ORIGINAL ARTICLE

# EGFR T790M detection in formalin-fixed paraffin-embedded tissues of patients with lung cancer using RNA-based in situ hybridization: A preliminary feasibility study

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## Abstract

**Background:** Following drug resistance in patients with lung cancer treated by EGFR TKIs, a biopsy is required to obtain sufficient cancer tissue for T790M detection in order to select potential beneficiaries suitable for third-generation EGFR TKIs, such as osimertinib. The purpose of this study was to explore the feasibility of using a new in situ analysis technique based on RNA target sequences to detect EGFR T790M in lung cancer.

**Methods:** A total of 28 formalin-fixed paraffin-embedded (FFPE) samples from 24 lung adenocarcinoma patients archived in Peking Union Medical College Hospital from 2015 to 2016 were collected. The BaseScope T790M detection technique by in situ hybridization on FFPE slides was used to analyze the mutation of EGFR T790M, and then the results were compared with the data acquired by Scorpions ARMS assay, which is the so-called gold standard for EGFR gene mutation testing. The sensitivity and specificity of the BaseScope T790M detection technique were preliminarily evaluated.

**Results:** Of the 28 FFPE specimens, the average proportion of T790M-positive cells was 35.78% ± 17.68% in 18 samples with EGFR T790M, confirmed by Scorpions ARMS assay, Compared with real-time PCR assay, the sensitivity and specificity of BaseScope T790M were all 100% in our cohort.

**Conclusion:** BaseScope T790M assay could be completed on only one FFPE slide and the visualized molecular result overplayed with histomorphological information perfectly, so it may be the alternative method for EGFR T790M evaluation. BaseScope assay has potential clinical utility, and it will be necessary to carry out validation with a large number of cases.

## Introduction

Epidermal growth factor receptor (EGFR) gene mutation detection in patients suffering from lung cancer is the key step before EGFR tyrosine kinase inhibitor (TKI) therapy, which only targets potential beneficiaries with a specific gene mutant subtype, e.g., exon 21 L858R, exon 19 deletion, and exon 20 S768I.Therefore, the relevant consensus and guidelines in China have emphasized the necessity and importance of assessment on EGFR gene status, the standardization of experimental operations, the acquisition and processing of specimens, and the selection of methodologies and scenarios of implementation due to the high percentage of prevalence of EGFR mutations in non-small cell lung cancer (NSCLC) patients in China (approximately 60%) compared to that of Caucasians.[1,2] Along with the clinical application of third-generation EGFR TKIs, such as osimertinib, in clinical practice, it is optimal to obtain sufficient cancer tissue by rebiopsy from recurrent lesions for essential pathology diagnosis and biomarker measurement, including threonine-to-methionine substitution at position

790 of the EGFR gene (T790M), which resulted from EGFR TKI resistance during disease progression and is probably sensitive to third-generation EGFR TKIs.[3] However, the number of cancer cells available for molecular testing is usually very limited after morphological pathological diagnosis. Therefore, it is an extreme challenge to ensure the experiment is successful and that reliable results are subsequently achieved for every sample.

Nowadays, the standard techniques of EGFR gene mutation detection in lung cancer with formalin-fixed paraffin-embedded (FFPE) specimen sources are based on polymerase chain reaction (PCR) and DNA sequencing. Although the experiment procedures were well known and the corresponding results highly acceptable, with at least 4–6 FFPE slides (depending on the number of cancer cells) from surgical excisional samples or 8–10 FFPE slides from biopsy (e.g., fiberoptic bronchoscopy biopsy, CT-guided percutaneous lung biopsy, lymph node incisional or excisional biopsy, endobronchial ultrasound-guided transbronchial needle aspiration, etc) samples, in most cases it was difficult to acquire sufficient cancer cells to meet the basic requirements for the platforms. The percentage of specimens sufficient for molecular testing by rebiopsy during disease progression was very low because some specimens are almost exhausted after hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining. The minimum number of slides required for EGFR T790M mutation evaluation based on PCR and DNA sequencing is often not reached in the real world for rebiopsy samples, which is one of the main bottlenecks in molecular detection of lung cancer.[4]

In addition, to avoid the possible effect of the potential contamination of PCR products on the results, all PCR-based tests should be completed in specialized clinical laboratories.[5] The relevant molecular analysis cannot be conducted in some basic medical institutions on time, although EGFR TKIs have been applied in lung cancer therapy extensively so far.

To solve the above problems, we explored a new method of EGFR T790M mutation by in situ hybridization based on RNA target sequence, which only required a maximum of three FFPE slides (positive control, negative control, and testing). Specialized laboratories to complete PCR were not needed because there was no nucleic acid amplification during the experiment. The results on the slide could be viewed under the microscope, and partial RNA degradation was found to rarely affect the results due to the lack of RNA extraction. Moreover, paracancerous cells in the sections, which were "natural" internal controls, ensured a high reliability of the results. Moreover, it was easy to recheck or rereview the stained sections because of its suitability for routine storage at room temperature (RT) for a certain period.[6]

## Methods

### Sample collection and preparation

A total of 28 cases of formalin-fixed paraffin-embedded consecutive remaining samples (after clinical diagnosis) with T790M and T790W determined by qPCR (primary or acquired resistance for EGFR TKIs) from 24 patients with lung adenocarcinoma in Peking Union Medical College Hospital between 1 January 2015 and 31 December 2016 were selected. Of these, four patients had primary or metastases lesions and recurrent tumor at the same time, seven patients had only primary or metastases lesion, and 13 patients had only recurrent tumor. All patients were in clinical Phase IV, and almost all of the specimens were obtained by biopsy (only one sample was acquired by surgical operation). The clinical characteristics of the patients are listed in Tables 1 and 2.

### DNA extraction and quantification

Each tissue block was serially prepared on 8–10 slides, one for H&E staining, and then areas rich in cancer cells were labeled; the rest of the slides were prepared for deparaffinization and rehydration. The tissue from the marked area rich in cancer cells on the slides was collected

**Table 1** Clinical characteristics of 20 cases (from 20 patients) suffering from lung cancer with only primary or recurrent tumors in our cohort

| Characteristics (N = 20) | No. of patients (%) |
| --- | --- |
| Age | |
| > = 70 | 16 (80.0%) |
| <70 | 4 (20.0%) |
| Gender | |
| Female | 14 (70.0%) |
| Male | 6 (30.0%) |
| Smoking | |
| Smokers | 3 (15.0%) |
| Nonsmokers | 17 (85.0%) |
| Histology at diagnosis of advanced lung cancer | |
| Adenocarcinoma | 20 (100.0%) |
| Nonadenocarcinoma | 0 (0%) |
| Tumor sample location (%) | |
| Lung | 13 (65.0%) |
| Lymph node | 4 (20.0%) |
| Others | 3 (15.0%) |
| Tumor sample by (%) | |
| Biopsy | 19 (95.0%) |
| Operation | 1 (5.0%) |
| Year cancer tissue acquired (%) | |
| 2016 | 7 (35.0%) |
| 2015 | 13 (65.0%) |
| Location of the tumor tissue acquired (%) | |
| Primary tumor | 7 (35.0%) |
| Recurrent tumor | 13 (65.0%) |

**Table 2** Clinical characteristics of eight cases from (four patients) suffering from lung cancer with primary and recurrent lesions in our cohort

| Case | Gender | Smoking | Year cancer tissue acquired (the first) | Year cancer tissue acquired (the second) | Tumor tissue location | Tumor sample acquisition | Histology at diagnosis of advanced lung cancer | Primary or recurrent tumor |
|---|---|---|---|---|---|---|---|---|
| 21 | F | N | 2015 | | Lymph node | Biopsy | Adenocarcinoma | Primary tumor |
| | | | | 2016 | Lung | Biopsy | Adenocarcinoma | Recurrent tumor |
| 22 | F | N | 2015 | | Lung | Biopsy | Adenocarcinoma | Primary tumor |
| | | | | 2016 | Lung | Biopsy | Adenocarcinoma | Recurrent tumor |
| 23 | M | N | 2015 | | Lung | Biopsy | Adenocarcinoma | Primary tumor |
| | | | | 2016 | Lung | Biopsy | Adenocarcinoma | Recurrent tumor |
| 24 | M | Y | 2015 | | Lung | Biopsy | Adenocarcinoma | Primary tumor |
| | | | | 2016 | Lung | Biopsy | Adenocarcinoma | Recurrent tumor |

by macrodissection and placed into the buffer with protease (Qiagen, Hilden, Germany) for digestion overnight. Genomic DNA was extracted using a QIAapm DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). The DNA is then dissolved in 50 μL of deionized water and 1 μL liquid samples were used for the DNA yield and absorbance ratio at 260/280 measurement using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Each DNA specimen was then diluted to a suitable concentration and stored at −20°C for real-time quantitative (q) PCR assays as follows.

## qPCR assays

According to the manufacturer's protocol, Scorpions ARMS assays were performed on Rotor-Gene Q Real-time PCR platform using therascreen EGFR RGQ PCR kit (Qiagen, Hilden, Germany).[7] The sequence-specific PCR primers that allow the detection of the common hotspot mutations of EGFR gene as follows: exon-19 deletion, exon-21 L858R (2573T > G), exon-20 T790M (2369C > T), exon-20 L861Q (2582T > A), exon-18G719A (2156G > C), exon-20 S768I (2303G > T), and exon-20 insertions (2319_2320insCAC and 2310_2311insGGT).

## BaseScope assay

A total of three 5 μm tissue sections of each sample were prepared onto Superfrost Plus slides (Erie Scientific LLC, New Hampshire, USA). Two slides were evaluated for RNA quality by using positive control probe (housekeeping gene POLR2A) and negative control probe (bacterial mRNA dapB), respectively. After QC tests, the third slide was performed for EGFR T790M tests using BaseScope probe (BaseScope Probe-BA-Hs-EGFR-T790M) which targeting on EGFR T790M correlated RNA sequences (Advanced Cell Diagnostics, Newark, CA). T790M samples from four patients with primary or metastases lesions and recurrent tumors simultaneously and two patients with recurrent tumors as well as 18 of T790M samples from five

patients with primary or metastases lesions and 13 patients with recurrent tumors detected by Scorpions ARMS were subjected to BaseScope assay according to the manufacturer"s instructions.[8] Briefly, slides were sequentially incubated with Pretreat 1 (hydrogen peroxide) for 10 minutes at RT, Pretreat 2 (target retrieval) for 15 minutes at 100°C, and Pretreat 3 (protease) for 30 minutes at 40°C after FFPE tissue sections on glass slides were dewaxed and hydrated. BaseScope EGFR T790M probe was loaded onto the slides and hybridized with the sections for two hours at 40°C in a HybEZ oven (Advanced Cell Diagnostics, Newark, CA). The slides were then incubated with AMP1 reagents for 30 minutes at 40°C, AMP2 for 30 minutes at 40°C, AMP3 for 15 minutes at 40°C, AMP4 for 30 minutes at 40°C, AMP5 for 30 minutes at 40°C, AMP6 for 15 minutes at 40°C, AMP7 for 30 minutes at RT and AMP8 for 15 minutes at RT. The signals were developed using BaseScope Fast Red. The slides were counterstained by hematoxylin and mounted using VectaMount permanent mounting medium (Vector Laboratories, Burlingame, CA). A whole slide image was scanned and stored under a 40 times objective lens using Aperio CS2 Digital Pathology Scanning System (Leica, San Diego, US). The number of cancer cells with ≥1 red color signal (positive cancer cells) was counted in at least 200 cancer cells. The percentage of positive cancer cells was calculated with the aid of Aperio Imagescope software (Leica, San Diego, US). EGFR L858R assay by BaseScope (BaseScope Probe-BA-Hs-EGFR-L858R) was performed to further confirm the method using 10 cases of EGFR L858R samples which were detected by qPCR. The procedure was the same as T790M staining described above.

## Data analysis

The sensitivity and specificity of BaseScope T790M assay were estimated compared with Scorpions ARMS assay, which is a standard method for EGFR gene mutation detection.

True positives were considered when the samples with T790M were revealed by BaseScope and also by Scorpions ARMS assay; true negatives were determined when the samples with T790 wild-type (T790W) were identified by BaseScope and also by Scorpions ARMS assay; false positives were determined when the tumors with T790M were found by BaseScope with T790W by Scorpions ARMS assay; false negatives were considered when the samples with T790W were detected by BaseScope, and those with T790M were detected by Scorpions ARMS assay. Sensitivity of BaseScope T790M assay = true positive/true positive + false negative; specificity of BaseScope T790M assay = true negative/true negative + false positive.

Statistics: Data analysis was performed using SPSS version 22.0 software. Results are established as mean $\pm$ 95% confidence intervals.

## Results

To save space in the laboratory and scarce samples that are often difficult to obtain, we preliminarily evaluated the feasibility of BaseScope assay by comparing it with the classical Scorpions ARMS assay using archived FFPE samples from lung cancer for EGFR T790M detection. The red color signals of T790M and blue nuclei of cells, including cancerous and non-cancerous cells, in the slide were easy to recognize under the conventional bright-field microscope. The cytoplasm or nuclei of tumor cells with numbers of red color signal $\geq 1$ was deemed to be T790M positive cell. The results of BaseScope assay from our cohort are listed in Table 3. The average rate of 35.78% $\pm$ 17.68% BaseScope -positive cells were found in all of the T790M samples were determined by Scorpions ARMS assay, of which the highest expression in the samples was 72.7%, and the lowest expression in the samples was 16.87%. Figure 1 visualizes representative images from five samples of lung cancer with the distinct red color signals by in situ hybridization with BaseScope T790M probe-targeted RNA sequences in cancer cells. The average percentage of 0.46% $\pm$ 0.70% cells with red color signals was found in all T790W samples (Fig 2). Compared to Scorpions ARMS detection, the BaseScope T790M assay showed 100% sensitivity and 100% specificity. According to our data, the minimal limit of detection for BaseScope T790M signals was approximately 1%, i.e., if $\leq 1$ positive cancer cell (with $\geq 1$ red color signals) was shown per 100 cancer cells, this kind of sample was considered as T790W. Otherwise, it should be defined as T790M, even though the potential clinical significance requires further exploration; for example, whether the patients with $\geq 1$% T790M positive cancer cells were third-generation EGFR TKI-responders as well as whether BaseScope T790M assay was more sensitive than conventional methods than PCR-

**Table 3** The validation of 28 cases with EGFR T790M and T790W by BaseScope in our cohort

| Case | Tumor sample location | Positive cells by BaseScope (%) | Scorpions -ARMS | Year tissue acquired |
|---|---|---|---|---|
| 1 | Cervical lymph node | 44.79 | T790M | 2015.04 |
| 2 | Lung | 37.28 | T790M | 2015.11 |
| 3 | Cervical lymph node | 25.4 | T790M | 2016.09 |
| 4 | Liver | 69.94 | T790M | 2016.07 |
| 5 | Lung | 17.63 | T790M | 2016.04 |
| 6 | Inguinal lymph nodes | 22.24 | T790M | 2015.05 |
| 7 | Cervical lymph node | 26.51 | T790M | 2015.11 |
| 8 | Lung | 16.87 | T790M | 2016.12 |
| 9 | Lung | 26.79 | T790M | 2015.10 |
| 10 | Shank | 58.39 | T790M | 2015.02 |
| 11 | Lung | 23.91 | T790M | 2015.11 |
| 12 | Lung | 19.32 | T790M | 2015.11 |
| 13 | Lung | 23.74 | T790M | 2016.08 |
| 14 | Lung | 32.52 | T790M | 2015.05 |
| 15 | Lung | 54.2 | T790M | 2015.07 |
| 16 | Lung | 27.59 | T790M | 2016.11 |
| 17 | Lung | 44.14 | T790M | 2016.11 |
| 18 | Liver | 72.7 | T790M | 2015.05 |
| 19 | Cervical lymph node | 0.6 | T790W | 2015.12 |
| 20 | Lung | 0 | T790W | 2016.11 |
| 21 | Lung | 0 | T790W | 2015.06 |
| 22 | Lung | 0.17 | T790W | 2016.02 |
| 23 | Lung | 0 | T790W | 2015.08 |
| 24 | Lung | 0 | T790W | 2016.09 |
| 25 | Lung | 0 | T790W | 2015.06 |
| 26 | Lung | 1.64 | T790W | 2016.12 |
| 27 | Lung | 1.82 | T790W | 2015.07 |
| 28 | Lung | 0.33 | T790W | 2015.07 |

based and DNA sequencing for EGFR T790M detection for FFPE samples from lung cancer. The average rate of 83.07% $\pm$ 10.74% BaseScope-positive cells were found in all of the EGFR L858R samples which were detected by Scorpions ARMS assay (Fig 3).

## Discussion

BaseScope showed the high sensitivity, high specificity, and low background noise for visual mRNA expression signals by in situ hybridization analysis, which has already been demonstrated in several studies,[6] such as the detection of EGFR L858R point mutations on FFPE specimens from human lung cancer cell lines (H2229 and H1975), based on its unique oligonucleotide probes design (about 6–20 double Z probes) and signal amplification strategies.[8] In clinical practice, the scarcity of specimens obtained by rebiopsy during first-generation of EGFR TKI resistance is relatively more prominent, and more effective detection methods need to be explored more urgently. This is the first report to explore the feasibility of EGFR T790M point
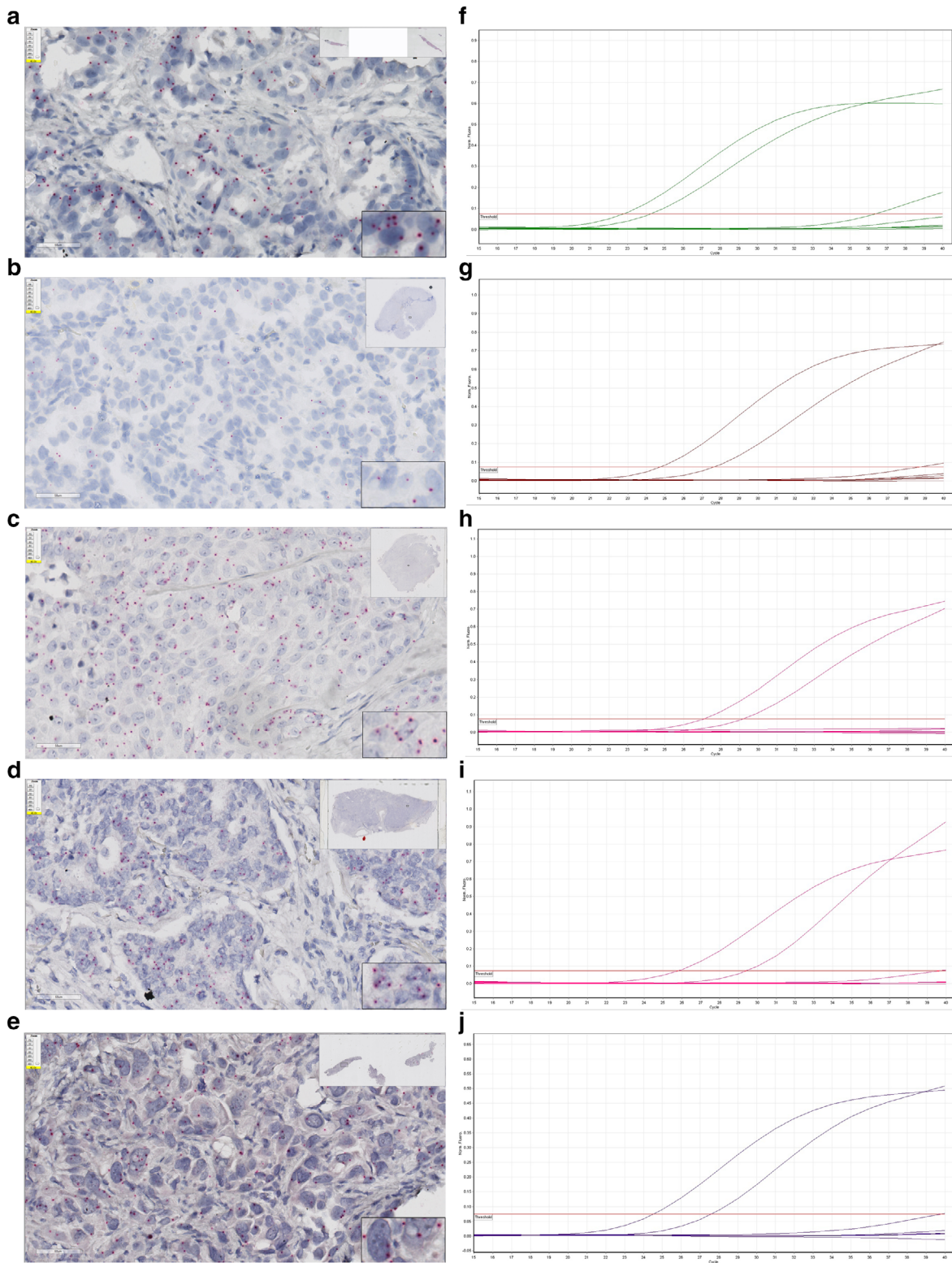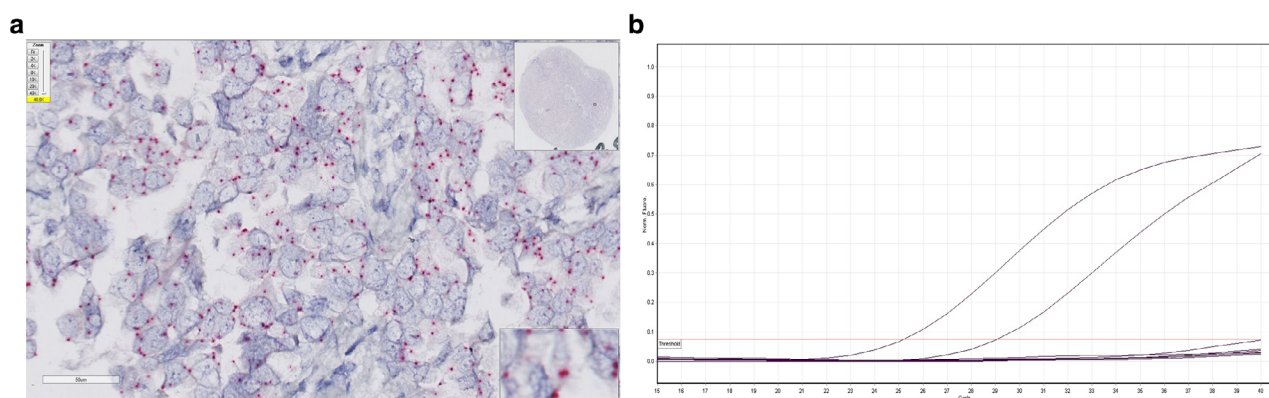
**Figure 1** Representative images from EGFR T790M tumor samples validated by BaseScope T790M probes and Scorpions-ARMS assay in five specimens from lung adenocarcinoma. There are distinct red color signals in (**a**) 69.94%, (**b**) 22.24%, (**c**)58.39%, (**d**)54.20%, and (**e**)72.70% cancer cells respectively, on the left images by BaseScope assay in every column, and the corresponding results are confirmed by Scorpions-ARMS assay on the right (**f–j**).
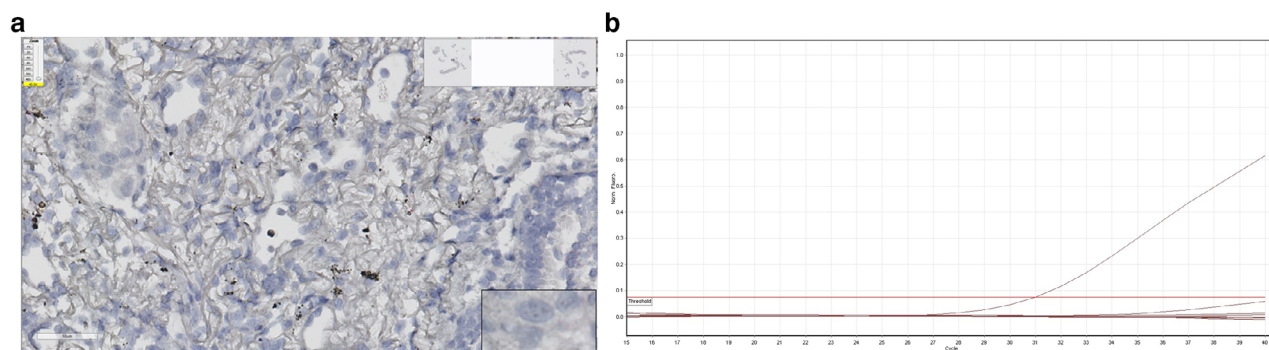
**Figure 2** Representative images from EGFR T790W tumor samples validated by BaseScope T790M probes and Scorpions-ARMS assay in a specimen from lung adenocarcinoma. There are a few red color signals in 0.17% of cancer cells on the left image (**a**) by BaseScope assay, and the corresponding results are confirmed by Scorpions-ARMS assay on the right (**b**).



**Figure 3** Representative images from EGFR L858R tumor samples validated by BaseScope L858R probes and Scorpions-ARMS assay in a specimen from lung adenocarcinoma. There are distinct red color signals in 89.90% cancer cells on the left image (**a**) by BaseScope assay, and the corresponding result was determined by Scorpions-ARMS assay on the right (**b**).

mutation detection in archived FFPE samples from human lung cancer using BaseScope. The experiment protocol has been previously described in detail,[8] so it has not been included in this article.

Our data showed that BaseScope T790M detection by in situ hybridization is highly sensitive and specific in comparison to the classic Scorpions ARMS assay. Accordingly, the tissue sections in the slides retained the original histological morphological information, which was helpful in identifying the target area of tumor, and then accurately recognized molecular biomarker signals of T790M probes in contrast to the noncancerous areas (e.g., stoma of tumor) as an internal control in the same tissue section, which was more reliable for visualization results compared with the common non-in situ method for gene mutation detection.

The consensus of most medical institutions is long-term storage for tumor pathological records, including stained slides if available, because of their necessity for the management of patients with advanced NSCLC by long-term follow-up and performing essential new biomarker testing and treatment during the tumor progression. Because of the visualization results of BaseScope EGFR T790M assay, the morphology images of tumors with the information of molecular biomarkers in the same interested cells within the same slide were revealed via multiview microsystems or digital pathology by remote mutiviewer for complicated clinical cases; it also provides the possibility for archiving the slides after BaseScope T790M testing at RT for a longer time to meet the extended requirement of laboratory quality assurance (QA) and scientific research for necessary review or rechecking any data at any time.

Although EGFR TKIs have been widely used for lung cancer therapy, EGFR gene mutations have been involved in the guidelines for the diagnosis and treatment of lung cancer in recent years. There are still challenges in the standard PCR laboratory space and professional staff

according to the mandatory requirement in some basic hospitals. The situation may only improve if the BaseScope T790M assay can be further verified as one of the potential feasibility evaluations for clinical application.

Our study showed that the average rate of positive cells with BaseScope T790M signals was 35.78% ± 17.68% by BaseScope assay in EGFR T790M cases detected by Scorpions ARMS assay, whereas the average rate of the false positive signals was 0.46% ± 0.70% in T790W specimens, proven by Scorpions ARMS assay. The minimal limit of detection for BaseScope T790M probes was speculated to be 1% reasonably, but it is not clear whether the cases with ≥1% and ≤ 17% of T790M-positive tumor cells exist or not because no such samples were observed in our cohort, or what is the implied meaning of such specimens in the real world. In other words, our research does not answer whether the samples with cutoffs as low as 1% T790M positive cells could be regarded as T790M features in tumors, or imply whether the patients will be third-generation EGFR TKIs-responders or not. We are looking forward to the accumulation of related data from large samples research in the future.

More than 200 ng of cancer tissue is generally necessary for genomic DNA extraction was adequate to be applied to EGFR gene status analysis using PCR-based and DNA sequencing detection platforms. In particular for FFPE samples, the bias of the EGFR gene mutation evaluation resulting in false positive or false negative results may be inevitable because of some degree of fragmentation of template DNA and linkage between protein and DNA, as well as inadequate specimen processing, and is difficult to completely avoid.[9,10] In addition, the cancer cell enrichment had to be administered by macrodissection initially and DNA extracted subsequently to eliminate the potential of false negative results in most laboratories. Thus, at least several slides with 4 to 5 μm thicknesses are required (4–6 slides for surgical specimens, 8–10 slides for biopsy specimens).[11,12] Most epidemiological studies have disclosed that the EGFR T790M subtype in the NSCLC patients could be attributed to EGFR TKI-acquired resistance. Herein, rebiopsy is considered from the recurrent tumor before the therapy with third-generation EGFR TKIs that targeted T790M and EGFR sensitive mutation simultaneously, and also because of its necessity for acquisition of tumor tissue for pathological diagnosis (several studies showed NSCLC transform to small-cell lung cancer after treatment with EGFR-TKIs), even though it is difficult to obtain adequate tissue from the recurrent tumor of every patient. Therefore, performing a liquid biopsy (e.g., circulating tumor DNA, ctDNA) as a substitute for cancer tissue was not appropriate in any case.[13] On the other hand, the cancer tissue was almost exhausted after the H&E and IHC staining of the specimens in cases of rebiopsy, so there was no tissue left

for further EGFR T790M testing, and ctDNA had to be used as a surrogate for determination of EGFR status. Unfortunately, because every liquid biopsy technique had its own limitations which lead to a certain percentage of false negative results, cancer tissue remains the preferred alternative for EGFR T790M testing by expert consensus so far, although the intratumor heterogeneity may affect the accuracy of the final results and is also not perfect for biopsy tissue.[14,15] A maximum of three sections of tumor tissue were sufficient for BaseScope T790M assay, leading to a decrease in the quantity of tissue slides required for gene mutation testing compare with the classical techniques. Furthermore, RNA extraction was not necessarily conducted from tissue block for RNA expression analysis by BaseScope assay, and the results were rarely affected by partial RNA degradation from FFPE slides. Some cases had failed tests due to inadequate processing of the samples occasionally, and the report of "no information" detected by the PCR-based method was remedied by BaseScope assay eventually in some cases.

The proportion of positive rate of EGFR T790M by BaseScope assay is quite different, the average prevalence of cancer cells with T790M signals was 35.78% ± 17.68%, with the highest being 72.70% and the lowest being 16.87% in our total 18 T790M patients because of cell truncation of FFPE samples, potential heterogeneity and partial degradation of RNA. In sharp contrast, the average rate of cancer cells with nonspecific BaseScope signals in 10 cases of T790W samples was 0.46% ± 0.70%, and background noise was also very rare in stroma of tumors, either in T790M or T790W samples. Our data indicate the BaseScope T790M assay possesses high sensitivity and specificity.

There are still some limitations in our study. Some issues that require further investigation are summarized as follows: (1) Approximately up to 75% of the patients with advanced-stage (IIIB or IV) non-small cell lung cancer were in the first contact care and a surgical operation failed to yield histological specimens, so extremely limited samples can be obtained through biopsy for the most of cases. In our study, all NSCLC patients with stage IV were collected, and some of the specimens were obtained from the recurrent lesions during EGFR TKI-acquired resistance by rebiopsy for EGFR T790M assay that was similar to daily work scenarios. Moreover, it was preferred for EGFR gene status detection by real-time quantitative PCR assay to provide the information of molecular typing for determination of strategy about therapeutic measurement after H&E and IHC stain for histopathology diagnosis indispensably, so that there were a limited number of cases with sufficient specimens for our research. Therefore, the results need to be further verified with a large sample size.[16,17] (2) Because the specimens involved in our study had already been

archived for three to four years, different degrees of RNA degradation may have resulted from FFPE blocks stored in RT conditions, resulting in the observation of a greater variation in the percentage of T790M signals (16.87%–72.70%) by BaseScope assay between the samples. Meanwhile, the frequency of positive cancer cells with T790M varied extensively in different individuals, which may be caused by EGFR-TKI-acquired resistance that can be attributed to intratumor heterogeneity. This mystery could not be explored further in our research.

FFPE specimens archived within three to four years were used in this study, and the expected results were obtained, suggesting that BaseScope assay for EGFR T790M detection may have a wider latitude and can be reliably implemented for the specimens prepared by routine procedure in the clinical laboratory from our preliminary data. The morphological features and point mutation of genes were revealed synchronously in the same slide in an in situ manner. Measuring the gene mutation, exploring subclonal evolution, and exposing tumor heterogeneity on the tissue section visualized illustrates advantages, but is difficult to achieve using non-in situ techniques. Baker *et al.* successfully revealed the multiple point mutations of BRAF, KRAS, and PIK3CA genes in colorectal cancer and a spatial distribution of rare subclones of tumor cells was drawn together with molecular characteristics on the slides by BaseScope and then displaced the intratumor heterogeneity visually.[6] Compared with high-throughput sequencing, the experimental conditions for only one or few of genes are better designed and optimized, e.g., BaseScope, ARMS PCR. Slides for multiple molecular biomarkers with different fluorescent labeled probes have been released separately in a relevant study on breast cancer[18] and may imply expansive application in cancer research and molecular diagnosis in the future.

In conclusion, our data showed, compared with real-time PCR assay, the sensitivity and specificity of BaseScope T790M were all 100% in our cohort. Because the visualized molecular signals of T790M overplayed with intact histomorphological information in the same slide, BaseScope T790M assay may be the alternative method for T790M assessment and innovative perspective for intratumor heterogeneity evaluation.

## Acknowledgments

## Disclosure

There are no conflicts of interest.

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