RESEARCH ARTICLE

Clinical Significance of Serum p53 and Epidermal Growth Factor Receptor in Patients with Acute Leukemia

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Abstract

Background: Pretreatment serum p53 and epidermal growth factor receptor (EGFR) were assessed using enzyme-linked immunosorbent assay (ELISA) in patients with acute leukemia to analysis their roles in characterization of different subtypes of the disease. Materials and Methods: Serum samples from thirty two patients with acute myeloid leukemia (AML) and fourteen patients with acute lymphoid leukemia (ALL) were analysed, along with 24 from healthy individuals used as a control group. Results: The results demonstrated a significant increase of serum p53 and EGFR in patients with AML ($p<0.0001$) compared to the control group. Also, the results showed a significant increase of both markers in patients with ALL ($p<0.05$, $p<0.0001$ respectively). Sensitivities and specificities for these variables were 52% and 100% for p53, and 73.9%, 95.8% for EGFR. Serum p53 and EGFR could successfully differentiate between M4 and other AML subtypes, while these variables failed to discriminate among ALL subtypes. A positive significant correlation was noted between p53 and EGFR. Negative significant correlations were observed between these variables and both of hemoglobin (Hg) content and RBC count. Conclusions: Mutant p53 and EGFR are helpful serological markers for diagnosis of patients with AML or ALL and can aid in characterization of disease. Moreover, these markers may reflect carcinogenesis mechanisms.

Keywords: Acute myeloid leukemia - acute lymphoid leukemia - p53 - EGFR - ELISA - ROC

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Introduction

Leukemia pathogenesis has not been yet completely clear, the conversion of the proto-oncogenes, tumor suppressor gene aberration and apoptosis inhibition may play an important role in the pathogenesis of disease (Xiaoming and Weiping, 2009). p53 is a tumor suppressor gene encoding a nuclear phosphoprotein that plays an important role in controlling the normal cell proliferation (Konikova et al., 1999). The suppression of p53 protein results in interruption of DNA repair mechanisms in dividing malignant cells thereby increasing the DNA damage and activating p53-independent mechanisms of apoptosis (Alachkar et al., 2012), so p53 inactivation is a key factor in human tumorigenesis and chemotherapy resistance. The traditionally described mechanisms of p53 inactivation in AML include TP53 mutations and abrogation of p53 pathway (Prokocimer and Peller, 2012).

The epidermal growth factor receptor (EGFR) family belongs to type I receptor tyrosine kinases. Overexpression or mutation of EGFR/ErbB1 gene has been detected in a large number of human solid tumors. According to some previous reports, this gene is not expressed in hematological malignancies. However, two recent clinical case reports showed that erlotinib caused complete remission of AML-M1 in patients who had both AML-M1 and non-small-cell lung cancer (Sun et al., 2012).

The rationale of this study was to analyze the pretreatment serum p53 and EGFR levels using ELISA in patients with acute leukemia and to investigate their correlations with hematological data. Analysis the roles of these variables for characterization of different subtypes of acute leukemic patients were also studied.

Materials and Methods

Study subjects

The present study had been conducted in cooperation with National Cancer Institute, (Cairo University) and Laboratory Research Unit (Gastroenterology Surgical Center, Faculty of Medicine, Mansoura University). A total of 46 patients with hematologically diagnosed acute leukemia, 32 patients were diagnosed as acute myeloid leukemia (19 men and 13 women; mean age 32.61 years, range 14-53 years) and 14 patients were acute lymphoid leukemia (9 men and 5 women; mean age 27.38 years, range 18-41 years). Patients with AML and ALL were classified into subtypes according to French-American-British (FAB) classification that based on the type of cell from which the leukemia developed and how mature the

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cells are. This was based largely on how the leukemia cells looked under the microscope after routine staining. The subtypes of AML involved in this study were M1 (26%), M2 (19.5%), M3 (15.2%), and M4 (8.7%), while the subtypes of ALL were ALL1 (15.2%) and ALL2 (15.2%). The control group included 24 healthy individuals (18 men and 6 women; mean age 33 years, range 24-42). The control individuals were selected without a clinical history of any chronic diseases and without symptoms or signs of acute or chronic leukemia. Peripheral blood samples were obtained from the patients and those from healthy subjects in the control group and sera were promptly separated and stored at –20˚C till use. The study was approved by the local Research and Ethics Committee of Mansoura and Cairo Universities. An informed consent was obtained from the child's parent or guardian before inclusion in the study.

Analysis of hematological data

Peripheral blood samples were obtained from all studied groups (healthy individuals, acute myeloid leukemia, and acute lymphoid leukemia) to analyze the hematological parameters as hemoglobin content (Hb), red blood cells (RBCs), white blood cells (WBCs), and platelets count according to routinely investigated laboratory tests.

Serum p53 and EGFR analysis using ELISA

A home-ELISA method was optimized to obtain the optimum reaction conditions. Polystyrene microtiter plates were coated with 50 µl/well of each serum sample diluted 1:1000 in carbonate/bicarbonate buffer (pH 9.6). The plates were incubated overnight at room temperature and washed three times using 0.05% (v/v) PBS-T20 (pH 7.2) and then incubated for 1h at room temperature with 200 µl/well of 0.2% (w/v) non-fat milk in carbonate/bicarbonate buffer (pH 9.6). After washing, 50 µl/well of mouse monoclonal antibody Bp53-12 (Sigma), diluted 1:100 in PBS-T20 or monoclonal anti-EGFR, clone 29.1 (Sigma) diluted 1:1000 were added and incubated at 37˚C for 2 h. After washing, 50 µl/well of anti-mouse IgG alkaline phosphatase conjugate (Sigma), diluted 1:250 in PBS-T20, was added and incubated at 37˚C for 1 h. Excess conjugate was removed by extensive washing and the amount of coupled conjugate was determined by incubation with 50 µl/well p-nitrophenyl phosphate (Sigma) for 30 min at 37˚C The reaction was stopped using 25 µl/well of 3M NaOH and absorbance was read at 405 nm using microplate autoreader (Bio-Tek Instruments. WI, USA). Cut-off level of ELISA above or below which the tested samples were considered positive or negative was calculated as the mean concentrations of 24 serum samples from healthy individuals +2SD.

Statistical analysis

Results were expressed as mean±SD. and were analyzed by using X^2-test, Mann–Whitney U–test, Fisher's exact test, Spearman correlation as appropriate. The Mann–Whitney U-test was used to compare different groups for continuous variables including the serum levels of mutant p53 and EGFR. The correlations between serum levels of mutant p53, EGFR, and hematological data of patients were assessed by Spearman correlation. $p \leq 0.05$ was considered significant. These statistical procedures were performed using SPSS software, version 11 for windows (SPSS Inc., USA). Receiver operating characteristic curves (ROCs), area under curve (AUC) calculations, and one-way analysis of variance (ANOVA) to compare among different subtypes of patients with acute leukemia were performed using MedCalc software, version 12 for windows (Belgium).

Results

Hematological data of the studied groups

The hematological data of all studied groups (HI, AML, and ALL) were listed in Table 1. Hg content, RBCs, and platelets count of patients with AML or ALL were significantly lower ($p<0.0001$ for all except platelets count in ALL group, $p<0.05$) than those in healthy individuals, while WBCs count was significantly higher ($p<0.0001$ in both of AML and ALL groups) than those in healthy individuals.

Serum levels of p53 and EGFR

As shown in Table 2, the results demonstrated a significant difference between the serum levels of mutant p53 and EGFR in patients with AML compared to that of controls ($p<0.0001$ for both p53 and EGFR). Also, serum EGFR and p53 levels were increased significantly in patients with ALL compared to the control group ($p<0.0001$ for EGFR, $p<0.05$ for p53). Our results showed that the positivities of p53 and EGFR in patients with AML were 60% and 78.12% respectively, while the positivities of these variables in patients with ALL were 8.69% and 61.53%. It is observed from our results that the serum level of p53 in patients with ALL was significantly lower ($p<0.01$) than those in patients with AML, but there was

Table 1. Age, Sex, and Hematological Data of Patients with Acute Leukemia

Variable	HI			AML			ALL		
	Mean±SD	Range	p value	Mean±SD	Range	p value	Mean±SD	Range	p value
Sex (M/F)	18/6			19/13			9/5		
Age (y)	33±5.3	24-42		32.61±10.95	14-53	NS	27.38±7.27	18-41	NS
Hb (g/dl)	12.52±0.78	11.7-14.0		6.97±2.31	2.7-12.9	<0.0001*	7.99±2.44	4.5-13	<0.0001*
RBCs×10^6/ml	4.62±0.44	3.8-5.2		2.46±0.82	1.2-4.9	<0.0001*	2.79±0.85	1.4-4.2	<0.0001*
WBCs×10^3/ml	6.4±1.22	3.7-8.1		37.19± 45.62	1-190	<0.0001*	52.13±60.18	1-180	<0.0001*
Platelet×10^3/ml	209±25	175-250		41.14±42.7	6-161	<0.0001*	48.71±47.73	3-165	<0.05*

*HI, healthy individuals, AML, acute myeloid leukemia, ALL, acute lymphoid leukemia

Table 2. Serum p53 and EGFR Levels in Patients with Acute Leukemia

Study group	p53 (ng/ml)			EGFR (Optical density)		
	Positivity (%)	Mean±SD	p value	Positivity (%)	Mean±SD	p value
Healthy individuals (HI)	0	0.14±0.022		0	0.25±0.045	
Acute leukemia (total patients) (AL)	52	0.21±0.08	<0.0001*	73.91	0.43±0.14	<0.0001*
Acute myeloid leukemia (AML)	60	0.22±0.087	<0.0001*	78.12	0.45±0.15	<0.0001*
Acute lymphoid leukemia (ALL)	8.69	0.18±0.07	<0.05* <0.01**	61.53	0.38±0.098	<0.0001* NQS**

*significance compared to control group, **significance compared to AML group. p value<0.05 is considered significant. NS: not significant. NQS: not quit significant

Table 3. Correlation Amongst Serum p53, EGFR and Hematological Data

Variable	p53	EGFR
	Spearman's correlation (p)	Spearman's correlation (p)
EGFR	0.85 (<0.0001*)	-
Hb	-0.57 (<0.0001*)	-0.42 (<0.01*)
RBCs	-0.5 (<0.001*)	-0.43 (<0.01*)
WBCs	0.03 (NS)	0.14 (NS)
Platelet	-0.07 (NS)	-0.08 (NS)

*Asterisks denote that p value is significant. NS, not significant

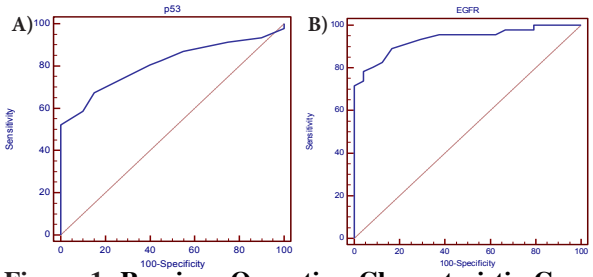

Figure 1. Receiver Operating Characteristic Curve (ROC). A) p53 Protein. The Area Under Curve (AUC) is 0.8 and B) Epidermal Growth Factor Receptor. The Area Under Curve (AUC) is 0.93

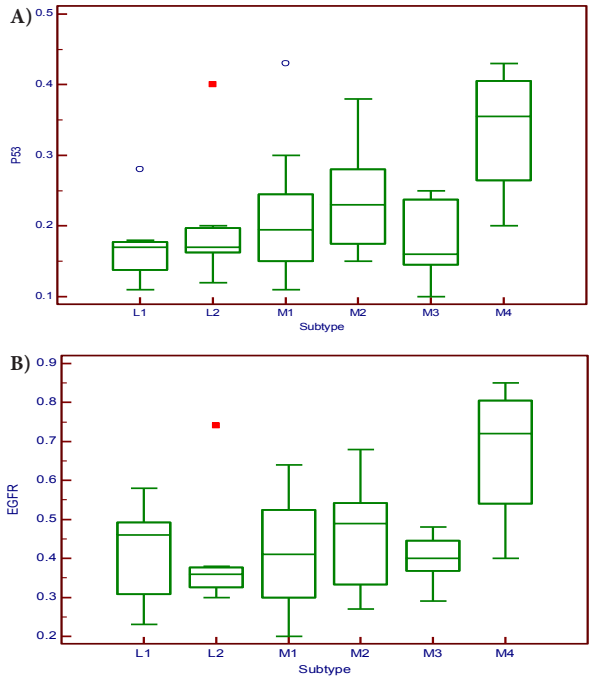

Figure 2. Box Plot Comparing Mean Serum Levels. A) p53 and B) EGFR in Different Subtypes of Patients with Acute Leukemia (L1, L2, M1, M2, M3, and M4) Using One-Way Analysis of Variance (ANOVA)

no significant difference in EGFR between patients with ALL and patients with AML. The receiver operating characteristic curves (ROCs) were plotted (Figures 1). It was found that the areas under curves (AUC) for p53 and EGFR are 0.8 and 0.93 respectively. This result indicates the good validity of p53 and EGFR to discriminate the positive from the negative samples.

Correlation amongst serum p53, EGFR and hematological parameters

As shown in Table 3, data statistical analysis showed a positive strong correlation between serum p53 and EGFR (r=0.85, p<0.0001). Thus, it seems clear that the p53 and EGFR are dependent variables. Both of p53 and EGFR were negatively correlated with Hg content [r=-0.57 (p<0.0001), -0.42 (p<0.01) respectively] and RBCs count [r=-0.5 (p<0.001), -0.43 (p<0.01) respectively], while there was no correlation between these two variables and both of WBCs and platelets count.

Serum p53 and EGFR levels in different subtypes of AML and ALL

Data showed no significant difference for p53 and EGFR with ALL subtypes (ALL1 vs ALL2) but these variables were able to discriminate between subtype M4 and both of M1, M2, and M3 (for p53, p=0.03, for EGFR, p=0.028). On the other hand, both of p53 and EGFR were not differentiate among M1, M2, and M3 subtypes (Figures 2).

Discussion

p53 biosignatures contain useful information for cancer evaluation and prognostication (Anensen et al., 2012). In the present work, there were significant increase in the serum levels of p53 in patients with AL, AML (p<0.0001, positivity 52%, 60% respectively), and ALL (p<0.05, positivity 8.69%) compared to control group. Also, the serum p53 levels in patients with AML were higher than those in ALL group with a statistically significant difference (p<0.01). These results indicate that, the expression of p53 protein may have a different mechanism in the pathogenesis progress in these two types of acute leukemia and it may be considered as a helpful marker to differentiate between them. Several studies revealed that the mutation of p53 gene has been reported only 5-10% of patients with AML (Diccianni et al., 1994; Hsiao et al., 1994; Wattel et al., 1994; Zhu et al., 1999). In contrast, the results of Sahu and Jena (2011) showed that 91% patients with AML were p53 immunopositive using

immunocytochemistry. Also, measurement of p53 protein expression by flow cytometry showed higher percentage of p53 expression in cells of AML patients at the time of diagnosis opposite to the controls (Konikova et al., 1999). Furthermore, the results of Park et al. (2000) revealed that the overexpression of p53 protein was found in 38% of patients with AML, while 25% of patients with ALL were p53 immunopositive using immunohistochemical technique. Significant increase of serum p53 protein in different human cancers were reported by several authors (Segawa et al., 1997; Suwa et al., 1997; Shim et al., 1998; Sobti and Parashar, 1998; Morita et al., 2000; Charuruks et al., 2001; Chow et al., 2001). In addition, our previous reports revealed an increasing level of serum p53 protein using ELISA in different gastrointestinal tumors (Attallah et al., 2003), hepatocellular carcinoma (Abdel-aziz et al., 2005), and colorectal cancer (Abdel-aziz et al., 2009).

Epidermal growth factor (EGF) and its receptor (EGFR) are one of the most important ligands/receptors of mammalian cells. EGFR possesses intrinsic tyrosine kinase activity, and its overexpression is associated with malignant transformations (Schlessinger and Ullrich, 1992; Rajkumar, 2001). Previous studies reported that EGF/EGFR binding plays an important role in the carcinogenesis of several human tumors because EGF stimulates proliferation of malignant cells through its receptor, EGFR (Yamazaki et al., 1998).

In the present study, there was a significant increase in the EGFR level in both of AL and AML patients groups compared to the control group ($p<0.0001$, positivity 73.91% and 78.12% respectively). Furthermore, EGFR levels in ALL patients were significantly increased ($p<0.0001$) with a high positivity (61.5%) compared to control group, while there was a not quit significant difference in EGFR levels between AML and ALL patients groups.

The present work showed that the optimized home – ELISA technique allows the serological quantitative analysis of these markers (p53 and EGFR) to give different sensitivities for each with good specificities (for p53, 52%, 100%, for EGFR, 73.91%, 95.8%). AUC for each marker was calculated according to their ROCs and it was found that the AUC for p53 and EGFR are 0.8, and 0.93 respectively. These results indicate the good validity for p53 and EGFR to discriminate the seropositive from the seronegative samples of AL patients and indicate that our optimized ELISA method is a reliable diagnostic technique for differentiation between positive and negative cases.

Furthermore, our results showed significant positive correlation between p53 and EGFR. Thus, it seems clear that these markers are dependent variables. On the other hand, there were significant negative correlations between these variables and some hematological data of AL patients as hemoglobin content and red blood cells count, while there were no correlations with white blood cells and platelets count.

The most important rationale of this study is to analyze the serum levels of the these variables in different subtypes of both AML and ALL patients. Our results showed that both of serum p53 and EGFR levels in M4 subtype are higher than those in M1, M2, and M3 (for p53, $p=0.03$, for EGFR, $p=0.028$), while there were no significant differences among the subtypes M1, M2, and M3. These results showed that the serological analysis of these markers have a significant role for characterization of AML subtype. In contrast, serum levels of these markers failed to discriminate between the two subtypes of ALL (ALL1 vs ALL2).

In conclusion, our optimized ELISA technique is a valid reliable assay for determination of serum p53 and EGFR and these markers are helpful serological markers for diagnosis of both AML and ALL patients and can discriminate between different types of AL patients. Furthermore, these variables can differentiate among the different subtypes of AML patients and aid for disease characterization. Our results encourage us and others to investigate the efficacy of these markers to monitor patients with AL during and after treatment.

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