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There is still a role for cytology in the 'liquid biopsy' era. A lesson from a TKI-treated patient showing adenocarcinoma to squamous cell carcinoma transition during disease progression

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ABSTRACT

Non-small cell lung carcinoma harbouring epidermal growth factor receptor (*EGFR*) mutation, usually progress after an initial response to tyrosine-kinase inhibitors (TKI). Liquid biopsy enables with a simple blood draw the accurate detection of *EGFR* p.T790M mutation, the most common resistance mechanism, avoiding the more invasive tissue re-biopsy. However, in a subset of cases, resistance mechanisms are more complex featuring both genetic and morphological changes. Here we report the case of a 67 years-old woman, affected by an *EGFR* mutated lung adenocarcinoma and treated by TKI. At disease progression, the patient developed a morphological transition to squamous cell carcinoma in association to the arising of a *PIK3CA* p.E542K mutant subclone. This case illustrates that, even in the "liquid biopsy" era, cytology can have still a role by providing an overall assessment of both morphology and genetic TKI resistance mechanisms.

INTRODUCTION

Non-small cell lung cancer (NSCLC) is often diagnosed at an advanced stage on small tissue biopsy samples, including fine-needle aspiration (FNA) specimens. NSCLC morphological subtyping, identification of adenocarcinoma (ADC) component and epidermal growth factor (*EGFR*) mutational testing are crucial to select those patients who could benefit from the tyrosine kinase inhibitors (TKI) administration.[1 2] Whenever the tissue specimen is scant and not adequate for molecular testing or tumour sampling is risky or not feasible, *EGFR* mutational testing on the cell-free DNA (cfDNA) obtained by blood draw (liquid biopsy) enables treatment decision making at the baseline.[3] In addition, the liquid biopsy is also useful in patients developing progressive disease, who are too debilitated for a tissue re-biopsy, even by the minimal invasive FNA procedure; in fact, the demonstration on cfDNA of the *EGFR* p.T790M-resistant mutation selects these patients for third-generation irreversible TKI administration.[4] However, only microscopy of tissue obtained through histological biopsy or FNA can demonstrate the morphological transitions from ADC to small-cell or squamous cell carcinoma, a less frequent resistance mechanism.[5 6] Detecting morphological transition could be relevant for the patient management, leading to a more effective attempt to stabilise tumour progression.[5 6] Here, the case of a 67 year-old woman with a lung ADC harbouring a *EGFR* mutation and featuring ADC to squamous cell carcinoma transition during TKI treatment is reported to highlight the need to combine morphology assessment and molecular tumour profiling.

CASE REPORT

A 67-year-old woman presenting with cough and dyspnoea was admitted in November 2015 at this study Institution. The thoracic CT scan revealed at the apex of the right upper lung lobe a 30 mm mass, infiltrating the mediastinal pleura (figure 1A) with concurrent multiple bilateral lung lesions and secondary involvement of mediastinal and right cervical lymphnodes. CT-guided FNA was performed on the primary lesion. An experienced pathologist (EV) performed rapid on-site evaluation (ROSE) on a Diff-Quik (Bio-Optica, Milan, Italy) stained smear, ensuring for sample adequacy. The microscopy showed a moderately cellular smear with honeycomb and papillary-like groups of cells featuring pleomorphic, atypical nuclei and abundant cytoplasms (figure 1B). To perform ancillary stainings for morphological subtyping and molecular predictive testing, the material aspirated from a second pass was formalin fixed and dedicated to paraffin-embedded cell-block preparation. On H&E staining, tumour cells showed cytoplasms featuring a secretive appearance with microvacuoles and macrovacuoles. Immunostainings displayed a strong nuclear TTF1 (clone: 8G7G3/1, Ventana, Roche, Basel, Switzerland) signal (figure 1C), whereas p63 (clone: 4A4, Ventana, Roche, Basel, Switzerland) was negative. On the overall, morphological and immunocytochemical features were diagnostic of lung ADC. The recent international guidelines recommended that any newly diagnosed IIIB/IV disease stage lung ADC should undergo predictive molecular testing.[2] To this end, our Institution validated a next-generation sequencing (NGS) assay on the Ion Torrent Personal Genomic Machine (PGM, Thermofisher, Waltham, Massachusetts, USA), developing a custom NGS panel to detect mutations in relevant lung cancer genes such as *EGFR*, *KRAS*, *NRAS*, *BRAF* and *PIK3CA*.[7]

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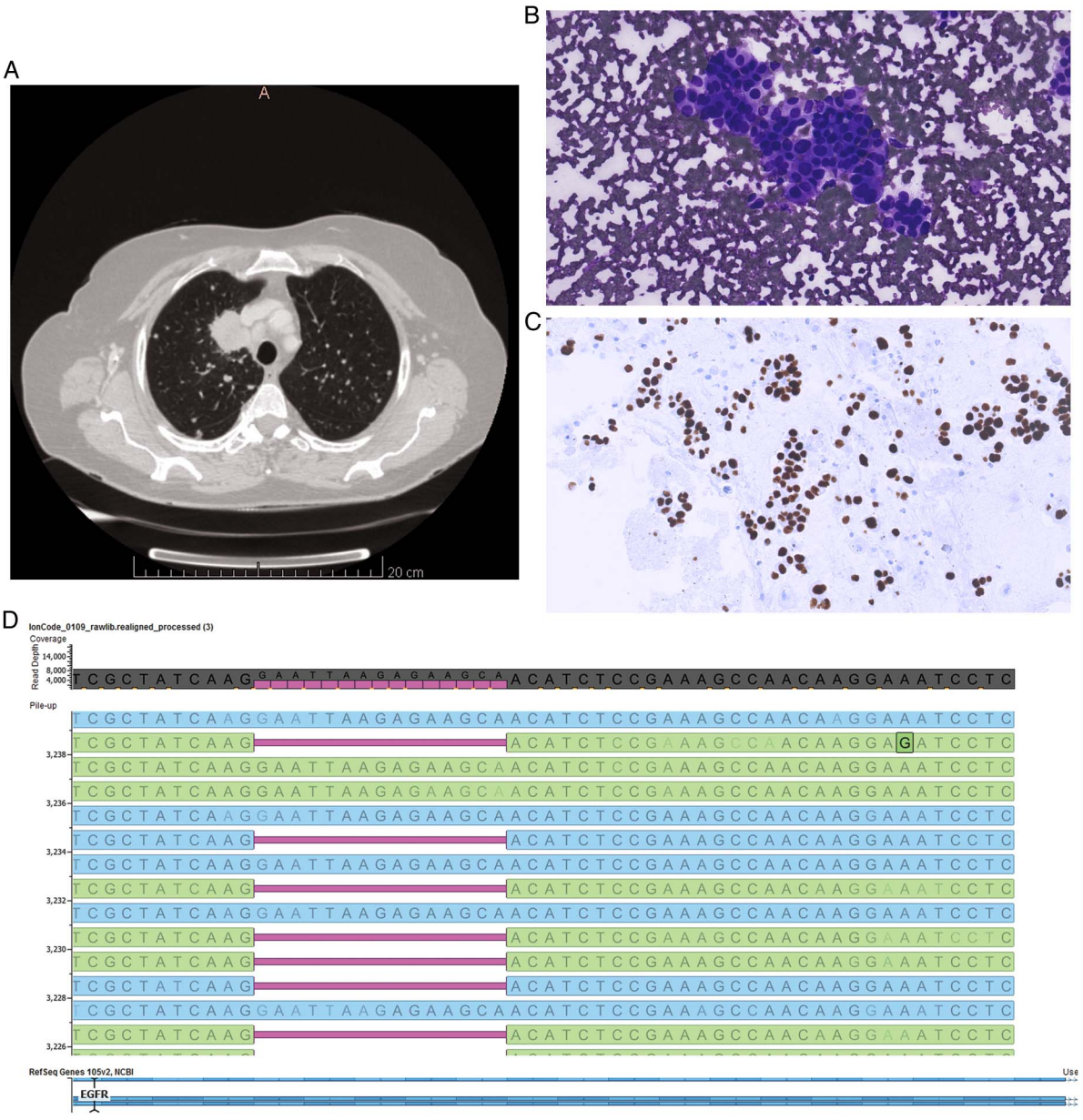

Figure 1 Thoracic scan (A) showing a 30 mm mass at the right upper lung lobe, infiltrating the mediastinal pleura (CT); the smear obtained from the CT-guided fine-needle aspiration (B) showed papillary-like groups featuring pleomorphic, atypical nuclei (B, Diff-Quik stain, ×200); on immunocytochemistry (C), the neoplastic cell showed a diffuse, strong TTF1 nuclear signal (C, haematoxylin counterstain, ×200). Visual inspection of BAM file showing the *EGFR* p.E746_A750del ELREA deletion in 73.4% of alleles (D).

The DNA was extracted from three unstained 5 μm cell-block sections after the H&E staining and microscopy review of a last additional section, to ensure a sufficient number of neoplastic cells. The unstained sections were scraped by a sterile scalpel from the slides and genomic DNA was extracted (2 ng/μL) by using the QIAamp DNA Mini Kit (Qiagen, Crawley, West Sussex, UK). Then, a gene panel library was produced by dispensing 15 μL of DNA on Ion Code plates and amplified using Ion AmpliSeq DL8 (Life Technologies). We used 20 cycles for DNA amplification and 4 cycles for library reamplification after barcoding, under the thermal conditions defined by the manufacturer. The pooled libraries were re-loaded into the Ion Chef instrument, and templates were prepared using the Ion PGM Hi-Q IC Kit (Life Technologies). The final templates were loaded into the 316v2 chip and sequenced on PGM. Signal processing, base calling and coverage analysis were carried out on Torrent Suite (v.5.0.2), and BAM files were visually inspected via the Golden Helix Genome Browser v.2.0.7 (Bozeman, Montana, USA). Only variants with ≥10X allele coverage and a quality score ≥50, within an amplicon covered at least 250X, were called; the frequency of each mutant allele was recorded. The NGS variant call software and visual inspection revealed, in this case, the *EGFR* exon 19 p.E746_A750 ELREA deletion in 73.4% of 336 sequencing reads (figure 1E). Based on this finding, the patient underwent TKI treatment and afatinib (Boehringer Ingelheim, Ingelheim am Rhein, Germany) was administered at 40 mg dose once daily for 6 months. However, in October 2016 the patient developed a right cervical lymph-node enlargement (figure 2A) and underwent ultrasound (US)-guided FNA. At our Institution, a dedicated

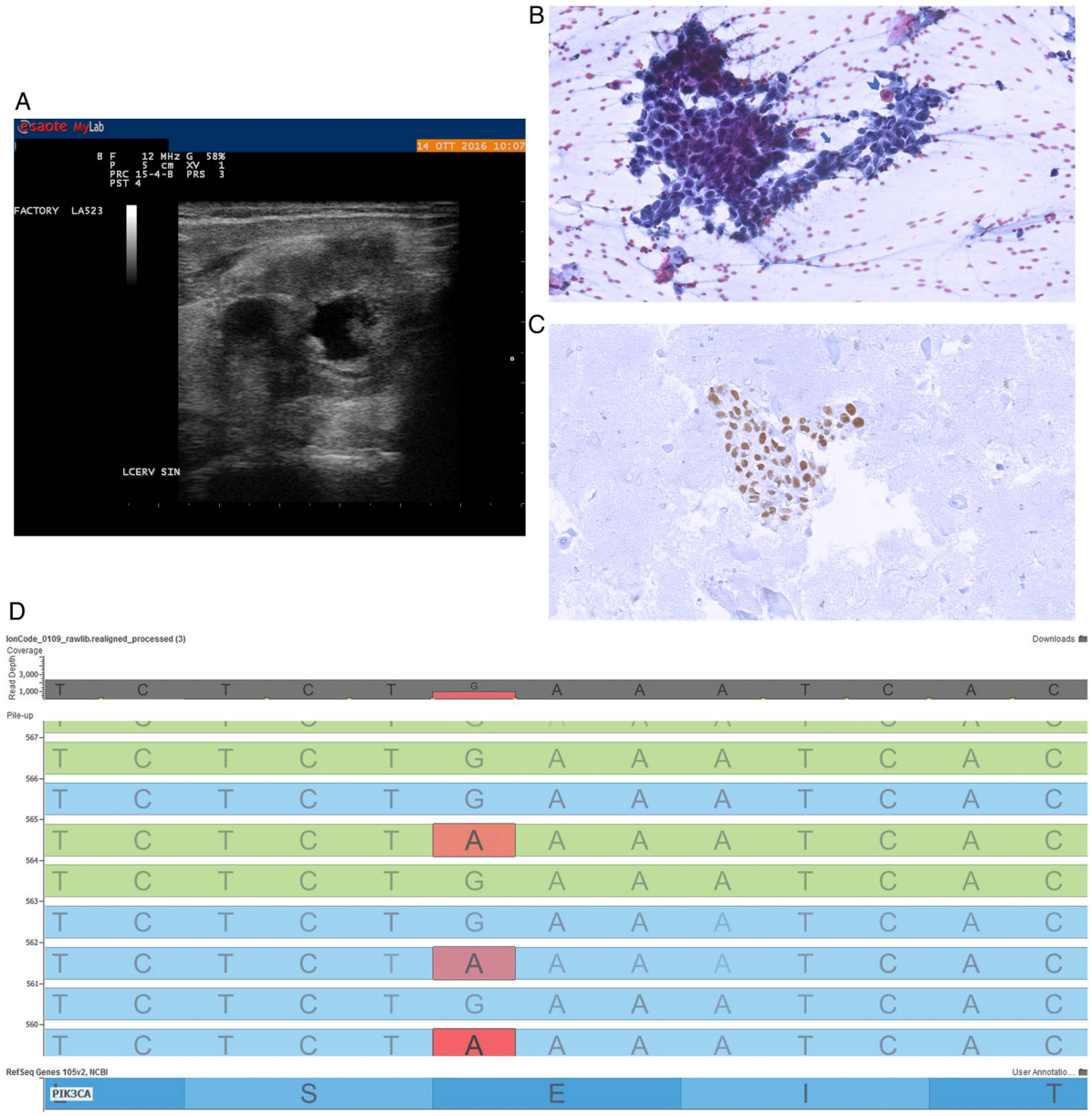

Figure 2 After 6 months of tyrosine kinase inhibitors therapy, the ultrasound (US) scan (A) showed a 3 cm right cervical lymphnode featuring a colliquated anechoic centre; the Papanicoalou-stained smears obtained from the US-guided fine-needle aspiration (B) revealed crowded and highly cohesive cell groups showing stratification (arrow) and dense cytoplasmic orangeophilia (arrow point, ×200); the immunocytochemistry showed a p63 positivity (C), confirming the squamous cell morphology observed on Papanicolaou (haematoxylin counterstain, ×200). Visual inspection of BAM file showing an additional *PIK3CA* p.E542K point mutation in 38.1% of alleles (D).

cytopathologist (CB) performs the FNA under US guidance independently, assessing at same time the specimen adequacy by ROSE and, as above described, taking additional passes to prepare the cell block. Microscopic examination of the lymph-node FNA showed squamous cell carcinoma, which was evident on alcohol-fixed, Papanicolaou-stained smears (figure 2B). The tumour cells displayed strong p63 nuclear positivity by immuno-histochemistry, whereas TTF1 was negative (figure 2C). DNA was extracted (15.4 ng/μL) and NGS was performed according to the protocol above described. The NGS variant call software and visual inspection revealed the original *EGFR* exon 19 mutation (detected in 42.6% of the 8695 sequencing reads) suggesting that this secondary deposit shared the same genotype origin from the ADC primary. In addition, a concurrent *PIK3CA* p.E542K mutation (detected in 38.1% of the 2370 sequencing reads) was observed (figure 2D). Changing in therapy was based on the morphological subtype identified by the FNA. Afatinib administration was discontinued, and a platinum plus vinorelbine-based regimen was adopted. At the time of the submission of this manuscript, the patient was alive with stable disease.

DISCUSSION

The invariably development of resistance to TKI treatment is associated in nearly half of NSCLC to the detection of *EGFR* p.T790M mutation.[4] This makes the neoplastic cells responsive to the third-generation irreversible TKI, and thus that its timely detection is mandatory to effectively treat patients showing disease progression. Body fluids, such as peripheral blood, can be tested by highly sensitive molecular techniques to overcome

Table 1 Clinical, morphological, molecular and therapeutic data of the adenocarcinoma to squamous cell carcinoma transition cases reported in the literature to date[8–14]

	Baseline						Progression							
Case id	Age/sex	Morphology (±IHC)	Sampling method	Anatomic site	*EGFR* mutation	Therapy	Progression time (months)	Morphology (±IHC)	Sampling method	Anatomic site	*EGFR* mutation	Additional mutation	Therapy (patient status)	Ref.
1	66/F	ADC (TTF1+, p63−)	B; PE	LLL	p.E746_A750del	Carbo/pem then erlotinib	8	SqCC (TTF1−, p63+)	B	LLL	p.E746_A750del	–	(DOD)	Levin *et al*[13]
2	74/F	ADC (TTF1+, p40−)	B	LL	p.L858R	Gefitinib	10	SqCC (p40+, TTF1−)	B	LL	p.L858R+p.T790M		Carbo/Vino (SD)	Jukna *et al*[8]
3	79/F	ADC (TTF1+, p40−)	B; PE	RLL	p.E746_A750del	Gefitinib	15	SqCC (p40+, TTF1−)	B	RL	p.E746_A750del +p.T790M		Gefitinib (SD)	Jukna *et al*[8]
4	58/F	ADC	CT-guided FNA	RUL	p.E746_A750del	Carbo/pem+RT then erlotinib	19	SqCC (p63+, CK 5/6 +, TTF1−)	CT-guided FNA	LL	p.E746_A750del	–	–	Scher *et al*[9]
5	51/F	ADC (TTF1+, napsin A+, p40−, CK 5/6-)	EB	RL	p.E746_A750del	Gefitinib	4	SqCC (p40+, CK5/6 +, TTF1−, napsin A-)	Lobectomy	RLL	p.E746_A750del	–	Gem/cis (SD)	Hsieh *et al*[12]
6	61/F	ADC (TTF1+, p40−)	B	RUL	p.L858R	Gefitinib then pem/platinum	18	SqCC (p40+)	B	RP	p.L858R	–	Erlotinib (D)	Hsieh *et al*[12]
7	48/F	ADC	B	LL	p.E746_A750del	Gefitinib (2 years) then platinum	30	SqCC (p40+, TTF1−)	B	LL	p.E746_A750del	–	(DOD)	Haratani *et al*[14]
8	64/F	ADC	B	RL	p.L858R+p.T790M	Gefitinib+cytotoxic agents	–	SqCC (p40+, TTF1−)	B	RL	p.L858R+p.T790M	–	Rociletinib (SD)	Haratani *et al*[14]
9	63/F	ADC (TTF1+, CK5/6−, p63−)	PE	RUL	WT*	Carb/pem/bev (12 months), doc (9 months), erlotinib (22 months)	43	SqCC (TTF1−, p40+)	B	RUL	–	p.L858R+p.T790M	–	Bugano *et al*[10]
10	63/F	ADC (TTF1+, p63−)	B	LLL	p.L858R	Erlotinib then cis/pem	5	SqCC (p63+, TTF1−, PASD-)	B	LLL	p.L858R	p.H1047R (*PIK3CA*)	Gefitinib (D)	Kuiper *et al*[11]
11	67/F	ADC	CT-guided FNA	LSD	p.E746_A750del	Afatinib	6	SqCC (p63+, TTF1−)	US-guided FNA	SL	p.E746_A750del	p.E542 K (*PIK3CA*)	Carb/vin (SD)	Clery *et al* 2017

*Low cellularity on cytological samples.
ADC, adenocarcinoma; B, biopsy; Bev, bevacizumab; Carbo, carboplatinum; cis, cisplatinum; D, dead (other causes); Doc, docetaxel; DOD, dead of disease; EB, excisional biopsy; FNA, fine-needle aspiration; IHC, immunohistochemistry; LL, left lobe; LLL, lower left lobe; PE, pleural effusion; Pem, pemetrexed; RL, right lobe; RLL, right lower lobe; RP, right pleura; RUL, right upper lobe; SD, stable disease; SL, supraclavicular lymphnode; SqCC, squamous cell carcinoma; Vin, vinorelbine.

the need of a tissue re-biopsy. However, as shown in this report, the TKI resistance can also be linked to the morphological shift from ADC to either small cell or squamous cell histotypes.[5 8] In particular, the transition to small cell carcinoma has been well described in sizeable series of cases. As an example, Sequist *et al* reported the transition to small cell carcinoma in 7/37 (14%) patients. Conversely, the morphological transition to squamous cell carcinoma has only been described in single case reports and mostly on histological material,[8–14] for a total of 11 cases including the present one (table 1). More in detail, in addition to the original *EGFR* sensitising mutation, three cases also harboured the p.T790M substitution (cases #2, #3 and #8), whereas one case harboured a *PIK3CA* (p.H1047R) mutation (case #10, table 1). Interestingly, also in our case a *PIK3CA* mutation (p.E542K) was detected together with the morphological shift suggesting that there may be a link between *PIK3CA* and the acquired squamous phenotype. Accordingly, squamous cell lung carcinoma is associated to *PIK3CA* mutations more frequently (8.9%) than ADC (2.9%).[15] However, a *PIK3CA* mutation has been demonstrated also in one case of ADC to small cell carcinoma transition,[5] questioning its specific association to the squamous cell morphological shift. Since the microscopical demonstration of a morphological transition impacts patient management, the present case may suggest that re-biopsy is still worth doing. In fact, following therapy scheme changes, patients (#2, #5 and this report #11 case, table 1) featured temporarily stable disease basing on the available follow-up. Noteworthy, two of the three patients (#3, #6 and #10, table 1), who despite the morphological shift were still treated with reversible TKI died. Interestingly, only one (#8) of the three cases (#2, #3 and #8, table 1) that also developed the additional *EGFR* p.T790M mutation was treated with a third-generation irreversible TKI, demonstrating a stable disease during the 10 months of available follow-up.[14]

The biological basis of morphological transition and TKI resistance mechanisms are still to be elucidated, and further investigation is required to assess the role of the primary tumour heterogeneity. In fact, it can be argued that tumours with concurrent adenomatous and squamous components had been misdiagnosed as 'pure' ADC, due to the inherent inability of small-sized biopsies to fully reflect tumour heterogeneity, as it was demonstrated in a patient affected by an *EGFR* mutated adenosquamous carcinoma who developed a brain metastasis that featured only the squamous cell component but still harboured the original *EGFR* mutation.[16]

CONCLUSION

In the present report, we have shown a case in which the FNA cytology accurately diagnosed an *EGFR* mutated lung ADC and, on progression, its transition to a squamous cell histotype harbouring the same *EGFR* alteration and a concurrent, additional *PIK3CA* mutation. Thus, in monitoring patients treated by TKIs there is still a role for cytology. In fact, besides the TKI resistance molecular mechanisms well detectable also by the liquid biopsy, the demonstration of a morphological transition can lead to appropriate therapeutic management.

Take home messages

- Liquid biopsy is a new non-invasive tool for the monitoring of TKI resistance mechanisms.
- However, rare resistance mechanisms (i.e. morphological shift) can be only identified by microscopy.
- In these cases, aspiration cytology may provide both molecular and morphological information, avoiding more invasive approach.

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Competing interests None declared.

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