HHS Public Access

Author manuscript

Mod Pathol. Author manuscript; available in PMC 2021 March 05.

Published in final edited form as:

Mod Pathol. 2020 November ; 33(11): 2280–2294. doi:10.1038/s41379-020-0571-7.

Invasive Squamous Cell Carcinomas and Precursor Lesions on UV-Exposed Epithelia Demonstrate Concordant Genomic Complexity in Driver Genes

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L.L.V, N.B., S.A.T, R.C.R., and P.W.H. substantially contributed to conception or design of the work. L.L.V., N.B., K.H., S.E.R., C. D., S.M., P.P., X.W., A.S., H.K.S., S.I.M., D.R.R., A.M.C., H.D., A.B.D., F.W., C.G.E., S.A.T., R.C.R., AND P.W.H contributed to acquisition, analysis, or interpretation of data. L.L.V, N.B., S.A.T, R.C.R., and P.W.H. drafted the work and significantly revised it. All authors have approved the submitted version, and have agreed both to be personally accountable for the authors' own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

Conflict of Interest: S.A.T. has had a prior sponsored research agreement with ThermoFisher Scientific that provided access to the OCP. S.A.T. is a co-founder of, prior consultant to, equity holder in, and current employee of Strata Oncology. A.M.C. is a consultant and SAB member of Tempus.

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Abstract

Although squamous cell carcinomas (SCC) are the most frequent human solid tumor at many anatomic sites, the driving molecular alterations underlying their progression from precursor lesions are poorly understood, especially in the context of photodamage. Therefore, we used high-depth, targeted next-generation sequencing (NGS) of RNA and DNA from routine tissue samples to characterize the progression of both well- (cutaneous) and poorly-studied (ocular) SCCs. We assessed 56 formalin-fixed paraffin-embedded (FFPE) cutaneous lesions (n= 8 actinic keratosis [AK], n= 30 carcinoma *in situ* [CIS], n= 18 invasive) and 43 FFPE ocular surface lesions (n= 2 conjunctival/corneal intraepithelial neoplasia [CIN], n= 20 CIS, n= 21 invasive), from institutions in the US and Brazil. An additional 7 cases of advanced cutaneous SCC were profiled by hybrid-capture-based NGS of >1,500 genes. The cutaneous and ocular squamous neoplasms displayed a predominance of UV signature mutations. Precursor lesions had highly similar somatic genomic landscapes to invasive SCCs, including chromosomal gains of 3q involving *SOX2*, and highly recurrent mutations and/or loss of heterozygosity events affecting tumor suppressors *TP53* and *CDKN2A*. We identify a novel molecular subclass of CIS with *RB1* mutation. Among *TP53* wild-type tumors, human papillomavirus (HPV) transcript was detected in one matched pair of cutaneous CIS and invasive SCC. Amplicon-based whole-transcriptome sequencing of select 20 cutaneous lesions demonstrated significant upregulation of pro-invasion genes in invasive cutaneous SCCs relative to precursors, including *MMP1*, *MMP3*, *MMP9*, *LAMC2*, *LGALS1*, and *TNFRSF12A*. Together, ocular and cutaneous squamous neoplasms demonstrate similar alterations, supporting a common model for neoplasia in UV-exposed epithelia. Treatment modalities useful for cutaneous SCC may also be effective in ocular SCC given the genetic similarity between these tumor types. Importantly, in both systems, precursor lesions possess the full complement of major genetic changes seen in SCC, supporting non-genetic drivers of invasiveness.

Keywords

actinic keratosis; Bowen’s disease; squamous cell carcinoma; ocular surface squamous neoplasia; intraepithelial neoplasia; carcinoma *in situ*

Keywords

squamous cell carcinoma; precursors; *TP53*; *CDKN2A*

Introduction

Squamous cell carcinoma (SCC) is a major cause of cancer mortality, and is the most common form of human solid tumor in many anatomic sites (1). Although some SCCs are associated with human papillomavirus (HPV), studies suggest that most SCCs are driven by recurrent somatic alterations such as mutations and copy number alterations. Specifically, cutaneous SCCs harbor a high mutation rate caused by UV damage; recurrent mutations in *TP53, CDKN2A,* and *NOTCH1/2*; focal or arm-level gains affecting chromosomes 3q26, 5p, 7q21, and 11q22; and *CDKN2A* loss (on chromosome 9p21) (1, 2) (3–8). In contrast to these well-characterized tumors, SCC precursors and invasive lesions from other sites, such as the ocular surface (ocular surface squamous neoplasms [OSSN], referred to as ocular SCCs in this study), are not as well understood (2, 9). Ocular SCC is the most common cancer of the ocular surface (cornea/conjunctiva) in the U.S. and can be locally destructive and blinding. Though unusual, lethal metastatic ocular SCC cases have been described (10). Recurrence rates are high despite surgery and adjunctive radiation or chemoradiotherapy. Proposed risk factors for ocular SCCs, such as HPV, remain controversial (11).

SCCs are thought to develop from hyperplastic precursor lesions. Cutaneous and ocular SCCs arise in the setting of environmental factors (UV damage for cutaneous lesions), transforming normal squamous epithelium into actinic keratosis (AK) on the epidermis and intraepithelial neoplasia on the ocular surface (conjunctiva and cornea, CIN). AKs are likely the most prevalent precancerous lesions in humans (12). Although some AKs undergo regression, a subset progress to carcinoma *in situ* (CIS), a preinvasive stage characterized by full-thickness atypia, or to invasive SCC (13, 14). Ocular SCC occurs in two forms: pre-invasive (i.e. conjunctival/corneal intraepithelial neoplasia [CIN] or CIS) and invasive subtypes. Thus far, only one study has profiled ocular tissues and this was limited by analysis of only CIN and CIS lesions, small cohort size, lack of treatment-naïve tumors, and failure to identify actionable targets (9). To our knowledge, neither treatment naïve, pre-invasive ocular lesions (CIN/CIS) nor invasive ocular SCCs has been molecularly profiled previously. The lack of these studies limits our understanding of how these cancers form and hampers our ability to develop molecular therapies against these highly recurrent squamous cancers.

As described in calls to generate a Pre-Cancer Genome Atlas (PCGA), the molecular progression of precursor SCC lesions to invasive cancer is not completely understood, in part due to technical challenges posed by the profiling of small areas of interest often only available in routinely processed formalin-fixed paraffin-embedded (FFPE) tissues (15). To date, the genetic alterations underlying epithelial *in situ* lesions have only been comprehensively profiled in a limited number of cancers, the largest being a recently published study regarding lung cancer (16). Obstacles to defining stepwise models for tumorigenesis in cutaneous malignancies include high burdens of passenger mutations, variability in driver mutations within a given tumor type, and lack of identifiable precursor lesions for some tumor types such as basal cell carcinoma. Despite these challenges, progression of precursor lesions to malignancy has been correlated with tumor suppressor gene inactivation events for a subset of sweat gland carcinomas and some melanomas; in contrast, oncogene activation events can be observed in both benign and malignant tumors

(17–19). Genetic events associated with progression in cutaneous squamous neoplasms are less clear. AKs harbor mutations and methylation profiles similar to invasive cutaneous SCC (6, 20–22). Although AKs display chromosomal aberrations and loss of heterozygosity events (23), these appear to be less numerous than in cutaneous SCC (24, 25). Some have proposed that tumor suppressor loss of heterozygosity might be a critical step in transition from AK to cutaneous SCC (21, 26); however, this hypothesis has not been rigorously addressed. Another area of uncertainty relates to genetic changes in CIS, which is premalignant but has distinct microscopic appearance and clinical management from AK. Finally, although ocular epithelium is a UV-exposed site and displays a similar spectrum of precursor neoplasms and invasive SCC, the genomic changes in treatment naïve or invasive ocular neoplasms remain hitherto uncharacterized, and to our knowledge mutational signatures in ocular surface lesions have not been described.

To better understand genomic changes associated with malignant progression in UV-exposed squamous lesions, we characterized the genomic landscape of cutaneous and ocular neoplasms, and respective precursor lesions at these anatomic sites from routine FFPE tissue samples.

Materials and Methods

Cohort

The study was conducted with local IRB approval. Our cohort was composed of 56 cutaneous tissues (n= 8 AK, n= 30 CIS, n= 18 invasive SCC), and 43 ocular tissues (n= 2 CIN, n= 20 CIS, n= 21 invasive SCC), from institutions in the US and Brazil with available archived formalin-fixed, paraffin-embedded (FFPE) tissues suitable for NGS analysis (Tables 1 & S1 & S2). Additional cutaneous invasive SCC cases from the Mi-Oncoseq program (n= 7) are described below (Table S3). For each case, regions of interest were identified on hematoxylin and eosin (H&E) stained slides and classified as AK or CIN, CIS, or invasive SCC (Figure 1) and given a histology-based tumor content by board certified anatomic pathologists. FFPE blocks were cut to make 4–8 10-μm sections. Although most areas with high tumor purity were macro-dissected using a scalpel, lesions classified as AK or CIN were mainly dissected under the microscope. DNA and RNA from each sample were co-isolated as described (Supplementary Methods).

Multiplexed PCR-based DNA next generation sequencing

We performed DNA based next generation sequencing (NGS) using a highly scalable approach optimized for routine FFPE material. To identify oncogenic and tumor suppressive somatic mutations and copy number aberrations, we performed multiplexed PCR-based DNA NGS (mxDNAseq) on spatially-defined, minute cell populations using the Oncomine Cancer Panel, which targets 134 cancer-related genes, including nearly all genes known to be recurrently mutated or amplified/deleted in SCCs. Targeted mxDNAseq NGS was performed using 20 ng of DNA from each sample. DNA library preparation, sequencing, and analysis was done as described in Supplementary Methods. All samples underwent variant analysis, with the exception of eight which only underwent copy number analysis due to mutation signatures indicative of over-fixation/low library complexity (Table S2). Sample-

level variant allele frequencies were used to determine tumor content. Variants were then classified as homozygous, heterozygous, or germline according to estimated tumor content. Most germline variants had a variant allele frequency between 40% and 60% or >90%; however, if the sample had a tumor content of ~50% or >80%, these thresholds were not applicable. Variants classified as germline and present in population databases were excluded unless occurring at a well-supported somatic mutation hotspot in COSMIC (https://cancer.sanger.ac.uk/cosmic). Validation of selected variants was conducted through Sanger Sequencing with custom designed primers (Supplementary Methods; Table S4; Figure S1).

For most cases, estimated tumor contents based upon variant allele frequency were used to correct copy number estimates to account for variability between samples. The exceptions were 10 ocular samples, for which copy number estimates were corrected by histology-based tumor content since we did not conduct variant analysis on eight samples (as described above), and there were no driver mutations to provide variant allele frequency data for two additional samples (Table S2). After correction of copy number estimates, the following copy number thresholds were used: loss (1-copy loss), deep deletion (2-copy loss), gain (1 or 2 copy gain), amplification (>2-copy gain). Further details are in Supplementary Methods.

Mi-Oncoseq

Seven cases of advanced cutaneous SCC were identified from the Mi-Oncoseq program, which performs comprehensive somatic and germline sequencing for patients with rare or advanced cancers to guide clinical trial enrollment and precision medicine approaches. Clinical grade, hybrid-capture-based exome or targeted sequencing of >1,500 cancer-related genes in tumor and normal tissue was performed to identify somatic mutations, fusions, copy number aberrations, and viral transcripts, as previously described (27).

cBioPortal

Selected prioritized variants for all samples were visualized using the public OncoPrinter tool available from the cBioPortal for Cancer Genomics. Additionally, the MutationMapper tool was used to map *TP53* (NM_000546), *RB1* (NM_000321), and *CDKN2A* (NM_000077) mutations across all samples (28, 29).

Amplicon-based whole-transcriptome sequencing and analysis

We performed amplicon-based whole-transcriptome sequencing of samples in singlicate, as previously described (30). The linear models used to fit the contrasts for AK versus invasive SCC, *in situ* versus invasive cutaneous SCC, and *RB1* Mut vs WT *in situ* samples did not have an intercept term and followed the model "~0 + factor." Overlapping differentially expressed genes of AK versus invasive SCC and *in situ* versus invasive cutaneous SCC were used for heatmap visualization. Functional analysis of differentially expressed transcripts between *RB1* Mut vs. WT populations was performed using Gene Set Enrichment Analysis version 3.0, developed by the Broad Institute (Cambridge, MA) (31, 32). The enrichment was done using a pre-ranked list with the ranking metric being the corrected p-value divided by the sign of the fold-change. The expression data was tested against the hallmark gene set.

RNAscope HPV

To determine HPV status of ocular SCCs and *TP53* wild-type cutaneous cases with adequate remaining tumor material, we used the RNAscope 2.5 HD Red Reagent Kit and target probes HPV-HR18 (pool probe of 18 high-risk HPV strains, 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82) and HPV-LR10 (pool probe of 10 low-risk HPV strains, 6, 11, 40, 43, 44, 54, 70; 69, 71, 74) (Advanced Cell Diagnostics Inc., Newark, CA), according to manufacturer's instructions. Cervical SCC and cutaneous verrucae were used as positive control samples for HR-HPV and LR-HPV, respectively. Positive (Hs-PPIB) and negative (DapB) control probes were also used as sample quality control and assay background control. DapB was uniformly negative. Samples without Hs-PPIB staining were excluded. FFPE tissue blocks were cut into 5-μm sections. After deparaffinization and pretreatments, tissue sections were hybridized with target probes, followed by a series of signal amplification steps and chromogenic staining with Fast Red dye. Stained slides were then evaluated for HR and LR HPV infection according to the staining results.

Results

Ocular and cutaneous SCCs: clinical features

Our final cohort included 106 samples from 87 distinct clinical lesions (Tables 1, S1, & S3). Patients had a mean age at diagnosis of 72.4 years (for cutaneous lesions) or 65.2 years (for ocular lesions), with no significant differences among subgroups. Cutaneous lesions in our cohort were relatively evenly divided between men and women, whereas ocular lesions were strongly skewed toward men. Altered immune status (related to iatrogenic immunosuppression, lymphoma, or human immunodeficiency virus) was present in 27% of patients with cutaneous lesions and 38% of patients with ocular lesions. Of patients with ocular lesions, 4 had a known history of human immunodeficiency virus (Table S1).

Comparison of chromosomal aberrations present in ocular and cutaneous SCCs

In this study, we observed a combination of alterations involving those previously reported in non UV-driven SCCs and UV-driven SCCs. Invasive cutaneous and ocular SCCs harbored *CDKN2A* copy number loss (n= 12/25 [48%] cutaneous, n= 17/21 [81%] ocular SCCs) and 3q gain (n= 5/25 [20%] cutaneous, n= 4/21 [19%] ocular SCCs), with additional focal *SOX2* gains. Furthermore, we observed *CCND1*, *MYC*, and *EGFR* gains in a minority of invasive ocular and cutaneous SCCs at similar frequencies (Figures 2, 3, S2 & S3). We also observed copy number gains in chromosomes 7, and 11q as well as copy number loss in chromosome 11q in both cutaneous (Figure 2A & S3) and ocular (Figure 2B & S2B) SCCs. Hence, cutaneous and ocular SCCs display striking similarity in patterns of chromosomal aberrations, including genomic loss of *CDKN2A*, 3q gains, and amplification of other oncogenes.

Copy number aberrations found in invasive SCC lesions are also recurrent in in situ lesions

After correcting for tumor content, we observed similar, recurrent copy number aberrations within cutaneous and ocular lesions that were present in all three types of lesions (AK, CIS,

and invasive SCC), indicating that these precursor and invasive lesions were essentially indistinguishable at the genomic level (Figure 2 & S2). *MYC*, *CCND1*, and *EGFR* gains were also present in some precursor lesions. In addition, *CDKN2A* loss was observed in 8/8 (100%) AK and in 4/30 (13%) CIS lesions. Notably, pair 9, composed of *in situ* (ID51) and invasive (ID50) regions from a single tumor, harbored *CDKN2A* copy number loss in both components accompanied by distinct mutations, suggesting that *CDKN2A* loss in this case was an early event (Figure 1; Table S5), although we could not exclude the possibility that this might represent collision of unrelated squamous malignancies. Similarly, shared *CDKN2A* loss was observed in pair 16 composed of ocular lesions IE140A and 140B (Figure 2B; Table S5). Additionally, as shown in Figures 2B & S2B, both *in situ* and invasive ocular SCCs can harbor 3q gains, an arm-level gain characteristic of SCCs. Therefore, here we show that many of the copy number aberrations presumed to be characteristic of invasive SCC are also found prior to invasion and overt malignant cytology.

Prioritized somatic variants recurrent in SCCs

After sequencing, we used filtering criteria as specified in the methods section to identify and prioritize somatic variants (Tables S6 &S7). Across all cutaneous and ocular tumor types with the exception of CIN (considering each group in aggregate), we observed a predominance of C > T transitions at dipyrimidine sites among single nucleotide variants, as well as a predominance of CC > TT tandem substitutions among dinucleotide substitutions, consistent with UV-mediated DNA damage. Despite a reported association with tobacco smoking, we found that C > A substitutions characteristic of tobacco signature mutations (33) are rare in ocular neoplasms. Prioritized mutations were highly conserved in multiple samples collected from the same clinical lesion (Table S5), supporting the clonal nature of driver mutations, with the exception of one SCCIS-SCC pair from separate blocks (ID50, ID51) as noted above. As previously reported in SCC, we observed recurrent mutations in genes such as *TP53*, *CDKN2A*, *NOTCH1*, *PIK3CA*, and *EGFR* (Figure 3). *TP53* is among the most frequently mutated genes in non-HPV driven SCCs from all anatomic sites, including 83% of lung SCC, 71% of head and neck SCC, and 48% of penile SCC. However, second *TP53* mutations are infrequent to absent at other sites, identified in 0% of lung SCC, 16% of head and neck SCC, and 8% of penile SCC [data downloaded from TCGA PanCancer Atlas, accessed on March 31, 2019 (28, 29).] Interestingly, both of our SCC cohorts had a high frequency of *TP53* mutations but also had a significant frequency of a second or even third mutation across all types of lesions (Figure 3). Upon mapping the location of the mutations across lesions, we confirmed that *TP53* mutations in precursor and invasive disease affect similar hotspot (e.g. p.R248, p.P278) regions across all types of tissues (Figure S4).

TP53 copy-neutral loss of heterozygosity in SCC progression

Studies on photodamaged skin have reported the presence of multiple *TP53* mutations in the absence of clinical lesions, consistent with multiple small, clonally-independent cell populations largely defined by these mutations (34). Other studies have used the ratio of heterozygous to homozygous mutations within a region of copy-neutral loss of heterozygosity to identify the temporal order of genomic events (35), demonstrating that in

cutaneous SCC, *TP53* loss of heterozygosity is an early event and may gate most of the remaining mutations present in invasive tumors.

Of cases that were evaluable for mutation events, our analysis identified homozygous *TP53* mutations in 40% (n= 17/42) of invasive lesions and 23% (n= 13/56) of precursor lesions. An additional second or even third mutation at a heterozygous variant allele frequency was found in 8 out of the 17 invasive lesions and 4 out of the 13 precursor lesions (Figure S4). In cutaneous lesions, this event was more frequently observed in invasive SCC than precursor lesions; however, a similar trend was not observed for ocular lesions. Most samples lacked *TP53* copy number loss, consistent with *TP53* copy-neutral loss of heterozygosity through a duplication event following the initial loss of the *TP53* wild-type allele. Our data also support either 1) continued acquisition of *TP53* mutations after the duplication event or 2) the presence of multiple histologically indistinguishable clonal populations, as heterozygous *TP53* mutations were found in both precursor and invasive samples with homozygous *TP53* mutations. Taken together, these results support *TP53* copy-neutral loss of heterozygosity as an early event in SCC development, frequently occurring before invasion.

CDKN2A loss of heterozygosity in SCC progression

As described above, *CDKN2A* copy number loss is a recurrent event in cutaneous and ocular SCCs (n= 12/25 [48%] cutaneous, n= 1/7 [14%] copy-neutral loss of heterozygosity reported in MiOncoseq cutaneous SCCs, n= 17/21 [81%] ocular). Specifically, *CDKN2A* deep deletions (two-copy if diploid) was observed in cutaneous SCCs (n= 3/25) and ocular SCCs (n= 3/21). Additionally, we report that *CDKN2A* copy number loss is also present in cutaneous and ocular precursor lesions (Figure 4). Interestingly, cutaneous AKs had the highest frequency (n= 8/8, 100%) of copy number loss and the highest frequency (n= 2/8, 20%) of homozygous *CDKN2A* mutations. In fact, we found that most samples with a *CDKN2A* copy number loss harbor a *CDKN2A* mutation at a homozygous variant allele frequency (Figure 4A). Similarly, this observation was also seen in the ocular lesions, with both CIN and invasive samples displaying *CDKN2A* homozygous mutation and copy number loss (Figure 4B). Therefore, like *TP53*, our analysis supports *CDKN2A* loss of heterozygosity as occurring at the earliest stages of cutaneous and ocular squamous neoplasia; however. *CDKN2A* loss of heterozygosity more frequently occurs through copy loss.

RB1 nonsense and splice mutations enriched in cutaneous CIS lesions

Unexpectedly, one of the most frequent differences between cutaneous CIS and invasive SCC lesions was the frequency of *RB1* mutations (Figure 3A), which is frequently mutually exclusive with *CDKN2A* alterations in many cancers (36, 37). Not only did we observe the same mutual exclusivity, but *RB1* homozygous/heterozygous nonsense and splice site mutations were enriched in cutaneous CIS lesions (n= 0/8 [0%] AK, n= 8/30 [27%] cutaneous CIS, n= 2/25 [8%] cutaneous SCC) (Figure 3A & S5). Of invasive SCC, *RB1* mutations in our cohort were exclusively found in recurrent or metastatic tumors. Comparison to two TCGA studies of invasive cutaneous SCC confirmed that driving *RB1* mutations were infrequently found in either cohort (6/68 samples with deleterious *RB1* mutations) (Figure S5) (3, 4), and were associated with significantly increased bone invasion

and shorter overall survival (Figure S6)(3). Microscopically, *RB1*-mutated CIS were morphologically heterogeneous from tumor to tumor, with a tendency to display increased inflammatory infiltrate. The findings suggest that *RB1* mutations may characterize a distinct subclass of cutaneous SCC that is enriched for CIS, but may display aggressive behavior when present in invasive SCC.

HPV in SCC

Ocular lesions in our cohort with adequate quantity and quality of tumor material for RNA-ISH were uniformly negative (n= 21/21) for HPV, regardless of *TP53* status. Of cutaneous squamous neoplasms that lacked detectable *TP53* mutation, 3 matched samples from 1 clinical lesion (ID05, ID06, and ID27) demonstrated HPV-associated viropathic changes and were positive for high-risk HPV transcript expression. The remaining *TP53* wild-type lesions were negative for HPV and lacked characteristic HPV morphologic changes.

Transcriptome profiles distinguish precursor and invasive squamous cell neoplasias, and correlate with mutation events—As our mutation and copy number based comparison of precursor/invasive lesions in cutaneous and ocular SCCs did not identify alterations likely driving invasion, we pursued RNA-seq on 20 cutaneous SCC samples selected based on appropriate tumor content (n= 4 AK, n= 8 CIS, n= 8 invasive SCC) to determine whether differences were present at the transcriptome level (Table S8). We found that 129 genes were differentially expressed when comparing invasive SCC against AK and CIS. These included genes previously associated with invasiveness in SCC or other cancer types, including *MMP1*, *MMP3*, *MMP9*, *LAMC2*, *LGALS1*, and *TNFRSF12A* (Figure 5). *CDKN2A* mutation correlated with loss of transcript expression. Transcriptome profiling and gene ontology analysis of *RB1*-mutated versus wild-type CIS lesions revealed enrichment for interferon gamma/alpha, inflammatory response, and allograft rejection signatures (Figure S5).

Discussion

Our study defines the genetic landscape of ocular SCC and describes molecular alterations in precursor lesions at cutaneous and ocular sites. We find that squamous epithelium at both sites undergoes similar pathways of tumorigenesis characterized by UV-signature mutations, an accumulation of driver mutations in precursor lesions, and frequent detection of multiple TP53 mutations in a single lesion.

TP53 mutations and clonality in UV-associated squamous neoplasia

Although *TP53* alterations are a hallmark of non-HPV driven SCCs, we report a high percentage of samples harboring a second or even third *TP53* mutation in our cutaneous and ocular SCCs. Multiple *TP53* mutations, described in normal skin and vulvar intraepithelial neoplasias (34, 38, 39), have been interpreted as multiple intermingled clonal populations with distinct *TP53* mutations. While we acknowledge this possibility, our data suggest there may instead be a single clonal population harboring multiple *TP53* mutations. In fact, studies report that clinically normal sun-exposed skin already has a high mutation burden, and that mutations known to drive cutaneous SCC, such as *NOTCH1* and *TP53*, are already

under strong positive selection. Therefore, a clone must acquire the proper combination of somatic alterations to outcompete all the other clones present in the skin for malignant transformation to begin (34). Reeves *et al*, drawing upon observations from transgenic mouse models of squamous neoplasia, suggests that while a terminally benign papilloma has a small number of subclones driving growth, a malignant tumor develops after a clonal sweep followed by the development of additional subclones originating from the progressing clone (40). Since we identify *TP53* mutations at homozygous and heterozygous variant allele frequencies at all stages of squamous neoplasia, our analysis suggests that a similar selection process in photodamaged human epithelia occurs prior to the formation of microscopically identifiable neoplastic lesions. Furthermore, our findings predict that photodamage results in subclinical proliferations of mutated keratinocytes in ocular epithelium, similar to those described in sun-damaged epidermis.

As precursors likely already harbor the full complement of highly recurrent genomic aberrations associated with invasive disease, other epigenetic or non-genomic events may trigger a transition to invasive disease. In support of this, we find gene expression profile differences between precursor and invasive squamous lesions. However, the mechanism for the shift in transcriptional profile remains unclear.

RB1 mutations in cutaneous squamous cell carcinoma *in situ*

RB1 mutations have been associated with highly metastatic forms of cutaneous carcinoma such as porocarcinoma and virus-negative Merkel cell carcinoma (18, 41, 42), but have not been recognized as a major driver in cutaneous SCC. We identified *RB1* mutations in a substantial subset of cutaneous CIS, as well as a small fraction of cutaneous SCC. *RB1* mutations were mutually exclusive with *CDKN2A* mutations and were associated with inflammatory gene signatures suggestive of distinct changes in the tumor microenvironment. When present in cutaneous SCC, *RB1* mutations were uniformly associated with recurrent or metastatic disease in our cohort. Although the sample size of *RB1*-mutated cutaneous SCC is small, precluding rigorous outcomes analysis, our findings suggest that *RB1* mutations characterize a distinct molecular subclass of SCC with a propensity to form *in situ* lesions, with a decreased rate of progression to invasiveness, but paradoxically increased aggressiveness in the setting of invasive carcinoma.

Molecular alterations in UV-associated precursor lesions: previous reports— Similar to SCCs, AKs have been shown to harbor mutations in tumor suppressors such as *TP53* and *CDNK2A* (6, 21), as well as amplifications of *EGFR* and *MYC* (43, 44). In contrast to a previous report describing intratumoral heterogeneity in pre-invasive squamoproliferative lesions (45), we found that major genomic events were relatively consistent across multiple areas of a given lesion. Despite a previous hypothesis to the contrary (21, 26), we find that *CDKN2A* mutation and loss of heterozygosity are frequent events in AKs, and thus do not represent a likely candidate driver for transition to invasiveness. Similarly, despite studies suggesting p53 inactivation to be a late event (46, 47), we observed that the p53 inactivation is already being selected for at early stages of cancer development in cutaneous and ocular squamous neoplasia, as predicted by a whole genome based study of cutaneous SCC (35).

Published reports comparing expression patterns in AKs and SCCs have had mixed results (6). This may be due in part to the relatively low power of some studies. The largest such study identified significant differences in gene expression between AKs and SCCs (48). Our cohort is of similar size to Lambert *et al* and corroborates findings from that report. Although we cannot exclude the possibility that a subset of the gene expression differences detected between AK and SCC may be related to differences in background tissue rather than neoplastic cells, the similarity of our results with results obtained using microdissection (48), and the established role for many differentially expressed genes in promoting tumor invasiveness, supports these expression changes to be occurring in neoplastic cells.

Molecular profiles of ocular surface squamous neoplasia—Previous mutational studies in ocular neoplasms have been limited, with a disputed role for UV-associated *TP53* mutations (9, 49). A recent exome sequencing study did not comment on mutational profiles within their cohort (9). To our knowledge, this is the first NGS study to profile ocular lesions at pre-invasive, invasive, and treatment-naïve stages. Our results demonstrate the utility of an NGS-based approach, using small ocular surface biopsies and excisions, to nominate precision therapeutic approaches for ocular squamous neoplasms. The current therapies, surgical excision and topical chemotherapies (i.e. interferon-α2b, mitomycin C, 5-fluorouracil) and surgical excision, are not genetically tailored and are variably effective inasmuch as ocular squamous neoplasms has an unusually high relapse rate, even when surgical margins are negative. These treatments can also be associated with ocular pain, limbal stem cell loss, conjunctivitis and other ocular surface toxicities. Such features create an unmet need that could potentially be addressed by currently available oral or systemic therapies, or potential future topical adjunctive therapies, that target aberrant pathways related to genetic alterations in *EGFR, FGFR,* and *PIK3CA* genes present in ocular squamous surface neoplasms that we have identified for the first time in this study (50–52). Furthermore, the molecular similarity between ocular and cutaneous SCC, including a likely hypermutated UV-signature profile in many cases, suggests that therapeutic approaches with promise in cutaneous SCC, such as immunotherapy (53, 54), may also be effective in advanced ocular SCC.

In agreement with several previous studies, we did not find evidence of a role for HPV in ocular SCC (55–58). The discrepancy between this result, and other studies reporting significant rates of HPV detection in these lesions, might be related to differences in clinical cohorts such as the rate of atopy (59). However, the balance of evidence suggests a less prominent role for HPV in ocular SCC than oropharyngeal SCC.

Study limitations—Our study has several limitations. Most ocular CIS samples were from the United States (Ann Arbor, MI; Nashville, TN, and Baltimore, MD) and the invasive, from Sao Paulo, Brazil. One practical reason for this is that invasive ocular SCCs are relatively rare in the United States, but are more common in regions with increased UV radiation exposure, such as Sao Paulo, Brazil. The dearth of invasive samples from the United States precluded meaningful comparison of histopathologic differences, such as aggressiveness, by nation of origin. We acknowledge that the possibility that differences related to region of origin might influence comparisons between CIS and invasive ocular

SCCs in our study; however, this factor is unlikely to be a significant confounder, as we report fundamental similarities between these groups rather than differences.

Furthermore, there was limited follow-up for many patients and few episodes of recurrence, precluding robust associations between genomic profiles and outcome. Our targeted approach does not detect events that affect genes not included in our cancer panel, and therefore exome-wide sequencing might provide more definitive analysis of mutational spectra and chromosomal copy number aberrations in these lesions. However, findings from previous exome-wide sequencing studies indicate that our panel encompasses the highly recurrent drivers of cutaneous SCC. Of note, our panel does not include KMT2 and FAT family genes reported to display recurrent (but not universal) mutations in SCC. Another potential limitation is lack of comparison to normal germline DNA for many samples, which may impact individual mutation calls; however, we compared our findings to multiple large germline sequencing databases (see Materials and Methods) to minimize potential inclusion of germline variants. As noted above, we cannot exclude the possibility that background tissue may influence expression profiling results. Finally, our approach does not address tumor mutation burden or epigenetic alterations.

Conclusion

We find that ocular and cutaneous squamous neoplasms demonstrate a similar spectrum of genetic changes and hence represent parallel models for squamous neoplasia on UV-exposed epithelia. We have profiled invasive and treatment naïve preinvasive ocular lesions for the first time. In both ocular and cutaneous settings, precursor lesions already possess the full complement of major genetic changes that are seen in invasive SCC. By contrast, cutaneous precursor lesions demonstrate a distinct transcriptome profile from invasive SCC. In contrast to the stepwise accumulation of mutations proposed for some other malignancies, our findings support the hypothesis that transition to invasiveness in cutaneous SCC may be driven by changes in the transcriptional program or other epigenetic features rather than acquisition of additional genomic insults. Finally, the alterations we identify here are targetable and provide crucial insights toward novel precision therapies for ocular surface lesions, which frequently recur despite current treatment modalities of surgical resection and topical chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This research was supported in part by NIH K08EY026654 (to R.C.R.), NIH/NEI 5K08EY027464-02 (to A.B.D.) P30CA046592 (to the University of Michigan Comprehensive Cancer Center); the Research to Prevent Blindness (to the University of Michigan Kellogg Eye Center and R.C.R.), A. Alfred Taubman Medical Research Institute Leslie and Abigail Wexner Emerging Scholar Program (to R.C.R.), A. Alfred Taubman Medical Research Institute A. Alfred Taubman Emerging Scholar Program (to S.A.T.), Grossman Research Fund (to R.C.R.), Leonard G. Miller Professorship and Ophthalmic Research Fund at the Kellogg Eye Center (to R.C.R.), Barbara Dunn Research Fund (to R.C.R.), Roz Greenspon Research Fund (to R.C.R.), Beatrice & Reymont Paul Foundation (to R.C.R.), and March Hoops to Beat Blindness (to R.C.R.). A.M.C is an NCI Outstanding Investigator (R35CA231996), Howard Hughes Medical Institute Investigator, A. Alfred Taubman Scholar, and American Cancer Society Professor.

Financial Support: This research was supported in part by NIH K08EY026654 (to R.C.R.), P30CA046592 (to the University of Michigan Comprehensive Cancer Center); the Research to Prevent Blindness (to the University of Michigan Kellogg Eye Center and R.C.R.), A. Alfred Taubman Medical Research Institute Leslie and Abigail Wexner Emerging Scholar Program (to R.C.R.), A. Alfred Taubman Medical Research Institute A. Alfred Taubman Emerging Scholar Program (to S.A.T.), Grossman Research Fund (to R.C.R.), Leonard G. Miller Professorship and Ophthalmic Research Fund at the Kellogg Eye Center (to R.C.R.), Barbara Dunn Research Fund (to R.C.R.), Roz Greenspon Research Fund (to R.C.R.), Beatrice & Reymont Paul Foundation (to R.C.R.), and March Hoops to Beat Blindness (to R.C.R.). A.M.C is an NCI Outstanding Investigator (R35CA231996), Howard Hughes Medical Institute Investigator, A. Alfred Taubman Scholar, and American Cancer Society Professor.

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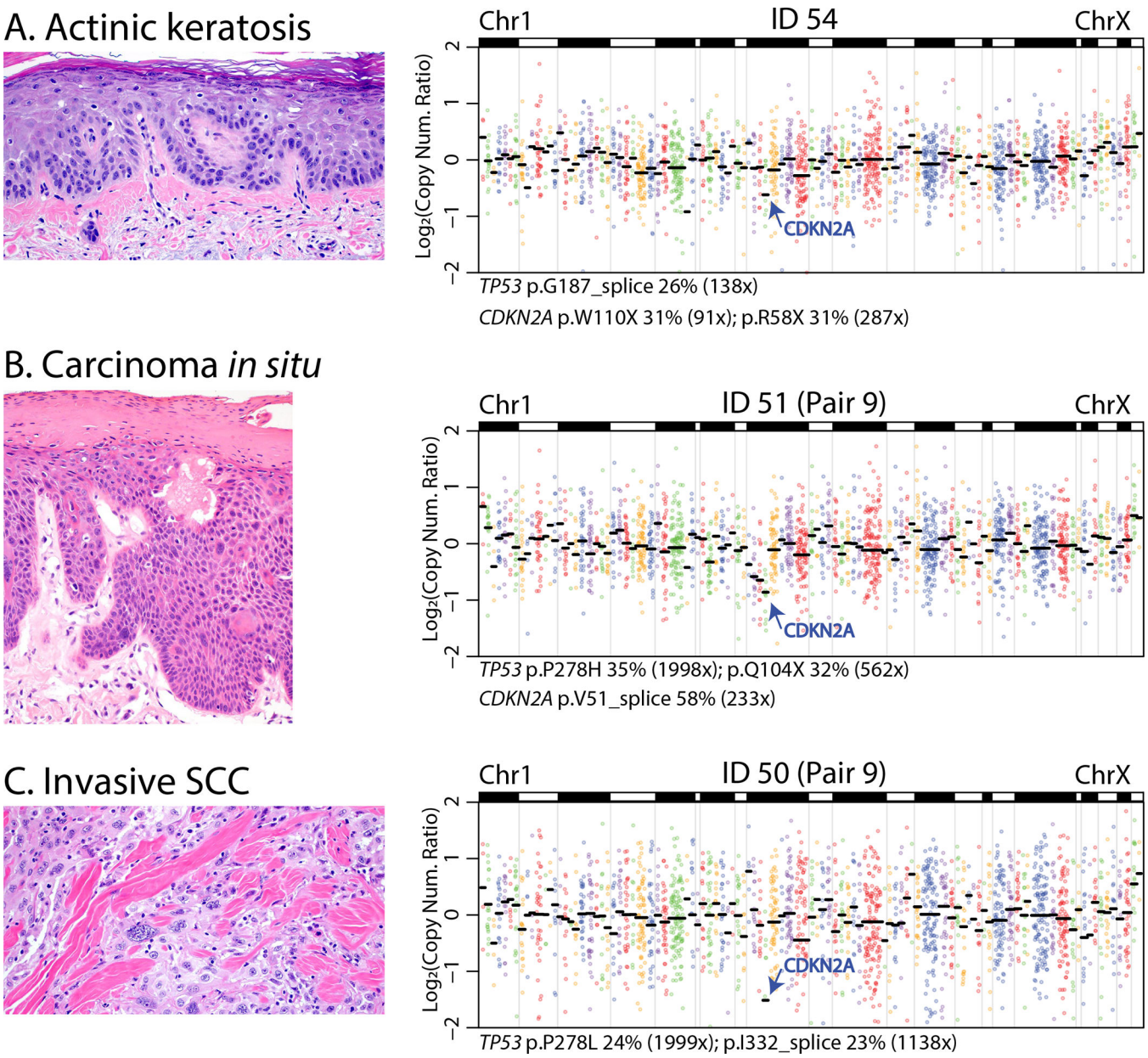

FIGURE 1. Cutaneous squamous carcinoma and precursor lesions by light microscopy (left) and molecular features (right).

(A) Actinic keratosis displaying atypia of the basal layer of the epidermis, with maturation in the upper layers. Copy number profiling demonstrated *CDKN2A* loss. Nonsynonymous mutations including truncating mutation of *TP53* and truncating mutations of *CDKN2A*. (B) Squamous cell *in situ*, demonstrating full-thickness squamous atypia without invasion. Molecular features include *CDKN2A* loss and mutation, accompanied by *TP53* mutations. (C) Invasive squamous cell carcinoma adjacent to the *in situ* lesion in the panel above, displaying malignant squamous cells infiltrating collagen. Molecular findings include *CDKN2A* loss and distinct *TP53* mutations.

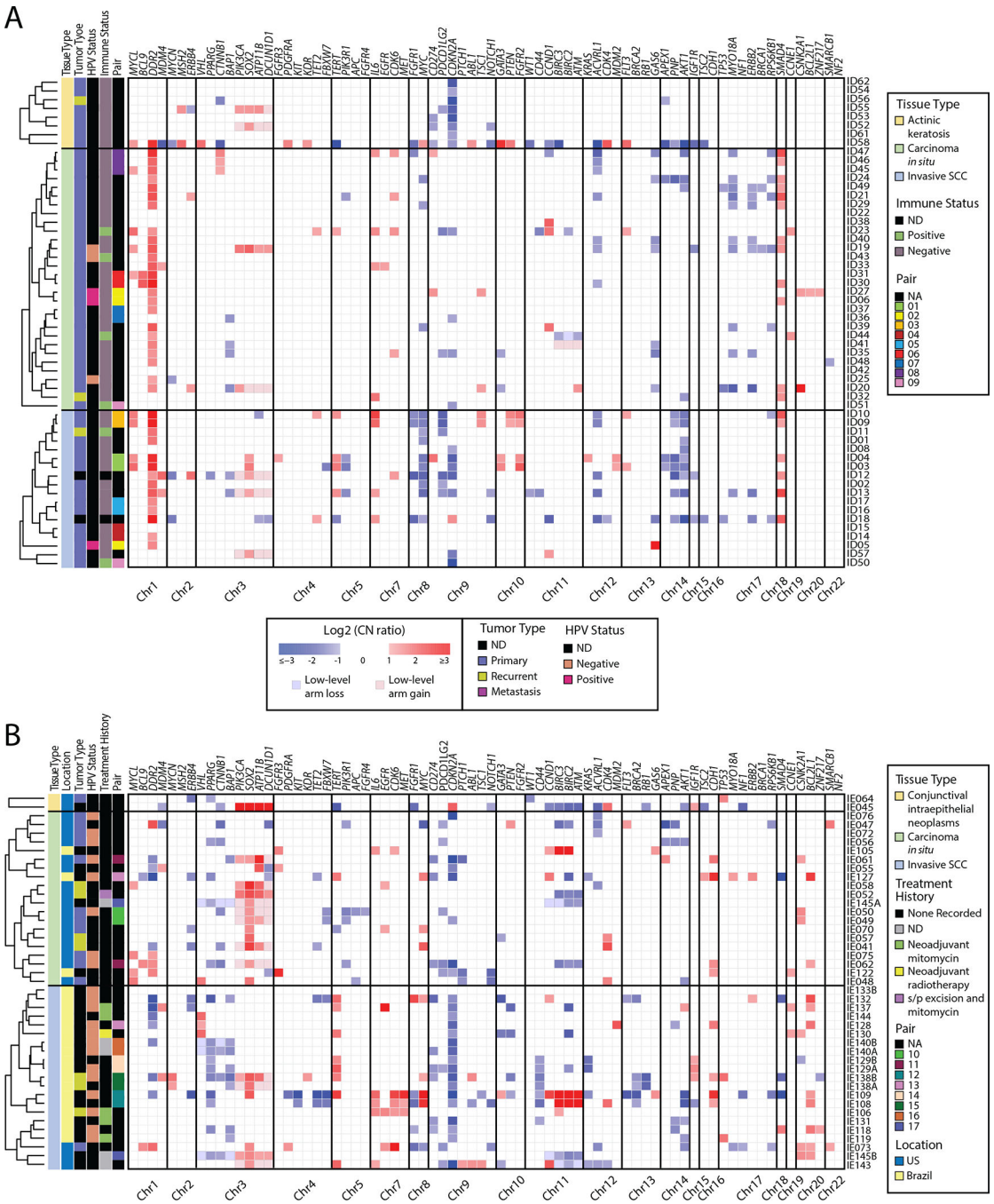

FIGURE 2. Somatic copy number profiles of (A) cutaneous and (B) ocular lesions generated by targeted next generation sequencing (NGS).

Somatic, autosomal copy number profiles are presented for (A) 63 cutaneous and (B) 43 ocular tissues. Each copy number profile was GC and tumor content corrected. Normalized read counts per amplicon were divided by those from composite normal tissue, yielding a copy number ratio for each gene (cancer/composite normal), with red and blue indicating gain and loss, respectively, according to the log2 color scale (right). Unsupervised clustering was used on all log2 copy number ratios within lesion groups. Copy number ratios between the range of −1 and 1 were not visualized. Genes part of low arm-level gains and losses are

shown with a different shade and border. Columns represent individual targeted genes in genome order (from chromosome 1 to 22). Clinicopathologic features are indicated in the figure legend. MI-Oncoseq cases are not shown due to differences in normalization. ND: Not Determined; NA: Not Available

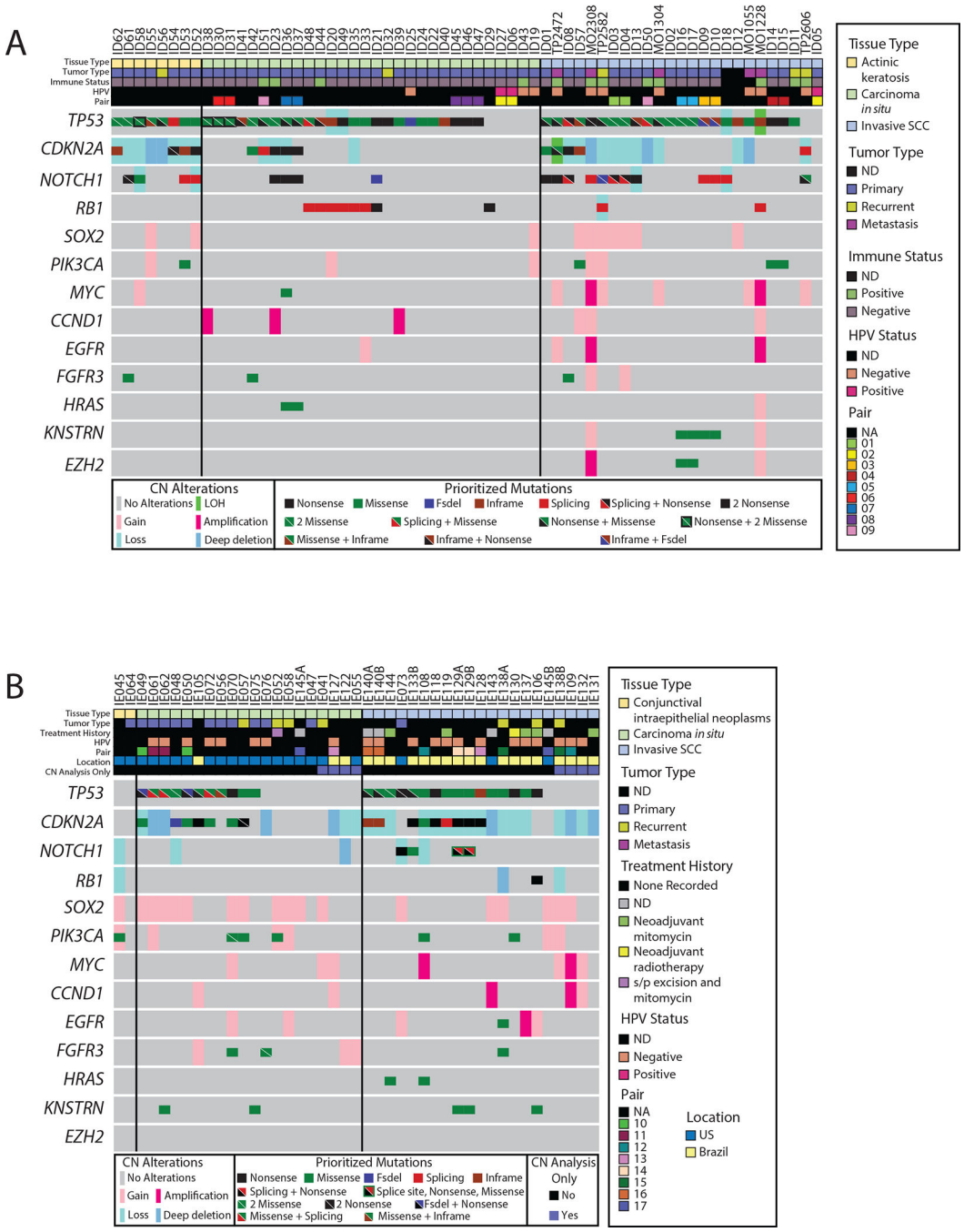

FIGURE 3. Integrated heatmap of prioritized mutations and copy number aberrations identified by next generation sequencing.

Integrated table of prioritized nonsynonymous mutations and copy number aberrations from (A) 63 cutaneous and (B) 43 ocular tissues. Rows represent genes and columns represent individual samples. Clinicopathologic features are indicated in the figure legend. Copy number aberrations and prioritized mutation types are indicated below the table. A total of eight ocular tissues samples were only analyzed for copy number aberrations and not for mutations, as labeled. Thresholds used: Loss (1-copy loss), Deep deletion (2-copy loss), Gain (1 or 2 copy gain), Amplification (>2-copy gain)

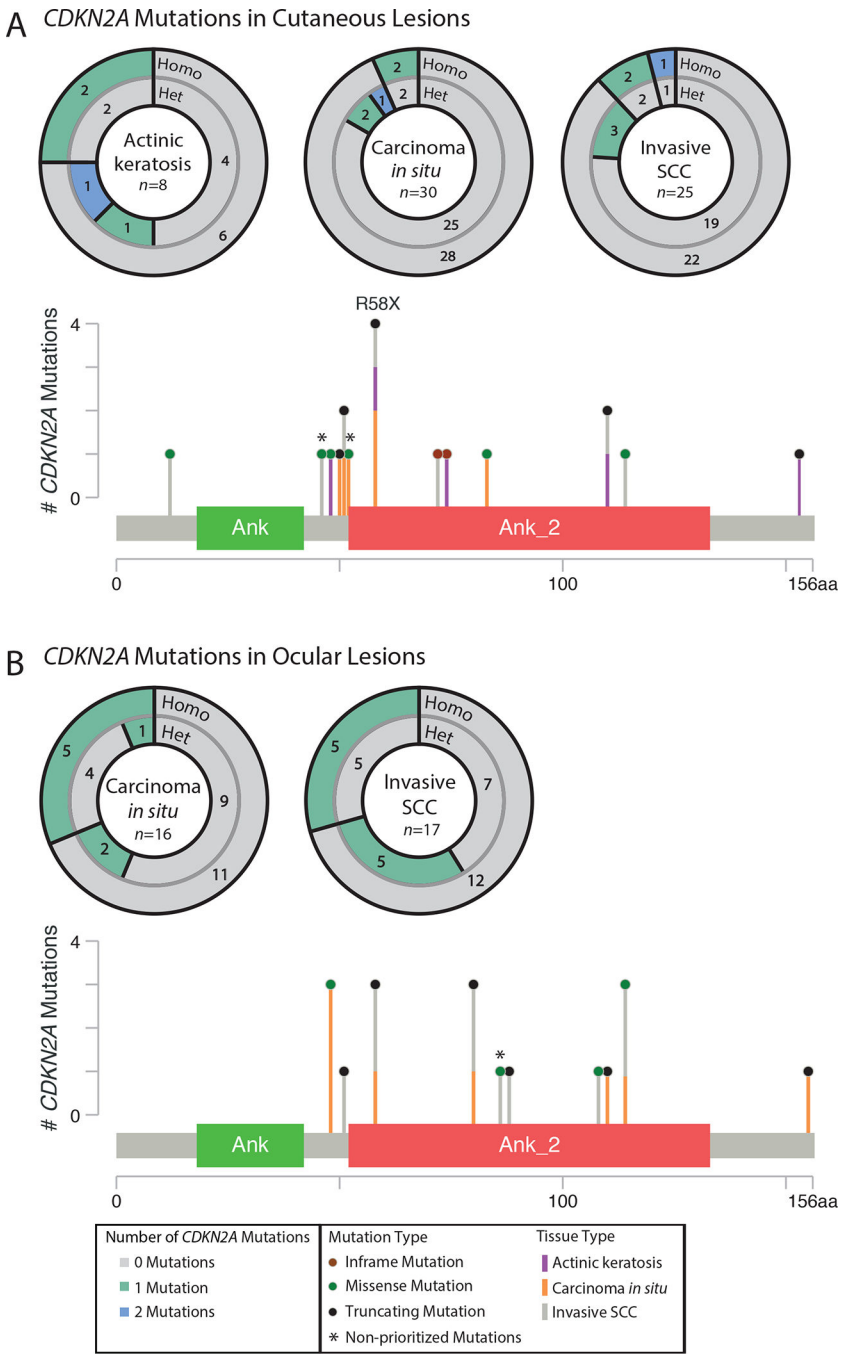

FIGURE 4. Two-level concentric pie charts and *CDKN2A* variant mapping.

Two-level concentric pie charts show zygosity (each level) and co-occurrence (overlapping regions of the two levels) of *CDKN2A* mutations and *CDKN2A* variant mapping across (A) AK, CIS, invasive cutaneous SCC and B) CIS and invasive conjunctival SCC. Outside circle gives the number of samples with a homozygous (Homo) mutations. Inside circle gives the number of samples with heterozygous (Het) mutations. The number of heterozygous/homozygous *CDKN2A* mutations in each section is denoted by shading. Overlapping regions of the pie chart indicate samples with multiple mutations at homozygous and

heterozygous mutations. *CDKN2A* (NM_000077) mutations in cutaneous and ocular SCCs were arranged by amino acid location. Histological classification is noted by the color of each post segment. Mutation type is labeled by the colored dot as according to the figure legend.

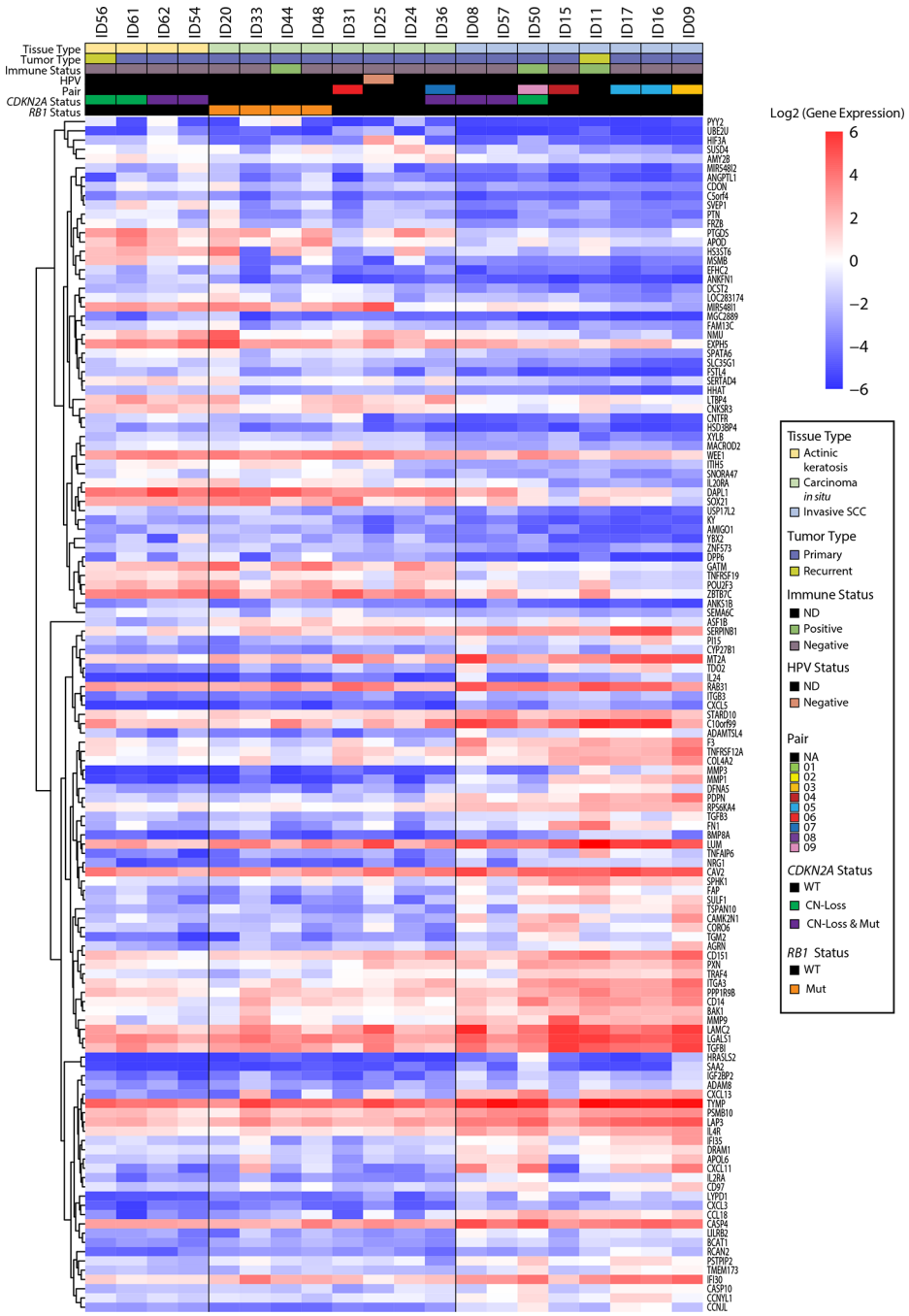

FIGURE 5. Gene expression heatmap generated from cutaneous SCC whole-transcriptome amplicon-based RNA-seq.

Heatmap of median-centered expression of 129 overlapping differentially expressed genes from the AK versus invasive SCC and *in situ* versus cutaneous invasive SCC comparison. Clinicopathologic features are indicated in the figure legend. ND: Not Determined; NA: Not Available

Table 1:

Summary of distribution of types of cutaneous and ocular lesions

	Tissue Type	Number of Samples	Number of Pairs	Number of Distinct Clinical Lesions	Notes
Cutaneous Lesions	Actinic Keratosis (AK)	8	0	52	Additional 2 pairs include *in situ* and invasive
	Carcinoma *in situ* (CIS)	30	3		
	Invasive SCC	25	4		
	Total	63	7		
Ocular Lesions	Conjunctival Intraepithelial Neoplasia (CIN)	2	0	35	Additional 2 pairs include *in situ* and invasive
	Carcinoma *in situ* (CIS)	20	2		
	Invasive SCC	21	4		
	Total	43	6		