**SUPPLEMENTARY MATERIALS**

**Supplementary Table S1.** Clinicopathologic characteristics of the cohort separated by death at the 5-year time point.

**Supplementary Table S2.** Primary and secondary antibody information and TSA dye pairings for multiplex IF panel.

**Supplementary Table S3.** Twenty highest ranking variables associating with overall survival by univariate Cox regression analysis.

**Supplementary Table S4.** Density and proximity variables selected by least absolute shrinkage and selection operator (LASSO) methods using a 5-fold cross-validation.

**Supplementary Figure S1.** Image cytometry to quantify immune cell biomarkers in metastatic melanoma.

**Supplementary Figure S2.** The expression of PD-L1 on tumor cells and ‘Other’ cells is determined by their proximity to CD8+ cells.

**Supplementary Figure S3.** Characterization of immune neighborhoods associated with overall survival.

**Supplementary Computational Methods.**  Survival and clinical statistical analysis - R markdown document.

**Supplementary Table S1. Clinicopathologic characteristics of the cohort separated by death at the 5 year time point**

|  |  |  |  |
| --- | --- | --- | --- |
| Median Age (years): 56 ± 23  Number of patients alive at last date of follow-up: 29 (31%) | | | |
|  | **Patients alive at 5 years**  **(n=34)** | **Patients deceased at 5 years**  **(n=59)** | **p-valuea** |
| **Stage** |  |  |  |
| III (n=39) | 18 (53%) | 21 (36%) | 0.13 |
| IV (n=54) | 16 (47%) | 38 (64%) |
| **Tumor mutation status** |  |  |  |
| BRAFV600E (n=2) | 1 (3%) | 1 (2%) | 1.0 |
| Negative BRAFV600E (n=61) | 22 (65%) | 39 (66%) |
| Unknown (n=30) | 11 (32%) | 19 (32%) | - |
| **Serum lactate dehydrogenase (range 140-280 U/L)** |  |  |  |
| <280 (n=35) | 12 (35%) | 23 (39%) | 0.40 |
| >280 (n=7) | 1 (3%) | 6 (10%) |
| unknown (n=51) | 21 (62%) | 30 (51%) | - |
| **Site of metastasis and biopsy** |  |  |  |
| Lymph node (n=45) | 18/34 (53%) | 27 (46%) | 0.79 |
| Skin / soft tissue (n=22) | 8 (24%) | 14 (24%) |
| Lung (n=13) | 5 (15%) | 8 (14%) |
| GI tract (n=7) | 1 (3%) | 6 (10%) |
| Salivary Gland (n=4) | 2 (6%) | 2 (3%) |
| Brain (n=2) | 0 (0%) | 2 (3%) |
| **Prior therapies** |  |  |  |
| Surgery (n=60) | 23 (68%) | 37 (63%) | 0.61 |
| Radiation (n=12) | 6 (18%) | 6 (10%) | 0.54 |
| Chemotherapy (includes temozolomide) (n=6) | 3 (9%) | 3 (5%) | 0.83 |
| Biologic therapy (IL-2b, INFc, or imiquimod) (n=12) | 5 (15%) | 7 (12%) | 0.95 |
| Immunotherapy (vaccine, checkpoint inhibitor) (n=2) | 0 (0%) | 2 (3%) | 0.76 |
| Unknown (n=31) | 11 (32%) | 20 (34%) | - |
| **Subsequent therapies** |  |  |  |
| Surgery (n=2) | 1 (3%) | 1 (2%) | 1.00 |
| Radiation (n=26) | 5 (15%) | 21 (36%) | 0.04 |
| Chemotherapy (includes temozolomide) (n=19) | 7 (21%) | 12 (20%) | 1.00 |
| Biologic therapy (IL-2b, INFc, and GM-CSF) (n=17) | 8 (24%) | 9 (15%) | 0.53 |
| Immunotherapy (vaccine, checkpoint inhibitor)(n=17) | 6 (18%) | 11 (19%) | 1.00 |
| Targeted therapy (BRAF or PARP inhibitor) (n=3) | 0 (0%) | 3 (5%) | 0.63 |
| Unknown (n=43) | 16 (47%) | 27 (46%) | - |

a Fisher’s Exact test, 2-sided; b interleukin-2; c interferon

**Table S2. Primary and secondary antibody information and TSA dye pairings for multiplex IF panel.**

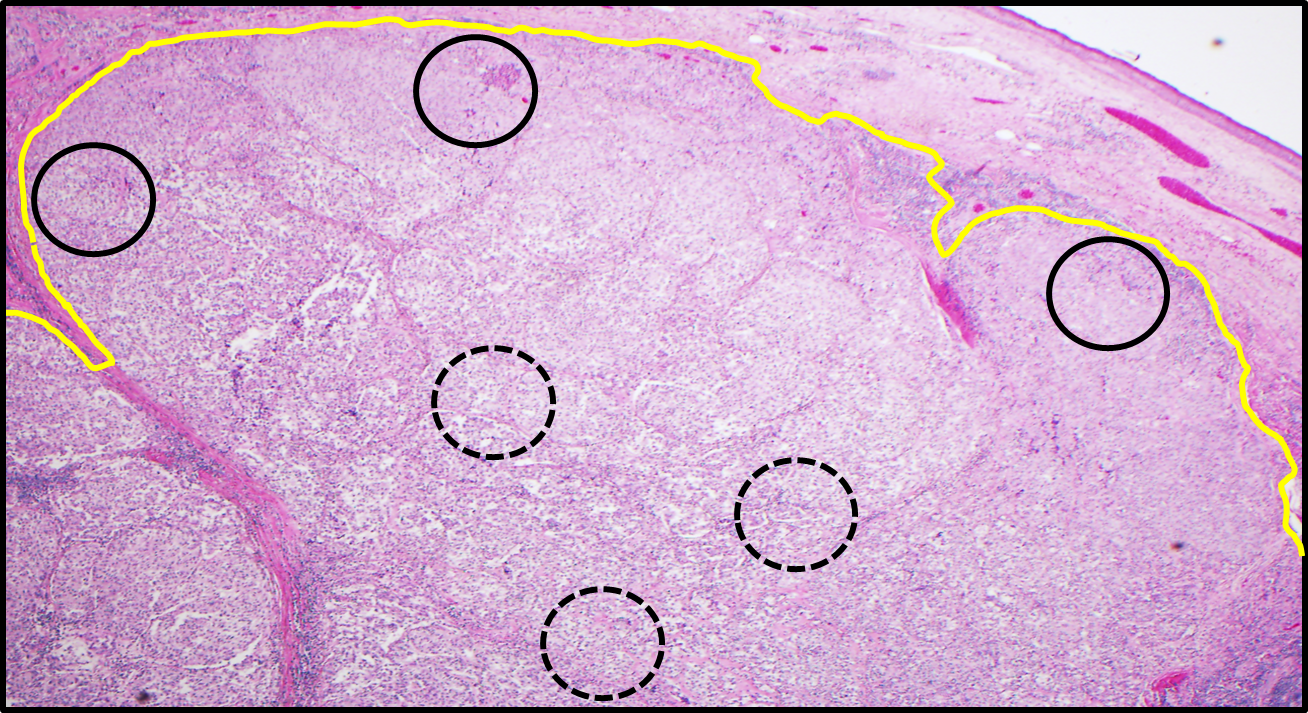
|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Position | Antibody | Clone (host)/Company | Dilution | Incubation (min) | Secondary/ | TSA dye | Dilution |
| Company |
| 1 | FoxP3 | 236A/E7(mouse)/ | 1:100 | 30 | Powervision 50%/Leica | 570 | 1:200 |
| Affymetrix |
| 2 | CD8 | 4B11(mouse)/BioRad | 1:100 | 30 | Opal Polymer/Akoya | 540 | 1:100 |
| 3 | Sox10/S100 | BC34(Mouse)/BioCare & 15E2E2+4C4.9 (Mouse)/LS Bio | 1:200/1:3200 | 60 | Opal Polymer/Akoya | 620 | 1:100 |
| 4 | PD-1 | EPR4877(2) (rabbit)/AbCam | 1:4000 | 30 | Powervision 50%/Leica | 650 | 1:100 |
| 5 | PD-L1 | SP142 (rabbit)/Spring Bio | 1:400 | 60 | Powervision 50%/Leica | 520 | 1:100 |
| 6 | CD163 | 10D6(mouse)/Leica | 1:100 | 120 | Opal Polymer/Akoya | 690 | 1:50 |
| 7 | DAPI | Akoya Opal 7-color kit | 2 drops/ml | 5 | N/A | N/A | 1:500 |

**Supplementary Table S3. Twenty highest ranking variables associating with overall survival by univariate Cox regression analysis.** Atotal of 557 clinicopathologic variables, cell density and proximity measurements were tested.

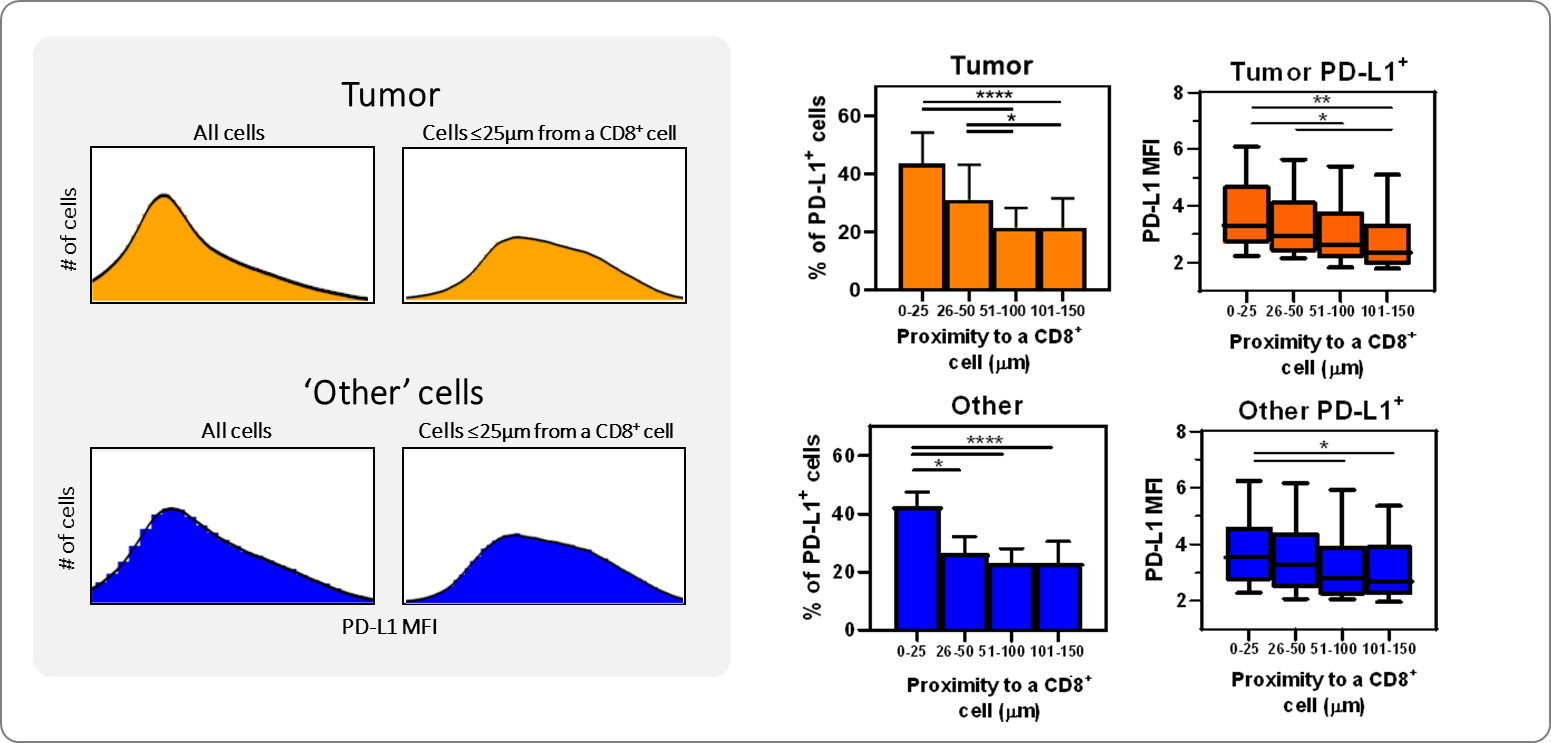
|  |  |  |  |
| --- | --- | --- | --- |
|  | **Variable** | **P value** | **HR** |
| 1 | CD163+PD-L1- cells/mm2 ≤25um from a CD163+ | 1.002 | 0.00004 |
| 2 | CD163+PD-L1- cells/mm2 ≤25um from a CD163+PD-L1- | 1.002 | 0.00005 |
| 3 | CD163+ cells/mm2 ≤25um from a CD163+PD-L1- | 1.001 | 0.00008 |
| 4 | CD8+PD-L1+ cells/mm2 ≤25um from a Tumor PD-L1- | 0.994 | 0.0008 |
| 5 | Tumor cells/mm2 ≤25um from a PD-1+ | 0.999 | 0.0008 |
| 6 | CD8+PD-L1+ cells/mm2 ≤25um from an 'Other' PD-L1- | 0.996 | 0.0009 |
| 7 | Tumor cells/mm2 ≤25um from an ‘Other’ PD-1+ | 0.998 | 0.0010 |
| 8 | CD8+ cells/mm2 ≤25um from a PD-L1+ | 0.999 | 0.0011 |
| 9 | CD163+PD-L1- cells/mm2 | 1.001 | 0.0011 |
| 10 | PD-1+ cells/mm2 ≤25um from a CD8+PD-L1+ | 0.999 | 0.0012 |
| 11 | CD163+PD-L1- cells/mm2 ≤25um from an ‘Other’ | 1.002 | 0.0012 |
| 12 | CD8+ cells/mm2 ≤25um from a CD8+PD-L1+ | 0.999 | 0.0012 |
| 13 | CD8+PD-L1+ cells/mm2 ≤25um from an 'Other' PD-1+ | 0.997 | 0.0012 |
| 14 | CD8+ cells/mm2 ≤25um from an 'Other' PD-L1+ | 0.999 | 0.0013 |
| 15 | CD8+PD-L1+ cells/mm2 ≤25um from an 'Other' | 0.998 | 0.0017 |
| 16 | CD8+PD-L1+ cells/mm2 ≤25um from a CD8+PD-L1- | 0.997 | 0.0017 |
| 17 | CD8+PD-L1+ cells/mm2 ≤25um from a PD-1+ | 0.998 | 0.0017 |
| 18 | CD8+PD-L1+ cells/mm2 ≤25um from a PD-L1+ | 0.998 | 0.0018 |
| 19 | CD8+ cells/mm2 | 0.999 | 0.0018 |
| 20 | CD8+PD-1- cells/mm2 ≤25um from a CD8+PD-L1+ | 0.996 | 0.0018 |

**Table S4. Density and proximity variables selected by least absolute shrinkage and selection operator (LASSO) methods using a 5-fold cross-validation.** A total of 555 clinicopathologic variables, cell density and proximity measurements were tested (columns with ‘NA’ values were excluded i.e., ‘size of largest tumor deposit’ and ‘presence of extracapsular extension’).

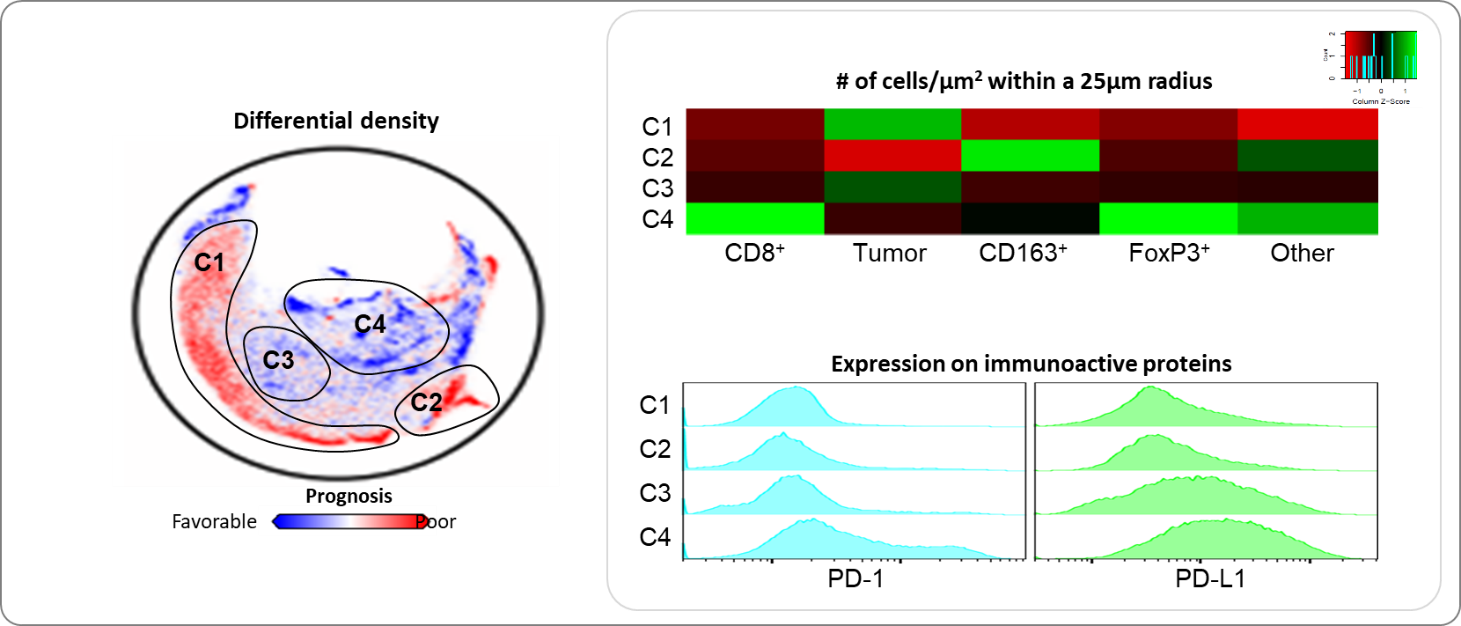
|  |  |
| --- | --- |
|  | **Variable** |
| 1 | CD163+PD-L1- cells/mm2 |
| 2 | CD163+PD-L1- cells/mm2 ≤25um from a CD163+ |
| 3 | Tumor cells/mm2 ≤25um from an 'Other' PD-1+ |
| 4 | CD8+PD-L1+ cells/mm2 ≤25um from an 'Other' PD-1+ |
| 5 | CD8+PD-1- cells/mm2 ≤25um from a CD8+PD-L1+ |
| 6 | Other' PD-L1- cells/mm2 ≤25um from a CD8+PD-L1+ |
| 7 | CD8+PD-L1+ cells/mm2 ≤25um from an 'Other' PD-L1- |
| 8 | CD8+PD-L1+ cells/mm2 ≤25um from a Tumor PD-L1- |

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**Supplementary Figure S1. Image cytometry to quantify immune cell biomarkers in metastatic melanoma.** Photomicrograph demonstrating approach to tissue microarray (TMA) construction. Representative hematoxylin and eosin-stained slide showing a melanoma metastasis in the dermis and subcutaneous tissue (below yellow line). Tissue microarrays were designed to include punches from both the central (dotted black lines) and peripheral (solid black lines) aspects of tumor deposits.

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**Supplementary Figure S2. The expression of PD-L1 on tumor cells and ‘Other’ cells is determined by their proximity to CD8+ cells.** Left upper panel: PD-L1 MFI histograms for all tumor cells (left) and tumor cells located ≤25μm from a CD8+ lymphocyte (right). Left lower panel: PD-L1 MFI histograms for all ‘Other’ cells (left) and ‘Other’ cells located ≤25μm from a CD8+ lymphocyte (right). Right panels: The percentage of tumor cells and ‘Other’ cells displaying PD-L1 as well as the PD-L1 expression intensity increased with proximity to a CD8+ cell.

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**Supplementary Figure S3. Characterization of immune neighborhoods associated with overall survival.** *Left panel:* Differences between the densities were used to identify spatial immune clusters with positive or negative prognostic impact. Numerous spatial clusters associated with clinical outcome were identified by this method, and four of the most prominent ones were selected (C1-C4). *Right panel:* These four clusters were further characterized for their mIF expression patterns. The heat map shows the average density (number of cells per μm2 within a 25 μm radius) of the five cell ‘lineages’ within each cluster. The histograms show PD-1 and PD-L1 expression for the cells in each cluster, highlighting the heterogeneous expression intensity across distinct spatial neighborhoods.

**Supplementary Computational Methods. Survival and clinical statistical analysis R markdown document**

## Cox regression

# read in mIF variables   
masterTab = read.csv('TMA\_Melanoma\_Master\_Table\_552variables\_5.11.2021\_de.identified.csv', row.names = 1, stringsAsFactors = F)  
dim(masterTab)

## [1] 93 552

# read in clinical covariates  
clinicalInfo = read.csv('Clinical.data\_5.12.2021\_de.identified.csv', row.names = 1, stringsAsFactors = F)  
dim(clinicalInfo)

## [1] 93 20

# check that all patients have clinical info  
all(rownames(masterTab) %in% rownames(clinicalInfo))

## [1] TRUE

# create survival object  
survObj5y = Surv(as.numeric(clinicalInfo[,"Follow\_up\_time\_5Y"]),  
 as.logical(as.numeric(clinicalInfo[,"Death\_5Y"])))  
rownames(survObj5y) = rownames(clinicalInfo)  
  
# convert stage to factor for the further analysis  
clinicalInfo$Stage = factor(clinicalInfo$Stage)  
clinicalInfo$Location = factor(clinicalInfo$Location)  
  
# clinical covariates to include in the analysis  
covToInclude = c("Location", "Age", "Stage", "Size", "Extracapsular")  
# markers to include in the analysis  
markers = cbind(clinicalInfo[,covToInclude], masterTab)  
  
  
# run cox regression for every marker  
tab = t(sapply(colnames(markers),function(x) {  
# print(x)  
 summary(coxph(survObj5y ~ markers[,x]))$coeff[1,c(2,5)]  
 }))  
  
tab = tab[order(tab[,2]),]  
tab = cbind(tab, FDR = p.adjust(tab[,2]))  
write.csv(tab, file = paste0('coxRes\_',Sys.Date(),'.csv'), quote = F)  
print(head(tab))

## exp(coef) Pr(>|z|) FDR  
## CD163.PDL1neg\_25um\_CD163 1.0019009 4.015989e-05 0.02236906  
## CD163.PDL1neg\_25um\_CD163PDL1neg 1.0018811 4.886338e-05 0.02716804  
## CD163\_25um\_CD163PDL1neg 1.0014520 8.565749e-05 0.04753991  
## CD8.PDL1pos\_25um\_TumorPDL1neg 0.9941012 7.669981e-04 0.42491697  
## Tumor\_25um\_PD1pos 0.9993547 7.950757e-04 0.43967689  
## CD8.PDL1pos\_25um\_OtherPDL1neg 0.9960540 9.026776e-04 0.49827805

#datatable(tab)

## LASSO analysis

#================  
# cox with lasso  
# convert to numeric matrix for further analysis  
dat = markers  
dat$Location = as.numeric(dat$Location)  
dat = t(do.call('rbind',dat))  
dat = t(apply(dat,1,as.numeric))  
colnames(dat) = colnames(markers)  
  
# remove columns with NAs  
s = apply(is.na(dat),2,sum)  
which(s>0)

## Size Extracapsular   
## 4 5

dat = dat[, -which(s>0)]  
  
  
# run LASSO  
glmRes = glmnet(as.matrix(dat), survObj5y, family = 'cox', alpha = 1)  
  
# run cross-validation  
set.seed(123456)  
glmRes\_cv = cv.glmnet(as.matrix(dat), survObj5y, family = 'cox', alpha = 1, nfolds = 5)  
# select threshold from cross-validation results  
c1 = coef(glmRes, s = glmRes\_cv$lambda.min)  
# select top probes using this threshold  
topLasso = rownames(c1)[which(c1 != 0)]  
print(topLasso)

## [1] "PDL1neg\_CD163" "CD163.PDL1neg\_25um\_CD163"   
## [3] "Tumor\_25um\_OtherPD1pos" "CD8.PDL1pos\_25um\_OtherPD1pos"   
## [5] "CD8.PD1neg\_25um\_CD8PDL1pos" "Other.PDL1neg\_25um\_CD8PDL1pos"  
## [7] "CD8.PDL1pos\_25um\_OtherPDL1neg" "CD8.PDL1pos\_25um\_TumorPDL1neg"

## Fisher's test of clinical covariates by death at the 5-year time point

# death at the 5-year time point  
table(clinicalInfo$Death\_5Y)

##   
## 0 1   
## 34 59

# summary of age  
summary(clinicalInfo$Age)

## Min. 1st Qu. Median Mean 3rd Qu. Max.   
## 8.20 46.00 57.00 56.36 69.00 87.00

# threshold for Serum\_LDH  
clinicalInfo$LDH = as.numeric(clinicalInfo$Serum\_LDH) < 280  
  
# calculate Fisher's test for each clinical covariate of interest  
# by the death status  
  
# save Fisher's test p-values  
pvals = c()  
# location (run separately because it's a character variable)  
i = "Location"  
m = table(data.frame(cov = (clinicalInfo[,i]), Death\_5Y = clinicalInfo$Death\_5Y), useNA = 'ifany')  
print(m)

## Death\_5Y  
## cov 0 1  
## Brain 0 2  
## GI\_tract 1 6  
## LN 18 27  
## Lung 5 8  
## Salivary gland 2 2  
## Skin 2 6  
## Soft tissue 6 8

# run Fisher's test  
res = fisher.test(m)  
# print p-value  
print(c("p-value:",res$p.value))

## [1] "p-value:" "0.778519359691425"

pvals = c(pvals,res$p.value)  
  
# covariates of interest  
cov = c(3,8,10:21)  
# iterate covariates, calculate Fisher's test and save p-values  
for(i in cov)  
{  
 print(colnames(clinicalInfo)[i])  
 # table of covariates by death status  
 m = table(data.frame(cov = as.numeric(clinicalInfo[,i]), Death\_5Y = clinicalInfo$Death\_5Y), useNA = 'ifany')  
 print(m)  
 # run Fisher's test  
 res = fisher.test(m)  
 # print p-value  
 print(c("p-value:",res$p.value))  
 pvals = c(pvals,res$p.value)  
}

## [1] "Stage"  
## Death\_5Y  
## cov 0 1  
## 1 18 21  
## 2 16 38  
## [1] "p-value:" "0.128399580749993"  
## [1] "BRAFV600E"  
## Death\_5Y  
## cov 0 1  
## 0 22 39  
## 1 1 1  
## <NA> 11 19  
## [1] "p-value:" "0.999999999999992"  
## [1] "Prior\_Surgery"  
## Death\_5Y  
## cov 0 1  
## 0 0 3  
## 1 23 37  
## <NA> 11 19  
## [1] "p-value:" "0.615567569689784"  
## [1] "Prior\_Radiation"  
## Death\_5Y  
## cov 0 1  
## 0 17 34  
## 1 6 6  
## <NA> 11 19  
## [1] "p-value:" "0.540574279031744"  
## [1] "Prior\_Chemotherapy"  
## Death\_5Y  
## cov 0 1  
## 0 20 37  
## 1 3 3  
## <NA> 11 19  
## [1] "p-value:" "0.826917104558948"  
## [1] "Prior\_BiologicTherapy"  
## Death\_5Y  
## cov 0 1  
## 0 18 33  
## 1 5 7  
## <NA> 11 19  
## [1] "p-value:" "0.953619667297429"  
## [1] "Prior\_Vaccine.ICPI"  
## Death\_5Y  
## cov 0 1  
## 0 23 38  
## 1 0 2  
## <NA> 11 19  
## [1] "p-value:" "0.759992633729355"  
## [1] "Post\_Surgery"  
## Death\_5Y  
## cov 0 1  
## 0 17 31  
## 1 1 1  
## <NA> 16 27  
## [1] "p-value:" "0.999999999999992"  
## [1] "Post\_Radiation"  
## Death\_5Y  
## cov 0 1  
## 0 13 11  
## 1 5 21  
## <NA> 16 27  
## [1] "p-value:" "0.0380821069746092"  
## [1] "Post\_Chemotherapy"  
## Death\_5Y  
## cov 0 1  
## 0 11 20  
## 1 7 12  
## <NA> 16 27  
## [1] "p-value:" "1"   
## [1] "Post\_Biologic"  
## Death\_5Y  
## cov 0 1  
## 0 10 23  
## 1 8 9  
## <NA> 16 27  
## [1] "p-value:" "0.534006416195805"  
## [1] "Post\_Vaccine.ICPI"  
## Death\_5Y  
## cov 0 1  
## 0 12 21  
## 1 6 11  
## <NA> 16 27  
## [1] "p-value:" "1"   
## [1] "Post\_TT"  
## Death\_5Y  
## cov 0 1  
## 0 18 29  
## 1 0 3  
## <NA> 16 27  
## [1] "p-value:" "0.631378379771334"  
## [1] "LDH"  
## Death\_5Y  
## cov 0 1  
## 0 1 6  
## 1 12 23  
## <NA> 21 30  
## [1] "p-value:" "0.403796709085223"

names(pvals) = c("Location",colnames(clinicalInfo)[cov])  
print(pvals)

## Location Stage BRAFV600E   
## 0.77851936 0.12839958 1.00000000   
## Prior\_Surgery Prior\_Radiation Prior\_Chemotherapy   
## 0.61556757 0.54057428 0.82691710   
## Prior\_BiologicTherapy Prior\_Vaccine.ICPI Post\_Surgery   
## 0.95361967 0.75999263 1.00000000   
## Post\_Radiation Post\_Chemotherapy Post\_Biologic   
## 0.03808211 1.00000000 0.53400642   
## Post\_Vaccine.ICPI Post\_TT LDH   
## 1.00000000 0.63137838 0.40379671

## R version 4.0.3 (2020-10-10)  
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## Running under: Windows 10 x64 (build 19041)  
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## Matrix products: default  
##   
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## [2] LC\_CTYPE=English\_United States.1252   
## [3] LC\_MONETARY=English\_United States.1252  
## [4] LC\_NUMERIC=C   
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##   
## other attached packages:  
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## [6] survival\_3.2-7  
##   
## loaded via a namespace (and not attached):  
## [1] knitr\_1.31 magrittr\_2.0.1 splines\_4.0.3 tidyselect\_1.1.0   
## [5] lattice\_0.20-41 R6\_2.5.0 rlang\_0.4.10 foreach\_1.5.1   
## [9] stringr\_1.4.0 tools\_4.0.3 grid\_4.0.3 xfun\_0.21   
## [13] DBI\_1.1.1 ellipsis\_0.3.1 htmltools\_0.5.1.1 iterators\_1.0.13   
## [17] assertthat\_0.2.1 yaml\_2.2.1 digest\_0.6.27 tibble\_3.0.6   
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## [25] xlsxjars\_0.6.1 htmlwidgets\_1.5.3 vctrs\_0.3.6 codetools\_0.2-18   
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