Supplemental information

Camrelizumab plus apatinib for patients with advanced mucosal melanoma: a single-arm study with biomarker analysis

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Biomarker sample detection methods

Immunohistochemistry (IHC)

PD-L1 expression was determined using a Dako PD-L1 IHC 22C3 pharmDx kit (Agilent Technologies) in combination with Dako Autostainer Link 48 system (Agilent Technologies) The PD-L1 expression was evavluated by tumor proportion score (TPS) and TPS ≥1% was defined as positive.

Tumor Mutation analysis

DNA extraction and targeted NGS

Formalin-fixed paraffin-embedded (FFPE) tumor samples obtained through biopsy or surgical excision were collected. 2ml of peripheral blood was collected in EDTA-coated tubes (BD) and centrifuged at 1800g for 10 min within 2h of collection to separate the plasma and white blood cells. White blood cell sediments were used for genomic DNA extraction as the germline controls.

Genomic DNA from FFPE sections and whole blood control samples were extracted with the QIAamp DNA FFPE Tissue kit and DNeasy Blood and Tissue Kit (Qiagen, USA), respectively.

For targeted-panel, customized xGen lockdown probes (Integrated DNA Technologies) targeting 425 cancer-relevant genes (Geneseeq) were used for hybridization enrichment. The target enriched library was then sequenced on the HiSeq4000 NGS platform (Illumina) with a mean coverage depth of 1000x for tumor tissue samples and 100x for matched normal blood control samples following the manufacturer’s instructions.

Sequence alignment and data processing

Base calling was performed on bcl2fastq V.2.16.0.10 (Illumina) to generate sequence reads in the FASTQ format (Illumina 1.8+encoding). Quality control was performed using the Trimmomatic software. High-quality reads were mapped to the human genome (hg19, GRCh37 Genome Reference Consortium Human Reference 37) using the Burrows-Wheeler Aligner (BWA) V.0.7.12 with BurrowsWheeler Aligner's maximal exact matches (BWA-MEM) algorithm and default parameters to create SAM files. Picard V.1.119 was used to convert SAM files to compressed BAM files, which were then sorted according to chromosome coordinates. The Genome Analysis Toolkit (GATK, V.3.4–0) was used to locally realign the BAM files at intervals with insertions/deletion (indels) mismatches and recalibrate base quality scores of reads in BAM files.

Mutation calling, CNV, TMB

Single-nucleotide variants (SNVs) and indels were identified using VarScan2, with a minimum variant allele frequency at 0.01. SNVs and indels were further filtered with the following parameters: (1) minimum mean dedup depth=30X (tissue and blood); (2) minimum base quality=15, (3) minimum variant supporting reads=3, (4) variant supporting reads mapped to both strands, (5) strand bias no greater than 10%, (6) if present in >1% population frequency in the 1000g or ExAC database and (7) through an internally collected list of recurrent sequencing errors using a normal pool of 100 samples. ANNOVAR was used to annotate mutations with variant type, dbSNP ID, clinical significance, and protein impact prediction with SIFT and PolyPhen.

Germline mutations were filtered out by comparing them to the patient's whole blood controls. Copy number variations (CNVs) were analyzed with CNVkit.14 Depth ratios of above 2.0 (tissue) and below 0.6 were considered as CNV gain and CNV loss. TMB was counted by summing all base substitutions and indels in the coding region of targeted genes, including synonymous alterations to reduce sampling noise and excluding known driver mutations as they are over-represented in the Panel, as previously described.

TCR analysis

TCR library preparation and sequencing

8ml of peripheral blood was collected in EDTA-coated tubes (BD) and centrifuged on Ficoll–paque gradients. Peripheral blood mononuclear cells (PBMC) were recovered and used for genomic DNA (gDNA) extraction with the DNeasy Blood Kit (Qiagen, USA) according to the manufacturer's instructions. The extracted gDNA samples were quantified with a Qubit 3.0 fluorometer (Thermo Fisher Scientific), and their purity was measured with a Nanodrop 2000 (Thermo Fisher Scientific). A multiplex PCR reaction was prepared using the Qiagen Multiplex PCR Plus Kit with a customized TCR primer mixture comprising 51 forward primers complementary to the V gene segments and 13 reverse primers complementary to the J gene segment. To correct for amplification bias from the multiplex PCR primers, 663 barcoded synthetic templates (i.e., a synthetic repertoire of all possible V-J combinations) were used to calibrate the PCR efficiency. These templates contain universal P5 and P7 ends for standard primer recognition, barcodes, and V and J gene segments flanking barcoded

internal markers. Amplified synthetic products and samples were then purified using the AxyPrep MAG FragmentSelect-I Kit (Axygen). Subsequently, the blood TCR library was prepared with the KAPA Hyper Prep Kit (KAPA Biosystems). Briefly, A-tailing and end-repair of fragments were performed before the ligation of index adaptors from the TruSeq DNA PCR-free Library Prep Kit (Illumina). Purified ligation products were then amplified with Illumina p5 and p7 primers with KAPA HiFi HotStartReadyMix (KAPA Biosystems), followed by a final purification step using Axygen beads. TCR libraries were sequenced using the Illumina HiSeq 4000 platform according to the manufacturer's instructions

TCR analysis and profiling.

Trimmomatic was used to remove adaptors and filter low-quality reads from FASTQ files. Non–V-J paired reads were further removed using Cutadapt (V 1.18). Next, paired-end read merger (PEAR, V 0.9.10) was employed to merge paired reads, and nonbarcoded reads were removed for synthetic standards. Clean reads were subsequently assembled using MiXCR (V 2.1.11). Reads were aligned to reference V or J gene segments according to the international ImMunoGeneTics database. Clonotypes, defined as unique antigen-recognizing CDR3 sequences assembled from specific usage of VJ gene segments, were then built from the alignments using the assemble pipeline of the software. TCR clonality describes the diversity of clonotypes of a TCR population. For final repertoire profiling, sample V/J counts, CDR3 counts, and clonality counts were calculated with normalization using corresponding counts from synthetic standards.

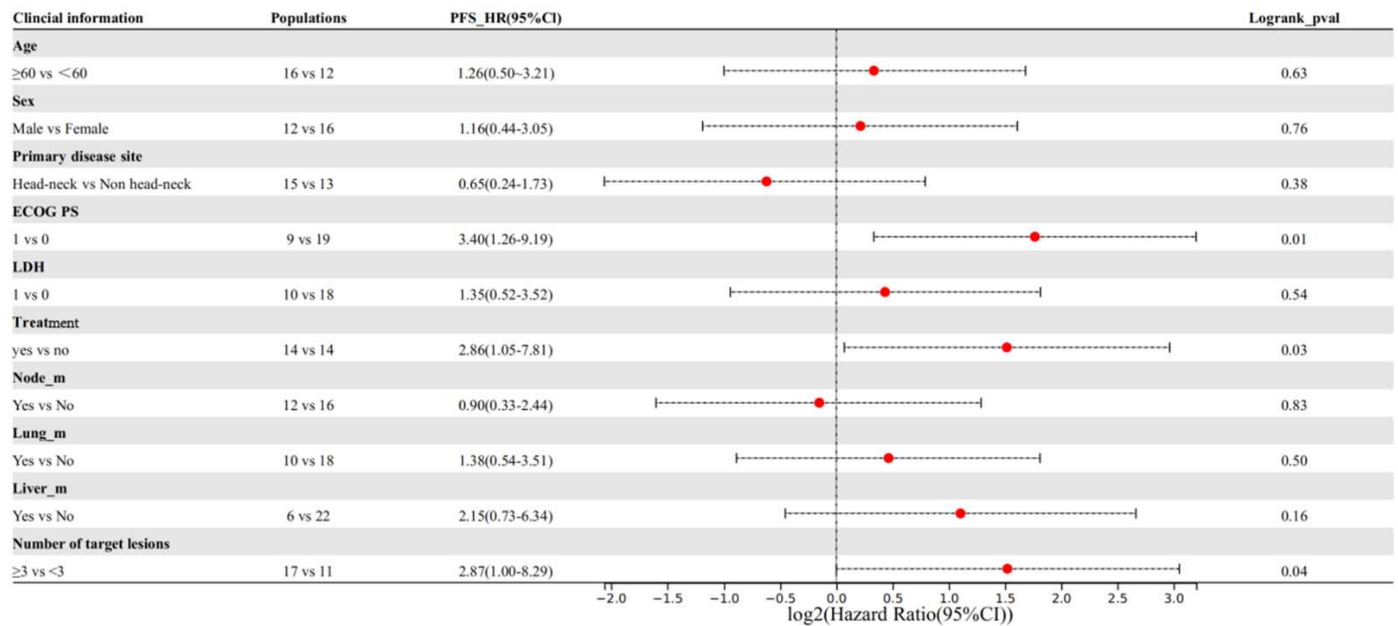
TCR clonality analysis was performed using common diversity measures from the R package vegan. Specifically, the **diversity** of the TCR repertoire was assessed by the Shannon index (*H*):

$$H = -\sum p_i \ln p_i$$

where p_i is the proportion of individuals found in the ith species and ln represents the natural logarithm. **Clonality** was defined as 1-Pielou's Evenness (J), where J is normalized Shannon's entropy to measure the distribution of the TCR population calculated by dividing the Shannon index (H') by the total number of species in a sample (S):

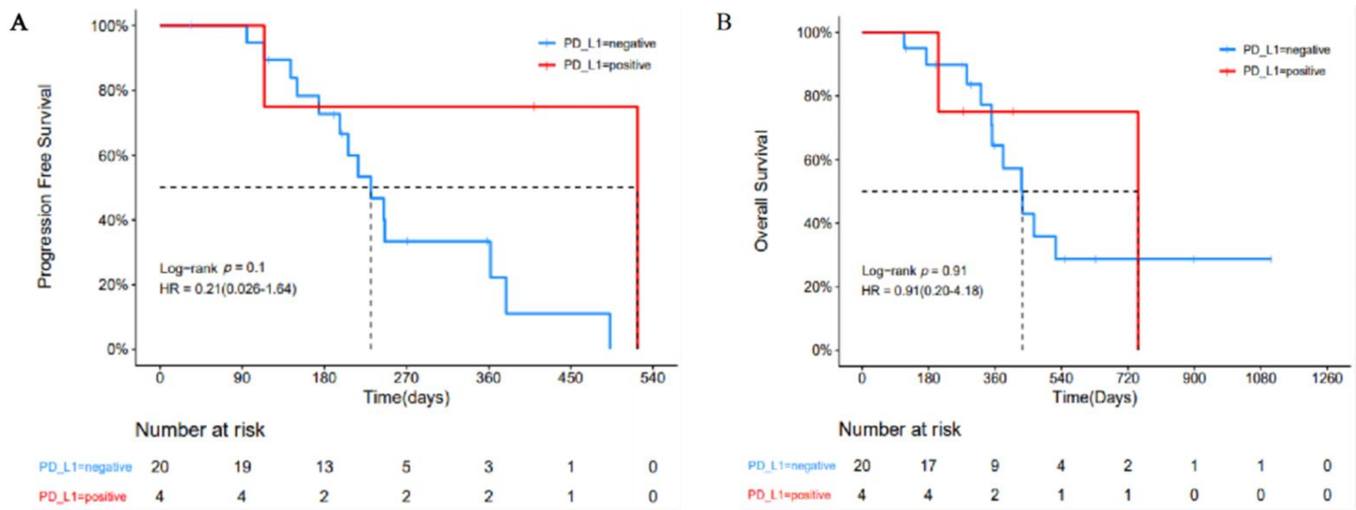
$$J = \frac{H'}{ln\,S}$$

Fig. S1 Forest plot of clinical variables with PFS



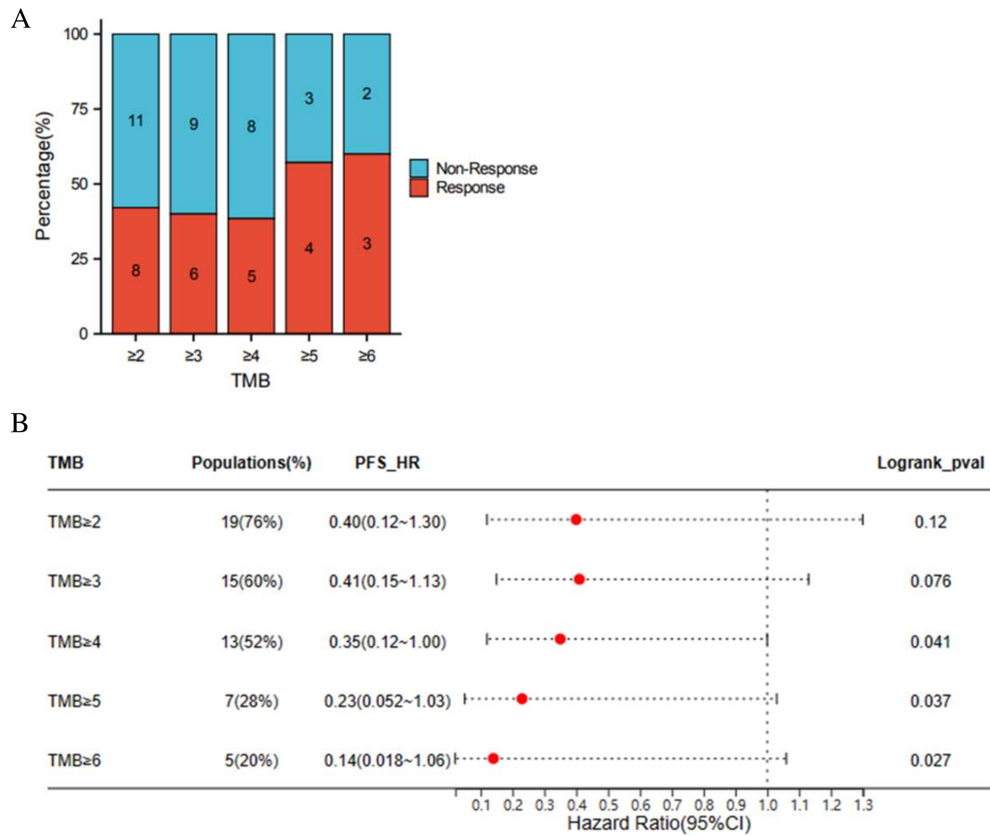
Patients with ECOG PS 0, treatment-naïve status, <3 target lesions showed prolonged PFS. ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; Node_m, lymphnode metastases; Lung_m, lung metastases; Liver_m, liver metastases.

Fig. S2 PFS and OS in relation to biomarkers include tumor PD-L1 expression



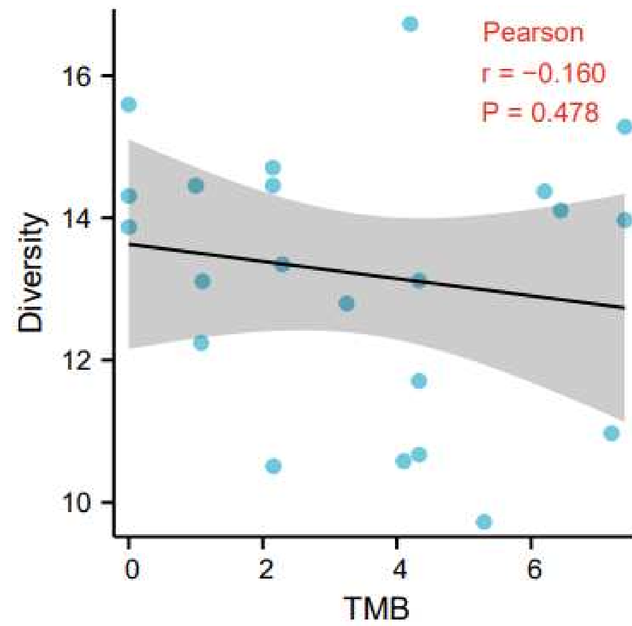
PD-L1 positive status was defined as the presence of membrane staining of any intensity in 1% or more of tumor cells or immune cells by 22C3 immunohistochemistry staining. TMB, tumor mutational burden; PFS, progression free survival; OS, overall survival; PD-L1, programmed death ligand-1.

Fig.S3 ORR and PFS in patients with TMB at cut-offs of 2, 3, 4, 5 or 6



TMB, tumor mutational burden; ORR, objective response rate. PFS, progression free survival.

Fig. S4 Correlation between TMB and TCR



No correlation was observed between TCR diversity in peripheral blood and TMB level in tumor tissue. TCR, T cell receptor; TMB, tumor mutational burden.

Table S1. Antitumor activity based on ITT (n=32)

Antitumor Activity	**N=32**
Confirmed ORR, No. (%)	12 (37.5)
95% CI	21.1-56.3
DCR, No. (%)	23 (71.9)
Best overall response, No. (%)	
CR	1 (3.1)
PR	11 (34.4)
SD	11 (34.4)
PD	5 (15.6)
NE	4 (12.5)

CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; NE: not evaluable; CI: confidence interval. ORR: objective response rate; DCR: disease control rate; ORR: the proportion of patients with a CR or PR. DCR: the proportion of patients with a CR, PR or SD. Cases not evaluable were those in which patients did not have at least one post-baseline tumor assessment.

Table S2. Antitumor response in treatment-naïve and previously treated patients

Antitumor Activity	**Untreated** **N=14**	**Treated** **N=14**
ORR, No. (%)	6 (42.9)	6 (42.9)
DCR, No. (%)	12 (85.7)	11 (78.6)
Best overall response, No. (%)		
CR	1（7.1）	0（0）
PR	5（35.7）	6（42.9）
SD	6（42.9）	5（35.7）
PD	2（14.3）	3（21.4）

Table S3. irAEs >10%

irAEs	**Grade 1-2**	**Grade 3**	**Total**
Hypothyroidism	14 (43.8)	0 (0.0)	14 (43.8)
Rash	10 (31.3)	4 (12.5)	14 (43.8)
Fevers	4 (12.5)	0 (0.0)	4 (12.5)

irAEs: immune-related adverse events. Data was shown as n (%). No ≥grade 4 irAEs was observed.

Table S4. Apatinib related AEs in >10% patients.

Apatinib related AEs	Grade 1-2	Grade 3	Total
Transaminase elevation	12 (37.5)	8 (25.0)	20 (62.5)
Hypertension	19 (59.4)	1 (3.1)	20 (62.5)
Fatigue	18 (56.3)	0 (0.0)	18 (56.3)
Thrombocytopenia	17 (53.1)	1 (3.1)	18 (56.3)
Leukocytopenia	17 (53.1)	0 (0.0)	17 (53.1)
Hand-foot syndrome	16 (50.0)	1 (3.1)	17 (53.1)
Decreased appetite	17 (53.1)	0 (0.0)	17 (53.1)
Proteinuria	13 (40.6)	2 (6.3)	15 (46.9)
Hypoalbuminemia	13 (40.6)	0 (0.0)	13 (40.6)
Neutropenia	13 (40.6)	0 (0.0)	13 (40.6)
Nausea	12 (37.5)	0 (0.0)	12 (37.5)
Mucositis oral	11 (34.4)	0 (0.0)	11 (34.4)
Hyperbilirubinemia	7 (21.9)	3 (9.4)	10 (31.3)
Epistaxis	8 (25.0)	0 (0.0)	8 (25.0)
Diarrhea	7 (21.9)	1 (3.1)	8 (25.0)
Vomiting	7 (21.9)	0 (0.0)	7 (21.9)

AEs: adverse events. Data was shown as n (%). No ≥grade 4 apatinib related AEs was observed.

Table S5. Correlation between ORR and TMB at cut-offs of 2, 3, 4, 5 or 6

TMB	ORR, n (%)
≥2	42.1% (8/19)
≥3	40.0% (6/15)
≥4	38.5% (5/13)
≥5	57.1% (4/7)
≥6	60.0% (3/5)
P-value	0.088

TMB, tumor mutational burden; ORR, objective response rate.

Table S6. Gene variants in correlation to survival outcomes.

Gene	Population(%)	ORR_OR(95% Cl)	pval	PFS_HR(95% CI)	Logrank_pval	OS_HR(95% CI)	Logrank_pval
MYC	9(34.6%)	0.58(0.07-3.88)	0.68	1.32(0.50~3.53)	0.58	1.38(0.49~3.91)	0.54
NRAS	8(30.8%)	0.76(0.09-5.42)	1.00	1.24(0.45~3.42)	0.68	0.90(0.28~2.82)	0.85
PTK2	8(30.8%)	1.54(0.21-11.52)	0.68	0.887(0.31~2.54)	0.82	2.27(0.80~6.46)	0.12
NF1	5(19.2%)	0.89(0.06-9.63)	1.00	1.03(0.29~3.71)	0.96	1.39(0.38~5.01)	0.62
ATM	4(15.4%)	1.42(0.09-23.13)	1.00	0.46(0.10~2.05)	0.30	1.27(0.35~4.58)	0.72
MCL1	4(15.4%)	4.92(0.33-294.24)	0.28	0.77(0.22~2.72)	0.68	1.23(0.38~3.91)	0.73
NSD1	4(15.4%)	1.42(0.09-23.13)	1.00	1.36(0.30~6.16)	0.69	1.66(0.35~7.86)	0.52
PTEN	4(15.4%)	0.41(0.01-6.12)	0.61	1.38(0.38~5.03)	0.63	2.12(0.56~8.08)	0.26
SDHD	4(15.4%)	4.92(0.33-294.24)	0.28	0.69(0.20~2.46)	0.57	1.25(0.39~3.96)	0.71
VEGFA	4(15.4%)	1.42(0.09-23.13)	1.00	1.32(0.37~4.74)	0.67	3.47(0.85~14.1)	0.06
CCND1	3(11.5%)	Inf(0.61-Inf)	0.06	0.65(0.14~2.99)	0.58	0.45(0.058~3.48)	0.43
KIT	3(11.5%)	Inf(0.61-Inf)	0.06	0.36(0.047~2.73)	0.30	0.99(0.13~7.87)	0.99

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TERT	3(11.5%)	0.66(0.01-14.43)	1.00	3.26(0.84~12.7)	0.07	1.68(0.47~6.03)	0.42
BRAF	2(7.7%)	1.38(0.02-117.52)	1.00	0.42(0.052~3.47)	0.41	0.78(0.17~3.56)	0.75
CDK4	2(7.7%)	Inf(0.26-Inf)	0.17	2.19(0.46~10.4)	0.31	1.32(0.29~5.96)	0.71
CDKN2A	2(7.7%)	1.38(0.02-117.52)	1.00	1.05e-07(0~Inf)	0.67	0(0~Inf)	0.25
CDKN2B	2(7.7%)	1.38(0.02-117.52)	1.00	1.61(0.20~12.6)	0.65	0.67(0.088~5.19)	0.70
ERBB3	2(7.7%)	Inf(0.26-Inf)	0.17	2.19(0.46~10.4)	0.31	1.32(0.29~5.96)	0.71
FGFR1	2(7.7%)	Inf(0.26-Inf)	0.17	2.58(0.55~12.2)	0.21	1.69(0.37~7.68)	0.49
KRAS	2(7.7%)	Inf(0.26-Inf)	0.17	0.35(0.044~2.79)	0.30	1.06(0.23~4.83)	0.94
MDM2	2(7.7%)	Inf(0.26-Inf)	0.17	2.19(0.46~10.4)	0.31	1.32(0.29~5.96)	0.71
MET	2(7.7%)	1.38(0.02-117.52)	1.00	0(0~Inf)	0.30	0(0~Inf)	0.17
NOTCH2	2(7.7%)	Inf(0.26-Inf)	0.17	0(0~Inf)	0.26	0(0~Inf)	0.45
TP53	2(07.7%)	1.38(0.02-117.52)	1.00	1.1(0.25~4.91)	0.90	0.67(0.088~5.19)	0.70