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Mutational analysis of pulmonary tumours with neuroendocrine features using targeted massive parallel sequencing: a comparison of a neglected tumour group

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Background: Lung cancer is the leading cause of cancer-related deaths worldwide. The typical and atypical carcinoid (TC and AC), the large-cell neuroendocrine carcinoma (LCNEC) and the small-cell lung cancers (SCLC) are subgroups of pulmonary tumours that show neuroendocrine differentiations. With the rising impact of molecular pathology in routine diagnostics the interest for reliable biomarkers, which can help to differentiate these subgroups and may enable a more personalised treatment of patients, grows.

Methods: A collective of 70 formalin-fixed, paraffin-embedded (FFPE) pulmonary neuroendocrine tumours (17 TCs, 17 ACs, 19 LCNECs and 17 SCLCs) was used to identify biomarkers by high-throughput sequencing. Using the Illumina TruSeq Amplicon-Cancer Panel on the MiSeq instrument, the samples were screened for alterations in 221 mutation hot spots of 48 tumour-relevant genes.

Results: After filtering > 26 000 detected variants by applying strict algorithms, a total of 130 mutations were found in 29 genes and 49 patients. Mutations in *JAK3*, *NRAS*, *RB1* and *VHL1* were exclusively found in SCLCs, whereas the *FGFR2* mutation was detected in LCNEC only. *KIT*, *PTEN*, *HNF1A* and *SMO* were altered in ACs. The *SMAD4* mutation corresponded to the TC subtype. We prove that the frequency of mutations increased with the malignancy of tumour type. Interestingly, four out of five *ATM*-mutated patients showed an additional alteration in *TP53*, which was by far the most frequently altered gene (28 out of 130; 22%). We found correlations between tumour type and IASLC grade for *ATM* ($P = 0.022$; $P = 0.008$) and *TP53*-mutated patients ($P < 0.001$). Both mutated genes were also associated with lymph node invasion and distant metastasis ($P \leq 0.005$). Furthermore, *PIK3CA*-mutated patients with high-grade tumours showed a reduced overall survival ($P = 0.040$) and the mutation frequency of *APC* and *ATM* in high-grade neuroendocrine lung cancer patients was associated with progression-free survival (PFS) ($P = 0.020$).

Conclusions: The implementation of high-throughput sequencing for the analysis of the neuroendocrine lung tumours has revealed that, even if these tumours encompass several subtypes with varying clinical aggressiveness, they share a number of molecular features. An improved understanding of the biology of neuroendocrine tumours will offer the opportunity for novel approaches in clinical management, resulting in a better prognosis and prediction of therapeutic response.

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Lung cancer is the leading cause of cancer-related deaths worldwide, with 15% of cases showing only a 5-year survival rate. Nearly one-fourth of all lung neoplasms originate from cells of the diffuse neuroendocrine system and are classified as neuroendocrine tumours (NET) (Rekhtman, 2010). This spectrum of neoplasms is composed of the high-grade small-cell lung cancer (SCLC), the large-cell neuroendocrine carcinoma (LCNEC), as well as the low-grade typical (TC) and atypical carcinoids (AC) (Takei *et al*, 2002).

Although smoking is not correlating with carcinoids, most of LCNEC and all SCLC patients exhibit a smoking history (Travis, 2009; Swarts *et al*, 2012). Even though tumours are detected at an early stage, SCLCs and LCNECs show the poorest clinical outcome as compared with other lung tumours because of their high malignancy (Takei *et al*, 2002). The 5-year survival rate of patients with SCLC is <5% (Rekhtman, 2010). The same holds true for LCNECs that show a similar progression with 5-year survival rates varying from 15% up to 57%, depending on the source of information (Asamura *et al*, 2006; Rekhtman, 2010). In contrast, patients suffering from TC show excellent survival rates of up to 100%. Nevertheless, some of them present with lymph node metastasis (4–15%) (Travis *et al*, 1998; Beasley *et al*, 2000; Fink *et al*, 2001; Thomas *et al*, 2001; Cardillo *et al*, 2004; Pelosi *et al*, 2005; Asamura *et al*, 2006; Garcia-Yuste *et al*, 2007; Rea *et al*, 2007; Travis, 2009). Atypical carcinoids are more aggressive and show a higher frequency of nodal metastasis (35–64%) with decreased survival rates ranging from 61% to 88% (Beasley *et al*, 2000; Fink *et al*, 2001; Scott, 2003; Cardillo *et al*, 2004; Pelosi *et al*, 2005; Asamura *et al*, 2006; Garcia-Yuste *et al*, 2007; Rea *et al*, 2007).

During lung carcinogenesis, cells stepwise accumulate multiple genetic and epigenetic abnormalities that lead to immortalisation, uncontrolled proliferation and spread of these altered cells (Brambilla *et al*, 2003). This is often initiated by the presence of mutations, but can also be an effect of chromosomal aberrations (Esteller *et al*, 1999; Zochbauer-Muller *et al*, 2001). In particular, the most common mutated gene in lung cancer is the tumour suppressor *TP53*, encoding a protein that induces cell growth arrest and apoptosis and mediates cell cycle regulation. In contrast to pulmonary carcinoids, which are rarely *TP53* altered, up to 50% of non-small-cell lung cancer (NSCLC) and >70% of SCLCs were associated with mutations in this gene (Travis *et al*, 2004). In addition, the crucial retinoblastoma (RB) pathway is often inactivated. Up to 100% of high-grade NETs like SCLCs or LCNECs are affected by the loss of the RB protein (Travis *et al*, 2004).

The advent of next-generation sequencing (NGS) systems allows a very sensitive analysis of multiple patients and targets simultaneously. Therefore, further mutational analysis of neuroendocrine lung tumours by a high-throughput sequencing approach helps to gain more insight into their genetic profile, enabling a further subdivision of these different groups. Here we subjected, for the first time, 70 neuroendocrine lung tumours to a targeted NGS approach based on sequencing-by-synthesis chemistry, feasible for formalin-fixed, paraffin embedded (FFPE) material.

MATERIALS AND METHODS

Demographic data and study design. The study is based on a collective of 70 representative, FFPE neuroendocrine lung tumours (17 TCs, 17 ACs, 19 LCNECs and 17 SCLCs) used for massive parallel sequencing purposes. The initial diagnosis was re-evaluated by two experienced pathologists (JW and TM). Inclusion criteria were sufficient tumour material (tumour cell count 50–80%) and a minimum of contamination by lymphocytic and stromal cells. All analysed cases comprise surgical resection. The surgical resected SCLCs were resected as initial unknown tumoural lesion and were afterwards diagnosed as SCLC. All investigated SCLCs were

classified as limited or very limited diseases (VLDL). Specimens were collected at the Institute of Pathology at the University Hospital Essen (Essen, Germany) from 2005 till 2012. The TNM staging was based on the *WHO Classification Of Tumours* guidelines (2004) (Travis *et al*, 2004). The mean age at date of diagnosis was 58.6 years (median age: 59.0 years; 95% CI: 50.8–66.9 months). Survival data were available for 34 patients with 12 reported deaths at the time of data collection. Survival data were collected for the high-grade group as well as overall survival (OS) for 3 carcinoid tumours showing an aggressive course with reported death after 4.4, 7.3 and 11.3 months, respectively. For the remaining carcinoids, no survival data were collected because of their excellent prognosis.

The study was conducted retrospectively for the identification of biomarkers. Patients who received chemotherapy before resection of tumour tissue specimens were excluded. The study was approved by the ethical committee of the University Hospital Essen (ID: 13-5382-BO). The investigations conform to the principles outlined in the declaration of Helsinki. Patient characteristics are summarised in Table 1.

DNA extraction. For DNA isolation, FFPE tissue slides of 4 µm were prepared using a microtome Cool Cut HM 355S (MICROM International, Walldorf, Germany). Three to five paraffin tissue slides per sample were deparaffinised with xylene before DNA extraction. Genomic DNA was isolated via Maxwell 16 Research (Promega, Madison, WI, USA) as recommended by the manufacturer. Following, DNA quantification was performed using

Table 1. Patient characteristics

	Absolute
Age at sampling (years)	
Median (range)	59 (20–84)
Gender	
Male	28
Female	35
Unknown	7
Regional lymph nodes (N-status)	
N0 (no regional lymph node metastases)	41
N1 (metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension)	9
N2 (metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s))	8
N3 (metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s))	2
Nx (unknown lymph node status)	10
Metastasis	
M0 (no distant metastasis)	53
M1 (metastasis in the contralateral lobe or pleura)	6
M2 (distant metastasis)	1
Mx (unknown appearance of distant metastasis)	10
Subtype	
Typical carcinoid (TC)	17
Atypical carcinoid (AC)	17
Large-cell neuroendocrine carcinoma (LCNEC)	19
Small-cell lung cancer (SCLC)	17
Progression	
Progressive disease	25
Stable disease	6
Partial remission	3
Complete response	0
Overall survival (month)	
Median (range) for the overall collective	6.4 (0.1–52.8)
Median (range) for the carcinoids only	7.3 (4.4–11.3)
Progression-free survival (month)	
Median (range)	4.6 (0.2–52.8)

Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) and Qubit (Life Technologies, Carlsbad, CA, USA).

Targeted next-generation sequencing. Sample preparation was done using the TruSeq Amplicon-Cancer Panel (Illumina, San Diego, CA, USA). Therefore, 424 target-specific probes, covering 221 mutation hot spots of 48 tumour-relevant genes, were used to capture the regions of interest (Table 2). Furthermore, each sample was labelled with a barcode sequence consisting of eight individual nucleotides, in a final PCR step. A total of 212 amplicons per sample with a mean length of 180 base pairs were sequenced paired-end on the MiSeq instrument (Illumina) using the v1 chemistry. Sample preparation and sequencing procedure was performed according to the standard protocols provided by the manufacturer.

Sequencing data analysis. Fastq files generated by the MiSeq reporter software (Illumina) were used for further analysis with a customised bioinformatics pipeline based on the general cancer genome analysis algorithm published by Peifer *et al* (2012).

Subsequently, data filtering was done by excluding variants with <25 effective reads (total reads \times (percent variant reads/100)) and by excluding variants with <5% allele frequency. Afterwards, synonymous alterations were removed and visual analysis of called variants was performed by means of the Integrative Genomic Viewer (IGV, Broad Institute, Cambridge, MA, USA). False positive variants, particularly in repetitive or highly homologous regions of the genome, variants in high background noise, as well as single-strand variants, were eliminated when they were clearly recognisable as artefacts. Finally, obtained variants were analysed for their functional impact on the protein by MutationAssessor (<http://mutationassessor.org>, release 2) (Reva *et al*, 2011) and Variant Effect Predictor (VEP, Ensembl release 78) (McLaren *et al*, 2010) implementing the bioinformatics tools SIFT (sorting intolerant from tolerant) (Kumar *et al*, 2009) and PolyPhen2 (polymorphism phenotyping v2) (Adzhubei *et al*, 2010). Only mutations with a functional impact as determined by at least two of the three applied algorithms as well as frameshift deletions/insertion and alterations leading to a stop codon were used for statistical analysis.

Statistical analysis. All statistical analyses were performed using the R environment for statistical computing and graphics (R Foundation, Vienna, Austria). For dichotomous factors and linear vectors the Wilcoxon Mann-Whitney rank sum test was applied. For variance analysis of variables with more than two categories, the Kruskal-Wallis test was performed. Double dichotomous contingency tables (e.g., mutational status to lymph node invasion status) were analysed using Fisher's exact test. To test dependence of ranked parameters with more than two groups (e.g., mutational status and tumour type) Pearson's χ^2 test was used. Kaplan-Meier analysis was performed to test associations between mutation status and OS as well as progression-free survival (PFS). The PFS was calculated from the first day of chemotherapy until radiologic progression, death from any cause or the last time of follow-up without progression. Overall survival was defined as the time from date of diagnosis until the date of

death or last follow-up. Patients were censored at the last follow-up if still alive or loss of follow-up. Surveillance of PFS and OS was stopped on 31 August 2014. Significant differences in PFS or OS between groups were verified by the Wald test, likelihood ratio test and Score (logrank) test (Supplementary Table S3). The level of statistical significance was defined as $P \leq 0.05$.

RESULTS

Run parameters and sequencing quality. A total of 70 neuroendocrine lung cancers were sequenced with 4 MiSeq runs, containing 23 or 24 samples per run. Samples showing coverages with <250 median read counts per amplicon (23 out of 70) were re-sequenced with an additional fifth run.

Bridge amplification of the target regions on the flow cell resulted in an average cluster density of 641 K mm⁻² with ~80% of the generated clusters passing the quality filter parameters. In total, the sequencing runs produced an average of 3.65×10^6 reads and an average output of 1.1 gigabases. The runs showed read quality parameters with average Q30 scores (equals a 0.1% chance of a wrong base call at this position) of ~63% in a range of 8–85% (Supplementary Table S1). Approximately 60% of the targets showed coverages between 500 and 2000 reads (Supplementary Figure S1).

Variant filtering and mutation detection. The sequencing analysis of all five runs revealed a total of 26 945 variants. Removal of variants with <25% effective reads and variants with <5% allele frequency resulted in 643 variants. Finally, additional removal of variants clearly recognised as artefacts resulted in 254 variants that were further analysed according to their functional relevance, leading to a total of 130 mutations in 29 genes (Table 3) detected in 49 of 70 FFPE neuroendocrine lung tumour samples (Figure 1 and Supplementary Table S2). No variants were found in the remaining 19 genes.

The mutation status of some genes was associated with a special subtype of the pulmonary NETs (Table 3 and Figure 2A). Mutations in *JAK3* (exon 16: p.W690*), *NRAS* (exon 2: p.G13D, p.S17N), *RB1* (exon 18: p.P595L; exon 20: H673Y) and *VHL1* (exon 3: Q164*) were exclusively found in SCLCs, whereas the detected *FGFR2* mutation p.A264T in exon 7 was only associated with LCNEC. The *KIT* (exon 10: p.K546delinsGG; exon 13 p.M638fs), *PTEN* (exon 8: p.P339S), *HNF1A* (exon 4: p.G288fs) and *SMO* (exon 5: p.L367R) alterations were only found in ACs and the *SMAD4* (exon 5: p.Q169*, exon 5) mutation corresponded to the TC subtype.

Furthermore, the total mutation frequency increased with the malignancy of the tumour type. Of the 130 mutations, 11 (9%) were related to TCs (mean: 0.6 per sample), 21 (16%) were found in ACs (mean: 1.2 mutations per sample) and 38 (29%) were related to LCNECs (mean: 1.9 per sample). Patients associated to SCLC showed the highest mutation frequency of 46% with 60 detected mutations (mean: 3.5 per sample). This effect was especially observed for *ATM*, *ERBB2*, *ERBB4*, *PIK3CA*, *RET* and *TP53* (Figure 2B). Interestingly, four of five *ATM* mutated patients

Table 2. Genes included in the TruSeq Amplicon-Cancer Panel (Illumina)

<i>ABL1</i>	<i>AKT1</i>	<i>ALK</i>	<i>APC</i>	<i>ATM</i>	<i>BRAF</i>	<i>CDH1</i>	<i>CDKN2A</i>
<i>CSF1R</i>	<i>CTNNB1</i>	<i>EGFR</i>	<i>ERBB2</i>	<i>ERBB4</i>	<i>FBXW7</i>	<i>FGFR1</i>	<i>FGFR2</i>
<i>FGFR3</i>	<i>FLT3</i>	<i>GNA11</i>	<i>GNAQ</i>	<i>GNAS</i>	<i>HNF1A</i>	<i>HRAS</i>	<i>IDH1</i>
<i>JAK2</i>	<i>JAK3</i>	<i>KDR</i>	<i>KIT</i>	<i>KRAS</i>	<i>MET</i>	<i>MLH1</i>	<i>MPL</i>
<i>NOTCH1</i>	<i>NPM1</i>	<i>NRAS</i>	<i>PDGFRA</i>	<i>PIK3CA</i>	<i>PTEN</i>	<i>PTPN11</i>	<i>RB1</i>
<i>RET</i>	<i>SMAD4</i>	<i>SMARCB1</i>	<i>SMO</i>	<i>SRC</i>	<i>STK11</i>	<i>TP53</i>	<i>VHL</i>

The panel consists of 2 \times 212 probes covering 221 mutation hot spots in 48 genes known to be important in human cancerogenesis.

Table 3. Distribution of mutations between the different tumour subtypes

	TC (%)	AC (%)	LCNEC (%)	SCLC (%)
JAK3 (n = 1)	0	0	0	100
NRAS (n = 2)	0	0	0	100
RB1 (n = 2)	0	0	0	100
VHL (n = 1)	0	0	0	100
ATM (n = 7)	0	0	14	86
TP53 (n = 31)	0	0	48	52
ALK (n = 2)	0	0	50	50
BRAF (n = 3)	0	0	67	33
FGFR2 (n = 1)	0	0	100	0
PIK3CA (n = 8)	0	13	13	75
ERBB4 (n = 5)	0	20	40	40
KDR (n = 3)	0	33	67	0
KRAS (n = 3)	0	33	67	0
MET (n = 3)	0	33	67	0
SMARCB1 (n = 2)	0	50	0	50
FGFR1 (n = 2)	0	50	50	0
KIT (n = 2)	0	100	0	0
PTEN (n = 1)	0	100	0	0
SMO (n = 1)	0	100	0	0
HNF1A (n = 1)	0	100	0	0
RET (n = 7)	14	14	14	58
EGFR (n = 7)	14	29	14	43
APC (n = 7)	14	29	14	43
ERBB2 (n = 11)	18	18	27	36
ABL1 (n = 5)	20	0	40	40
PDGFRA (n = 4)	25	0	25	50
IDH (n = 5)	40	0	0	60
GNAS (n = 2)	50	50	0	0
SMAD4 (n = 1)	100	0	0	0

Abbreviations: AC = atypical carcinoid; LCNEC = large-cell neuroendocrine carcinoma; SCLC = small-cell lung cancer; TC = typical carcinoid. JAK3, NRAS, RB1 as well as VHL variants were exclusively found in SCLCs, whereas the FGFR2 mutation was only detected in LCNEC. The KIT, PTEN and SMO alterations were determined in ACs and the SMAD4 variant in TC subtype only.

also showed an alteration in the tumour suppressor *TP53*, and this was by far the most frequently altered gene (31 out of 130; 24%) followed by *ERBB2* (11 out of 130, 9%), *PIK3CA* with 8 (6%) and *EGFR* present with 7 mutations (5%).

We found significant correlations between tumour type and IASLC grade for *ATM* ($P = 0.022$; $P = 0.008$) and *TP53* mutated patients ($P > 0.001$; $P < 0.001$). In addition, *TP53* showed significant more multiple mutations ($P < 0.001$). Both genes were also associated with lymph node invasion and distant metastasis when they were mutated (both $P \leq 0.005$).

Moreover, the frequency of variants in *ATM* and *APC* in high-grade neuroendocrine lung cancer patients was associated with PFS ($P = 0.020$; Figure 3A). Finally, *PIK3CA* mutated patients with high-grade tumours showed a reduced OS compared with their wild-type counterparts ($P = 0.040$; Figure 3B).

DISCUSSION

In contrast to NETs of other organ systems (e.g., gastrointestinal), pulmonary NETs form two prognostically different groups: the

low-grade carcinoid tumours (TC and AC) and the high-grade carcinomas (LCNEC and SCLC) (Rindi *et al*, 2014). The detailed molecular characteristic of these subgroups still remains unknown.

Here, for the first time, we describe a targeted high-throughput parallel sequencing approach used to analyse 70 neuroendocrine lung tumours for alterations in 221 hot spots of 48 genes suspected to play an important role in the tumourigenesis. Entity-specific mutations identified by this method can potentially serve as markers for classification of neuroendocrine lung tumours in diagnostics or therapy decision.

Our data confirm previous findings (Lohmann *et al*, 1993; Przygodzki *et al*, 1996; Couce *et al*, 1999; Leotlela *et al*, 2003; Swarts *et al*, 2012), showing that mutations in the *TP53* gene increase with malignancy of the tumour subtype. The *TP53* alterations detected in this study ranged from none in carcinoid tumours to 11 in the 17 analysed SCLC samples. Interestingly, the same trend was observed for *ATM* mutations. Furthermore, four out of five *ATM* mutated patients showed co-alterations in *TP53* (Figure 1). *ATM* is a serine/threonine kinase that coordinates an integrated cellular response to DNA damage by double-stranded DNA breaks and acts upstream of *TP53* where it leads to the activation of an apoptotic pathway (Pettitt *et al*, 2001). Furthermore, it activates several *TP53*-independent cellular pathways and it is conceivable that mutations in both genes could increase the survival advantage of a malignant tumour cell clone. Subsequently, additional mutations in *APC*, which is a downstream target of *TP53*, could possibly enhance the survival benefit for certain tumour subclones. Actually, two out of seven *APC* mutations detected in our study were observed coincidentally with an *ATM* or with *ATM* and *TP53* mutations, pointing out a second or third hit mechanism during tumourigenesis.

Interestingly, the group of R Thomas (Fernandez-Cuesta *et al*, 2014) identified mutations in genes related to chromatin remodelling (including *MEN1*, *ARID1A* and *EIF1AX*) as frequent events in pulmonary carcinoids. In addition, mutations in the histone methyltransferases (including *SETD1B*, *SETDB1* and *NSD1*) as well as demethylases (*KDM4A*, *PHF8*, *JMJD1C* and others) were determined (Fernandez-Cuesta *et al*, 2014). Likewise, his working group proofed the appearance of CREBBP and EP300 histone acetyltransferases as well as the histone-modifying enzyme MLL in high-grade lung cancers in another study (Peifer *et al*, 2012), indicating that histone modifications play a crucial role in neuroendocrine lung cancer tumourigenesis and progression.

Activating mutations in the *EGFR* gene have been widely studied in NSCLC (Engle and Kolesar, 2014; Karachaliou and Rosell, 2014; Revannasiddaiah *et al*, 2014). Unfortunately, although new therapeutic strategies are urgently needed for metastatic pulmonary carcinoid tumours, this subgroup of lung neoplasia is totally neglected, so far. Instead of common activating *EGFR* mutations, we found alterations located in the TOPO domain in all four tumour subtypes. In particular, the p.A859V in exon 21 may activate the EGF receptor similarly to the well-known therapeutically relevant activating p.L858R mutation (Peng *et al*, 2014). Of note, in one patient we observed that *EGFR* mutations were located in different regions of the gene. Importantly, these complex events are also under suspicion to predict a response to tyrosine kinase inhibitor (TKI) therapy (Peng *et al*, 2014).

Similar to *EGFR*, both other investigated members of the ERBB superfamily (*ERBB2* and *ERBB4*) also showed mutations in the extracellular TOPO domain (11 samples; 2 TCs and ACs, 3 LCNECs and 4 SCLCs). In addition, the growth factor receptor *PDGFRA* is impaired in four samples (one TC, one LCNEC and two SCLCs). Furthermore, *KDR* (VEGFR2) is mutated in one AC and two LCNECs. Of note, *KDR* p.Q427H variant in exon 11, located in the protein kinase domain, occurred in approximately one-third of all carcinoids and the half of all investigated carcinomas. Although all three applied algorithms predicted a benign or tolerated alteration of

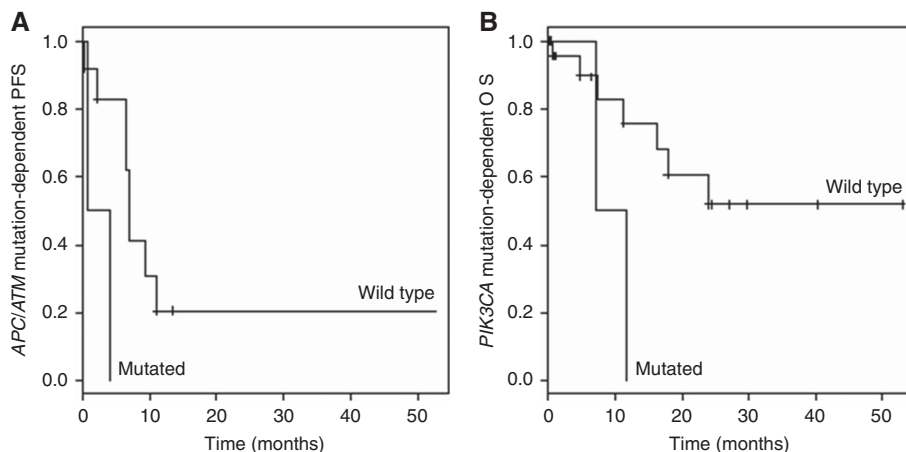


Figure 3. Kaplan–Meier curves for progression-free survival (PFS) (A) and overall survival (OS) (B) differentiated by the patients' mutational status are shown. Because of the excellent survival rate of pulmonary carcinoids, only high-grade cases are depicted. Of note, samples had mutations in both *ATM* and *APC*, showing an association to PFS ($P=0.020$). The relation of this double mutation has to be clarified. The *PIK3CA* mutated patients with high-grade tumours showed a reduced OS compared with their wild-type counterparts ($P=0.040$).

the protein, the *KDR* p.Q427H mutation is reported to be associated with an increased risk of coronary heart disease and has been detected in patients with NSCLC (Wang *et al*, 2007; Glubb *et al*, 2011). Glubb *et al* (2011) demonstrated that this alteration can modestly increase VEGFR-2 signaling and may be also associated with increased lung tumour vascularisation. The high proportion of neuroendocrine lung tumours showing this alteration underlines the hypothesis that it might be a potential marker for future risk assessment in a diagnostic setting.

Interestingly, downstream of these receptor tyrosine kinases, well-known activating mutations were observed in both *NRAS* and *KRAS*. Pathway activation downstream of EGFR, for example, by *KRAS* mutations, is known to be a resistance mechanism for EGFR-TKI therapeutic approaches (Engelman *et al*, 2006). Activation downstream of *BRAF*, primary by *NRAS* mutations, is reported to act as a potential resistance mechanism against vemurafenib therapy (Sos *et al*, 2014). Even continued activation of PI3K signalling by a *PIK3CA* oncogenic mutant was shown to be sufficient to abrogate gefitinib-induced apoptosis (Engelman *et al*, 2006). Additional studies confirmed mutations in the PI3K pathway to be involved in different *EGFR* resistance mechanisms (Sequist *et al*, 2011). Such mutations were detected in our cohort of NETs including variants affecting the C2-PI3K domain as well as the PI3K-ABD domain and the PI3K/PI4K domain of the *PIK3CA* gene locus. Our findings bear analogy to the previously published results of Capodanno *et al* (2012) who also found *PIK3CA* mutations in neuroendocrine lung cancers correlated to an increase of entities' malignancy. In contrast to our present study, the frequency of mutations detected by Capodanno *et al* (2012) is as twice as high, mainly consisting of C:G>T:A transversions (>81%). Considering the use of FFPE material, this high mutation frequency has to be assessed critically. To overcome this problem, we assessed a very stringent filtering mechanism for data analysis to avoid fixation artefacts.

The *RET* proto-oncogene, a member of the cadherin superfamily, encodes a receptor tyrosine kinase. Mutations in this gene are associated with the disorders multiple endocrine neoplasia (MEN) type IIA and IIB and medullary thyroid carcinoma (Krampitz and Norton, 2014). In line with previous findings in other endocrine tumour entities (Rotondi *et al*, 2009; Schulte *et al*, 2010; Colombo *et al*, 2015; Yeganeh *et al*, 2015), we determined *RET* variants in ~9% (6 out of 70) of pulmonary NET patients. By the way, the activating p.M918T *RET* mutation was previously assessed in one metastatic SCLC specimen (Dabir *et al*, 2014). In

contrast, Futami *et al* (2003) could not proof mutations but LOH of *RET* in the majority of SCLCs. Nevertheless, the analysed collective was very small (8 informative samples) (Futami *et al*, 2003). In carcinoid tumours, high gene expression of *RET* was associated with low long time survival but was not proven on protein level and also mutations in the *RET* oncogene could not be identified in any samples of the study (Swarts *et al*, 2013). In our collective we found one *RET* mutation in each TC and AC.

Summing up, the implementation of targeted high-throughput sequencing for the analysis of the neuroendocrine lung tumours has revealed that these tumours, even if they vary in their clinical aggressiveness, share a number of molecular features. The impact of these alterations for tumourigenesis and progression of the tumour has to be addressed in further studies, may be smoothing the way for enhanced therapeutic option in the near future.

AUTHOR CONTRIBUTIONS

All authors have read and approved the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

- Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR (2010) A method and server for predicting damaging missense mutations. *Nat Methods* 7(4): 248–249.
- Asamura H, Kameya T, Matsuno Y, Noguchi M, Tada H, Ishikawa Y, Yokose T, Jiang SX, Inoue T, Nakagawa K, Tajima K, Nagai K (2006) Neuroendocrine neoplasms of the lung: a prognostic spectrum. *J Clin Oncol* 24(1): 70–76.
- Beasley MB, Thunnissen FB, Brambilla E, Hasleton P, Steele R, Hammar SP, Colby TV, Sheppard M, Shimosato Y, Koss MN, Falk R, Travis WD (2000) Pulmonary atypical carcinoid: predictors of survival in 106 cases. *Hum Pathol* 31(10): 1255–1265.
- Brambilla C, Fievet F, Jeanmart M, de Fraipont F, Lantuejoul S, Frappat V, Ferretti G, Brichon PY, Moro-Sibilot D (2003) Early detection of lung cancer: role of biomarkers. *Eur Respir J Suppl* 39: 36s–44s.
- Capodanno A, Boldrini L, Ali G, Pelliccioni S, Mussi A, Fontanini G (2012) Phosphatidylinositol-3-kinase alpha catalytic subunit gene somatic

- mutations in bronchopulmonary neuroendocrine tumours. *Oncol Rep* **28**(5): 1559–1566.
- Cardillo G, Sera F, Di Martino M, Graziano P, Giunti R, Carbone L, Facciolo F, Martelli M (2004) Bronchial carcinoid tumors: nodal status and long-term survival after resection. *Ann Thorac Surg* **77**(5): 1781–1785.
- Colombo C, Minna E, Rizzetti MG, Romeo P, Lecis D, Persani L, Mondellini P, Pierotti MA, Greco A, Fugazzola L, Borrello MG (2015) The modifier role of RET-G691S polymorphism in hereditary medullary thyroid carcinoma: functional characterization and expression/penetrance studies. *Orphanet J Rare Dis* **10**: 25.
- Couce ME, Bautista D, Costa J, Carter D (1999) Analysis of K-ras, N-ras, H-ras, and p53 in lung neuroendocrine neoplasms. *Diagn Mol Pathol* **8**(2): 71–79.
- Dabir S, Babakoohi S, Kluge A, Morrow JJ, Kresak A, Yang M, MacPherson D, Wildey G, Dowlati A (2014) RET mutation and expression in small-cell lung cancer. *J Thorac Oncol* **9**(9): 1316–1323.
- Engelman JA, Mukohara T, Zejnullahu K, Lifshits E, Borrás AM, Gale CM, Naumov GN, Yeap BY, Jarrell E, Sun J, Tracy S, Zhao X, Heymach JV, Johnson BE, Cantley LC, Janne PA (2006) Allelic dilution obscures detection of a biologically significant resistance mutation in EGFR-amplified lung cancer. *J Clin Invest* **116**(10): 2695–2706.
- Engle JA, Kolesar JM (2014) Afatinib: a first-line treatment for selected patients with metastatic non-small-cell lung cancer. *Am J Health Syst Pharm* **71**(22): 1933–1938.
- Esteller M, Sanchez-Céspedes M, Rosell R, Sidransky D, Baylin SB, Herman JG (1999) Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer Res* **59**(1): 67–70.
- Fernandez-Cuesta L, Peifer M, Lu X, Sun R, Ozretic L, Seidel D, Zander T, Leenders F, George J, Muller C, Dahmen I, Pinther B, Bosco G, Konrad K, Altmüller J, Nurnberg P, Achter V, Lang U, Schneider PM, Bogus M, Soltermann A, Brustugun OT, Helland A, Solberg S, Lund-Iversen M, Ansen S, Stoelben E, Wright GM, Russell P, Wainer Z, Solomon B, Field JK, Hyde R, Davies MP, Heukamp LC, Petersen I, Perner S, Lovly CM, Cappuzzo F, Travis WD, Wolf J, Vingron M, Brambilla E, Haas SA, Buettner R, Thomas RK (2014) Frequent mutations in chromatin-remodelling genes in pulmonary carcinoids. *Nat Commun* **5**: 3518.
- Fink G, Krelbaum T, Yellin A, Bendayan D, Saute M, Glazer M, Kramer MR (2001) Pulmonary carcinoid: presentation, diagnosis, and outcome in 142 cases in Israel and review of 640 cases from the literature. *Chest* **119**(6): 1647–1651.
- Putami H, Egawa S, Takasaki K, Tsukada T, Shiraishi M, Yamaguchi K (2003) Allelic loss of DNA locus of the RET proto-oncogene in small cell lung cancer. *Cancer Lett* **195**(1): 59–65.
- García-Yuste M, Matilla JM, Cueto A, Paniagua JM, Ramos G, Canizares MA, Muguza I (2007) Typical and atypical carcinoid tumours: analysis of the experience of the Spanish Multi-centric Study of Neuroendocrine Tumours of the Lung. *Eur J Cardiothorac Surg* **31**(2): 192–197.
- Glubb DM, Cerri E, Giese A, Zhang W, Mirza O, Thompson EE, Chen P, Das S, Jassem J, Rzyman W, Lingen MW, Salgia R, Hirsch FR, Dziadziuszko R, Ballmer-Hofer K, Innocenti F (2011) Novel functional germline variants in the VEGF receptor 2 gene and their effect on gene expression and microvessel density in lung cancer. *Clin Cancer Res* **17**(16): 5257–5267.
- Karachaliou N, Rosell R (2014) Systemic treatment in EGFR-ALK NSCLC patients: second line therapy and beyond. *Cancer Biol Med* **11**(3): 173–181.
- Krampitz GW, Norton JA (2014) RET gene mutations (genotype and phenotype) of multiple endocrine neoplasia type 2 and familial medullary thyroid carcinoma. *Cancer* **120**(13): 1920–1931.
- Kumar P, Henikoff S, Ng PC (2009) Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc* **4**(7): 1073–1081.
- Leotlela PD, Jauch A, Holtgreve-Grez H, Thakker RV (2003) Genetics of neuroendocrine and carcinoid tumours. *Endocr Relat Cancer* **10**(4): 437–450.
- Lohmann DR, Fessler B, Putz B, Reich U, Bohm J, Prauer H, Wunsch PH, Hofler H (1993) Infrequent mutations of the p53 gene in pulmonary carcinoid tumors. *Cancer Res* **53**(23): 5797–5801.
- McLaren W, Pritchard B, Rios D, Chen Y, Flicek P, Cunningham F (2010) Deriving the consequences of genomic variants with the Ensembl API and SNP Effect Predictor. *Bioinformatics* **26**(16): 2069–2070.
- Peifer M, Fernandez-Cuesta L, Sos ML, George J, Seidel D, Kasper LH, Plenker D, Leenders F, Sun R, Zander T, Menon R, Koker M, Dahmen I, Muller C, Di Cerbo V, Schildhaus HU, Altmüller J, Baessmann I, Becker C, de Wilde B, Vandesompele J, Bohm D, Ansen S, Gabler F, Wilkening I, Heynck S, Heuckmann JM, Lu X, Carter SL, Cibulskis K, Banerji S, Getz G, Park KS, Rauh D, Grutter C, Fischer M, Pasqualucci L, Wright G, Wainer Z, Russell P, Petersen I, Chen Y, Stoelben E, Ludwig C, Schnabel P, Hoffmann H, Muley T, Brockmann M, Engel-Riedel W, Muscarella LA, Fazio VM, Groen H, Timens W, Sietsma H, Thunnissen E, Smit E, Heideman DA, Snijders PJ, Cappuzzo F, Ligorio C, Damiani S, Field J, Solberg S, Brustugun OT, Lund-Iversen M, Sanger J, Clement JH, Soltermann A, Moch H, Weder W, Solomon B, Soria JC, Validire P, Besse B, Brambilla E, Brambilla C, Lantuejoul S, Lorimier P, Schneider PM, Hallek M, Pao W, Meyerson M, Sage J, Shendure J, Schneider R, Buttner R, Wolf J, Nurnberg P, Perner S, Heukamp LC, Brindle PK, Haas S, Thomas RK (2012) Integrative genome analyses identify key somatic driver mutations of small-cell lung cancer. *Nat Genet* **44**(10): 1104–1110.
- Pelosi G, Scarpa A, Puppa G, Veronesi G, Spaggiari L, Pasini F, Maisonneuve P, Iannucci A, Arrighi G, Viale G (2005) Alteration of the E-cadherin/beta-catenin cell adhesion system is common in pulmonary neuroendocrine tumors and is an independent predictor of lymph node metastasis in atypical carcinoids. *Cancer* **103**(6): 1154–1164.
- Peng L, Song ZG, Jiao SC (2014) Efficacy analysis of tyrosine kinase inhibitors on rare non-small cell lung cancer patients harboring complex EGFR mutations. *Sci Rep* **4**: 6104.
- Pettitt AR, Sherrington PD, Stewart G, Cawley JC, Taylor AM, Stankovic T (2001) p53 dysfunction in B-cell chronic lymphocytic leukemia: inactivation of ATM as an alternative to TP53 mutation. *Blood* **98**(3): 814–822.
- Przygodzki RM, Finkelstein SD, Langer JC, Swalsky PA, Fishback N, Bakker A, Guinee DG, Koss M, Travis WD (1996) Analysis of p53, K-ras-2, and C-raf-1 in pulmonary neuroendocrine tumors. Correlation with histological subtype and clinical outcome. *Am J Pathol* **148**(5): 1531–1541.
- Rea F, Rizzardi G, Zuin A, Marulli G, Nicotra S, Bulf R, Schiavon M, Sartori F (2007) Outcome and surgical strategy in bronchial carcinoid tumours: single institution experience with 252 patients. *Eur J Cardiothorac Surg* **31**(2): 186–191.
- Rekhtman N (2010) Neuroendocrine tumors of the lung: an update. *Arch Pathol Lab Med* **134**(11): 1628–1638.
- Reva B, Antipin Y, Sander C (2011) Predicting the functional impact of protein mutations: application to cancer genomics. *Nucleic Acids Res* **39**(17): e118.
- Revannasiddaiah S, Thakur P, Bhardwaj B, Susheela SP, Madabhavi I (2014) Pulmonary adenocarcinoma: implications of the recent advances in molecular biology, treatment and the IASLC/ATS/ERS classification. *J Thorac Dis* **6**(Suppl 5): S502–S525.
- Rindi G, Petrone G, Inzani F (2014) The 2010 WHO classification of digestive neuroendocrine neoplasms: a critical appraisal four years after its introduction. *Endocr Pathol* **25**(2): 186–192.
- Rotondi M, Ercolino T, Fonte R, Lagonigro MS, Leporati P, Villani L, La Manna L, Mannelli M, Chiovato L (2009) Occurrence of medullary thyroid carcinoma, bronchial carcinoid tumor, and papillary thyroid carcinoma in a family bearing the RET G691S polymorphism. *J Endocrinol Invest* **32**(2): 115–118.
- Schulte KM, Machens A, Fugazzola L, McGregor A, Diaz-Cano S, Izatt L, Aylwin S, Talat N, Beck-Peccoz P, Dralle H (2010) The clinical spectrum of multiple endocrine neoplasia type 2a caused by the rare intracellular RET mutation S891A. *J Clin Endocrinol Metab* **95**(9): E92–E97.
- Scott WJ (2003) Surgical treatment of other bronchial tumors. *Chest Surg Clin N Am* **13**(1): 111–128.
- Sequist LV, Waltman BA, Dias-Santagata D, Digumarthy S, Turke AB, Fidias P, Bergethon K, Shaw AT, Gettinger S, Cospoer AK, Akhavanfard S, Heist RS, Temel J, Christensen JG, Wain JC, Lynch TJ, Vernovsky K, Mark EJ, Lanuti M, Iafrate AJ, Mino-Kenudson M, Engelman JA (2011) Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci Transl Med* **3**(75): 75ra26.
- Sos ML, Levin RS, Gordan JD, Oses-Prieto JA, Webber JT, Salt M, Hann B, Burlingame AL, McCormick F, Bandyopadhyay S, Shokat KM (2014) Oncogene mimicry as a mechanism of primary resistance to BRAF inhibitors. *Cell Rep* **8**(4): 1037–1048.
- Swarts DR, Henfling ME, Van Neste L, van Suylen RJ, Dingemans AM, Dinjens WN, Haesevoets A, Rudelius M, Thunnissen E, Volante M, Van Criekinge W, van Engeland M, Ramaekers FC, Speel EJ (2013) CD44 and OTP are strong prognostic markers for pulmonary carcinoids. *Clin Cancer Res* **19**(8): 2197–2207.

- Swarts DR, Ramaekers FC, Speel EJ (2012) Molecular and cellular biology of neuroendocrine lung tumors: evidence for separate biological entities. *Biochim Biophys Acta* **1826**(2): 255–271.
- Takei H, Asamura H, Maeshima A, Suzuki K, Kondo H, Niki T, Yamada T, Tsuchiya R, Matsuno Y (2002) Large cell neuroendocrine carcinoma of the lung: a clinicopathologic study of eighty-seven cases. *J Thorac Cardiovasc Surg* **124**(2): 285–292.
- Thomas Jr CF, Tazelaar HD, Jett JR (2001) Typical and atypical pulmonary carcinoids: outcome in patients presenting with regional lymph node involvement. *Chest* **119**(4): 1143–1150.
- Travis WD (2009) Lung tumours with neuroendocrine differentiation. *Eur J Cancer* **45**(Suppl 1): 251–266.
- Travis WD, Rush W, Flieder DB, Falk R, Fleming MV, Gal AA, Koss MN (1998) Survival analysis of 200 pulmonary neuroendocrine tumors with clarification of criteria for atypical carcinoid and its separation from typical carcinoid. *Am J Surg Pathol* **22**(8): 934–944.
- Travis WD. World Health Organization, International Agency for Research on Cancer, International Association for the Study of Lung Cancer, International Academy of Pathology (2004) *Pathology and Genetics of Tumours of the Lung, Pleura, Thymus and Heart*. IARC Press Oxford University Press (distributor): Lyon, Oxford.
- Wang Y, Zheng Y, Zhang W, Yu H, Lou K, Zhang Y, Qin Q, Zhao B, Yang Y, Hui R (2007) Polymorphisms of KDR gene are associated with coronary heart disease. *J Am Coll Cardiol* **50**(8): 760–767.
- Yeganeh MZ, Sheikholeslami S, Hedayati M (2015) RET proto oncogene mutation detection and medullary thyroid carcinoma prevention. *Asian Pac J Cancer Prev* **16**(6): 2107–2117.
- Zochbauer-Muller S, Fong KM, Virmani AK, Geradts J, Gazdar AF, Minna JD (2001) Aberrant promoter methylation of multiple genes in non-small cell lung cancers. *Cancer Res* **61**(1): 249–255.

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