RESEARCH ARTICLE

Prognostic Value of Prepro-Gastrin Releasing Peptide in Lung Cancer Patients; NCI-Prospective Study

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Abstract

Background: Prior series investigated the expression of prepro-gastrin releasing peptide (prepro-GRP) in the peripheral blood of lung cancer patients. Our aim was to assess any prepro-GRP role as a prognostic factor for small cell lung cancer (SCLC) and NSCLC and correlations with clinical presentation and treatment outcome. **Methods:** A prospective study was conducted during the time period from the beginning of January 2012 till the end of January 2014. Prepro-GRP expression was analysed using a nested RT-PCR assay in peripheral blood of 62 untreated lung cancer patients attending the National Cancer Institute (NCI), Cairo University, and 30 age and sex matched healthy volunteers. **Results:** Among the 62 lung cancer cases, there were 24 (38.7%) SCLC, and 38 (61.3%) NSCLC (10 squamous cell carcinomas, 12 adenocarcinomas, 11 large cell carcinomas, 4 undifferentiated carcinomas, and 1 adenosquamous carcinoma). Twenty six patients (41.9%) were prepro-GRP positive. Prepro-GRP expression was higher (58.3%) among SCLC patients compared to NSCLC (squamous cell carcinoma (15.4%), large cell carcinoma (36.4%), and adenocarcinoma (25%)). Mean OS among prepro-GRP negative cases was longer than that among preprogastrin positive cases (17.6 vs 14.9 months). The mean PFS durations among preprogastrin negative versus positive cases were 7.7 vs 4.6 months (p= 0.041). No difference in response to chemotherapy was identified between the groups (p=0.983). **Conclusion:** Prepro-GRP is suggested to be a useful prognostic marker for lung cancer patients, especially with the fast- growing, bad prognostic SCLC type. More studies should aim at detailed understanding of the mechanisms of prepro-GRP action and its use in monitoring the response to treatment in a larger cohort.

Keywords: Prepro-GRP- lung cancer- prognosis

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Introduction

World Health Organization (WHO) reported in 2012 that lung cancer causes 1.59 million deaths worldwide reflecting that it is the leading cause of death worldwide (De Martel et al., 2012). Lung cancer represents the second of incident cancer among both males and females in the United States (U.S.) with 224,390 new cases and 158,080 deaths expected in 2016 (American Cancer Society, 2016). In Egypt, lung cancer is the 3rd most common cancer in male (8.2%) and nearly 5.7% of all cancers in both sexes (Ibrahim et al., 2014). Cigarette smoking is the most important cause of lung cancer and lung cancer-related mortality and contributes significantly to lower 5 years survival (Prizment et al., 2014; Rahouma et al., 2015). However, other factors, such as asbestos, arsenic, environment pollution mainly air and excessive alcohol may also be contributing for Lung Cancer (Chen et al., 2004; Schmid et al., 2010; Markowitz et al., 2013).

Lung cancer histologically is classified into 2 major classes, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC is a heterogeneous group comprised of many subtypes the 2 most common are squamous cell carcinoma and adenocarcinoma (Beasley et al., 2005). The most aggressive type is small cell lung cancer with early and frequent development of widespread dissemination and it accounts for 15-18% of all lung cancer diagnosis (Planchard and Le Pechoux, 2011). In fact, the median survival of SCLC patients is about 15-20 months and the 5-year survival is less than 15% (Fruh et al.,2013; Pietanza et al., 2015; Cascone et al., 2016) due to the high propensity of SCLC to disseminate early via the blood stream (Sher et al.,2008).

Small cell lung carcinoma is a neuroendocrine tumor that produces a number of neuropeptides, including gastrin, gastrin-releasing peptide (GRP), calcitonin, vasopressin, and corticotropic hormone (Russel et al., 1990). Of these, GRP is an autocrine growth factor that was detected in SCLC cell lines (Cuttitta et al., 1985). It is a gut peptide hormone that was isolated from the porcine stomach. It is present in nerve fibers in non-antral stomach tissue, brain, and neuroendocrine cells in fetal lung and in pulmonary carcinoid and SCLC cells (Yanaihara et al., 1978). ProGRP, a precursor protein of GRP (Moody, 1998), was reported to be one of the reliable tumor markers for SCLC (Miyake et al., 1994). A precursor

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of GRP, prepro-GRP is an appropriate target of reverse transcriptase (RT)-PCR because it is not expressed in the hematopoietic or endothelial cells in blood vessels, nor in the epithelial cells of the human skin due its unique mammalian tissue distribution. Therefore, the risk of contamination during needle puncture of the skin can be avoided.

As there are limited researches concerning the relation between preprogastrin and lung cancer, we investigated prepro-GRP expression using a nested reverse transcription polymerase chain reaction (RT-PCR) assay to detect circulating tumor cells in peripheral blood and its utility in the early diagnosis and prognosis of lung cancer cases.

Patients and methods

This prospective study was conducted on 62 untreated lung cancer patients attending National Cancer Institute (NCI), Cairo University during the time period from the beginning of January 2012 till the end of January 2014 and 30 age and sex matched healthy volunteers. After Institutional Review Board (IRB) of National Cancer Institute (NCI) approval of this study, a written informed consent from each patient and volunteer before starting the data collection was done. For the sake of patient's privacy, they were given coded numbers.

Among our cohort there were 62 newly diagnosed lung cancer cases (24 histologically proven as SCLC and 38 histologically proven as NSCLC) and 30 age and sex matched healthy controls (smokers and nonsmokers). Routine clinical and imaging investigations were done. Histopathological diagnosis was performed on biopsy samples obtained during bronchoscopy to confirm the diagnosis. Tumor staging and grading were according to the 7th edition of the TNM classification of The American Joint Committee on Cancer (AJCC) (Edge et al., 2010). The range of follow up period of the patients was 0.3-23.1 months with a mean of 6.2 months.

Materials and Methods

Ten mL of peripheral blood on EDETA were obtained from patients at diagnosis as well as controls. Nucleated cells were isolated by the osmotic red blood cell lysis method. RNA was extracted using QIAamp RNA Blood Mini isolation kit (QIAGEN, Valencia, CA, and USA) following standard procedures according to the manufacturer's instructions. RNA concentration was determined by measuring the absorbance at 280 nm and stored at -80 º C. RNA integrity was checked by gel electrophoresis and ethidium bromide staining.

Two ug RNA were subjected to reverse transcription with random primers using the GeneAmp Gold RNA PCR Reagent Kit (Applied Biosystems, Carlsbad, CL, USA) according to the manufacturer's instructions in a total volume of 20 uL at 25°C for 10 minutes and a period of 12 minutes at 42°C. The cDNA integrity was checked using B-actin amplification as a control gene.

Outer primer and internal primer pairs encompassing exon 1 and exon 2 of prepro GRP18 were selected for nested RT-PCR (Spindel et al., 1984; Salto et al.,2003).

The nested PCR was performed as follows. In the first round PCR assay, 5 of 20 uL of the cDNA preparation was subjected to a first preproGRP-specific amplification in a total volume of 25 uL using 2.5 U Taq DNA polymerase, 1.5 mM MgCl2, 200 uM of each dNTP, and 10 pM of the outer primers. The outer primers were 5'TGC TGG CGC TGG TCC TCT GC 3' (outer forward) and 5' TGC TGC TAT CCT CTG AAT CC 3' (outer reverse). The PCR reactions were performed according to the following standard protocols, five minutes of initial denaturation at 94 °C; 45 cycles: 94 °C for 1 minute, 68°C for 1 minute, 72 °C for 1 minute; final elongation: 72°C for 7 minutes, yielding a 324-base pair (bp) PCR product.

After the first amplification, an aliquot was diluted 1:10 and 5 µL was submitted to the second amplification with nested (internal) primers using the same thermal cycling conditions. The internal primers were 5' GGA CCG TGC TGA CCA AGA TG 3' (internal forward) and 5' TCC CAC GAA GGC TGC TGA TT 3' (internal reverse), yielding a 244-bp PCR product (Figure 1).

Aliquots from amplified samples from the two PCR rounds were analyzed on 2% agarose gel electrophoresis and visualized by ethidium bromide staining under ultraviolet (UV) light. All experiments were carried out twice.

Statistical Methods

Different clinico-pathological variables were included in this study; age, gender, Eastern Cooperative Oncology Group- performance status(ECOG-PS), stage(TNM stage for NSCLC 7th edition (Edge et al., 2010) while either limited or extensive for SCLC), different symptoms; cough, dyspnea or chest pain, histopathology, grade, presence of metastasis using different imaging modalities(CT, bone scan). Pearson's chi (X2) test and student t-test were used to compare categorical and continuous variables respectively. Kaplan Meier curves were used for survival analysis and compared using Log-rank test. Values less than 0.05 were considered statistically significant. Variables were expressed as median and range for continuous variables and frequency percentages for categorical ones. All analyses were performed using SPSS version 22.0 (IBM, Armonk, NY).

Results

The patients' characteristics were shown in Table 1. Forty eight patients were complaining of cough, 50 dyspnea, 24 chest pain, 2 hemoptysis of 6 months duration. 26 cases underwent bronchoscopy and biopsy, 30 cases underwent CT guided biopsy, FNAC in 3 cases (pleural effusion, infra-clavicular mass, cervical LN).

Results of preproGRP-Specific Nested RT-PCR in PB samples of various patient groups and healthy donors are shown in Table 2.

The Relation between PreproGRP and clinico-demographic data of the patients is shown in table 3 indicating a statistical significant relationship between preprogastrin and smoking (P=0.010) and stage IV disease (p=0.042). Also, there was a statistical significant relationship between preprogastrin and SCLC (P=0.038)

Table 1. Characteristics of Patients with Lung Carcinoma

Disease type	(No. of patients; %)
SCLC	24 (38.7%)
NSCLC	38 (61.3%)
Adenocarcinoma	12 (19.4%)
Squamous cell carcinoma	10 (16.1%)
Large cell carcinoma	11 (17.7%)
Others	5 (8.1%)
Gender (M/F)	60/2
Age (median; range);in years.	57 (34-81)
Smokers	47 (75.8%)
Performance status	
PS I	15 (24.2%)
PS II	42 (67.7%)
PS III	5 (8.1%)
Clinical picture	
Cough	48 (77.4%)
Dysnea	50 (80.6%)
Disease stage	
SCLC	
LD	8 (12.9%)
ED	16 (25.8%)
NSCLC	
II	2 (3.2%)
III	10 (16.1%)
IV	26 (41.9%)
Grade	
Grade II	22 (35.5%)
Grade III	40 (64.5%)
Metastasis (No./%)	35 (56.5%)

SCLC, small cell lung carcinoma; NSCLC, non-small cell lung carcinoma; Others, 4 undifferentiated carcinoma and 1 adenosquamous carcinoma); LD, limited disease; ED, extensive disease

Among the lung cancer patients the median overall survival was 19.3 months; while the mean progression free survival was 6.3 months (Figure 2).

The mean PFS among the prepro-GRP negative versus positive cases was 7.7 months (SE=1.3, 95%CI=5.1-10.3) versus 4.6 months (SE=0.8, 95% CI=3.2 - 6.1 months) respectively with a p value= 0.041 (Figure 3).

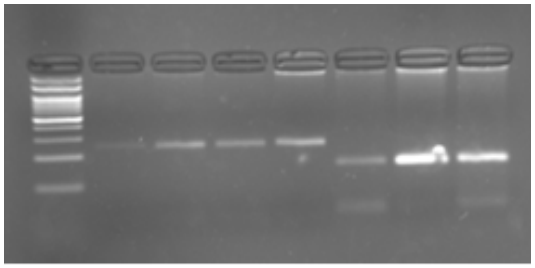

Figure 1. Prepro-Gastrin-Releasing Peptide (Prepro GRP)-Specific Nested Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Amplification Analysis. lane 1is a molecular weight marker 100bp, Lane 2,3,4,5 positive preprogastrin cases (244-base pairs), Lane 6, 7, 8 positive B- actin as control (160-base pairs)

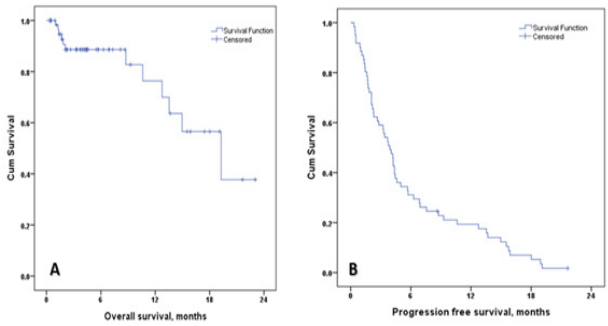

Figure 2 . Showing Overall Survival (A) and Progression Free Survival (B) of Lung Cancer Cases

Table 2. Results of PreproGRP-Specific Nested RT-PCR in PB Samples of Various Patient Groups and Healthy Donors

Subjects	PreproGRP RT-PCR-positive (%)
Patients with SCLC	14/24 (58.3)
Patients with NSCLC	
Adenocarcinoma	3/12 (25)
Squamous cell carcinoma	4/10 (40)
Large cell carcinoma	4/11 (36.4)
Others	1/5 (20)
Total	12/38 (31.6)
Healthy donors	0/30 (0)

Mean OS among preprogastrin negative cases (=17.6 months, standard error (SE) =1.6, 95% Confidence Interval (95% CI) =14.6 - 20.7 months) was longer than that among preprogastrin positive e cases (=14.9 months, SE=2.2, 95%CI=10.6-19.3 months) with a p value =0.158.

No difference in response to chemotherapy identified between both groups i.e. 19.4% in negative Prepro GRP groups vs 19.2% in positive PreproGRP group (p=0.983)

Discussion

Limited researches have been conducted about prepro-GRP expression among lung cancer cases (Lacrois et al., (2001); Salto et al., (2003); Shingyoji et al., (2003); Liu et al., (2008)). We studied its expression in 62 cases of lung cancer, and 30 age and sex matched healthy controls Twenty six (41.9%) cases were preprogastrin

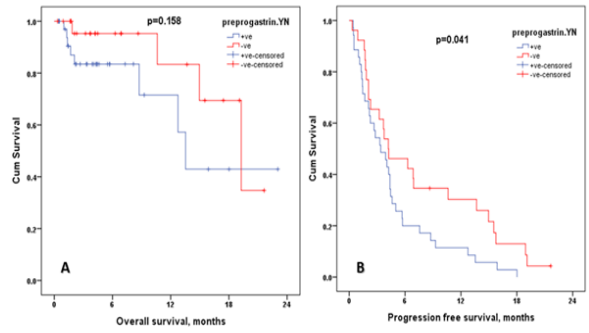

Figure 3. Showing OS (A) and PFS (B) Differences Among Prepro-GRP Negative Cases vs Prepro-GRP Positive Cases

Table 3. Relation between PreproGRP Expression and Clinico-Demographic Data of the Studied Lung Cancer Patients

	–ve PreproGRP (N=36)	+ve PreproGRP (N=26)	
Age (median; range); in years.	59 (34-81)	57 (39-74)	p=0.346
Smoking	23 (63.9%)	24 (92.3%)	P=0.010
Performance status	27 (57.4%)	20 (42.6 %)	P=0.861
Cough	25 (69.4%)	23 (88.5%)	P=0.077
Dyspnea	27 (75%)	23 (88.5%)	P=0.186
Chest pain	15 (41.7%)	9 (34.6%)	P=0.574
Pathology			
SCLC(n=24)	10 (41.7%)	14 (58.3%)	P=0.038
Squamous cell ca(n=10)	6 (16.7%)	4 (15.4%)	P=0.892
Adenocarcinoma(n=12)	9 (75%)	3 (25%)	P=0.186
Large cell carcinoma(n=11)	7 (63.6%)	4 (36.4%)	P=0.680
Grade III	22 (61.1%)	18 (69.2%)	P=0.790
Extensive stage	7 (19.4%)	9 (34.6%)	P=0.178
Stage IV disease	19(52.8%)	7 (26.9%)	P=0.042
Metastasis	23 (63.9%)	12 (46.2%)	P=0.165
Chemotherapy response	7 (19.4%)	5 (19.2%)	P=0.983

positive (53.8% SCLC, 15.4% squamous cell carcinoma, 15.4% large cell carcinoma, 11.5% adenocarcinoma and 3.8% undifferentiated carcinoma), while in the control group, 0 out of 30 healthy individuals (0%) expressed the prepro-GRP. Up to our Knowledge this is the first study in Egypt investigating the expression of prepro-GRP in the peripheral blood samples of lung cancer patients. A statistical significant relationship between preprogastrin and SCLC (P=0.038) in comparison to NSCLC was encountered.

Our results were nearly similar to the results of Lacrois et al (2001) who found that positive prepro-GRP RT-PCR in peripheral venous blood samples in 50% of the patients with confirmed SCLC, in 34% of the patients with squamous epithelial carcinoma, in 29% of patients with large cell anaplastic carcinoma, and in 20% of the patients with adenocarcinoma with 0% healthy controls.

These results were somewhat consistent with those of Salto et al.(2003), who found that prepro-GRP transcript was detected in 16 of 32 (50.0%) of SCLC patients, (5.9%) patients with adenocarcinoma, but in (0%) patients with squamous cell carcinoma, (0%) in patients with large cell carcinoma of the lung and (0%) healthy donors and the frequency of preproGRP among patients with SCLC was significantly greater than it was among patients with NSCLC ($p < 0.01$).

However Shingyoji et al., (2003) investigated expression of prepro-GRP, in peripheral blood from 40 untreated patients with SCLC, 5 NSCLC and 20 healthy volunteers. Prepro- GRP was detected in only 11% in SCLC patients, 0% in NSCLC and healthy volunteers. Also, Liu et al., (2008) investigated Prepro-GRP among 134 lung cancer patients, 106 benign pulmonary diseases and 80 healthy individuals. Prepro-GRP was detected in 34.3%, 5.7% and 0% in the studied groups respectively.

Up to our knowledge, this is the first study investigating the correlation of prepro-GRP with clinico-demographic data of lung cancer patients and it showed a statistically significant correlation between prepro-GRP and smoking (p=0.010).

There was statistically significant correlation between prepro-GRP and stage IV lung cancer patients (P= 0.042). This was in consistency with Lui et al (2008) who found that the detection rate of pre-proGRP was significantly higher in patients of stage III or IV than those of stage I or II ($P < 0.05$).

Limited research has been conducted regarding the correlation of prepro-GRP with overall survival and progression free survival. Shingyoji et al., (2003) stated that only prepro-GRP expression in bone marrow contributed to poor prognosis in patients with SCLC with statistical significance p value=0.038.

We investigated the prepro-GRP expression with the overall survival and progression free survival of lung cancer patients. Among the lung cancer patients the median overall survival was 19.3 months; while the mean progression free survival was 6.3 months. Those results were in consistency with Shokralla and Rahouma, (2015), who found that the median overall survival among Egyptian lung cancer patients was 18 months, while the median progression free survival was 6 months.

Though the mean OS among preprogastrin negative cases (17.6 months), was longer than that among preprogastrin positive cases (14.9 months) however the p value was 0.158. On the other hand, the mean PFS among the prepro-GRP negative versus positive cases showed statistical significance of p= 0.041 (7.7 months versus 4.6 months respectively).

In conclusion, the study indicated that Prepro-GRP can provide a useful tool for monitoring the overall survival and progression free survival of lung cancer patients after chemotherapy.

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