Case report

Case report of three EGFR TKI naïve lung adenocarcinoma containing double EGFR mutations (L858R/T790M or Exon 19 Deletion/T790M); Comparing genetic information and histology☆

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ABSTRACT

EGFR T790M mutation is a crucial gene alteration causing EGFR TKI resistance. However, the implication of T790M mutation is still unknown for the stepwise progression of EGFR TKI naïve lung adenocarcinoma. In this study, we studied site-related EGFR T790M mutation analysis in EGFR TKI naïve lung adenocarcinomas harboring double EGFR mutation (L858R and T790M or Exon 19 deletion (Del.19) and T790M) by droplet digital (dd) PCR method. We examined three resected lung adenocarcinoma cases harboring EGFR double mutation including T790M. These cases didn’t receive EGFR TKI treatment. We divided formalin-fixed and paraffin embedded (FFPE) unstained slide tissues into 11–18 areas in each tumor and extracted DNAs from each area separately. The DNAs were analyzed by ddPCR. T790M mutation ratio (T790M/L858R or T790M/Del.19) were calculated. For three cases, we also performed EGFR FISH for analyzing EGFR copy number. In Case 2 and 3, T790M mutation ratio were 100% and 30% homogeneously and showed increased EGFR copy number also homogeneously. However, in case 1, it was different between invasive and non-invasive areas. EGFR copy number was also heterogeneous and showed increasing only in invasive area. We indicated a peculiar case harboring T790M heterogeneity and only invasive area had T790M mutation even though the case was not treated by EGFR TKI. It suggests that T790M is possibly significant not only for EGFR TKI resistance but also the progression in lung adenocarcinoma.

1. Introduction

EGFR mutation is thought to be a driver gene mutation, since the mutation alone can produce adenocarcinoma in mouse models [1,2], and adenocarcinomas harboring EGFR mutations are very sensitive to treatment with EGFR Tyrosin kinase inhibitor [3]. However, almost all adenocarcinomas harboring EGFR mutation acquire resistance to EGFR TKI during the course of treatment.

Many previous studies have investigated the mechanism of EGFR TKI resistance. The major focuses of attention have been gate-keeper mutations such as T790M [4,5], alternative mechanisms for activation of downstream signaling such as MET amplification [6,7] and HGF overexpression [8], transformation to small cell carcinoma, micro RNA [9], BIM expression [10] and histone modification [11].

Among the various mechanisms of EGFR TKI resistance, T790M mutation is the most frequent and the best known [4,5]. Yun et al. showed that increased affinity for ATP caused drug resistance [12], in addition to binding of an interfering inhibitor [13]. Moreover, Mullloy et al. showed that the phosphorylation level of the EGFR harboring T790M/L858R mutation is much higher than that of EGFR harboring L858R alone [14]. In other words, EGFR T790M might cause not only drug resistance but also malignant progression through ATP affinity and promote the level of EGFR phosphorylation. Surgically resected and EGFR TKI naïve lung adenocarcinomas sometimes harbor EGFR T790M mutation [4]. However, it is still little known how T790M mutation is related to stepwise progression.

✉ All authors have contributed significantly, and that all authors are in agreement with the content of the manuscript.

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We examined three peculiar cases of surgically resected EGFR TKI naïve lung adenocarcinoma harboring double EGFR mutation indicating naïve T790M mutation. In order to examine the biological implications of T790M mutation heterogeneity in lung adenocarcinoma, we separated the tumor into 11–18 areas and subjected them to ddPCR (droplet digital PCR) [15,16], a technique that has been developed recently and examined the accurate mutation rates in each area.

In the present study, we performed site-related EGFR T790M mutation analysis of EGFR TKI naïve lung adenocarcinomas and considered the clinical implications of the mutation in stepwise progression of cancer, based on the heterogeneous mutation rates of EGFR T790M.

2. Materials and methods

2.1. Pathological examination

Resected surgical materials were fixed with 15% neutral buffered formalin for 24 h. After fixation, the tumor area was selected and paraffin-embedded blocks were prepared. Hematoxylin-eosin (HE) and elastica van Gieson (EVG) staining were used for pathological examination. Histological diagnosis and TNM grading were based on the WHO 2016 [17] and UICC classification 8th edition [18].

For immunohistochemistry, section was autoclaved in 10 mM tris-HCl (pH 8), 1 mM EDTA (1 × Tris-EDTA; TE buffer) at 105 °C for 15 min for antigen retrieval, then incubated with Ki-67 using the MIB-1 antibody diluted 1:100 (Dako, Glostrup, Denmark). Subsequently, the sections of Ki-67 was incubated with EnVision + Dual Link System–HRP Polymer Reagent (Dako, Carpinteria, CA, USA) for 30 min at room temperature. Immunoreactivity was detected with a diaminobenzidine substrate kit (Dako Japan, Tokyo, Japan), and the sections was counterstained with hematoxylin.

2.2. Examined cases

Pre-treated five hundred and ninety four lung adenocarcinoma cases were resected at University of Tsukuba Hospital (Ibaraki, Japan) between 2007 and 2016. Among them, 113 cases were examined EGFR gene mutation (Tsukuba i-laboratory), and we detected EGFR gene mutation in 56 cases (49.6%, Tsukuba i-laboratory, Tsukuba, Japan). Two cases (3.6%) of them showed double mutation including T790M. On the other hand, 518 cases were resected at Ibaraki-Higashi Hospital (Ibaraki, Japan) in the same period (2007–2016) and EGFR gene mutation was examined for 264 cases (Tsukuba i-laboratory). EGFR gene mutation was detected in 156 cases (59.1%) and 5 cases (3.2%) showed double mutation including T790M. Finally we collected 7 cases showing multiple mutations of EGFR including T790M and 3 cases which were succeeded for ddPCR analysis, were used for the study (Supplemental Fig. 1). The clinic-pathological characteristics of the cases were shown in Table 1. All specimens had obtained informed consent at University of Tsukuba Hospital and Ibarakihigashi National Hospital, and this project was approved by ethics committee in both hospitals.

2.3. DNA extraction

DNA was extracted using the MagLead system (Precision System Science Co., Chiba, Japan) from FFPE samples. First, FFPE unstained slide sections 7 μm thick were deparaffinized and scraped from divided areas each measuring roughly 1 × 1 cm. We then added ATL buffer and protein kinase K (QiAgen DNA Mini kit, QiAgen, Düsseldorf, Germany) and incubated the samples for 1 h at 56 °C. We then placed each tube in a nucleic acid extraction reagent cartridge and transferred it to a magLEAD 6gC (Precision System Science Co.). DNA quality and quantity were checked by nanoVue (Biochrom).

2.4. Droplet digital PCR

Seventy nanograms of DNA, 2xddPCR Supermix for probes (BIO-RAD Laboratories, Inc., Hercules, CA), the target probe, reference probe and UDG (Roche Diagnostics, Basel, Switzerland) were mixed and incubated for 20 min at 37 °C for UDG digestion in a thermal cycler (Takara Bio, Inc., Shiga, Japan). Droplet generator oil for the probes (BIO-RAD Laboratories, Inc.) and digested samples were applied separately to a DG80TM Cartridge (BIO-RAD Laboratories, Inc.) and added to a QX200 TM Droplet generator (BIO-RAD Laboratories, Inc.). After droplet generation, the droplets were subjected to thermal cycling (MyCycler, BIO-RAD Laboratories, Inc.). The PCR cycling parameters were 10 min at 95 °C, then 40 cycles of 94 °C for 30 s, 60 °C for 1 min, and 98 °C for 10 min.

The probes we used were a Bio-Rad EGFR WT and E746_A750del assay kit, a Bio-Rad EGFR WT and L858R Assay Kit, and a Bio-Rad EGFR WT and T790M Assay Kit (BIO-RAD Laboratories, Inc.). After PCR completion, the emulsion was loaded into a QX200 TM Droplet Reader (BIO-RAD Laboratories, Inc.) for fluorescence detection. QuantaSoftTM Software (BIO-RAD Laboratories, Inc.) was used for data acquisition and analysis in accordance with the manufacturer’s instructions. The concentration was expressed as copies/μl in the final 1x ddPCR reaction. Cell lines (P16T, H1975 and HCC827) were used for Positive and Negative control.

Fluorescence in situ hybridization

Formalin-fixed, paraffin-embedded serial sections 5 μm thick were subjected to dual-color FISH using an Vysis LSI EGFR Spectrum Orange/CEP7 SpectrumGreen probes (Abbott Molecular Inc., Des Plaines). After deparaffinization and dehydration, all slides were incubated in pretreatment solution for 15 min at 80 °C, and immersed in protease solution for 20 min at 37 °C. After co-denaturation of the probe, DNA in the tissue sections was hybridized by incubation at 73 °C for 3 min followed by incubation at 37 °C for 14–24 h using ThermoBrite (Abbott Molecular Inc.). After hybridization, the slides were counterstained with DAPI. Using a fluorescence microscope with single interference filter sets for green (SpectrumGreen), orange (SpectrumOrange), and blue (DAPI), FISH signals were enumerated in non-overlapping tumor cell nuclei.

Table 1

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Gender</th>
<th>Smoking status</th>
<th>Part</th>
<th>Histology</th>
<th>Size</th>
<th>V</th>
<th>ly</th>
<th>EGFR</th>
<th>TNM</th>
<th>Stage(UICC 8th)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case1</td>
<td>64</td>
<td>Female</td>
<td>non</td>
<td>RU</td>
<td>Lepidic adenocarcinoma</td>
<td>30 × 25 × 16 mm</td>
<td>1</td>
<td>0</td>
<td>L858R + T790M</td>
<td>pT1bN0M0x</td>
<td>pStageIA2</td>
</tr>
<tr>
<td>Case2</td>
<td>49</td>
<td>Female</td>
<td>non</td>
<td>RL</td>
<td>Papillary adenocarcinoma</td>
<td>22 × 18 × 13 mm</td>
<td>1</td>
<td>0</td>
<td>L858R + T790M</td>
<td>pT4N2M0</td>
<td>pStageIIIB</td>
</tr>
<tr>
<td>Case3</td>
<td>68</td>
<td>Female</td>
<td>non</td>
<td>LL</td>
<td>Acinar adenocarcinoma</td>
<td>28 × 28 × 18 mm</td>
<td>1</td>
<td>1</td>
<td>Ex19 del(L747-T751) + T790M</td>
<td>pT1cNxM0</td>
<td>pStage IA3 or more</td>
</tr>
</tbody>
</table>

RU upper lobe of right lung, RL: lower lobe of right lung, LL: lower lobe of left lung, v: vascular invasion, ly: lymphatic invasion, TMN and Stage was scored according to the UICC 8th edition.
3. Results

3.1. T790M heterogeneity analysis using droplet digital PCR (ddPCR)

To analyze T790M heterogeneity, we used ddPCR. First, we divided the tumor into roughly 1 × 1-cm divided areas, as shown in Fig. 1A. Case 1 was divided into 12 areas, Case 2 into 11 areas and Case 3 into 18 areas. After macroscopic dissection, we extracted the DNAs from each area and performed ddPCR for each DNA, respectively. As DNA extraction failed in one area in Case 3, 17 areas in total were analyzed.

In Case 2, we also examined metastatic carcinoma in a lymph node. For calculation of the T790M mutation ratio, L858R or Del.19 was used as a control (mutation ratio = T790M/L858R or T790M/Del.19).

In Case 1, the T790M mutation ratio was 0–50%, indicating a heterogeneous distribution of T790M mutation. However, Case 2 had a T790M mutation ratio about 95% and Case 3 about 30%, suggesting homogeneity (Fig. 1B, Supplemental Table 1).

3.2. EGFR copy number analysis using fluorescence in situ hybridization (FISH)

It is well known that in many oncogenes such as myc, amplification is a relatively late event during malignant progression. EGFR gene amplification is also thought to occur at a late stage during the course of malignant progression [19]. We therefore examined EGFR gene amplification using fluorescence in situ hybridization and compared the results with those for EGFR gene mutation examined by ddPCR.

We selected three different areas in tissue sections. In case 1, we

<table>
<thead>
<tr>
<th>A</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lepidic adenocarcinoma</td>
<td>Papillary adenocarcinoma</td>
<td>Acinar adenocarcinoma</td>
<td></td>
</tr>
<tr>
<td>Lepidic: 50%, papillary: 45% poorly: 5%</td>
<td>papillary: 100%</td>
<td>Acinar: 100%</td>
<td></td>
</tr>
<tr>
<td>Divide into 12</td>
<td>Divide into 11</td>
<td>Divide into 18</td>
<td></td>
</tr>
</tbody>
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B

![Graph showing mutation ratio distribution](image)

Fig. 1. (A) Slide images and histological diagnosis of the three cases. The tumor area was divided into a number of areas, as shown in the figure, and DNA was extracted from each area. (B) Distribution of the mutation ratio (T790M/L858R or Del.19). The mutation ratio in case 1 was heterogeneous, but homogeneous in cases 2 and 3 as with histology. One dot represents one area.
selected the non-invasive area, the invasive area and an area with a poorly differentiated component, whereas in case 2 we selected two invasive areas and one lymph node metastasis, and in case 3, three areas were selected randomly. We then performed EGFR FISH analysis of each area and compared the copy number among the three areas (Fig. 2).

In case 1, although EGFR gene amplification was not detected, the EGFR copy number was decreased relative to the degree of histological differentiation (Fig. 2A). In case 2, the tumor showed no EGFR amplification but had roughly 4–5 EGFR copies homogeneously (Fig. 2B). The copy number in the lymph node metastasis was slightly low, but almost the same as that for the primary tumor. In case 3, the tumor showed no EGFR amplification but had roughly 6–7 EGFR copies homogeneously (Fig. 2C).

3.3. Precise analysis of T790M mutation and EGFR copy number heterogeneous case (Case 1)

Next, we compared the mutation ratio data with the histological findings in Case 1. The case showed several histological patterns. Some areas (nos. 3, 8, 9, 11 and 12) mainly showed a lepidic (non-invasive) growth pattern, whereas others (nos. 1, 2, 4, 5) included an invasive (papillary) growth pattern (Fig. 3A). Moreover, area 5 had a poorly differentiated component (Fig. 3A). Interestingly, tumor cells in the non-invasive areas (nos. 3, 8, 9, 11, 12) contained L858R mutation but not T790M mutation. However, tumor cells in mainly invasive areas (nos. 1, 2, 4, 5, 6) contained both L858R and T790M mutations. The L858R mutation rate was uniform. The detected rate of L858R mutation (about 10–40%) was thought to be affected by contamination with normal cells. However, the T790M mutation ratio (T790M/L858R) (40%) was uniform in invasive areas except for area 6, which had a mixed invasive and non-invasive component. Moreover, even in area 5 containing poorly differentiated adenocarcinoma, the T790M mutation ratio was similar to that in the other invasive areas (Fig. 3A-B).

Histologically, there are clear front line between the area not having T790M mutation and the area having T790M mutation. Fig. 3C is the representative figure. In the low power field of view, T790M mutated area (left lower part) had structural atypia and more condensed and over-crowed cells whereas no T790M area (right upper part) had no structural destruction and low cell density. Ki-67 staining revealed the labeling index of 12% in invasive area but < 1% in non-invasive area, clear boundary line between no T790M area and T790M mutated. These finding concrete the idea that histology is related with gene alteration such as T790M.

Fig. 3D is representative figure showing that the case’s stepwise progression.

4. Discussion

T790M is a very well-known EGFR mutation that confers EGFR TKI resistance [4,5]. The mechanism of resistance is thought to be an increased affinity for ATP [12] and promotion of EGFR phosphorylation [14] in addition to binding of an interfering inhibitor [13]. If the mutation is functionally associated with EGFR activity, there is a possibility that T790M would be related to not only drug resistance but also malignant progression, especially in EGFR TKI naïve lung adenocarcinoma. In fact, some previous reports have indicated that the T790M proportion ratio is related to prognosis [20].

EGFR mutation is thought to be a homogeneous [21] and clonal event [22]. However, in terms of L858R or Del 19, some reports showed intratumoral heterogeneity of EGFR. These report analyzed proportion or existence of EGFR L858R or Del. 19 mutation area by area [23,24]. They showed the possibility of the heterogeneity but they didn’t mention about correlation between gene and histology and they didn’t show T790M mutation. Cai et al. report comparison between gene and histology and analyzed many EGFR mutated adenocarcinoma having T790M, but they found only cases which L858/Del.19 and T790M was dependent such as our Case 2 and Case 3. This is maybe because their cases rarely had lepidic component [25].

No previous reports have described intratumoral heterogeneity of EGFR T790M mutation in EGFR TKI naïve tumors.

Precise analysis of heterogeneity of gene alterations in tumors is challenging. One potential problem is contamination by normal cell. Even if DNA is extracted from a specific area within the tumor, this area...
Fig. 3. (A) Distribution of each histological subtypes and mutation ratio of each area which DNA was extracted separately. (B) Site-selected mutation data. Left bar graph represents calculated mutation data. Number is matched with area. Blue bar represents the L858R mutation rate \(\frac{\text{L858R mutated droplet (copies/μl)}}{\text{L858R mutated droplet (copies/μl)} + \text{EGFR wt droplet (copies/μl)}}\). Red bar represents the T790M mutation rate \(\frac{\text{T790M mutated droplet (copies/μl)}}{\text{T790M mutated droplet (copies/μl)} + \text{EGFR wt droplet (copies/μl)}}\). Green bar represents the mutation ratio \(\frac{\text{L858R or Del.19 mutation rate (Blue bar)}}{\text{T790M mutation rate (Red bar)}}\). (C) H. E. staining and ki-67 staining for the front line between no-T790M mutated area (right lower) and T790M mutated area (left upper). (D) Summary figure of heterogeneous case. (left) Lepidic component. Only L858R mutation was detected and EGFR copy number was 2.02. (middle) papillary component. Both L858R and T790M mutation were detected. EGFR copy number was 2.72. (right) poorly differentiated component. Both L858R and T790M mutation were detected. EGFR copy number was 3.62.
may contain many normal cells such as stromal cells, blood vessel endothelial and immune cells. Therefore, we calculated the T790M mutation ratio in terms of frequently reported mutations such as EGFR L858R and Del 19, because normal cells would not harbor these mutations. Hidaka et al. have reported that almost all T790M mutations are present on the same allele as activating mutations [26]. Therefore, it would be reasonable to use these mutations as a tumor control. In fact, EGFR mutations such as L858R and Del 19 showed good correlation with histological tumor cellularity (Fig. 3B). However, we cannot completely exclude the possibility that only some tumor cells harbored EGFR L858R mutation, or that some may have harbored only T790M mutation.

A further issue is the method used for estimating the proportion of mutated cells. Sequencing is the major method for detection of mutation, but it is not suitable for calculating the mutation ratio. Therefore, we used the ddPCR method for this purpose [14,15]. ddPCR creates a single droplet that in theory contains only one fragment of DNA, allowing very accurate estimation of the proportion of mutation. Moreover, to analyze the heterogeneity of mutation, we divided tumor sections into several areas and extracted the DNA from each area separately. In this way, we were able to obtain DNA from precisely delineated histological areas.

Using this approach, we found T790M mutation only in invasive areas in the 3 cases we studied. However, this did not, of course, clarify whether T790M caused invasion or was a result of invasion. However, we were able to demonstrate that in one case the presence of T790M mutation was related to invasion, and that the distribution of an important mutation such as T790M differed between non-invasive and invasive areas. Histologically, there is relatively histological boundary line between no T790M mutated area and T790M mutated area on H.E slides (Fig. 3C). It might show that histology was correlated with T790M mutation. The tumor cellularity and each cell nuclear atypia are different between the two areas. Moreover, Ki-67 staining index showed extremely different (< 1% vs 12%). Additional T790M mutation in invasive area is suspected that the mutation was cause of effect of the malignant progression of Case 1 adenocarcinoma. Of cause, T790M is very rare in EGFR TKI naïve adenocarcinoma even Stage IV, so we cannot say T790M is major mechanism of malignant progression. This is just one of the malignant progression mechanism. Moreover, biologically, this is very difficult to prove. As we showed, some reports showed EGFR is more phosphorylated in the case having T790M mutation. However, Chmielecki J et al. showed that cell line having acquired T790M mutation grew slowly. Anyhow, almost all study was about cell line derived from advanced cases. There is a possibility that the function of T790M is different between early cancer and advanced cancer.

In order to determine the EGFR copy number, we conducted FISH analysis of 3 cases. None of these cases showed amplification (EGFR/CEP7 > 2). In terms of copy number, even though the T790M ratio was homogeneous in invasive areas, the EGFR copy number was low in relation to the degree of histological differentiation in case 1. However, in cases 2 and 3, the EGFR copy number was uniform. Those two cases were very homogeneous, not only histologically but also genetically.

Case 3 was interesting from another viewpoint in that only 30% of all cases had T790M mutation homogeneously. There is a possibility that wild-type (WT) tumor cells and mutant tumor cells were mixed uniformly, although usually any subclonal proliferation would lead to the formation of a cell “colony”. In such a case, the mutation ratio would differ from area to area [22]. Therefore, we think it is still possible that individual tumor cells would possess the WT allele and the mutant allele, resulting in “intratumoral cellular heterogeneity”. If each tumor cell possessed only two alleles, this is difficult to consider. However, FISH analysis showed that individual tumor cells possessed many EGFR copies, and therefore intratumoral cellular heterogeneity would have been responsible for the uniform T790M mutation rate of 30%.

In this case report, we introduced three lung adenocarcinomas harboring two EGFR mutations including T790M naïve mutation. One of them (Case 1) contained tumor cells with L858R mutation homogeneously but T790M naïve mutation heterogeneously and only tumor cells in invasive area contained T790M mutation. This result indicated that T790M mutation is not only associated with EGFR-TKI resistance but also may play a functional role in the malignant progression of lung adenocarcinoma.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.prp.2018.05.016.

References


