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Research

Presence of PCR Artifacts in Sanger Sequencing in Formalin-fixed Paraffin-embedded (FFPE) Tissue – Experience in a Collective of 990 Advanced NSCLC

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Abstract

Background: Despite the rapid development of new molecular techniques such as Next Generation Sequencing (NGS), Sanger sequencing has been thus far the gold standard for mutation analysis. It is constantly used for daily routine diagnostics because it represents a quick and comprehensive available method for mutation analyses. Although Sanger sequencing is a good validated method, PCR artifacts may occur in formalin fixed paraffin embedded (FFPE) material. This constitutes a serious source of error.

Aims: To assess the prevalence of typical and atypical EGFR mutations in exon 19 and 21 in a collective of 990 advanced non-small cell lung cancer (NSCLC) patients, focusing especially on methodological issues and challenges concerning mutation analysis, particularly PCR artifacts.

Material and Methods: We examined 990 NSCLC (FFPE material) by Sanger sequencing for exon 19 and 21 of the EGFR gene. Four cases dropped out because of insufficient DNA quality (n =986).

Results: Beside 101 typical exon 19 and 21 mutations (99 cases, two double mutations) we found 45 additional cases with distinct peaks at atypical positions in exon 19 and 21 in our first

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analysis. This would have implied a mutation rate of 14.6 %. Only six of these putative atypical mutations (all exon 21 and none of the exon 19 mutations) could be validated by repeated mutation analysis. All other peaks were not reproducible, therefore considered as PCR artifacts and consequently as wild type. Altogether we found 105 cases (107 mutations, 10.6 % of cases) with typical/atypical mutations in exon 19 and 21 of the EGFR gene.

Conclusion: In our opinion it is in general important to detect and report all mutations even at atypical sites to discover their possible clinical relevance. However, one must always be aware of the possibility, reasons and prevention of PCR artifacts in FFPE tissue. Therefore, prior to reporting mutations at uncommon sites these must be validated by repeated analyses.

Keywords: Lung cancer, molecular analysis, Sanger sequencing, fixation artifacts, PCR artifacts, EGFR

Introduction

Lung cancer is the most frequent cancer associated cause of death worldwide [1-2]. It has an increasing incidence with approximately 50.000 new cases per year in Germany [3] and comprises a heterogeneous group of mainly carcinomas. These can be divided historically into small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). Both groups have different histomorphology and response to conventional chemotherapy. Therefore, different therapeutic concepts have been applied [4]. The NSCLC group includes adenocarcinoma (LAC), which comprises the largest group (approximately 40 % of all tumors), followed by squamous cell carcinoma (SCC) and large cell neuroendocrine carcinoma (LCNEC).

In the past decades NSCLC in advanced stages were treated like a homogenous group with conventional systemic chemotherapy regimens [5]. The discovery of tumor specific epidermal growth factor receptor (EGFR, ERBB1) mutations in certain subgroups of NSCLC and the subsequent introduction of EGFR-specific tyrosine kinase inhibitors (TKI) in 2003 provided for the first time the opportunity for a personalized targeted therapy approach in advanced NSCLC [6-10]. EGFR mutations are particularly prevalent in LAC, women, never smokers, and patients of younger ages, as well as in the Asian population [11-13]. Hence, accurate detection of EGFR mutations in advanced NSCLC, especially in LAC, is important for the therapeutic concept and - not least - for the clinical outcome.

To date, more than 250 EGFR mutations are known. Most of the mutations (80-90 %) are TKI-sensitive Exon 19 in-frame deletions (45 %, e.g. delE746_A750, del747_T751insS and delL747_P753insS) and a TKI-sensitive point mutation in exon 21 (41 %, L858R) [14].

Besides these so-called classic mutations, there are some less frequently observed mutations in exon 19, 20 and 21. These are for example the TKI-sensitive point mutations L861Q/R in

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exon 21, the TKI-resistance mutation T790M in Exon 20 [15] or the D761Y mutation in exon 19 [6, 8, 16-19] <Figure 1>.

In this study we assessed the prevalence of typical and atypical EGFR mutations in exons 19 and 21 in a collective of 990 advanced NSCLC. Hereby, we focused on methodological issues and challenges concerning mutation analysis, particularly fixative induced PCR artifacts.

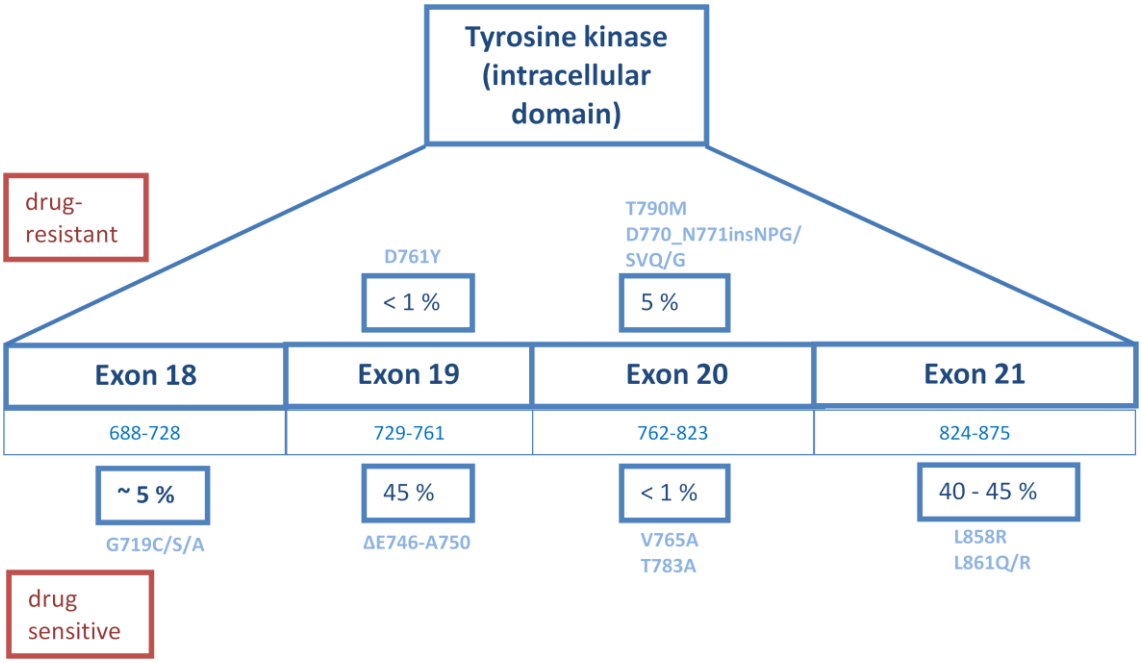

Figure 1: EGFR mutations: the (intracellular) tyrosine kinase domain consists of seven exons (18-24). Four of them (exon 18-21) span codon 688 to 875 and harbor the relevant EGFR mutations. The most common mutations (80-90 %, so-called classic activating mutations) are TKI-sensitive Exon 19 in-frame deletions (45 %) and a TKI-sensitive point mutation in exon 21 (L858R, 41 %) (modified by Sharma et al. 2007)

Material and methods

Cohort

All Sanger sequences for exons 19 and 21 of the EGFR gene (n = 1047) (FFPE tissue) performed at the Department of Surgical Pathology, University Hospital Freiburg in 2008-2012 were re-evaluated. The cohort consisted of 990 locally advanced and/or metastasized NSCLC as well as 21 adenocarcinomas from other sites and three SCLC. 986 NSCLC specimen could be evaluated, four dropped out because of insufficient DNA quality and 33 dropped out because of routinely performed repeated analysis. The evaluable NSCLC cohort (n = 986) consisted of 561 men and

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425 women with an age range from 30 to 90 years. The mean age at diagnosis of NSCLC was 64.4 years (median 65 years).

The re-evaluation was performed virtually, using blast tools and alignment programs by two experienced pathologists (CKA, GK). In discrepant cases (no consensus between the two pathologists) and cases with distinct sequence peaks outside the known hotspots mutation analysis was repeated by Sanger sequencing.

DNA extraction

Tumor tissue was delineated in H&E-slides and the amount of tumor cells was estimated by a pathologist. From each marked tumor tissue block, three to five sections (5 µm thick) were cut and microdissected for DNA extraction and collected in Eppendorf tubes. Dewaxing was performed with 1 ml Roticlear® at 60 ºC (ten minutes, two times, in between centrifugation at 10500 rotations per minute (rpm)) following ethanol (100 %) incubation (one minute, two times) and centrifugation at 10500 rpm. After removing the ethanol from the Eppendorf tubes 15 to 30 minutes of vaporization at 45 ºC lead to a completely dry pellet. According to the amount of tissue the pellet was soaked in 50-200 µl Proteinase-K-Buffer for ten minutes at 70 ºC, cooled down to room temperature and subsequently digested over night at 37 ºC by adding 5-10 µl Proteinase K (20 mg/ml). After digestion the Eppendorf tube was incubated at 100 ºC for 20 minutes, cooled down on ice and centrifuged at 10500 rpm minute for ten minutes. The DNA solution was then pipetted into a new DNase/ RNase free Eppendorf tube. DNA amount and quality was estimated by NanoDrop.

Polymerase chain reaction (PCR)

We used a nested PCR for exon 19 and 21 of the EGFR gene. EGFR-1- Mix <Table 1> and 1-10 ng/µl DNA as well as EGFR-2- Mix <Table 1> and 2 µl PCR 1 product were used for the PCR 1 and PCR 2 reaction, respectively. The primers used are shown in <Table 2>. The PCR 1 and 2 profiles were as follows: 3´95 ºC [20´´95 ºC, 20´´ 60 ºC, 40´´ 72 ºC] 40x and 3´95 ºC [20´´95 ºC, 20´´ 60 ºC, 40´´ 72 ºC] 30x, respectively. The PCR products were purified by Qiaquick purifying Kit (28106).

EGFR-Mix PCR 1 and PCR 2
1 x PCR-Buffer
1,5 mM MgCL2
200 mM/dNTP
0,4 µM Primer F
0,4 µM Primer R
1,0 U Taq-polymerase (Genaxxon)

Table 1: EGFR-Mix for PCR 1 and PCR2.

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Primer	
PCR 1	
Exon 19	Exon 21
GCAATATCAGCCTTAGGTGCGGCTC (F)	CTAACGTTCGCCAGCCATAAGTCC (F)
CATAGAAAGTGAACATTTAGGATGTG (R)	GCTGCGAGCTCACCCAGAATGTCTGG (R)
PCR 2	
Exon 19	Exon 21
GTAAAACGACGGCCAGTCCTTAGGTGCGGCTCCACAGC (F)	GTAAAACGACGGCCAGTCAGCCATAAGTCCTCGACGTGG (F)
TAATACGACTCACTATAGGGCATTTAGGATGTGGAGATGAGC (R)	TAATACGACTCACTATAGGGCATCCTCCCCTGCATGTGTTAAAC (R)
Sequencing PCR	
Exon 19	Exon 21
GTAAAACGACGGCCAGT (F)	GTAAAACGACGGCCAGT (F)
TAATACGACTCACTATAGGG (R)	TAATACGACTCACTATAGGG (R)

F = forward; R = reverse

Table 2: EGFR Exon 19 and 21 primer.

Sequencing PCR

25 ng purified PCR 2 product, 1.0 µl BigDye Terminator V1.1, 1.0 µl SeqSaver, 4.0 µl 1 µM from each primer (and water to reach 10 µl reaction volume) were used for the sequencing PCR. The primers used for the sequencing PCR are shown in <Table 2>. The PCR profile was as follows: 1´92 ºC, [10´´92 ºC, 5´´ 50 ºC, 4´ 60 ºC] 20x, 30´´ 25 ºC. The sequencing product was purified with DyeEx columns (Qiagen). Analysis was performed with the ABI-3130XL capillary sequencer.

Results

From 990 NSCLC samples 986 could be evaluated. Four cases dropped out because of insufficient DNA quality. The distribution and the characterization of the mutations are shown in <Table 3>.

Out of the NSCLC 986 samples 105 had all in all 107 typical/atypical exon 19 and/or 21 EGFR mutations (10.6%), as demonstrated in <Table 3>. Two samples had reproducible double mutations (case 6 and 15, labelled with * in <Table 3>). One had a typical and a non-typical exon 21 mutation and another case had a typical exon 21 and a typical exon 19 mutation. Three samples showed hemizygous mutations (case 40, 68 and 98; labelled with + in <Table 3>); one typical Exon 21, two typical exon 19 mutations. We found 19 complex exon 19 mutations (delins). One of these mutations was an insertion (case 93; c.2217_2234dupl;

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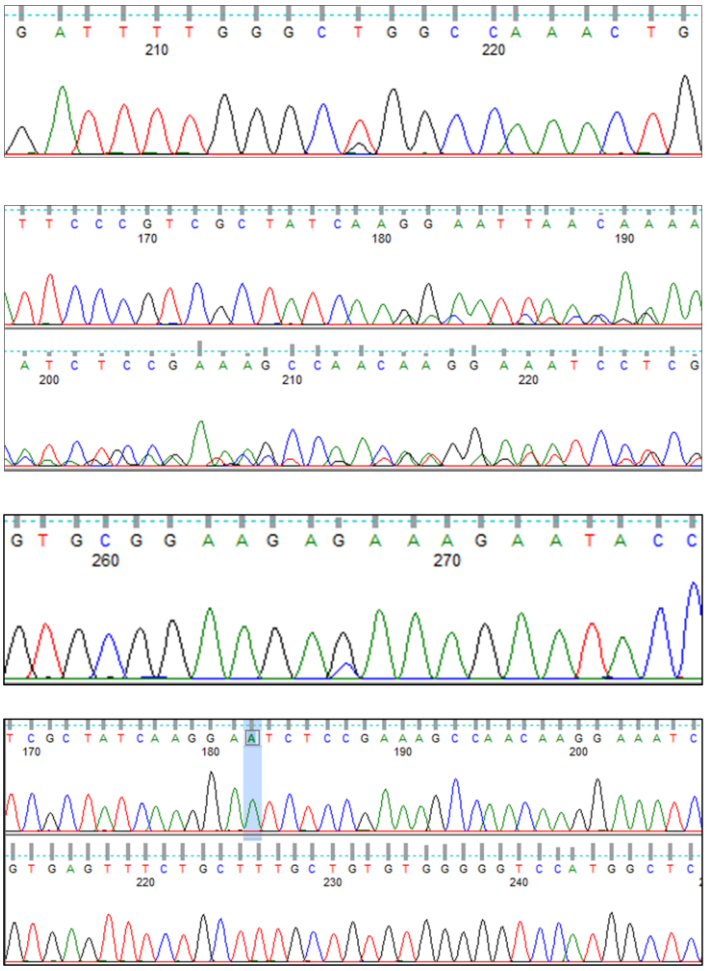
p.K745_E746insIPVAIK) previously described by our research group [20]. Some examples for detected EGFR exon 19 and 21 mutations are shown in <Figure 2> (A-D).



A:
Exon 21
c.2573G>T, pL858R

B:
Exon 19
c.2235_2249del15;
p.E746_A750del

C:
Exon 21
c.2598G>T;
p.E866D

D:
Exon 19
[c.2239_2253del15][c.2239_2253del15];
[p.L747_T751del] [p.L747_T751del]

Figure 2: Typical and atypical EGFR mutations: A) typical Exon 21 point mutation, B) typical Exon 19 deletion, C) atypical Exon 21 point mutation, D) hemizygous appearing typical Exon 19 mutation.

Besides the typical mutations (66 exon 19 mutations and 35 exon 21 mutations) in our first analysis we found that 45 additional samples had distinct or debatable peaks at non-hotspot positions in exon 19 and 21. Some cases showed more than one, mainly distinct peaks (up to four). However, only six of these 45 putative atypical mutations - all in exon 21 and none in exon 19 - could be validated by repeated Sanger sequencing mutation analysis <Table 3>. Two of these mutations (L861Q/R) have now been detected more frequently in large collectives of NSCLCs. Two other mutations have not yet been reported in the current literature (G874A and E829D) and one (E866D) has been described in one collective in a SCLC patient [21]. The V834L mutation has been observed several times in NSCLC. Out of these 45 cases five even remained

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doubtful because repeated mutational analysis provided always different results. Due to the lack of proof these five cases were included in the wild type group. The remaining 34 cases also had been considered as wild type because the peaks could not be validated by repeated sequencing.

Consequently, 39 of the 45 cases with sequence peaks at non-hotspot positions in exon 19 and 21 could not be verified. The main artificial base change found in our study was G>A (14 times) (example <Figure 3>, A-D) followed by A>G and C>T (ten times, respectively), T>G (eight times), C>G (five times) and G>T, C>A, T>C, G>C and T>A (once, respectively). Some cases had more than one artificial peak (up to four). One case showed an artificial TT>CC base change and two others an artificial GT>AG base change.

The mean age at diagnosis was 66.3 years (median 69 years) in the population with mutation. The typical mutations were less often found in men (n = 36) than in women (n = 63). Altogether 97 cases with genuine mutation were LAC, three SCC and five classified as NSCLC, NOS. None of the 21 adenocarcinoma from other sites or the SCLC showed an EGFR exon 19 or 21 mutation.

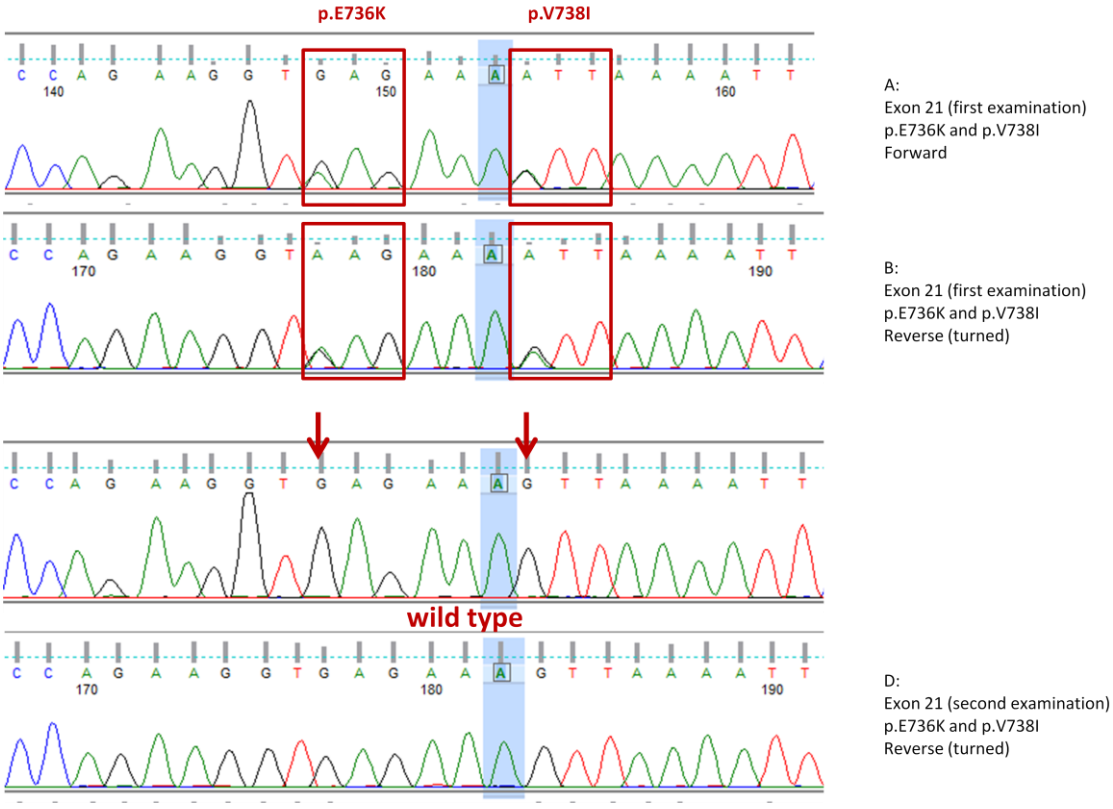

Figure 3: Example for a non reproducible Exon 19 peak (base change G>A) considered as PCR artifact.

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No.	ID	Exon 21 atypical mutation	Exon 21 typical mutation	Exon 19 typical mutation	sex	age	histology
1	18		c.2573G>T, pL858R		f	67	LAC
2	27			c.2235_2249del15; p.E746_A750del	m	68	LAC
3	30			c.2240_2257del18; p.L747_T751del	m	67	LAC
4	34			c.2235_2249del15; p.E746_A750del	f	43	LAC
5	37		c.2573G>T, pL858R		f	45	LAC
6	40		c.2573G>T, pL858R*	c.2235_2249del15; p.E746_A750del*	f	76	LAC
7	44			c.2235_2249del15; p.E746_A750del	f	73	LAC
8	50			c.2237_2253del17ins5; p.E746_T751delinsVN	m	69	LAC
9	59	c.2582T>G; p.L861R			m	61	LAC
10	72			c.2235_2249del15; p.E746_A750del	f	63	LAC
11	76			c.2235_2252del18; p.L747_S752del	f	59	LAC
12	77			c.2235_2249del15; p.E746_A750del	f	47	LAC
13	90	c.2487G>C, p.E829D			m	66	LAC
14	98			c.2239_2248del10insC; p.L747_A750delinsP	f	57	LAC
15	100	c.2500G>T; p.V834L*	c.2573T>G; p.L858R*		f	78	LAC
16	106			c.2235_2249del15; p.E746_A750del	f	55	LAC
17	111			c.2235_2249del15; p.E746_A750del	m	78	LAC
18	122		c.2573G>T, pL858R		f	70	LAC
19	131			c.2237_2255delinsT; p.E746_S752delinsV	m	69	LAC
20	134		c.2573G>T, pL858R		m	67	LAC
21	141		c.2573G>T, pL858R		m	64	LAC
22	144			c.2235_2249del15; p.E746_A750del	f	56	LAC
23	148			c.2235_2249del15; p.E746_A750del	f	45	LAC
24	156			c.2235_2249del15; p.E746_A750del	m	76	LAC
25	163			c.2235_2249del15; p.E746_A750del	f	70	LAC
26	183	c.2621G>C p.G874A			f	59	LAC
27	184		c.2573G>T, pL858R		f	76	LAC
28	185		c.2573G>T, pL858R		f	76	LAC
29	193		c.2573G>T, pL858R		f	73	LAC
30	206			c.2235_2249del15; p.E746_A750del	f	72	LAC
31	224		c.2573G>T, pL858R		f	73	LAC
32	240			c.2235_2249del15; p.E746_A750del	f	56	LAC
33	244		c.2573G>T, pL858R		f	76	LAC
34	265			c.2239_2258del18,p.L747_S752del	m	59	LAC
35	273			c.2235_2249del15; p.E746_A750del	m	64	LAC
36	282			c.2239_2253del15,p.L747_T751del	f	67	LAC

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No.	ID	Exon 21 atypical mutation	Exon 21 typical mutation	Exon 19 typical mutation	sex	age	histology
37	302		c.2573G>T, pL858R		f	83	LAC
38	311	c.2598G>T; p.E866D			m	64	LAC
39	327			c.2236_2255del20insAT; p.E746_S752delinsI	f	71	LAC
40	335		c.[2573G>T][2573G>T] , p.[L858R][L858R] +		m	52	LAC
41	338		c.2573G>T, pL858R		m	71	LAC
42	349			c.2239_2248del10insC,p .L747_A750delinsP	f	75	LAC
43	352			c.2239_2248del10insC,p .L747_A750delinsP	f	75	LAC
44	362			c.2239_2253del15; p.L747_T751del	f	83	LAC
45	369		c.2573G>T, pL858R		f	59	LAC
46	372			c.2237_2248del12insCA C, p.E746_E749delinsP	f	73	LAC
47	374			c.2236_2250del15; p.E746_A750del	f	66	LAC
48	383			c.2236_2250del15; p.E746_A750del	m	66	LAC
49	389			c.2235_2249del15; p.E746_A750del	f	60	LAC
50	404			c.2235_2249del15; p.E746_A750del	f	40	LAC
51	408		c.2573G>T, pL858R		f	67	LAC
52	458			c.2235_2249del15; p.E746_A750del	m	72	LAC
53	462			c.2235_2249del15; p.E746_A750del	m	83	LAC
54	470			c.2235_2249del15; p.E746_A750del	m	63	LAC
55	476			c.2236_2250del15; p.E746_A750del	f	58	LAC
56	480			c.2235_2249del15; p.E746_A750del	m	71	SCC
57	493		c.2573G>T, pL858R		m	74	LAC
58	501			c.2235_2249del15; p.E746_A750del	f	63	NSCLC, NOS
59	504		c.2573G>T, pL858R		m	74	LAC
60	511			c.2237_2240del4insCCC C; p.E746_L747delinsAP	m	49	LAC
61	516			c.2239_2248delinsC, p.L747_A750delinsP	f	63	LAC
62	536			c.2235_2249del15; p.E746_A750del	m	54	LAC
63	539			c.2236_2250del15; p.E746_A750del	m	76	LAC
64	541			c.2235_2249del15; p.E746_A750del	f	74	LAC
65	558		c.2573G>T, pL858R		f	69	NSCLC, NOS
66	566		c.2573G>T, pL858R		f	67	LAC
67	582			c.2237_2253del17insCT; p.E746_T751delinsA	m	80	SCC
68	590			[c.2237_2253del17insCT] [p.E746_T751delinsA] +	f	51	LAC
69	601		c.2573G>T, pL858R		f	59	LAC
70	609			c.2235_2249del15; p.E746_A750del	m	30	LAC

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No.	ID	Exon 21 atypical mutation	Exon 21 typical mutation	Exon 19 typical mutation	sex	age	histology
71	611		c.2573G>T, pL858R		f	77	LAC
72	620			c.2236_2250del15; p.E746_A750del	m	77	LAC
73	635			c.2235_2249del15; p.E746_A750del	m	70	LAC
74	683		c.2573G>T, pL858R		f	81	LAC
75	687			c.2253_2276del24;p.S752_I759delSPKANKEI	f	76	LAC
76	696			c.2240_2257del18;p.L747_S753delinsS	m	55	LAC
77	775			c. 2235_2249del15; p.E746_A750del	f	65	LAC
78	776			c. 2235_2249del15; p.E746_A750del	f	71	LAC
79	782	c.2582T>A p.L861Q			f	80	LAC
80	784			c.2237_2255del19insT; p.E746_S752delinsV	m	72	LAC
81	799		c.2573G>T, pL858R		m	60	LAC
82	826			c.2235_2249del15; p.E746_A750del	m	72	LAC
83	859			c.2235_2249del15; p.E746_A750del	m	55	LAC
84	864		c.2573G>T, pL858R		f	47	LAC
85	872		c.2573G>T, pL858R		f	70	NSCLC, NOS
86	888		c.2573G>T, pL858R		f	71	LAC
87	901		c.2573G>T, pL858R		f	68	LAC
88	930			c.2235_2249del15; p.E746_A750del	f	50	LAC
89	939			c.2235_2249del15; p.E746_A750del	f	58	NSCLC, NOS
90	947			c.2253_2276del24 p.S752_I759delSPKANKEI	f	70	LAC
91	956		c.2573G>T, pL858R		f	79	LAC
92	957			c.2236_2250del15; p.E746_A750del	m	83	LAC
93	961			c.2217_2234dupl; p.K745_E746insIPVAIK	f	79	LAC
94	962		c.2573G>T, pL858R		f	76	LAC
95	966		c.2573G>T, pL858R		f	79	NSCLC, NOS
96	969		c.2573G>T, pL858R		f	78	LAC
97	977			c.2235_2249del15; p.E746_A750del	f	54	LAC
98	989			[c.2239_2253del15][c.2239_2253del15]; [p.L747_T751del] [p.L747_T751del] +	f	62	LAC
99	990		c.2573G>T, pL858R		f	74	LAC
100	994			c.2239_2248del10insC; p.L747_A750delinsP	m	53	LAC
101	996		c.2573G>T, pL858R		m	53	LAC
102	997			c.2235_2249del15; p.E746_A750del	f	78	LAC
103	1003			c.2239_2248del10insC; p.L747_A750delinsP	m	70	SCC
104	1019			c.2235_2249del15; p.E746_A750del	f	82	LAC

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No.	ID	Exon 21 atypical mutation	Exon 21 typical mutation	Exon 19 typical mutation	sex	age	histology
105	1042			c.2237_2253delinsTTGCT; p.E746_T751delinsVA	m	41	LAC

f = female; m = male; age = age at diagnosis; LAC = lung adeno carcinoma; SCC = squamous cell carcinoma; NSCLC, NOS = non-small cell lung cancer, *not otherwise specified;* * = double mutation; + = hemizygous mutation.

Table 3: Distribution and characterization of the EGFR exon 19 and 21 mutations.

Discussion

Although new molecular technologies like next generation sequencing (NGS) are rapidly developing and entering routine diagnostics, until recently Sanger sequencing has been the gold standard for mutation analysis and it is still one of the most frequently used techniques for this purpose in daily practice [22].

Nevertheless, in FFPE material, it is known that deamination artifacts frequently occur [23-27] and that there has to be a relatively high amount of tumor cells (usually > 40 %) to achieve valid results. Low tumor cell amount and therefore lower template DNA can lead to higher rate of PCR artifacts [23-25, 27].

In our large cohort of 986 evaluable advanced NSCLC initially 144 samples showed distinctive or debatable sequence peaks by Sanger sequencing in exon 19 and/or 21 of the EGFR gene. This would then have implied a mutation frequency of 14.6 %, that is slightly higher than the current value in literature for EGFR mutations in NSCLC in Europe [13, 28-30]. By repeated Sanger sequencing of doubtful cases and those with peaks at atypical sites only 105 (10.6 %) samples harbored one or two distinct and reproducible EGFR exon 19 and/or 21 mutations <Table 3>. This is in concordance with the data in current literature concerning EGFR mutations in different European populations [13, 28-30].

We found in our collective a significant number of sequence peaks in EGFR mutation analyses that could not be validated in repeated sequencing procedures and consequently had to be considered as PCR artifacts and therefore as wild type. Five cases remained unclear, although mutation analysis was performed several times. In our opinion these peaks are likely to represent formalin deamination artifacts caused by DNA degradation, a phenomenon that is issue of ongoing discussion [23-25, 27].

The artificial peaks occurred especially in exon 19 at atypical sites and represented mainly base changes G>A followed by A>G and C>T. In literature the occurrence of G>A and C>T base changes (like in our study) seem to accumulate [23,27], but C>A and G>T base changes are also on record [24, 27]. These artificial base changes are due to deamination of adenine or cytosine. This can produce hypoxanthine or uracil residues, respectively [26]. These structural changes lead to losses of hydrogenbonds needed for accurate matching of DNA-basepairs. In

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this context, Hofreiter et al. could prove the occurrence of artificial G>A and C>T base changes in ancient animal bones caused by deamination artifacts [31].

Although Gillio-Tos et al. could demonstrate that high quality DNA could also be isolated from 25 years old FFPE blocks, they discussed that parameters like the used fixative (Bouin´s fixative and unbuffered formalin is unfavorable), fixation time (long fixation time leads to decrease of the DNA quality) and tissue treatment before fixation is crucial for received DNA quality [32]. However, if the evaluation of older tissue blocks is necessary, the result of the analysis should be interpreted with caution. We use 4% buffered formalin by default in our department and have a standard fixation time ranging from four hours (for small biopsies) to 36 hours for larger specimen. However, due to the fact that we examined FFPE tissue blocks from 1047 patients from several years as well as paraffin material from other German and eastern European pathology departments (consultation investigation request) we cannot exclude differences in the pre-analytic-treatment of each individual case, retrospectively.

Williams et al. directly compared the results of Sanger sequencing from fresh frozen material with FFPE material analysis. They found a high PCR artifact rate in FFPE material, especially in cases with low DNA input. These potential mutations were not present in corresponding fresh frozen tissue [27]. As we re-examined the EGFR molecular analyses of our routinely diagnosed cases of 2008 until 2012 unfortunately we could not compare our results of FFPE analysis with fresh frozen tissue. However, repeated mutation analyses revealed that the non-reproducible peaks in our collective are to be interpreted as PCR artifacts, most likely induced by formalin fixation.

Marchetti et al. found 45 rare EGFR mutations that proved to be PCR artifacts [26]. Lohinai et al. reported rare EGFR mutations that were not associated with response to TKI treatment and better survival but with positive smoking status [33]. In this setting those results may suggest that these rare mutations could also have been caused by PCR artifacts. Domingues et al. pointed out in this context that reported rare EGFR mutations in general may very often represent PCR artifacts. They recommended microdissection preparation of the tissue blocks and in case of the necessity for using small samples, the addition of uracil-N-glycosylase to the DNA before the PCR reaction [25]. Uracil-N-glycosylase leads to removal of Uracil from the DNA and subsequently to single base gaps. Hence, this biochemical reaction prevents mismatched base pairing caused by cytosine-deamination [26]. As in our routine diagnostics microdissection is performed, the addition of uracil-N-glycosylase to the DNA of small biopsies could be a further improvement.

Costello et al. even found oxidation-specific artifacts in FFPE material also in NGS analyses. They also reported a higher artifact rate when less DNA template was used [24]. As we have already begun to get experience with NGS sequencing we are aware that also in this new and promising technique PCR artifacts can occur. One has to be even more cautious in conjunction with artificial sequence alterations because of the high sensitivity of this technique.

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Furthermore, because the reasons for the artifacts are mainly caused by fixation, the artificial peaks might also cluster in some regions simulating mutations.

Of the six reproducible exon 21 mutations other than L858R, we found two L861Q/R mutations that were detected more frequently in huge collectives of NSCLC [34-37] presenting approximately 1 - 2 % of all EGFR mutations in NSCLC [38]. Chiu et al. found in a large multicenter study (together 639 patients with mutations in the EGFR gene) 57 L861Q mutations out of 161 uncommon mutations. These mutations were less sensitive to TKI inhibitor treatment in comparison with exon 19 deletions and exon 21 L858R [34, 39].

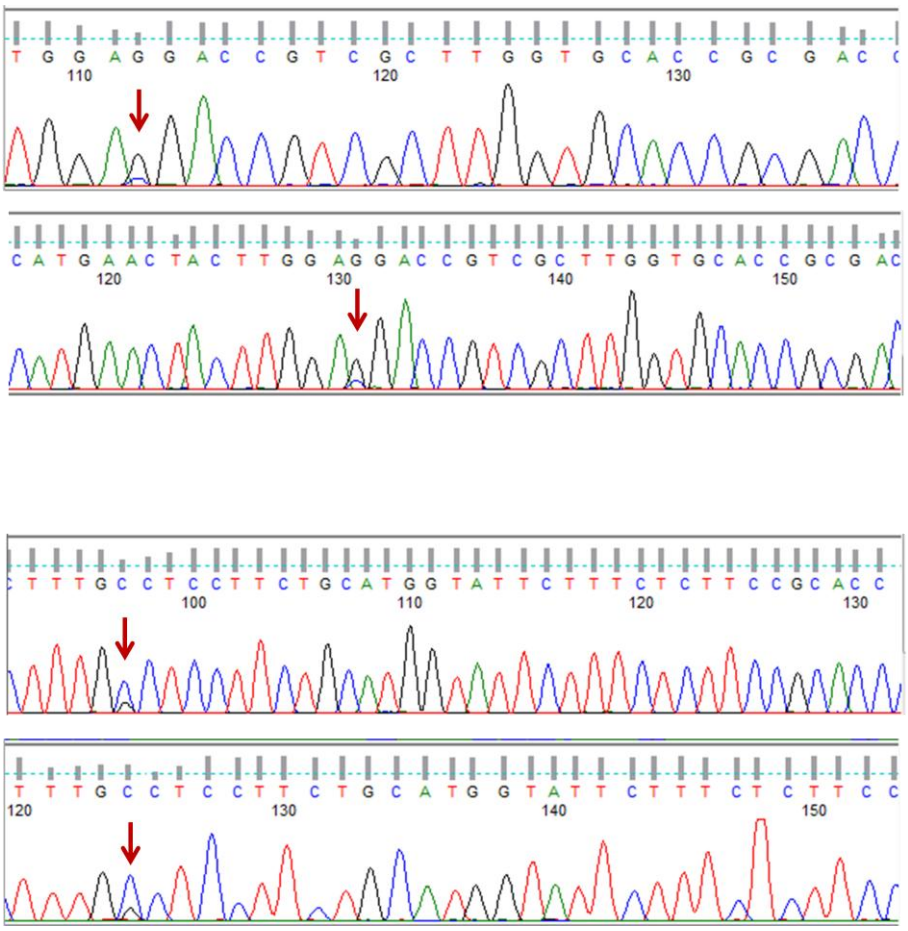

Figure 4: Two reproducible EGFR Exon 21 mutations (A-B: p.E829D; forward and reverse strand, C-D: p.G874A; forward and reverse strand).

The detected E866D mutation has been reported in one collective in a SCLC patient [21] but to our knowledge not in NSCLC. Therefore the biological value of this mutation is not known for NSCLC. The V834L mutation has been reported several times in the literature in NSCLC. It

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mainly occurs as double mutation together with L858R (as in our case 15) or together with an exon 19 deletion leading to response to TKI inhibitor therapy [40, 41]. Two of the six mutations in our collective have not been reported in the current literature, thus far (G874A and E829D) <Figure 4, A-D>. It would be interesting to correlate this finding with the corresponding clinical data.

Reviewing the literature we found double mutations to represent a relatively frequent event in EGFR analysis [35, 37, 40, 41]. Our data confirm this finding since we have detected two double mutations (1.9 %) in our collective (exon 19 deletion + exon 21 L858R; as in case 6 and exon 21 L858R and exon 21 V834L). The constellation of one of our two double mutations (case 6; exon 19 deletion + exon 21 L858R) has been described in the literature also resulting in TKI sensitivity [41]. Hemizygous appearing mutations seem to be a relatively frequent event in NSCLC (three cases in our collective; case 40, 68 and 98) and can easily be missed and may also represent a loss of heterozygocity. Therefore it is important to use different evaluation tools such as blast and alignment programs in routine diagnostics.

In summary we believe that it is important to report all detected genuine (EGFR) mutations, even at atypical sites and even if they have not been reported in special genomic databases, to discover their eventual clinical relevance. But one should be aware of the possibility, potential reasons and prevention of deamination artifacts which could easily simulate point mutations. Our recommendation therefore is that one should in general be skeptical if a new point mutation (in our study especially in EGFR exon 19, where deletions and complex delins predominate) occurs. Because of the explicit therapeutic relevance of the result one should in any case verify these non-typical mutations by repeated analyses to rule out PCR artifacts.

As we could detect two uncommon EGFR exon 21 mutations (G874A and E 829D) that have not been described until now, it will be very interesting to correlate this finding with the clinical data. Furthermore our technical experience in a large collective of 990 advanced NSCLC contributes clearly to a better understanding of PCR processes and challenges in FFPE material and subsequently to a sufficient treatment for patients with NSCLC, particularly when using new emerging techniques like next generation sequencing (NGS).

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