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Original article

Significance of MUC4 in Epithelioid Mesothelioma of the Pleura, Adenocarcinoma and Squamous Cell Carcinoma of the Lung (Immunohistochemical Study)

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Abstract:

Background: Lung cancer is the second most common cancer, and the leading cause of cancer mortality. Malignant pleural mesothelioma is a highly aggressive malignant neoplasm, and epithelioid mesothelioma is the commonest histological subtype. MUC4 is a high-molecular weight glycoprotein that encoded by MUC4 gene. **Aim**: The aim of this study was to evaluate MUC4 expression in LAC, LSqCC, and PEM. **Materials and Methods**: This retrospective study was done upon 70 different lung and pleural lesions designated as; 30 cases of LAC, 20 cases of LSqCC and 20 cases of PEM. MUC4 immunostaining was done and assessed for each case. **Results:** There is high significant statistical correlation between MUC4 expression with LAC, LSqCC, and PEM cases (P value=0.0001). 90% and 95% of LAC and LSqCC respectively are positive. All PEM cases are negative. The sensitivity and specificity of MUC4 in differentiating PEM from lung cancer including LAC and LSqCC are 92% and 100% respectively. Significant statistical correlation is found between MUC4 and histological subtype (P value < 0.05), grade (P < 0.01), and TNM stage (P < 0.05) of LAC. Significant statistical inverse correlation is found between MUC4 expression and grade of LSqCC (P value < 0.05). **Conclusions:** MUC4 IHC can differentiate PEM from LAC and LSqCC. MUC4 expression increases with aggressive LAC, and increases with well-differentiated LSqCC.

Keyword: lung cancer, pleural mesothelioma, MUC4.

Abbreviations: Lung adenocarcinoma (LAC), Lung squamous cell carcinoma (LSqCC), Pleural epithelioid mesothelioma (PEM).

Introduction:

Lung carcinoma is the second most common cancer and the leading cause of cancer mortality worldwide, comprising almost 20% of all cancer deaths **(1).** In Egypt, lung cancer ranks the 5th among all cancer, and according to the National Cancer Institute's Surveillance, primary lung tumors represented 2.23% of all primary malignant tumors diagnosed during the period 2000-2011 **(2).** Lung cancer is more common in males than females, and most people diagnosed at the age of 65 years or older. The main risk factor of lung malignancy is tobacco smoking **(1).**

Malignant mesothelioma is an aggressive neoplasm. In US, about 3,000 cases of mesothelioma are diagnosed each year **(3).** In Egypt, pleural mesothelioma is the most frequent primary pleural malignant tumor, and epithelioid subtype is the most common subtype forming 76.20% of all mesotheliomas **(2).** It occurs commonly in men due to more asbestos exposure **(4).** The median age at diagnosis of mesothelioma is 72 years **(5).**

Some of peripheral LAC or LSqCC present with pleurotropic growth like mesothelioma. The prognosis and management of epithelioid mesothelioma differ from lung carcinoma, so rapid correct diagnosis of mesothelioma is important **(6).**

MUC4 is a high-molecular weight glycoprotein which encoded by MUC4 gene. It consists of beta subunit which is ErbB2 ligand, and alpha subunit that is glycosylated and causes anti-adhesion effect to the cell, causing cell-cell and cell–matrix detachment **(7).** Expression of MUC4 was detected normally in respiratory tract epithelium, glandular epithelium of digestive tract, cervix, and prostate **(8).**

The aim of this study is to evaluate the IHC expression of MUC4 in LAC, LSqCC and PEM, and correlate the results with clinico-pathological data, to clarify its diagnostic and prognostic role.

Material and Methods:

Study Groups:

This study was conducted retrospectively on selected formalin-fixed, paraffin-embedded biopsy specimens from 70 different lung and pleural lesions designated as; 30 cases of LAC (18 cases were excisional biopsy, and 12 cases were bronchoscopic biopsy), 20 cases of LSqCC (one case was excisional

biopsy, and 19 cases were bronchoscopic biopsy), and 20 cases of PEM (all of them were open pleural biopsy). Cases were obtained through collection of archived formalin fixed, paraffin embedded blocks from Department of Pathology; Benha faculty of medicine, and Early Cancer Detection Unit of Benha University Hospital, during the period from 2011 to 2019. Cases were selected on basis of availability of demographic data and clinicopathological data. This research plan was approved by ethical committee.

A- Histopathological Examination:

Hematoxylin and eosin-stained slides on all cases were revised by two observers simultaneously to confirm the diagnosis and to classify the lesions. LAC cases were classified according to WHO 2015 into lepidic, acinar, papillary, solid, and invasive mucinous adenocarcinoma (IMA) **(9),** and were graded to well, moderate, and poorly-differentiated tumors depending on conventional histological criteria **(10).** LSqCC cases were graded to well, moderate, and poorly-differentiated tumors according to percentage of keratinization. **(11)** LAC and LSqCC cases were staged by TNM staging to stage I, II, III, IV **(12),** and staging was applied only to 18 cases of LAC and one case of LSqCC which were excisional biopsies. PEM cases were graded as grade I, II, and III according to Three-tier Nuclear Grading System depending on nuclear atypia and mitotic count **(13)** and were staged by TNM staging to stage I, II, III, IV **(14).**

B-Immunohistochemical Procedure:

According to manufacture instructions, 3-4 micron tissue sections were obtained from formalin-fixed, paraffin-embedded tissue blocks on coated slides. After xylene deparaffinization, the sections were rehydrated in descending grades of alcohol then in distilled water. Antigen retrieval was done by using 10 mmol/L citrate monohydrate buffers (pH 6.0) and heated for 15 minutes in microwave. The endogenous peroxidase activity was inactivated by incubation in 3% hydrogen peroxide (H2O2) for 15 minutes then washing by distilled water. Slides then were immunostained for MUC4 polyclonal antibody ***(Chongqing, 400039, China)*)** at a dilution of 1:50, at room temperature overnight. Immunodetection was executed using a standard labeled streptavidin-biotin system (***Dako Cytomation, Denmark, A/S).*** Immunoreaction was seen by adding DAB as a chromagen. Counterstaining of slides

was done with Mayer hematoxylin for 1-2 minutes and dehydrated in ascending alcohol. The slides were cleared in xylene for three changes and cover slides were applied.

Negative & positive controls:

Apparently normal bronchial epithelium at resected surgical margins for lung carcinoma cases was used as positive control for MUC4. For negative controls, omitting the primary antibody and replacing it with normal rabbit serum IgG.

Immunostaining evaluation:

MUC4 expression was detected as cytoplasmic or cytoplasmic and membranous homogeneous brown coloration. Immunoreactivity was assessed by evaluating the extent and intensity of stained cells **(15) (16).** As regard 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 colour reaction, 1 = mild reaction, 2 = moderate reaction, 3 = intense reaction. 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). **Table (1)** Then immunoreactivity was assessed according to IRS score as following: 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 **(17).** Expression of MUC4 was then correlated with histopathological data in studied cases.

Table 1: IRS and IRS-Classification Scoring System.

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

IRS: Immunoreactive score

Statistical analysis: Results were analyzed by SPSS (version 16) statistical package for Microsoft windows. The Pearson correlation coefficient was used for statistical analysis. P value <0.05 was considered statistically significant, and highly statistically significant when it was <0.01.

Results:

1-Clinical result:

Significant statistical correlation is found between smoking and different histological types of studied cases (P value < 0.05). No significant statistical correlation is found between gender and different histological types of studied cases (P value > 0.05) as shown in **table (2).**

Table 2: Correlation between different studied cases and clinical data:

Different studied cases	Mean age	
LAC	61 year	
LSqCC	61.6 year	
PEM	66.8 year	
	Gender	
	Male	**Female**
LAC	50%	50%
LSqCC	80%	20%
PEM	65%	35%
	P value = 0.2	
	Smoking status	
	Smoker	**Non smoker**
LAC	50%	50%
LSqCC	75%	25%
PEM	15%	85%
	P value = 0.03	

2-Histopathological result:

Table 3: Histopathological features of different histological types of studied cases

	Variable	No (%)
Histological subtypes Of LAC	**Acinar adenocarcinoma**	15 (50%)
	Lipidic adenocarcinoma	1 (3.3%)
	Papillary adenocarcinoma	4 (13.3%)
	Solid adenocarcinoma	6 (20%)
	Invasive mucinous adenocarcinoma	4(13.3%)
Grade of LAC	**Grade II**	21 (70%)
	Grade III	9 (30%)
TNM stage of LAC	**Stage I**	4 (22.2%)
	Stage II	6 (33.3%)
	Stage III	3 (16.7%)
	Stage IV	5 (27.8%)
Grade of LSqCC	**Grade II**	8 (40%)
	Grade III	12 (60%)
Grade of PEM	**Grade I**	5 (25%)
	Grade II	10 (50%)
	Grade III	5 (25%)
TNM stage of PEM	**Stage I**	0 (0%)
	Stage II	8 (40%)
	Stage III	11 (55%)
	Stage IV	1(5%)

N.B: As regard LSqCC, only one case was excisional biopsy and it was stage I, while the other 19 cases were bronchoscopic biopsy so TNM staging cannot be applied.

Table 4: Correlation between histopathological subtype and TNM stage of LAC

Histopathological subtype of LAC	Total	TNM stage of LAC			
		Stage I	Stage II	Stage III	Stage IV
	NO. (%)	NO. (%)	NO. (%)	NO. (%)	NO. (%)
Acinar	9 (50%)	3 (33.3%)	3 (33.3%)	1 (11.1%)	2 (22.2%)
Papillary	3 (16.7%)	1 (33.3%)	2 (66.7%)	0 (0%)	0 (0 %)
Solid	2 (11.1%)	0 (0 %)	0 (0 %)	1 (50 %)	1 (50 %)
Lipidic	1 (5.5%)	0 (0 %)	1 (100%)	0 (0%)	0 (0 %)
Invasive mucinous	3 (16.7%)	0 (0 %)	0 (0%)	1 (33.3%)	2 (66.7%)
Total	**18 (100%)**	**4 (22.2%)**	**6 (33.3%)**	**3 (16.7%)**	**5 (27.8%)**
		P Value = 0.03			

There is a significant statistical correlation between histopathological subtype and TNM stage of the LAC (P value< 0.05). No significant statistical correlation is found between grade of LAC and histopathological subtype or between grade and TNM stage of LAC (P value >0.05).

3-Immunohistochemical results:

There was high significant statistical correlation between MUC4 expression and different studied types (P value = 0.0001), as all cases of PEM were negative, while 90%, 95% of LAC and LSqCC respectively were positive. Regarding LAC, MUC4 expression is significantly statistical correlated with histological subtype, grade, and TNM stage (P value < 0.05), while in LSqCC, there is significant statistical inverse correlation between MUC4 expression and grade (P value < 0.05). **Table (5) (Figure 1; a, b, c, d)**

Table 5: Correlation between MUC4 and different clinicopathological variables of studied cases

Clinico-pathological variants		MUC4 expression				
		Negative	**Mild**	**Moderate**	**Strong**	**P value**
Studied cases	**Lung adenocarcinoma**	10%	36.7%	20%	33.3%	0.0001 **Highly significant**
	Lung squamous cell carcinoma	5%	45%	35%	15%	
	Pleural epithelioid mesothelioma	100%	0%	0%	0%	
Histological subtype of lung adenocarcinoma	**Acinar adenocarcinoma**	20%	46.7%	13.3%	20%	<0.05 **Significant**
	Papillary adenocarcinoma	0%	75%	25%	0%	
	Solid adenocarcinoma	0%	0%	0%	100%	
	Lipidic adenocarcinoma	0%	0%	100%	0%	
	Invasive mucinous adenocarcinoma	0%	25%	50%	25%	
Grade of lung adenocarcinoma	**Grade II**	14.3%	47.6%	28.6%	9.5%	0.0001 **Highly significant**
	Grade III	0%	11.1%	0%	88.9%	
TNM stage of lung adenocarcinoma	**Stage I**	25%	75%	0%	0%	<0.05 **Significant**
	Stage II	0%	50%	33.3%	16.7%	
	Stage III	0%	66.7%	0%	33.3%	
	Stage IV	0%	20%	40%	40%	
Grade of lung squamous cell carcinoma	**Grade II**	0%	12.5%	62.5%	25%	<0.05 **Significant**
	Grade III	8.3%	66.7%	16.7%	8.3%	

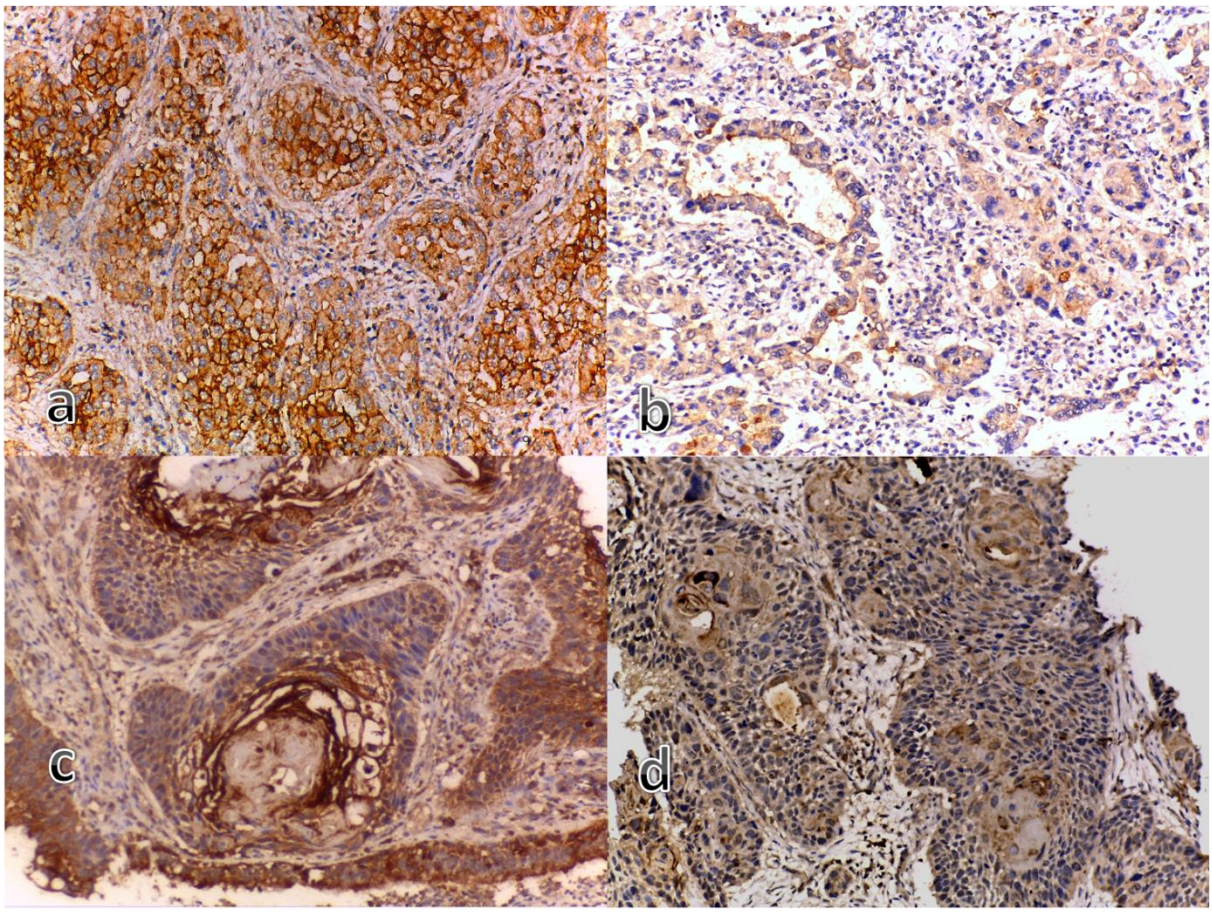

Figure (1): a) Lung adenocarcinoma, solid subtype, grade III, showing strong positive MUC4 IHC, with cytoplasmic and membranous expression. (ABC X 200). b) Lung adenocarcinoma, acinar subtype, grade II, showing moderate positive MUC4 IHC, with cytoplasmic and membranous expression. (ABC X 200). c) Lung squamous cells carcinoma; grade II, showing strong positive MUC4 IHC, with cytoplasmic and membranous expression. (ABC X 200). d) Lung squamous cells carcinoma; grade II, showing moderate positive MUC4 IHC, with cytoplasmic and membranous expression. (ABC X200)

Discussion:

Lung cancer is the second most common cancer and leading cause of cancer mortality worldwide. In Egypt, primary lung tumors represented 2.23% of all primary malignant tumors. **(1) & (2).** Malignant pleural mesothelioma is an aggressive malignant tumor**.** In US, about 3,000 new cases of mesothelioma are diagnosed each year. In Egypt, pleural mesothelioma is the commonest primary pleural malignancy forming 53.08%. **(2) & (3)**.

This retrospective study was done on 70 different lung and pleural cases including LAC, LSqCC, and PEM. MUC4 was immunohistochemically stained and evaluated for each case. Then its expression

was correlated with different clinical and histopathological variables.

Regarding age distribution in our study, the mean age of LAC, LSqCC, and PEM cases was 61, 61.6, 66.8 year respectively. Maximum age group for LAC and LSqCC was in the seventh decade, while for PEM, it was in the eighth decade. This is close to a study reported that the mean age of lung cancer was 60 years **(18)**. Other study reported that mesothelioma incidence increases in the ≥75-year age group **(19)**. This may be explained by that mesothelioma occurs after latency periods range from 20 to more than 50 years after asbestos exposure **(20)**.

In our study, there was equal gender distribution in LAC, while 80% of LSqCC cases showed male predominance. Other study explained male predominance in LSqCC could be due to majority of smoker patients are men **(21)**. 65% 0f pleural epithelioid mesothelioma cases showed a male predominance. Male predominance in pleural mesothelioma is explained by occupational exposure to asbestos in male **(22)**.

There was significant statistical correlation between smoking and different histological type of studied cases. 75%, 50%, and 15% of studied LSqCC, LAC, and PEM cases respectively were smokers. This runs parallel with a study reported that smoking is the main cause of lung carcinoma **(23)**.

In our study, there was a significant statistical correlation between histopathological subtype and TNM stage of LAC (P value = 0.03). The acinar, papillary, and lipidic subtypes associated with stage I and II, while solid and mucinous subtype associated with advanced stage (Stage III and IV). This is close to a study in which nodal metastasis was high in solid adenocarcinoma and distant metastasis was more in mucinous and solid types, while acinar, lipidic, and papillary subtypes have lower nodal and distant metastasis risk **(24)**. Some studies reported that invasive mucinous type presented with advanced stage **(25) (26)**. Other study reported that solid adenocarcinomas were significantly correlated with advanced stage, and lipidic subtype had low TNM stage **(27)**.

In contrast, other study reported that invasive mucinous subtype was associated with low stage **(28)**. This could be explained by that invasive mucinous is not aggressive neoplasm as it has common KRAS mutation, but rare TP53 mutation, and low mutation burden overall **(29)**.

MUC4 is a high-molecular weight glycoprotein which encoded by MUC4 gene which is located at chromosomal locus 3q29. Beta subunit acts as a ligand for HER2 **(30).** HER2 binds to EGF-like domain of MUC4 then activates AKT, MAPK, and ERK pathways causing cell proliferation, survival, and invasion **(7).**

The current study evaluated MUC4 expression in LAC, LSqCC, and PEM aiming to evaluate its diagnostic role in distinguishing PEM from LAC and LSqCC, and evaluate the relation between MUC4 expression and aggressiveness of LAC, and LSqCC.

Our study revealed a highly significant statistical relation between the MUC4 expression and different histological subtype of studied cases (P value=0.0001). Out of the studied LAC cases, 90% were positive, while 95% of studied LSqCC cases were positive. All studied cases (100%) of PEM were negative.

This result runs parallel to a study that described the value of MUC4 immunostaining for differentiation between reactive mesothelial cell and metastatic adenocarcinoma of variable primary sites including lung adenocarcinoma in pleural and peritoneal effusions. 88.4% of metastatic lung adenocarcinoma cases were positive MUC4, while only 9.8% of reactive mesothelial cells cases were positive **(31)**.

This study is parallel to a study reported that MUC4 can differentiate epithelioid mesothelioma from LAC or LSqCC, as MUC4 expression was positive in 83.3% of lung adenocarcinoma, 89.3% of LSqCC, and 0% of PEM **(16)**.

This can be explained by that the mesothelial cells, which have mesodermal origin, cannot express apomucins, which expressed in adenocarcinoma of variable origins and normal tissues which arise from endoderm **(32)**.

This current study showed significant statistical correlation between different histological subtype of LAC and MUC4 expression (P value < 0.05). 100% of solid cases were strong positive for MUC4, while the mild positivity was detected in 46.7% of acinar and 75% of papillary subtypes. This can be explained by that MUC4 inhibits cell differentiation which mediated by MUC4 induced ErbB2 and ErbB3 phosphorylation. This finding runs parallel to a study which reported that MUC4 tend to be positive in solid adenocarcinoma **(15)**.

In our work, there was highly significant statistical correlation between the

histological grading of LAC and MUC4 expression (P value= 0.0001). 47.1% of grade II lung adenocarcinoma were mild positive, while 88.9% of grade III lung adenocarcinoma were strong positive. This finding runs parallel to a study which reported that MUC4 expression increases with poorly differentiated adenocarcinoma **(15).**

The current study showed significant statistical inverse correlation between the histological grading of LSqCC and MUC4 expression (P value <0.05) as MUC4 expression was lower in grade III LSqCC. This runs parallel with a study which reported that, MUC4 was highly expressed in well-differentiated squamous cell carcinoma cells located in the center of tumor nests and in squamous pearls, and weak positive or negative in periphery and less-differentiated cells of the nests **(33)**. Other study reported that MUC4 gene expression during differentiation of cultured airway epithelial cells was high in well-differentiated cells compared to low-differentiated cells **(34)**.

Our current study showed significant statistical correlation between the TNM stage of LAC and MUC4 expression (P value < 0.05), as expression increased with high stage. This finding is close to a study which reported that over-expression of MUC4 was significantly correlated with invasion, nodal metastasis, and high stage **(35)**. While other study showed that MUC4 expression was insignificantly correlated with lung adenocarcinoma stage **(15)**.

The oncogenic role of MUC4 can be explained by that beta subunit acts as ligand for HER2, and when HER2 bind with EGF-like domain of MUC4, it activates AKT, MAPK, and ERK pathways causing cell proliferation, apoptosis inhibition, and invasion **(36)**. HER2 cause apoptosis inhibition by deactivation of protein Bad causing activation of antiapoptotic Bcl-XL and Bcl-2 **(37)**. MUC4 increases epithelial-to-mesenchymal transition, leading to tumor invasion **(38)**.

In contrast, a study revealed that lower MUC4 expression in NSCLC was associated with higher stage, and explained that MUC4 has tumor suppressor role in NSCLC as it inhibits proliferation and metastasis of lung carcinoma **(39)**. Also other study reported that MUC4 expression was significantly high in stage I and II lung adenocarcinoma, and lower expression was detected in adenocarcinoma with nodal metastasis **(33)**.

Such divergent results regarding MUC4 correlations with different variables

explained by that MUC4 may be an indicator for differentiation or mediator for tumor growth and aggressiveness. MUC4 is ligand for ErbB2, and it causes phosphorylation of ErbB2, and there are two pathways of ErbB2 tyrosine phosphorylation by MUC4 and prognostic role of MUC4 depends on which of the two pathways is working.

The first pathway: ErbB2-MUC4 complex phosphorylation may happen without heterodimerization of other ErbB2 receptor families, and with absence of other soluble ligand that causes phosphorylation of ErbB2 receptor tyrosine kinase only at special site 1248, that results in increasing p27 expression (cyclin dependent kinase inhibitor) leading to cell cycle arrest and promoting tumor differentiation, and this pathway associated with better prognosis.

The second pathway: Alternately MUC4 can complex with ErbB2 and form heterodimer with ErbB3 with presence of neurogulin. MUC4-ErbB2-ErbB3-neurogulin complex causes high degree phosphorylation of ErbB2 and ErbB3 leading to inactivation of p27 and activation of MAPK and PI3K dependent AKT pathway, leading to inhibition of differentiation and increasing cell proliferation causing progression and worse prognosis of tumors **(40)**.

Conclusion:

The present work reveals that MUC4 IHC may help to differentiate pleural epithelioid mesothelioma from lung adenocarcinoma and lung squamous cell carcinoma. The study reveals that MUC4 is a good negative marker for PEM. As regarding LAC, MUC4 expression increases with more aggressive tumors which have solid predominant histological pattern, high grade, and advanced stage, so it could be considered as an independent prognostic factors.

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