ERJ Express. Published on August 29, 2013 as doi: 10.1183/09031936.00018013

EGFR, KRAS, BRAF and ALK Gene Alterations in Lung Adenocarcinomas: Patient Outcome, Interplay with Morphology and Immunophenotype

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Conflict of interest statement: We (all authors) have no conflict of interest to disclose.

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Abstract

Background: Numerous studies have been published on single aspects of pulmonary adenocarcinoma (ADC). To comprehensively link clinically relevant ADC characteristics, we evaluated established morphologic, diagnostic, and predictive biomarkers in 425 resected ADC.

Patients and Methods: Morphology was reclassified. CK7, TTF1, napsin A, thymidylate synthase (TS), and ERCC1 expression, *ALK* rearrangements as well as *EGFR*, *KRAS* and *BRAF* mutations were analyzed. All characteristics were correlated with clinical and survival parameters.

Results: Morphologic ADC subtypes were significantly associated with smoking history and distinct patterns of diagnostic biomarkers. KRAS mutations were prevalent in male smokers while EGFR mutations were associated with female sex, non-smoking and lepidic as well as micropapillary growth patterns. TTF1 expression (HR for OS=0.61, p=0.021) and BRAF mutations (HR for DFS=2.0, p=.046) were found as morphology- and stage-independent predictors of survival in multivariate analysis. Adjuvant radio-/chemotherapy in some instances strongly impacted on the prognostic effect of both diagnostic and predictive biomarkers.

Conclusion: Our data draw a comprehensive picture of the prevalence and interplay of yet established histological and molecular ADC characteristics. This data will help to develop time and cost effective diagnostic and treatment algorithms for ADC.

Introduction

As the leading cause of cancer related mortality lung cancer is a major health issue in developed countries [1]. Non-small cell lung cancer (NSCLC) accounts for ~80% of all cases, approximately 60% of NSCLC are adenocarcinomas (ADC). ADC are a complex, heterogeneous disease showing various clinicopathological and molecular characteristics with significant prognostic and predictive impact [1-10]. Especially, mutations in *V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog* (*KRAS*), *epidermal growth factor receptor* (*EGFR*), and *v-Raf murine sarcoma viral oncogene homolog B1* (*BRAF*), and translocations of the *anaplastic lymphoma kinase* (*ALK)* gene locus have been identified as oncogenic drivers of ADC with potential predictive value for targeted therapies. Selected patients treated with respective inhibitors have a significantly improved outcome compared to standard chemotherapy [3, 5]. Furthermore, thymidylate synthase (TS) and excision repair cross-complementing rodent repair deficiency, complementation group 1 protein (ERCC1), were identified as putative predictive biomarkers for pemetrexed- and platinum-based therapies, respectively [11, 12] [13] [14]. Most recently, exome and genome sequences of ADC have been mapped, revealing novel potential therapeutic targets [15, 16], which, however, have mostly not entered clinical decision making yet.

The majority of ADC patients present with inoperable tumor stages at initial diagnosis. Thus, only small biopsies or cytological specimens are available for diagnostic and predictive assessment; in up to one third of all cases diagnostic immunohistochemistry is additionally required for reliable tumor subtyping [8]. To set up effective patient stratification and reliable treatment strategies in the limited tissue setting linkage of histomorphological, immunohistochemical, molecular, and clinical data is crucial to understand the interplay between all relevant parameters.

In order to comprehensively assess yet established diagnostic, prognostic, and predictive ADC characteristics, their associations with each other and with patient outcome, we retrospectively analyzed a Caucasian cohort of 425 subsequently resected ADC with available clinical data for histomorphology, diagnostic immunomarkers, genetic alterations of *KRAS, EGFR*, *BRAF*, and *ALK*, as well as protein expression of TS and ERCC1.

Patients and Methods

Patients

Only invasive ADC surgically resected between 2002 and 2008 with available clinicopathological data were included. Diagnoses and subtyping were made according to the 2004 WHO classification for lung cancer [17] and the novel IASLC/ATS/ERS classification [18]. The 7th Edition International Union Against Cancer/American Joint Committee on Cancer (UICC) TNM classification was applied. Overall survival (OS) and disease free survival (DFS) were recorded. For DFS an event was defined as any definite clinical or pathological evidence of local or distant recurrence.

Clinical Characteristics

Morphological, immunohistochemical, molecular, and clinical data sets were available from 425 cases. Cases with single missing data points were not included into the specific analyses. 9 patients underwent wedge resection (2.1%), 2 segmentectomy (0.5%), 340 lobectomy (80%), 11 bilobectomy (2.6%), and 63 pneumonectomy (14.8%), accompanied by systematic lymph node dissection. 264 patients were male

(62.1%). Median age at resection was 62.6 years (range: 38.3-84.8). 107 patients (25.2%) received adjuvant chemotherapy, 73 stage III/IV patients (48.7%) received adjuvant mediastinal radiotherapy. Adjuvant platinum-based chemotherapy or radiotherapy was administered, unless contraindications were present, according to the guidelines in effect at the time of resection and the clinical status of the patient. None of the patients received biomarker-based targeted therapies. Mean follow-up for patients alive at the endpoint of OS analysis (n=246, 57.9%) was 48.2 months. Never smokers were defined as having smoked <100 cigarettes/life, former and active smokers were designated as smokers. Clinicopathological characteristics of the patients included into the analyses are given in Table 1.

Histomorphological Evaluation

All conventional ADC were subjected to pattern analysis according to the criteria of the IASLC/ATS/ERS classification as described previously in detail [9], recording the percentage of each histological component (lepidic, acinar, solid, papillary, and micropapillary) in 5% increments. The predominant pattern was defined as the pattern covering the largest tumor area.

Tissue Microarray Construction and Immunohistochemistry

For immunohistochemical analyses of diagnostic (cytokeratin 7 (CK7), thyroid transcription factor 1 (TTF1), and napsin A) and predictive immunomarkers (TS and ERCC1) a tissue microarray (TMA) described previously in detail [8] was used. Use of all tissues was approved by the local ethics committee (No. 206/2005). After H&E-based selection of appropriate areas, a TMA machine (ATA 27, AlphaMetrix Biotech, Rödermark, Germany) was used to extract tandem 1.0 mm cylindrical core samples from tissue donor blocks. Immunohistochemical stainings were performed by the

Tissue Bank of the National Tumor Center Heidelberg (NCT), Germany, using commercially available antibodies. All standard routine diagnostic antibodies were applied according to quality controlled protocols consistently tested in round robin trials (www.nordiqc.org) and in an accredited setting. TMA slides were deparaffinized and pre-treated with an antigen retrieval buffer. Subsequent steps were carried out in a staining machine (DAKO Autostainer). Expression of diagnostic immunomarkers was evaluated according to a dichotomous scoring scheme [8]. Nuclear and cytoplasmic TS and ERCC1 expression were analyzed separately using the H-score (H = A (% Tumor 1+) + B (% Tumor 2+) + C (% Tumor 3+)). For overall assessment of TS and ERCC1 expression the highest nuclear and/or cytoplasmic H-score for the respective marker was noted. For further details on the antibodies used see Supp. Table 1.

Molecular Analyses

All cases were analysed for mutations in *KRAS* (exon 1), *EGFR* (exons 18-21), and *BRAF* (exon 15) by Sanger sequencing. Extraction of genomic DNA was performed after manual microdissection [10] by proteinase K digestion using a fully automated purification system (QIASymphony SP, Qiagen, Hilden, Germany). For reliable sequencing analyses [10] only microdissected tissue material with >40% tumor cell content was used. DNA content was measured using a NanoDrop (Thermo Scientific, Wilmington, USA). For PCR amplification the following primers were used: *EGFR*: 5'-gctgaggtgacccttgtctc-3' (Exon 18 forward), 5'-acagcttgcaaggactctgg-3' (Exon 18 reverse); 5'-gctggtaacatccacccaga-3' (Exon 19 forward), 5'-gagaaaaggtgggcctgag-3' (Exon 19 reverse); 5'-catgtgcccctccttctg-3' (Exon 20 forward), 5'-gatcctggctccttatctcc-3' (Exon 20 reverse), 5'- cagagcttcttcccatgatga-3' (Exon 21 forward), 5'-cctggtgtcaggaaaatgct-3' (Exon 21 reverse). *KRAS:* 5'-gtgtgacatgttctaatatagtca-3'

(Exon 1 forward) and 5'-gaatggtcctgcaccagtaa-3' (Exon 1 reverse). *BRAF:* 5'-cctaaactcttcataatgcttgctc-3' (Exon 15 forward) and 5'-ccacaaaatggatccagaca-3' (Exon 15 reverse). Direct sequencing of the PCR amplicons was carried out for both strands on a 3500 Genetic Analyzer using the BigDye® Terminator v1.1 Cycle Sequencing Kit (both Applied Biosystems).

To identify cases with *ALK* rearrangements all cases were screened by immunohistochemistry using a sensitive antibody for the detection of ALK positive NSCLC [19]. Positive cases were subjected to fluorescence *in situ* hybridization (FISH) using a break-apart probe (Vysis, Abbott Laboratories, Illinois, USA) as described previously [6]. Only cases with FISH confirmed ALK rearrangement were considered ALK positive.

Statistics

Correlation of categorical biomarkers with clinicopathologic data was done by Fisher's exact test, χ^2 test and χ^2 test for trends as indicated. Semi-quantitatively evaluated biomarkers (ERCC1 and TS) were compared with clinicopathological data using Mann-Whitney-U-Test and Kruskal-Wallis-Test. OS and DFS were estimated using the Kaplan-Meier method, with a log-rank test to probe for significance. Hazard ratios for univariate and multivariate survival analyses were calculated with the Cox proportional hazard model. All statistical analyses were performed using SPSS Statistics 20 (IBM, Ehningen, Germany). P-values <0.05 were considered significant.

Results

Distribution of pathologic, diagnostic and molecular biomarker characteristics in pulmonary ADC

The final cohort consisted of 416 (97.9%) conventional and 9 (2.1%) invasive mucinous ADC. Of the conventional ADC 30 were lepidic (7.5%), 176 acinar (43.9%), 21 papillary (5.2%), 25 micropapillary (6.2%), and 149 solid predominant (37.2%). In 15 cases the existing archival tissue was not sufficient for a reliable morphological reclassification.

Concerning the diagnostic biomarkers, 96.7%, 87.6%, and 75.5% of ADC cases expressed CK7, TTF1, and napsin A, respectively.

ALK translocations were identified in 6 cases (1.4%). *KRAS*, *EGFR*, and *BRAF* mutations were detected in 160 (37.6%), 66 (15.5%), and 17 cases (4%), respectively.

Double mutations (mutation 1/mutation 2) occurred in *KRAS/KRAS* (n=8; 1.9%), *EGFR/EGFR* (n=6, 1.4%), *KRAS/EGFR* (n=6; 1.4%), and *EGFR/BRAF* (n=2; 0.5%). One out of 6 *ALK* translocated ADC showed an additional *KRAS* mutation. Mean H-Scores for ERCC1 and TS were 57.9 and 45.5. For further details on the distribution of clinicopathological characteristics see Table 1.

Association of selected clinical and morphologic parameters

Distribution of UICC stages and TNM classification parameters significantly differed when compared to the dominant growth pattern. Furthermore, predominant histomorphology correlated with smoking status; acinar (91.2% of cases), solid

(95.7% of cases) and papillary (89.5% of cases) tumors were significantly more likely to occur in smokers or ex-smokers than lepidic (69.2% of cases) or micropapillary (81% of cases) predominant ADC ($p<0.001$). For a comprehensive overview of further findings compare Figure 1 and Table 1.

Association of diagnostic and predictive biomarkers with clinical parameters

Apart from TTF1, whose expression was significantly more prevalent in tumors with low pT stages ($p=0.035$), there were no significant associations of the other diagnostic immunomarkers (CK7, napsin A) with staging parameters (Table 1).

EGFR mutations were significantly more frequent in tumors of female patients while *KRAS* mutations were more frequent in men (Table 1, Figure 2). TS expression was significantly higher in tumors of older patients. ERCC1 expression was significantly higher ($p=0.023$) in tumors associated with smoking history. Of note, smoking history was also associated with higher rates of *KRAS* mutations ($p=0.003$) and lower rates of *EGFR* mutations ($p=0.001$); the other analyzed biomarkers showed no significant association with smoking (Table 1).

ALK translocations, *EGFR*, *KRAS,* and *BRAF* mutations were not significantly associated with staging parameters (Table 1, Figure 2).

Association of diagnostic and predictive biomarkers with morphologic characteristics

Napsin expression was significantly associated with predominant histomorphological pattern ($p<0.001$, Supp. Fig. 1), with micropapillary carcinomas showing the highest

expression rate, while solid and lepidic carcinomas were more likely to be negative. *KRAS* mutations were more frequent in invasive mucinous ADC, while no other types of the analyzed driver mutations were found in this ADC subtype (Table 1). Of all analyzed molecular alterations only *EGFR* mutation frequency was significantly different with respect to growth pattern with lepidic and micropapillary predominant ADC showing higher *EGFR* mutation rates (Table 1), mainly due to differences in the frequency of Exon 19 mutations (Supp. Fig. 2). *ALK* translocations were exclusively seen in acinar and solid predominant ADC (Supp. Fig. 2) and *BRAF* mutations were predominantly found in micropapillary but not in papillary or lepidic predominant ADC (Figure 1; Table 1).

Association of diagnostic and predictive biomarkers with each other

Both TS and ERCC1 expression were significantly higher in TTF1 positive ADC (p=0.026 and p=0.007, respectively; Table 1). Furthermore, higher expression levels of ERCC1 and TS were associated with *ALK* translocations and wildtype *KRAS* (ERCC1: p=0.005 and 0.008, TS p=0.075 and 0.049, respectively; Supp. Fig. 3).

Prognostic value of diagnostic and predictive biomarkers in pulmonary adenocarcinoma

TTF1 and napsin A expression were associated with prolonged survival with a stronger prognostic value for TTF1 (Table 2). Furthermore, TTF1 expression was a stage- and pattern-independent predictor of OS (HR=0.61, p=0.021). The survival effect was specifically evident in patients without adjuvant chemotherapy (Supp. Table 2). TS expression was a significant predictor of better patient survival for OS

and DFS (Table 3, Supp. Fig. 4). However, when survival impact of TS expression was adjusted for stage and dominant histomorphological pattern in a Cox regression model, TS failed to show independent impact on patient survival (OS (HR=0.82, p=.233), DFS (HR=0.73, p=.073)). The presence of *BRAF* mutations was a negative prognostic factor for DFS (p=0.009) but not for OS (Supp. Fig. 5). Interestingly, although overall no significant differences in survival were noted, patients with *EGFR* mutations receiving adjuvant chemotherapy clearly showed an improved outcome compared to patients without such therapy (Supp. Table 3). A comprehensive overview of the strength of prognostic associations of all morphologic, clinical, and molecular biomarkers is given in Table 2 and in Figure 3.

Discussion

Although a wealth of details on clinical, morphological, and molecular biomarkers in pulmonary ADC has been published, comprehensive studies covering all clinical and pathological characteristics relevant for the current routine diagnostic setting are lacking so far. Here, we demonstrate that several specific clinical, histomorphological, immunohistochemical, and molecular parameters are tightly linked or occur almost mutually exclusive, which may have significant impact on the development of rational, tissue sparing diagnostic algorithms as well as an optimized patient stratification.

The last decade in lung cancer research was dominated by large scale molecular approaches to identify prognostic and predictive markers for personalized medicine. Up to now, however, only *EGFR* mutations and *ALK* translocations were successfully translated into the diagnostic setting; several other potential biomarkers have failed to achieve this goal. Molecular characterization and subsequent clinical trials underlined

that morphologic features (e. g. squamous versus non-squamous) are crucial for therapy selection. Today it is known that the group of pulmonary ADC is more heterogeneous than expected with diverse biological behavior and prognosis. Therefore, re-classification of ADC based on histomorphology [18] was a logic and essential step with highly significant prognostic and, likely, predictive value [9]. With this novel and largely reproducible [20, 21] tool linkage of the different ADC histotypes to diagnostic and predictive biomarkers as well as clinical characteristics is essential for a comprehensive interdisciplinary classification of ADC in the future [18]. Furthermore, different therapeutic targets were found to be associated with each other, implying combined inhibitory strategies for optimized treatment algorithms. For example, *ALK* translocations were found to be associated with TS expression [22], *EGFR* mutations were reported more frequently in ERCC1-negative tumors [23], and EGFR inhibitors are known to down-regulate TS [24, 25]. Moreover, novel agents like lapatinib, a dual EGFR and Her2 tyrosine kinase inhibitor (TKI), crizotinib (ALK/c-Met inhibitor) or sorafenib (multikinase inhibitor) are directed against more than one target and may thus successfully prevent tumor escape mechanisms. Acquired resistance to targeted therapies is also closely linked to specific molecular alterations [26]. Hence, combined treatment approaches require correlative prevalence data of the respective predictive biomarkers.

ERCC1 and TS are involved in DNA synthesis and repair and their loss of expression was considered as predictive for response to platinum-based [27] and pemetrexed-based [28] chemotherapies, respectively. However, in addition to the potentially negative predictive value of both proteins high expression levels have also been reported to be associated with an improved outcome [27, 29-31], which was confirmed, at least for TS, by this study. This is explained by the hypothesis that by preventing mutagenesis, DNA repair does not only prevent cancer but also inhibits

molecular events related to tumor progression. Thus, high expression of the respective markers may indicate an improved outcome in untreated patients by identifying tumors that have only slowly progressed at the molecular level [32], which is also reflected by our finding that high TS and ERCC1 expression levels were more prevalent in early tumor stages (Table 1). The association of TS expression with invasive mucinous ADC, TTF1 positivity, older age, and *ALK* translocations [22], but also the resulting TS down-regulation by TKI [24, 25] might be used for the stratification of patients towards combined therapies with pemetrexed.

Several studies reported on the predictive value of ERCC1 for platinum-based chemotherapy, including a large trial of the International Adjuvant Lung Cancer Trial (IALT) on 761 NSCLC [11]. However, subsequent studies indicate that this association might be specifically prominent in SQCC but not in ADC [33] [Warth et al., unpublished]. In our ADC cohort ERCC1 expression was also associated with reduced survival in those patients receiving adjuvant platinum based chemotherapy, however, these differences failed to reach statistical significance, which might indicate a comparably small predictive value of this marker but on the other side might also be attributed to the relatively small sample size in this subcohort. Most recently, Friboulet et al. [34] reported that they were not able to validate the predictive effect of immunostaining for ERCC1 in several large NSCLC cohorts including the cohort from the IALT trial, which might be due to the fact that commercially available antibodies, including the one used in this study, do not seem to specifically detect the unique functional ERCC1 isoform. Thus, the suggested predictive value of ERCC1 might be disputed until more specific antibodies allow for a validation of the predictive effect of ERCC1 expression.

EGFR mutations have been reported to accumulate in young, female, Asian, and never smoking patients. However, most of the studies published to date reported on selected cohorts in the context of clinical trials. In the herein analyzed unselected Caucasian cohort we could confirm the predominance of female sex and, in addition, found a higher prevalence in lepidic and micropapillary predominant ADC. Age was not associated with differences in the prevalence of EGFR mutations. Contradictory, others found a higher prevalence of *EGFR* mutations in older patients and an association to acinar predominant ADC [35]. These differences might be explained by different ethnical backgrounds of the respective cohorts. In any case, morphological criteria seem to be helpful if a pre-selection of patients for *EGFR* mutation testing is desired.

Although no specific targeted therapies exist so far, *KRAS* mutations are perceived as a potential negative predictive factor for TKI-based ADC treatment and a prognostic factor for surgically resected early stage ADC [36, 37]. However, there is also evidence that *KRAS* mutations, which are more prevalent in males, smokers, and invasive mucinous ADC [15, 35, 38] (Figure 2), are not an “a priori” negative factor for TKI administration [39]. Agents targeting downstream effectors of the *KRAS* pathway may provide treatment options for this large ADC subgroup. Patient stratification for *KRAS* mutation testing could be performed based on the above outlined characteristics.

In first trials *BRAF* inhibitors showed apparent antitumor activity in NSCLC [40] and specific *BRAF* mutations also rendered tumors responsive to Dasatinib [41]. *BRAF* mutations were reported to be associated with female sex and smoking. Furthermore, *BRAF* mutated tumors have been suggested to belong to an aggressive histotype, characterized by micropapillary features and shorter DFS and OS [42, 43]. We could

confirm the high prevalence of *BRAF* mutations in micropapillary predominant ADC and also the significant correlation of *BRAF* mutations with worse DFS, but not the proposed association to female sex or smoking.

Among the diagnostic markers TTF1 was most recently reported as an independent predictor of survival [44], which was confirmed by our study. Others also described an independent prognostic effect for napsin A and an association of napsin A expression to the presence of *EGFR* mutations in an Asian ADC cohort [45]. However, we could only see borderline associations of napsin A with survival parameters and no significant association to *EGFR* mutations. Again, these differing findings might be attributed to differences with respect to the ethnical background and underlines the need for reliable prevalence data of large ADC cohorts from various geographical regions.

One limitation of this study is its retrospective nature. The assessment of multiple clinicopathologic characteristics necessarily results in small groups for comparison and thus hampers multivariate analyses in some instances. Furthermore, predictive biomarkers are usually not analyzed using resection specimens but in advanced tumor stages or recurrent tumors where only sparse tissue is available. Although TMA-based assessment of immunomarkers may largely reflect the biopsy constellation, we cannot exclude a slight prevalence shift of the molecular alterations compared to the daily routine setting.

Perspectively, considering the growing number of clinically relevant biomarkers, not only the establishment of tissue-sparing diagnostic algorithms but also the development of time- and cost-effective multitesting platforms for molecular alterations with respective implementation into routine diagnostics seems to be mandatory in order to fulfill the requirements of evidence-based decision making

within the context of personalized treatment of lung cancer. Novel subgenomic massive parallel sequencing (MPS) strategies, which allow for a comprehensive mutational screen of tumor material in just one sequencing run are especially promising in this regard. However, prior to a widespread routine diagnostic use each of the MPS technologies applied must be adapted to the specific needs in lung cancer diagnosis, specifically, the robustness of the respective methods must be shown on small bronchial biopsy samples and paraffin embedded material.

Taken together, this is the first large scale study covering in parallel yet established morphological, diagnostic and predictive biomarkers as well as clinical characteristics of pulmonary ADC. The herein presented data of a largely unselected Caucasian cohort not treated with biomarker-based targeted therapies may form a basis in the development of rational diagnostic stratification algorithms for the selection of appropriate therapies and may also serve as a source of prevalence data for the design of clinical trials.

Acknowledgements

Jenny Schmitt, Angelika Brüntgens, Christina Hofherr, Waltraud Schmitz, Marie Karipidis, Meike Viole (Institute for Pathology, Heidelberg, Germany), and Christa Stolp (Thoraxklinik Heidelberg, Germany) are acknowledged for excellent support of this study.

Funding: Parts of this study were supported by the LungSys Consortium and a research grant by Novartis.

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Figure legends

Figure 1: Interplay between clinicopathological variables, diagnostic, and predictive biomarkers in pulmonary ADC. Lines indicate a positive association of the respective parameters.

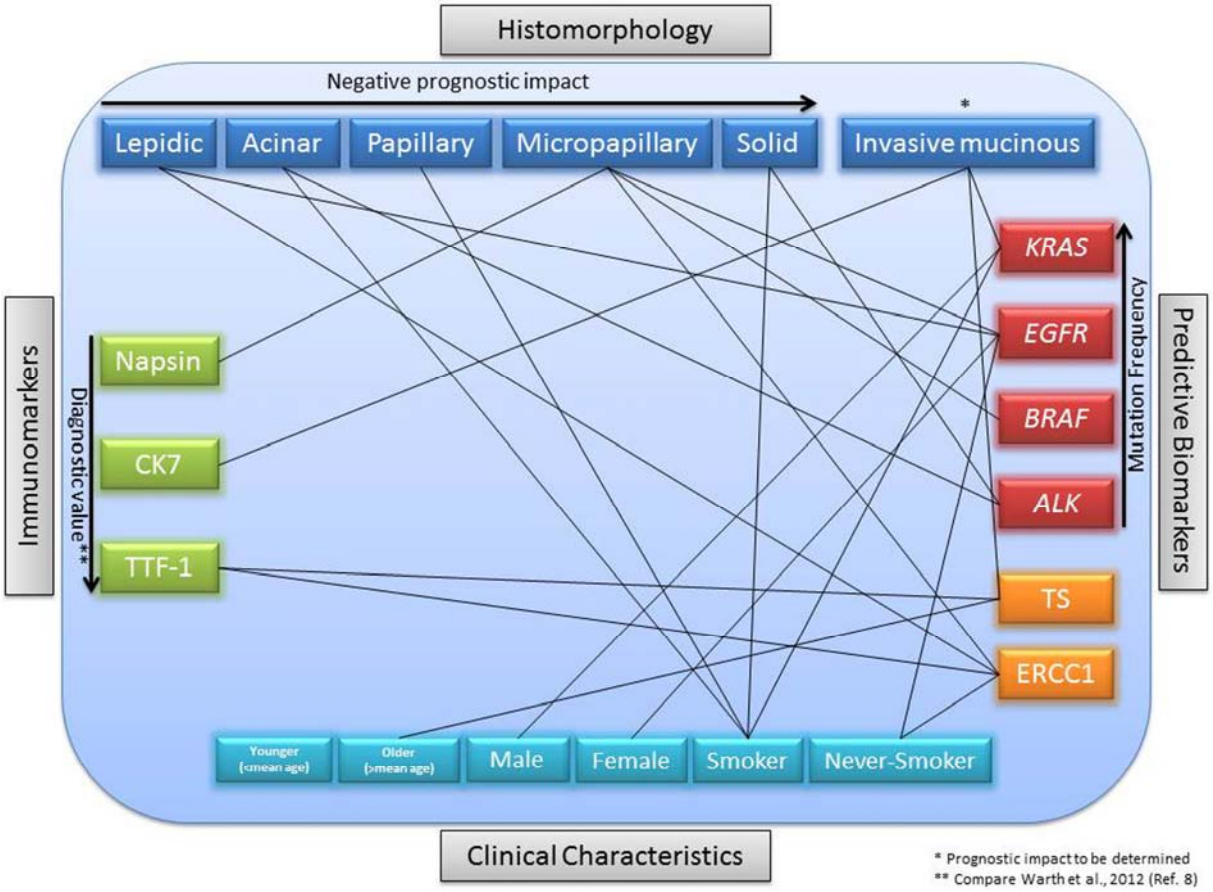


Figure 2: Distributions of common oncogenic driver mutations according to stage and sex in 425 pulmonary adenocarcinomas.

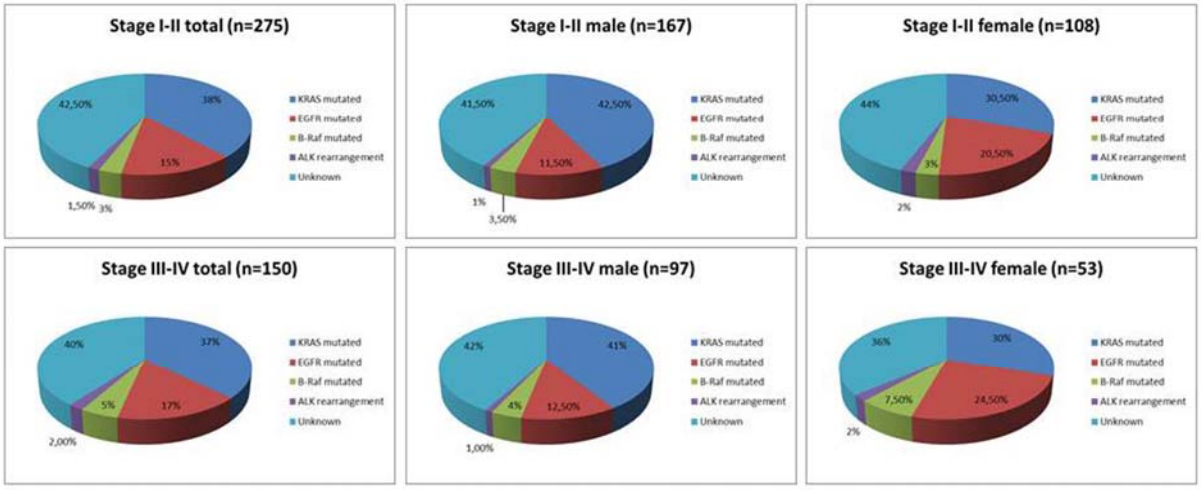
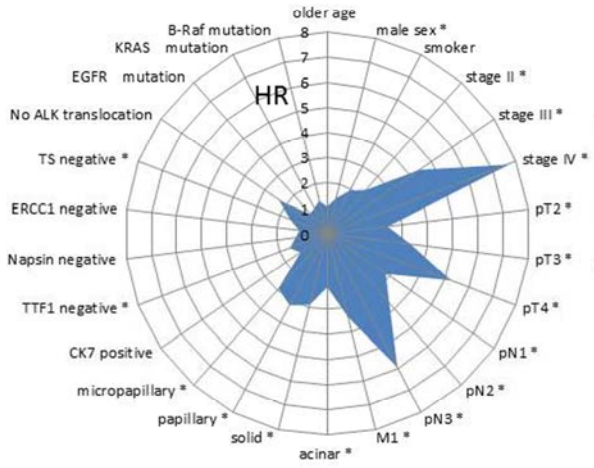


Figure 3: Spider web diagrams depicting the hazard ratios (HR) from univariate survival analysis of the analyzed parameters including older age (HR reference: younger age), male sex (female sex), smoker (never-smoker), stages II-IV (stage I), pT2-4 (pT1), pN1-3 (pN0), M1 (M0), acinar, papillary, micropapillary, and solid predominant pattern (lepidic predominant), CK7 positive (CK7 negative), TTF-1 negative (TTF-1 positive), napsin negative (napsin positive), ERCC1 negative (ERCC1 positive), TS negative (TS positive), no *ALK* translocation (*ALK* translocation), *EGFR* mutation (*EGFR* wt), *KRAS* mutation (*KRAS* wt), and *BRAF* mutation (*BRAF wt*). Significant differences for overall (OS) and disease free survival (DFS) are indicated with an asterix.

OS


HR
older age
male sex *
smoker
stage II *
stage III *
stage IV *
pT2 *
pT3 *
pT4 *
pN1 *
pN2 *
pN3 *
M1 *
acinar *
solid *
papillary *
micropapillary *
CK7 positive
TTF1 negative *
Napsin negative
ERCC1 negative
TS negative *
No ALK translocation
EGFR mutation
KRAS mutation
B-Raf mutation
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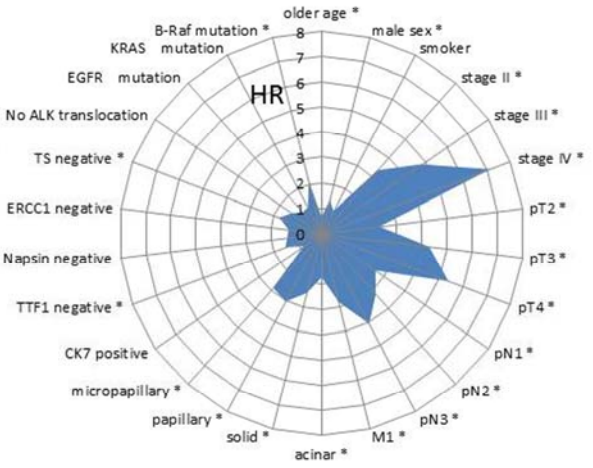
DFS


HR
older age *
male sex *
smoker
stage II *
stage III *
stage IV *
pT2 *
pT3 *
pT4 *
pN1 *
pN2 *
pN3 *
M1 *
acinar *
solid *
papillary *
micropapillary *
CK7 positive
TTF1 negative *
Napsin negative
ERCC1 negative
TS negative *
No ALK translocation
EGFR mutation
KRAS mutation
B-Raf mutation *
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Tables

Table 1: Association of clinicopathologic variables and diagnostic immunomarkers with common predictive biomarkers in pulmonary adenocarcinomas.

Characteristic	All cases	ERCC1 mean	ERCC1 SE	p-value	TS mean	TS SE	p-value	Alk negative	Alk positive	p-value	EGFR wt	EGFR mutated	p-value	KRAS wt	KRAS mutated	p-value	BRAF wt	BRAF mutated	p-value
All cases	425 (100%)	57,9	4,1		45,5	2,9		419 (98.6%)	6 (1.4%)		359 (84.5%)	66 (15.5%)		265 (62.4%)	160 (37.6%)		408 (96%)	17 (4%)	
Age																			
below mean	196 (46.1%)	51,7	6,0	*0.090**	37,9	4,0	*0.045**	194 (99%)	2 (1%)	*0.691#*	170 (86.7%)	26 (13.3%)	*0.283#*	119 (60.7%)	77 (39.3%)	*0.548#*	189 (96.4%)	7 (3.6%)	*0.806#*
above mean	229 (53.9%)	64,5	5,8		52,3	4,3		225 (98.3%)	4 (1.7%)		189 (82.5%)	40 (17.5%)		146 (63.8%)	83 (36.2%)		219 (95.6%)	10 (4.4%)	
Sex																			
male	264 (62.1%)	60,3	5,5	*0.995**	45,6	3,8	*0.989**	261 (98.9%)	3 (1.1%)	*0.677#*	233 (88.3%)	31 (11.7%)	*0.008#*	153 (58%)	111 (42%)	*0.018#*	254 (96.2%)	10 (3.8%)	*0.802#*
female	161 (37.9%)	56,3	6,3		45,8	4,9		158 (98.1%)	3 (1.9%)		126 (78.3%)	35 (21.7%)		112 (69.6%)	49 (30.4%)		154 (95.7%)	7 (4.3%)	
Smoking status																			
never-smoker	34 (9.3%)	87,5	16,1	*0.023**	55,3	10,6	*0.171**	33 (97.1%)	1 (2.9%)	*0.388#*	22 (64.7%)	12 (35.3%)	*0.001#*	29 (85.3%)	5 (14.7%)	*0.003#*	32 (94.1%)	2 (5.9%)	*0.629#*
smoker	332 (90.7%)	55,7	4,8		43,8	3,4		328 (98.8%)	4 (1.2%)		290 (87.3%)	42 (12.7%)		195 (58.7%)	137 (41.3%)		320 (96.4%)	12 (3.6%)	
UICC stage																			
I	176 (41.4%)	68,5	6,9		60,1	5,2		172 (97.7%)	4 (2.3%)		147 (83.5%)	29 (16.5%)		115 (65.3%)	61 (34.7%)		171 (97.2%)	5 (2.8%)	
II	94 (22.1%)	37,7	6,8	*0.106+*	36,7	6,0	*<0.001+*	94 (100%)	0 (0%)	*0.483§*	83 (88.3%)	11 (11.7%)	*0.841§*	53 (56.4%)	41 (43.6%)	*0.715§*	90 (95.7%)	4 (4.3%)	*0.154§*
III	143 (33.6%)	58,4	7,0		34,1	4,0		141 (98.6%)	2 (1.4%)		120 (83.9%)	23 (16.1%)		88 (61.5%)	55 (38.5%)		137 (95.8%)	6 (4.2%)	
IV	12 (2.8%)	67,3	35,5		33,2	13,4		12 (100%)	0 (0%)		9 (75%)	3 (25%)		9 (75%)	3 (25%)		10 (83.3%)	2 (16.7%)	
Tumor stage																			
T1	78 (18.4%)	79,7	11,5		51,1	7,9		76 (97.4%)	2 (2.6%)		64 (82.1%)	14 (17.9%)		53 (67.9%)	25 (32.1%)		76 (97.4%)	2 (2.6%)	
T2	268 (63.1%)	57,9	5,3	*0.106+*	50,3	3,8	*0.004+*	264 (98.5%)	4 (1.5%)	*0.183§*	224 (83.6%)	44 (16.4%)	*0.319§*	171 (63.8%)	97 (36.2%)	*0.072§*	255 (95.1%)	13 (4.9%)	*0.587§*
T3	67 (15.7%)	33,7	6,8		20,6	4,4		67 (100%)	0 (0%)		62 (92.5%)	5 (7.5%)		33 (49.3%)	34 (50.7%)		67 (100%)	0 (0%)	
T4	12 (2.8%)	67,3	21,2		35,9	15,5		12 (100%)	0 (0%)		9 (75%)	3 (25%)		8 (66.7%)	4 (33.3%)		10 (83.3%)	2 (16.7%)	
Nodal status																			
N0	230 (54.1%)	60,8	5,8		54,3	4,5		226 (98.3%)	4 (1.7%)		200 (87%)	30 (13%)		144 (62.6%)	86 (37.4%)		222 (96.5%)	8 (3.5%)	
N1	76 (17.9%)	47,5	8,0	*0.732+*	38,5	6,3	*0.031+*	76 (100%)	0 (0%)	*0.807§*	63 (82.9%)	13 (17.1%)	*0.102§*	45 (59.2%)	31 (40.8%)	*0.791§*	73 (96.1%)	3 (3.9%)	*0.515§*
N2	116 (27.3%)	61,6	8,5		33,2	4,4		115 (99.1%)	1 (0.9%)		94 (81%)	22 (19%)		73 (62.9%)	43 (37.1%)		110 (94.8%)	6 (5.2%)	
N3	3 (0.7%)	45,0	45,0		30,0	30,0		2 (66.7%)	1 (33.3%)		2 (66.7%)	1 (33.3%)		3 (100%)	0 (0%)		3 (100%)	0 (0%)	
Distant Metastases																			
M0	414 (97.4%)	58,3	4,2	*0.509**	46,1	3,0	*0.372**	338 (83%)	69 (17%)	*0.699#*	351 (84.8%)	63 (15.2%)	*0.388#*	256 (61.8%)	158 (38.2%)	*0.221#*	399 (96.4%)	15 (3.6%)	*0.067#*
M1	11 (2.6%)	67,3	35,2		33,2	13,4		10 (90.9%)	1 (9.1%)		8 (72.7%)	3 (27.3%)		9 (81.8%)	2 (18.2%)		9 (81.8%)	2 (18.2%)	
Pattern																			
lepidic	30 (7.5%)	90,4	18,4		58,2	12,9		30 (100%)	0 (0%)		23 (76.7%)	7 (23.3%)		19 (63.3%)	11 (36.7%)		30 (100%)	0 (0%)	
acinar	176 (43.9%)	63,1	6,6	*0.017+*	51,2	5,0	*0.111+*	171 (97.2%)	5 (2.8%)	*0.406$*	143 (81.2%)	33 (18.8%)	*0.020$*	119 (67.6%)	57 (32.4%)	*0.630$*	169 (96%)	7 (4%)	*0.459$*
solid	149 (37.2%)	47,0	6,5		39,9	4,1		148 (99.3%)	1 (0.7%)		135 (90.6%)	14 (9.4%)		88 (59.1%)	61 (40.9%)		141 (94.6%)	8 (5.4%)	
papillary	21 (5.2%)	41,9	17,1		29,4	16,4		21 (100%)	0 (0%)		18 (85.7%)	3 (14.3%)		13 (61.9%)	8 (38.1%)		21 (100%)	0 (0%)	
micropapillary	25 (6.2%)	73,9	19,9		48,0	13,9		25 (100%)	0 (0%)		17 (68%)	8 (32%)		16 (64%)	9 (36%)		23 (92%)	2 (8%)	
Type																			
conventional	416 (97.9%)	58,6	4,2	*0.964**	45,6	3,0	*0.498**	410 (98.6%)	6 (1.4%)	*1.000#*	350 (84.1%)	66 (15.9%)	*0.366#*	262 (63%)	154 (37%)	*0.087#*	399 (95.9%)	17 (4.1%)	*1.000#*
invasive mucinous	9 (2.1%)	59,3	25,7		52,9	18,4		9 (100%)	0 (0%)		9 (100%)	0 (0%)		3 (33.3%)	6 (66.7%)		9 (100%)	0 (0%)	
CK7																			
negative	14 (3.3%)	22,5	7,6	*0.310**	32,9	15,8	*0.580**	14 (100%)	0 (0%)	*1.000#*	13 (92.9%)	1 (7.1%)	*0.707#*	9 (64.3%)	5 (35.7%)	*1.000#*	14 (100%)	0 (0%)	*0.554#*
positive	404 (96.7%)	57,8	4,2		45,9	3,1		398 (98.6%)	6 (1.5%)		339 (83.9%)	65 (16.1%)		250 (61.9%)	154 (38.1%)		387 (95.8%)	17 (4.2%)	
TTF1																			
negative	52 (12.4%)	29,1	8,7	*0.007**	28,5	6,6	*0.026**	52 (100%)	0 (0%)	*1.000#*	43 (82.7%)	9 (17.3%)	*0.685#*	32 (61.5%)	20 (38.5%)	*1.000#*	50 (96.2%)	2 (3.8%)	*1.000#*
positive	366 (87.6%)	60,6	4,4		47,9	3,3		360 (98.4%)	6 (1.6%)		310 (84.7%)	56 (15.3%)		226 (61.7%)	140 (38.3%)		351 (95.9%)	15 (4.1%)	
Napsin																			
negative	103 (24.5%)	43,4	7,0	*0.080**	38,8	5,9	*0.104**	103 (100%)	0 (0%)	*0.343#*	93 (90.3%)	10 (9.7%)	*0.061#*	64 (62.1%)	39 (37.9%)	*1.000#*	101 (98.1%)	2 (1.9%)	*0.263#*
positive	317 (75.5%)	61,9	4,9		47,8	3,5		311 (98.1%)	6 (1.9%)		261 (82.3%)	56 (17.7%)		197 (62.1%)	120 (37.9%)		302 (95.3%)	15 (4.7%)	

For smoking status (n=59), growth pattern (n=15), CK7 (n=7), TTF1 (n=7), napsin (n=5), ERCC1 (n=41), and TS (n=30) data were missing in few cases.

* Mann-Whitney-U test + Kruskal-Wallis test # Fisher's exact test § χ^2-test for trends $ χ^2-test

Table 2: Overall (OS) and disease free (DFS) survival in dependence of clinicopathological, diagnostic, and predictive biomarkers. P-values were calculated with a log-rank test.

	Overall survival					Disease free survival				
	Cases	Events	Survival (months)	Standard error	p-value	Cases	Events	Survival (months)	Standard error	p-value
Age										
below mean	196	80	65,32	3,43	0,644	196	101	50,67	3,46	0,006
above mean	229	99	60,91	3,17		229	84	63,12	3,52	
Sex										
male	264	123	59,76	2,94	0,024	264	124	53,00	3,06	0,045
female	161	56	69,35	3,77		161	61	64,06	4,12	
Smoking status										
non-smoker	34	8	57,05	3,44	0,068	34	15	41,39	5,03	0,959
smoker	332	144	62,23	2,68		332	148	55,04	2,80	
UICC stage										
I	176	41	83,07	3,17		176	42	79,18	3,28	
II	94	43	61,05	4,60	<0.001	94	51	47,99	4,89	<0.001
III	143	87	41,65	3,68		143	85	34,70	3,83	
IV	12	8	20,25	4,06		12	7	16,10	5,20	
Tumor stage										
T1	78	15	82,12	4,92		78	17	77,23	5,35	
T2	268	122	61,91	2,85	<0.001	268	120	56,95	3,04	<0.001
T3	67	34	54,69	5,83		67	41	37,47	5,70	
T4	12	8	37,32	11,14		12	7	17,50	4,14	
Nodal status										
N0	230	63	79,27	2,89		230	74	70,70	3,11	
N1	76	44	49,44	5,04	<0.001	76	41	45,17	5,84	<0.001
N2	116	71	40,36	4,03		116	69	31,66	3,51	
N3	3	1	13,13	0,00		3	1	8,82	1,87	
Distant Metastases										
M0	413	171	64,42	2,37	<0.001	413	178	58,12	2,52	0,006
M1	12	8	20,25	4,06		12	7	16,10	5,20	
Pattern										
lepidic	30	8	70,21	6,60		30	10	65,74	7,00	
acinar	176	72	65,96	3,52	0,071	176	72	60,92	3,78	0,051
solid	149	72	56,36	3,83		149	71	50,68	4,22	
papillary	21	10	46,16	7,45		21	12	35,12	6,63	
micropapillary	25	11	47,19	6,96		25	13	38,20	7,29	
Type										
conventional	416	178	62,64	2,37	0,046	416	185	56,11	2,53	0,009
invasive mucinous	9	1	87,04	8,51		9	0	/	/	
CK7										
negative	14	5	48,66	5,85	0,569	14	7	35,45	7,14	0,701
positive	404	171	63,27	2,40		404	177	57,10	2,55	
TTF1										
negative	52	26	49,80	61,09	0,030	52	26	43,61	6,51	0,045
positive	366	150	64,75	24,99		366	157	58,50	2,67	
Napsin										
negative	103	48	58,04	4,68	0,160	103	51	50,67	4,96	0,056
positive	317	129	64,84	2,69		317	133	58,97	2,89	
ERCC1										
below mean	198	84	62,24	3,51	0,509	198	94	52,30	3,62	0,072
above mean	186	79	63,98	3,40		186	78	59,44	3,75	
TS										
below mean	208	95	56,35	3,31	0,005	208	102	49,29	3,53	<0.001
above mean	187	66	71,84	3,43		187	66	65,85	3,88	
Alk										
negative	419	178	63,30	2,35	0,387	419	183	57,41	2,52	0,615
positive	6	1	47,88	7,84		6	2	50,05	4,22	
KRAS										
wt	265	111	64,28	2,90	0,464	265	114	58,96	3,10	0,302
mutant	160	68	59,76	3,55		160	71	52,24	3,94	
BRAF										
wt	408	170	63,81	2,40	0,398	408	172	59,19	2,55	0,009
mutant	17	9	55,13	10,92		17	13	29,47	6,16	
EGFR total										
wt	359	150	63,85	2,55	0,777	359	154	57,78	2,71	0,529
mutant	66	29	60,38	5,72		66	31	54,92	6,46	
EGFR Exon 18										
wt	419	174	64,13	2,36	0,025	419	181	57,80	2,52	0,108
mutant	6	5	30,79	10,24		6	4	17,98	5,09	
EGFR Exon 19										
wt	398	169	63,16	2,43	0,543	398	170	58,19	2,59	0,214
mutant	27	10	67,82	8,73		27	15	46,96	9,07	
EGFR Exon 20										
wt	413	172	63,88	2,38	0,317	413	179	57,48	2,54	0,626
mutant	12	7	52,41	11,95		12	6	55,90	13,95	
EGFR Exon 21										
wt	398	167	63,62	2,43	0,912	398	174	57,10	2,58	0,705
mutant	27	12	54,23	6,77		27	11	54,28	7,45	