificity). A tumour index (S + G2/M) higher of 10% had a 97.4% sensitivity and 90%| specificity for the detection of neoplastic lesions [Fig. 1].

In 4 neoplastic cases we additionally analyzed specimens from resection margins in order to evaluate the extent of excision. Pathology verified that the margins of the excised lesions were free of tumour in 3 cases and infiltrated in 1 case. In the 3 cases with clear resection margins the G0/G1, S-phase and G2/M phase fraction of the marginal tissue verified the absence of tumour cells. In the fourth case pathology showed infiltrated margins and flow cytometry analysis showed the presence of aneuploid/hyperploid cells that could be readily discriminated from normal tissue [Fig. 2].

Discussion

Head and neck squamous cell carcinoma is a grave disease with high morbidity and mortality in advanced stages [8]. At the time of diagnosis more than 50% of patients have advanced disease and thus a poor prognosis. Pathological reviews showed infiltrated surgical margins in up to 40% of the cases [9,10]. Overall survival has not been significantly improved. The 5-year survival rate is approximately 40% and

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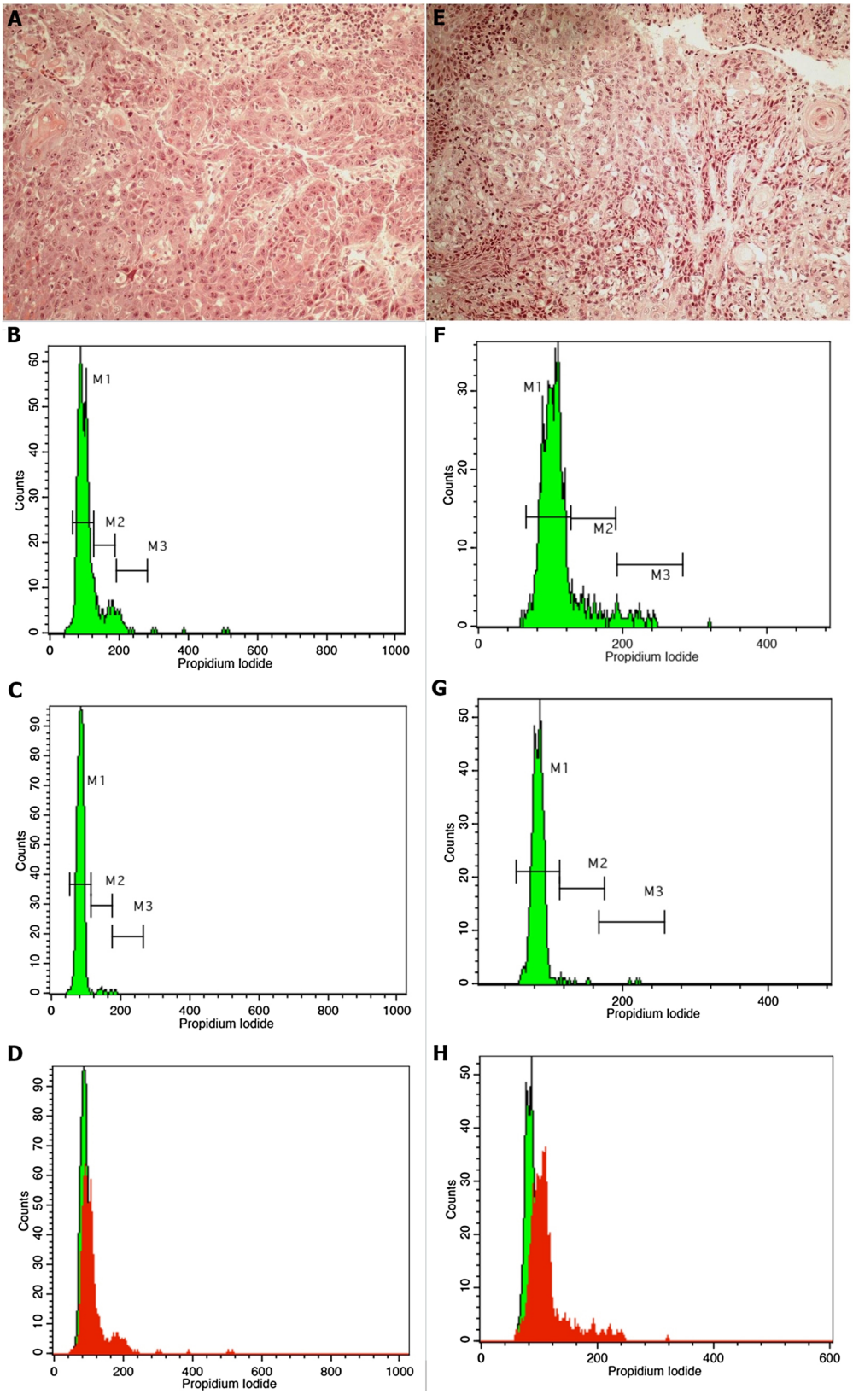

Fig. 1. A. Histopathology microscopical image of an invasive squamous cell carcinoma of oral mucosa (H&E X200). B. DNA histogram acquired after intraoperative flow cytometry analysis of a tissue specimen from the tumour showing a G0/G1 phase (M1) of 85%, S-phase fraction (M2) of 12%, G2/M phase fraction (M3) of 3%. TI = 15%. C. DNA histogram acquired after flow cytometry analysis of normal blood cells used as a control sample. D. Overlay of DNA histograms from the tumour (red) and from normal blood (green) indicating a diploid tumour with pathological tumour index (S-phase + G2/M). E. Histopathology microscopical image of an invasive squamous cell carcinoma of larynx (H&E X200). F. DNA histogram acquired after intraoperative flow cytometry of a tissue specimen from the tumour indicating a G0/G1 phase (M1) of 86%, S-phase fraction (M2) of 10%, G2/M phase fraction (M3) of 4%. TI = 14%, DI = 1,22. G. DNA histogram acquired after flow cytometry analysis of normal bood cells used as a control sample. H. Overlay of DNA histograms from the tumour (red) and normal blood cells (green) indicating an aneuploid tumour. The cancerous tissue can be easily identified from the different place of the histograms.

local recurrence is the main cause of death. Despite aggressive surgery, in combination with chemotherapy and radiotherapy, many patients will die because of local or regional recurrences. A very important prognostic factor, which has been well documented as a predictor of recurrence, is the status of resection margins in head and neck squamous cell carcinoma [8]. Increased local recurrences and low survival rates are related to the presence of microscopic tumour cells in the resection margins [8].

Frozen section analysis during surgery remains the “gold standard” technique to determine the extent of tumour resection. Byers et al.

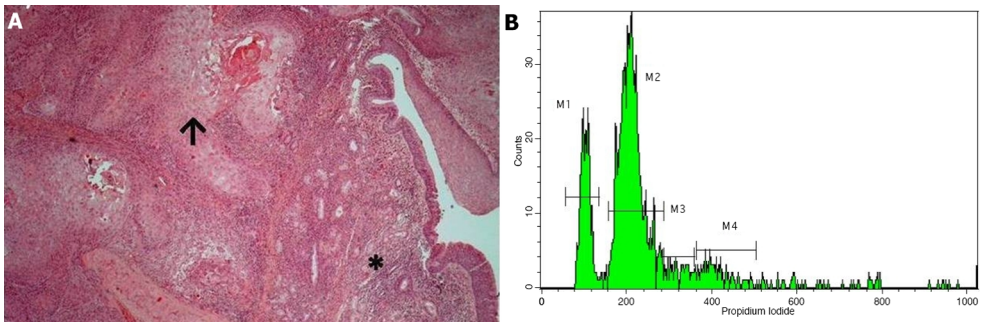

Fig. 2. A 79 year old men presented with a lesion of the left vocal cord. The patient underwent microlaryngoscopy and intraoperative flow cytometry was performed showing a aneuploid/hyperploid tumour. A. Histopathology microscopical image of an invasive squamous cell carcinoma of larynx (black arrow) and the adjacent normal mucosa of larynx (asterisk).(H&E x40) B. DNA histogram acquired after intraoperative flow cytometry analysis of a tissue specimen from the resection margins showing the presence of two distinct cell populations:normal mucosa of larynx (M1 = G0/G1) and cancerous aneuploid/hyperploid tissue (M2 = G0/G1). Thus, the surgeon should continue the excision of the tumour until the aneuploid population in the histogram disappears or in case of a diploid tumour until the G2/M phase fraction reach normal tissue values.

showed that the use of intraoperative frozen sections reduced the probability of local recurrence in head and neck cancer [11]. However, this method is time consuming and can evaluate only a small part of the tumour's resection margins. Thus, the evaluation of several surgical margins by frozen sections may require analysis of many samples and prolongs duration of surgery. Several studies using different methods have been performed for the intraoperative characterization of head and neck cancer. Unfortunately, there may be time consuming or require the administration of drugs to the patients [12–14]. Recently, tumour-targeted fluorescent tracers have been shown to successfully visualize tumours and their margins intraoperatively. The combination of γ-glutamyl hydroxymethyl rhodamine green and a clinical fluorescence imaging system had 80% sensitivity and 87% specificity on frozen tissue samples [15]. Additionally, biological characteristics of tumours may contribute to the surgical resectability. Saidak et al. showed that in head and neck squamous cell carcinomas three genes (*CCDC66*, *ZRANB2* and *VCPKMT*) had significantly higher mRNA levels in tumour of the tongue with positive resection margins compared to tumours with negative resection margins [16].

A future goal of our research is to investigate the possible prognostic role of intraoperative flow cytometric analysis and identification of the exact tumour type using immunophenotypic analysis. One limitation of the present study was the limited number of patients in which surgical margins were assessed. Nevertheless, with the results so far cell cycle analysis provided important intraoperative information for the characterization of head and neck tumours. More importantly, this novel application of flow cytometry may guide the surgeon to identify the ''true'' resection margins that ultimately has a great impact on patients' prognosis. It is obvious that future studies with more patients are needed in order for safe conclusions to be reached and we call for an international collaboration in order to verify our preliminary results.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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George Vartholomatos[a], Lentiona Basiari[b], George Exarchakos[b], Ioannis Kastanioudakis[b], Ioannis Komnos[b], Maria Michali[b], Georgios S. Markopoulos[c], Anna Batistatou[d], Alexandra Papoudou-Bai[d], George A. Alexiou[e,*]

[a] *Haematology Laboratory – Unit of Molecular Biology, University Hospital of Ioannina, Greece*
[b] *Department of Otorhinolaryngology, Head and Neck Surgery, University Hospital of Ioannina, Greece*
[c] *Laboratory of Biology, Department of Medicine, Faculty of Medicine, School of Health Sciences, Ioannina, Greece*
[d] *Department of Pathology, University Hospital of Ioannina, Greece*
[e] *Department of Neurosurgery, University Hospital of Ioannina, Greece*
E-mail address: galexiou@cc.uoi.gr (G.A. Alexiou).

* Corresponding author at: University of Ioannina, Department of Neurosurgery, Ioannina 45500, Greece.