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Detection of Ki67 and AgNORs among Esophageal Tumors

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Abstract:

This study aimed to detect Ki67 expression and its index, and mean Nucleolar Organizer Regions (AgNORs) count in esophageal tumors. Forty paraffin embedded blocks (FFPB) previously diagnosed as esophageal tumors were selected for this study. The patient sex showed that 19 (47.5%) were male and 21 (52.5%) were female. Their ages ranged between 14-89 years with mean age 55.9 years. Samples include 21(52.5%) malignant tumors, and 19 (47.5%) benign tumors. Tumor grade among malignant samples revealed 10 (47.6%) well differentiated tumors, 10 (47.6%) moderately differentiated tumors, and one (4.8%) poorly differentiated tumors. Tissue

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sections were stained using two methods; immunohistochemical method (new indirect method) for detection of Ki67, and silver impregnation method for AgNORs detection. Data and results obtained were analyzed using SPSS computer program. Malignant esophageal tumors revealed positive expression of ki67 in 19 (90.5%) samples, and negative expression in 2 (9.5%) samples, while benign tumors showed positive expression in 4 (21.1%) samples, and negative expression in 15 (78.9%) samples, this result showed significant statistical difference (P= 0.000). Ki67 index among samples represented (40.21±18.94) in malignant tumors, and (14.60±14.85) in benign tumors, the differences showed significant result (P= 0.000). Association between Ki67 index and the grade of tumor showed (29.80±13.22) in well differentiated tumors and (45.96±15.10) in moderately differentiated tumors with significant association (p= 0.020). The mean AgNORs count per cell was (5.13±1.17) in malignant tumors, and (2.78±0.77) in benign tumors, with significant differences (P= 0.000). The mean AgNORs count in well differentiated tumors and moderately differentiated tumors was (4.36±0.48) and (5.65±0.79) respectively, with statistically significant correlation (P= 0.001). Positive correlation was found between mean AgNORs count and Ki67 index over expression in esophageal tumor (P= 0.01, r= 0.783).

Conclusion: *The study concluded that both Ki67 index and mean AgNORs count increase in malignant tumor more than benign, with association with tumor grade.*

Key words: Ki67, AgNORs, esophageal tumors.

INTRODUCTION:

Esophageal cancer is the eighth most common incident cancer in the world because of its extremely aggressive nature and poor survival rate [(1)], esophageal carcinoma affects more than

450000 people worldwide and the incidence is rapidly increasing [2].

In Sudan, according to Sudan National Cancer Registry (NCR), during the period 2009 - 2010, 6771 cases were recorded for Khartoum State, in which esophageal cancer rate 5.8 per 100,000 of the population [3].

The risk factors for squamous cell carcinoma (SCC) and adenocarcinoma (AC) include tobacco use, alcohol consumption, dietary insufficiencies, hot food and beverages, gastro-esophageal reflux disease (GERD), *Helicobacter pylori* infection, human papilloma virus (HPV), radiation, age and obesity [4] and genetic factors [5].

Diagnosis of esophageal cancer is done by esophagram, upper endoscopy, endoscopic ultrasound (EUS), bronchoscopy, biopsy, computed tomography scan (CT), magnetic resonance imaging (MRI), positron emission tomography scan (PET) [6].

The treatment options for esophageal cancer include chemotherapy, radiation therapy, chemo-radiotherapy, biological/targeted therapy and surgery [5].

Ki67 is a marker of cell proliferation. It is present in all periods of the cell cycle except for G_0 and provides a reliable method to characterize malignant tumors, there is a strong correlation between Ki67 proliferative activity and esophageal carcinomas [8].

Argyrophilic nucleolar organizer regions (AgNORs) are loops of ribosomal DNA located in the short arms of acrocentric chromosomes 13, 14, 15, 21 and 22 and transcribe to ribosomal RNA. AgNORs vary in size and shape according to nucleolar transcription. They are intimately related to the cell cycle and may be related to proliferation and ploidy [9]. The AgNOR number is a good indicator of the malignant potentiality of esophageal carcinoma [10].

MATERIALS AND METHODS:

Study sample:

Forty paraffin embedded tissue blocks previously diagnosed as esophageal tumors were randomly collected from different hospital in Khartoum State.

Patient identification and other information obtained from patients files, includes age, sex and diagnosis.

Sample processing:

Two sections of 3 μm in thickness were obtained from formalin fixed paraffin wax embedded tissue using rotary microtome, section for IHC were mounted into coated slide.

Immunohistochemical staining for detection Ki67:

Sections were dewaxed in oven and cleared in two changes of xylene for 2 minutes for each then rehydrated through descending concentration of ethanol (100%, 90%, 70%, and 50%) then water 2 minutes for each, then antigen retrieved using PT link for 20 minutes at 95°C in tris buffer EDTA (pH 9.0), then washed in phosphate buffer saline (PBS) pH 7.4 for 3 minutes, then treated with hydrogen peroxide solution and methanol for 10 minutes then washed in PBS (pH 7.4) for 3 minutes. Then the sections were treated with primary antibodies (anti Ki67) for 20 minutes in a moisture chamber, then washed in PBS (pH 7.4) for 3 minutes, then treated with secondary polymer conjugated antibodies for 20 minutes, then washed in PBS (pH 7.4) for 3 minutes, then treated with DAB for 5 minutes, then washed in phosphate buffer saline (pH7.4), then counter stained in Mayer's haematoxylin for 1 minute then washed and blued in running tap water, then dehydrated through ascending concentrations of ethanol, cleared in xylene and mounted using dextrin plasticizer xylene (DPX) [(11)].

Silver impregnation technique for detection of AgNORs:
Sections were dewaxed in oven and cleared in two changes of xylene then rehydrated through descending concentration of ethanol (100%, 90%, 70%, and 50%) to distilled water, and then sections were inoculated in freshly prepared AgNOR staining solution for 45 minutes at room temperature in dark place, then washed with distilled water and dehydrated through graded ethanols to xylene [(11)].

RESULT INTERPRETATION:

All quality control measure was adopted. In IHC negative controls were completed by omission of the primary antibody. Positive staining of normal, non-neoplastic epithelial cells, fibroblasts and lymphocytes within analyzed samples served as internal positive control for Ki67. Cells that displayed a brown nuclear stain were considered to be Ki67 positive. The final result was assessed by the proliferation index for every case, which is calculated from the average of stained cells in relation to the total analyzed cells, with a minimum count of 500 cells (Negative expression 0% - 25% and positive expression 26% - 100%), as described by Bianto *et al*., [(12)].

Histochemically stained section for AgNORs count were examined under x100 oil immersion lens. AgNORs were clearly evident as brown-black dots of varying size. Dots were counted in 100 cells in each section randomly, as described by Morita *et al*., [(10)].

DATA ANALYSIS:

Data was analyzed using SPSS 11.5 computer program. Frequencies, means, independent T test, correlation, scatter graph and chi-squire test were calculated.

RESULTS:

A total of 40 samples of patients with esophageal tumors were investigated, 21 (52.5%) of them were malignant esophageal tumors and 19 (47.5%) were benign, table (1). The description of tumor grade among malignant samples revealed that well differentiated tumors in 10 (47.6%) samples, moderately differentiated tumors in 10(47.6%) samples, and poorly differentiated tumors in one sample (4.8%), table (2). The age of study population ranged between 14 to 89 years with mean age 55.9 years. Less than 39 years were 8 (20%) patients, 39-54 years were 9 (22.5%) patients, and older than 54 years were 23 (57.5%) patients, table (3). The description of sex showed that 19 (47.5%) were male and 21 (52.5%) were female, table (4). Malignant esophageal tumors revealed positive expression of ki67 in 19 (90.5%) samples, and negative expression in 2 (9.5%) samples, while benign tumors showed positive expression in 4 (21.1%) samples, and negative expression in 15 (78.9%) samples, (P= 0.000), table (5). The Ki67 index among samples (Mean ± Standard deviation (SD)), in malignant tumors was (40.21±18.94), while in benign tumors was (14.60±14.85), (p value=0.000), table (6). Association between Ki67 index and the grade of tumor showed that the mean of Ki67 index was (29.80±13.22) in grade I and (45.96±15.10) in grade II (P= 0.020), table (8). The AgNORs count per cell (Mean±SD) in malignant tumors was (5.13±1.17), while in benign tumors was (2.78±0.77), (P= 0.000), table (7). The mean AgNORs count in grade I and grade II was (4.36±0.480) and (5.65±0.79) respectively, (P= 0.001), table (8). Positive correlation was found between mean AgNORs count and Ki67 index over expression (P= 0.01, r= 0.783), graph (1).

Table (1): Distribution of sample among the study population:

Sample	Frequency	Percent
Malignant	21	52.5
Benign	19	47.5
Total	40	100

Table (2): Distribution of cancer grade among malignant esophageal tumor:

Tumor grades	Frequency	Percent
Well differentiated tumor	10	47.6%
Moderate differentiated tumor	10	47.6%
Poorly differentiated tumor	1	4.8%
Total	21	100%

Table (3): Distribution of age group among study population:

Age group	Frequency	Percent
Less than 39 years	8	20%
39 – 54 years	9	22.5%
More than 54	23	57.5%
Total	40	100%

Table (4): Distribution of sex among study population:

Sex	Frequency	Percent
Male	19	47.5%
Female	21	52.5%
Total	40	100%

Table (5): Immunohistochemical expression of Ki67 among the study samples:

Expression of Ki67			Positive	Negative	Total	P.value
Histopathology result	Benign	N	4	15	19	0.000
		%	21.1	78.9	100	
	Malignant	N	19	2	21	
		%	90.5	9.5	100	

Table (6): Relation between Ki67 index and histopathological results:

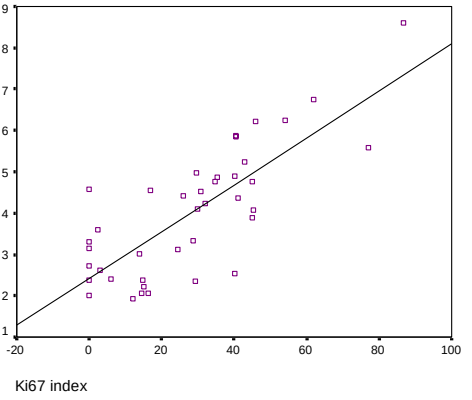
Sample	Mean	Standard deviation	P.value
Benign	14.60	14.85	0.000
Malignant	40.20	18.94	

Table (7): Relation between mean AgNOR and histopathological results:

Sample	Mean	Standard deviation	P.value
Benign	2.78	0.77	0.000
Malignant	5.13	1.17	

Table (8): Relation of Ki67 index and mean AgNOR with the grade of tumor:

Grade of tumor		Grade I	Grade II
Ki67 index	**Mean**	29.80	45.96
	SD	13.22	15.10
	P.value	0.02	
Mean AgNOR	**Mean**	4.36	0.48
	SD	5.65	0.79
	P.value	0.001	



Graph (1): shows strong positive Correlation between Ki67 indexes and mean AgNORs count (P= 0.01, r= 0.783).

DISCUSSION

Esophageal cancer is one of the most common cancers in the world because of its extremely aggressive nature and poor

survival rate [1]. The age of study population ranged between 14 to 89 years with mean age 55.9 years, most patient aggregating in the age more than 54 years representing 57.5% meaning that individual more than 50 years are more susceptible to esophageal cancer, this result is similar to study of Saeed *et al*., [3], they reported that the incidence of the esophageal cancer increase with aging particularly after 55 years.

Ki67 protein is a marker of cell proliferation, it is present in all periods of the cell cycle (G_1, S, G_2, and mitosis except for G_0), and provides a reliable method to characterize malignant tumors [7]. This study revealed that Ki67 index is higher in malignant tumor than in benign tumor, (40.21±18.94), (14.60±14.85) respectively, this result is similar to result observed by Khattab *et al*., [8], they reported that Ki67 index was significantly different between malignant and benign tumor.

This study showed that Ki67 index increase with high grade of tumor (29.80±13.22), in grade I and (45.96±15.10) in grade II, this result is consistent with the study of Huang *et al*., [13], they reported that there was significant correlation between Ki67 index and the histological grade of tumor.

AgNOR are argyrophilic protein seen as black intranuclear granules by silver stainig technique, it's number correlates with both cellular kinetics and ploidy [9], in this study the mean AgNORs count per cell increase in malignant tumor in comparison to benign tumor, (5.13±1.17), (2.78±0.77) respectively, this result is similar to result of Kuwano *et al*., [14], they reported that there was significant differences between mean AgNORs count in malignant and benign tumor.

The study revealed that mean AgNORs count increase with high grade of tumor, grade I (4.36±0.48) and grade II (5.65±0.79), this finding is similar to result of Siddique *et al*., [15], they reported that there was significant correlation between mean AgNORs count and histological grade of tumor, this

finding is inconsistent with the study of Morita *et al*., [10], they reported that there is no significant correlation between mean AgNORs and tumor grade.

The study showed that there was strong positive correlation between mean AgNORs count and Ki67 over expression (P= 0.01, r= 0.783), same result observed by Kakeji *et al*., [16], they reported that there was a significant correlation between the Ki67 index and mean AgNORs count.

CONCLUSION

On basis of the results this study conclude that Ki67 index and mean AgNORs are higher in malignant rather than benign tumors and increase with high grade of tumors.

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