Lead exposure and plasma mRNA expression in ERBB2 gene

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Abstract. Epidemiologic data for carcinogenicity in those exposed to lead (Pb) suggests relations with cancers although the totality of the evidence is inconsistent. Alterations in the expression of ERBB receptors have been studied during the development and malignant transformation of different kinds of human tumors where they induce proliferation, angiogenesis and metastasis generation. Relevant clinical data demonstrate the role of ERBB2 receptors in the development and malignancy of human cancers. Therefore, the objective of the present investigation is to give more information on the link between plasma mRNA expression in ERBB2 gene and lead blood levels in a healthy population. Blood samples, socio-demographic, exposure and health data were obtained from 48 healthy men. Real-time polymerase chain reaction assays were performed to detect ERBB2 gene transcripts, $\Delta\Delta Ct$ method was used to quantify gene expression. Pb blood level was assayed using high-resolution sector field inductively coupled mass spectrometry and is expressed in $\mu g/dl$. Plasma mRNA expression in ERBB2 gene was 6.44 ± 3.07 $\Delta\Delta Ct$; Pb blood levels was 16.07 ± 6.74 $\mu g/dl$. Regression analysis revealed a significant association ($r^2 = 0.5345$; $p < 0.0001$) between Pb levels and mRNA expression in ERBB2. So far, it has still not been established if the expression of ERBB2 receptors is influenced by Pb exposure. On the base of the above reported data, we believe an *in vitro* study might be useful, to understand the molecular mechanisms implicated.

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Introduction

Lead (Pb) is a metal that is frequently used in many manufacturing settings worldwide and it is a significant environmental pollutant (1). The environmental occurrence of Pb, in developed countries, has decreased significantly in recent decades because of the abolition of most leaded gasoline; however, occupational exposures continue (2-4). Occupations that have had regular exposures include battery-production, battery-recycling, foundry, lead chemical, lead smelter and refinery, leaded-glass, pigment, construction and radiator-repair workers (5-12).

It has been reported that Pb can cause adverse health effects on the nervous, renal, immune, and reproductive tissues and produce harmful effects on the cardiovascular and hematological systems (1). Pb compounds are also capable of evoking a definite response in various assays including tests for enzyme inhibition, fidelity of DNA synthesis, mutagenicity, carcinogenicity and teratogenicity (2-7).

Pb also is a suspected carcinogen, with inorganic lead compounds currently identified by the IARC as probably carcinogenic (Group 2A), based on limited evidence in humans and sufficient evidence in animals (12).

Several human cancers are characterized by high levels of proteins that regulate cell cycle progression and proliferation. The HER tyrosine kinase receptor family includes four homologous epidermal growth factor (EGF) receptors: EGFR/ERBB1, HER2/ERBB2, HER3/EHBB3 and HER4/ERBB4 (13). In addition, the HER family of receptors are among the most examined cell signaling families in cancer biology (14). Deregulation of growth-factor signaling attributable to hyperactivation of the ERBB receptors is seen in a wide variety of cancers: breast, bladder, prostate, pancreatic, colon, ovary and non-small cell lung cancers (15-17).

The ERBB2 encodes a 185 kDa glycoprotein and its overexpression occurs primarily as an outcome of gene amplification. It occurs in many cancers such as ovarian, stomach, lung and prostate cancer as well as uterine serous endometrial carcinoma (18-25). ERBB2 is known to form clusters which may be a factor in tumorigenesis (26,27).

Therefore, the objective of the present study is to give more insight into the link between plasma mRNA expression in ERBB2 gene and Pb blood levels in a healthy population.

Materials and methods

Ethics statement. The research protocol was approved by the Ethics Committee of Catania University Hospital (Catania, Italy) and the written informed consent of all subjects was acquired prior to their inclusion in the study.

Study population. During periodic occupational surveillance, 48 refinery workers were visited and invited to participate in this study; none had chronic pathology such as diabetes, hypertension, thyroid, liver, kidney, lung and hematological diseases.

A questionnaire was administered by a trained interviewer to acquire information regarding occupational history, smoking and drinking habits, body mass index, duration of current employment and history of Pb exposure.

The blood samples were drawn into 2 vacuum tubes, one with EDTA one specific NH Trace Elements Sodium Heparin (Vacuette®; Greiner Bio-One International AG, Kremsmünster, Austria).

RNA extraction and ERBB2 mRNA detection. The peripheral blood was centrifuged at 2,850 x g for 10 min at 4°C and the supernatants (plasma) were collected and stored at -80°C until analysis.

RNA was extracted from 1 ml plasma from 5 ml peripheral blood using TRIzol reagent (Invitrogen, Paisley, UK) according to manufacturer's instructions. The total and quality of RNA were calculated by spectrophotometric analysis at 260 nm.

Thirty nanograms of RNA from the plasma or 1 μg RNA from mononuclear cells was transcribed to cDNA by a reverse transcriptase in a total 20 μl RT reaction solution comprising 4.0 μl 5X First strand buffer, 2.0 μM DTT, 20 U RNase inhibitor, 1 mM dNTP, 1 μM random primer and 200 U SuperScript™ II Reverse Transcriptase (Invitrogen).

Each PCR reaction (final volume of 20 μl) contained 0.5 μM primers, 1.6 mM Mg^{2+}, 1X LightCycler® FastStart DNA Master SYBR-Green I (Roche Diagnostics GmbH, Mannheim, Germany). Amplifications were carried out using a LightCycler® 1.5 (Roche Diagnostics GmbH) with the subsequent setting: i) cDNA denaturation (1 cycle: 95°C for 10 min); ii) quantification (45 cycles: 95 °C for 10 sec, 57°C for 7 sec, 72°C for 10 sec); iii) melting curve analysis (1 cycle: 95°C for 60 sec, 65°C for 15 sec, 95°C for 60 sec); and iv) cooling (1 cycle: 40 °C for 30 sec). Each amplification was made out in triplicates in three different experiments. The condition transition rate was 20°C/sec, except for the third segment of the melting curve analysis where it was set to 0.1°C/sec. Quantification was obtained by comparing the fluorescence emitted by PCR products at unknown concentration with the fluorescence emitted by external standards at known concentration. In this investigation, fluorescence values were estimated with the second derivative maximum method using LightCycler Data Analysis software (version 1.5; Roche Diagnostics GmgH). PCR products specificity was assessed

Table I. Primer sequences.

Primer	PCR product (bp)	Sequence
ERBB2 outer	338	5'-ATGTGAAGATGATGAGGATGA-3'
		5'-TAGAAGAGTTGGACAGAATGG-3'
ERBB2 inter	211	5'-GTGATGATCCACTTCCACTTA-3'
		5'-CACTGAGCCATCCTTCTCCTG-3'

by melting curve analysis followed by gel electrophoresis. The sequences of primers are presented in Table I.

To evaluate the different expression levels we employed a ΔCt method. We analyzed the mean of the crossing points (or crossing threshold = Ct) of each sample. The Ct performs the number of cycles needed to detect a fluorescence over a specific threshold level and it is inversely correlated to the quantity of the nucleic acid template. The ΔCt was calculated by normalizing the mean Ct of each sample to the mean Ct of reference gene measured in the equivalent experimental condition. The $\Delta\Delta$Ct of each sample was designed by subtracting calibrator ΔCt to sample ΔCt. The formula $2^{-\Delta\Delta Ct}$ was used to calculate the fold change.

Pb analysis. All samples were diluted 1:10 in a solution containing 0.5% of sub-distilled HNO_3 (VWR, Radnor, PA, USA) and 0.005% (v/v) of Triton™ X-100 (Sigma, St. Louis, MO, USA) and analyzed according to Batista *et al* (28). Certified reference materials were analyzed in each batch and were in agreement with reference values Seronorm™ Trace Elements Whole Blood (Sero AS, Billingstad, Norway). Iridium at 10 ng ml^{-1} was used as internal standard.

Statistical analysis. Data were summarized as mean ± SD for continuous variables and frequencies for categorical variables. Normality was checked by Kolmogrov-Smirnov test and homogeneity of variance by Levene's test. The correlation analysis was applied with logarithmic conversion to identify the correlation between mRNA expression of the ERBB2 gene and blood lead values. P-values ≤0.05 were considered significant. Data analysis was performed using GraphPad Prism version 7 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

A total of 48 male workers were included in the study with a mean age of 41.1±6.6 years. Years of working was 17.4±7.7 years. A total of 40% of subjects were smokers and 55% were non-smokers. Mean Pb blood levels was 16.07±6.74 μg/dl; only two subjects presented Pb values greater than the reference value (30 μg/dl).

The mRNA expression in the plasma for ERBB2 gene was detected in all subjects with a mean value of 6.44±3.07 $\Delta\Delta$Ct. Table II summarizes the characteristics of the studied population.

Regression analysis revealed a significant association (r^2=0.5345; p<0.0001) between Pb levels and the expression of mRNA in ERBB2. Fig. 1 shows the scatter plot of correlation analysis.

Table II. Population characteristics.

Workers, n (%)	48 (100%)
Gender (%)	M (100%)
Age	41±6.6
Years of working	17±7.7
Smokers	19 (40%)
Non-smokers	16 (55%)
Blood Pb levels (μg/dl)	16.07±6.74
Plasma mRNA expression in ERBB2 gene ($\Delta\Delta$Ct)	6.44±3.07

Figure 1. Correlation between blood lead levels plasma mRNA expression in ERBB2 gene.

Discussion

Data showed a correlation between blood lead levels and the expression of plasma mRNA in ERBB2 gene. This is the first study in literature that assesses the association between Pb and ERBB2.

ERBB receptors have an extracellular ligand-binding region, a single membrane-spanning region and a cytoplasmatic tyrosine-kinase-containing domain. The ERBB receptors are expressed in different tissues of epithelial, mesenchymal and neuronal derivation (29). Futhermore, ERBB2 have been assessed in the development of many human tumors. The structure of ERBB's extracellular region is radically different to that of others. ERBB2 has a fixed conformation that resembles the ligands-activated state: the domain II and IV interaction is absent and the dimerization loop in domain II is exposed (30,31). This structure is consistent with the information that ERBB2 is the favorite partner for the other activated ERBBs, as its interaction is permanently balanced with another ligand-bound receptor (29).

Amplification of ERBB2 leading to overexpression of the receptor, at first observed in a subset of breast cancers, occurs also in other human tumors (29).

The carcinogenic potential of Pb could be influenced by the activation of intracellular signaling pathways that change cell proliferation, survival, migration and differentiation. Pb is known to induce extracellular signal-regulated kinase 1 and 2 (ERK1/2) in numerous cell lines including CL3 human non-small cell lung adenocarcinoma cells (30), 1321N1 human astrocytoma cells (31) and PC12 rat pheochromocytoma cells (32). Pb can stimulate the α isoform of protein kinase C (PKC) membrane translocation and activation, which functions upstream of the ERK1/2 signaling in CL3 (33) and 1321N1 (31). It has been reported that activation of the PKCα → ERK1/2 signaling contributes to DNA synthesis stimulation in Pb-treated 1321N1 cells (31). In contrast, this signaling increases nucleotide excision repair, promoting survival and preventing mutagenicity in CL3 cells following Pb introduction (30,33).

Cell-surface receptor tyrosine kinases (RTK) participate in the activation of intracellular signaling pathways, as well as the PKC → ERK1/2 cascade with subsequent variety of extracellular stimuli (34,35).

The PKC family of serine/threonine protein kinases has been contemplated to be a key target of Pb (36-39).
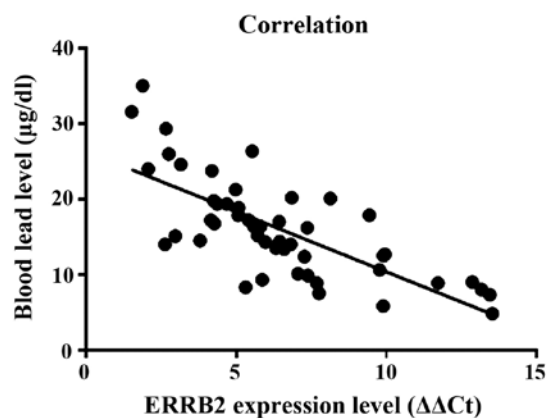
Studies indicate that Pb activation of PKCα can be upstream of ERK1/2 signaling that involves in DNA repair, survival, anti-mutagenesis (30,33) and DNA synthesis (31). Though, no evidence has been shown for the participation of receptor/non-RTKs in the activation of PKCα → ERK signaling cascade subsequent to Pb exposure.

A range of growth factors have been shown to stimulate ERBB2 kinase activity and promote Ras-mediated stimulation of a downstream kinase cascade, which includes the ERK pathway leading to tumor cell growth and migration (40-42).

Future examination is essential to explain whether the PKCα activation by Pb is mediated via such an indirect mechanism.

This study thus becomes the basis for addressing the prevention of damage caused by exposure to Pb related to occupation. Workplace risk prevention and safety rely predominantly on eliminating the risk itself known as primary prevention. However, where this is not technically feasible, technical, organizational, and procedural measures have to be enacted to reduce risk of exposure to a minimum (43,44).

When chemical agents are concerned, primary prevention entails replacing a toxic agent with a non-toxic one. Though, certain mutagenic/carcinogenic agents can be produced in synthetic processes as intermediates or as waste products (45-54).

When risk assessment determines the existence of a health risk, adequate risk control systems must be implemented. These systems are divided into general and personal protection devices (PPD). The former includes adoption of technical and procedural measures, for instance the reduction of environmental pollutants, whereas PPD largely consist of devices worn by employees (such as masks and gloves), preventing direct contact with vapors, fumes and/or potentially polluted material.

In workplaces where risks are recognized, safety procedures must be instituted in accordance with national guidelines. In case of flaws or deficiencies in such guidelines, those in charge of workplace safety are required to refer to the guidelines of internationally recognized organizations, such as the American Conference of Governmental Industrial Hygienists (ACGIH®) or National Institute for Occupational Safety and Health (NIOSH).

Epidemiological studies have demonstrated a causal association between Pb exposure and the occupational origin of certain types of human cancer (2-7,55-57). The identification of the environmental causes of human cancers has been a long and difficult process. The role of specific components and the interaction of different risk factors in the etiology of human cancer remains to be determined. Regardless of the progress achieved in understanding the cancer process and the impact of this knowledge on treatment, primary prevention remains the most effective approach to reduce cancer mortality in developed and developing countries.

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