MATERIAL AND METHODS

This is a retrospective study performed upon selected formalin-fixed, paraffin-embedded biopsy specimens from 70 different lung and pleural lesions designated as; 30 cases of adenocarcinoma of the lung (18 cases were excisional biopsy, and 12 cases were bronchoscopic biopsy), 20 cases of squamous cell carcinoma of the lung (one case was excisional biopsy, and 19 cases were bronchoscopic biopsy), and 20 cases of epithelioid mesothelioma of the pleura (all of them were open pleural biopsy). Ten cases of apparently normal bronchial tissue were taken as positive control for MUC4 IHC expression.

The material included archival formalin-fixed, paraffin-embedded blocks processed during the years from 2011 to 2019. Blocks were collected from Department of Pathology; Benha faculty of medicine, and Early Cancer Detection Unit of Benha University Hospital. Clinicopathological data were collected from the files of patients. The study was approved by the Ethical committee of faculty of Medicine, Benha University.

From each block, two sections were obtained. One section for histopathological study using hematoxylin and eosin (H&E) and the other section was cut on positive charged slide for immunohistochemical stain.

(A) Histopathological study:

a) In lung adenocarcinoma cases:

1-Determining the histological subtypes of lung adenocarcinoma:

Each case of adenocarcinoma of the lung was assessed regarding the histopathological type according to WHO 2015 **(Tazelaar and Lilenbaum., 2019)**

- Lepidic adenocarcinoma.
- Acinar adenocarcinoma.
- Papillary adenocarcinoma.
- Solid adenocarcinoma.
- Invasive mucinous adenocarcinoma**.**

2- Determining the grade of lung adenocarcinoma:

Grading is based on conventional histological criteria. Grades 1, 2, and 3 refer to well-, moderately, and poorly differentiated tumors, respectively **(Yasukawa et al., 2019).**

3-Determining the stage of lung adenocarcinoma:

Lung adenocarcinoma cases were staged according to TNM staging system into stage I, II, III, IV **(Goldstraw et al., 2016).**

TNM staging was applied only to 18 cases of lung adenocarcinoma which were excisional biopsy, while the other 12 cases were bronchoscopic biopsy, so can't be staged.

b) In lung squamous cell carcinoma cases:

1- Determining the grade of lung squamous cell carcinoma:

The grading system defined the different grades as follow; grade 1 (well differentiated) in tumor with 75-100 % keratinized cell nests, grade 2 (moderately differentiated) in tumor with 50-75% keratinized cell nests, grade 3 (poorly differentiated) in tumor with 25-50% keratinized cell nests **(Kadota et al., 2014).**

2-Determining the stage of lung squamous cell carcinoma:

Squamous cell carcinoma of the lung cases were staged according to TNM staging system into stage I, II, III, IV **(Goldstraw et al., 2016).**

TNM staging was applied only to one case of lung squamous cell carcinoma which was excisional biopsy, while the other 19 cases were bronchoscopic biopsy, so can't be staged.

c) In pleural epithelioid mesothelioma cases:

1-Determining the grade of pleural epithelioid mesothelioma:

According to Three-tier Nuclear Grading System; the nuclear grading system based on the two independent prognostic factors: nuclear atypia and mitotic count. For nuclear atypia, tumors were scored as 1 for mild atypia (uniform small nuclei with inconspicuous nucleoli), 2 for moderate atypia (nuclei intermediate in size with variability in shape and variably prominent nucleoli), and 3 for severe atypia (bizarre, enlarged nuclei, multinucleation, and macronucleoli in >5% of tumor cells) **(Habougit et al., 2017).**

For mitotic count, tumors were scored as 1 for low (0−1/10 HPF), 2 for intermediate (2−4/10 HPF), and 3 for high (≥5/10 HPF) **(Alì et al., 2018).**

A total score was done by the summation of the two-parameter scores, ranging from 2 to 6. The score was simplified into a three-tier grade: grade I for total scores 2 or 3, grade II for total scores 4 or 5, and grade III for a total score 6 **(Rosen et al., 2018).**

2-Determining the stage of pleural epithelioid mesothelioma:

Pleura epithelioid mesothelioma cases were staged according to TNM staging system into stage I, II, III, IV **(Proto et al., 2019).**

TNM staging was applied to all 20 pleural epithelioid mesothelioma cases which were open pleural biopsy.

(B) Immunohistochemical study:

For immunohistochemical study, anti MUC4 immunostaining was performed for all biopsies, using Avidin-Biotin complex technique **(Hsu et al., 1981)**.

Sections were incubated with the diluted primary polyclonal antibody (1:50) for MUC4 **(*Chongqing, 400039, China*)** overnight.

Positive external control:

Apparently normal bronchial epithelium was used as positive control **(Mawas et al., 2018)**

Negative control:

Omitting the step of the primary was done and replacing it with normal rabbit serum IgG.

Interpretation of MUC4 expression:

Slides were screened on low power, MUC4 expression was detected as cytoplasmic or cytoplasmic and membranous homogeneous brown coloration. Immunoreactivity was assessed by evaluating the extent and intensity of the stained cells **(Rokutan-Kurata et al., 2017) (Mawas et al., 2018).**

As regard for the extent of staining, percentage of positive cells was scored as: 0=no positive cells, 1= <10% of positive cells, 2= 10-50% positive cells, 3=51- 80% positive cells, 4=>80% positive cells. Intensity of staining was scored as: 0= no color reaction, 1= mild reaction, 2= moderate reaction, 3=intense reaction **(Abidullah et al., 2019).**

An immunoreactive score (IRS) which consecrate a range of 0-12 was calculated by multiplication of percentage of positive cells score (0-

4), and intensity of staining score (0-3). Then Immunoreactivity was assessed according to IRS score as following **(Abidullah et al., 2019)**:

- **Negative**: IRS score was 0-1.
- **Mild positive**: IRS score was 2-3.
- **Moderate positive:** IRS score was 4-8.
- **Strong positive**: IRS score was 9-12.

Table (6): IRS and IRS-Classification Scoring System (Abidullah et al., 2019).

Percentage of positive cells (0-4)	Intensity of staining (0-3)	IRS Score (Multiplication of A and B)
0= no positive cells 1= < 10% of positive cells 2= 10-50 % positive cells 3= 51-80 % positive cells 4= > 80% positive cells	0= no color reaction 1= mild reaction 2= moderate reaction 3= intense reaction	0-1 = negative 2-3= mild 4-8= moderate 9-12= strongly positive

(C) Statistical analysis

All statistical analyses were carried out using Statistical Package for the Social Science (SPSS) program, version 16 for Microsoft windows, on a personal computer. The Pearson correlation coefficient was used for statistical analysis. P value of 0.05 or less was considered to be statistically significant, and highly statistically significant when it was <0.01.

(D) Sensitivity and specificity

The following calculations were performed to determine MUC4 Diagnostic specificity and sensitivity**: (McNamara and Martin, 2018).**

- Sensitivity = $\frac{\text{True positive}}{\text{True positive+False negative}}$
- Specificity = $\frac{\text{True negative}}{\text{True negative+False positive}}$
- Positive predictive value = $\frac{\text{True positive}}{\text{True positive+False positive}}$
- Negative predictive value = $\frac{\text{True negative}}{\text{True negative+False negative}}$
- Accuracy rate =

$$\frac{\text{True positive} + \text{True negative}}{\text{True positive} + \text{False positive} + \text{True negative} + \text{False negative}}$$