***TP53* mutations predict sensitivity to adjuvant gemcitabine in pancreatic ductal adenocarcinoma: next-generation sequencing results from the CONKO-001 trial**

Sinn et al. Online-only Supplement

**Supplemental methods**

**NGS panel development and sequencing**

A customized sequencing panel for PDAC was designed using Ion AmpliSeq Designer based on mutation data of pancreatic cancer from the COSMIC data base. To focus on potentially clinical relevant alterations, mutational hotspots that were reported in the COSMIC database in at least two tumors were included for panel design, when the genes were also mutated in at least eight tumors.

The PDAC panel IAD85732\_169 included 197 amplicons located in 37 genes: *ACTN4, ACVR1B, APC, ARID1A, ARID1B, ARID2, ATM, BRAF, CDK6, CDKN2A, CDNK2B, CNDP2, CREBBP, CTNNB1, ERBB2, FGFR1, GATA6, KDM6A, KMT2C, KMT2D, KRAS, MAP2K4, MET, MYC, PBRM1, PIK3CA, PREX2, RNF43, RPA1, SF3B1, SMAD4, SMARCA2, SMARCA4, SOX9, STK11, TGFBR2, TP53.*

The panel includes the most important driver mutations for PDAC (*KRAS*, *TP53*, *SMAD4*, *CDKN2A*). The genes reflect different molecular motifs like the G1/S checkpoint machinery (*TP53, CDKN2A*), histone modification (*KDM6A*), genes of the ASCOM-TP53-complex (KMT2C/MLL3, KMT2D/MLL4, TP53), SWI/SNF complex (*ARID1A/BAF250A, ARID1B/BAF250B, ARID2/BAF200, PBRM1/BAF180*, *SMARCA2/BRM, SMARCA4/BRG1*), BRCA pathway (*ATM*), WNT signaling defects RNF43), and RNA processing (SF3B1).

**Preparation of samples**

For microdissection three serial 5µm-thick sections were prepared,[[1]](#endnote-1) the first section was stained with H&E and the tumor area was marked by a pathologist. DNA was extracted from the corresponding area in the unstained sections by Versant® kPCR (Siemens) using DNAext protocol according to the manufacturer’s instructions.

**NGS library preparation and sequencing**

DNA quantities were measured using Qubit™ HS DNA Assay (Thermo Fisher Scientific) and TaqMan® RNase P Detection Reagents Kit (Thermo Fisher Scientific). Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific) was used to perform library preparation with 5 µL or 10ng of genomic DNA with the customized sequencing panel IAD85732\_169 and Ion AmpliSeq Sample ID Panel (Thermo Fisher Scientific). The final library was quantified with Ion Library Quantitation Kit (Thermo Fisher Scientific). Samples were 8-fold multiplexed and amplified on Ion Spheres Particles either manually using the Ion OneTouch™ 200 Template Kit v2 DL (Thermo Fisher Scientific) or automatically with Ion PGM™ IC 200 Kit (Thermo Fisher Scientific). Samples were sequenced using Ion 318 chip v2 BC chips (Thermo Fisher Scientific) with an adapted standard protocol using 330 flows. [[2]](#endnote-2)

**NGS data processing strategy**

Semiconductor sequencing raw data were processed using Torrent Suite version 5.0.3. For 107 samples that were analyzed using targeted sequencing, 101 passed the quality criterion of coverage 200 in at least 50% of the amplicons and were analyzed further. The median number of amplicon with coverage ≥ 200 in a sample was 191 of 197 amplicons (97%). Variant calling was performed using the Torrent Variant Caller version 5.0.3.5-5.2.1.39 and the protocol “somatic mutations, low stringency”. VCF files were imported into Ion Reporter and annotated with the workflow “Annotate variants single sample, version 5.0”. For specific variant calling in the FFPE samples of this clinical cohort, only annotated COSMIC variants with a minimum coverage of 200 at the locus under consideration were taken into account.

**Variant analysis strategy**

Since DNA quality varied considerably between samples different variant allele frequencies (VAF), ranging vom 5% to 15%, were applied. For some of the samples, a large number of mutations were called at low VAFs, most of them being C>T substitutions. For these samples, we increased the VAF threshold to 10% or 15%, if the number of detected mutations exceeded n=7 or n=20 at VAF 5%. In summary, there were n=70 high quality DNA samples for which we applied a threshold of 5%, n=18 medium DNA quality samples for which we increased the threshold to 10% and n=17 low DNA quality samples, with >20 variants at VAF5% for which we increased the threshold to 15%. For the high and medium DNA quality samples, we required a minimal tumor area of 10%, for the low DNA quality samples we required a minimum tumor area of 20%. The study cohort was comprised of the 101 samples with sufficient tumor content according to this criterion.

**Supplemental Tables**

**Supplemental table 1:**

Comparison of the clinical parameter for the NGS cohort and the overall CONKO-001 ITT cohort

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **NGS cohort**  N=101 (%) | **Non-NGS cohort**  **N=253 (%)** | **p-value**  **(chi²)** | **CONK0-001**  **Overall study population**  N=354 (%) |
| **T stage** (%)  pT1-T2  pT3-4 | 12 (12)  89 (88) | 37 (15)  216 (85) | p=.500 | 49 (14)  305 (86) |
| **N stage** (%)  pN0  pN1/2 | 23 (23)  78 (77) | 77 (30)  176 (70) | p=.148 | 100 (28)  254 (72) |
| **Grading** (%)  G1-2  G3  missing | 50 (49.5)  50 (49.5)  1 (1) | 168 (66)  80 (32)  5 (2) | **p=.002** | 218 (61)  130 (37)  6 (2) |
| **Resection margin** (%)  R0  R1 | 81 (80)  20 (20) | 212 (84)  41 (16) | p=.418 | 293 (83)  61 (17) |
| **Age**  Median  Range | 62  37-81 | 62  34-82 |  | 62  34-82 |
| **Gender** (%)  Female  Male | 47 (46.5)  54 (53.5) | 104 (41)  149 (59) | p=.351 | 151 (43)  203 (57) |
| **Karnofsky Performance State**  Median  Range | 80  50-100 | 80  60-100 |  | 80  50-100 |
| **Treatment Arm** (%)  Gem  Observation | 48 (47.5)  53 (52.5) | 131 (52)  122 (48) | p=.470 | 179 (51)  175 (49) |
| **Survival Gem group** (95% CI)  Median DFS  Median OS | 13.8 (11.2-16.4)  24.4 (13.7-35.2) | 13.1 (11.3-15.0)  22.4 (17.4-27.3) |  | 13.4 (11.6-15.3)  22.8 (18.5-27.2) |
| **Survival Obs group** (95% CI)  Median DFS  Median OS | 5.8 (4.3-7.3)  18.2 (12.5-24.0) | 7.0 (5.6-8.3)  20.3 (17.5-23.1) |  | 6.7 (6.0-7.5)  20.2 (17.7-22.8) |

**Supplemental table 2:**

Comparison of the clinical parameters for patients with *TP53*mut vs. *TP53*wt tumors

|  |  |  |  |
| --- | --- | --- | --- |
|  | **TP53 wildtyp**  **N= 40 (%)** | **TP53 mutated**  **N=61 (%)** | p-value  (chi²/\*fisher’s test) |
| **Treatment Arm**  Gem vs Obs | 19 (47.5) vs 21 (52.5) | 29 (47.5) vs 32 (52.5) | p=.997 |
| **T stage**  T1/2 vs T3/4 | 4 (10.0) vs 36 (90.0) | 8 (13.1) vs 53 (86.9) | p=.759\* |
| **Lymph node involvement**  N0 vs N1/2 | 11 (27.5) vs 29 (72.5) | 12 (19.7) vs 49 (80.3) | p=.359 |
| **Grading**  **G1/2 vs G3** | N=40  22 (55.0) vs 17 (42.5) | 28 (45.9) vs 33 (54.1) | p=.305 |
| **Resection margin**  R0 vs R1 | 30 (75.0) vs 10 (25.0) | 51 (83.6) vs 10 (16.4) | p=.288 |
| **Gender**  Female vs male | 17 (42.5) vs 23 (57.5) | 30 (49.2) vs 31 (50.8) | p=.510 |
| **Age**  Median (range) | 62 (37-78) | 63 (39-81) |  |
| **Karnofsky Performance State (%)**  Median (range) | 80 (50-90) | 80 (60-100) |  |

**Supplemental table 3A-C:**

Univariate and multivariate\* analysis of overall survival for the most common mutations – separate analysis for the complete cohort, the gemcitabine arm and the observation arm

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Complete cohort - univariate** | | | | | | **Complete cohort - multivariate** | | | |
|  | **HR** | **lower.95** | **upper.95** | **p** | **Interact\*\*** | **HR** | **lower.95** | **upper.95** | **p** |
| **TP53** | 1,215 | 0,794 | 1,858 | 0,370 | 0,08001 | 1,203 | 0,775 | 1,867 | 0,411 |
| **ASCOM** | 1,314 | 0,854 | 2,021 | 0,214 | 0,47597 | 1,315 | 0,842 | 2,052 | 0,228 |
| **KRAS** | 0,929 | 0,574 | 1,503 | 0,764 | 0,20821 | 0,902 | 0,548 | 1,485 | 0,685 |
| **SMAD4** | 0,712 | 0,344 | 1,474 | 0,361 | 0,27393 | 0,900 | 0,424 | 1,893 | 0,774 |
| **CDKN2A** | 1,085 | 0,542 | 2,173 | 0,817 | 0,37749 | 1,173 | 0,574 | 2,396 | 0,662 |
| **SWI/SNF** | 0,617 | 0,308 | 1,236 | 0,173 | 0,0825 | 0,688 | 0,330 | 1,434 | 0,319 |

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Gemcitabine - univariate** | | | | | **Gemcitabine - multivariate** | | | |
|  | **HR** | **lower.95** | **upper.95** | p | **HR** | **lower.95** | **upper.95** | **p** |
| **TP53** | 0,856 | 0,456 | 1,606 | 0,628 | 0,651 | 0,327 | 1,296 | 0,222 |
| **ASCOM** | 0,924 | 0,489 | 1,744 | 0,807 | 0,718 | 0,360 | 1,429 | 0,345 |
| **KRAS** | 0,966 | 0,482 | 1,939 | 0,923 | 0,967 | 0,449 | 2,083 | 0,931 |
| **SMAD4** | 1,148 | 0,449 | 2,939 | 0,773 | 1,836 | 0,651 | 5,176 | 0,251 |
| **CDKN2A** | 1,629 | 0,491 | 5,406 | 0,426 | 1,279 | 0,358 | 4,568 | 0,705 |
| **SWI/SNF** | 0,614 | 0,255 | 1,477 | 0,276 | 0,614 | 0,221 | 1,704 | 0,349 |

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Observation group – univariate** | | | | | **Observation group -multivariate** | | | |
|  | **HR** | **lower.95** | **upper.95** | **p** | **HR** | **lower.95** | **upper.95** | **p** |
| **TP53** | 1,835 | 0,997 | 3,380 | 0,051 | 2,406 | 1,218 | 4,750 | 0,011 |
| **ASCOM** | 2,006 | 1,077 | 3,735 | 0,028 | 2,665 | 1,337 | 5,315 | 0,005 |
| **KRAS** | 0,643 | 0,320 | 1,291 | 0,215 | 0,634 | 0,295 | 1,359 | 0,241 |
| **SMAD4** | 0,413 | 0,127 | 1,341 | 0,141 | 0,453 | 0,132 | 1,555 | 0,208 |
| **CDKN2A** | 0,745 | 0,316 | 1,756 | 0,501 | 0,839 | 0,332 | 2,115 | 0,709 |
| **SWI/SNF** | 1,225 | 0,376 | 3,992 | 0,736 | 1,104 | 0,314 | 3,884 | 0,877 |

\* Multivariate Cox regression analysis adjusted for patient age (< 60 years vs. <= 60 years), tumor stage (T1-2 vs. T3-4), nodal status (N+ vs. N-), grading (G1-2 vs. G3) and resection status (R0 vs. R1). \*\* test for interaction p-value derived from Cox regression analysis for overall survival for all patients including mutational alterations and treatment arms .

**Supplemental Table 4**

To further evaluate samples with multiple mutations, we performed bivariate interaction models with TP53 and KRAS, SMAD4 and CDKN2A, respectively.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Gemcitabine** |  | **Observation** |  |
|  | **HR (95% CI)** | **p** | **HR (95% CI)** | **p** |
| **TP53** | 1.143 (0.302-4.328) | 0.844 | 1.535 (0.465-5.064) | 0.482 |
| **KRAS** | 1.452 (0.416-5.068) | 0.559 | 0.607 (0.212-1.736) | 0.352 |
| **Interaction** | 0.519 (0.113-2.372) | 0.398 | 1.847 (0.462-7.383) | 0.385 |
|  |  |  |  |  |
| **HR** | HR (95% CI) | P | HR (95% CI) | P |
| **TP53** | 0.649 (0.336-1.253) | 0.198 | 2.036 (1.088-3.81) | **0.026** |
| **SMAD4** | 1.488 (0.551-4.02) | 0.433 | 0.203 (0.027-1.549) | 0.124 |
| **Interaction** | NA | NA | 11.422 (1.068-122.137) | **0.044** |
|  |  |  |  |  |
|  | HR (95% CI) | P | HR (95% CI) | P |
| **TP53** | 0.719 (0.377-1.37) | 0.316 | 2.38 (1.245-4.551) | **0.009** |
| **CDKN2A** | 19.172 (1.694-216.936) | **0.017** | 0.63 (0.181-2.2) | 0.469 |
| **Interaction** | 0.071 (0.004-1.205) | 0.067 | 1.112 (0.197-6.276) | 0.904 |

1. Weichert W, Schewe C, Lehmann A, Sers C, Denkert C, Budczies J, Stenzinger A, Joos H, Landt O, Heiser V, Röcken C, Dietel M. KRAS genotyping of paraffin-embedded colorectal cancer tissue in routine diagnostics: comparison of methods and impact of histology. J Mol Diagn. 2010 Jan;12(1):35-42. [↑](#endnote-ref-1)
2. Budczies J, Bockmayr M, Treue D, Klauschen F, Denkert C. Semiconductor sequencing: how many flows do you need? Bioinformatics. 2015 Apr 15;31(8):1199-203 [↑](#endnote-ref-2)