EGFR MUTATIONS IN NON-SMALL CELL LUNG CANCER: LOCAL EPIDEMIOLOGY AND CLINICAL IMPORTANCE


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EGFR MUTATIONS IN NON-SMALL CELL LUNG CANCER: LOCAL EPIDEMIOLOGY AND CLINICAL IMPORTANCE (Abstract): Introduction: Lung cancer's dismal prognosis led to new therapeutic approaches among which TKIs being among most promising; Material and method: Retrospective study at the Regional Institute of Oncology Iasi of non small cell lung cancer patients which underwent molecular investigations between November 2013 – September 2014. EGFR mutation status (positive, negative, undetermined) was assessed with an Entrogen EGFR kit using DNA extracted from paraffin embedded samples (surgical or endobronchial biopsies) with the Macherey-Nagel "NucleoSpin FFPE DNAkit" and then amplified on a Applied Biosystem 7500 Real Time PCR System. Results: There were 63 adenocarcinoma samples (17 females, mean age 60.9+/−9 years): 49 primary lung tumors and 14 secondary lesions (brain, lymph nodes, pleural). There was insufficient biotic material for three cases. TTF1 status was determined for 46 patients — six were negative. There were twelve mutations identified (7 female subjects, 5 male) - six L858R, five Del 19 and one G719X; ten were TTF1 positive for the remaining two TTF1 status was unknown. Female sex predominance was statistically significant (p=0.02, chi squared). Mean age for mutation positive patients was 64+/−10 years; there were three never smokers, three active smokers and no data on smoking status was available for six subjects. Conclusion: Although small dimension of the study group precludes statistical significance EGFR mutations seem to correlate with TTF1 status. Keywords: MUTATIONS, LUNG CANCER, EPIDEMIOLOGY.

Lung cancer is a public health problem being the most important neoplastic cause of death – each year 1.3 million deaths are registered (1). Overall the 5 year survival is less than 15% despite therapeutical efforts (surgery, radiotherapy and chemotherapy either used alone or as a combination).

Non small cell lung carcinoma accounts for almost 80% of overall lung cancers and most predominant subtypes are adenocarcinomas, squamos cell carcinomas, adenosquamos carcinomas and large cell carcinomas. Small cell and squamos cell carcinomas incidence has decreased in the last years for both sexes and many countries presumably because of reduction of tobacco consumption. Presently adenocarcinomas are the most prevalent non small cell
carcinoma subtype (almost 50% of all lung cancer cases) (2).

Lung cancer treatment is dictated by disease stage and patients general health status and comorbidities. Stage I, II and IIIa non small cell lung cancer patients are usually offered a surgical solution frequently associated to an adjuvant therapy (chemotherapy and sometimes radiotherapy); this approach has a reasonable chance of achieving cure (3). However 50 to 70% of NSCLC patients either have an advance stage at diagnosis or relapse after a curative-intent treatment. Standard chemotherapy may improve survival, alleviate symptoms and improve quality of life for these patients. EGFR Tyrosine kinase inhibitors may be a therapeutic option as they may significantly increase relapse free survival and possibly global survival (4). Still, using these compounds require identification of some specific genetic abnormalities that drive tumor development.

Current oncogenetics is considered a discrete process characterized by progressive accumulation of genetic abnormalities involving both oncogenes (promoting tumoral development) and suppressor genes (exercising a limiting effect). Suppressor genes may be classified a "caretakers" and "gatekeepers"; both alleles have to be inactivated to allow tumor development. On the other hand single allele oncogene mutations are sufficient to stimulate cell proliferation. Bioinformatics research show an average of 120 genetic anomalies for a cancer cell. Among those a limited number can be considered driver mutations for tumor development; the others are dubbed silent mutations and their involvement is limited (passenger genes) (5, 6).

There is genetic data suggesting frequent involvement of KRAS, EGFR and BRAF mutations in lung cancer. As well as ALK, ROS1, RET and RON translocations and less frequent newly described JAK2 or ErbB4 (7).

EGFR is a transmembrane protein coded on a chromosome 7 gene (short arm) and belonging to the ErbB receptor family whose members are: HER1/ErbB1 (EGFR), HER2/neu/ErbB2, HER3/ErbB3 and HER4/ErbB4. It consists of a single 1186 aminoacid polypeptide chain. EGFR can be found on the surface of almost any normal cell but it is also intensely expressed on skin and digestive tract; this explains the TKI side effects. EGFR is also largely expressed on epithelial bronchial cells and moderately on alveolar epithelial cells. Three regions may be described – an extracellular one involved with ligand interaction; a transmembrane region characterized by hidrophoby and an intracellular region associating tyrosine kinase activity (8).

Inactive EGFR can be found as a monomer on membrane level. Following ligand interaction EGFR will activate and form homodimers. Several ligands have been described: TGF-α, EGF, amphyregulin, heparin-binding EGF-like growth factor, betacellulin and epiregulin (9, 10).

EGFR may also interact with another ErbB family member such as ErbB2/Her2/neu forming an active heterodimer.

EGFR dimerization induces conformational changes thus increasing intrinsic intracellular tyrosine kinase activity. This results in multiple signal pathways activation; best known are: the RAS/RAF/MAPK pathway involved with cell proliferation, tumor invasion and metastasis, PI3K/AKT pathway leading to cell survival by inhibiting apoptosis thru nuclear transcription factors activation and the JAK/STAT
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pathway similarly involved in transcription activation (11, 12).

Under normal conditions tyrosine kinase activity is balanced by tyrosine phosphatase; there are various mechanisms leading to tyrosine kinase dysfunctions – mutations are probably the most important. Extracellular domain mutations may induce ligand independent cell proliferation; such is the case for some glioblastomas, ovarian cancers and non small cell lung cancers. Point mutations of the extracellular domain have been described for myeloma and some somatic mutations were associated with cervix and bladder cancers (8, 13, 14).

The vast majority however involve the intracellular tyrosine domain; there have been no reports on transmembrane region mutations involved in ligand independent receptor activation.

Historically all advanced stage NSCLC patients were offered first line therapy – a combination of two agents (one being a platinum compound), docetaxel or pemetrexed as second line therapy and erlotinib as a second or third line option.

The development of tyrosine kinase inhibitors (TKI) such as gefitinib and erlotinib has brought some minor survival improvements if whole NSCLC patient population was considered. Later research showed that some characteristics such as race (Asian), histology (adenocarcinoma), sex (female) and smoking status (non smoker) associate a greater response if EGFR TKI are used (7, 15, 16). This data lead the discovery of EGFR activating mutations which are predictive for response to TKI.

Mutation status can be assessed by either direct sequencing or targeted approaches.

Sequencing techniques have the main advantage to identify any mutation (previously known or not); their main drawback is the less sensitivity – samples with tumoral content over 20% are required and the method is work intensive including a PCR amplification stage prior to sequencing(1).

Targeted approaches are mainly used to detect a limited number of high clinical significance mutations – usually therapy response predictors. Sensitivity is higher and are reliably used for small biopsy or cytology samples (with down to 1% tumoral cell content).

All EGFR activating mutations were found on exon 18-21(3). Three mutation classes have been described: class I – exon 19 deletions almost exclusively involving the 747 – 749 aminoacid sequence accounting for around 44% of total EGFR mutations. Class II contains point mutations leading to aminoacid substitutions. Most frequent point mutation involves exon 21 (codon 858) leading to arginine>leucine substitution and accounts for 41% of activating TK changes. Less frequent (around 2%) class II mutations involves exon 18 -the 719 glicine is replaced by serine, alanine or cysteine (G719X) and L861Q for exon 21. Class III mutations are exon 20 duplicates or inserts and account for around 5% of total EGFR mutations (15).

To sum up, exon 19 deletions and exon 21 point mutations account for 90% of total activating EGFR mutations and are dubbed „classic” activating mutations. Their activating effect is achieved by ligand independent tyrosine kinase domain conformational changes. Exon 20 inserts associate with primary TKI resistance and T790M point mutation with secondary resistance. Activating mutations positive status is the best response predictor for TKI therapy;
recent data showed gene amplification and/or hyperexpression alone not to be effective independent predictors. Thus EGFR sequencing is the best approach to predict TKI response and progression free survival (1).

Some pharmacological agents have been developed to block different signaling pathways; almost every single one belong to the tyrosine kinase inhibitor group and EGFR targeted agents are included to every day practice (17-19).

MATERIAL AND METHOD
Retrospective study including IRO Iasi non-small cell lung cancer patients which underwent molecular investigations starting November 2013 until September 2014.

Main aim was to retrospectively evaluate the EGFR mutations prevalence in non-small cell lung cancer in IRO Iasi patients during November 2013 – September 2014.

Surgical, endobronchial or minimally invasive transthoracic biopsy samples were collected from primary tumors and secondary sites (cerebral, pleural, lymphatic).

Sample processing
Samples were formalin fixed and paraffin embedded; tissue blocks were microtomed to 3-5 μm sections. Diagnosis was reached using classical stains and immunohistochemistry approaches (TTF-1, p63, CK7, CK5/6 were the most frequently used markers).

DNA extraction
The pathologist marked the high tumoral cell percentage regions on hematoxylin eosin stained 3 μm sections – then manual macrodissection on relevant 10 μm sections was performed to reduce non malignant cell content. Paraffin was removed (toluene 5 min, ethanol 3 min, ethanol 2 min) and DNA extraction was performed using Machery-Nagel "NucléoSpin FFPE DNA Kit" and then amplified on a Applied Biosystem 7500 Real Time PCR System. The DNA quality (A260/280) and quantity (ng/μl) was evaluated by UV spectrophotometric method on a Eppendorf BiophotometerPlus and a HellmaTrayCell-Light path 1mm and a second measure was performed by fluorimetric method on a Qubit® 2.0 Fluorometer with the Qubit® dsDNA HS Assay Kit. The method that we used for the detection of EGFR mutations requires a 5-10 ng of genomic DNA per reaction (40-90 ng for a sample), with a limit of detection between 0,1-1% diluted in the wild-type genomic DNA. EGFR mutation status (positive, negative, undetermined) was assessed on Entrogen mutation EGFR kit using 10 ng of DNA extracted from paraffin embedded samples (surgical or endobronchial biopsies).

Demographic/clinical data was also collected – age, smoking status
Each patient signed an informed consent form.

RESULTS
There were 63 adenocarcinoma samples (17 females, mean age 60,9+/-9 years): 49 primary lung tumors and 14 secondary lesions (brain, lymph nodes, pleural). There was insufficient biotic material for three cases (5%). TTF1 status was determined for 46 patients – forty were positive, 6 were negative and 17 had no data provided.

There were twelve mutations identified (7 female subjects, 5 male) - six L858R, five Del 19 and one G719X; ten were TTF1 positive for the remaining two TTF1 status was unknown. Female sex predominance was statistically significant (p=0.02, chi
Mean age for mutation positive patients was 64 +/- 10 years; there were three never smokers, three active smokers and no data on smoking status was available for six subjects.

**DISCUSSION**

Our data reveals 19.04% mutation positive; the majority are point mutations L858R and del19, there was only one G719X case.

From the six L858R cases 4 were females; exon 19 deletions were present for three females and 2 males, the G719X mutation patient was male.

There were more females among mutation positive patients but less than literature data; this may be interpreted as a selection bias and insufficient addressability.

Our study group was characterized by frequent exon 21 mutations (50% of total mutation positive); this may have practical implications given the fact that best TKI response is seen with exon 19 mutations (20). There is available data reporting on worse survival for exon 21 mutation patients as compared to exon 19 mutation subpopulation mainly treated with afatinib but possibly also with first generation TKI (21, 22).

There are reports correlating TTF-1 expression and mutation status – EGFR mutations seem to be frequent in TTF-1 positive tumors (23, 24). Our data is similar as mutations were found almost exclusively in TTF-1 positive tumors. Published data suggests different mutation incidence – Asians associates greater mutation frequency (35-40%) as compared to Caucasians (11-13%) while black ancestry falls between (25, 26). Smoking status associations could not be assessed because history data was not always available or reliable.

EGFR mutations were identified for 12 out of 63 tested patients; to our knowledge this is the first Romanian study of this type up to date.

**CONCLUSION**

Although small dimension of the study group precludes statistical significance EGFR mutations seem to correlate with TTF1 status. More data from large patient numbers is necessary to profile EGFR mutation spectrum in local population. Limited data available seems similar to literature data. Local standard of practice could probably benefit from more intensive testing.

**REFERENCES**

4. *J Clin Oncol* 32:5s, 2014 (suppl; abstr 8004^)