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Methods

Establishment and Characterization of a Novel Human Ocular Adnexal Sebaceous Carcinoma Cell Line

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Purpose: Sebaceous carcinoma (SC) is a malignant eyelid tumor of the ocular adnexa that is primarily treated via surgical excision. Few therapies exist in advanced cases, and medical therapy is limited because of our incomplete understanding of SC biology. Herein, we describe a technique to culture human ocular adnexal SC for use as an in vitro model.

Methods: Human ocular adnexal SC tumor cells were isolated from a patient undergoing orbital exenteration surgery and named Bascom Palmer 50 (BP50). They were cultured in Dulbecco's modified Eagle medium/nutrient mixture F-12 supplemented with 10% fetal bovine serum and antibiotics and were maintained at 37°C in humidified 5% CO_2. The cells were characterized by immunohistochemistry, exome sequencing, and short tandem repeats analysis. In vitro drug screening against mitomycin-C (MMC) was performed using a cell viability assay.

Results: BP50 grew past 40 passages with a doubling time of 52.3 hours. Immunocytochemical staining revealed expression of SC-associated markers adipophilin, epithelial membrane antigen, p53, and androgen receptor. Whole exome sequencing showed a significant carryover in somatic mutations between the tumor tissue and corresponding cell line, revealing genetic markers consistent with SC. MMC affected cell viability in a dose-dependent manner.

Conclusions: BP50 displays characteristics of ocular adnexal SC and therefore may facilitate improved understanding of SC biology and the high throughput assessment of novel therapeutic compounds and new drug combinatorial approaches targeted for this disease.

Translational Relevance: Drug screening with MMC against these cells shows in vitro evidence to support its continued clinical use in SC.

Introduction

Sebaceous carcinoma (SC) is a malignant neoplasm that most commonly originates in the ocular adnexa where there is a high density of sebaceous glands. It is the second most common malignant eyelid neoplasm in the United States, trailing only basal cell carcinoma in frequency.[1] SC accounts for 5% of all eyelid tumors in the United States, or about 0.5 cases per million persons.[2,3] Over the past two decades, increased physician awareness and surveillance has decreased patient mortality in advanced disease from 30% of all cases to 1% to 6%.[4–6] In contrast, therapeutic advances for this

condition have been stagnant: surgical excision continues to be the mainstay therapy and the 5-year mortality rate for metastatic cases remains high at 50% to 67%.[5,7] Effective medical therapy for locally advanced or metastatic SC is lacking, especially in comparison to recent therapeutic breakthroughs for advanced basal and squamous cell carcinomas.[8,9]

Our understanding of SC tumor biology and the development of new therapies have been hampered by the lack of laboratory platforms and models in which the cellular and molecular determinants of SC can be elucidated. In this study, we report the establishment of an SC cell line, Bascom Palmer 50 (BP50), which was derived from a patient with histologically-confirmed ocular adnexal disease. Our in vitro analyses indicate that BP50 retains the immunophenotypic and molecular features of SC and serves as a valuable tool for drug screening.

Materials and Methods

Ethics Statement and Sample Collection

This study was approved by the University of Miami Institutional Review Board and was conducted in a Health Insurance Portability and Accountability Act of 1996–compliant manner in accord with the tenets of the Declaration of Helsinki. Written informed consent was obtained from the patient.

Primary Tumor Culture and Cell Lines

Fresh tumor tissues were washed in 1× phosphate-buffered saline solution (Thermo Fisher Scientific, Waltham, MA, USA), finely minced, and digested with 400 U/mL of collagenase A (Millipore Sigma, St. Louis, MO, USA) in Dulbecco's modified Eagle media, high glucose (DMEM; Thermo Fisher Scientific) supplemented with 1% trypsin-ethylenediamine tetra-acetic acid and 1% fetal bovine serum (Premium US Source Heat Inactivated; Thomas Scientific, Swedesboro, NJ, USA) for four hours at 37°C under constant agitation. The tumor cells were then pelleted at 300*g* for five minutes and cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12; Thermo Fisher Scientific) containing 10% fetal bovine serum (Thomas Scientific) supplemented with 1% penicillin/streptomycin. The cells were cultured at 37°C in humidified 5% CO_2 as monolayers. The cells were routinely subcultured at a 1:4 ratio, and the new line was successfully established with more than 40 passages.

SC Tumor Immunohistochemistry

Surgical specimens were sent to the Florida Lions Ocular Pathology Laboratory at Bascom Palmer Eye Institute (Miami, FL, USA). Specimens were fixed in formalin and microscopic glass slides were prepared. Sections were stained with hematoxylin and eosin. Immunostaining was performed at the Diagnostic Pathology Laboratory of the University of Miami Hospital using an automated immunostainer (Leica Biosystems, Buffalo Grove, IL, USA) with appropriate controls to validate antibody specificity. The following probes were used: epithelial membrane antigen (EMA; mouse monoclonal; Cell Marque, Millipore Sigma; prediluted); p53 (mouse monoclonal; Leica Biosystems; prediluted); adipophilin (rabbit polyclonal; BioCare Medical, Concord, CA, USA; prediluted); androgen receptor (AR; rabbit monoclonal, Cell Marque, Millipore Sigma; prediluted). Slides were read by an experienced ophthalmic pathologist (S.D.).

Immunocytochemistry (ICC)

Cytospin preparations of BP50 between passages 7 to 10 were prepared for ICC. Each cuvette was loaded with 100 µL of cell suspension (0.5×10^6 cells/mL) and spun at 1000 rpm for five minutes. The preparations were fixed using 4% paraformaldehyde for two minutes. ICC was performed with the antibodies detailed above.

Short Tandem Repeat Analysis and Viral Screening

PowerPlex 16 HS short tandem repeat (STR) DNA analysis (Promega Corporation, Madison, WI, USA) for 15 independent genetic sites and amelogenin (the sex identity locus) was carried out on BP50 cells (passage 5 and passage 24) by Genetica Cell Line Testing (LabCorp, Burlington, NC, USA). Screening for mycoplasma, Hepatitis B/C, HIV, and HPV 16/18 was carried out by polymerase chain reaction using the h-IMPACT oncogenic profile (IDEXX Bioanalytics, Columbia, MO, USA).

Growth Curve

The growth curve for BP50 was measured with an MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability assay (Biotium, Fremont, CA, USA) according to the manufacturer's protocol. Briefly, cells were plated at a density of 2000 cells per well in a 96-well tissue culture plate with complete medium. MTT reagent was added to the wells, and the cells were incubated for four hours

at 37°C. Formazan crystals were solubilized using 20% sodium dodecyl sulfate solution, and the amount of formazan was measured by absorbance at 570 nm with a SpectraMax i3 multimode plate reader (Molecular Devices, San Jose, CA, USA). Background absorbance was measured at 630 nm. Triplicate readings were recorded for each time point.

Whole Exome Sequencing (WES) and Bioinformatic Analysis

DNA from snap-frozen tissue and cell lines (passage 5) was extracted using the QIAamp DNA Mini Kit (Qiagen, Germantown, MD, USA). Isolated blood, tumor, and cell line DNA was submitted for whole exome sequencing to the John P. Hussman Institute for Human Genetics Center for Genome Technology Sequencing Core (Miami, FL, USA). Libraries were prepped according to manufacturer's protocols. In brief, 200 ng of gDNA quantitated via the Qubit dsDNA Assay was used as input for library preparation and target enrichment according to the Agilent SureSelect XT Low Input Target Enrichment protocol with the Human All Exon V7 baits. Cluster generation and sequencing took place on the Illumina NovaSeq 6000 in paired end 150bp sequencing reactions on an S1 flow cell. Overall, 47.8 million pass-filter paired-end 150 base reads were generated per sample, providing an average depth of ×161 to the Agilent Whole Human Exome v7 target region. Bioinformatic data processing was performed using the University of Miami's Institute for Data Science and Computing Pegasus 2 compute cluster using the GATK best practices workflow[10,11] for somatic variation including mapping to human reference genome GRCh38 with BWA mem, duplicate removal and base quality recalibration[12] followed by variant detection with the tumor-normal paired function in MuTect2.[13] Variants were annotated using ANNOVAR and somatic variants filtered for those that were covered at least ×10 in both the tumor (or cell) and normal tissue, a greater than 5% alternate allele count in tumor (or cell), and <1% alternate allele fraction in normal, and an overall population frequency in the gnomAD database <0.01. Variants were considered to affect protein function if they were annotated as exonic or splicing and any category but synonymous. Single base pair mutational signature was determined using the package MutationPatterns (v1.10.0).[14]

Drug Screening

Cells were seeded at a density of 10,000 cells per well in a 96-well tissue culture plate with culture medium. Once the cells reached 80% confluence, they were treated with various concentrations of mitomycin-C (1.5625–100 µM) for 72 hours and maintained at 37°C in humidified 5% CO_2. Normal saline solution was used as the vehicle control. Cell viability was assessed using the MTT assay (Biotium). The intensity of the dissolved formazan crystals was quantified with a SpectraMax MiniMax 300 Imaging Cytometer (Molecular Devices) at 570 nm. Background absorbance was measured at 630 nm. Quadruplicate readings were recorded.

Results

Establishment of SC Cell Line

SC cell line BP50 was derived from a female patient in her 50s with T4aN0M0[15] disease who underwent orbital exenteration with subsequent control of disease. Notably, her medical history was significant for bilateral retinoblastoma treated with cryoablation and external beam radiation during her childhood. She was otherwise healthy before the diagnosis of SC, which was found incidentally on clinical examination.

More than 40 serial passages of the cells have been carried out successively supporting an immortalized line. The cells grow as an adherent monolayer with a polymorphic fibroblast-like morphology that remained constant throughout passaging (Fig. 1). BP50 grew steadily under standard culture conditions and had a doubling time of 52.3 hours (Fig. 2). The cells did not show any abnormal growth changes after

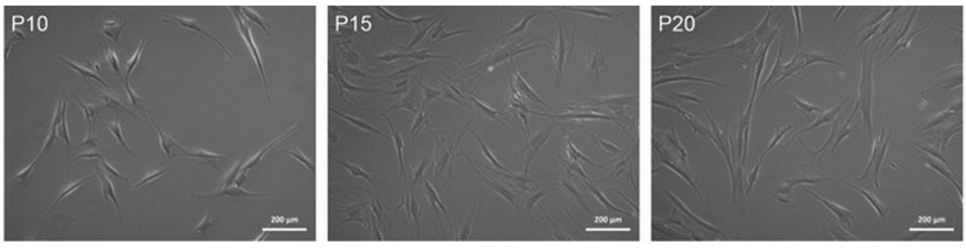

Figure 1. BP50 morphology under inverted phase-contrast microscopy. The cell line maintains a polymorphic fibroblast-like morphology that remains unchanged throughout each passage and after cryopreservation.

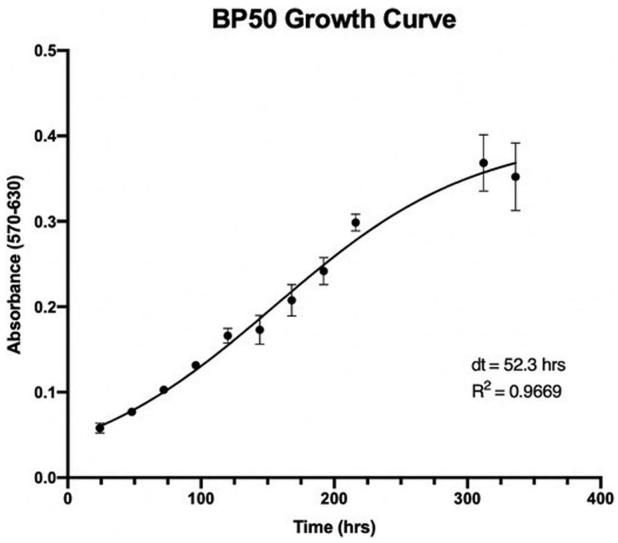

Figure 2. Growth curve for BP50 indicates a doubling time of about 52.3 hours. Error bars: standard deviations.

cryopreservation and recovered from liquid nitrogen. Mycoplasma, Hepatitis B/C, HIV1/2, and HPV 16/18 could not be detected via polymerase chain reaction. STR analysis of early and late passages of BP50 are identical, indicating that the cell line is stable regarding the analyzed DNA regions for the time frame of these passages (Table 1).

Immunophenotypic Characterization

Typical SC morphology is seen on hematoxylin and eosin staining of the BP50 tumor tissue. Immunophenotypic characterization of the BP50 tumor sample and cells express a panel of known SC markers, including adipophilin, EMA, p53, and AR (>Fig. 3).

Table 1. STR Profile of BP50 (Passage 5 and Passage 24)

Regions	Passage 5	Passage 24
D3S1358	14	14
D7S820	8, 13	8,13
vWA	18, 20	18, 20
FGA	25	25
D8S1179	13	13
D21S11	28, 31.2	28, 31.2
D18S51	17	17
D5S818	11,12	11,12
D13S317	8,12	8,12
D16S539	11,13	11,13
TH01	8, 9.3	8, 9.3
TPOX	8,9	8,9
CSF1PO	12	12
Amelogenin	X	X
Penta D	10,13	10,13
Penta E	7,16	7,16

Mutational Analysis of Primary Tumor and Derived Cell Line

We performed whole exome sequencing in the cell line, primary tumor, and whole blood. When comparing all genotype calls across the three samples,

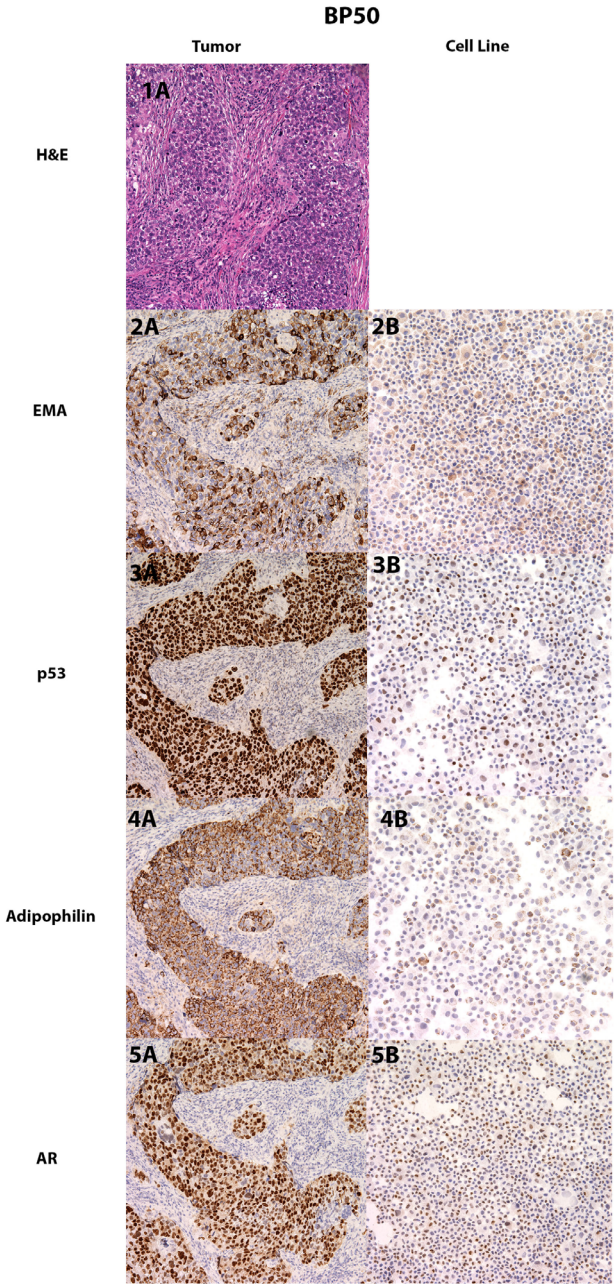

Figure 3. BP50 tumor and cell line at magnification × 20. The left hand column (1A-5A) denotes tumor specimen and right hand column (2B-5B) denotes cell line. 1A: Hematoxylin-eosin stain. 2A,B: EMA stain. 3A,B: p53 stain. 4A,B: Adipophilin stain. 5A,B: AR stain.

Table 2. Shared Protein Altering Mutations in the Cell Line and Tumor

Chr	Start	Ref	Alt	Gene	Amino Acid Change
chr1	152214049	C	T	HRNR	p.R2527Q
chr1	152214059	C	T	HRNR	p.G2524S
chr1	152216488	C	T	HRNR	p.S1714N
chr1	201210045	G	A	IGFN1	p.E1718K
chr2	60780710	A	C	PAPOLG	p.E279D
chr4	152863664	A	C	ARFIP1	p.H51P
chr5	141331101	G	T	PCDHGA1	p.E139D
chr16	20685363	T	G	ACSM1	p.N78T
chr17	44904914	A	C	FAM187A	p.H362P
chr19	8886805	CC	AT	MUC16	p.R13647H
chrX	21984392	T	G	SMS	p.V227G

we identified 98.5% genotype concordance, confirming the identity of the blood, tumor, and cell line from the same individual. Also, all three samples carry a predicted splice site altering variation in RB1 (c.264+2T>C) thought to be a pathogenic variant.

To determine potential mutations driving tumorigenesis, we identified putative somatic mutations in the paired tumor-blood samples. We identified a total of 131 putative somatic mutations meeting our criteria (see Methods) with 66 predicted to alter protein sequence (See Supplementary Table S1 for a list of mutations). We performed the same analysis in the cell line-blood pair and identified a total of 119 somatic mutations with 83 disrupting protein function (See Supplementary Table S2 for a list of mutations). Among the total number of protein altering mutations, 11 were shared between the tumor and cell line (Table 2). Analysis of the mutational signatures for the tumor and cell line showed similar signatures, with C>A, C>T, and T>C being the most predominant (Fig. 4). Notably, a somatic missense *p53* mutation was identified in the tumor, but not in the cell line. Otherwise, somatic mutations within the *ZNF750*, *PCDH15,* or *NOTCH1* genes or related pathways were not identified in the tumor or cell line.[16–18]

Drug Screen to Mitomycin-C

To evaluate the utility of BP50 as an in vitro drug screening platform, we investigated the dose-dependent

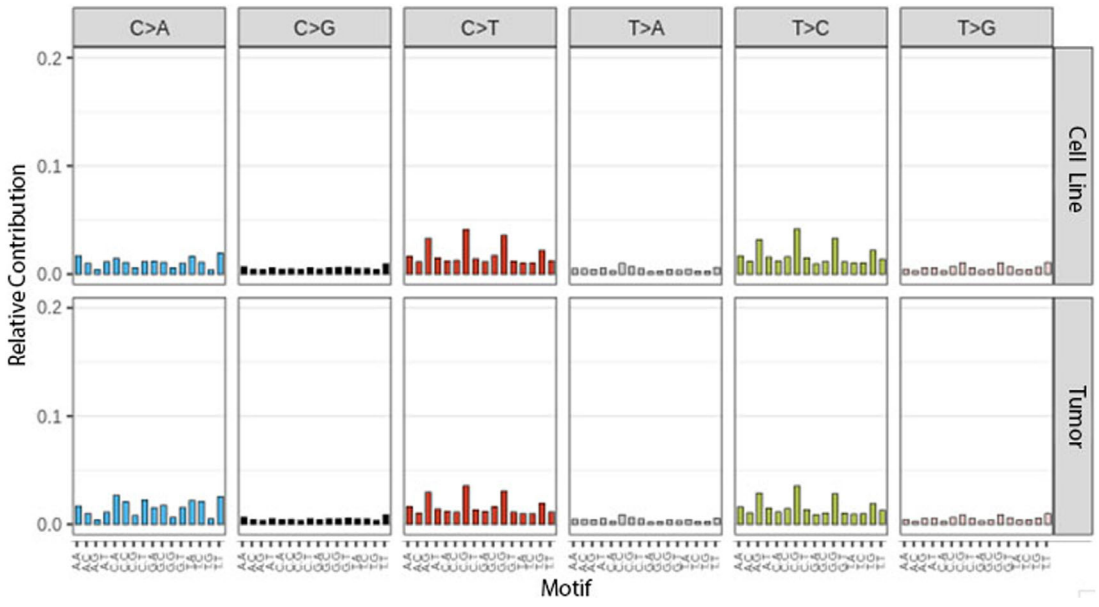

Figure 4. Mutational signature of BP50 and corresponding tumor.

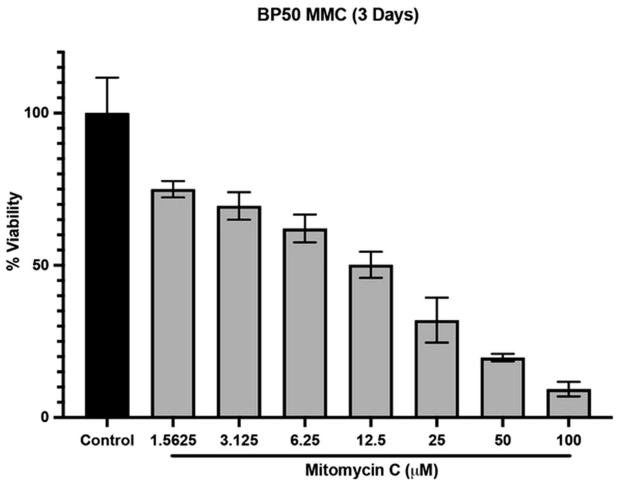

Figure 5. Cytotoxic effects of MMC on BP50 (EC_{50} of 10.4 μM). Error bars: standard deviations.

response in cell viability to mitomycin-C (MMC), a previously reported topical treatment for sebaceous carcinoma. Over a 72-hour period, MMC affected the viability of BP50 in a dose-dependent manner with a half maximal effective dose (EC_{50}) of 10.4 μM (Fig. 5).

Discussion

We report the derivation and characterization of a novel ocular adnexal SC cell line, named Bascom Palmer 50 (BP50). Human sebocyte cultures have long been established to study various dermatologic/ocular conditions and, like most other post-mitotic somatic cells, can only be passaged three to six times unless they are synthetically immortalized.[19–22] On the other hand, we show that its neoplastic counterpart can undergo more than 40 passages, consistent with the natural self-renewal and immortalization that precedes the malignant transformation of cancer cells. Our results confirm that BP50 cells retain immunophenotypic and molecular features of ocular adnexal SC.

SC has historically been characterized by the use of oil-red-O, a burdensome stain to perform with overall poor sensitivity.[23] Recently, immunostaining with adipophilin, EMA, p53, and AR has been shown to represent a sensitive and reliable marker panel for SC.[24,25] BP50 showed concordant staining of the four markers when compared to the original tumor specimen, bolstering evidence that the cells were derived from SC.

Genomic testing of BP50 cells and the corresponding tumor sample was performed. These results show that BP50 shares a high percentage (98.5%) of identical mutations to its original tumor, providing additional support that the cells were derived from their respective source individual and specimen. Notably, WES of the tumor identified a somatic *TP53* mutation not found in the cell line. Upon further examination of the tumor sample data, only a fraction of the reads (15 alternate reads vs. 118 reference reads) identified the *TP53* variant from the reference. This most likely reflects tumor heterogeneity, with only a small portion of cells holding the *TP53* mutation, and the majority of cells maintaining the wild-type genotype. This may account for the discordance regarding *TP53* between the tumor and derived cell line. Interestingly, despite this genotypic difference, immunostaining for nuclear p53 protein was positive in both the tumor and cell line (Fig. 3).

Our patient's history was significant for hereditary retinoblastoma treated with childhood cryoablation and external beam radiation. We identified a germline splice site mutation within the *RB1* gene, which was propagated into the BP50 cell line. Historically, patients with hereditary retinoblastoma have an elevated risk of developing ocular adnexal SC following the effects from external beam radiation.[26,27] However, cases of ocular adnexal SC developing in hereditary retinoblastoma patients without a history of irradiation may highlight the importance of the Rb protein inactivation in SC tumorigenesis. In fact, recent work shows that of the mutations identified by WES, mutations in *RB1* were some of the most common and unique to ocular SC.[17,18] Given the high incidence of *RB1* mutation found in SC, the propagation of the mutation into BP50 supports its use as a *RB1*-specific SC model.

Current medical therapies for ocular SC such as topical MMC, PD-1 inhibitors, cryotherapy, and radiotherapy have been adopted despite a lack of rigorous scientific or biologic data to support their effectiveness in the disease.[28–32] Topical use of MMC 0.04% for locally advanced SC remains controversial, with limited evidence due to the small sample size of treated patients and the lack of evidence of biologic efficacy.[28] Our preliminary drug screening on BP50 with topical MMC shows in vitro efficacy in SC cells. Although further in vivo studies in the setting of a patient-derived xenograft model is indicated, we provide the first biologic evidence for these agents in the use against ocular SC. Future studies include comparing the efficacy and concentration of MMC against a variety of topical chemotherapeutics used in clinical practice.

In conclusion, we present an SC cell line which was derived from a patient with confirmed ocular adnexal SC. A reliable in vitro model will further our understanding of SC tumor biology, improve the ability to identify drug resistance mechanisms, and lead to the

development of new drug combinations. Given the relatively low incidence of this neoplasm, prospective studies would take significant time or large multicenter support. Thus BP50 serves as a valuable preclinical model to further our goal of developing a globe-sparing therapy for advanced ocular SC.

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