Ki-67 expression in cytologic scrapes from oral squamous cell carcinoma before and after 24 Gray radiotherapy- a study on 43 patients

Prashant Sharma [1], Neeta Kumar [2], Anil Kumar Bahadur [3], Ashish Kumar Mandal [2]

(1) Senior Resident Doctor in Pathology
(2) Professors of Pathology
(3) Professor and Head of Department of Radiotherapy
Affiliations: Departments of Pathology and Radiotherapy, Maulana Azad Medical College and associated Lok Nayak Hospital, New Delhi 2, India

Correspondencia / Address:
Dr. Prashant Sharma
Flat 188, Block C2A, Pocket 16,
Janak Puri, New Delhi- 110058 India
Telephones 91-11-25508126, 9810661394
E-mail: prashant_sharma@msn.com

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SUMMARY

OBJECTIVE: To study the Ki-67 labeling indices in surface scrape smears from patients with oral squamous cell carcinoma before and after 24 Gray radiotherapy.

STUDY DESIGN: Forty three patients with histologically documented squamous cell carcinoma of the oral cavity were sampled by means of surface scrape smears prior to therapy and after receiving 24 Gray fractionated radiotherapy. These smears were stained for Ki-67 expression using the avidin biotin alkaline phosphatase technique.

RESULTS: Ki-67 expression was seen in an extremely small number of cells. Only 10 tumors showed positive cells, and the labeling index in them varied from 0.1 % to 0.01 %. After 24 Gray irradiation, no case showed any Ki-67 positive cells.

CONCLUSIONS: The overall yield of malignant cells in surface smears is low even prior to therapy and their number decreases further after irradiation. This, along with other factors including low concentration of proliferating cells on the surface of the lesion and obscuring inflammatory cells, anucleate squames, bacterial colonies and proteinaceous material could have accounted for the low labeling indices obtained. Radiation induced decline in proliferation has been described previously. The major conclusion, in balance, is that conventional oral scrape cytology may not be the optimal tool for immunocytochemical evaluation of proliferation in oral squamous cell cancer.

Key words: *Ki-67, oral smears, squamous cell carcinoma, scrape cytology, radiotherapy, head and neck cancer.*

INTRODUCTION

Precise assessment of the behavior and progression of oral squamous cell carcinoma (SCC) with a view to predict prognosis remains an elusive goal. Radiation induced morphologic changes in oral scrape smears have been explored in recent years as potential tools towards this end (1,2). However, the application of immunohistochemical techniques to these smears is largely unexplored.

The present study aimed at evaluating the Ki-67 labeling indices (LI) in surface scrapes from oral cancer. Additionally, the effect of irradiation on these indices was also assessed. The Ki-67 antibody is used extensively as a measure of proliferative activity in a variety of neoplastic lesions. Its application to a large range of cytological specimens has been shown to be technically feasible giving reproducible results (3).

MATERIAL AND METHODS

The study group comprised of 43 patients receiving fractionated radiotherapy at the rate of 2 Gray per day for histologically confirmed SCC of the oral cavity. Multiple scrape smears were taken from the tumour surface using a pre-wetted wooden spatula once before irradiation (day zero) and subsequently after the 24 Gray dose (day 12). The smears were air-dried and fixed for five minutes in cold acetone. The unstained smears were screened microscopically for the most cellular areas which were circled using a glass marking pencil. Unused air-dried smears were stored in an airtight wrapping at 0 degrees Celsius for future use if repeat staining was needed.

The smears were stained using a prediluted Ki-67 rabbit anti-human primary antibody (Dako's, Glostrup, Denmark). The technique and reagents have been standardized and are in routine use in our department by both cytology and surgical pathology services. With all batches of slides, known positive controls

in the form of similarly fixed and stored smears of fine needle aspirates from breast carcinoma and non-Hodgkin lymphoma were stained in parallel. No retrieval or pretreatment was done. Incubation time for the primary antibody was one hour; secondary antibody (biotinylated goat anti-polyvalent, Dako's Universal LSAB® Kit) was 30 minutes, followed by alkaline phosphatase conjugated streptavidin for 30 minutes. Washing in between these steps comprised 2 changes of Tris HCl buffer (pH 7.6) for 5 minutes each. Color development was done using a Fast-red chromogenic system (tablets of Fast red + Naphthol Phosphate buffer). Slides were counterstained with Mayer's hematoxylin and mounted in glycerol jelly.

Ki-67 positive cells were counted at 400x magnification and the LI calculated as the percentage of positively stained cells out of total epithelial cells counted. A minimum of 500 cells were counted per slide. Only sharp nuclear staining was taken as a positive stain (Figure 1) while cytoplasmic staining was ignored. In cases where no Ki-67 positive cells were seen, additional smears were subjected to repeat staining.

RESULTS

The clinical and histologic data of the patient group is summarized in Table 1. The exfoliated cells in the oral scrapes comprised of dysplastic to malignant epithelial cells, mature (nucleate and anucleate) squames, inflammatory cells and red blood cells. A morphologic continuum was seen to exist between the clearly benign cells to the frankly malignant forms. The overall cellularity as well as the numbers of dysplastic cells decreased drastically as the radiation dose imparted increased till the 24 Gray fraction when most scrapes showed predominantly anucleate squames, inflammatory cells and rare bizarre degenerating forms.

Table 1. *Clinical and histological data of study group*

Age [mean ± SD (range)] in years	53 ± 11 (36-77)
M : F ratio	31 : 12
Intraoral site	n (%)
Anterior 2/3 tongue	12 (27.9)
Buccal mucosa	10 (23.3)
Hard palate	4 (9.3)
Floor of mouth	4 (9.3)
Lower alveolar ridge	3 (6.9)
Upper alveolar ridge	1 (2.3)
Indeterminate	9 (20.9)
Clinical Stage (AJCC)	
I	12 (27.9)
II	7 (16.3)
III	17 (39.5)
IV	7 (16.3)
Histologic grade	
Well differentiated	14 (32.6)
Moderately differentiated	19 (44.2)
Poorly differentiated	10 (23.2)

An extremely low level of Ki-67 expression was seen in the pretreatment samples. Scrapes from only 10 tumours (23.3%) showed Ki-67 positive cells. LI varied from 0.01 to 0.1 percent, i.e. only 1 to 10 positive cells were seen per thousand cells counted. These cells invariably had high nuclear/cytoplasmic ratios and a tendency to cluster together. (Figure 1) The remaining 33 tumors did not show any Ki-67 positive cells even on repeat staining and diligent screening.

None of the smears taken after the 24 Gray dose of radiation showed any positive cells. While the decline in the number of Ki-67 positive cells was statistically significant for the 10 cases with pretherapy positive cells, the overall number of cells stained was too minuscule to arrive at meaningful statistical conclusions.

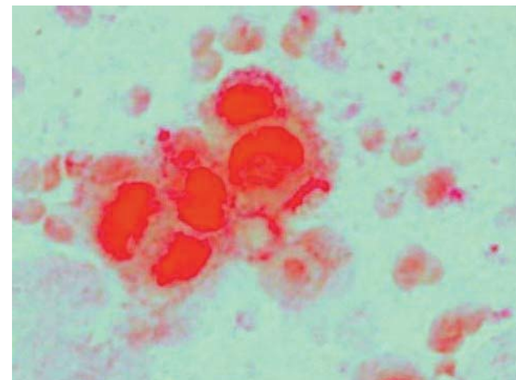

Fig. 1. *Cluster of Ki-67 positive cells in smear. (Avidin biotin alkaline phosphatase immunostain, Mayer's Haematoxylin counterstain)*

DISCUSSION

The Ki-67 antibody was first developed by Gerdes and coworkers (4,5) who demonstrated the antigen to be present in G1, S, G2 and M phases of continuously cycling cells, but absent in G0 cells. Since then, its utility as a proliferative marker for both diagnostic and research purposes has increased progressively.

To the best of our knowledge there are no previous publications on the application of Ki-67 immunostaining to oral scrape smears even though histologic material from the oral cavity and cytologic smears from the uterine cervix have been extensively studied (6-11). The present pilot study was carried out as a part of a larger project to study the feasibility of oral cytology in predicting response to radiotherapy. Cancer of the head and neck is amongst the commonest malignancies presenting to radiotherapy clinics in India, and the demand for economically viable and technically feasible prognostic tests is therefore intense.

Histologic studies show variable Ki-67 expression in oral SCC with most cancers showing LI between 5 to 60% (7,8). The comparatively much lower expression of Ki-67 in oral cytologic smears may have multiple reasons, the most obvious of which is the overall low yield of malignant cells in scrape smears. This has also been observed by other researchers (1,2,12,13). Umiker et al (13), for instance, found in an early series of 55 cases that scrapes from half the patients had 25% or less morphologically

malignant cells. An additional reason could be that the Ki-67 labeling indices at the deep invasive fronts of tumors are higher than those at the centre or surface of mucosal cancers (9,10). This indicates that actively proliferating cells are concentrated at the deep tumour margin, an area that obviously cannot be sampled by the superficial oral scrape. And finally, obscuring inflammation and blood, necrotic or proteinaceous debris and bacterial contamination often hamper cytologic evaluation in these smears, a limitation that may be surmountable by the use of liquid based cytology.

Prior histologic studies on oral SCC have documented a radiation induced decline in the number of proliferating cells (11,14). The complete absence of any positive cells in our post therapy smears appears to corroborate this. However, it needs to borne in mind that the overall cellularity and especially the yield of malignant or dysplastic cells in the post-24 Gray smears was extremely low. The few bizarre radiation altered cells that were present in these post-treatment smears have been considered to be genetically damaged and hence mitotically inactive cells by Bhattathiri et al (2). Their hypothesis is supported by our study to the extent that no such cell was Ki-67 positive in our smears.

In conclusion, Ki-67 expression is low in scrape smears from oral SCC. Considering that nearly 77% (33 out of 43) cases had a labeling index of zero even pretreatment and all positive ones were in the range of 0.1 to 0.01% indicates that Ki-67 immunostaining on conventional oral scrape cytology, though previously unreported in literature, is probably not the optimal tool for evaluation of proliferative status of oral squamous cell cancer.

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