**Supplementary Information**

**Supplementary Materials and Methods**

**Cell sorting on a high-throughput platform**

Each section was collected in a nylon biopsy bag inside a 50-ml conical tube, dewaxed by three sequential 10 min incubations with xylene and then rehydrated by ethanol washes with a decreasing concentration. After washing with deionized water, the sections were immersed in 10 mM sodium citrate buffer (pH 6.4) for 5 min at room temperature and heat-treated in the same prewarmed buffer for 1 h at 80 °C. After cooling to room temperature, the sections were washed with three sequential 5 min incubations with RPMI medium (Life Technologies, Carlsbad, California, United States). To obtain the cell suspension, the section was incubated in a solution of 10 ml 0.1% collagenase I-A (Sigma-Aldrich, St. Louis, Missouri, United States) and 0.1% dispase (Life Technologies) at 37 °C. The dissociation process was stopped after 45 min by placing the sample tube on ice. The cell suspension was resuspended by pipetting and transferred through a 30-μm mesh nylon filter into a 15-ml conical tube. The cell suspension was washed two times with ice-cold PBATw (PBS-1% BSA-0.05% Tween 20 buffer) by centrifugation at 1,000 g for 5 min.

The cell pellet was resuspended in 1 ml of ice-cold PBATw, and an aliquot of 5 × 105 cells was incubated with 100 μl of the primary monoclonal antibody mixture containing anti-keratin MNF116, IgG1 (DAKO, Glostrup, Denmark) (final concentration 3.2 μg/ml); anti-keratin AE1/AE3, IgG1 (Millipore–Chemicon, Burlington, Massachusetts, United States) (final concentration 10 μg/ml); and anti-vimentin 3B4, IgG2a (DAKO) (final concentration = 3.1 μg/ml) in PBATw. After 30 min at 4 °C, the cells were washed twice with ice-cold PBATw and 100 μl of the following premixed secondary reagents were added to the pellet: Alexa Fluor® 488 Goat Anti-Mouse IgG1 (Life Technologies) at a final concentration of 2.5 μg/ml for keratin detection; Alexa Fluor® 647 Goat Anti-Mouse IgG2a (Life Technologies) at a final concentration of 2.5 μg/ml for vimentin detection; and PBATw. The incubation occurred for 60 min in the dark at 4 °C and was followed by two washes with ice-cold PBATw. A DNA staining solution containing 10 μM DAPI (Sigma-Aldrich) in PBATw was added, and after an incubation of 30 min at 37 °C, the cells were washed twice with PBATw with a 5-min centrifugation at 1,000 g, and the pellet was resuspended in the same buffer.

For DEPArray™ cell sorting, a small amount of the labeled cell suspension was washed twice with 1 ml of SB115 buffer (Menarini Silicon Biosystems, Bologna, Italy). The pellet was resuspended in the same buffer, and an aliquot corresponding to approximately 24,000 cells was loaded into the DEPArray™ A300K cartridge (Menarini Silicon Biosystems). The DNA integrity in the cell suspension was determined using the DEPArray™ FFPE QC Kit (Menarini Silicon Biosystems).

**OncoSeek panel analysis of sorted cells**

After the identification and selection of cell populations showing the desired fluorescence patterns based on antibodies against vimentin and pan-cytokeratin and based on the DAPI signal, the precise number of homogeneous cells in the tumor and stromal populations, together with pools of mixed cells, were recovered in different PCR tubes. After lysis, reagents were added to the same tubes to prepare the DEPArray™ OncoSeek libraries.15,16

Illumina-compatible targeted NGS libraries were obtained from the sorted cell lysates using the OncoSeek panel (Menarini Silicon Biosystems) with 63 clinically relevant oncology-related genes.9

Each DEPArray™ OncoSeek library was diluted 1:10,000 and then quantified in triplicate by qPCR using the KAPA Library Quantification Kit (Hoffmann-La Roche, Basilea, Switzerland) following the user manual’s instructions. Finally, the quantification was adjusted for an average library size of 243 bp. All libraries were pooled, and NGS was performed using MiSeq v2 (150 PE) reagents on a MiSeq instrument (Illumina, St Diego, California, United States) according to the manufacturer’s protocol.

The FASTQ paired-end reads were trimmed using Cutadapt (cutadapt.readthedocs.io) according to the manufacturer’s protocol to remove synthetic primers from overlapping amplicons. The trimmed reads were aligned to the human reference genome (hg19) using the BWA software (bio-bwa.sourceforge.net). The alignment and coverage statistics were obtained using SAMtools (samtools.sourceforge.net) and BEDTools (bedtools.readthedocs.io/en/latest) packages. After filtering to discard the partial, poorly aligned and unmapped reads, variant calls were obtained using LoFreq software (csb5.github.io/lofreq). The resulting variants were annotated using the Ensembl Variant Effect Predictor (http://www.ensembl.org/info/docs/tools/vep/index.html). All identified variants were filtered according to their presence/absence in public databases (1000 Genomes, gnomAD, and COSMIC) and their pathogenic effect.

The copy number alteration analysis of the OncoSeek data was performed using sorted populations as tests and a set of stromal cell pools from different samples as controls. For the copy number calling, the reads mapping to the target amplicons of the DEPArray™ OncoSeek panel were counted. Then, the read counts were normalized using the following 2-step procedure: 1) between-sample normalization using the total number of aligned reads and 2) within-sample normalization using a LOWESS fitting of read counts with respect to the first component explaining >90% variation between regions in the control samples. The fold changes were computed by dividing the normalized counts in the test samples by the baseline and are represented by the median value of the normalized counts per amplicon across the control samples. The final copy number calls per gene were obtained by calculating the median fold changes in all gene-specific amplicons.

**Whole-genome low-pass sequencing for copy number alterations (CNA) analysis**

Recovered cells were lysed in the recovery tube using the SB LysePrep™ Kit (Silicon Biosystems), and 46 μl of low TE buffer was added to the tube. The sample was then transferred into a microTUBE-50 AFA Fiber Screw-Cap for fragmentation by Covaris M220 instrument for 3 min and 52 sec (pick power: 50, duty factor: 20, cycles/burst: 200) to obtain a 150-200 bp fragment size. Libraries were prepared using an Accel-NGS® 2S PCR-Free DNA Library kit (Swift Biosciences) according to the manufacturer’s instructions.

Twenty microliters of library were amplified as follows: 6 μM of amplicon PCR forward primer (5′-AATGATACGGCGACCACCGAGATC-3′), 6 μM of amplicon PCR reverse primer (5′-CAAGCAGAAGACGGCATACGA-3′) and 2× KAPA HiFi HotStart Ready Mix (Kapa Biosystems). The PCR cycling conditions were 98 °C initial denaturation for 45 sec, followed by 16 cycles and 15 cycles for ~100 cells and ~300 cells, respectively, at 98 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 1 min. The products were cleaned up with 0.75X Agencourt AMPure XP beads (Beckman Coulter Genomics) according to the manufacturer’s protocol and eluted in 20 μl low TE (Swift Biosciences).

Libraries were normalized and pooled to 4 nM based on qPCR quantification. Pooled samples were denatured and diluted to a final concentration of 12 pM. All samples were multiplexed and sequenced in a single lane on the MiSeq using 2 × 100 bp paired-end sequencing using the MiSeq Reagent Kit V3.

The BWA algorithm was used to align the 98.7% reads to the hg19 human reference genome. PCR duplicates and secondary alignments were filtered out using Picard MarkDuplicates and SAMtools. The control-FREEC algorithm was used to obtain copy number calls using the mode using the mode without control sample independently for all libraries. Read counts were corrected by GC content and mappability (uniqMatch option), and the main ploidy level was estimated for each library based on best fitting of profiles to the underlying copy number levels.

**Sanger sequencing**

Genomic DNA was extracted from different FFPE tissue sections of the same tumor tissue block with a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). KAPA HiFi HotStart (Roche, Mannheim, Germany) was used for genomic DNA amplification, and the purified PCR products were analyzed by Sanger sequencing according to the previously described method.17 Primer sequences are available on request from the authors.

**Droplet digital PCR (ddPCR)**

The ddPCR experiments were performed for TP53\_R273H and CDKN2A\_R58\* assays using the QX100/QX200 Droplet Digital PCR (ddPCR) system (Bio-Rad, Hercules, California, United States). The ddPCR mixture consisted of 10 μl of a 2X ddPCR Supermix for Probes no dUTP (Bio-Rad), 1 μl of 20X primer/probe mix and 50 ng of sample DNA from unsorted tissue material in a final volume of 20 μl. Mutant target and wild-type assays were provided together in a single tube, with the FAM probe targeting the mutant allele and the HEX probe targeting the wild-type allele. The entire reaction mixture was loaded into the sample well of a DG8 cartridge (Bio-Rad) with 70 μl of droplet generation oil (Bio-Rad) and placed in the droplet generator (Bio-Rad). After processing, the droplets generated from each sample were transferred into a 96-well plate that was heat-sealed with a foil seal and placed in a conventional thermal cycler. Thermal cycling consisted of a 10 min at 95 °C followed by 40 cycles of a two-step thermal profile of 30 s at 94 °C (denaturation) and 1 min at 55 °C (for combined annealing-extension) and 1 cycle of 98 °C for 10 min. The ramp rate for each step was set to 2 °C/s. Droplets were read using a QX100/QX200 Droplet Reader, and data were analyzed using QuantaSoft software. No-template controls were performed using water in place of template in every experiment; in all no-template controls, no amplification occurred.

**Statistical Analysis**

Data are represented as the median and interquartile range (IQR) for continuous variables and as n (%) for categorical variables unless otherwise stated.

Differences in frequency data were analyzed in contingency tables using Chi-square (*χ2*) or Fisher’s tests as appropriate. We used the Mann-Whitney test to analyze continuous variables. *P*-values <0.05 were considered significant. Data were analyzed using SPSS (version 15.0; SPSS Inc., Chicago, IL, USA). Cancer-specific survival was assessed using the Kaplan-Meier method and log-rank test. Comparison between histological subtypes defined according to Lauren’s classification and distribution of *TP53* mutations was performed with the Chi-square test for given probabilities (R software package; R Project for Statistical Computing, Vienna, Austria), Bonferroni's correction was applied, and *P-*values <0.025 were considered significant.

**Supplementary Figure Legends**

Supplementary Figure 1|**Point mutations detected through sorted cells sequencing and unsorted cells sequencing. A.** Number of point mutations, distinguishing between missense (blue), nonsense (orange) and frameshift (grey), identified by targeted sequencing in sorted and unsorted cells. **B**. Number of point mutations identified in sorted (blue) and unsorted (orange) cells, clustered in 4 categories according to the number of reads supporting the alternative allele.

Supplementary Figure 2| ***TP53* and *CDKN2A* mutations identified in different patients with OncoSeek panel and validated by droplet digital PCR (ddPCR) specific assays. A.** *TP53* hotspot mutation (p.R273H) identified by the targeted panel in the sorted pure populations of the tumor with hyperdiploid (violet) and pseudodiploid (brown) DNA content and in unsorted fractions (gray) of 3 EAC samples (EAC6, EAC11 and EAC26). Values represent the alternative allele frequency. The graph shows *TP53* mutant allele fractional abundances (%) identified with ddPCR in the DNA from unsorted material of 3 EACs, a known wild-type sample and a no-template control (NTC). **B.** *CDKN2A* nonsense mutation (p.R58\*) identified by targeted panel in the sorted pure hyperdiploid cell population of tumor (violet) and in unsorted fractions (gray) of sample EAC4. Values represent the alternative allele frequency. The graph shows the *CDKN2A* mutant allele fractional abundance (%) identified with ddPCR in the DNA from unsorted material of EAC4, a known control wild-type sample (CTR wt) and a no-template control (NTC).

Supplementary Figure 3| **Kaplan-Meier plot of p53 IHC and *TP53* mutational status and clinical outcomes.** **A**. Cancer-specific survival, as stratified for patients with p53 overexpression (1) or with normal immunohistochemical profiles (0). Kaplan-Meier test; log-rank test *P=*0.206). **B**. Disease-free survival, as stratified for patients with p53 overexpression (1) or with normal immunohistochemical profiles (0) (Kaplan-Meier test; log-rank test *P=*0.459). **C.** Kaplan-Meier plot of cancer-specific survival, as stratified for patients carrying mutated or wild-type *TP53* (Kaplan-Meier test; log-rank test *P=*0.028). **D.** Kaplan-Meier plot of disease-free survival, as stratified for patients carrying mutated or wild-type *TP53* (Kaplan-Meier test; log-rank test *P=*0.037).

**Supplementary Tables**

**Supplementary Table 1: Clinical and epidemiological information for EAC cases included in the study.**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **ID** | **Age** | **Sex** | **Cancer**  **Specific**  **Survival**  **1=dead** | **Follow-up (months)** | **Recurrence**  **(1=yes)** | **Lauren** | **BIM/GIM** | **Stage (7th ed)** |
| EAC1 | 53 | F | 0 | 72 | 1 | intestinal | BIM+/GIM- | II |
| EAC2 | 43 | M | 0 | 60 | 0 | diffuse | BIM+/GIM- | II |
| EAC3 | 76 | F | 0 | 1 | 0 | diffuse | BIM+/GIM- | III |
| EAC4 | 66 | M | 1 | 36 | 1 | intestinal | BIM+/GIM- | II |
| EAC5 | 71 | M | 1 | 11 | 1 | intestinal | BIM+/GIM- | III |
| EAC6 | 82 | F | 0 | 84 | 0 | intestinal | BIM+/GIM- | III |
| EAC7 | 83 | M | 0 | 27 | 0 | intestinal | BIM+/GIM- | III |
| EAC8 | 86 | M | 1 | 13 | 1 | intestinal | BIM-/GIM+ | III |
| EAC9 | 62 | M | 1 | 3 | 1 | intestinal | BIM-/GIM+ | III |
| EAC10 | 72 | M | 1 | 58 | 1 | diffuse | BIM-/GIM+ | I |
| EAC11 | 76 | M | 0 | 12 | 0 | intestinal | BIM-/GIM- | III |
| EAC12 | 58 | M | 0 | 53 | 0 | intestinal | BIM-/GIM- | III |
| EAC13 | 28 | F | 1 | 22 | 1 | intestinal | BIM-/GIM- | IV |
| EAC14 | 83 | F | 0 | 0 | 0 | intestinal | BIM-/GIM- | III |
| EAC15 | 60 | F | 0 | 84 | 0 | intestinal | BIM-/GIM- | II |
| EAC16 | 78 | M | 1 | 36 | 1 | intestinal | BIM-/GIM- | II |
| EAC17 | 59 | M | 0 | 24 | 1 | intestinal | BIM-/GIM- | II |
| EAC18 | 75 | M | 1 | 8 | 1 | intestinal | BIM-/GIM- | III |
| EAC19 | 44 | F | 1 | 35 | 1 | diffuse | BIM-/GIM- | III |
| EAC20 | 79 | M | 0 | 0 | 0 | diffuse | BIM-/GIM- | IV |
| EAC21 | 63 | M | 0 | 56 | 0 | intestinal | BIM-/GIM- | II |
| EAC22 | 84 | M | 0 | 84 | 0 | intestinal | BIM-/GIM- | II |
| EAC23 | 77 | F | 0 | 4 | n/a\* | intestinal | BIM-/GIM- | III |
| EAC24 | 78 | F | 1 | 10 | 1 | intestinal | BIM-/GIM- | III |
| EAC25 | 80 | M | 0 | 71 | 0 | diffuse | BIM-/GIM- | I |
| EAC26 | 74 | M | 0 | 19 | 0 | intestinal | BIM-/GIM- | III |
| EAC27 | 68 | M | 0 | 0 | 0 | intestinal | BIM-/GIM- | III |
| EAC28 | 72 | M | 0 | 6 | 0 | intestinal | BIM-/GIM- | III |
| EAC29 | 67 | M | 0 | 14 | 0 | intestinal | BIM-/GIM- | II |
| EAC30 | 82 | M | 0 | 14 | 1 | intestinal | BIM-/GIM- | III |
| EAC31 | 66 | M | 1 | 33 | 1 | intestinal | BIM-/GIM- | III |
| EAC32 | 54 | M | 1 | 15 | 0 | intestinal | BIM-/GIM- | III |
| EAC33 | 87 | M | 0 | 0 | 0 | intestinal | BIM-/GIM- | II |
| EAC34 | 61 | M | 1 | 12 | 1 | intestinal | BIM-/GIM- | III |
| EAC35 | 82 | M | 1 | 6 | 1 | intestinal | BIM-/GIM- | IV |
| EAC36 | 65 | M | 1 | 29 | 1 | intestinal | BIM+/GIM- | III |
| EAC37 | 62 | M | 1 | 5 | 1 | intestinal | BIM+/GIM- | II |
| EAC38 | 54 | F | 0 | 6 | 0 | diffuse | BIM-/GIM- | II |

F=Female; M=Male. \*n/a= not available.

**Supplementary Table 2: p53 immunohistochemistry and *TP53* mutation status.**

We report in black the missense mutation; in red stop codon (\*) and frameshift (fs) mutations. wt=wild-type.

TA classes and GVGD scores, according to IARC TP53 Database (http://p53.iarc.fr/) are based: a) for TA classes, on the overall transcriptional activity (TA) on 8 different promoters as measured in yeast assays.38 For each mutation, the median of the 8 promoter-specific activities (expressed as percent of the wild-type protein) is calculated and missense mutations are classified as "non-functional" if the median is <=20, "partially functional" if the median is >20 and <=75, "functional" if the median is >75 and <=140, and "supertrans" if the median is >140; b) for the GVGD scores, classification is based on alignments obtained with Align-GVGD tool for *TP53* missense variant prediction.39 C15 is considered the best cut-off of pathogenicity.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **EAC\_ID** | **p53 immunostaining** | ***TP53* mutation** | **TA classa** | **GVGD classb** |
| EAC1 | Overexpression | p.R282W | non-functional | C65 |
| EAC2 | no overexpression | wt | - | - |
| EAC3 | Overexpression | p.C176F | partially functional | C65 |
| EAC4 | no overexpression | p.P75L | non-functional | C0 |
| EAC5 | no overexpression | p.P191Sfs\*18 | - | - |
| EAC6 | no overexpression | p.R273H | non-functional | C25 |
| EAC7 | no overexpression | p.A79G /  p.A74Efs\*45 | Functional  - | C0  - |
| EAC8 | Overexpression | p.A138V /  p.V73Rfs\*76 | partially functional  - | C55  - |
| EAC9 | Overexpression | p.R273C | non-functional | C65 |
| EAC10 | no overexpression | p.S303Afs\*42 | - | - |
| EAC11 | Overexpression | p.R273H | non-functional | C25 |
| EAC12 | Overexpression | p.R273C | non-functional | C65 |
| EAC13 | no overexpression | wt | - | - |
| EAC14 | no overexpression | wt | - | - |
| EAC15 | no overexpression | p.R248Q | non-functional | C35 |
| EAC16 | Overexpression | p.C275F | non-functional | C65 |
| EAC17 | Overexpression | p.R175H | non-functional | C25 |
| EAC18 | no overexpression | p.R342\* | - | - |
| EAC19 | no overexpression | wt | - | - |
| EAC20 | Overexpression | p.P278S | non-functional | C65 |
| EAC21 | Overexpression | p.R273C | non-functional | C65 |
| EAC22 | Overexpression | p.R175H | non-functional | C25 |
| EAC23 | no overexpression | wt | - | - |
| EAC24 | no overexpression | wt | - | - |
| EAC25 | Overexpression | p.R175H | non-functional | C25 |
| EAC26 | Overexpression | p.R273H | non-functional | C25 |
| EAC27 | Overexpression | p.R196\* | - | - |
| EAC28 | no overexpression | p.S15Rfs\*28 | - | - |
| EAC29 | no overexpression | p.L43V | Functional | C0 |
| EAC30 | Overexpression | p.D259V | non-functional | C15 |
| EAC31 | Overexpression | p.R248W | non-functional | C65 |
| EAC32 | Overexpression | p.R267G | non-functional | C65 |
| EAC33 | no overexpression | p.Q167\* | - | - |
| EAC34 | no overexpression | wt | - | - |
| EAC35 | no overexpression | wt | - | - |
| EAC36 | Overexpression | p.Y220C | non-functional | C65 |
| EAC37 | no overexpression | wt | - | - |
| EAC38 | Overexpression | wt | - | - |

**Supplementary Table 3: Evaluation of the predictive value of the correlation between p53 IHC staining and presence of mutations in the *TP53* gene.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sensitivity** | **Specificity** | **Positive predictive value** | **Negative predictive value** | **Accuracy** |
| 64% | 90% | 95% | 47% | 71% |

**Supplementary Table 4: Crosstab showing the relationship between *TP53* mutational status and Lauren’s classification.** The chi-squared test for given probabilities and Bonferroni's correction were applied to calculate the P-values (*P*) showed in table.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | ***TP53*** | | Total | *P* |
| **Lauren** | **wt** | **mutant** |  |
| **Intestinal** | 7  (22.6%) | 24  (77.4%) | 31  (100%) | 0.002 |
| **Diffuse** | 3  (42.9%) | 4  (57.1%) | 7  (100%) | 0.705 |
| Total | **10**  (26.3%) | **28**  (73.7%) | **38**  (100%) |  |