

Transcriptomic Analyses of Obesity-driven Oral Cancer Progression

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Abstract

The relationship between oral squamous cell carcinoma (OSCC) and dietary habits, nutritional status, and body mass index (BMI) have been assessed in many studies. Similar to other epithelial tumors, obesity is associated with worse outcomes in OSCC. Obesity has been associated with metabolic dysfunction, including insulin resistance and altered adipokine profiles, which promotes tumor cell proliferation and survival. The purpose of this study was to begin to understand how obesity can alter the immune cell infiltrate and associated expression profile during oral cancer progression. Syngeneic murine MOC2 oral cancer tumor cells were injected into the flank of lean (low-fat diet; 10% energy from fat) and obese (high-fat diet; 45% energy from fat) C57BL/6J mice (n=6/group). To understand how obesity alters gene expression profiling in the TME, single cell RNA sequencing (scRNAseq; 10X Genomics) was used to analyze immune cell and RNA gene expression changes in tumor-bearing lean and obese mice. Flow cytometry was used to measure immune cell changes in visceral and subcutaneous adipose tissues of tumor-bearing mice and CD45+ infiltrating immune cells determined by histopathology. Oral cancer tumor burden was significant higher in obese compared with lean mice ($P<0.05$; unpaired Student's t-test). Obese mice had increased macrophage infiltrate with increased M2 polarization ($P<0.05$; unpaired Student's t-test) in visceral and subcutaneous adipose tissues. scRNAseq analysis indicated that multiple immune cell populations and mRNA profiles within each cell population were different between lean and obese mice. In obese tumor-bearing mice, there was an increased CD45+ infiltrate and inflammation in tumor tissues compared to lean tumor-bearing mice. Obesity drives oral cancer progression in this syngeneic mouse model through multiple changes in immune cell infiltrate within the tumor microenvironment.

Methods

Animal Models: C57BL/6J mice were fed a low-fat diet (LFD; 3.82 kcal/g, 10% fat, 20% protein, 70% carbohydrate; D12450Hi, Research Diets, New Brunswick, NJ) or high-fat diet (HFD; 4.7 kcal/g, 45% fat, 20% protein, 35% carbohydrate; D12451). MOC-2 mouse oral cancer cells syngeneic for C57BL/6J were mixed with 50 μ l of Matrigel (Corning, USA) and subcutaneous injected over dorsum using 26G needles (5 $\times$ 10⁵ in 50 μ l RPMI-1640, Sigma, USA). Tumor burden was calculated as (length $\times$ width²)/2 with digital caliper every other day from 4th day. Animals were sacrificed if tumor volume exceeded 1,500 cm³ or tumors developed extensive non-healing ulcerations. 4NQO model mice first fed with 4NQO water for 16 weeks and changed to regular water for 12-16 weeks starting at 7 weeks of age with LDF or HFD-fed mice. All animal experiments were approved by the State University of New York at Buffalo Institutional Animal Care and Use Committee.

Flow cytometry after magnetic cell sorting: Briefly, after harvest visceral adipose tissues (VAT) and subcutaneous adipose tissues (SAT) of MOC-2 mice, adipose tissue into small pieces of 2-4 mm in gentle MACS Dissociator (Miltenyi Biotec, Germany) according to tumor dissociation kit (130-096-730, Miltenyi Biotec, Germany), then use ACK lysing buffer (A10492-01, Gibco, USA) to lysis red blood cells and then added CD45 microbeads (130-052-301, Miltenyi Biotec, Germany) per 10⁷ total cells and sorted the cells by autoMACS (Miltenyi Biotec, Germany) After CD45+ magnetic cell sorting, fluorescently labeled antibodies diluted in staining buffer were added following incubation with CD16/CD32 (553141, Clone 2.4G2, BD, USA). After several steps wash and centrifuge, the cells were fixed by examined by LSR FortessaTM cell analyzer (BD, USA). For intracellular FOXP3 were incubated in BD Cytotfix/CytoPerm solution (51-2090KZ, BD, USA), then wash twice with BD Perm/wash buffer (51-2091KZ, BD, USA). Resuspended in FOXP3 antibody on ice, wash twice with BD Perm/Wash, then fixed by and examined by LSR Fortessa TM cell analyzer (BD, USA). The results were analyzed by FlowJo (BD, USA).

Immunohistochemistry: After harvest, MOC-2 tumors were sectioned along the long axis stored in OCT media immediately, with the remaining tumor tissues fixed for paraffin and H&E staining. OCT tissues were sectioned at 5 μ m thickness. Sections were incubated with 3% H₂O₂, washed by PBS, then blocked in 10% goat serum for 30 min at R.T, incubated with CD45, Ki67, CD31 and Caspase-3 antibodies overnight. After washed by PBS, slides were incubated with biotinylated secondary antibody (1:200, ab7089, Abcam, USA), ABC reagent (PK-6100, VECTOR, USA), DAB peroxidase substrate solution (SK-4100, VECTOR, USA), and Rinsed in tap water, Ehrlich's hematoxylin, graded ethanol in turn, finally mount slides with Cytoseal 60 (Thermo scientific, USA). Staining was scored by two research pathologists blinded to animal identification and experiment group. Immunohistochemistry scores were calculated by the Quick-score method of multiplying the intensity coefficient and the frequency of positive coefficient. The intensity coefficient was scored as 0 (negative), 1 (low), 2 (moderate) or 3 (strong), and the positive coefficient was scored based on the percentage of positively staining cells. The resulting product was categorized from 0-5 yielding the immunohistochemistry staining score as follow: 0= negative, 1=1-3, 2=4-6, 3=7-9, 4=10-12, 5=13-15. If there are few positive cells, only count the number of positive cells.

Single Cell RNA sequence (10X Genomics): Single Cell RNA sequencing (scRNA-seq) approaches have provided unprecedented detail of the heterogeneity of the cellular composition, and increasingly being used to characterize both the abundance and functional state of tumor-associated cell and all kinds of immune cell types. MOC-2 single cell samples were processed using 10X Genomics as per the manufacturer's recommendations. Briefly, after harvest tumor at MOC-2 mice flank, and cut tumor tissue into small pieces of 2-4 mm in gentle MACS Dissociate (Miltenyi Biotec, Germany) according to tumor dissociation kit (130-096-730, Miltenyi Biotec, Germany), then use ACK lysing buffer (A10492-01, Gibco, USA) to lysis red blood cells, followed by a dead cell removal kit (Miltenyi Biotec, Germany), resulting in 80%-90% purity. Single-cell suspensions were loaded onto a Chromium Single Cell Chip (10X Genomics) at a target capture rate of around 6000 cells per sample. We used captured mRNA by bar code during cDNA synthesis and transformed barcoded cDNA into a combined single cell RNA seq library, to Illumina sequence using Chromium Single Cell 5' Solution (10X Genomics). All samples of a given donor were processed simultaneously with the Chromium Controller in a batch and the resulting library was prepared in parallel. We have collected all libraries of a given donor, and each library is barcode encoded with a unique Illumina sample index for sequencing in a single Illumina flow pool. Before gene expression analysis, the original sequencing data has been corrected for index exchange. For each reading that has a unique, chain specific alignment with the exon sequence, we constructed an address composed of cell identification barcode, unique molecular identifier (UMI) barcode and gene identifier and the number of reads associated with each address in each sample. After correcting the index exchange, we used UMI to fold the amplified repeat segments and correct the errors in the cell identification barcode and UMI barcode to generate a preliminary matrix of the molecular count of each cell as a cluster. All data are summarized in the cell Ranger Pipeline software (10X Genomics) after each cluster identification by Immune Cell Gene Signatures (ImSig) Loupe Cell Browser (10X Genomics) were used to fast and interactive visualization and analysis of single cell data, but gene expression data of Ranger Pipeline software were analyzed by t-Distributed Stochastic Neighbor Embedding (t-SNE) and cluster through Seurat (R-language). All data showed by 2d-plot at different colors and violin map.

Background

Tumor Associated Macrophages

