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Harmonization of Epidermal Growth Factor Measurements: Paving the Way of Finding a Biomarker in Non-Small Cell Lung Cancer

Idania González-Pérez[1,2]* and Kalet León Monzón[1]

[1]Center of Molecular Immunology (CIM), Systems Biology, Zip Code 11600, Havana, Cuba.
[2]National Institute of Oncology and Radiobiology, Clinical Pharmacology Department,Zip Code 10400Havana, Cuba.

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Literature reports only a few contradictory findings regarding the capacity of serum EGF concentrations to differentiate between healthy individuals andpatients sufferingnon-small cell lung cancer (NSCLC). Therefore, thepossible diagnostic capacity of serum EGF levels, suggestive of dependency on this growth factor in NSCLC patients/tumors and hence indicative of possible response to therapies directed to EGF/EGFR, is controversial. Inconsistencies likely derive from the lack of harmonization and even standardization in methodologies for blood and sera processing. This manuscript is a mini-review of a recently published study, where the control of the key factors that influence theconcentration of EGF in serum, along with the normalization of EGF concentrations by platelets count,allowed to clarify the diagnostic value of serum EGF levels.Several EGF-related variables were identified as potential biomarkers in NSCLC, particularly those normalized by platelets, which highlighted the differences between patients and controls. Additionally, the study revealed thatNSCLC patients differ from healthy individuals not by the total stock of EGF, but byits higher accessibility to serum. The increase in free/accessible EGF in blood circulation is probably relevant to the biology of NSCLC, most likely because it reflects a higher accessibility to this tumoral growth factor.

Keywords: non-small cell lung cancer, epidermal growth factor, platelets, Stratification; diagnostic biomarker,predictive biomarker.

The Epidermal Growth Factor (EGF), one of the ligands of EGF receptor (EGFR), was first isolated from submandibular glandsof male mice[1]. Known to stimulate the growth of several types of epithelial tissue, possesses strong mitogenic activity on tumor cells that converts this factor in an attractive target for designing antitumor strategies[2]. One of these EGF-targeted therapies is the Cuban vaccine CIMAvax-EGF® [3],a proven effective treatment for advanced non-small cell lung cancer (NSCLC). The vaccineinduces anti-EGF antibodies that recognize the EGF in circulation, preventing its binding to EGFR, and disrupting this waythe associated signal transduction cascade in cancer

*Corresponding author E-mail: idania.gp@gmail.com

patients and ultimately cell proliferation.Studies of serum EGF concentrations ([EGF])in Cuban patients treated with this vaccine revealed that high serum EGF levels are a factor of bad prognosis for NSCLC and at the same time a predictive biomarker of CIMAvax-EGF® efficacy[4,5,6].However, high EGF concentrations in serum have been able to explain the bad prognosis and the good response to this vaccine not in all patients.Furthermore, thequestion about the capacity of EGFconcentrations to discriminate between NSCLC patients and healthy individuals (its diagnosticvalue), suggestive of dependency on EGF in patients (tumors) and hence indicative of possible response to therapies directed to this growth factor or its receptor, has not been reliably answered by the scientific community. Thus far there are only a few reports available on this topic, some of which have published discrepant findings[7,8].

This manuscript is a mini-review of a recently published study, where the standardization of methodologies for blood and sera processing, along with the normalization of the estimates of EGF by platelets count, achieved the control of the key factors that influence the concentration of EGF in serum, helping to clarify its diagnostic value.

DISCUSSION

Serum EGF concentrations: the causes of its variability

The variability in published serum EGF levels and studies is presumably caused by the lack of harmonization and sometimes of standardization in the methodologies used for blood processing, sera collection, and EGF quantification.This methodologicaldeficiency has a variety of expressions.Essentially, the majority of reports are unaware of the known dependency between serum EGF concentrations and the process of clot formation, during the incubation of blood for sera separation.Do not controlthe duration of this step[9,10], neither the type of tubes employed for blood collection, which affects clotting times and then the release of EGF by platelets[11,12,13],not even the temperature at which blood coagulates, which also influences this process.Another aspect that has probably increased the variability in reported values, thus contributing to the observed differences, is thesingle nucleotide polymorphism (SNP)[14,15,16] of the EGF promotor gen. This polymorphism is functional, and modulates the expression levels of the molecule, thus provoking natural differences among individuals, which are not associated to their healthy condition or illness.This SNP, although extensively approached in specializedliterature and linked to the risk of suffering the disease[14,15,17,18], its severity[19,20,21],prognosis[20,21,22]and response to treatment[23],in LC particularly, has not been considered in the comparison of serum EGF concentrations in patients, for the evaluation of its potential in prognosisand prediction; neither for its comparison with the respectiveconcentrations in healthy individuals, for the estimation of its possible diagnostic value.Differences in the selection of controls for the different cohorts of patients(the lack of control of confounding factors as age and gender) have also contributed to discrepant results[8].Environmental factors could additionally contribute according to Pantsulaia*et al.*[24].

Finally, the analysis of published data is more complicated due to the coexistence of different quantification platforms (ELISA, LUMINEX, microarrays, among others), some of which producenot comparable results.

Other studies have also revealed similar problemsfor several potential predictive biomarkers, which have slowed the functional transition of these markers to the oncology clinic[25,26].The identified problems include, among others, inadequate attention to: the details of specimen collection; the definition of standard operating procedures; the requirements for analytical validation of assays and the statistical evaluation of the sources of assay variability[27]. Only the overcoming of these issues will facilitate the identification of predictive biomarkers and the development of predictive tests, capable of guiding novel systemic therapies of fundamental importance for advancing in the field of precision oncology.

Standardization and normalization: the solution for a comprehensive harmonization

Recently, the evaluation of serum EGF levels and platelets counts in 25 NSCLC patients(at diagnosis and after first-line therapy)[28], employing an standardized methodology for separationof the sera[10]and its quantification[29],along with the normalization of estimated concentrations by

platelets count, has allowed to elucidate platelet´s contribution to serum EGF concentrationsin healthy individuals and NSCLC patients, aiding to clarify the diagnostic value of EGF levels in NSCLC. For quantification it was employed a validated ELISA[29], calibrated against the EGF international standard 91/530 from National Institute for Biological Standards and Control (NIBSC).It is noteworthy that the UMELISA EGF® kit exhibits similar characteristics to other commercially available assays, in terms of precision, accuracy and dynamic range[29].Furthermore, its estimations correlated very well with those obtained with the Human EGF Immunoassay Quantikine® ELISA (R&D Systems, Minneapolis, MN, USA), which is probably the most widely used kit for EGF estimations at the moment.

In this studyeach phlebotomy provided two sera, separated at 1h and 4h after venipuncture, and therefore two EGF concentrations: $[EGF]_{1h}$ and $[EGF]_{4h}$, respectively[28].The $[EGF]_{1h}$, was interpreted as a good estimate of the actual concentration of free EGF in blood circulation, while the$[EGF]_{4h}$representeda good measure of the average total stock of EGF in the blood sample of an individual. From these primary variables were constructed and studied several EGF-related variables,which were also interpreted in a simple manner from thebiological point of view.The variableratior=$[EGF]_{1h}/[EGF]_{4h}$ was interpreted as the EGF fraction from the total stock which is available in circulation.The stratification of patients using the variable rremoves the variability associated to stratification by absolute serum EGF concentrations, which is derived from the natural differences among individuals, andinherent even to measurements obtainedunder standardized procedures.Due to these inter-individual differences,the conceptshigh/low regarding toserum EGF levelsare actually relative (r). These conceptscontain information which is not included in absoluteEGF concentrations[10,28]. An estimated EGF concentration could be considered high/low depending on the percentage it represents from the total EGF of the individual. The variable difference $d=[EGF]_{4h}-[EGF]_{1h}$,which offers different but complementary information to variable r,was interpretedas the EGF stored in platelets (not available to circulation).Among the variablesnormalized by platelets count, $[EGF]_{1h}$/platelets/L was interpreted as the average EGF contributed to circulation per platelet, andd/platelets/L as the average EGF stored per platelet (not in circulation).

Diagnostic capacity of studied EGF-related variables

In the commented study[28] several variables achieved a successful discrimination between healthy individuals and NSCLC patients, at diagnosis and after chemoradiotherapy, when were evaluated by ROC (Receiver Operating Characteristic) analysis[30,31]. Differences werefound between NSCLC patients and healthy individuals regarding the accessibility of EGF to circulation, but not regarding the total stock of EGF. It was observed a higher fraction of free EGF in the circulation of patients (r) and consequently a lower amount of EGF stored in platelets (d). Similarly, the analysis of normalized variables showed that the EGF per platelet accessible to circulation ($[EGF]_{1h}$/platelets/L) was significantly higher in patients, before/after chemoradiotherapy. Conversely, the average total stocks of EGF per platelet ($[EGF]_{4h}$/platelets/L) were equal in healthy controls and patients, also before/after chemoradiotherapy.

The comparison of cohorts through the normalized variables made more evident the differences between them, suggesting an altered relationship between EGF and platelets in NSCLC patients, as contrasted with healthy controls. Overall the results of this study suggest that the increase in free/accessible EGF in blood circulation is relevant to the biology of NSCLC, most likely because it reflects a higher accessibility to this tumoral growth factor.

Inference of EGF-dependency in NSCLC

According toRodriguez´s*et al.*results[6], those NSCLC patients with high EGF concentrations have a poor prognosis andrespond better to therapy with the CIMAvax-EGF® vaccine, which reducesthe free EGF in the blood of treated patients. Thissuggests the existence of NSCLC variants with different underlying biology of the EGF/EGFR system, and patients with different levels of dependency on the availability of EGF in serum. Inspired in these findings, in Gonzalez-Perez *et al.* work[28]the studied EGF-related variables were used for stratification purposes, trying to infer the dependency on EGF in different NSCLC patients. It was reasoned that those variables with a higher

capacity for discrimination between patients and healthy controls might better capture the aberrant EGF biology in cancer patients. Therefore, these variables might be better for the identification of those patients probably more sensitive to therapies attempting to normalize EGF/EGFR interactions.

Stratification of patients with study variables

Patients were stratified using the optimal cut-off valuesobtainedaccording to Youden[32],in ROC analysis for each study variable.Patients were predicted as highly EGF-dependent or vice versa, in each specific case, depending on its ranking with respect to the selected thresholds(cut-off values). To compare alternative stratifications, itspercentages of overlapping in predictions were calculated by pairs of variables[28].For the sake of comparison, the stratification method reported by Rodriguez*et al.*[6] for the identification of patients more benefitted from CIMAvax-EGF® vaccine, was also included. In Rodriguez´s method, patients with [EGF] above the median of the studied population appear to carry tumors apparently more EGF-dependent. Making a parallel with Rodriguez´s method, in the revised study the cut-off values were also set according to the medians of either the [EGF] at 1h and 4h. Although in Rodriguez´sstudy the time of sera separation was not controlled, it was likely close to the 4h processing in González-Pérez*et al.* study, given the similarity between the correspondingreported medians of [EGF] after chemoradiotherapy (873pg/mLand 829pg/mL, respectively).

Interestingly, the classifications by the medians of [EGF] were quite different to those obtained with the normalized variables at diagnosis. However, the variable $[EGF]_{1h}$/ platelets/L appears to classify patients quite similarly to variables d/platelets/L and $[EGF]_{1h}$. After first-line chemoradiotherapy, the normalized variables showed a remarkably high coincidence in patient´s classification, and moderate overlappings with the classification by the median of $[EGF]_{1h}$. However, the classification by the median of $[EGF]_{4h}$showed a very low overlapping with the selections of anyother study variable, including the classification achieved by the median of$[EGF]_{1h}$. Therefore, the normalized variablesare quite complementary and therefore will provide similar classifications of patients, but different to those obtained when the median of [EGF] is used as cut-off, as proposed Rodriguez *et al.*[6], especially when using $[EGF]_{4h}$, a variable representing a good measure of the average total stock of EGF in the blood sample of individuals, which was not able to discriminate cases from controls in the revised study. Therefore, although in Rodriguez´s approach $[EGF]_{4h}$ could explain in some measure the prognosis of patients and the vaccine´s efficacy after chemoradiotherapy[6], the normalized variables, which were able to discriminate in that scenery, might be more valuable than $[EGF]_{4h}$for these purposes.

CONCLUSIONS

By accepting that the EGF levels in serum are influenced by several factors, which are mainlyexpressed in the process of sera separation, is comprehensible that the standardization of this procedure is crucial to guarantee results valid, reliable and comparablebetween laboratories. Therefore, besides a validated quantitative assay, calibrated against the current approved international standardfor EGF,standardization and harmonization ofkey procedures are needed.

The methodology applied in the discussed manuscriptfor the separation of the sera, the estimation ofserumEGF levels, its normalization and interpretation of results,allowed to elucidate the diagnostic value of EGF in NSCLC. Additionally, the study revealed that patients suffering NSCLCdiffer fromhealthy individuals not by the total stock of EGF, but byits higher accessibility to serum. Furthermore, the proposed approach added value to the efforts of finding an efficacy biomarker for CIMAvax-EGF® vaccine,so farapproved as a second-line therapy. Overall, the studyrevealed that the normalizedvariables might bepotential biomarkers in NSCLC, and good candidate biomarkers ofefficacy forCIMAvax-EGF®immunotherapy.Further studies are needed to evaluate the usefulness of these markerson its predictive value to select good responders to treatment with therapies targeting the EGF/EGFR system, and alsoto estimate its effectivenessin prognosis, monitoring of therapy and evaluation of response, in NSCLC and other epithelial cancers. Finally, the proposed methodology, and particularly the normalization of EGF levels by platelets count,might help to better understandthe role of

EGF in other diseases where this growth factor is also involved[33,34,35,36,37,38,39].

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