Targeted next-generation sequencing of locally advanced squamous cell carcinomas of the head and neck reveals druggable targets for improving adjuvant chemoradiation

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Supplementary information

Methods

Genomic DNA isolation

All tissue blocks were subjected to haematoxylin and eosin (HE) staining for confirmation of histological diagnosis and for estimation of the squamous cell carcinoma content. After deparaffinization using xylol and ethanol (three times each), tissue pellets were dried at 37 °C for 30 min. Genomic DNA extraction was performed using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction and stored at -20°C until required.

Sequencing

Amplicon library preparation was performed using approximately 10ng of genomic tumor DNA. Briefly, DNA was mixed with the primer pool, containing all primers and the AmpliSeq HiFi Master Mix. Multiplex PCR cycling conditions were as follows: Initial denaturation, 99°C for 2 min; cycling, 21 cycles at 99°C for 15 sec and 60°C for 4 min. Primer end sequences were partially digested using FuPa reagent as instructed, followed by the ligation of barcoded sequencing adapters (Ion Xpress Barcode Adapters, Life Technologies). The final library was purified using AMPure XP magnetic beads (Beckman Coulter, Krefeld, Germany) and quantified using qPCR (Ion Library Quantitation Kit, Life Technologies) on a StepOnePlus Instrument (Life Technologies). The individual libraries were diluted to a final concentration of 100pM and eight to ten libraries were pooled and processed to library amplification on Ion Spheres using the Ion OneTouch 2 instrumentation with the 200-bp chemistry. Unenriched libraries were quality-controlled using Ion Sphere quality control measurement on a QuBit instrument. After library enrichment (Ion OneTouch ES), the library was processed for sequencing using the Ion Torrent 200-bp sequencing chemistry and a pool of eight barcoded libraries was loaded onto a 318 chip.

Sequencing data analysis

Raw data analysis of sequencing results was performed using Ion Torrent Software Suite (version 4.0). The reads were aligned to the human reference sequence build 38 (hg19) using the TMAP aligner implemented in the Torrent Suite software and subsequently filtered against the NCBI dbSNP and COSMIC databases since matched normal tissue was not available for this study. By this approach a discrimination between germline and somatic variants should be possible in most cases. Variants with exclusive entry in the COSMIC database were retained whereas those with exclusive entry in the NCBI dbSNP database were removed. However, several variants were listed in both databases. These variants could represent natural genetic variations or somatic variants with oncogenic activity. Independent of their status as germline or somatic mutation these variants might have important clinical implications. We therefore retained them for further analysis.

Identified somatic mutations were annotated for effect of the mutation on the protein product by using Oncotator, a comprehensive parsing script for mutation annotation. Only nonsense, missense and frameshift mutations in exons or at splice sites were included in further analysis. Missense mutations were classified into neutral, low, medium and high impact using the MutationAssessor functional impact score (release 2, Sep 2012, mutationassessor.org).

HPV status by p16 immunohistochemistry and PCR array-based analysis of HPV DNA

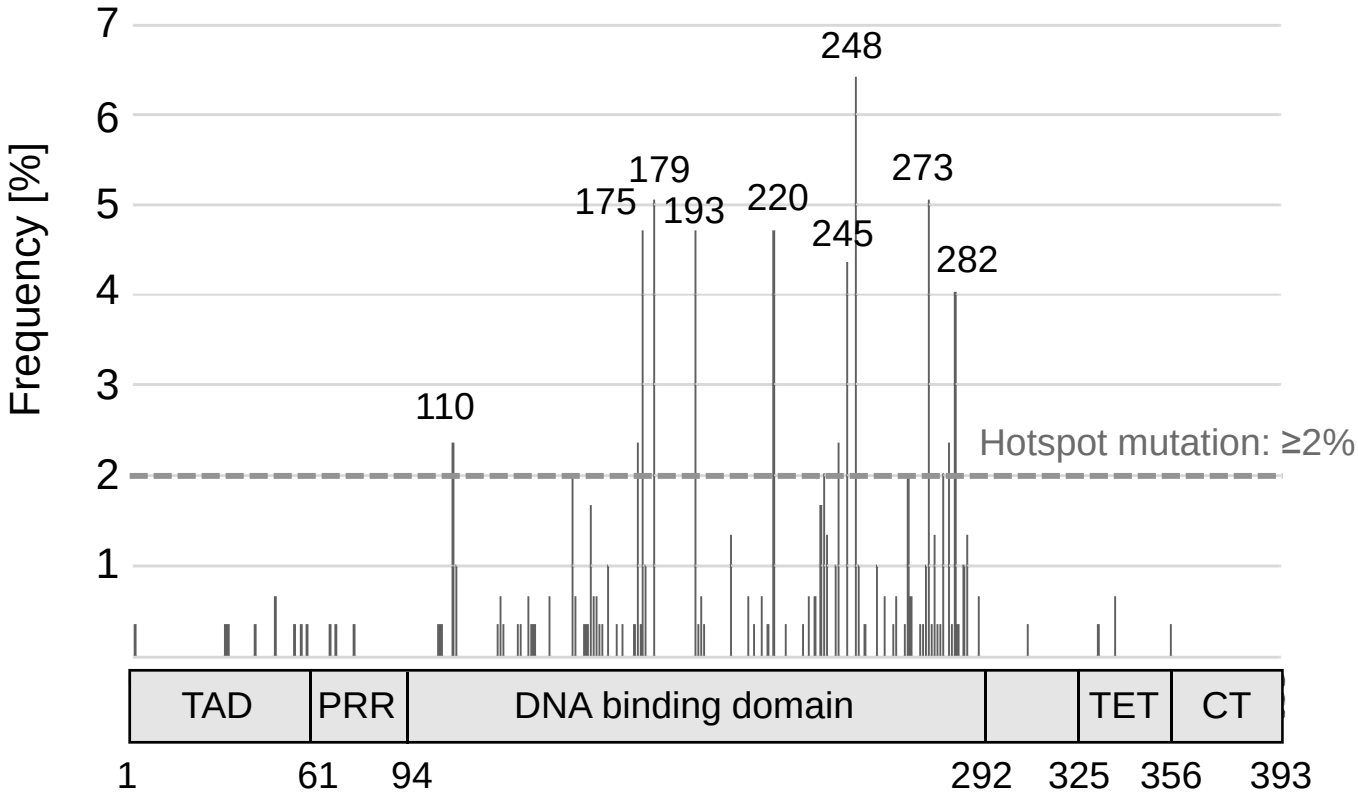
HPV status was determined as previously described (Lohaus-F et al, Radiother Oncol 2014). Briefly, p16 immunohistochemistry was performed using the CINtec® Histology Kit (Roche mtm laboratories AG, Basel, CH) according to the manufacturer's instructions. Tumors with strong, diffuse cytoplasmic and/or nuclear p16 staining in ≥70% tumor cells were classified as p16+ (Ang-KK et al, New Engl J Med 2010). HPV DNA analyses including genotyping were carried out using the LCD-Array HPV 3.5 kit (CHIPRON GmbH, Berlin, DE) according to the manufacturer's instructions. For five samples HPV DNA analysis was not possible due to too low DNA yield and these samples were excluded from the final analysis set. Only oropharyngeal cases positive for both HPV DNA and p16 expression were classified as HPV+ (N=47), all others (HPV DNA-/p16-, N=104; HPV DNA+/p16-, N=8; HPV DNA-/p16+, N=14; HPV DNA+/p16+ non-oropharyngeal carcinomas, N=6) as HPV- cases.

Immunohistochemical staining of p53

Staining of p53 was performed as previously described (Lohaus-F et al, Radiother Oncol 2014). Briefly, sections were incubated with the monoclonal mouse anti-human p53 antibody (Clone DO-7; Dako) or the corresponding IgG antibody control (Dako). Staining was visualised by DAB immunostaining (Dako REAL EnVision Detection

System, Peroxidase/DAB, Rabbit/Mouse). Staining intensity was scored (-, +, ++, +++). Tumors with moderate (++) or strong (+++) immune staining in ≥70% of nuclei were classified as p53 positive.

***TP53* missense mutation spectrum in SCCHN**
707 patients* -> 437 cases with alterations in *TP53* (61.8%)
Cases with *TP53* missense mutations: N=268
Total number of missense mutations: N=301
Mutations in DNA binding domain: N=282/301 (93.7%)
Mutations at hotspot sites (frequency ≥2%): 56.6%



TAD	transactivation domain	TET	tetramerization domain
PRR	proline-rich region	CT	C-terminal regulatory domain

*cohorts (TCGA, Nature 2015, N=306; Agrawal et al., Science 2011, N=32; Stransky et al., Science 2011, N=74; ARO0401 phase III study, Tinhofer et al., ASCO2014, abstract 6002, N=110; current study N=184)

Supplementary Figure 1, Tinhofer et al. 2015

Supplementary Table 1: Targeted NGS gene panel (covered exons are given in brackets)

AKT1 (3,6)	**ERBB4** (3,4,6,7,8,9,15,23)	**KRAS** (2,3)	**PTEN** (1,3,5,6,7,8)
ALK (23,25)	**FAT1** (2,6,10,19,25)	**LRP1B** (11,42,81)	**PTPN11** (3,13)
BRAF (11,15)	**FBXW7** (5,8,9,10,11)	**MET** (2,11,14,16,19)	**RB1** (2,3,6,11,13,16, 17,18,20,21,22,23)
CASP8 (3,9,10)	**FGFR1** (5,7,10,14)	**MLL2** (11,48)	**RET** (10,11,13,15,16)
CCND1 (1,3)	**FGFR2** (7,9,12)	**MYC** (2,3)	**SMAD4** (3,4,5,6,8,9,10,11,12)
CCNE1 (5,10)	**FGFR3** (7,9,14,16,18)	**NFE2L2** (2)	**SMO** (3,5,6,9,11)
CDH1 (2,3,4,5,6,7,9,10, 11,12,13,14,16)	**FLT3** (11,14,16,20)	**NOTCH1** (3,6,7,8,12,15,18,20, 22,23,25,26,27,32,34)	**SRC** (14)
CDKNA2 (1,2)	**HRAS** (2,3)	**NRAS** (2,3,4)	**STK11** (1,2,4,5,6,8)
CSMD3 (5,14,63,65)	**JAK2** (12,14,25)	**NSD1** (5,14,19)	**TP53** (4,5,6,7,8,9,10)
CTNNB1 (3,5,9)	**JAK3** (4,13,16)	**PCDH15** (11,14,19,27)	
EGFR (3,7,15,18,19,20, 21)	**KDR** (6,7,11,19,21,26, 27,30)	**PDGFRA** (5,10,11,12,14,15,18)	
ERBB2 (19,20,21)	**KIT** (2,9,10,11,13,14, 15,17,18)	**PIK3CA** (2,5,8,10*,14,21*)	

*According to the general definition, exons were defined as any nucleotide sequences encoded by a gene that remain present within the final mature RNA product of that gene after introns have been removed by RNA splicing. In the case of *PIK3CA* the exon which is currently exon 2 was originally classified as exon 1. Many publications reporting on *PIK3CA* hotspot mutations have thus referred to exon 9 and exon 20, which according to the correct definition would represent exons 10 and 21, respectively.

Supplementary Table 2. Clinicopathologic characteristics of SCCHN patients (N=179), according to the HPV status

	non-HPV-driven SCCHN N=132	HPV+ OPC N=47	*P* value
Parameters	**No. of patients (%)**	**No. of patients (%)**	
Sex			0.83
Male / Female	107 (81) / 25 (19)	39 (83) / 8 (17)	
Age (years)			0.14
Median / Range	55.8 / 32-75	58.3 / 24-76	
Smoking history*			<0.001
Current smokers	86 (78)	19 (53)	
Ex-Smokers	18 (16)	6 (17)	
Never smokers	7 (6)	11 (31)	
Tumor site			<0.001
Oropharynx	56 (42)	47 (100)	
Oral cavity	52 (39)	0 (0)	
Hypopharynx	24 (18)	0 (0)	
UICC stage			0.47
II	6 (5)	2 (4)	
III	24 (18)	5 (11)	
IV	102 (77)	40 (85)	
pT stage			0.18
T1	25 (19)	7 (15)	
T2	55 (42)	27 (57)	
T3	30 (23)	10 (21)	
T4	22 (16)	3 (6)	
pN stage			0.63
N0	15 (11)	3 (6)	
N1	19 (14)	6 (13)	
N2	85 (64)	32 (68)	
N3	13 (10)	6 (13)	

*Information on smoking habits was available for 147 patients only.

Supplementary Table 3. Mutation frequency, according to the HPV status

Gene	non-HPV-driven SCCHN N=132 No. of cases (%)	HPV+ OPC N=47 No. of cases (%)	*P* value
CASP8	1 (0.8)	0 (0)	1.0
CCND1	0 (0)	1 (2.1)	0.26
CDH1	3 (2.3)	2 (4.2)	0.23
CDKN2A	24 (18.2)	0 (0)	**0.001**
EGFR	3 (2.3)	2 (4.2)	0.24
ERBB4	0 (0)	1 (2.1)	0.26
FAT1	3 (2.3)	2 (4.3)	0.61
FBXW7	3 (2.3)	4 (8.5)	**0.062**
FGFR1	1 (0.8)	0 (0)	1.0
FGFR3	7 (5.3)	5 (10.6)	0.16
HRAS	0 (0)	1 (2.1)	0.26
JAK2	0 (0)	1 (2.1)	0.26
JAK3	2 (1.5)	1 (2.1)	1.0
KDR	2 (1.5)	0 (0)	1.0
KIT	20 (15.2)	7 (14.9)	0.22
KRAS	2 (1.5)	3 (6.4)	**0.11**
LRP1B	1 (0.8)	0 (0)	1.0
MET	19 (14.4)	4 (8.5)	0.50
MLL2	1 (0.8)	0 (0)	1.0
MYC	17 (12.9)	5 (10.6)	0.69
NFE2L2	3 (2.3)	1 (2.1)	1.0
NOTCH1	17 (12.9)	7 (14.9)	0.24
NRAS	0 (0)	2 (4.3)	**0.068**
NSD1	2 (1.2)	0 (0)	0.69
PDGFRA	26 (19.7)	5 (10.6)	0.18
PIK3CA	16 (12.1)	14 (29.8)	**0.012**
PTEN	3 (2.3)	3 (6.4)	0.18
RB1	1 (0.8)	1 (2.1)	0.21
RET	1 (0.8)	1 (2.1)	0.21
SMAD4	1 (0.8)	0 (0)	1.0
SMO	1 (0.8)	0 (0)	1.0
STK11	9 (6.8)	2 (4.3)	0.73
TP53	89 (67.4)	2 (4.3)	**<0.001**

Supplementary Table 4: p53 expression profile of *TP53* genotype subgroups in non-HPV-driven SCCHN

***TP53* alterations**	**p53 positive [%]***	***P* value**
a) Hotspot criteria		**<0.001**
wt (n=45)	31	
hotspot missense (n=34)	82	
any other alteration (n=53)	49	
b) 'Poeta' criteria		**<0.001**
wt (n=45)	31	
disruptive (n=49)	47	
non-disruptive (n=38)	82	
c) EAp53 criteria		**<0.001**
wt (n=45)	31	
nonsense/indels (n=31)	32	
low-risk missense (n=23)	74	
high-risk missense (n=33)	82	
d) Any alteration		**0.001**
wt (n=45)	30	
any *TP53* alteration (N=87)	62	

* Tumors with moderate (++) or strong (+++) immune staining in ≥70% of nuclei were classified as p53 positive.

Supplementary Table 5: Mutation profile of *TP53*^{wt} cases (N=90), according to the HPV status

Affected genes / pathways	**non-HPV-driven SCCHN (N=45)** **No. of patients (%)**	**HPV+ OPC (N=45)** **No. of patients (%)**	***P* value**
CDKN2A	9 (20)	0 (0)	**0.003**
PIK3CA	5 (11)	12 (27)	0.15
PTEN	0 (0)	3 (7)	0.24
Oncogenes (*HRAS, KRAS, NRAS, CCND1, MYC*)	5 (11)	10 (22)	0.17
NOTCH pathway (*NOTCH1, FBXW7*)	4 (9)	9 (20)	0.23
PI3K pathway (*PIK3CA, PTEN*)	5 (11)	15 (33)	**0.021**