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Background

Activating mutations of epidermal growth factor receptor (EGFR) predict sensitivity to tyrosine kinase inhibitors (TKI) in non-small cell lung cancer (NSCLC). However, despite a very high response rate (58-82%) to first line treatment with EGFR-TKI (erlotinib or gefitinib), tumors invariably progress after a median of 5-10 months from the beginning of treatment. Therefore, acquired resistance (AR) to TKIs is a clinically relevant issue to address. The knowledge of the molecular basis of AR and its early detection is instrumental to discontinue ineffective treatment and to migrate to effective second line treatments. Indeed, the dependence on EGFR signaling is maintained in most cases that develop AR, suggesting that failure of EGFR-TKIs is dependent on mechanisms of cell proliferation additional to EGFR-driven growth and insensitive to EGFR-TKIs. In vitro, several mechanisms responsible of AR have been described. In re-biopsies of tumors progressing under EGFR-TKI, the molecular aberrations include second mutation of EGFR (T790M, ~50%), MET (5-15%) and HER2 amplification (12%), PIK3CA (4.1%) and BRAF (1%) mutations and transformation into small cell histology (3%). Secondary mutations in the EGFR domains or in cross-talking pathways have been detected in biopsies of resistant cancers). NSCLC is an heterogeneous disease and the molecular fingerprint can drive therapeutic decisions; therefore, tissue availability is increasingly recognized as a crucial issue. The location of the tumor and the risk of complications are serious limitations to re-biopsies. Alternatively, detection of somatic mutations in cell-free circulating tumor DNA (cftDNA) in plasma could be instrumental to better understand the dynamics of the genetic adaptation of tumors under stress conditions caused by drug treatments. The analysis of gene mutations conferring AR on cftDNA released into the peripheral blood from primary tumor and metastatic sites and the periodic monitoring of the genetic evolution of the tumor, unfeasible with repeated biopsies, will greatly contribute to a better understanding and clinical management of drug resistance in cancer patients. Interestingly, approximately 15–25% of patients with NSCLC have KRAS mutations (mut-KRAS), resulting in constitutive activation of KRAS signaling pathways. mut-KRAS is a negative predictor of bene t to anti-EGFR antibodies in colo-rectal cancer, while it seems to be a negative predictor of response to EGFR-TKIs in EGFR wild type (wt-EGFR) NSCLC patients. In a previous study on a large collection of NSCLC tissues from patients with acquired resistance to EGFR-TKI, NRAS or KRAS mutations were not demonstrated. Despite these negative results, we employed a sensitive ddPCR-based platform

to investigate the presence of mut-KRAS alleles in plasma of patients resistant to EGFR-TKIs and we were able to demonstrate a potential role of mut-KRAS in acquired resistance to EGFR-TKI, besides the p.T790M-EGFR.Nucleic acids have been described in plasma and serum for more than 30 years. Usually, are short fragment (<1000 nt) constituted either by DNA or RNA. The concentration of cftDNA in human plasma usually ranges between 1-100 ng/ml and is dependent on the type and the size and tumor cell mass. Several hypotheses have been postulated that cftDNA derives from active release of DNA from proliferating cells, exosomes production, the leakage after tumor necrosis or apoptosis and the lysis of circulating tumor cells.

Aim

The present study is aimed at evaluating the potential importance of development of KRAS mutation as a cause of AR to EGFR-TKI in addition to the EGFR T790M and the need of molecular monitoring of the disease to improve our understanding of the biology of the disease and our ability to personalise treatment. cftDNA represents an optimal approach for a time-dependent monitoring of occurrence of secondary mutations in subjects treated with target therapy. Therefore, the aim of the study will be to prospectively assess the frequence of mutations developing during treatment and their association with resistance and to monitoring treatment AR for the emergence of well known mutations.

Material and methods

This is a prospective, translational, pharmacogenetic biomarker study in patients with EGFR-mutated NSCLC treated with EGFR-TKI as per approved (in-label) indication.

Study population

A total of 33 NSCLC patients with EGFR activating mutations (exon 19 deletion [ex19del], exon 21 c.2573T>G [p.L858R] or exon 19 c.2240T>C [p.L747P]), receiving EGFR-TKI (gefitinib or erlotinib) as per approved indication were included in this study. The analysis of EGFR mutations in primary tumors was performed following the standard diagnostic procedures (i.e., EGFR TKI response®, Diatech, Italy; Therascreen®, Qiagen, USA) adopted by each centre. mutKRAS were not examined at the time of diagnosis because mutually exclusive with EGFR mutations. Plasma and/or re-biopsy samples were taken at the time of disease progression. The analysis of mutKRAS and EGFR c.2369C>T (p.T790M) in plasma was not part of the routine clinical management and for this reason the study was submitted and approved by the Ethics Committee of Pisa University Hospital

and conducted in accordance to the principles of the Declaration of Helsinki; all patients gave their signed informed consent before blood collection and cftDNA analysis.

Plasma collection and cftDNA extraction

Six ml of blood were collected in EDTA and centrifuged at 4°C for 10 min at 3000 rpm within two hours after blood drawing. Plasma samples were stored at -80°C until analysis. cftDNA was extracted using a QIAmp Circulating nucleic acid Kit (Qiagen®, Valencia, CA) from 1 to 3 ml of plasma following the manufacturer's protocol and the DNA was eluted in 100 µl of buffer.

Analysis of cftDNA

The investigational part of this study was the assessment of KRAS codon 12 and EGFR c.2369C>T (p.T790M) mutations in cftDNA. Other mutations potentially associated with EGFR-TKI resistance were not examined because of the limited amount of cftDNA available. The analysis of cftDNA was performed by digital droplet PCR (ddPCR, BioRad®, Hercules, CA, USA) and ddPCR Mutation Assay (BioRad®). The analytic procedure was unable to discriminate the nature of the KRAS mutations detected because the analysis was performed with a ddPCR KRAS Multiplex assay. PCR reactions were assembled into individual wells of a single-use injection molded cartridge, according to the following protocol: 20 ng of template DNA (4 µl), 1 µl of 20X target primer/probe assay (FAM), 1 µl of 20X wild type primer/probe assay (HEX), 10 µl of 2X ddPCR Super Mix and 4 µl of DNAse/RNAse-free water up to a total volume of 20 µl. Droplet generation oil (70 µl) was then loaded and the cartridge was placed into the droplet generator. Using vacuum, sample and oil were mixed, generating mono-disperse droplets. Thereafter, 40 µl of packed droplets were transferred into a 96-well PCR plate for thermal cycling amplification. The protocol was standardized for all mutations to the following conditions: 95°C x 10 min, 94°C x 30 s and 55°C x 60 s (35 cycles), 98 °C x 10 min, and 4°C hold. The droplet reader (BioRad®) was used for fluorescence signal quantification. The concordance between KRAS and c.2369C>T (p.T790M) mutational status was assessed on pairwise cftDNA and tissue DNA of 8 patients who underwent re-biopsy for diagnostic purposes. DNA was extracted from formalin-fixed paraffin-embedded biopsies using the QIAmp DNA Mini Kit (Qiagen®) and analyzed using conventional diagnostics as reported above. As a positive control for mutKRAS, the cftDNA from 30 patients with known KRAS mutated pancreatic cancer was used, while the DNA extracted from plasma of 43 healthy blood donors was employed as negative control for KRAS and p.T790M mutations.

A 0,1% and 0,2% cut-off was setted for the detection of p.T790M and KRAS mutant alleles, respectively, as per manifacturers manual.

Results

Of 33 patients, 20 (60.6%) were female and 13 (39.4%) male. Median age was 62 years (range 41 – 75); 32 patients were affected by a stage IV disease, while one was a stage IIIB NSCLC. The frequency of EGFR activating mutations was as follows: 20 patients (60.6%) showed ex19del, 10 patients (30.3%) p.L858R, 2 patients (6.1%) p.L747P and 1 patient presented exon 19 insertion (3%). As expected, the majority of them (66.7%) was never-smokers, while 9 (27.2%) and 2 (6.1%) patients were former- and current-smokers, respectively. Twenty-seven (81.8%) subjects received gefitinib and 6 (18.2%) erlotinib; the treatment was administered as first-line in 23 (69.7%) (including 2 as maintenance), second-line in 6 (18.2%) and third or further lines in 4 patients (12.1%). The majority of them (66.7%) presented partial response to TKI treatment and only 1 patient showed complete response. Stable and progressive diseases were observed in 4 (12.1%) and 6 subjects (18.2%), respectively. Patients who underwent to progression of disease on EGFR-TKI treatment presented the following molecular profile in their primary tumors: p.L747P and ex19del (n=1 each) and p.L858R (n=4); these patients were receiving gefitinib. Median time to progression (TTP) was 13.6 months (95%CI, range 8.0 – 19.2) and median overall survival (OS) was 40.2 months (95%CI, range 25.8 – 54.7) for the overall population.

In 16 patients (48.5%), a codon 12 mut-KRAS was detected in cftDNA. In addition to this, the EGFR c.2369C>T (p.T790M) second site mutation was present in the cftDNA of 24 patients (72.7%). Interestingly, 13 patients (39.4%) had both the KRAS and p.T790M mutations, while 3 (9.1%) and 11 (33.3%) subjects displayed only mut-KRAS or p.T790M, respectively. In 6 subjects, no mutation was detected.

In 8 patients, paired re-biopsies and cftDNA were available. The analysis of re-biopsies by standard methods and ddPCR demonstrated EGFR c.2369C>T (p.T790M) mutation in 4 (standard) vs. 2 (ddPCR) samples and mut-KRAS in none (standard) vs. 3 (ddPCR) specimens. p.T790M was detected in 7 cftDNA specimens and mut-KRAS in 5 of them. The concordance between tissue (standard methods) and plasma (ddPCR), calculated by combining positive and negative results, was 62,5% for p.T790M and 37,5% for KRAS. Moreover, three paired samples found positive for the p.T790M on re-biopsies (standard methods) and on cftDNA (ddPCR), were negative by ddPCR on tissue.

Finally, the analysis of survival data in our patients, stratified on the basis of KRAS mutational status, showed that TTP and OS in wild-type vs. mut-KRAS were not statistically different, despite a

trend towards a better survival in wild-type vs. mut-KRAS, i.e., TTP 14.4 mo. vs 11.4 mo ($p=0.97$); OS 40.2 mo vs 35.0 mo ($p=0.56$), respectively.

Discussion

The present study demonstrates the presence of MUTKRAS in the cftDNA of a significant proportion of patients progressing after EGFR-TKI treatment. In addition to this, the present study provides evidence that sensitizing mut-EGFR and mut-KRAS can coexist after the selective pressure of EGFR-TKI treatment. p.T790M-EGFR determines acquired resistance by increasing the affinity of EGFR to ATP. p.T790M-EGFR has been described in re-biopsies of 50–63% of tumors progressing under EGFR-TKI treatment and in the cftDNA at a frequency similar to our study. Because drugs active on p.T790M-EGFR, such as AZD9291 and rociletinib, are under clinical study and will be available soon in the clinical practice, the identi cation of this molecular marker is of utmost clinical relevance.

In our study, p.T790M-EGFR was more frequent in p.L858R-EGFR patients than in ex19delEGFR ones (80% vs. 67%); on the contrary, mut-KRAS in cftDNA was detected in 55% of patients with ex19delEGFR vs. 30% of patients with L858REGFR. To our knowledge, a mechanism of resistance depending on activating mut-EGFR has not been previously reported; however, this cohort is too small to draw any conclusion.

Mut-EGFR and mut-KRAS are mutually exclusive in primary NSCLC and only anecdotal case reports described their coexistence. Mut-KRAS occurs in approximately 20% of NSCLC cases at diagnosis, more frequently in Caucasian population, adenocarcinomas, males and current smokers. About 90% of KRAS mutations occur in exon 2 (codon 12 and 13), while exon 3 (codon 61) is less frequently involved; in never-smokers with lung adenocarcinoma, mut-KRAS is more frequently a transition (G to A) compared to transversion in current smokers. Colo-rectal cancer cells with mut-KRAS treated with anti-EGFR monoclonal antibodies are able to escape growth inhibition by several mechanisms, including mut-RAS [28]. While the role of mut-KRAS in primary resistance to EGFR-TKIs in molecularly unselected NSCLC is quite well established, its development and role in acquired resistance to EGFR-TKIs in mut-EGFR patients has not been explored in detail. In a previous work on a large collection of NSCLC tissues from patients with acquired EGFR-TKI resistance, mut-NRAS or mut-KRAS were not demonstrated. However, comparison with the present results is not possible because detailed information were not provided neither on the timing of sampling with respect of development of TKI resistance nor on the type of tissue

analysed. Therefore, we addressed this issue and a sensitive ddPCR-based platform was employed to investigate the presence of mut-KRAS alleles besides the well-known p.T790M-EGFR. Due to its high sensitivity, ddPCR is able to identify small amounts of mut-KRAS and many methodological issues need to be addressed prospectically, particularly the threshold level of both mut-KRAS and p.T790M-EGFR to be considered clinically relevant. However, a mechanism of drug resistance does not necessarily reflect biologic aggressiveness and the lack of difference in OS between KRAS wild-type and mutated patients it is therefore not surprising. The numeric dimension of the cell clone bearing mut-KRAS should be taken into consideration as well. Nevertheless, despite the low proportion of smokers in our cohort, the high prevalence of mut-KRAS could support its role of as mechanism of acquired resistance.

Eight patients underwent re-biopsy after tumor progression during EGFR-TKI, allowing a comparison between tissue and cftDNA. The detection of mutations in cftDNA but not in re-biopsy, using both standard methods and ddPCR, could suggest the presence of heterogeneity within metastatic sites or the lower performance of ddPCR in the presence of paraffin. Nevertheless, the detection of mutations in both plasma and tissue by ddPCR, but not by standard methods, could be due to the higher sensitivity of ddPCR analysis. Two patients, initially diagnosed wt-KRAS by standard method, were re-analysed by ddPCR and were found mut-KRAS in the primary biopsy, suggesting that the mut-KRAS clone co-existed with activating mut-EGFR since the beginning, as also demonstrated in previous reports. In these patients, mut-KRAS cannot strictly be considered a mechanisms of resistance but it could be possible that EGFR-TKI treatment may have favored the expansion of mut-KRAS-positive clones. However, conclusions cannot be drawn as pre-treatment cftDNA was not available. Our observation is a pivotal evidence of the presence of mut-KRAS in cftDNA of tumors with sensitizing mut-EGFR resistant to EGFR-TKIs. A small percentage of our patients received EGFR-TKI as third line therapy and a new biopsy was not repeated at this time. It could be possible that mut-KRAS appeared before the initiation of TKI as a mechanism of resistance to previuos therapy. This hypotesis is weakened by the evidence that patients given second or further lines of therapy showed TTP and OS similar to patients treated in first line, although a mechanism of resistance does not necessarily affect survival. The presence of mut-KRAS has been recently reported using next generation sequencing analysis of tumor re-biopsies after progression under EGFR-TKI treatment [31], similarly to colo-rectal cancer treated with EGFR antibodies. It remains to be determined if the presence of p.T790M-EGFR and mut-KRAS coexist in the same tumor cell or arise in different subclones.

Targeting mut-KRAS proteins is still a challenge. Theoretically, it is possible that MUTKRAS identify a less responsive subgroup of as pre-treatment cftDNA was not available. Our observation is a pivotal evidence of the presence of mut-KRAS in cftDNA of tumors with sensitizing mut-EGFR resistant to EGFR-TKIs. A small percentage of our patients received EGFR-TKI as third line therapy and a new biopsy was not repeated at this time. It could be possible that mut-KRAS appeared before the initiation of TKI as a mechanism of resistance to previuos therapy. This hypotesis is weakened by the evidence that patients given second or further lines of therapy showed TTP and OS similar to patients treated in first line, although a mechanism of resistance does not necessarily affect survival. The presence of mut-KRAS has been recently reported using next generation sequencing analysis of tumor re- biopsies after progression under EGFR-TKI treatment [31], similarly to colo-rectal cancer treated with EGFR antibodies. It remains to be determined if the presence of p.T790M-EGFR and mut- KRAS coexist in the same tumor cell or arise in different subclones.

Targeting mut-KRAS proteins is still a challenge. Theoretically, combined treatment with KRAS and EGFR inhibitors can be administered to patients to prevent mut-KRAS-dependent resistance or restore sensitivity to EGFR-TKIs, as recently demonstrated co-targeting EGFR and MEK. To date, p.T790M-EGFR remains the most important predictor of efficacy of third generation EGFR-TKIs. Moreover, it was found that the coexistence of both activating MUTEGFR and mut-KRAS was not necessarily a negative predictor for EGFR-TKI therapy [23]. With these evidences in mind, all patients with p.T790M-EGFR should receive third generation EGFR-TKI, even in the presence of mut-KRAS. Theoretically, it is possible that MUTKRAS identify a less responsive subgroup of patients but this hypothesis should be validated by monitoring patients prospectically during second-line therapy. Beside p.T790M-EGFR and mut-KRAS, other mechanisms of acquired resistance not evaluated in our study have been described in tumor re-biopsies after EGFR-TKI progression, including actionable mutations of MET, HER2, PIK3CA or transformation into small cell hystology (3%).

In conclusion, despite the small number of patients involved, the retrospective analysis and the low rate of re-biopsies, our results confirm the importance of cftDNA analysis for the monitoring of secondary mutations associated with EGFRD-TKI resistance in NSCLC and underline the role of a highly sensitive approach, i.e., ddPCR, for the detection of low-level mutations. The clinical relevance of these findings, expecially for what concerns mut-KRAS, needs to be evaluated prospectively. These observations open new perspectives in the molecular mechanisms of

acquired resistance, indicating a possible role of mut-KRAS in tumor escape from pharmacologic treatment. The effect of mut-KRAS in NSCLC with activating mut-EGFR needs to be further elucidated at the molecular level and encurages the development of mut-KRAS inhibitors for an optimal treatment of patients.