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Is the current diagnostic algorithm reliable for selecting cases for *EGFR*- and *KRAS*-mutation analysis in lung cancer?

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ABSTRACT

Objectives: Adenocarcinoma (ADC) of the lung may harbor *EGFR*- or *KRAS*-mutations, which are relevant for treatment decisions. There is no consensus on the percentages of *EGFR*- and *KRAS*-mutations that are allowed to be missed by a diagnostic algorithm, although a percentage of less than 1% for *EGFR*-mutations has been suggested. The current guidelines do not advise to perform *EGFR*-mutation analysis in unequivocal squamous cell carcinoma (SqCC). For *KRAS*-mutations no threshold for missing cases is suggested yet. To improve segregation between ADC and SqCC in small samples, the classification of lung cancer was updated in 2011, adding immunohistochemistry (IHC) for p63 and TTF-1 to the diagnostic algorithm. In this study we examined how many cases with an *EGFR*- or *KRAS*-mutation in our database would have been missed, if the current guideline for selecting cases for mutation analysis would have been applied.

Materials and methods: From an institutional lung cancer database of specimens analyzed for *EGFR*- and *KRAS*-mutations ($n = 816$), cases harboring a mutation without being treated prior with an EGFR-TKI were selected ($n = 336$). Corresponding original histological diagnoses and IHC for TTF-1, p63 and PAS-D were collected. Cases with SqCC on HE or with an IHC pattern favoring SqCC were reassessed according to the criteria of the 2011-classification.

Results: From the 336 cases 70% had a *KRAS*-mutation and 30% an *EGFR*-mutation. The number of cases with SqCC on HE and/or an IHC-profile favoring SqCC was 12. After the reassessment six specimens (1.8%) would not have been tested for EGFR-/KRAS-mutations, if the current diagnostic algorithm had been used: 2.0% of *EGFR*-mutations and 1.7% *KRAS*-mutations. All six cases were NSCLC with an IHC-profile favoring SqCC.

Conclusion: Most NSCLC-cases with *EGFR*- and *KRAS*-mutations are selected by the current diagnostic algorithm. As a small but relevant fraction is missed, there is room for improvement.

1. Introduction

Lung cancer is the leading cause of cancer-related death [1]. It is a heterogeneous disease, morphologically divided into two categories: SCLC (less than 15%) and NSCLC with several subcategories [2–4]. ADC is the most common NSCLC-type, accounting for 30–40% of all lung cancer cases. Specific molecular changes, like *EGFR*- and *KRAS*-mutations, can be found in lung ADC, which are relevant for treatment decisions [5–8].

Although it has been reported that these mutations can also be found in SqCC of the lung [9–25], the current general assumption is that SqCC does not harbor *EGFR*- or *KRAS*-mutations [26].

Abbreviations: SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; ADC, adenocarcinoma; SqCC, squamous cell carcinoma; NSCLC-NOS, non-small cell lung cancer not-otherwise-specified; LCC, large cell carcinoma; IHC, immunohistochemistry; HRM, high resolution melting; AdSq, adenosquamous carcinoma; PAS-D, diastase periodic acid–schiff.

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This implies that it is not necessary to analyze SqCC samples for the presence of *EGFR*- or *KRAS*-mutations, in line with the recent guideline for molecular testing in NSCLC [27]. If ADC and SqCC could be segregated unequivocally, no *EGFR*- or *KRAS*-mutations would be missed. However, in biopsies with poorly differentiated lung cancer, these subtypes are often difficult to separate on standard HE-staining alone. In order to distinguish ADC from SqCC, the 2004 WHO-classification recommended the use of additional mucin stains [2]. PAS-D staining for mucin is a marker for ADC, which has a moderate sensitivity and high specificity. In the latest classification of lung cancer immunohistochemical staining for TTF-1 and p63 was added [3]. These stains should be applied in case HE-staining shows NSCLC-NOS in small samples. TTF-1 is present in approximately 75% of lung adenocarcinomas [28–36]. This percentage varies, depending on the clone used for staining [37]. It is not expressed in SqCC, despite suggestions in previous reports [38]. P63 is highly sensitive for SqCC, up to 100%, but it can also be, usually weaker, positive in samples of ADC, up to 30% of cases. This is why some advocate a high cut-off point in assessing the p63-staining in relation to SqCC phenotype [39]. The assumption is that division of lung cancer subtypes, based on the classification, reliably segregates cases which may harbor *EGFR*- or *KRAS*-mutations. Consequently, it is advocated that cases of NSCLC should not be analyzed for *EGFR*- or *KRAS*-mutations, if HE-staining clearly shows SqCC or if it shows NSCLC-NOS in combination with an unequivocal SqCC IHC-pattern [40].

The aim of our study was to investigate whether this diagnostic algorithm is reliable to detect *EGFR*- and *KRAS*-mutations in lung cancer patients. To this end we examined a large series of cases harboring a *EGFR*- or *KRAS*-mutation and analyzed how many cases would have been diagnosed as SqCC and consequently not have been tested for mutations according to the guidelines.

2. Materials and methods

A database was constructed of lung cancer cases on which *EGFR*- and *KRAS*-mutation analysis had been performed. The database consisted of specimens from patients treated at the VU University Medical Center (VUMC) in Amsterdam and of samples referred for *EGFR*- and/or *KRAS*-mutation analysis from other Dutch hospitals from May 2004 until December 2010 ($n = 816$). The selection of patients for mutation analysis was made by the treating pulmonologist or pathologist. Cases which were treated with an EGFR-TKI prior to mutation analysis were excluded, because of possible aberrations due to clonal selection by the targeted therapy. The original histological diagnosis, which was recorded in the database, was performed according to the 2004 WHO classification [2]. Immunohistochemical analysis was done at the VUMC. Cases with a histological diagnosis of SqCC ($n = 7$) and cases with IHC 'positivity for p63 only' ($n = 6$) were reviewed by a pathologist (E.T.) and reclassified according to the 2011 classification [3]. From six cases with an IHC-profile of 'p63+ only' the cover slip was removed from an original negative control slide and subsequently restained for p40. All samples were analyzed in compliance with the respective institutional ethical regulations.

2.1. EGFR- *and KRAS- mutation analysis*

Mutation analysis was performed as described before [41–43]. From 2004 till October 2008 PCR-sequencing was used, and later HRM was used as selection procedure and abnormal melting curves were examined, confirming mutations by sequencing. For *EGFR*-mutations exons 19, 20 and 21 and for *KRAS*-mutations exons 1 and 2 were analyzed. Some mutations, which are known to be not correlated with malignancy, were excluded, using the website MoKCa [44]. These excluded cases ($n = 8$) comprised of mutations P848L, R832H, R776H and V843I.

2.2. Immunohistochemical staining

Data of immunohistochemical analysis for TTF-1, PAS-D and p63 were obtained for all selected cases. In case data were missing and tumor material was still available, additional staining was performed. IHC was done and interpreted as described before [39,45]. IHC for TTF-1 was performed with clone 8G7G3/1 and for p63, 4A4 alone or in combination with Y4A3 was used. Mucin staining was performed by PAS-D and Alcian blue methods according to routine procedures. The WHO advocates the use of five tumor cells in at least two high power fields in resection specimens [2]. In our analysis, for practical reasons, biopsies with the presence of at least two tumor cells with distinct intracytoplasmatic mucin droplets were considered to be positive for mucin staining [39].

A negative and an external positive control were used with every immunohistochemical staining procedure. Staining intensity for TTF-1 and p63 were scored according to the method of Ruschoff et al. [46], ranging from 0 to 3: 0 = negative, 1 = weak, 2 = moderate and 3 = strongly positive, and the percentage of positive tumor cells was examined. For each immunohistochemical staining a total score (H-score) was obtained by multiplying intensity and percentage positive tumor cells [47]. Specimens were considered positive for TTF-1 and p63 with H-scores ≥ 30 and ≥ 240, respectively.

For p40-staining a p40-monoclonal antibody (BC26, BioCare Medical, USA) was used, according to the manufacturer's manual (http://biocare.net/wp-content/uploads/3066.pdf).

2.3. Statistical analysis

The relation between *EGFR*- and *KRAS*-mutations and (i) the histological diagnosis and (ii) additional (immuno)histochemical staining (TTF-1, p63 and PAS-D) was assessed with the McNemar test, SPSS version 20.0. The analysis was performed for all cases and separately for resection specimens (BW). A p-value <0.05 was considered to be significant.

3. Results

3.1. Clinical data

Out of 816 specimens examined for an *EGFR*- or *KRAS*-mutation, 343 positive cases were identified. Cases which were treated with an EGFR-TKI prior to mutation analysis ($n = 7$) were excluded, leaving 336 cases eligible for this study. Patients were female in 56% and male in 44%. *EGFR*-mutations were more commonly observed in women (69%; $p < 0.001$), whereas *KRAS*-mutations were distributed equally over the sexes (female 51%, male 49%). The median age of the patients was 62 (range: 18–88). The mean age of female patients was lower than of male patients (59.5 years versus 63.3; $p < 0.001$). Among *EGFR*-mutations no significant difference in age was found between women and men (mean age 59.0 versus 56.3 years respectively, $p = 0.321$). Female patients with a *KRAS*-mutation were on average younger than the male patients (mean age 59.7 versus 65.0 years, $p < 0.0001$). Data on smoking status were regrettably lacking for most patients.

Specimens were biopsies in 71%, resection samples in 24% and cytological material in 5%. For *EGFR*-mutations these percentages were 71%, 22% and 7% respectively. For KRAS-mutations the distribution was 70%, 26% and 4% respectively.

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