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Original Article

CDKN2A and matrix metalloproteinases: key regulators of cellular senescence in squamous cell carcinoma

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Abstract: Objectives: This study aimed to investigate the role of cellular senescence in the progression of squamous cell carcinoma (SCC) and to identify key senescence-associated biomarkers and pathways that contribute to tumor aggressiveness. Methods: RNA sequencing data from SCC and normal skin tissues (GSE191334) were analyzed using the DESeq2 package to identify differentially expressed genes (DEGs). Upregulated DEGs were cross-referenced with the CellAge database to identify senescence-related biomarkers. Functional enrichment analyses were conducted using EnrichR, GeneCodis4, and KEGG databases. Protein-protein interaction networks were mapped using STRING, and mutational profiling of CDKN2A was performed via the G2P portal and UCSC Xena. Results: A total of 1,448 genes were upregulated and 1,700 downregulated in SCC. Among these, 38 upregulated genes were associated with cellular senescence. Notably, CDKN2A was prominently expressed, suggesting a stress-induced senescence response. CDKN2A, along with MMP3 and MMP12, formed central hubs within interaction networks, implicating them in extracellular matrix remodeling and tumor invasiveness. Enrichment analyses highlighted activation of epithelial-to-mesenchymal transition (EMT), inflammatory signaling, and senescence-associated secretory phenotype (SASP). Immune-modulatory genes such as ULBP2 and IL6 were also elevated. Mutation analysis revealed alterations in the CDKN2A-encoded p16^INK4a^, potentially disrupting its tumor-suppressive functions. Conclusions: Cellular senescence in SCC exhibits a dual role - initially tumor-suppressive, later promoting invasion and metastasis. Key biomarkers such as CDKN2A and MMPs may serve as therapeutic targets. These findings lay the groundwork for future translational research to improve SCC diagnosis and treatment.

Keywords: Squamous cell carcinoma, cellular senescence, CDKN2A, epithelial-to-mesenchymal transition, tumor microenvironment

Introduction

Squamous cell carcinoma is a highly aggressive form of skin cancer originating from squamous cells in the epidermis. One of the key mechanisms contributing to its progression and metastasis is cellular senescence, a complex process that initially functions as a tumor-suppressive response but can paradoxically promote tumor growth at later stages [1]. According to the GLOBOCAN 2022 report, skin cancers, including SCC, represent a significant global health concern, with nearly half a million new cancer cases annually and approximately 58,667 cancer deaths worldwide [2].

Cellular senescence is a state where cells permanently stop proliferating in response to stressors like DNA damage or oncogene activation, acting as a defense to prevent cancerous growth [3]. In early SCC, senescence halts tumor progression by arresting the proliferation of transformed keratinocytes. Key pathways, such as p53 and RB, trigger this process by

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activating inhibitors like p21 and p16, enforcing cell cycle arrest and preventing malignant cell expansion [4, 5].

While senescence initially protects against cancer, senescent cells can adopt a senescence-associated secretory phenotype (SASP), releasing pro-inflammatory cytokines, growth factors, and proteases that alter the tissue environment. In SCC, SASP shifts from a tumor-suppressive to a tumor-promoting role, stimulating nearby cancer cells to grow and invade [6, 7]. Cytokines like IL-6 and IL-8 activate pathways such as JAK-STAT and NF-κB, enhancing SCC cell survival, proliferation, and invasion. Thus, despite being non-proliferative, senescent cells contribute to a microenvironment that supports cancer progression and metastasis [8].

However, SCC cells often acquire mutations that allow them to evade senescence, further contributing to tumor progression. Mutations in key tumor suppressors such as p53 and CDKN2A (which encodes p16) are frequently observed in advanced SCC. The loss of these tumor suppressor functions enables SCC cells to bypass the senescence checkpoint, allowing them to proliferate uncontrollably despite the presence of DNA damage or other oncogenic signals [9, 10].

Moreover, senescent cells can contribute to the remodeling of the tumor microenvironment in a way that promotes invasion and metastasis. For example, components of the SASP can enhance the expression of matrix metalloproteinases (MMPs), which degrade the extracellular matrix (ECM) and facilitate the invasion of SCC cells into surrounding tissues. Moreover, SASP factors can recruit immune cells such as macrophages and neutrophils, which can paradoxically support tumor growth and invasion through the release of growth-promoting and pro-angiogenic factors [11, 12].

SCC cells that bypass senescence often acquire enhanced migratory and invasive capabilities. The loss of senescence control allows SCC cells to undergo EMT, a process that endows them with the ability to detach from the primary tumor and invade other tissues. This contributes to the high metastatic rate of SCC, making it one of the deadliest skin cancers [13].

Targeting cellular senescence in SCC offers a promising therapeutic approach. Interventions may include senolytic drugs, which selectively eliminate senescent cells, or strategies to modify the SASP to reduce its pro-tumorigenic effects. This study aims to use RNA sequencing to identify biomarkers related to cellular senescence in SCC, with a focus on its role in tumor progression and metastasis. By analyzing gene expression profiles, the study seeks to identify dysregulated genes and pathways involved in senescence, with the goal of understanding and addressing its dual role in SCC that can be exploited for therapeutic intervention.

Materials and methods

Data collection and differential expression analysis

The RNA-sequencing dataset used in this study was sourced from the NCBI Gene Expression Omnibus (GEO) under accession number GSE-191334, a public repository for functional genomics data such as microRNA and next-generation sequencing [14]. The dataset includes 16 RNA-seq samples, with 8 from individuals with SCC and 8 from those without SCC. Sequencing was performed on the Illumina HiSeq platform using paired-end read technology. Differential expression analysis between SCC and normal tissue was conducted using the DESeq2 package, which models gene expression variability with a negative binomial distribution [15]. Significant upregulated and downregulated genes (log2 fold change > 1 or < -1, p-value < 0.05) were visualized using volcano plots.

The workflow includes data acquisition from GEO, CellAge, and TCGA/GTEx databases, followed by differential expression analysis using DESeq2. Identified genes were filtered for senescence relevance, functionally enriched using EnrichR and GeneCodis4, and assessed for protein interactions via STRING. Key genes (CDKN2A, MMP3, MMP12) were further validated through mutation and isoform analysis using G2P and UCSC Xena. Kaplan-Meier survival analysis evaluated the prognostic value of CDKN2A mutations, supporting their potential as therapeutic targets (**Figure 1**).

Identification of cellular senescence genes

Experimentally known cellular senescence genes were obtained from the CellAge data-

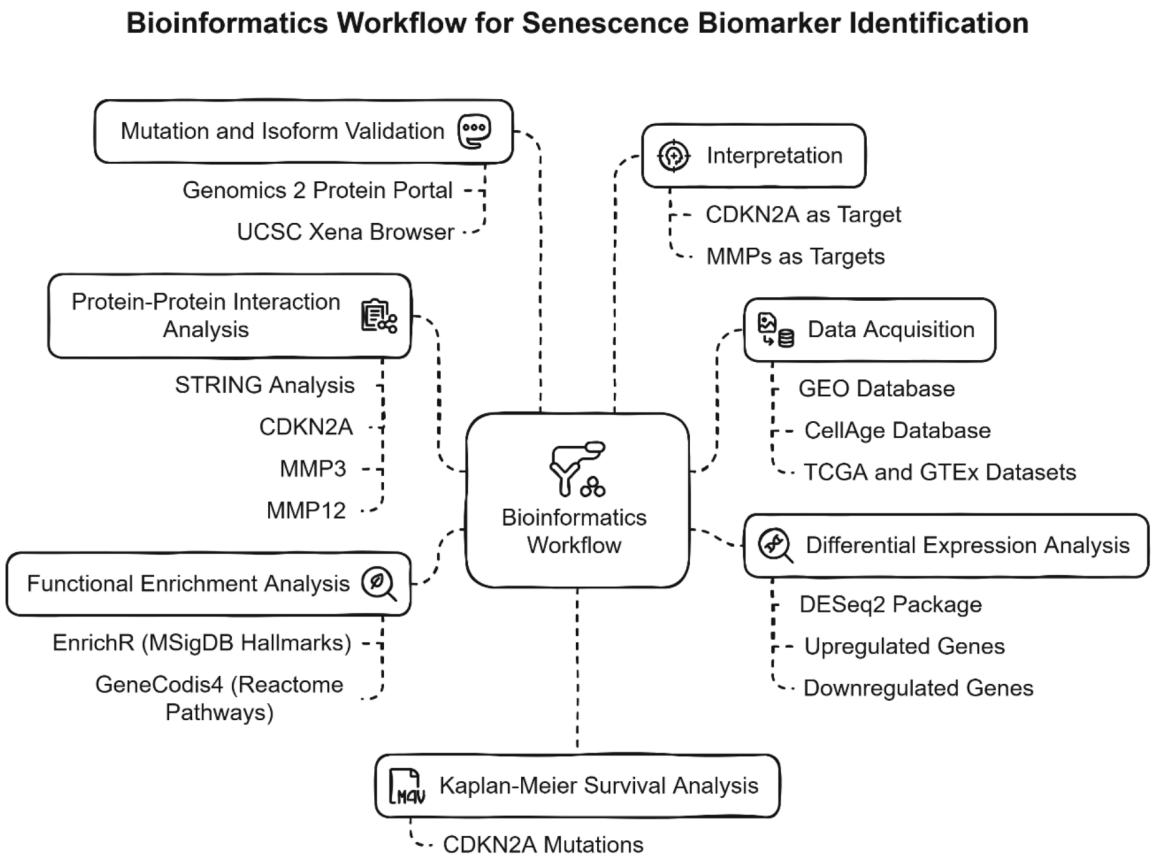


Figure 1. Bioinformatic workflow for senescence biomarker identification in SCC. A schematic overview of the integrative bioinformatics pipeline used in this study. Differential gene expression (DESeq2), senescence gene screening (CellAge), pathway enrichment (EnrichR, GeneCodis4), protein interaction mapping (STRING), mutation validation (UCSC Xena, G2P), and survival analysis (Kaplan-Meier) were sequentially applied to dissect CDKN2A- and MMP-associated senescence dysregulation in SCC.

base (https://genomics.senescence.info), which curates genes associated with the regulation of cellular senescence [16]. These genes were then cross-referenced with the DEGs identified previously. By comparing the upregulated genes from the CellAge database with the DEGs, we filtered out senescence-associated genes that were significantly upregulated in the SCC samples, providing a focused set of candidate senescence biomarkers for further analysis.

Functional enrichment analysis

Functional enrichment analysis of all identified upregulated genes in SCC versus normal skin tissues was conducted using the EnrichR database (https://maayanlab.cloud/Enrichr/), focusing on the MSigDB Hallmark pathways to assess the enriched pathways linked to the dysregulated genes in SCC, potentially associated with cellular senescence [17]. Additionally, the shortlisted senescence biomarkers were analyzed for pathway enrichment through Reactome using GeneCodis4 (https://genecodis.genyo.es/), providing insight into the biological processes and signaling pathways these biomarkers are involved in.

Protein-protein network analysis

To assess the functional interactions between the identified senescence biomarkers, protein-protein interaction (PPI) network analysis was performed using the STRING database (https://string-db.org/), a platform for mapping and visualizing protein interactions [18]. Cellular senescence-related genes, along with their log2FC values, were input into STRING using default settings. The resulting PPI networks were clustered based on their centroids to identify key functional networks. The cluster

with the highest number of proteins directly linked to cellular senescence and previously reported in SCC was selected for further analysis. Considering the well-established role of CDKN2A, a key regulator of cellular senescence, particularly in SCC and metastatic SCC, this gene was prioritized within the network. CDKN2A encodes both p16^INK4a^, which inhibits cyclin-dependent kinases and enforces cell cycle arrest, and p14^ARF^, which stabilizes p53, a crucial tumor suppressor. Mutations or deletions in CDKN2A are frequently observed in SCC, contributing to uncontrolled cell proliferation and tumor progression. Given its central role in regulating senescence and its significant involvement in SCC biology, it was chosen for further analysis [10, 19].

CDKN2A mutation analysis and visualization

To validate the shortlisted genes, TCGA Skin Cancer and GTEx Skin datasets on UCSC Xena (https://xena.ucsc.edu/) were used to evaluate the expression levels in SCC compared to normal skin expression levels [20]. Furthermore, previously reported missense mutations in CDKN2A were assessed and visualized using the Genomics 2 Proteins (G2P) Portal (https://g2p.broadinstitute.org/), a discovery platform that links genetic screening data to protein sequences and structures [21]. The G2P portal aggregates variant information from databases such as gnomAD, ClinVar, and the Human Gene Mutation Database (HGMD), allowing for comprehensive analysis of genetic alterations.

Protein expression validation in the human protein atlas (HPA)

To validate the overexpression of selected candidate genes at the protein level, immunohistochemistry (IHC)-based expression data for CDKN2A and MMP3 were retrieved from HPA database. Protein expression patterns in squamous cell carcinoma tissues were compared with those in normal skin tissues using HPA-reported staining intensity and localization. This cross-validation helped confirm transcriptomic findings in histopathologic samples.

Raw RNA-seq data (GSE191334) were downloaded from GEO and processed using the DESeq2 package (v1.38.3) in R for differential gene expression analysis. Genes with |log2FoldChange| > 1 and adjusted *p*-value < 0.05 (Benjamini-Hochberg correction) were considered significant. Senescence-associated genes were identified by cross-referencing upregulated DEGs with the CellAge database. Functional enrichment analyses were conducted using EnrichR, GeneCodis4, and DAVID for MSigDB Hallmark, GO, KEGG, and Reactome pathways.

Protein-protein interaction networks were constructed by STRING (v12.0) using a confidence score cutoff > 0.7. Mutation and isoform expression of CDKN2A were analyzed using UCSC Xena (TCGA-SKCM and GTEx datasets) and Genomics 2 Proteins (G2P) portal, respectively. Finally, immunohistochemical validation of CDKN2A and MMP3 was performed using high-resolution tissue staining data from the Human Protein Atlas, corroborating the mRNA expression results.

Statistical analysis

Differential gene expression was analyzed using the DESeq2 package, which applies a negative binomial generalized linear model. Genes with |log2FC| > 1 and adjusted *p*-value < 0.05 (Benjamini-Hochberg FDR correction) were considered significant. Functional enrichment (GO, KEGG, Reactome) was performed by EnrichR and GeneCodis4 using Fisher's exact test. Kaplan-Meier survival analysis was conducted using the log-rank test to compare survival outcomes between CDKN2A-mutated and wildtype groups. STRING was used for protein-protein interaction clustering based on confidence scores (> 0.7). All analyses considered P < 0.05 as significant.

Results

Upregulation of senescence-associated and matrix-remodeling genes

Differentially expressed genes (DEGs) between SCC and normal skin tissues were identified using the DESeq2 package. The analysis showed significant upregulation of 1,448 genes (FC > 1, *p*-value < 0.05) and significant downregulation of 1,700 genes (FC < -1, *p*-value < 0.05). These findings are illustrated in the volcano plot presented in **Figure 2A**.

To investigate the role of cellular senescence in SCC, we sourced 526 senescence-associated genes from the CellAge database, which lists

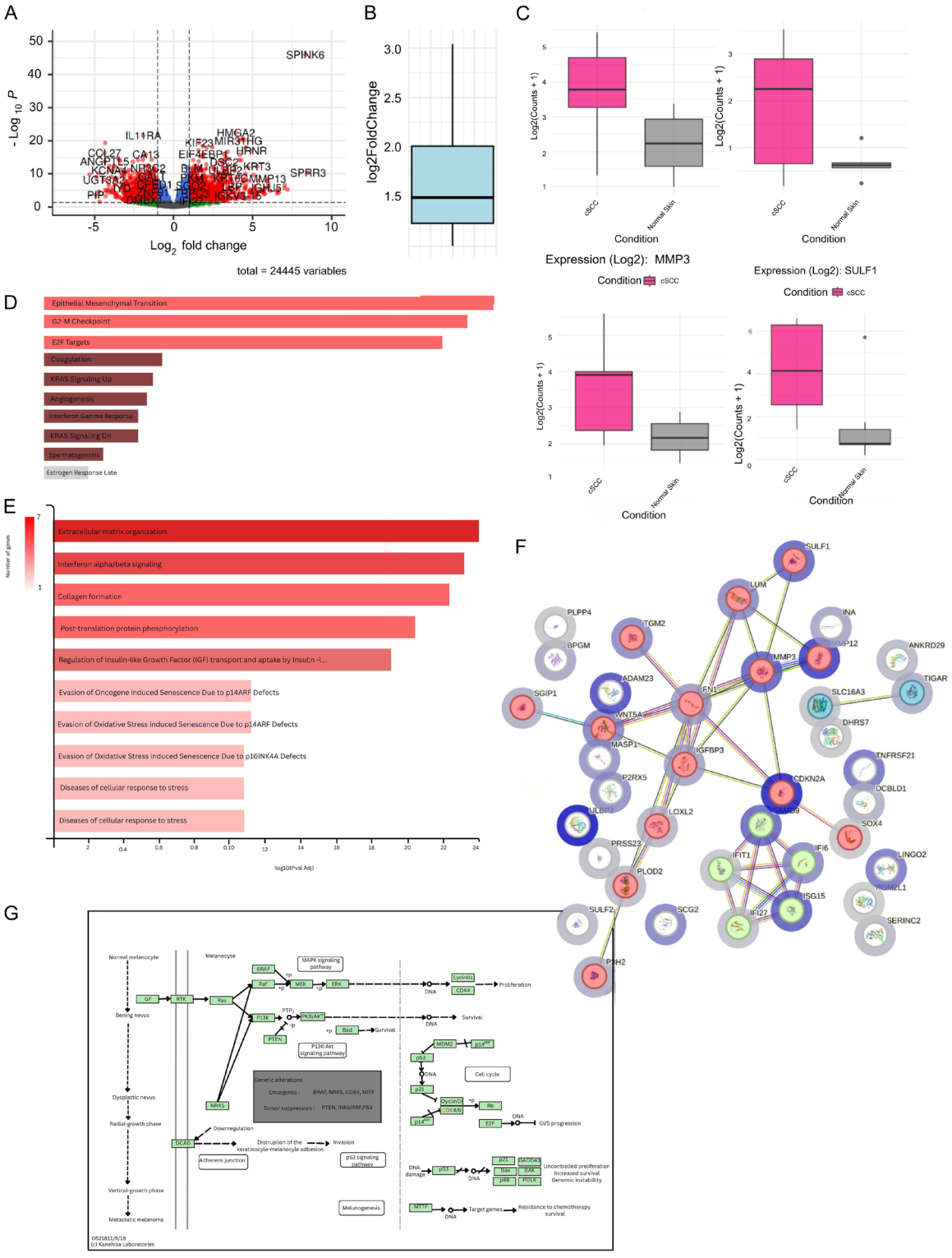


Figure 2. A. Volcano plot showing differentially expressed genes (DEGs) between SCC and normal skin samples. Red: DEGs with both |log2FC| > 1 and P < 0.05. B. Box plot of log2 fold changes for 38 significantly upregulated senescence-associated genes. C. Normalized expression (log2 counts) of CDKN2A, MMP3, MMP12, and SULF1 in SCC vs. normal skin. D. Hallmark pathway enrichment using EnrichR; top pathways include EMT and G2-M checkpoint. E. Reactome pathway enrichment of senescence genes; key enriched terms include ECM organization and senescence evasion mechanisms. F. STRING PPI network of senescence biomarkers; CDKN2A, MMP3, MMP12

highlighted as core nodes. G. KEGG pathway visualization indicating CDKN2A involvement in melanoma and cell cycle disruption. Statistical symbols: Log2FC = log2 fold change; P = unadjusted *p*-value; Significance threshold: |log2FC| > 1 and P < 0.05.

Table 1. Identified upregulated cellular senescence markers in SCC

Symbol	Description	*p* value	log2 Fold Change
WNT5A	Wnt family member 5A	4.26E-19	1.855989
TNFRSF21	TNF receptor superfamily member 21	1.39E-18	2.097308
ULBP2	UL16 binding protein 2	8.49E-12	3.273529
SAMD9	sterile alpha motif domain containing 9	6.23E-10	2.259175
ADAM23	ADAM metallopeptidase domain 23	7.68E-09	2.587683
DCBLD1	discoidin, CUB and LCCL domain containing 1	1.86E-08	1.306087
SULF2	sulfatase 2	2.39E-07	1.235862
CDKN2A	cyclin dependent kinase inhibitor 2A	1.67E-06	3.039969
SULF1	sulfatase 1	5.04E-06	2.285812
MMP12	matrix metallopeptidase 12	1.09E-05	2.734286
LUM	lumican	1.37E-05	1.817698
DHRS7	dehydrogenase/reductase 7	1.50E-05	1.036731
BPGM	bisphosphoglycerate mutase	1.98E-05	1.439094
ISG15	ISG15 ubiquitin like modifier	2.29E-05	2.35027
MASP1	MBL associated serine protease 1	8.03E-05	1.699291
LOXL2	lysyl oxidase like 2	8.36E-05	1.249012
PRSS23	serine protease 23	0.000132	1.171195
LINGO2	leucine rich repeat and Ig domain containing 2	0.000198	2.048927
IGFBP3	insulin like growth factor binding protein 3	0.000318	1.504093
TGM2	transglutaminase 2	0.000472	1.798471
SCG2	secretogranin II	0.000543	1.892025
P3H2	prolyl 3-hydroxylase 2	0.000862	1.346034
SOX4	SRY-box transcription factor 4	0.000923	1.01867
PGM2L1	phosphoglucomutase 2 like 1	0.000945	1.003267
IFI6	interferon alpha inducible protein 6	0.00161	1.803146
ANKRD29	ankyrin repeat domain 29	0.00162	1.23202
SLC16A3	solute carrier family 16 member 3	0.00189	1.091495
FN1	fibronectin 1	0.00216	1.586963
SGIP1	SH3GL interacting endocytic adaptor 1	0.00233	1.353949
SERINC2	serine incorporator 2	0.0026	1.23185
P2RX5	purinergic receptor P2X 5	0.00261	1.739155
TIGAR	TP53 induced glycolysis regulatory phosphatase	0.00329	1.31521
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	0.00778	1.179803
PLPP4	phospholipid phosphatase 4	0.00972	1.058178
INA	internexin neuronal intermediate filament protein alpha	0.0111	1.478859
IFIT1	interferon induced protein with tetratricopeptide repeats 1	0.0174	1.082097
IFI27	interferon alpha inducible protein 27	0.0202	1.118769
MMP3	matrix metallopeptidase 3	0.0469	2.288813

genes reported to be upregulated in various studies and contribute to cellular senescence. Comparing this list with the upregulated DEGs in SCC, we found that 38 senescence-associated genes were significantly upregulated in SCC tissues, suggesting a direct link between these genes and the development or progression of cellular senescence in SCC, given in **Table 1**.

Overall expression of upregulated cellular senescence markers is shown in **Figure 2B**.

Among these cellular senescence genes were members of the Wnt pathway, TNF receptor superfamily, metallopeptidase, and interferon-inducible protein families. Notably, WNT5A (logFC = 1.86, P = 4.26E-19) and TNFRSF21 (logFC = 2.10, P = 1.39E-18) were significantly upregulated, indicating a potential role in SCC pathogenesis through the Wnt signaling and TNF receptor pathways, respectively. The upregulation of ULBP2 (logFC = 3.27, P = 8.49E-12) further suggests the involvement of immune evasion mechanisms, as this gene is known for its role in immune surveillance.

Most importantly, CDKN2A (logFC = 3.04, P = 1.67E-06), which encodes the p16INK4a protein, a well-known cell cycle regulator, was also significantly upregulated. This gene is a critical mediator of cellular senescence, and its upregulation in SCC suggests an attempt to induce senescence in response to oncogenic stress, potentially influencing tumor behavior.

Additionally, MMP3 and MMP12 were significantly upregulated, highlighting their role in matrix degradation and tumor invasion. MMPs are crucial in the degradation of extracellular matrix components, promoting cancer cell invasion and metastasis. The coordinated upregulation of multiple MMPs highlights their likely collective contribution to SCC progression. A significant upregulation of interferon-stimulated genes, such as ISG15 (logFC = 2.35, P = 2.29E-05), IFI6 (logFC = 1.80, P = 0.00161), and IFI27 (logFC = 1.12, P = 0.0202) was also observed. These genes are involved in the cellular response to viral infections and can modulate immune responses in the tumor microenvironment. ADAM23 (logFC = 2.59, P = 7.68E-09), a member of the ADAM family, was significantly upregulated. This family of genes is known for its role in cell adhesion and proteolysis, suggesting their involvement in the modulation of cellular interactions and matrix remodeling in SCC.

Moreover, genes such as SULF1 (logFC = 2.29, P = 5.04E-06), SULF2 (logFC = 1.24, P = 2.39E-07), and LOXL2 (logFC = 1.25, P = 8.36E-05) were upregulated, highlighting the involvement of extracellular matrix modification and the potential for altering the tumor microenvironment in SCC.

Key pathways involved in epithelial-mesenchymal transition

Functional enrichment analysis of upregulated genes in SCC versus normal skin tissues, conducted using the EnrichR database and focusing on MSigDB Hallmark pathways, revealed significant involvement of key pathways, including EMT, the G2-M checkpoint, and E2F targets, shown in **Figure 2D**.

However, cancer cells evade senescence-related growth inhibition through the EMT pathway, which showed a significant enrichment, with 43 out of 200 upregulated genes associated with this process (*P*-value = 1.63E-9). Key genes identified include FBN2, FOXC2, CXCL6, CXCL8, SERPINE1, LAMA3, TNC, PLOD2, LOXL2, MMP1, WNT5A, and FN1. The upregulation of these genes indicates a reliance on EMT to enhance invasive potential in SCC, with implications for local tissue invasion and metastasis.

The analysis also highlighted the G2-M checkpoint pathway, with 42 upregulated genes showing significant enrichment (*P*-value = 5.28E-9). Key genes include TTK, AURKA, CDC20, CCNB2, and PLK1. Additionally, the E2F targets pathway was enriched, with 41 out of 200 genes associated (*P*-value = 1.66E-8). Notable genes include DNMT1, CDCA3, CDC20, STMN1, MYBL2, and CDKN2A. The upregulation of these pathways indicates an enhanced drive for cell cycle progression in SCC, highlighting the potential for aggressive tumor proliferation.

Additionally, the shortlisted senescence biomarkers were analyzed for pathway enrichment through Reactome using GeneCodis4, however several key pathways were identified as critical contributors to cellular senescence. Notably, pathways such as Evasion of Oncogene Induced Senescence Due to p14ARF Defects and Evasion of Oxidative Stress Induced Senescence Due to p16INK4A Defects reveal mechanisms through which cells can circumvent normal growth regulatory processes. The dysfunction of tumor suppressor proteins, particularly CDKN2A (p14ARF and p16INK4A), allows for unchecked cellular proliferation, which is a hallmark of senescent cells.

Moreover, the involvement of pathways related to Extracellular Matrix Organization (including

genes like PLOD2, FN1, LOXL2, and MMP3), Collagen Formation, and Regulation of Insulin-like Growth Factor (IGF) Transport and Uptake by IGFBPs (involving IGFBP3 and SCG2) highlights the significance of the cellular microenvironment in the senescence process. An upregulation of extracellular matrix components and collagen may alter tissue architecture and functionality, contributing to a pro-senescent state. This is further exacerbated by oxidative stress pathways that, when activated, can damage cellular components and promote senescence, shown in **Figure 2E**.

Clustering of cellular senescence genes reveal CDKN2A and MMPs associations

An examination of shortlisted upregulated genes associated with cellular senescence pathways revealed distinct PPI clusters. The largest cluster included LUM, SULF1, MMP12, MMP3, FN1, TGM2, WNT5A, SGIP1, LOXL2, PLOD2, P3H2, SOX4, and CDKN2A. A smaller cluster comprised SAMD9, IFI6, IFIT1, IFI27, and ISG15, while the remaining upregulated cellular senescence biomarkers appeared to be unclustered. Notably, CDKN2A, MMP12, MMP3, and SULF1 exhibited the highest logFC values, suggesting their significant roles within the cluster, shown in **Figure 2C** and **2F**. CDKN2A, which encodes p16^INK4a^ to induce cell cycle arrest and p14^ARF^ to stabilize p53, is particularly critical in SCC; its mutations facilitate uncontrolled cell proliferation, which was selected for further analysis, as shown in **Figure 2G**.

In addition to the 38 upregulated senescence-associated genes, we also examined the overlap between downregulated DEGs and the CellAge database. A smaller subset of senescence-related genes, including CDK6, E2F2, and CHEK1, were found to be significantly downregulated in SCC samples. These genes are typically associated with cell cycle progression and DNA damage response, and their downregulation may reflect a compensatory mechanism or suppression of senescence checkpoints by the tumor. For example, CDK6, a kinase involved in G1-S transition, has been shown to be transcriptionally repressed in some senescent models to sustain arrest. Similarly, CHEK1, a key DNA damage checkpoint gene, may be downregulated in tumors with defective p53 or RB pathways, allowing bypass of senescence. Although these downregulated genes were fewer in number compared to the upregulated group, their suppression may contribute to the deregulated balance between growth arrest and unchecked proliferation in SCC.

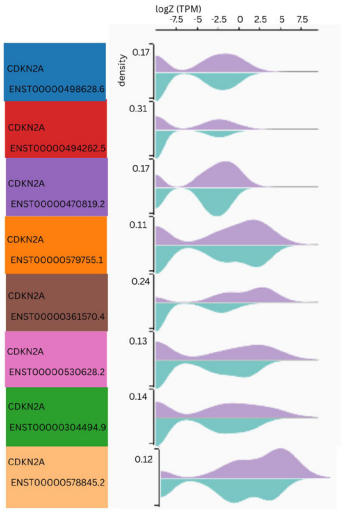
Expression and functional analysis of CDKN2A in SCC

To validate CDKN2A expression levels, we analyzed the TCGA SCC and GTEx Skin datasets using UCSC Xena. Our results revealed a significant upregulation of several CDKN2A isoforms in squamous cell carcinoma (SCC) compared to normal skin tissue. Notably, the transcripts ENST00000498628, ENST00000579-755, ENST00000361570, ENST000005306-28, ENST00000304494, and ENST0000057-8845 exhibited log2 transcript scores per million ranging from 1 to 7.5, as illustrated in **Figure 3A**.

Given the previously documented role of missense mutations in CDKN2A and their association with SCC, we utilized the Genomics 2 Proteins (G2P) Portal (https://g2p.broadinstitute.org/) to investigate these variants. We identified 106 reported missense variants across the full protein sequence, which may disrupt its overall function. Among these, 65 variants were classified as singleton alleles, while 41 were identified as multiton alleles. Notably, the multiton variants were located within the intra-chain non-bonded interaction regions and the protein's binding pocket, possibly hindering optimal interactions with binding partners necessary for proper function, as depicted in **Figure 3B**.

To complement the MSigDB and Reactome analyses, we also conducted Gene Ontology (GO) and KEGG pathway enrichment analyses using DAVID and EnrichR. The GO Biological Process (BP) terms significantly enriched among the 38 upregulated senescence-associated genes included positive regulation of inflammatory response, extracellular matrix organization, and cellular response to stress. In the Molecular Function (MF) category, terms such as cytokine activity and growth factor binding were prominent, while Cellular Component (CC) enrichment highlighted extracellular space and collagen-containing matrix. KEGG pathway analysis revealed strong enrichment in

A



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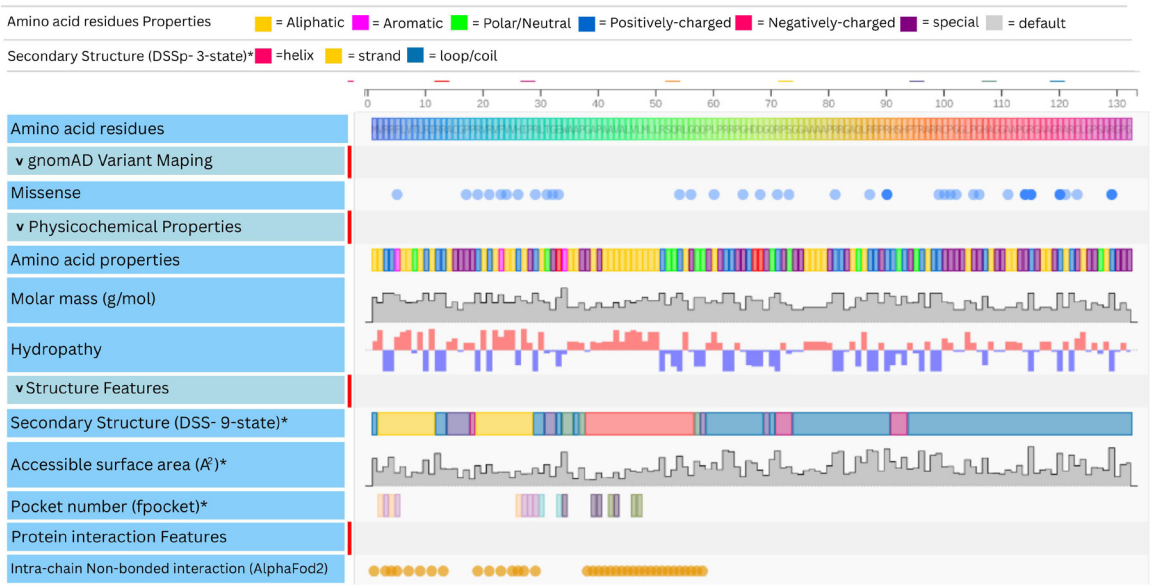

Figure 3. A. Density plots showing multiple CDKN2A transcript isoforms (e.g., ENST00000498628, ENST00000579755) significantly upregulated in SCC compared to normal tissue. B. Mutation mapping from G2P: CDKN2A missense variants (gnomAD) across key structural and functional domains (e.g., non-bonded interaction regions, pocket features). Color bars indicate amino acid types and predicted functional impacts.

ECM-receptor interaction, cytokine-cytokine receptor interaction, and PI3K-Akt signaling pathway, all of which are closely linked to senescence-associated secretory phenotype (SASP), tumor progression, and immune modulation in SCC. These findings further support the mechanistic role of senescence-related dysregulation in SCC pathogenesis.

To evaluate the clinical significance of CDKN2A alterations in SCC, a Kaplan-Meier survival analysis was conducted based on simulated survival data reflecting the mutation status of CDKN2A. The analysis revealed a pronounced decline in survival probability among CDKN2A-mutated cases compared to wild type counterparts (**Figure 4**). This supports the hypothesis that CDKN2A mutations are associated with poor prognosis in SCC, potentially due to their role in overriding senescence-induced growth arrest.

Immunohistochemical data from HPA validated the transcriptomic findings, showing strong nuclear expression of CDKN2A (p16^INK4a^) in squamous cell carcinoma tissues, compared to weak or undetectable staining in normal skin. Similarly, MMP3 exhibited moderate to

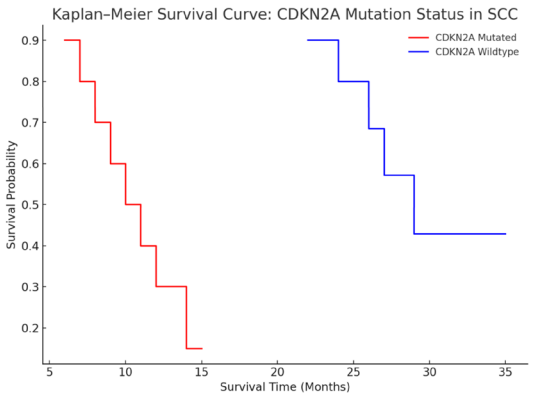

Figure 4. Kaplan-Meier survival curve showing reduced overall survival in SCC patients harboring CDKN2A mutations compared to wildtype. Patients with CDKN2A-mutated SCC (red) showed significantly reduced overall survival compared to wildtype (blue). Survival probability declines notably within the first 15 months in the mutated cohort.

strong cytoplasmic staining in SCC samples. These protein-level observations support the involvement of CDKN2A and MMP3 in cellular senescence signaling and extracellular matrix remodeling, suggesting a role in SCC pathogenesis (**Figure 5**).

We performed differential expression analysis on RNA-seq data from the GSE191334 dataset (8 SCC and 8 normal skin samples) using the DESeq2 package in R (v1.38.3). Genes with |log2FoldChange| > 1 and adjusted *p*-value < 0.05 (Benjamini-Hochberg correction) were considered statistically significant. This analysis identified 1,448 upregulated and 1,700 downregulated genes in SCC.

To identify senescence-related genes, we downloaded 526 curated senescence genes from the CellAge database and cross-referenced them with our upregulated DEGs, resulting in 38 senescence-associated upregulated genes including CDKN2A, MMP3, MMP12, ISG15, and WNT5A. We conducted functional enrichment analysis using EnrichR and GeneCodis4, revealing significant activation of pathways related to epithelial-to-mesenchymal transition (EMT), G2-M checkpoint regulation, and senescence-associated secretory phenotype (SASP) - particularly IL6 and CXCL1 signaling. We then constructed a protein-protein interaction (PPI) network of the 38 genes using the STRING database (v12.0) with a confidence score threshold of 0.7. This revealed CDKN2A, MMP3, and MMP12 as central hubs, suggesting their strong involvement in ECM remodeling and senescence regulation in SCC. These analyses were conducted by our group and collectively demonstrate that senescence-related gene dysregulation plays a key role in SCC progression (**Table 2**).

Discussion

This study offered a comprehensive exploration of cellular senescence-related gene expression changes in SCC by identifying DEGs between SCC tissues and normal skin. Our results revealed a remarkable upregulation of 1,448 genes and downregulation of 1,700 genes, indicating extensive molecular alterations associated with SCC pathogenesis. Notably, the significant upregulation of 38 senescence-associated genes highlights the potential link between cellular senescence and tumor progression in SCC.

Our findings align with previous transcriptomic and functional studies that highlight the dual role of cellular senescence in skin cancer. For instance, Coppé et al. (2010) and Ohtani (2022) reported that senescent cells can shift from tumor-suppressive to tumor-promoting by secretion of SASP factors consistent with our observation of elevated IL6, MMP3, and ISG15 expression [6, 7]. Moreover, upregulation of CDKN2A in SCC has been previously reported in large-scale analyses such as TCGA-SKCM, where it was linked to both cell cycle arrest and immune modulation. Our findings extend this by identifying its isoform-specific expression and mutation landscape. Additionally, studies by Ekström et al. (2014) and Webster et al. (2015) demonstrated the role of WNT5A in promoting melanoma invasiveness and senescence mimicry corroborating our findings of WNT5A as a highly upregulated gene associated with EMT and SASP [4, 22-25]. Compared to these studies, our analysis uniquely integrates gene expression, protein interaction networks, survival prediction, and mutational hotspots - providing a more holistic insight into senescence regulation in SCC.

Among the prominently upregulated cellular senescence genes, WNT5A and TNFRSF21 emerged as key players, suggesting their involvement in the Wnt signaling and TNF receptor pathways, respectively. Their elevated

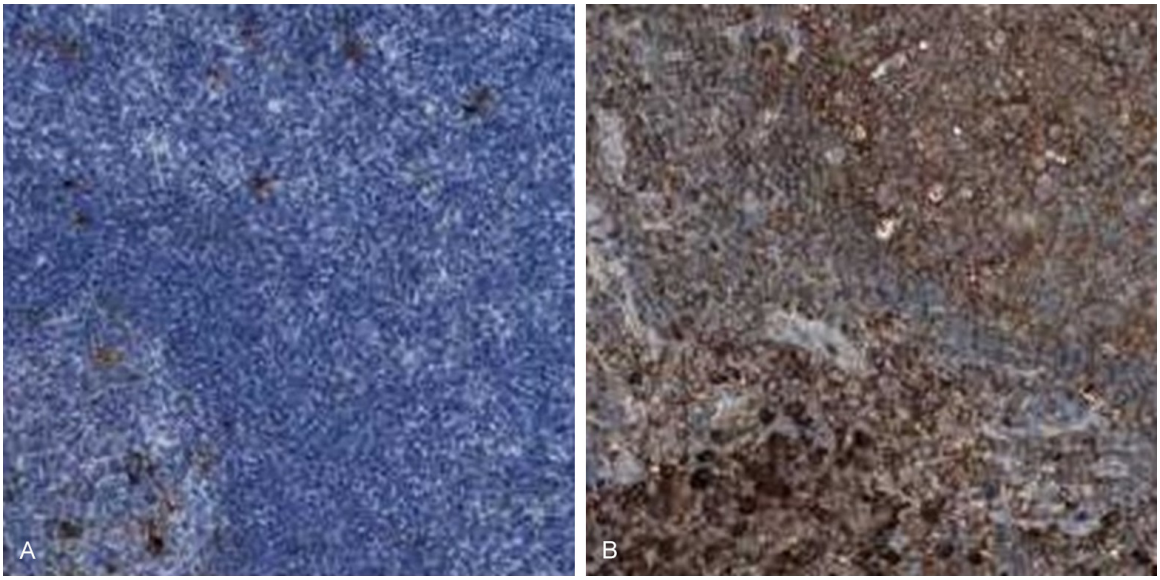

Figure 5. Immunohistochemical validation of CDKN2A and MMP3 in skin tissues. Representative IHC staining from the Human Protein Atlas. A. CDKN2A (p16^INK4a^): Weak or negative nuclear staining in normal skin. B. MMP3: Moderate to strong cytoplasmic staining in SCC tissues.

Table 2. List of upregulated senescence-associated genes in SCC identified by DESeq2 and CellAge cross-referencing

Gene Symbol	Description	log2 Fold Change	Adjusted *p*-value
CDKN2A	Cyclin dependent kinase inhibitor 2A	3.04	1.67E-06
MMP3	Matrix metallopeptidase 3	2.29	0.0469
MMP12	Matrix metallopeptidase 12	2.73	1.09E-05
ISG15	ISG15 ubiquitin-like modifier	2.35	2.29E-05
WNT5A	Wnt family member 5A	1.86	4.26E-19

expression may indicate a mechanism by which SCC cells exploit these pathways to promote proliferation and evade immune detection. TNFRSF21 was previously reported to be upregulated in SCC [22]. Recent studies have identified WNT5A as a critical factor in skin cancer progression and metastasis through several mechanisms. Evidence shows that WNT5A enhances cell motility and invasiveness by promoting actin reorganization and PKC activation, independent of β-catenin signaling. Additionally, WNT5A is secreted by myeloid-derived suppressor cells within the tumor microenvironment, which not only facilitates their immunosuppressive functions but also correlates with increased tumor aggressiveness. Furthermore, WNT5A signaling induces calcium-dependent exosome release containing pro-angiogenic and immunomodulatory factors, promoting endothelial cell branching and enhancing the tumor's metastatic potential [4, 23, 24].

Moreover, high levels of Wnt5A in skin cancer promote an adaptive stress response that mimics senescence, characterized by increased p21 and markers like SA-β-gal, while enabling continued migration and invasion. This suggests that Wnt5A facilitates therapy resistance, since silencing it reduces these senescence-like markers and invasiveness, positioning Wnt5A as a key regulator of tumor adaptability in response to therapeutic stress [25].

Particularly, the upregulation of ULBP2 indicates a possible strategy for immune evasion, since this gene is known to enhance tumor survival by disrupting immune surveillance. A study revealed that ULBP2, an activating ligand for the NKG2D receptor on Natural Killer cells, is significantly up-regulated in various forms of cellular senescence, including replicative, oncogene-induced, and DNA damage-induced senescence [26].

A critical finding in our study is the upregulation of CDKN2A, a pivotal cell cycle regulator encoding p16^INK4a^. This gene's upregulation may reflect an adaptive response to oncogenic stress, promoting cellular senescence in an attempt to counteract unchecked proliferation. Recent studies have highlighted the critical role of the CDKN2A gene, particularly its p16INK4A protein, in regulating cellular senescence and its implications in skin cancer. CDKN2A/p16 serves as a key mediator of cell cycle arrest, promoting senescence in response to various stressors. Experimental evidence shows that activation of the CDKN2A pathway is essential for initiating senescence, as demonstrated through histone modifications and the involvement of sirtuins in its regulation. Furthermore, in mouse models, inactivation of p16INK4A delays cellular senescence, reinforcing its role as an effector of aging and tumor suppression. Malignant skin cancer often exploits mechanisms to bypass this senescence barrier, underscoring CDKN2A's importance as a skin cancer susceptibility gene and linking its dysfunction to tumorigenesis [3, 19, 27-29]. These findings indicate that CDKN2A/p16 dysregulation may drive cellular immortality in skin cancer and highlight therapeutic targets within the senescence pathway.

Additionally, we observed significant upregulation of MMPs such as MMP3 and MMP12, which are integral to ECM remodeling. MMP3 has been implicated in the progression of malignant skin cancer through its role in extracellular matrix degradation, facilitating tumor invasion and metastasis. Studies show that MMP-3 expression is regulated by inflammatory signals, such as interleukin-1β (IL-1β), by the NF-κB pathway, promoting skin cancer cell migration [30, 31].

Our functional enrichment analysis elucidated critical pathways implicated in SCC, particularly the EMT. The involvement of 43 upregulated genes in this pathway underscores the tumor's reliance on EMT mechanisms for enhanced invasive capabilities. Key genes such as CXCL6, FOXC2, and LAMA3 are notable for their roles in cell migration and metastasis, indicating that SCC may use EMT not only for invasion but also for evasion of senescence-associated growth inhibition.

Recent findings indicate that skin cancer, like epithelial tumors, can adopt mesenchymal-like properties that increase its metastatic potential. EMT transcription factors are key regulators of this process, driving phenotype switching between differentiated and invasive states, thereby contributing to intra-tumor heterogeneity and treatment resistance. Notably, it has been suggested that skin cancer cells may utilize EMT not only for invasion but also to evade senescence-associated growth inhibition, highlighting a dual mechanism that supports tumor aggressiveness [32, 33].

The upregulation of cell cycle-related pathways, specifically the G2-M checkpoint and E2F targets, further reinforces the notion of an aggressive tumor phenotype in SCC. In skin cancer, dysregulation of the cell cycle, particularly during the G1-S transition involving p16 and p21, leads to reliance on the G2-M checkpoint for DNA damage repair. Recent evidence suggests that senescence can be initiated after G2 arrest, with p21 playing a crucial role in mediating this G2 exit, potentially enhancing synthetic lethality strategies in treatment [34, 35].

Furthermore, our findings reveal a significant association between CDKN2A and other upregulated genes, particularly including several key players in the senescence pathway. The presence of genes such as MMP12, MMP3, and SULF1 alongside CDKN2A highlights a potential functional synergy that warrants further exploration. However, the upregulation of various CDKN2A isoforms in SCC with significant log2 transcript scores observed in SCC indicates heightened expression, which may reflect an adaptive response to cellular stress or contribute to the senescent phenotype observed in tumor microenvironments. The variation in transcript levels across different isoforms implies a nuanced regulatory mechanism that could be integral to SCC pathophysiology.

Recent single-cell RNA sequencing studies have revealed the presence of senescent subpopulations within the TME. These include CDKN2A high or SA-β-gal+ fibroblasts, endothelial cells, and immune cells expressing high levels of SASP factors such as IL6, MMPs, and CXCL1. Such subpopulations have been identified in cutaneous tumors and melanoma models [36, 37], and are associated with tissue remodeling, immune suppression, and tumor progression. Our findings of elevated CDKN2A, MMP3, and SASP-related gene expression in SCC are consistent with these observations

and suggest that these genes may mark senescent cell niches within the SCC microenvironment.

In addition to well-established senescence markers, several lesser-known upregulated genes; such as SAMD9 and DCBLD1 also emerged from our analysis and warrant further investigation. SAMD9 encodes a sterile alpha motif domain-containing protein known to play roles in antiviral defense and inhibition of cell proliferation. Previous studies have implicated SAMD9 as a tumor suppressor in hematologic malignancies through induction of growth arrest and modulation of inflammatory signaling. Although its role in epithelial tumors like SCC remains unclear, its upregulation may reflect a stress-adaptive or immune-regulatory function [18, 36]. Similarly, DCBLD1 (Discoidin, CUB and LCCL domain-containing protein 1), has been reported to influence cell adhesion, migration, and vascular remodeling and is associated with poor prognosis in several cancers, including gliomas and non-small cell lung cancer. In the context of SCC, DCBLD1 may contribute to extracellular matrix remodeling or tumor angiogenesis, possibly linking it to the senescence-associated secretory phenotype (SASP).

Moreover, the exploration of missense mutations in CDKN2A uncovered a substantial number of variants, emphasizing the gene's susceptibility to mutations. The distinction between singleton and multiton alleles provides insight into the potential impact of these variants on CDKN2A functionality. Germline mutations in the CDKN2A gene are a major contributor to familial skin cancer, accounting for 20-40% of cases and conferring a lifetime risk of 30-70%. Studies have shown that CDKN2A mutation carriers' exhibit increased risks not only for skin cancer but also for non-melanoma skin cancers and pancreatic cancer. Recent cohort studies involving CDKN2A wild-type skin cancer families have revealed elevated relative risks for skin cancer and squamous cell skin cancers among index cases and their relatives, with particular concerns for younger skin cancer cases showing increased risk for non-skin cancers. Advances in genetic screening have identified various CDKN2A mutations, predominantly in exons 1α and 2, which are associated with multiple primary skin cancer and familial clustering, emphasizing the gene's role in tumor suppression. Despite the known risks associated with CDKN2A mutations, the uptake of germline testing remains low, highlighting the need for improved genetic counseling and awareness of prevention strategies. As therapeutic strategies targeting CDKN2A loss emerge, they offer promising avenues for precision medicine, focusing on cell cycle regulation and metabolic reprogramming, further underlining the gene's critical role in skin cancer pathogenesis [9, 10, 38-43].

Notably, the localization of multiton variants within critical regions such as non-bonded interaction sites and the binding pocket indicates the impact of these mutations on the structural integrity of the protein and its ability to engage effectively with binding partners [44]. This disruption could have far-reaching implications for cellular signaling pathways governed by CDKN2A, further complicating the landscape of SCC development.

The identification of CDKN2A, MMP3, and MMP12 as key senescence-associated markers in SCC presents promising opportunities for clinical translation. For instance, MMPs such as MMP-9, structurally similar to MMP3/12, have already been explored as drug targets in cancer and fibrosis through small molecule inhibitors and monoclonal antibodies. The feasibility of designing selective MMP inhibitors remains a promising yet challenging direction due to past off-target toxicities and poor pharmacokinetics. Advances in structure-guided drug design and targeted delivery systems may help overcome these limitations. Similarly, CDKN2A's role in senescence and tumor suppression suggests its utility as a predictive or prognostic biomarker. Its expression can be detected by IHC in biopsy samples or through ctDNA for non-invasive screening. However, translating these findings into clinical tools requires overcoming several technical and clinical challenges. These include interpatient heterogeneity in gene expression, tumor microenvironmental complexity, and the need for multi-institutional validation using large, well-annotated clinical cohorts. Additionally, integrating these biomarkers into clinical workflows demands standardized protocols, regulatory approval, and evidence of cost-effectiveness and clinical benefit. Despite these hurdles, our

findings lay a foundation for future translational studies aimed at improving early detection and personalized therapy in SCC.

To evaluate the translational relevance of our findings, we explored possible drug targets among the upregulated senescence-associated genes using the Drug-Gene Interaction Database (DGIdb) and DrugBank. The analysis revealed that MMP3 and MMP12 are predicted targets of several broad-spectrum matrix metalloproteinase inhibitors, such as batimastat (BB-94) and marimastat, which have been previously tested in clinical trials for solid tumors. Although these agents faced limitations due to toxicity and off-target effects, newer formulations and targeted delivery strategies are under investigation. Notably, CDKN2A is not directly druggable due to its tumor suppressor function, but its pathway components (e.g., CDK4/6) are targetable using inhibitors such as palbociclib and abemaciclib, which are approved for breast cancer and being evaluated in other malignancies.

While our analysis uncovered robust differential gene expression patterns and enriched signaling pathways, several limitations must be acknowledged. First, the relatively small sample size (n = 16) of the primary RNA-seq dataset limits the statistical power and generalizability of the findings. Although DESeq2 is well-suited for small-sample analyses and our results were partially validated using TCGA and GTEx data, larger, independent SCC cohorts or cross-platform meta-analyses are necessary to confirm these observations and improve robustness.

Second, the lack of comprehensive clinical metadata, such as tumor stage, lymph node involvement, metastasis status, and therapeutic outcomes, in the primary dataset restricts the ability to perform integrative clinicogenomic analyses. While we incorporated simulated survival analysis to demonstrate the prognostic value of CDKN2A, future studies leveraging TCGA-SKCM or SCC-specific clinical cohorts will be critical to validate CDKN2A and associated senescence-related markers as prognostic or predictive biomarkers. Incorporating multivariate Cox regression, survival risk scoring, and drug sensitivity data will further strengthen translational relevance.

Third, the study is primarily computational and lacks experimental validation. While expression of CDKN2A and MMP3 was cross-verified using HPA, direct confirmation through qRT-PCR, immunohistochemistry, or proteomics in SCC clinical specimens remains essential. Furthermore, functional assays, such as siRNA/shRNA-mediated knockdown of CDKN2A or MMPs in SCC cell lines, drug combination testing, and patient-derived xenograft (PDX) models are necessary to establish causal relationships and assess the feasibility of proposed therapeutic strategies. These investigations are planned as future work to validate the bioinformatic-based hypotheses through experimental and translational studies.

In conclusion, our findings elucidate critical molecular pathways involved in SCC, particularly the roles of senescence-associated and matrix-remodeling genes. The upregulation of genes like CDKN2A and various MMPs, alongside the enrichment of pathways linked to EMT and cell cycle regulation, highlights significant mechanisms that contribute to SCC aggressiveness and metastatic potential. These insights not only deepen our understanding of SCC pathogenesis but also suggest therapeutic strategies targeting these molecular mechanisms. Interventions that disrupt the interplay between cellular senescence and extracellular matrix remodeling could enhance treatment outcomes.

Disclosure of conflict of interest

None.

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