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Clinical significance of EGFR mutation subtypes in lung adenocarcinoma : A multi-center Korean study

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ABSTRACT

Background: Adenocarcinoma is the most common type of non-small cell lung cancer (NSCLC). Some causative genomicalterations, including deletion of exon (E) 19 and point mutation of E 21, are known to have a favourable prognoses due to sensitivity to tyrosine kinase inhibitors (TKIs); however, the prognoses of other uncommon mutations are unclear. The goal of this study was to analyse the clinical significance of epidermal growth factor receptor (EGFR)mutation subtypes in lung adenocarcinoma.

Methods: We retrospectively reviewed 1,020 subjects (mean age: 66.8 years, female: 41.7%) who were diagnosed with advanced (stage III–IV) lung adenocarcinoma, had EGFR mutation data, and did not undergo surgery from five medical institutes between 2010 and 2016. Subjects were classified according to EGFR mutation status, particularly for exon-specific mutations. It was defined to EGFR positivity for the presence of mutation or insertion/deletion and EGFR negativity for wild type EGFR.

Results: The median follow-up period was 13.3 months. EGFR positivity was 38.0%, with the incidence of mutations in E 18, 19, 20, and 21 being 3.6%, 51.0%, 3.4%, and 42.0%, respectively. The EGFR positive group survived significantly longer than the negative group ($p<0.001$), and there was a significant difference in survival among the four EGFR subtypes ($p=0.003$): univariate and multivariate analysis showed that E 19 deletion was the only significant factor that lowered mortality (HR:0.678, $p=0.002$), while mutations in E 21 was the poorer prognostic factor that increased mortality (HR:1.365, $p=0.015$). Amongst EGFR-positive cases, the proportion of E19 deletion in TKI responders was significantly higher than that of non-responders, and the proportion of E21 mutations was significantly lower.

Conclusion: In advanced EGFR-mutant lung adenocarcinoma, E 19 deletion predicts a good prognosis, while mutations in E 21 significantly increase mortality. Unlike previous reports, mutations in E 18 and 20 were not worse factors than mutations in E 21 L858R.

EXTERNAL LINK

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GUIDELINES

Inclusion criteria

- Between January 2010 and December 2016, 1491 lung adenocarcinoma patients who had EGFR sequencing data from five secondary or tertiary medical institutes

Exclusion criteria

- non-advanced stage (n = 326)
- underwent surgery (n = 128)
- the presence of anaplastic lymphoma kinase (ALK) mutation (n = 7)
- double EGFR mutation (n = 9)
- EGFR 19 insertion (n = 1)

BEFORE STARTING

All patients were initially classified into EGFR-negative and -positive groups. Then, the EGFR-positive group was divided according to EGFR mutation subtypes (mutations in E 18, 19, 20, or 21) and analysed. Also, EGFR-positive lung adenocarcinoma patients who received more than four TKI cycles were defined as TKI responders and separately analysed

1 **Patient enrolment**

1,020 patients were included in our study

2 **Study design**

Demographics and clinical information of subjects at time of diagnosis were obtained through medical records. The following variables were collected: age, sex, low body mass index (BMI <18.5 kg/m2), EGFR mutation type, stage according to 7th TNM, date of diagnosis, survival status, date of death, status of TKI treatment, other treatments (surgery, radiotherapy, others), forced expiratory volume in 1 second (FEV1), and forced vital capacity (FVC) at diagnosis. Variables for Charlson Comorbidity Index (CCI) were also collected to evaluate baseline comorbidities. OS was defined as the time from diagnosis to death or last follow-up and was evaluated for all subjects.

3 **Tissue preparation and DNA extraction**

When tissue is obtained from cancer cells, it is stored in a paraffin fixed tissue or in a tissue slide containing tumor cells. When lung cancer is diagnosed in this state, DNA extraction and amplification from tissue proceed as follows. Tumor cells were scraped with a 26-gauge needle. 50–100 µL DNA extraction buffer solution (50 mM Tris buffer, pH 8.3, 1 mM EDTA, pH 8.0, 5% Tween-20, and 200 µg/mL proteinase K) with 10% resin was added to the scraped cells. After incubation at 56 °C for at least 1 h, each tube was heated to 100 °C for 20 min followed by centrifugation at 12,000 rpm for 10 min at 4 °C to pellet the debris

4 **EGFR Mutation Testing**

The analysis was conducted in two ways according to the facilities and timing of each institution. Between 2010 and 2013, mutation testing was conducted by ISU ABXIS Co Ltd (Seoul, Korea), an independent commercial laboratory. From 2013 to 2016, PNAClampTMEGFR Mutation Detection Kit (Panagene, Daejeon, Korea) was used to identify EGFR mutation in the department of pathology at each institute according to manufacturer's instructions. Complete data analysis and quality control according to their own specific protocols were performed. The target somatic mutations included E 19 deletion mutations, E 21 L858R point mutations, E 18 G719X mutations, E 20 S768I mutations, E 20 insertions, E 20 T790M mutations, and E 21 L861Q mutations. The subtypes of detected mutations are described in S1 Table.

4.1 **Direct sequencing by ISU ABXIS Co. (Seoul, Korea).**

The mutational analyses of EGFR were performed by directional sequencing using polymerase chain reaction (PCR) fragments amplified with genomic DNA. The DNA was amplified with standard polymerase chain reaction technique using each exon-specific primer. PCR products were electrophoresed on 2% agarose gels and were purified with a QIAquick PCR purification kit (QIAGEN, Germany). Bidirectional sequencing was performed using the BigDye Terminator v 1.1 kit (Applied Biosystems, CA,USA) on an ABI 3130xl DNA analyzer (Applied Biosystems, CA, USA)[28].

4.2 **PNA clamping method**

The principle of the technology is that peptide nucleic acid (PNA) inhibits amplification of wild type DNA by hybridizing wild type sequences, and therefore mutant DNA is dominantly amplified. The amplified mutant type DNA is detected by intercalating dye. PNAClamp analysis was performed using PNAClampTMEGFR Mutation Detection Kit (Panagene, Deajeon, Korea) following the manual provided by the manufacturer. Both kits have the same procedure and protocol. In summary, 7 µl of DNA template, 3 µl of each PNA mix, and 10 µl of 2X premix are mixed for a single amplification reaction. The amplification of the mixture (20 µl) was performed in CFX96 real-time PCR instrument (Bio-Rad, CA, USA) with the following thermal program: a pre-incubation at 94°C for 5 min. and 40-cycles amplification (94°C for 30 sec.; 70°C for 30 sec.; 63°C for 30 sec.; and 72°C for 30 sec). Detection of signal of intercalating dye was measured at every 63°C steps. Ct value for the reaction (Sample Ct value) is determined based on the fluorescence values measured at every 63°C steps. If a mutation occurs in a specific codon site, it is not hybridized and amplified so that the Ct value is low. Assessment of the result was determined according to the delta(Δ): Ct value which was calculated according to each kit manual. The ΔCt is obtained from [standard Ct value minus Ct value obtained from an unknown sample]. The presence of each codon mutation is confirmed by the unique value of ΔCt.

5 **Statistical analysis**

All continuous variables were presented as mean ± standard deviation (SD) and categorical variables were presented as number (%). Among EGFR mutation subtypes, continuous variables were analysed by one-way analysis of variance (ANOVA) test or Kruskal-Wallis test and categorical variables were analysed by chi-square test or Fisher's exact test, when appropriate. Student t-test or Mann-Whitney test were used for comparison of continuous variables between two groups. Bonferroni post hoc test was applied with p value < 0.01 in variables which showed significant differences in ANOVA for the comparison of 5 institutions.OS was estimated by using the Kaplan-Meier methods, and difference between groups was assessed by log-rank test. A Cox proportional hazard regression model was used to identify independent factors of OS in EGFR mutated subjects, presenting hazard ratio (HR) with 95% confidential interval (CI). P-value < 0.05 (two-tailed) was considered statistically significant. All statistical analyses were calculated using SPSS version 24.0 (IBM Corporation, Armonk, NY, USA).