

## P53 mRNA in- Situ Hybridization analysis and Immunohistochemical Expression in Lung Cancer: A Comparative Study.

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### Summary:

**Background and objectives:** P53 gene mutation and deletion are among the important molecular markers linked to lung cancer. In most cases, the inactivating mutations affecting both p53 alleles are acquired in somatic cells. Less commonly, the mutations are inherited ones. The aim of the present study was to analyze the frequency of having a wild and/or a mutated p53 gene in lung cancer compared to benign lung lesions and to relate these findings to different morphological types and grades of lung cancer.

**Patients, materials and methods:** In this retrospective study, the histopathology blocks of 30 lung cancer cases covering the period from 2002 to 2007 were obtained from the archives of the histopathology section of the Special Surgeries Hospital Laboratories. Twenty cases of non-malignant lung diseases served as a control group. Sections made on charged slides were subjected to p53 mRNA in-situ hybridization and p53 protein immunohistochemical staining.

**Results:** Positive p53 in situ hybridization signal was detected in 29 cases of carcinoma. The highest percentage score was score- 3 being detected in 16(53.3%) cases. High intensity of hybridization signal was seen in 17(56.7%) cases. All control cases revealed positive hybridization signals (100%). Seven cases revealed score-3 and of these 5 revealed high intensity of hybridization. Immunohistochemical expression of p53 protein was seen in 21(70%) cases of carcinoma with score-3 being found in 11(36.7%) cases, 7 cases revealed score-3 in situ hybridization signals as well. High intensity was found in 11(36.7%) cases, 10 of them showed high intensity of hybridization signal. Only two control cases (10%) revealed positive p53 expression. They showed score-2 and low intensity of expression. Significant statistical correlations were found between in situ hybridization signaling and immunohistochemical expression scores and intensities in carcinoma cases with p value < 0.05.

**Conclusion:** The relations of tumor grade to the score and intensity of ISH signaling and IHC expression were significant suggesting the importance of having higher scores and intensities of positive cells which is an indication of tumor progression and prognosis. Studying p53 gene integrity or expression of a mutated protein is important for predicting tumor prognosis and establishing a proper therapeutic approach.

**Keywords:** lung cancer, p53 immunohistochemistry, p53 in situ hybridization, p53 gene, p53 expression.

*Fac Med Baghdad  
2010; Vol. 52, No. 3  
Received: June 2010  
Accepted: June 2010*

### Introduction:

Lung cancer is regarded as the 1<sup>st</sup> common cancer in Iraqi males and the 2<sup>nd</sup> most common cancer in both sexes according to the latest Iraqi cancer registry. (1) Many molecular markers have been linked to lung cancer among them is the mutation in the cancer suppressor gene p53. (2) Mutations in p53 have been documented in more than 50% of human cancers. In most cases, the inactivating mutation affecting both p53 alleles are acquired in somatic cells. Less commonly, the mutation is an inherited one and the patient is a victim of Li-Fraumeni syndrome. (3) P53 gene is located on chromosome 17p13.1.(4) It encodes a 53 kilo Daltons protein localized in the nucleus.(5)

p53 protein is now widely held to be central to the cellular reaction to a variety of stressful stimuli such as DNA damage, hypoxia, heat shock, hypotension, sepsis and certain cytokines. These stimuli activate p53 protein which in turn switches on a series of cellular events that lead to cell cycle arrest or apoptosis. The major physiological role of p53 is to suppress the development of cancer.(6, 7) Deletion at the p53 locus often correlates with a point mutation in the other allele and this results in the total inactivation of the p53 suppressor gene, the most common molecular change in cancer patients.(8) Mutations or deletions of the p-53 gene may facilitate the transmission of a genetic damage and the emergence of neoplastic clones with a survival advantage.(9) Tumor cells with mutant p53 have high levels of the protein, to such an extent that immunohistochemical

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detection of p53 has been used as a surrogate for the presence of a p53 mutation. (4)Molecular methods are now available to study different genes and their expression including in-situ hybridization, PCR and microarrays. (10) The aim of the present study was to analyze the frequency of having wild and/or mutated p53 gene in lung cancer compared to benign lung lesions and to relate these findings to different morphological types and grades of lung cancer.

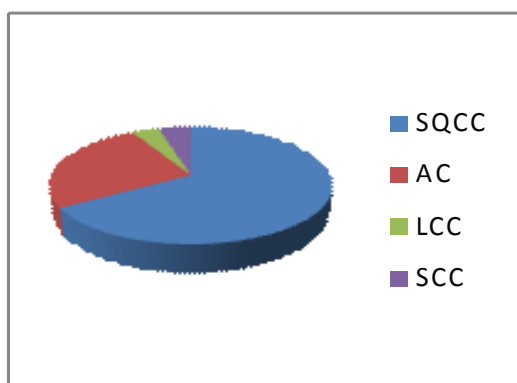
#### **Materials and methods:**

This is a retrospective study whereby randomly collected paraffin blocks of 30 lung cancer cases covering the period from 2002 to 2009 were recruited at the Histopathology section of the Special Surgeries Hospital laboratories. Twenty cases of non-malignant lesions served as a control group. Two new 4 micrometer sections were made on charged slides (Fisher brand) from each block. One of the sections was subjected to in-situ hybridization (ISH) analysis of p53 gene mRNA. The second was subjected to immunohistochemical (IHC) study to detect p53 protein. Biotinylated p53 cDNA probe together with ISH detection kit were purchased from Maxim biotech (USA). The kit also contains a biotinylated human house-keeping genome probe as a positive control. Monoclonal anti human p53 antibody (against mutant p53) and the detection kit were purchased from United States Biological (USA). Slide preparation for in-situ hybridization and immunohistochemistry involved their baking in a vertical position at 60 C for overnight, deparaffinization and rehydration at room temperature (RT) (25°C) by dipping slides in xylene, serial dilution of ethanol and de-ionized water. For ISH, after heating at 98° C in citric buffer, tissue sections were deproteinized using 1X proteinase-k enzyme solution for 10 minutes at RT. Slides were dehydrated at RT by sequential dipping in ethanol (70%, 95% and 100%) each for 1 minute and dried by incubation at 37°C for 5 minutes. P53 DNA probe was diluted to 8%, denatured at 95°C for 5 minutes and ice-quenched immediately. One drop of the probe was placed on the tissue section, covered by a cover slip and heated in an oven at 70° C for 10 minutes. Hybridization was carried out in a humidity chamber at 37°C for 3 hours, followed by soaking in 1X detergent wash, addition of RNase- A and washing with 3X protein block at 37°C for 3 minutes. The hybridized probe was detected by streptavidin-alkaline phosphatase (streptavidin-AP) conjugate. Upon addition of the substrate solution which is 5-brom-4 chloro-3 indolyl phosphate/Nitro blue tetrazolium (BCIP/NBT), an intense blue signal appeared at the specific site of the hybridized probe. Eosin was used as a counter stain. Slides were

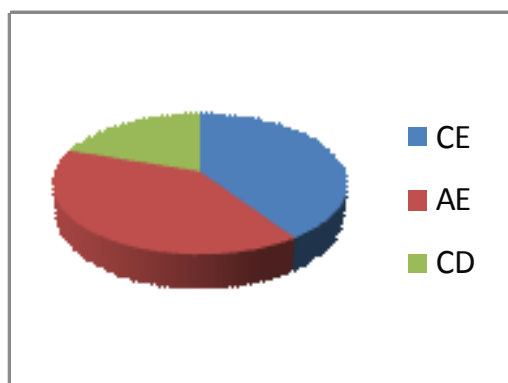
dehydrated and mounted. Using the light microscope, scoring was done at X400 to determine positive hybridization signals. Positive cells were counted out of 100 nucleated cells in 10 different high power fields. The mean percentage of positive cells was determined assigning cases to one of the four following score categories: Score 1: 1-25%, Score 2: 26-50%, Score 3: 51-75% and Score 4: > 75%. The ISH signaling intensity was assessed using a scale of negative, low, moderate and high intensities of signaling. (11) For immunohistochemical detection of p53 protein, blocking of endogenous peroxidase by 0.3% H<sub>2</sub>O<sub>2</sub> was followed by antigen retrieval in a microwave oven for 12 minutes. Application of primary antibody, secondary antibody, streptavidine and DAB were all conducted at room temperature. Hematoxyline was used as a counter stain. Slides were dehydrated by serial dipping in ethanol of different grades and mounted with DPX. Positive reaction was confirmed with the presence of a brown nuclear precipitate. The percentage score of positive cells in 100 malignant cells was performed at X400 in 10 HPF as follows: Score 1: 1-25%, Score 2: 26-50%, Score 3: 51-75% and Score 4: > 75%. Intensity score included negative, low, intermediate and high. (11) Statistical analysis was performed with the SPSS version 17. Demographic features were expressed as frequencies as tables or charts. Analytical data included cross tabulation between categorical variables with Pearson's Chi square test. Fisher-exact test was applied whenever data were invalid for Pearson's Chi square test. P value was regarded significant if < 0.05 in all tests.

#### **Results:**

Among the lung cancer cases, twenty five cases (83.3%) were males and 5 cases (16.7%) were females. The male to female ratio was 5:1. Patients' ages ranged from 38-72 years. The mean age  $\pm$  S.D. was 54.5  $\pm$  9.5. Histopathological examination of lung cancer cases revealed non-small cell types of lung cancer in 26 cases (68.7%). These included squamous cell carcinoma (SQCC) in 18(60%) cases, adenocarcinoma (AC) in 7(23.3%) cases and large cell carcinoma (LCC) in one case (3.3%). Small cell carcinoma (SCC) was found only in 4 cases (13.3%), figure-1 A.



A

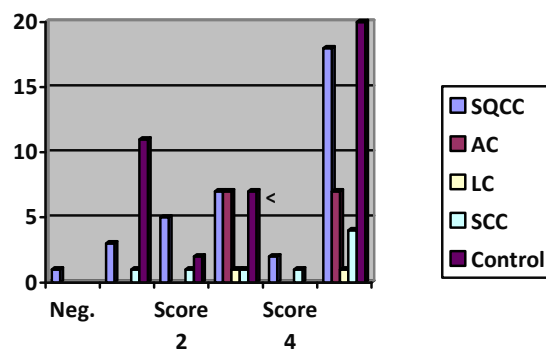


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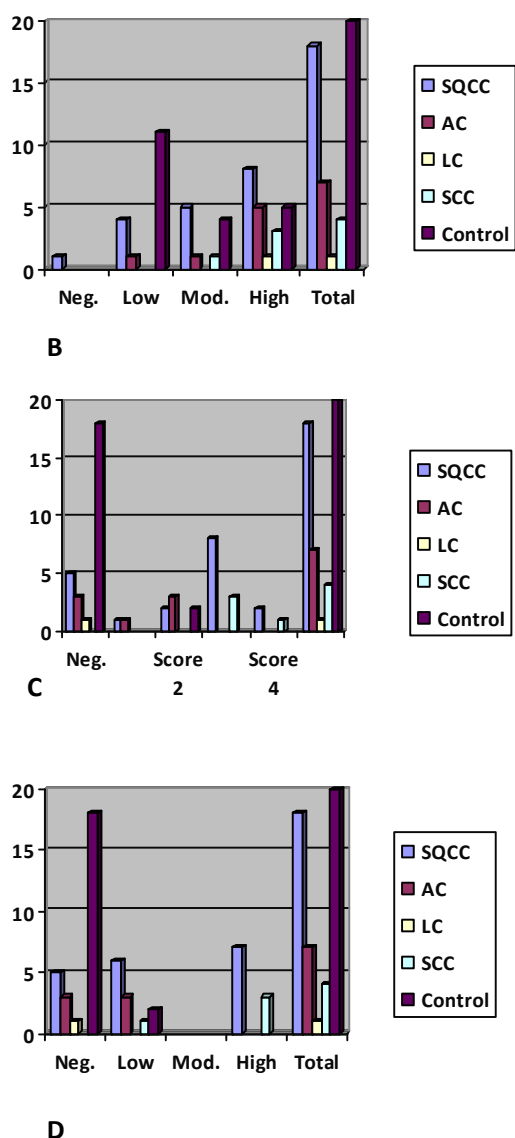
**Figure-1: The frequency distribution of different histological types of lung cancer (A) and control cases (B). A includes: SQCC: 18 (60%), AC: 7 (23.3%), LCC: 1 (3.3%) and SCC: 4 (23.3%). B includes: CE: 8 (40%), AE: 8 (40%) and CD: 4 (20%).**

Histopathological grading of non-small cell carcinomas revealed moderately differentiated carcinomas in 11(42.3%) poorly differentiated carcinomas in 14(53.8%) and undifferentiated LCC in 1 (3.8%). The control group included 8 (40%) cases of congenital emphysema, 8 (40%) cases of adult emphysema and 4 (20%) cases of chronic bronchitis, figure-1 B. ISH signals for p53 mRNAs were detected both in the nucleus and in the cytoplasm, while positive IHC p53 expression was regarded positive only when it was intra-nuclear. Positive p53 ISH signal was detected in 29 (96.6%) cases of carcinoma. The highest percentage score was score- 3 being detected in 16(53.3%) cases. High intensity of hybridization signal was seen in 17(56.7%) cases. Only one case of SQCC did not show any

hybridization signal. All control cases revealed positive hybridization signals (100%). Seven cases revealed score-3 and of these 5 revealed high intensity of hybridization, figure-2 A & B. The differences between the study group and control group regarding both ISH scores and intensities were statistically significant  $p= 0.024$  and  $0.03$  respectively. The test was 96% sensitive but not specific. When score 1, which was the predominant in control cases, was regarded as a cut- off point,  $p$  value became 0.004, the sensitivity decreased to 83% and the specificity became 55%. The positive predictive value (pv+) was 73% and the negative one (pv-) was 68%, figure-2 A. Similarly, on using low intensity as a cut- off point, the test was 80% sensitive, 55% specific, (pv+) was 72%, (pv-) was 64% and  $p$  value= 0.01, figure-2 B. Immunohistochemical expression of p53 protein was seen in 21(70%) cases of carcinoma with score-3 being found in 11(36.7%) cases, 7cases revealed score-3 ISH signals as well. High intensity was found in 11(36.7%) cases, 10 of them showed high intensity of hybridization signal. Only two control cases (10%) revealed positive p53 expression. They showed score-2 and low intensity of expression. By ISH they revealed high score and intensity, figure-2 C & D. The difference between the study group and control group regarding IHC scores and intensities were significant,  $p= 0.01$  and  $0.000$  respectively. The test's sensitivity was 70%, specificity was 90%, (pv+) was 91% and (pv-) was 66%. On using score 1 as a cut-off point they became 63%, 90%, 90% and 62% respectively, figure-2 C. On using low intensity as a cut-off point, the test's sensitivity was 36%, specificity was 100%, (pv+) was 100% and (pv-) was 51%, figure-2 D.



A



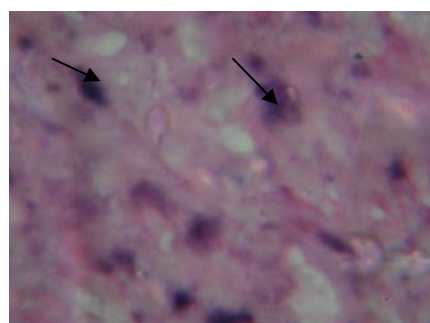
**C:**  $p = 0.01$  sensitivity= 70%, specificity = 90%, PV+ = 91% and PV- = 66%.

**Score 1 as a cut-off point:**sensitivity= 63%, specificity=90%, PV+=90% and PV- =62% .

**D:**  $p=0.000$ , sensitivity=70% and specificity=90%.

**Low intensity as a cut-off point:**  $p=0.002$ , sensitivity= 36%, specificity= 100%, PV+ = 100% and PV- =51%.

The frequency distribution of p53 mRNA ISH signal and IHC expression percentage scores and intensities according to histopathological types is shown in figure-2. Twenty five (96.1%) cases of non-SCC were positive for p53 mRNA ISH signals, including 17 out of 18 (94.4%) SQCC, 7 (100%) AC and 1 (100%) LCC as well as all 4 (100%) of SCC cases. Seventeen out of 26 (65.4%) cases of non-SCC including 13 out of eighteen (72.2%) SQCC and 4 out of 7 (57.1%) AC as well as all 4 (100%) cases of SCC were positive for mutated p53 by IHC detection. Figure-3 and 4 show different histological types of lung cancer and control cases with positive p53 mRNA ISH signals and p53 IHC expression respectively. Correlation of tumor grade with p53 mRNA ISH signals and p53 protein IHC expression percentage scores are shown in figure-5. They were both significant as well as correlations with the intensities p values were 0.028, 0.001, 0.025 and 0.000 respectively. Pearson Chi square test revealed significant statistical correlations between positive ISH signaling and positive immunohistochemical expression scores and intensities in carcinoma cases with p value = 0.003 and 0.001 respectively, table-1 and 2.



**A**

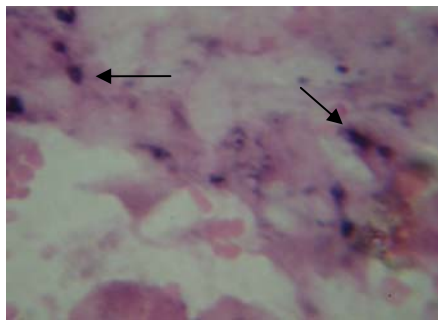
**Figure-2: Frequency distribution of different types of lung cancers and control cases according to A: ISH score, B: ISH intensity, C: IHC score and D: IHC intensity.**

**A:**  $p = 0.024$ , sensitivity = 96%, specificity = 0, PV+=59% and PV- =0.

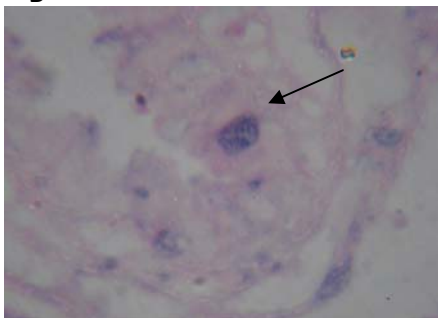
**Score 1 as a cut-off point:**  $p = 0.004$ , sensitivity= 83%, specificity= 55%, PV+ = 73% and PV- = 68%.

**B:**  $p = 0.03$ , sensitivity=96% and specificity= 0.

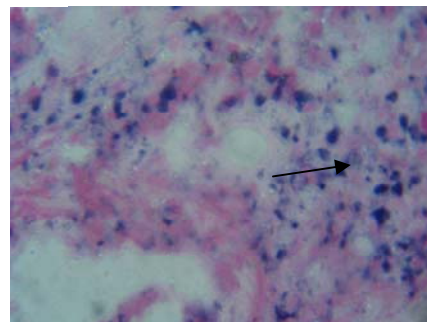
**Low intensity as a cut-off point:**  $p = 0.01$ , sensitivity=80%, specificity= 55%, PV+ =72% and PV- = 64% .



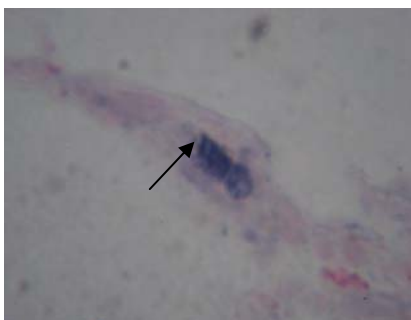
**B**



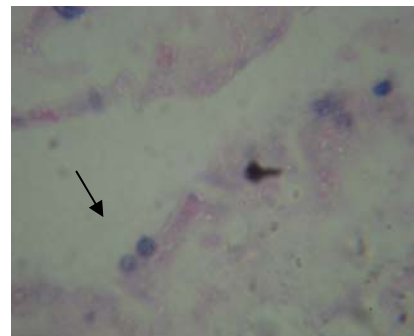
**C**



**D**

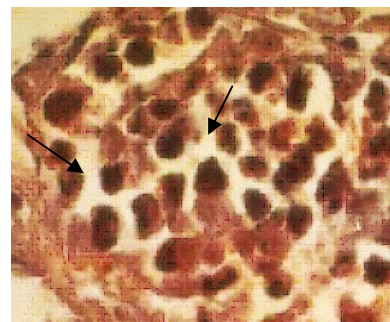


**E**

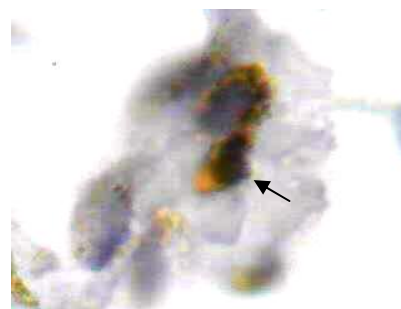


**F**

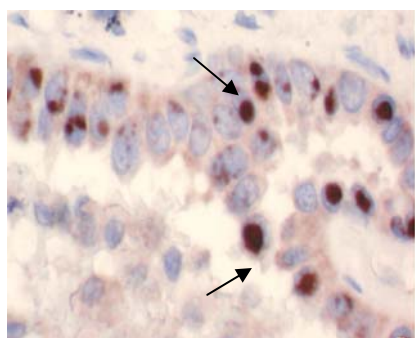
**Figure-3: mRNA ISH signals, high intensity. A: SQCC (mod. diff.), B: SQCC (poor. diff.), C: AC (mod. diff.), D: SCC, E: LCC and F: Emphysema (adult). (X1000).**



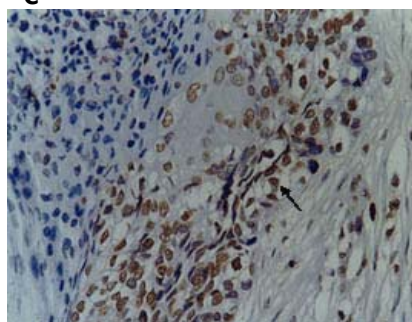
**A**



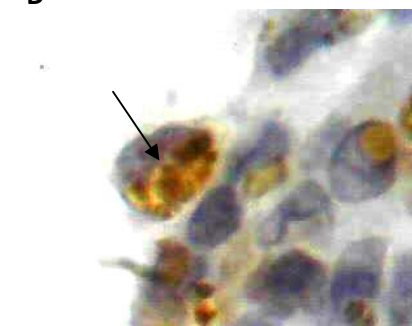
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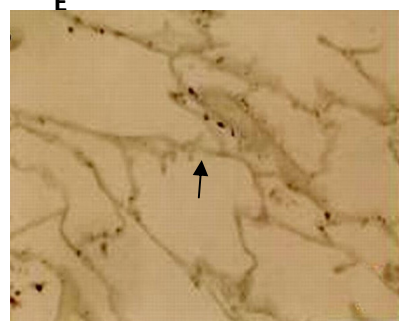
**C**



**D**

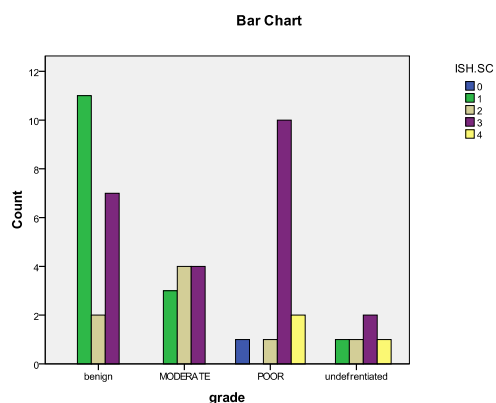


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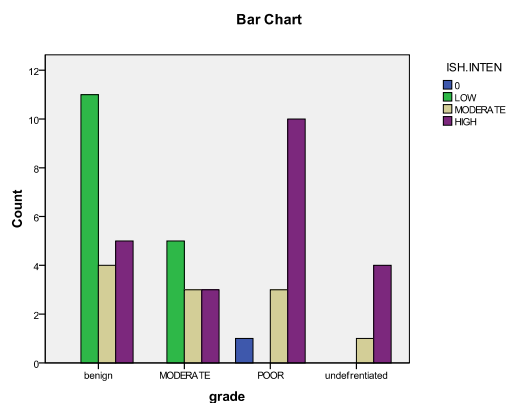


**F**

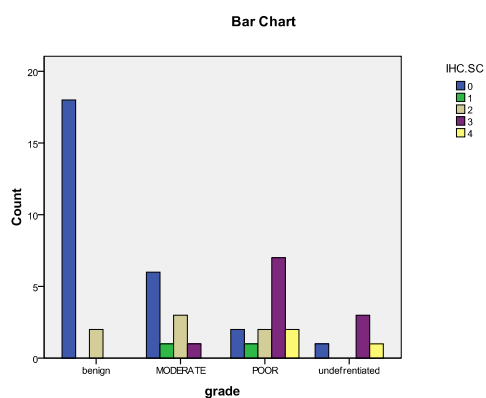
**Figure-4: IHC expression of p53 mutated protein.**  
**A: SQCC (mod. diff.) (X1000), B: SQCC (poor. diff.) (X1000), C: AC (mod. diff.) (X1000), D: SCC (X400), E: SQCC (mod. diff.)(X1000) and F: emphysema (adult) (X100).**



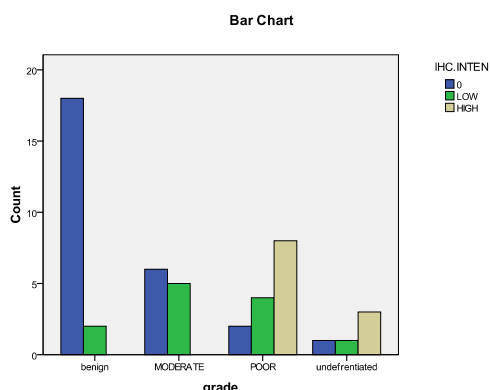
**A**



**B**



**C**



D

Figure-5: Frequency distribution of different grades of lung cancer according to A: ISH score, p=0.028, B: ISH intensity, p=0.001, C: IHC score, p=0.025 and D: IHC intensity, p=0.000.

Table-1: Cross tabulation between ISH score and IHC score in lung cancer cases.

Score		IHC					Total
		Neg.	Score 1	Score 2	Score 3	Score 4	
ISH	Neg.	1	0	0	0	0	1
	Score 1	2	0	0	2	0	4
	Score 2	1	1	2	2	0	6
	Score 3	5	1	3	7	0	16
	Score 4	0	0	0	0	3	3
Total		9	2	5	11	3	30

$\chi^2=36.141$   
P=0.003

Table-2: Cross tabulation between ISH intensity and IHC intensity in lung cancer cases.

Intensity		IHC			Total
		0	LOW	HIGH	
ISH	0	1	0	0	1
	LOW	5	0	0	5
	MODERATE	1	5	1	7
	HIGH	2	5	10	17
Total		9	10	11	30

$\chi^2=22.819$   
P=0.001

### Discussion:

P53 cancer suppressor gene expression abnormality was studied to determine its relation to lung cancer in Iraqi patients. P53 mRNA was absent only in one case suggesting either gene deletion or probably a defect in gene expression involving mRNA transcription. (12) However, 21 (70%) cases revealed the presence of a mutated protein. The remaining 8 cases of lung cancer were positive for p53 mRNA, which appears to be a wild type since they were negative for a mutated p53 by immunohistochemistry. Increased score and intensity of hybridization signals in the cases with high score and intensity of mutated p53 expression are explained by the fact that mutation of p53 gene leads to a loss of function and to increased protein half-life resulting in nuclear accumulation of the mutant p53 protein. (13) Approximately 80% of the p53 point mutations present in human cancers are located in the DNA-binding domain of the protein. However, the effects of different point mutations vary considerably; in some cases there is complete abrogation of transcriptional capabilities, whereas other mutants retain the ability to bind to and activate a subset of genes. In addition to somatic and inherited mutations, p53 functions can be inactivated by other mechanisms. It has been found that within malignancies where the p53 gene is not mutated, other mechanisms may exist to attenuate its function as a tumor suppressor independently of p53 gene mutation. For instance, overexpression of the negative regulators, MDM2 or MDMX, negates the requirement of cells to mutate p53. (14, 15,16,17,18, and 19). Deletion or inactivation of p53 is reported to be present in more than 70% and up to 90% of SCC and 50% in non-SCC. (20, 21). In the present study, a mutated p53 expression was found in 70% of cases including 72% of SQCC and 57% of AC and 100% of SCC. Saad et al reported 64% of CA lung to be p53-positive by immunohistochemistry. (22) Passlick et al reported positive expression in 45.2% of non-small-cell lung cancers. (23) Lee et al reported that 55% of non-SCC to be positive for P53 overexpression. (24) Gebitekin et al observed positive expression in 50% of SQCC and 46% of AC. (25) while Melhem et al. reported about 60% and 40% of SQCC and AC respectively. (26) The majority of lung cancer cases are related to cigarette smoking, including SCC and SQCC. (3) It should also be pointed out that 25% of lung cancers worldwide arise in nonsmokers and these are pathogenetically distinct. They occur more commonly in women, and most are ACs. They tend to show p53 mutations less commonly. The natures of the p53 mutations are also distinct. (27) In the present study, 3 cases of AC were negative for p53 expression, 2 of them were females. However, history of cigarette smoking was not

available in the patients' case sheets to correlate with the results. The relations of tumor grade to the score and intensity of ISH signaling and IHC expression were significant which is an indication of tumor progression. Those cells are more likely to be of higher grade and associated with high invasion potential (advanced stage). It has been documented that p53 gene alteration is not an early event in a category of lung cancer such as AC, whereby it is present in invasive types and correlates with poor prognosis.(28, 29, 30) Integrity of p53 gene as detected by its positive mRNA ISH signals was evident in all control cases. This emphasizes the need for an active p53 in cells with DNA insults including those created by hypoxia, a common observation in emphysema and chronic lung diseases. (3) In the present study 2 (10%) of cases demonstrated positive IHC expression of a mutated type which raises a couple of questions, is it present in a dysplastic cell or is it an inherited gene mutation? This requires further genetic and molecular studies. (21) Sasano et al related p53overexpression in the vicinity of SQCC of the esophagus to the presence of dysplasia. (31) However, no dysplastic changes were seen in those 2 cases. On the other hand, positive cells were alveolar and bronchial. The most acceptable explanation would be that there is a mutation in one p53allele (either inherited or predisposed by smoking) (32) but the other allele is still wild and capable of preventing cancer appearance as demonstrated by the high score and intensity of mRNA ISH signals in comparison to the score 2 and low intensity of IHC expression.

#### **Conclusion:**

Studying p53 gene integrity or expression of a mutated protein is important for predicting tumor prognosis and establishing a proper therapeutic approach. Irradiation and chemotherapy, the two common modalities of cancer treatment, mediate their effects by inducing DNA damage and subsequent apoptosis. Tumors that retain normal p53 are more likely to respond to such therapy than tumors that carry mutated alleles of the gene, while tumors such as lung cancers and colorectal cancers, which frequently carry p53 mutations, are relatively resistant to chemotherapy and irradiation. Various therapeutic modalities aimed at increasing normal p53 activity in tumor cells that retain this type of activity or selectively killing cells with defective p53 function are being investigated. (11, 33)

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