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Research Paper

Multi Egyptian Centers Study

Medical Science

KEYWORDS : Dickkopf protein 1 (DKK1), Bronchogenic carcinoma, Diagnosis

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ABSTRACT

Dickkopf protein 1 (DKK1), a secreted protein played an important role in cellular proliferation, differentiation, survival, apoptosis and cell motility Objective:-This study was conducted to evaluate diagnostic utility of Dickkopf-1level in bronchogenic carcinoma patients and its relation with clinicopathological parameters in those patients .Patients and methods:- Ninety patients thirty of them with benign lung diseases as bronchial asthma, their ages ranged between 40 and 76 years with a mean ± SD of 57.06 ± 10.35 years and rest were bronchogenic carcinoma patients their ages ranged between 31 and 84 years with a mean ± SD of 61.1 ± 10.51years, also fifteen healthy volunteer their age ranged between 41 and 68 years with a mean ± SD of 55.53±8.20 years were used as control group. All patients were subjected to full medical history including smoking status with laboratory investigation including determination of serum carcinoembryonic antigen by immunoassay using enzyme linked fluorescent assay and detection of serum Dickkopf-1using ELISA technique, also for each bronchogenic carcinoma patients histopathological classification of malignant tissues and clinical staging of patients were done Results:- There was a highly significant association of Dickkopf protein 1level with control, patients group, histopathological type and advanced stage of the disease (F was 8.8,6.2, 7.7 reversely and p value 0.001),when correlation coefficient was applied a positive correlation between DKK1 level and carcinoemberyonic antigen (r was 0.009 and p value 0.001) Sensitivity of carcinoemberyonic antigen was 36.7% in patient group and area under curve was 0.64 and 95% Confidence Interval was (0.48-0.81) but the Sensitivity of DKK1 was 73.3% and increased to 86.7% when we used DKK1togather with CEA in diagnosis of bronchogenic carcinoma while area under curve was 0.73 and 95% Confidence Interval was (0.58-0.88).Conclusion:-Dickkopf protein 1 (DKK1) could be considered as a valuable diagnostic marker in diagnosis of bronchogenic carcinoma either alone or in combination with currently used conventional marker.

Introduction

Lung cancer is the most common cause of cancer-related death in the United States. Based on estimates published by the Surveil - lance, Epidemiology, and End Results (SEER) database**, [1]**

Novel biomarkers are urgently needed for early diagnosis and predicting treatment response and prognosis bronchogenic carcinoma. Many types of samples are possible for this purpose, but cytological fluid from a NAB (percutaneous needle aspiration biopsy) is obtained directly from tumor tissue, therefore many biomarker candidates will be present in high concentrations **[2].**

Dickkopf protein 1 (DKK1) , a secreted protein, is a known negative regulator of the Wnt signalling pathway, which plays an important role in a variety of cellular processes, including proliferation, differentiation, survival, apoptosis and cell motility **[3].**

Dickkopf protein 1 (DKK1) gene was frequently hyper methylated in colorectal cancers, whereas over expression of DKK-1 reduced the growth of colorectal carcinoma cells**.** The expression of DKK-1 is also reportedly down regulated in melanoma cells **[4].**

Patients and methods

This study had been carried out at inpatient and outpatient clinic of chest department of Aswan university, hematology/ oncology unit of internal medicine department, clinical pathology department, faculty of medicine, zagazig university and zagazig insurance, chest hospitals in period between January 2012 and January 2015, ninety patients thirty of them with benign lung diseases as bronchial asthma, their ages ranged between 40 and 76 years with a mean ± SD of 57.06 ± 10.35 years and rest were bronchogenic carcinoma patients their ages ranged between 31 and 84 years with a mean ± SD of 61.1 ± 10.51years. All patients were subjected to full medical history including smoking status with laboratory investigation including determination of serum CEA by immunoassay on VIDAS immunoanalyzer (Bio MERIEUX) using enzyme linked flurocent assay and detection of serum Dickkopf-1(DKK-1) using ELISA technique, also for each bronchogenic carcinoma patients histological classification of malignant tissues and clinical staging of patients according to TNM classification . All patients given well informed consent with approval of local ethical committee

Method of assay

1. 100 ul of standards, quality control, dilution buffer (= blank) and diluted samples were pipetted into the appropriate wells.
2. The wells were incubated at room temperature (25°C) for 1 hour, shaking at 300 rpm on an orbital micro plate shaker.
3. The wells were washed 5-times with wash solution. After final wash .the plate was inverted and taped strongly against paper towel.
4. 100 ul of biotin labelled antibody solution was added into each well.
5. The wells were incubated at room temperature (25°C) for 1 hour, shaking at 300 rpm on an orbital micro plate shaker.
6. The wells were washed 5-times with wash solution. After final wash, invert and tap the plate strongly against paper towel.
7. 100 ul of Streptavidin-HRP Conjugate was added into each well.
8. The plate was incubated at room temperature (25°C) for 30 min, shaking at 300 rpm on an orbital micro plate shaker.
9. The wells were washed 5-times with wash solution. After final wash, the plate was inverted and taped strongly against paper towel.
10. 100 ul of substrate solution was added into each well. Wells were protected from exposure to direct sunlight by covering

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the plate with aluminum foil.

11. The plate was incubated for 10 minutes at room temperature. The incubation time may be extended up to 20 minutes if the reaction temperature was below than 20°C. Shaking the plate was avoided during the incubation.
12. The colour development was stopped by adding 100 ul of stop solution.
13. The absorbance of each well was determined using a micro plate reader set to 450 nm with the reference wavelength set to 630 nm .The absorbance was read within 5 minutes following step 12. The steps are shown in figure (1).

Standard curve was constructed by plotting the mean absorbance (Y) for standards against the known concentration (X) of standards in logarithmic scale and results were reported as concentration of DKK-1 ng/ml in samples

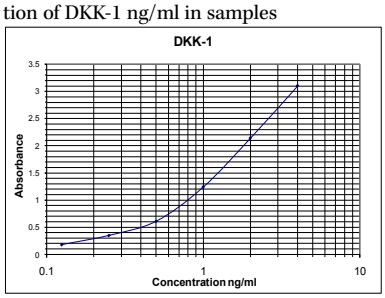

Figure (1) Standard curve for Calculation of Dickkopf-1concentration

Statistical analysis

Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data of scores were expressed as mean and standard deviation or median and range as appropriate. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For not normally distributed quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric t-test). Spearman-rho method was used to test correlation between numerical variables. P value less than0.05 was significant

Results

Ninety patients thirty of them with benign lung diseases as bronchial asthma, their ages ranged between 40 and 76 years with a mean ± SD of 57.06 ± 10.35 years and rest were bronchogenic carcinoma patients their ages ranged between 31 and 84 years with a mean ± SD of 61.1 ± 10.51years, also fifteen healthy volunteer their age ranged between 41 and 68 years with a mean ± SD of 55.53±8.20 years were used as control group, sixty eight of patients them were male and rest were female .(table,1)

Table (1): Demographic and clinical characteristics of studied groups:

variable	N	%	Test	P value
Age Control group (41-68) 55.53±8.20	15	30%		
Patients group Benign (40-76) 57.06±10.35 Bronchogenic carcinoma (31-84) 60.1±10.51	30 60	30% 60%	F 1.2	0.31
Sex				
Male Control group	12	80%		
Patients group Benign Bronchogenic carcinoma	24 44	80% 73.3%		
Female Control group Patients group Benign Bronchogenic carcinoma	3 6 16	20% 20% 27.7%	X^2 0.3	0.518

There was a highly significant association of DKK1 level with control, patients group, histopathological type and advanced stage of the disease (F 8.8,6.2, 7.7 reversely and p value 0.001) (table 2)

Table (2): DKK-1 values in different demographic and clinical characteristics in patients group

	N=90	DKK-1 (mean ± SD)	Test of Significance	P
Sex Male Female	 44 16	 3.42±1.81 4.60±2.15	 t=1.50	 0.14
Control group **Patient group** Benign Bronchogenic carcinoma	 15 30 60	 1.94±0.61 2.39±0.84 3.73±1.94	 F =8.8	 0.001*
Smoking Nonsmokers Smokers	 22 38	 4.24 ±2.10 3.44 ±1.84	 t=1.09	 0.28
MALIGNANT HISTOPATHOLOGY Adenocarcinoma Squamous cell carcinoma Small cell lung cancer	 28 20 12	 3.65 ±2.06 2.99 ±2.07 1.49 ±1.71	 F=6.2	 0.001*
Stage IIIA IIIB IV	 10 16 34	 1.52 ±0.47 3.24 ± 1.0 4.63 ±1.97	 F=7.7	 0.001 *

Correlation coefficient was applied and positive correlation between DKK1 level and carcinoemberyonic antigen (r was 0.009 and p value 0.001) (figure 1)

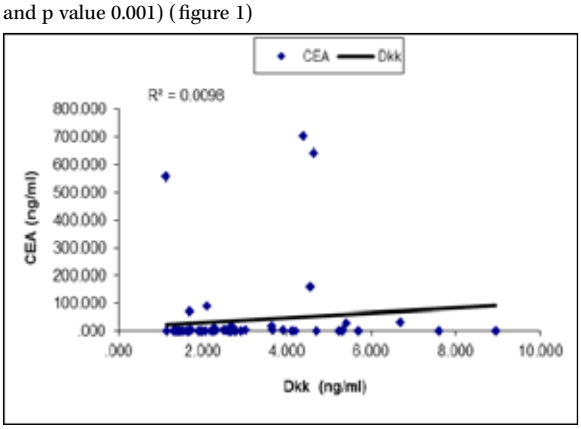

Figure (1): correlation of DKK-1 with CEA (r + 0.009 and p value 0.001)

Sensitivity of carcinoemberyonic antigen was 36.7% in patient group and area under curve (AUC) was 0.64 and 95% Confidence Interval (95%CI) was (0.48-0.81) but the Sensitivity of DKK1 was 73.3% and increased to 86.7% when we used DKK1togather with CEA in diagnosis of bronchogenic carcinoma while area under curve (AUC) was 0.73 and 95% Confidence Interval (95%CI) was (0.58-0.88). (Table 2.3 .4 figure 2.3.4)

Table (3): Diagnostic Performance of CEA in diagnosis of patients group (cut off 5ng/ml):

CEA	Benign disease n=30	Bronchogenic carcinoma n=60	Sensitivity	specificity	positive predictive value	negative predictive value	kappa	P*
<5 ng/ml	15	22	36.7%	100%	100%	61%	0.27	0.007
≥5ng/ml	0	38						

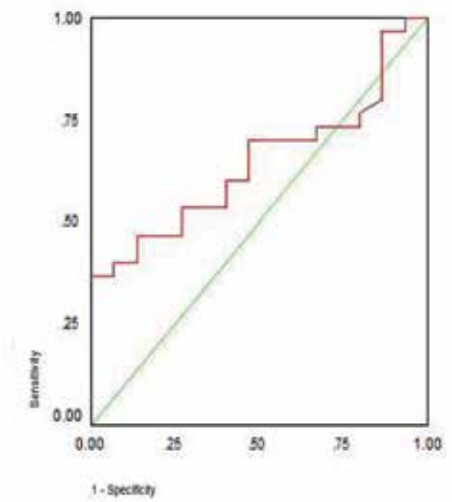

Figure. (2): ROC curve of CEA in diagnosis of patient group. Area under curve (Auc) is 0.64 and 95% Confidence Interval (95%CI) is (0.48-0.81)

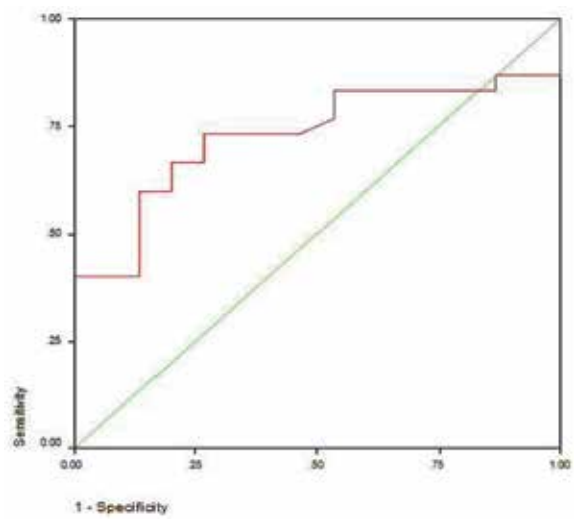

Figure. (3): ROC curve of DKK-1 in diagnosis of patients group. Area under curve (AUC) is 0.73 and 95% Confidence Interval (95%CI) is (0.58-0.88).

Table (4): Diagnostic performance of DKK-1 in diagnosis of patients group (cut off 2.5ng/ml):

DKK-1	Benign disease n=30	bronchogenic carcinoma n=60	Sensitivity	Specificity	positive predictive value	negative predictive value	kappa	P*
<2.5ng/ml	11	16	73.3%	73.3%	84.6%	57.9%	0.437	0.003
≥2.5ng/ml	4	44						

* Significant.

Table (4): Performance of combined CEA and DKK-1 in diagnosis of patients group

	Benign disease n=30	Bronchogenic carcinoma n=60	Sensitivity	Specificity	positive predictive value	negative predictive value	kappa	P*
DKK-1<2.5 ,**CEA**<5ng/ml	11	8	86.7%	73.3%	86.7%	73.3%	0.60	<0.001
DKK-1≥2.5 ,**CEA**≥5ng/ml	4	52						

*Significant.

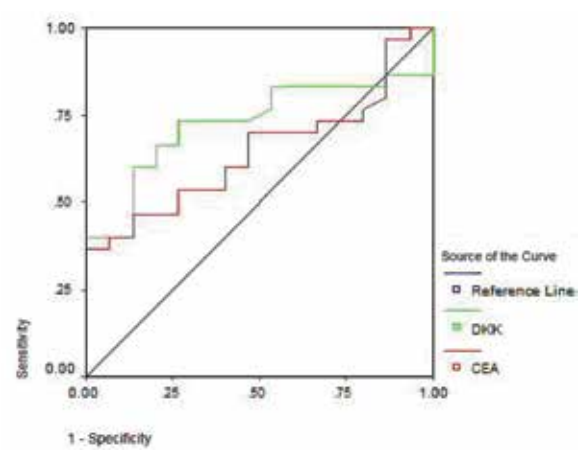

Figure. (4): Combined ROC curve of CEA and DKK-1 in diagnosis of patients group

Discussion

Lung cancer remains the most common cause of cancer-related death in the world. Based on estimates published by the Surveillance, Epidemiology, and End Results (SEER) database, 160,340 American men and women died of a malignancy of the lung or bronchus in 2012 .Deaths attributable to lung cancer accounted for 29% of all male-related cancer mortality and 26% of all female-related mortality in 2012 **[1][5]**.

The Wnt signaling pathways were a group of signal transduction pathways made of proteins that pass signals from outside of a cell through cell surface receptors to the inside of the cell. Three Wnt signaling pathways had been characterized: the canonical Wnt pathway, the Non canonical planar cell polarity pathway, and the Non canonical Wnt/calcium pathway played an important role in cancer **[6][7].**

The Wnt pathway played a role in development and in regulating adult stem cell systems, and aberrant activation of the pathway was a major trait of many human cancers, including colorectal cancer, melanoma, non-small cell lung cancer, leukemia, and

bladder cancer **[8].** A variety of cellular processes were mediated by Wnt signaling, including proliferation, differentiation, survival, apoptosis, and cell motility **[9]**.

The vast majority of lung cancers are directly attributable to cigarette abuse**,** furthermore, a substantial number of lung cancers in never-smokers are due to environmental tobacco exposure (passive smoking).So smoking is one of the strongest risk factors for the development of lung cancer**[10][11].**

Four cases (26.7%) of control group, twenty two cases (73.3%) of benign lung disease and thirty eight cases (63.3%) of cancer lung patients had positive smoking history and there was significant association between smoking history and benign or malignant lung disease when compared to control.

There was not any significant relation between DKK-1 results and age, sex and smoking status, while DKK-1 level significantly increased with advancement of cancer TNM stage and non-small cell lung cancer and these consistent with **Sheng et al;** and **Dong et al;** who reported that DKK-1 levels did not differ significantly with age ,sex but significantly associated with advanced stage and histologic types ,lymph node involvement and distant metastases . Patients with a high levels of DKK-1 tended to have shorter survival than patients with lower levels, indicating that high DKK-1 level was a marker of poor prognosis for patients with non-small cell lung cancer, also there was significant positive correlation between DKK-1 and CEA while **Yamabuki et al;** reported that there was not any significant association correlation between DKK-1 and carcinoemberyonic antigen (CEA**) [12][13][14].**

Carcinoembryonic antigen (CEA) was an onco-fetal protein considered to be involved in cell-cell recognition and modulate cellular processes **[15]** and this biomarker had been extensively studied in a variety of neoplasms, such as colorectal **[16]**,gastric **[17]**,esophageal**[18],** pancreatic **[19]** and breast cancer **[20]**.

In bronchogenic carcinoma serum CEA levels had been widely reported to be correlated with advanced disease, early relapse, pathological upstaging, poor therapeutic response and survival **[21[22].**

There was significant relation between its level either in heathy volunteer or patients with bronchogenic carcinoma and benign lung disease patients and had significantly higher CEA compared to healthy control group and this was consistent with Charalabopoulos et al; reported that serum CEA levels were significantly higher in malignant lung diseases compared to benign lung lesion and healthy persons and carcinoemberyoic antigen(CEA) concentrations were particularly high in adenocarcinoma and large cell lung cancer. Asymptomatic average-risk patients who present with raised CEA level should be reviewed by bronchoscope and radiological investigations for commonly-associated cancers, and there after followed up for at least two years, because up to 7.4% present with a subsequent malignancy **[23][24].**

Area under the curve at cutoff value 5 ng/ml showed that carcinoembryonic antigen had 36.7% sensitivity, 100% specificity in diagnosis of lung cancer,and this matched with Ishiguro et al., [25]

Okamura et al. reported that serum level of carcinoembryonic antigen was elevated and cut-off levels of 3.2 ng/mL with 69% sensitivity and 68% specificity **[26]**

Use of serum carcinoembryonic antigen in lung cancer was limited due to the facts that its elevation was common in conditions that frequently coexist in the population risk for lung cancer, especially in smokers and in a variety of benign pulmonary disease **[27].** The utility of estimation carcinoembryonic antigen was doubted for its low sensitivity for diagnosis and treatment of non-small cell lung cancer cases**.** So there was a need of other tumor marker to diagnose non-small cell lung cancer either alone or in combination with carcinoembryonic antigen. [28][29]

The Wnt signaling pathways were a group of signal transduction pathways made of proteins that pass signals from outside of a cell through cell surface receptors to the inside of the cell. Three Wnt signaling pathways had been characterized, the canonical Wnt pathway, the non-canonical planar cell polarity pathway, and the Non canonical Wnt/calcium pathway **[30]**

Wnts had a negative (DKK-1) and positive regulators, which acted either intra cellular to modulate components of the signal transduction machinery or extra cellular to modulate ligand receptor interactions. **[31].**

The expression of DKK-1 was also reportedly down regulated in melanoma cells **[32],** lung cancer cells with neuroendocrine differentiation **[33]** over expression of DKK-1 had also been found in some tumors, such as Wilms' tumor, hepatocellular carcinoma and ovarian adenocarcinoma **[34]** and gene expression profiles had revealed that DKK-1 was serving as a diagnostic serologic and prognostic biomarker **[14].**

DKK-1 was elevated either in benign lung disease or lung cancer when compared to heathy volunteer and cancer lung patients had significantly higher DKK-1 level and this data was consistent with Dong et al. who reported that levels of serum of DKK-1 were significantly increased in patients with bronchogenic carcinoma and high level of DKK-1 expression on tissue and serum level were associated with poor prognosis for patients with non-small cell lung cancer **[13]**.

Serum DKK-1 level was lower in patients with small cell lung cancer and early stage non-small cell lung cancer patients had reduction in serum DKK-1and DKK-1 level increased after therapy in non-small cell lung cancer patients and these might attributed to multifactorial. First, since these studies in different laboratories used different ELISA assays, some being commercially available and others being developed in house, it is possible that the assays are not measuring the same form of DKK-1 protein. Second, all serum DKK-1 studies were primarily based on a case/control study design, which can be heavily influenced by the potential mismatching of cases and controls in confounding variables. Therefore, spurious associations may be found in studies based on small number of samples and to avoid mismatching issues, longitudinal data provides more solid evidence **[35].**

Area under the curve of DKK-1 in diagnosis of bronchogenic carcinoma was 0.73 and 95% confidence interval was higher than of carcinoemberyonic antigen CEA and at cutoff value 2.5 ng/ml, DKK-1 had 73.3 % sensitivity, 73.3% specificity in diagnosis of bronchogenic carcinoma and its sensitivity was higher than that of CEA in diagnosis it and this matched with Yamabuki et al;and Sheng et al; while Lu et al**;** reported that DKK-1 sensitivity was 69.50% and specificity was 92.20% in the patients' serum with lung cancer but by combining the results of CEA and DKK-1 the sensitivity increased to 86.7 % in diagnosis of bronchogenic carcinoma **.[14][12][36]**

Conclusion

Dickkopf protein 1 (DKK1) could be considered as a valuable diagnostic marker in diagnosis of bronchogenic carcinoma either alone or in combination with currently used conventional marker**.**

Disclosure

There was no conflict to authors

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