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PSEUDOMONAS AERUGINOSA FUSION PROTEIN VACCINES CONTAINING THE TH17-STIMULATING ANTIGEN POPB IMPROVE MUCOSAL IGA RESPONSES AND PROTECTIVE EFFICACY IN MICE AFTER INTRANASAL IMMUNIZATION

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Introduction: *Pseudomonas aeruginosa* continues to be a significant opportunistic pathogen, with growing antibiotic resistance and no available vaccine. Previous work showed that the *P. aeruginosa* type III secretion system (T3SS) protein, PopB, elicits a protective Th17 response after intranasal immunization using the Th17 adjuvant curdlan, but the protection is of low potency. In those studies, PopB was co-purified with its chaperone PcrH. In the current studies, we tested whether fusing or mixing PopB with PcrV, a T3SS protein known to elicit protective antibodies, would enhance protective efficacy. We also hypothesized that adding a Th17 antigen (PopB) to PcrV would improve mucosal IgA responses to PcrV.

Experimental Design: Fusion proteins to the avidin homolog rhizavidin (Rhavi) were constructed to achieve multimeric antigen presentation as dimers and also for future coupling to biotinylated polysaccharides. A fusion protein of Rhavi to full-length PcrV and PopB (denoted RVB) was purified from *E. coli* using a pACYCDuet-1 vector co-expressing His-PcrH, where untagged RVB bound to His-PcrH was pulled down by nickel affinity resin, followed by ion exchange chromatography and then negative affinity chromatography with nickel resin using elution with 0.1% LDAO to dissociate RVB from His-PcrH. Recombinant His-tagged Rhavi-PcrV, PcrV, and PopB/PcrH were prepared by published methods. Mice were immunized intranasally using curdlan as adjuvant. IgA titers in bronchoalveolar lavage fluid (BALF) were assayed by ELISA, and Th17 responses were assessed by measuring IL-17 (ELISA) in supernatants of splenocytes stimulated with antigens in vitro. Protective efficacy was evaluated in a murine acute pneumonia model using *P. aeruginosa* strain N13.

Results: Our results show significantly higher IgA titers to PcrV in BALF of mice immunized with RVB when compared to mice immunized with either PcrV or Rhavi-PcrV, where IgA titers were undetectable. Th17 responses to PopB were significantly higher in RVB-immunized mice compared to mice immunized with PopB/PcrH. In the *P. aeruginosa* acute pneumonia model, RVB-immunized mice displayed 100% survival, whereas PcrV- and Rhavi-PcrV-immunized mice had approximately 60% survival (logrank P=0.06 compared to RVB), all significantly higher than curdlan-immunized mice (0% survival).

Conclusion: These results suggest that integrating PopB with PcrV into the same rhizavidin fusion protein elicits improved IgA titers to PcrV while maintaining a robust Th17 response to PopB, ultimately conferring broad and potent protection against *P. aeruginosa* lung infection.

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Title: THE EFFECTS OF MATERNAL IMMUNE ACTIVATION AND AGING ON ASTROCYTE MITOCHONDRIA STRUCTURE AND CONNECTIONS WITH NEURONAL DENDRITIC SPINES

Abstract

Introduction: Prenatal exposure to infections is a potential risk factor for developmental and neurodegenerative disorders. Mitochondrial dysfunction and altered morphology have been implicated in various neurodegenerative disorders. Evidence has shown that astrocytes have brain region-specific morphological and functional changes throughout development and aging. During development astrocytes influence synaptic growth and maturation through the release of factors. Astrocytes have various roles including metabolic and neurotransmitter homeostasis; and within their unique spatial territory, astrocytes communicate with many neuronal processes and cell bodies. Neuronal synaptic stability is conferred by astrocytes through astrocytic perisynaptic processes and astrocytic contact with dendritic spines. This study aimed to assess the effect of maternal immune activation (MIA) in mice by examining astrocyte mitochondria morphology and astrocytic-dendritic spine interactions of aged mice.

Materials and Methods: Pregnant wildtype mice were injected with the viral mimetic polyyinosinic-polycytidylic (PIC) at 20mg/kg or saline at embryonic day 12.5. Both male and female offspring at 9 months of age received intracranial injections of AAV2/5 gfaABC1D-mitoGFP to label astrocytic mitochondria and with AAV1.CAG.FLEX.tdTomato combined with AAV1.CaMKII.Cre to label pyramidal neurons in layer 2/3. Mice were fixed between 12-15 months of age. Sections were imaged between cortical layers 1-3 on a confocal microscope to analyze astrocytic mitochondria morphology and astrocytic mitochondria- dendritic spine proximity. Mitochondria morphology was analyzed in ImageJ assessing average volume, surface area, sphericity, and branches with the Mitochondria Analyzer Plugin. Mitochondria-spine contact was assessed using Imaris version 10.0.0. software to identify and mitochondria within 1um of dendritic spines. Mitochondria were normalized to the total number within 1 um and reported as percent mitochondria within a specified distance.

Results: Analysis is ongoing for astrocytic mitochondria morphology. Preliminary analysis of astrocytic mitochondria-spine contact suggests MIA aged mice have a greater percentage of astrocytic mitochondria within 0.5um of dendritic spines compared to aged saline injected mice. Future analysis will also be separated by sex of mice and completed in a younger age cohort.

Conclusion: While analysis needs to continue for astrocytic mitochondria morphology and astrocytic mitochondria-spine contact, the preliminary results of astrocytic mitochondria-spine contact differences suggest that MIA has impacts on astrocyte-dendritic spine interactions in aged mice. Analysis is ongoing in younger mice to determine if this effect persists throughout aging or emerges as the mice age.

CHARACTERIZING THE EFFECTS OF CANCER-ASSOCIATED MISSENSE MUTATIONS IN SWI/SNF CHROMATIN REMODELING

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Chromatin accessibility is pivotal in regulating gene expression. ATP-dependent chromatin remodelers such as SWI/SNF play essential roles in creating and maintaining accessible chromatin regions to facilitate the binding of transcription factors and RNA Polymerase II (RNAPII), thereby being pivotal in regulating gene expression. In this project, we focus on the mammalian SWI/SNF complex also known as BRG1/BRM associated factor (BAF).

BAF regulates chromatin structure by repositioning and evicting genomic nucleosomes. BAF is also known to oppose the functions of Polycomb Repressive Complexes (PRC) at transcriptionally repressive genomic regions. Our previous work shows that BAF along with RNAPII dynamically probe both transcriptionally active and repressive chromatin and partially unwraps nucleosomes, but is only able to evict nucleosomes to generate accessible chromatin at transcriptionally active regions with the help of transcription factors.

Consistent with its critical role in regulating chromatin structure and gene expression, subunits of the BAF complex are mutated in more than 20% of cancers. While most of these mutations result in a defective BAF complex (loss of function, LOF), several substitution mutations within the catalytic BRG1 subunit have been found to be biochemically gain-of-function (GOF), and genetically dominant. Even though most studies on cancer-associated perturbations of BAF have focused on LOF mutations, how cancer-associated GOF mutations regulate BAF remains poorly understood.

We hypothesize that gain-of-function (GOF) mutations within BRG1 convert the abortive probing mechanism of BAF at Polycomb-repressed chromatin regions into productive nucleosome eviction. Gain of function mutations may increase residence time as well as chromatin remodeling efficiency, thereby resulting in nucleosome eviction without transcription factors.

To test our hypothesis, we will ectopically express naturally occurring cancer-associated mutations (from the COSMIC database) in *BRG1* (also known as *SMARC4*) in mouse embryonic stem cells (mESCs). Additionally, we will delete the post-HSA region within BRG1 (ΔpostHSA) and express the mutant protein in mESCs. The post-HSA region is known to be a negative regulator of SWI/SNF ATPase activity, but it's specific role in regulating chromatin structure is unknown. As several GOF mutations are found within post-HSA, we hypothesize that this region is a regulator of dynamic abortive-to-productive chromatin remodeling by BAF.

We will conduct time-resolved chromatin profiling experiments to determine occupancy of BRG1, Polycomb complexes (PRC1 and PRC2), and histone post-translational modifications (PTM) using Cleavage Under Targets and Tagmentation (CUT&Tag). Along with that we will also conduct Cleavage Under Targeted Accessible Chromatin (CUTAC) targeting RNA Polymerase II phosphorylated on serine 5 (RNAPIIS5P- mark of paused RNA polymerase II which indicates transcriptionally active genes) to view changes in chromatin accessibility. Lastly, we will also determine changes in gene expression via Precision nuclear Run-On Sequencing (PRO-seq).

This is an ongoing project therefore there is no data to be reported yet.

UNDERSTANDING THE FUNCTIONAL ROLE AND TARGETING POTENTIAL OF B7H3 IN SMALL CELL LUNG CANCER

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Abstract: Lung cancer is the leading cause of cancer-related deaths worldwide. Smal cell lung cancer (SCLC) is an aggressive subtype of lung cancer. The overall survival of SCLC is dismal owing to early metastasis, chemoresistance, higher rate of recurrence, and lack of available treatment options. Although some immunotherapeutic drugs have been approved for SCLC, they are effective only in a small fraction of the patient population. This necessitates the quest to identify promising vulnerabilities in SCLC. Bioinformatic analysis on SCLC data set in the backdrop of various immune checkpoint regulators revealed B7H3 as a promising target. The bioinformatics data was recapitulated in SCLC cell lines and human SCLC tissues. To delineate the effects of B7H3 targeting, we performed CRISPR-Cas9 mediated B7H3 knockout in SCLC cell lines. B7H3 knockout in SCLC cells showed decreased colony formation, migration, and proliferative properties. B7H3 knockout also resulted in decreased tumor growth in SCLC xenograft models. Our results suggest that deletion of B7H3 decreases the functional activities of SCLC cells and activates the oncogenic signaling pathways, such as Erk, and Akt. We then targeted B7H3 in spontaneous SCLC mouse model (RPM: $Rb1^{fl/fl}$, $Trp53^{fl/fl}$, LSL-Myc^{T58A}) using B7H3-specific antibody-drug conjugate (ADC) m276-SL-PBD. We observed that treatment of SCLC mouse models with m276-SL-PBD resulted in decreased tumor growth, stemness, and angiogenesis and increased T-cell infiltration. Altogether, our data indicate that B7H3 plays a crucial role in SCLC pathogenesis and is a compelling therapeutic target for SCLC.

ANALYSIS OF RECOMBINANT ANTI-PHOSPHATIDYLCHOLINE ANTIBODIES FROM VH12-TRANSGENIC MICE

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Background: B and T lymphocytes form the foundation of our adaptive immune system, which is based on specific recognition of foreign molecules by structurally diverse surface antigen receptors.
Structural diversity in these receptors originates through site-specific rearrangement of antigen receptor genes during lymphocyte development, called V(D)J recombination, which is initiated when the RAG1/2 proteins cleave antigen receptor gene segments. In the classical model, B cell repertoire diversity is restricted by the number of functional V, D, and J gene segments in the immunoglobulin (Ig) heavy and light chain gene loci that encode the B cell receptor (BCR). In B cell development, primary, antigen-independent V(D)J recombines sequentially with the immunoglobulin heavy (H) and light (L) chain. This process usually requires multiple rounds of L rearrangements to achieve a permissive pairing with the rearranged H chain. The resulting pre-B and B cell receptors undergo multiple selection events, the result of which is in part determined by the specificity of the BCR. The antigen-dependent selection steps trigger receptor editing, modifying their specificities and enforcing self-tolerance.

Significance: Classically, innocuous BCR specificities are allowed to persist while autoreactive specificities trigger receptor editing causing further, secondary, L rearrangements, ensuring antigen reactivity and self-tolerance. These outcomes have been investigated extensively in several laboratories by enforcing BCR specificity to different model antigens through expression of conventional or site-directed H and/or L transgenes. However, a subset of innate-like B cells called B-1 B cells often have BCR specificities to various self-antigens. These B cells are responsible for production of most natural antibodies (abs), which do not require stimulation to be expressed. One B-1 B cell BCR specificity is against phosphatidylcholine (PtC), a common component of mammalian and bacterial cell membranes. VH12 transgenic mice express a H chain that, if paired with the KV4-91 L chain, confers reactivity to PtC. Selection mechanisms for autoreactivity remain unclear. Bulk sequencing of VH12 KV4-91 PtC+ B cells shows skewing towards distal J kappa (JK) gene usage, especially JK5, which is more pronounced in receptor editing deficient (dnRAG1) VH12 mice.

Hypothesis: *We hypothesize that the VH12 H chain paired with KV4-91 joined to JK5 results in stronger PtC reactivity than other JK genes, triggering receptor editing more frequently.* To test this, we cloned, expressed, and purified recombinant VH12 KV4-91 mouse antibodies paired with JK2, JK4, or JK5, analyzed their PtC reactivity, and computationally modeled their structures.

Experimental Design: Antibodies from sorted VH12 transgenic mouse B cells are cloned into an expression vector and expressed in HEK293T cells. A control ab is expressed and tested using the same vector backbone. Harvested supernatants are screened for binding to fluorescent PtC liposomes by flow cytometry, purified using Protein A/G, and analyzed for PtC binding by indirect ELISA. Computational modeling of the recombinant antibodies' variable domains was performed by RosettaAntibody and docking with PtC by AutoDock Vina.

Results: Flow cytometry shows PtC binding by all KV4-91 antibodies, with JK2 and JK4 showing low levels and with JK5 being markedly higher for both the supernatants and purified abs. ELISAs using the purified abs shows titratable effects of binding to PtC and non-specific binding to phosphatidyl ethanolamine. There is no significant difference between VH12 abs in PtC binding by ELISA. Computational modeling shows highly flexible loops in VH12 and minor differences in predicted binding affinity with PtC.

Conclusions: Results from the flow cytometry assay and PtC ELISAs suggest that VH12 antibodies containing KV4-91 joined to JK5 are more PtC reactive than those joined to other JK genes. The results of the ELISA suggest that antigen conformation may play a role in the different reactivity profiles seen by the flow cytometry assay. Efforts are ongoing to model the docking of PtC, to identify and validate essential residues for PtC reactivity, and to profile cross-reactivity to various phospholipids.

Title: KNOCKOUT OF KERATINOCYTE FLOWER PROTEIN IN CUTANEOUS SQUAMOUS CELL CARCINOMA

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Background

Studies have suggested that cellular fitness markers are used as communication between competing cells to direct cellular growth and elimination[1]. One such class of these fitness fingerprints is the human flower isoform proteins (hFWE), which are thought to influence the progression of cutaneous squamous cell carcinoma (cSCC). In humans, *hFWE* encodes four isoforms, hFWE1-4, however current research suggests that within skin, predominantly hFWE 3 and 4 are expressed. Research surrounding Flower isoform proteins' role in skin cancer tumor growth is limited yet may have therapeutic potential. Consequently, the aim of our research was to investigate how knockout of Flower proteins in cSCC affected proliferation and differentiation of keratinocytes.

Significance of Problem

There are an estimated 1.8 million cases of cSCC diagnosed worldwide each year, and over 15,000 American deaths alone are caused by the disease[2,3]. Additionally, there was an overall 263% increase in the incidence of cSCC between 1976-1984 and 2000-2010 periods[4]. Although there are molecular therapies for cSCC under investigation, there is currently no therapy specifically designed for cSCC[5]. Characterizing mechanisms governing the selection for, or against, aggressive cell populations within developing cSCCs may uncover novel therapeutic targets.

Question

How does the genetic deletion of Flower (hFWE) affect keratinocyte proliferation and differentiation in cutaneous squamous cell carcinoma?

Experimental Design

This research utilized three clonally expanded hFWE knockout (KO) and control cell populations derived from cSCC13 cell lines using CRISPR editing. One million cells from each cell line were implanted subcutaneously into each of four NCG mice. The mice were euthanized after enlargement and the tumors were collected, fixed, and stained. Cell proliferation was assessed using Ki67 immunofluorescence, a well-known marker of proliferation, (Abcam – 1:150) with biotinylated anti-rabbit 647 (1:1000). Differentiation was detected using an anti- Keratin 10 (K10) antibody (Abcam – 1:150), and loricrin (Abcam – 1:150) antibody with biotinylated anti-rabbit 647 (1:1000). Both K10 and loricrin are proteins expressed in superficial layers of normal skin. Images were digitized using Olympus VS 120 Virtual Slide Scanner.

Results/Data

Loricrin signal was consistently stronger throughout the superficial layers of the cSCC epithelium compared to the epidermis lacking hFWE (KO). Regarding K10 expression, KO samples exhibited greater signal intensity as cells moved medially toward the center of keratin pearls in the epidermis. This pattern was not observed in wild-type (WT) samples, where signal was greatest on the periphery of the pearls and decreased as cells moved medially. In regions lacking keratin pearls, KO tissue showed a more disorganized K10 signal compared to a more uniform localization in WT tissue. Ki67 positivity was slightly greater in the WT tumors, with similar patterns of premature basal cell signal in both genotypes.

Conclusions

Genetic deletion of hFWE in cSCC13 xenografts resulted in reduced keratinocyte differentiation in the tumor epithelium, as shown by diminished loricrin and aberrant K10 localization within KO tissue compared to WT samples. Although both WT and KO showed similar patterns of Ki67 expression, overall intensity was putatively higher in WT cSCC. These results suggest that hFWE may play a significant role in keratinocyte differentiation in cSCC and normal epidermis.

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Abstract Title: Resorbable Polymeric Gel for Junctional Hemorrhage Control in a Porcine Model

Background & Significance of Problem. One of the leading causes of preventable death in war fighters is uncontrolled hemorrhage, including junctional hemorrhage. Currently, the only approved direct treatment for junctional hemorrhage is XStat® pellets; however, this product is non-degradable and must be surgically removed, which can cause rebleeding. The objectives of this study were to perform benchtop characterization studies on novel, injectable, expansile, and resorbable pellet technologies (gels), and to gather proof-of-principle data in a non-survival porcine model of junctional hemorrhage.

Hypothesis. We hypothesized that our experimental gels would have hemostatic efficacy comparable to XStat® in the porcine junctional hemorrhage model.

Experimental Design. Through the use of electrospinning, cryogelation, crosslinking, and polymer entanglement physics, we developed two novel gel types: (1) nanofiber-reinforced microfiber hybrid aerogels; and (2) polyacrylamide-based cryogels. Anesthetized domestic swine underwent right neck arterial/venous line placement, laparotomy and splenectomy, and post-splenectomy crystalloid replacement. Fifteen minutes after crystalloid replacement completion, the right femoral artery and vein were transected via an 8 cm right groin incision, and free hemorrhage was allowed for 30 seconds. Local treatment (XStat® or test gels) then was administered and manual compression over the incision was maintained for three minutes. Subjects were followed for a maximum of three hours.

Results. In benchtop studies with porcine blood, gels expanded from their compressed state five times faster than XStat® pellets and attained their maximum absorption capacity within five seconds of blood contact. Administration of compressed gels into the groin of the swine with junctional hemorrhage produced immediate hemostasis and three-hour survival with a final MAP of 49 ± 6 mmHg. At necropsy, each gel type was observed to have expanded and conformed to the wound cavity. Preliminary results suggested that hemostatic efficacy of the experimental gels was equivalent to XStat® pellets. In this model, no treatment typically produced fatal exsanguination within 15 minutes.

Conclusions. Both hybrid aerogels and polyacrylamide-based cryogels have beneficial properties in comparison to XStat®. After injection into a junctional wound, the compressed gels expanded rapidly and effectively tamponaded the hemorrhage almost immediately. Currently, we are engaged in a powered acute hemostatic efficacy trial using the porcine junctional hemorrhage model to compare our experimental gels to XStat®.

TITLE: EFFECTS OF PH ON AUTORESUSCITATION EFFICACY IN A MOUSE MODEL OF SUDDEN UNEXPECTED DEATH IN EPILEPSY

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Abstract:

Background: During a generalized convulsive seizure (GCS), patients often stop breathing due to tonic muscle paralysis. The autoresuscitation reflex is engaged by the resulting hypoxia and hypercapnia (HH) decreasing pH and triggering a chemoresponse to increase ventilation, expel excess CO_2, and increase pH. If pH is stabilized, eupnea ensues. If too much CO_2 is expelled, respiratory alkalosis-induced hypocapnia triggers apnea to increase CO_2 again (apparent as post-convulsive central apnea). The hypercapnia/hypocapnia oscillation will continue, lessening each cycle until CO_2 stabilizes and eupnea ensues. In sudden unexpected death in epilepsy (SUDEP), individuals fail to autoresuscitate. **Hypothesis:** We hypothesize that the starting pH has a critical role in determining autoresuscitation efficacy. **Experimental Design:** We tested the effects of decreasing or increasing *in vivo* pH on ventilatory chemoresponses and autoresuscitation of wild-type mice and *Kcna1*-null littermates, a well-studied model of SUDEP. For 48 hours, mice were supplemented with ammonium chloride (280 mM) to decrease, or sodium bicarbonate (150 mM) to increase pH. Mice were placed in a whole-body plethysmography and subjected to either a combination of hypoxia (6% O_2) and hypercapnia (3-9% CO_2) to test for ventilatory chemoresponses**,** or an anoxia challenge (97% N_2 and 3% CO_2) to test for autoresuscitation. **Results:** *Kcna1*-null ventilation response to the HH challenge was variable indicating chemoresponse dysfunction. We found only 25% of *Kcna1*-null mice (3 of 12) mounted a successful autoresuscitation response, whereas 75% of wild-type mice succeeded (9 of 12). In contrast, increasing pH improved autoresuscitation and survival in 72% of *Kcna1*-null (5 of 7) and 66% wild-type mice (6 of 9). In contrast, decreasing pH reduced autoresuscitation success to 0% of *Kcna1*-null (0 of 11) and 62% wild-type mice (5 of 8). Next, we tested whether daily treatment influenced lifespan. Daily treatment with ammonium chloride decreased lifespan, whereas treatment with sodium bicarbonate increased lifespan. **Conclusions:** These data suggest that baseline pH may affect the ability to commence a proper chemoresponse and autoresuscitation, which may be particularly detrimental in individuals at high-risk for SUDEP.

OSTEOSARCOMA IN THE LONG BONES OF THE LOWER LIMB: SURVIVAL ANALYSIS CLASSIFIED BY DEMOGRAPHIC FEATURES

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Background:
Osteosarcoma is a malignancy of bone with infiltration of mesenchymal cells. It affects males more frequently than females, and the distal femur is the most commonly affected location. The average age of diagnosis is 15. Some of the symptoms of osteosarcoma include pain and decreased range of motion. Elevations in non-specific markers such as serum alkaline phosphatase and lactate dehydrogenase may be observed. Imaging may be performed, but biopsy is necessary to confirm the diagnosis. For treatment, both surgery and chemotherapy are recommended.

Significance of Problem:
Osteosarcoma is a highly malignant cancer, and there have been recent developments in the management of this cancer. For instance, there has been a shift from amputations to limb-salvage surgery. Given the progress in treatment interventions, analyzing trends in the survival of osteosarcoma patients in a specific region can offer greater insights into management and effectiveness of treatment.

Hypothesis:
We hypothesize that five-year survival rates for osteosarcoma in the long bones of lower limb and associated joints may vary based on patient characteristics such as age or sex.

Experimental Design:
Using the Surveillance, Epidemiology, and End Results (SEER) Program, we have selected osteosarcoma cases from 2000-2018 using the ICD code 9180/3. The site was also specified to be the “Long bones of lower limb and associated joints”. In total, 1,028 patients were identified using these two criteria. For the analysis, the SEERStat software was used. Both frequency and five-year survival tables were generated using the demographic variables included in the database. This includes information such as median income, race, sex, and age.

Results:
Between male and female patients, the overall five-year survival rates were 54.0% and 64.3% respectively. When comparing males and females with an age of diagnosis between 15-19 years, the five-year survival rates were 63.2% and 74.2% respectively (Table 1). This group was studied since this age range covers the average age of diagnosis. Lastly, there are differences in survival by race. The five-year survival rate was highest in the Unknown group (100%, n=8), followed by White (59.5%, n=750), Asian (53.6%, n=86), Black (53.5%, n=176), and Native American/Alaska Native (18.8%, n=8). This represents a future direction of analysis.

Table 1. Differences in survival rate between males and females between 15-19 years at age of diagnosis. Groups are organized according to SEER classification.

Sex	1-Year Survival	2-Year Survival	3-Year Survival	4-Year Survival	5-Year Survival
Male	93.10%	83.30%	74.80%	68.30%	63.20%
Female	96.30%	91.70%	81.10%	76.90%	74.20%

Conclusion:
There is no statistically significant difference in the overall survival between males and females with osteosarcoma in the long bones of the lower limb. However, other demographic variables may impact the five-year survival rate.