Supplemental methods

Early recurrence

Early recurrence was defined as recurrence <12 weeks after surgery in patients not treated with adjuvant therapy and as recurrence before planned start of adjuvant therapy in patients intended to receive adjuvant therapy. This cut-off was based on our institutional guidelines stating that adjuvant therapy should start <12 weeks after surgery. Additionally, this was supported by data from our cohort (the 75th percentile of time from surgery date to start adjuvant therapy is 11.7 weeks), and the EORTC 1325 trial with a median of 11.3 weeks to start of adjuvant therapy (personal communication, EORTC).

RNA sequencing

Transcriptome sequencing was performed by CeGaT GmbH (Tübingen, Germany). Transcriptome libraries were generated using the KAPA RNA HyperPrep with RiboErase (HMR), according to manufacturer's instructions. The libraries were sequenced with 2x 100-bp reads on a NovaSeq 600 system according to manufacturer's protocols, with a sequence quality with a Q30 value of >93%.

Data were analyzed in CeGaT transcriptome analysis pipeline. Briefly, demultiplexing of the sequencing reads was performed with Illumina bcl2fastq (2.20). Adapters were trimmed with Skewer (version 0.2.2).[33] The quality of FASTQ files was analyzed with FastQC (version 0.11.5-cegat).[34] Plots were created using ggplot2 in R (version 3.6.1).[35]

FASTQ files were mapped to the human reference genome (Homo.sapiens.GRCh38.v82) using STAR(2.6.0c)[36] with default settings. Count data generated with HTseq-count[37] was analyzed with DESeq2.[38] For the total dataset, centering of normalized gene expression was performed by subtracting the row means and scaling by dividing the columns by the standard deviation.

Immunohistochemistry

Immunohistochemistry (IHC) of the FFPE tumor samples was performed on a BenchMark Ultra (PD-L1 stain) or a Discovery Ultra (CD20-CD3 double stain) automated stainer (Ventana Medical Systems). Briefly, paraffin sections were cut at 3 µm, heated at 75°C for 28 minutes and deparaffinized with EZ prep solution (Ventana Medical Systems). Heat-induced antigen retrieval was carried out using Cell Conditioning 1 (CC1, Ventana Medical Systems) for 48 minutes at 95°C (PD-L1) or 32 minutes (CD20-CD3 double staining).

The PD-L1 clone 22C3 (DAKO) was detected using 1/40 dilution, 1 hour at RT (DAKO). Bound antibody was visualized using the OptiView DAB Detection Kit (Ventana Medical Systems). Slides were counterstained with Hematoxylin II and Bluing Reagent (Ventana Medical Systems). Both tumor and

immune cells were scored separately. Membranous coloring was used to determine PD-L1 positivity, immune cells were in addition scored as positive when cytoplasmic coloring was present. Scoring was performed on the online platform SlideScore (www.slidescore.com).

For the double staining CD20 (yellow) followed by CD3 (purple) the CD20 was detected in the first sequence using clone L26 (1/800 dilution, 32 minutes at 37°C, Agilent/DAKO). CD20 bound antibody was visualized using Anti-Mouse NP (Ventana Medical Systems) for 12 minutes at 37°C followed by Anti-NP AP (Ventana Medical Systems) for 12 minutes at 37°C, followed by the Discovery Yellow detection kit (Ventana Medical Systems). In the second sequence of the double staining procedure CD3 was detected using clone SP7 (1:100 dilution, 32 minutes at 37°C, Thermo Scientific) and visualized using Anti-Rabbit HQ (Ventana Medical Systems) for 12 minutes at 37°C followed by Anti-HQ HRP (Ventana Medical Systems) for 12 minutes at 37°C, followed by the Discovery Purple Detection Kit (Ventana Medical Systems). Slides were counterstained with Hematoxylin and Bluing Reagent (Ventana Medical Systems). After staining, the slides were scanned with the P1000 (Sysmex).

RNA sequencing of validation cohort MIA

Haematoxylin and eosin-stained sections were assessed by a clinical pathologist and samples with >100 viable tumor cells were included for downstream analysis. The tumor location was circled for DNA/RNA microdissection. RNA was extracted using the AllPrep DNA/RNA FFPE kit (#80234, Qiagen). Quality control was assessed through RNA quantity using a Qubit 4 Fluorometer (#Q33238, Invitrogen) and quality via the TapeStation (Agilent). Samples with less than 50% RNA fragments longer than 200 nucleotides (DV200) were re-extracted using the High Pure FFPET RNA isolation kit (06650775001, Roche) before NGS library preparation.

Libraries were generated using the TruSeq RNA Exome kit (#20020189, Illumina) and quality assessed via the TapeStation system to measure the quality and quantity of libraries. Libraries with a DV200 of <50% were not sequenced. Sequencing was performed on a NovaSeq instrument (NovaSeq S2 2x100bp, Illumina) and FASTQ filed generated.

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