**Hypothesis:** Sex-based heterogeneity in the TME and antitumor response exists and contributes to more favorable outcomes in males after ICI therapy

**Analysis Plan**

* Focus on primary cutaneous melanoma (N=366):
  + Exclude: ocular (N=6), mucosal (N=6), unknown (N=1)
* Tumor microenvironment
  + Primary cutaneous melanoma with bulk RNA sequencing (N=?): TODO: Does primary cutaneous melanoma have bulk RNA sequencing if there is a corresponding row in clinical molecular linkage data with a value in column “RNA Batch”?
    - Immune cell infiltrate
    - CD8+ T cell phenotype
    - GSEA of immune-related gene signatures
    - TIDE assessment
  + Primary cutaneous melanoma with whole exome sequencing (N=352)
    - Tumor mutational burden
* Clinical outcomes

|  |  |  |  |
| --- | --- | --- | --- |
| **Characteristic, N (%)** | **Male (N=)3** | **Female (N=)3** | **Total (N=)** |
| **Sequencing data1** |  |  |  |
| **WES only1** |  |  |  |
| **RNAseq only1** |  |  |  |
| **WES and RNAseq1** |  |  |  |
| **Specimen collection site1** |  |  |  |
| **Skin and other soft tissues1,a** |  |  |  |
| **Lymph node1** |  |  |  |
| **Lung1** |  |  |  |
| **Abdominal viscera1,b** |  |  |  |
| **Brain1** |  |  |  |
| **Bone1** |  |  |  |
| **Melanoma driver mutations2** |  |  |  |
| **BRAF2** |  |  |  |
| **NRAS2** |  |  |  |
| **PTEN2** |  |  |  |
| **Agec (years)1** |  |  |  |
| **< 201** |  |  |  |
| **20-291** |  |  |  |
| **30-391** |  |  |  |
| **40-491** |  |  |  |
| **50-591** |  |  |  |
| **60-691** |  |  |  |
| **70-791** |  |  |  |
| **80-891** |  |  |  |
| **90+1** |  |  |  |
| **Mean** |  |  |  |
| **Median** |  |  |  |
| **Stagec** |  |  |  |
| **II** |  |  |  |
| **III** |  |  |  |
| **IV** |  |  |  |
| **ICB Status**c |  |  |  |
| **Naïve** |  |  |  |
| **Experienced** |  |  |  |
| **Anti-PD1 only** |  |  |  |
| **Anti-CTLA4 only** |  |  |  |
| **Anti-PD1 and anti-CTLA4** |  |  |  |

**Table 1. Sequencing and clinicopathological characteristics of patient tumor specimens.**

1 Use `../../Clinical\_Data/24PRJ217UVA\_Normalized\_Files/24PRJ217UVA\_20241112\_ClinicalMolLinkage\_V4.csv`.

2 Use column `TMarkerTest` in `../../Clinical\_Data/24PRJ217UVA\_Normalized\_Files/24PRJ217UVA\_20241112\_TumorMarket\_V4.csv`.

3 Use column `PatientMaster` in `../../Clinical\_Data/24PRJ217UVA\_Normalized\_Files/24PRJ217UVA\_20241112\_PatientMaster\_V4.csv`.

a Includes muscle and chest wall: TODO: “chest wall” is column `SpecimenSiteOfCollection` of clinical molecular data but not in list of keywords identifying a specimen site of collection of “Skin and other soft tissues”. Resolve this discrepancy.

b Includes organs of the retroperitoneum and genitourinary tract

c At tumor specimen collection

|  |  |  |  |
| --- | --- | --- | --- |
| **Characteristic, N (%)** | **Male (N=)** | **Female (N=)** | **Total (N=)** |
| **Age (years)** |  |  |  |
| **< 20** |  |  |  |
| **20-29** |  |  |  |
| **30-39** |  |  |  |
| **40-49** |  |  |  |
| **50-59** |  |  |  |
| **60-69** |  |  |  |
| **70-79** |  |  |  |
| **80-89** |  |  |  |
| **90+** |  |  |  |
| **Mean** |  |  |  |
| **Median** |  |  |  |
| **Race** |  |  |  |
| **[options]** |  |  |  |
| **Ethnicity** |  |  |  |
| **[options]** |  |  |  |
| **AJCC v8 Stagea** |  |  |  |
| **IA** |  |  |  |
| **IB** |  |  |  |
| **IIA** |  |  |  |
| **IIB** |  |  |  |
| **IIC** |  |  |  |
| **IIIA** |  |  |  |
| **IIIB** |  |  |  |
| **IIIC** |  |  |  |
| **IIID** |  |  |  |
| **IV** |  |  |  |
| **Primary diagnosis site** |  |  |  |
| **Head and neck** |  |  |  |
| **Upper extremity** |  |  |  |
| **Trunk** |  |  |  |
| **Lower extremity** |  |  |  |
| **Vulva** |  |  |  |
| **Skin, NOS** |  |  |  |
| **ECOG PS** |  |  |  |
| **0** |  |  |  |
| **1** |  |  |  |
| **2** |  |  |  |
| **3+** |  |  |  |

**Table 2. Patient baseline characteristics**. Demographic and clinical characteristics at the time of diagnosis.

*AJCC*, American Joint Committee on Cancer 8th edition; *NOS*, not otherwise specified; *ECOG* PS, Eastern Oncology Cooperative Group Performance Status

a Pathologic stage reported if known; otherwise, clinical stage reported

**Aim 1. Characterize the immune cell infiltrate in the melanoma tumor microenvironment.**

*1.1. Evaluate sex differences in immune cell infiltrate composition.*

* Include all tumor specimens with RNAseq data
* Immune cell enrichment scores using xCell
  + **Check with Dr. Shakeri - he wrote some technical details in the grant application for our plans that seem better than what I propose below, so you can edit this to be what you and Dr. Shakeri actually do**
    - Consider comparing enrichment scores by sex (FDR ≤ 0.05, two-sided Wilcoxon rank-sum test with Benjamini-Hochberg correction)
      * FDR ≤ 0.05 considered significant, but consider also showing 0.05 < FDR ≤ 0.20
      * Can also compare ICB naïve tumors to ICB experienced tumors within each sex
      * Consider adjusting for age and stage
* Summary publication of different methods (including xCell): 31510660
* Example publications for xCell: PMID 34016641, PMID 32286310, PMID 32102984
  + Can enter these PMID numbers into the search on Pubmed to see how others have used xCell and reported the results.
* <https://github.com/dviraran/xCell>
* User guide: <https://link.springer.com/protocol/10.1007/978-1-0716-0327-7_19>

*1.2. Evaluate sex differences in phenotypes of T cell populations*.

* CD8+ tumor infiltrating lymphocytes (TILs) using reference gene signatures published from single cell datasets of melanoma TILs
  + Sade-Feldman (Cell 2019; PMID 30388456): metastatic melanoma prior to or during ICB treatment (**this is the same gene signature set that you previously worked with; can also check with Dr. Shakeri on the technical details he wrote in the grant for this part, which may have been what you’ve already done; edit to what you and Dr. Shakeri actually do**)
    - CD8\_B (enriched in ICB non-responders) vs CD8\_G (enriched in ICB responders) by sex
      * CD8\_B = CD8\_1, CD8\_2, CD8\_3
      * CD8\_G = CD8\_4, CD8\_5, CD8\_6
      * Can also explore by fine clusters (CD8\_1 through CD8\_6)
    - Include all tumor specimens with RNAseq data
  + Can also consider Oliveira (Nature 2021; PMID 34290406) – *hold off for now*
    - 5 tumor-specific clusters
    - Include only ICB naïve tumor specimens with RNAseq data

**Aim 2. Characterize key features of the antitumor response in the melanoma tumor microenvironment.**

*2.1. Evaluate sex differences in immune signaling pathways*.

*2.2. Evaluate sex differences in immune evasion mechanisms*.

*2.3 Evaluate sex differences in the tumor mutational burden.*

**Aim 3**. **Investigate sex differences in clinical outcomes after immune checkpoint inhibitor therapy.**

*3.1. Evaluate sex as an independent predictor for clinical outcome after immune checkpoint inhibitor therapy.*

**Details for Table 1 (Specimens): only for AssignedPrimarySite = cutaneous**

**Sequencing Data**

* WES only = Counts of specimens with value for “WES” on Molecular Linkage file
* RNAseq only = Counts of specimens with value for “RNASeq” on Molecular Linkage file
* WES and RNAseq = Counts of specimens with values for both “WES” and “RNASeq” on Molecular Linkage file

**Specimen Collection Site**

* Counts for each category using the “SpecimenSiteOfCollection” on Molecular Linkage file
  + Skin and other soft tissues: contains “skin” OR [“soft tissue” and does not contain “lymph node”] OR “breast” OR “ear” OR “eyelid” OR [“head” and does not contain “lymph node”] OR “muscle” OR “thorax” OR “upper limb, NOS” OR “vulva”
  + Lymph node: contains “lymph node” OR “parotid”
  + Lung: contains “lower lobe” OR “lung” OR “trachea” OR “upper lobe”
  + Abdominal viscera: contains “anus” OR “adrenal” OR “colon” OR “gallbladder”, “ileum” OR “jejunum” OR “kidney” OR “liver” OR “retroperitoneum” OR “small intestine” OR “peritoneum” OR “spleen” OR “ureter” OR “vagina”
  + Brain: contains “brain” OR “cerebellum” OR “frontal lobe” OR “occipital lobe” OR “temporal lobe”
  + Bone: contains “bone” OR “spine” OR “vertebral”

**Melanoma Driver Mutations**

* I’m not sure how one identifies driver mutations from the available tumor and germline WES data, so you’ll need to discuss with Dr. Shakeri. Common melanoma driver mutations that are of interest to us are *BRAF*, *NRAS*, and *PTEN*.

**Age**

* Counts for each age group and the mean and median using “Age At Specimen Collection” on Molecular Linkage file
  + For calculating the mean, will have to approximate by assigning age 90 for the age 90+ group

**Stage**

* Counts for each stage using the “AssignedStage” from your pipeline

**ICB Status**

* Naïve = “Age of Specimen Collection+0.005” on Molecular Linkage file is before any “AgeAtMedStart” entries for ICBs on the Medications file
  + Tumor collected prior to starting any ICB medications
* Experienced = “Age of Specimen Collection+0.005” on Molecular Linkage file is after any “AgeAtMedStart” entries for ICBs on the Medications file
  + Tumor collected after starting an ICB medication
* ICB Medications
  + Anti-PD1 = nivolumab (includes nivolumab-relatlimab-rmbw), pembrolizumab, atezolizumab
  + Anti-CTLA4 = ipilimumab

**Details for Table 2 (Diagnosis): only for AssignedPrimarySite = cutaneous**

* This table uses the fields associated with the diagnosis selected by your pipeline except for the race and ethnicity fields that are from the Patient Master file

**Age**

* Counts for each age group and the mean and median using “AgeAtDiagnosis”
  + For calculating the mean, will have to approximate by assigning age 90 for the age 90+ group

**Race**

* Counts for each category reported for “Race” on the Patient Master file

**Ethnicity**

* Counts for each category reported for “Ethnicity” on the Patient Master file

**Stage**

* Counts for each stage
  + Use “PathGroupStage” when known
  + If “PathGroupStage” = “Unknown/Not Reported” OR “Unknown/Not Applicable” OR “No TNM applicable for this site/histology combination”, then use “ClinGroupStage”
  + If “PathGroupStage” and “ClinGroupStage” are both unknown, list as a new category in table as “Unknown”
  + I started the table at stage IA with the assumption that none of our patients have a diagnosis of stage 0, but if there are some with that initial stage, just add as a row to the table.
* Pull these stages as they are, regardless of the associated “TNMEditionNumber”. This means you can ignore the header text AJCC v8. I can re-assign those that were previously classified under a prior version (before 8th edition) if there is enough information.
  + If possible, could you pull the list of patients with “TNMEditionNumber” that does not contain “Eighth Edition” into a separate Excel file for me to review (AvaterKey, Group (from your pipeline: A, B, C, D), ClinTStage, ClinNStage, ClinMStage, ClinGroupStage, PathTStage, PathNStage, PathMStage, PathGroupStage, TNMEditionNumber)

**Primary Diagnosis Site**

* Use “PrimaryDiagnosisSite”
  + Head and neck: contains "ear" OR "eyelid" OR "lip" OR "scalp" OR "face"
  + Upper extremity: contains "upper limb"
  + Trunk: contains "trunk"
  + Lower extremity: contains "lower limb"
  + Vulva: contains "Vulva, NOS"
  + Skin, NOS: contains "Skin, NOS"

**ECOG PS**

* Use “PerformStatusAtDiagnosisScale” and “PerformStatusAtDiagnosis” fields
* I haven’t seen what this data looks like, but the goal is to pull the Eastern Cooperative Oncology Group (ECOG) performance status (PS) score (range 0-5). It looks like ORIEN may also report a different score (Karnosfky), but my hope is that the majority have an ECOG status.
* Counts for each ECOG score (0, 1, 2, and then combine the others into 3+) if “PerformStatusAtDiagnosisScale” contains “ECOG” (you’ll have to see if they abbreviate or spell out the name)
* If “PerformStatusAtDiagnosisScale” does not contain “ECOG”, then add a row for Unknown.