Female Immunity Protects from Cutaneous Squamous Cell Carcinoma

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ABSTRACT

Purpose: Cancer susceptibility and mortality are higher in males, and the mutational and transcriptomic landscape of cancer differs by sex. The current assumption is that men are at higher risk of epithelial cancers as they expose more to carcinogens and accumulate more damage than women. We present data showing women present with less aggressive primary cutaneous squamous cell carcinoma (cSCC) and early strong immune activation.

Experimental Design: We explored clinical and molecular sexual disparity in immunocompetent and immunosuppressed patients with primary cSCC ($N = 738$, $N = 160$), advanced-stage cSCC ($N = 63$, $N = 20$) and FVB/N mice exposed to equal doses of DMBA, as well as in human keratinocytes by whole-exome, bulk, and single-cell RNA sequencing.

Results: We show cSCC is more aggressive in men, and immunocompetent women develop mild cSCC, later in life. To test whether sex drives disparity, we exposed male and female mice to equal doses of carcinogen, and found males present with more aggressive, metastatic cSCC than females. Critically, females activate cancer immune-related expression pathways and CD4 and CD8 T-cell infiltration independently of mutations, a response that is absent in prednisolone-treated animals. In contrast, males increase the rate of mitosis and proliferation in response to carcinogen. Women's skin and keratinocytes also activate immune-cancer fighting pathways and immune cells at UV radiation–damaged sites. Critically, a compromised immune system leads to high-risk, aggressive cSCC specifically in women.

Conclusions: This work shows the immune response is sex biased in cSCC and highlights female immunity offers greater protection than male immunity.

Introduction

Many diseases show sex disparity in their epidemiology, clinical course, and outcome (1), and cancer incidence is higher in men even after adjusting for risk factors. Cancer mortality also affects men disproportionately, and women respond better to some cancer therapies (2–4). Squamous cell carcinomas (SCCs) arise in epithelia from the head and neck, lung, bladder, esophagus, and skin, have a strong male bias and are primarily caused by carcinogens such as tobacco, alcohol, and UV radiation (UVR). The higher SCC male incidence and mortality is thought to reflect the increased lifetime exposure of men to carcinogens and delayed clinical care, and molecular studies show a higher burden of carcinogen-driven mutations in male tumors (5–9). SCCs arising on the skin, cutaneous SCC (cSCC; refs. 10–12), are more frequent in men, the second most frequent malignancy in humans, and the most common malignancy in patients with a compromised immune system (10, 13, 14). Previous studies indicate male mice may be inherently more susceptible to cSCC (15, 16), so we examined whether the higher incidence and mortality of cSCC in men is due to increased vulnerability to epithelial neoplasia reflected in the molecular landscape, rather than due to increased exposure to a carcinogen. To test this hypothesis, we used the most frequent cSCC mouse model driven by the topical cutaneous application of 7,12-Dimethylbenz[a]anthracene (DMBA), a polycyclic aromatic hydrocarbon that induces epidermal *Hras* mutations, followed by topical tetradecanoyl-phorbol acetate (TPA), which induces inflammation and epidermal proliferation, leading to epidermal papillomas and cSCC (17). The advantages of this model are that the genomic landscape of DMBA/TPA-induced cSCC displays significant overlap with human cSCC, and that it allows the *in vivo* study of cSCC controlling for age, strain, susceptibility, and dose of carcinogen (18). We explored the clinical and molecular sex bias of cSCC carcinogenesis in animals exposed to the same dose of carcinogen, and confirmed the molecular findings in human skin and keratinocytes. In addition, we examined the relationship between age at diagnosis, histologic grade of cSCC and immune status in human cSCC patient cohorts to explore the role of immunity by sex.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Sex bias affects cancer incidence, mortality, and therapy response; and the molecular landscape of cancer differs by sex. However, it is not known whether the sex discrepancy is due to a difference in behavior and exposure to carcinogens, or due to sex-linked susceptibility. This work reveals men are more susceptible to cutaneous aggressive squamous cell carcinoma, in contrast to women who activate stronger immune responses when challenged with the same carcinogens. The loss of immunity particularly affects women. Personalized medicine approaches stratify patients with cancer by genotype; however, to date, the potential for cancer stratification, prevention strategies, and therapy by sex and immune competency has not been explored. We show men and immunosuppressed women are at higher risk of aggressive disease and may benefit from targeted prevention programs, closer surveillance, and stratification for adjuvant therapy.

Materials and Methods

Animal experiments

Experiments were performed in 4-week-old FVB/N male and female mice and DMBA (25 mg/mL) and TPA (0.02 mg/mL) in acetone applied once a week, 2 days apart for 6 weeks, followed by TPA weekly for 10 weeks or until tumor development. All procedures involving animals were performed under the Home Office–approved project license P8ADED6C8, and UK Home Office regulations under the Animals (Scientific Procedures) Act 1986. The study received ethical approval by the Cancer Research UK Manchester Institute's Animal Welfare and Ethics Review Body.

Molecular analyses

DNA from the largest sized mouse tumor, adjacent treated skin, and a kidney was sequenced, patterns of single-nucleotide variation, insertion, and deletions in tumors and skin were analyzed and oncoplots generated of the top 20 frequently mutated genes by sex and histology to identify patterns of mutations. Paired-end RNA sequencing (RNA-seq) was performed from fresh whole DMBA/TPA-treated skin from the back and normal skin from the abdomen. Human single-cell RNA-seq data from male and female keratinocytes were analyzed from a study of SCC (19), downloaded from Gene Expression Omnibus (GEO) database (GSE144236).

Histology

Mouse tumors were classified as papillomas, keratoacanthoma, well, moderately, and poorly differentiated cSCC. Visceral organs were stained with hematoxylin and eosin (H&E) and pan-keratin to confirm metastasis. IHC of tumors and skin was performed with anti–phospho-Histone H3 (Ser10) (06-570) CD4 Antibody (14-9766-82) Thermo Fisher Scientific/eBioscience and CD8a Antibody (14-0808-82) Thermo Fisher Scientific/eBioscience.

Human clinical data

A retrospective, noninterventional review of clinical data: age, sex, immune status, and histologic grade of human primary cSCC, metastatic cSCC to the lymph node, and cSCC treated with systemic therapy from three UK NHS centers and one French hospital, from immunocompetent and immunosuppressed patients diagnosed by dermatologists and pathologists under routine diagnostic practice were collected. The review at Salford Royal (Salford, United Kingdom) is part of a larger skin cancer study integrated research application system (IRAS) approval: 216310, REC reference: 16/LO/2098. The Christie and St. George's clinicians collected age, sex, and immune status within the SCC care audit. No patient consent of retrospectively obtained, anonymized variables was required by local hospital and National Health Research Authority United Kingdom. The clinical data collection in the French cohort was approved by tumor board local, national regulations, given Comité de Protection des Personnes Sud Méditerranée (Marseille, France), and Aix-Marseille University Hospital (Marseille, France) approval. Publicly available genomic data from single-cell RNA-seq analysis of human skin was accessed by GEO database (GSE144236). cSCCs were classified as keratoacanthoma/well-differentiated invasive SCC (1), moderately differentiated SCC (2) and poorly differentiated SCC (3). The immune status of patients was retrieved from clinical histories, and immunosuppressed patients were organ transplant recipients on immunosuppressive medication, with white blood cell dyscrasia, systemic cancer treatment with immunotherapy, radiotherapy, or chemotherapy in the past 10 years, chronic inflammatory disorders and autoimmune disorders on systemic immunosuppressive medication.

Results

Immunocompetent men develop more aggressive cSCC than women

To explore whether men are more susceptible to aggressive cSCC than women, we studied the relationship between sex, age, and histologic grade in consecutive cSCC samples excised from immunocompetent patients ($n = 738$; men: 459, women: 279; Supplementary Table S1A). We confirmed a male preponderance of cSCC in this cohort of immunocompetent patients (ref. 10; 62.2% men, Z-test, $P < 0.0001$), and found the median age of diagnosis to be similar in men and women (median age men = 79, women = 81, Mann–Whitney test, $P = 0.10$; **Fig. 1A**). Strikingly, men more frequently presented cSCC that was less differentiated, of higher histologic grade compared with cSCC of women (**Fig. 1B**; Mann–Whitney test, $P = 0.0002$). Furthermore, when we restricted our study by sex to the more aggressive variants of cSCC, which have the highest risk of metastasis (14), we found that immunocompetent females diagnosed with higher grade cSCC were older than men (median age men = 80, median age women = 83, $P = 0.04$; **Fig. 1C**). In addition, a review of the prevalence of metastatic cSCC to the lymph nodes in a tertiary cancer hospital revealed male preponderance (men: 25, women: four, Z-test, $P = 0.0001$; Supplementary Table S1B), and men developed aggressive primary cSCC at a younger age than women [women: 86 (71–91), men: 73 (41–91), $P = 0.01$; **Fig. 1D**]. Furthermore, we investigated sex bias in a cohort of patients with metastatic or advanced-stage cSCC that required systemic treatment, which showed more men received systemic treatment than women (men: 62.2%, Z-test, $P = 0.0011$; Supplementary Table S1C; Supplementary Fig. S1E), and intriguingly, female cSCC was histologically more aggressive in this cohort of poor outcome (Mann–Whitney test, $P = 0.01$; **Fig. 1F**). These data show that men are at higher risk of aggressive cSCC.

Male mice are more susceptible to chemically induced aggressive cSCC

To determine whether the increased susceptibility to aggressive cSCC in men is due to a greater exposure to carcinogens or due to biological sex differences, we exposed immunocompetent male and

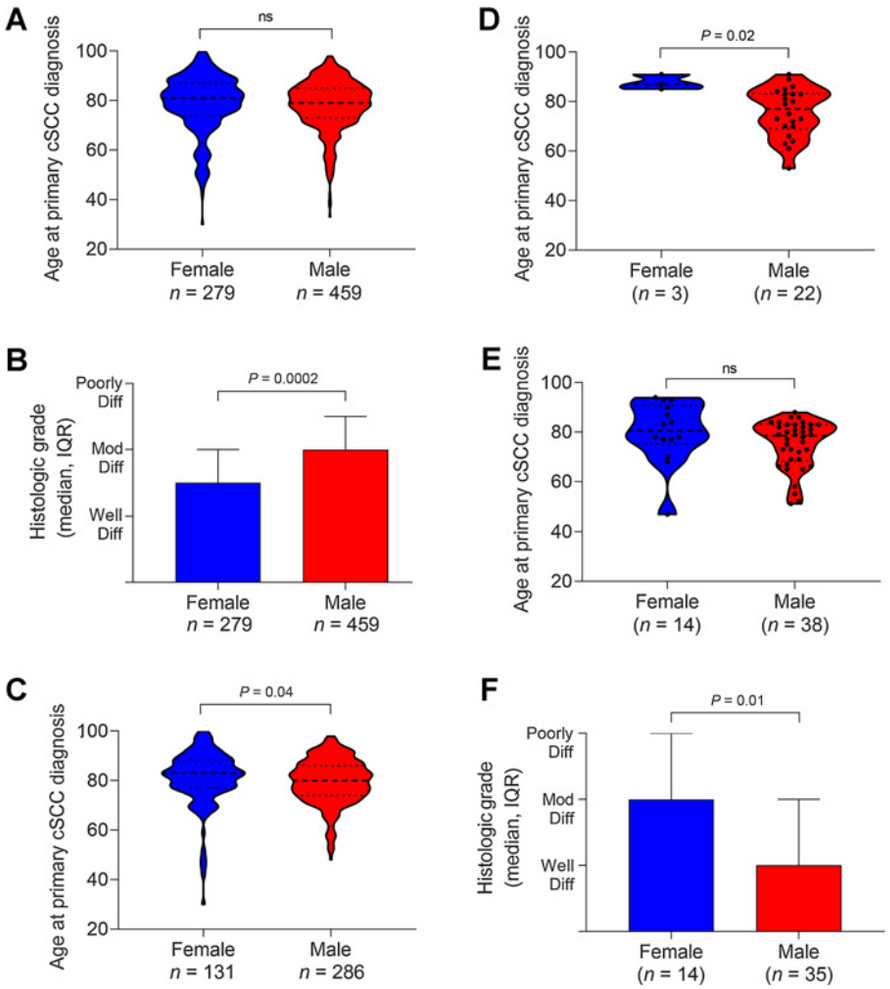

Figure 1.

Men have more aggressive cSCC. **A,** Age of immunocompetent patients diagnosed with primary cSCC by sex, n.s not significant, Mann–Whitney $P = 0.1$. **B,** Histologic grade of primary cSCC by sex in immunocompetent patients [median, interquartile range (IQR)]. **C,** Age of immunocompetent women and men diagnosed with moderately and poorly differentiated primary cSCC. **D,** Lymph node metastatic cSCC by sex and age at time of primary cSCC diagnosis in immunocompetent patients. **E,** Median age at primary diagnosis of a cohort of advanced-stage cSCC treated with systemic agents. **F,** Histologic grade of cSCC by sex at the time of primary tumor diagnosis (all tests Mann–Whitney).

female mice, matched for age and strain, to equal doses of the carcinogen DMBA/TPA, which promotes cSCC in mice. We recorded earliest lesion incidence and burden, and found males developed more papillomas and cSCC earlier than female animals, although this difference was not statistically significant ($P = 0.10$; **Fig. 2A**). Animals presented a range of squamoproliferative lesions, including epidermal hyperplasia, papillomas, well-differentiated, invasive SCC and more aggressive, undifferentiated SCC. However, compared with females, males presented more advanced, aggressive subtypes of disease (Fisher exact test, $P = 0.04$; **Fig. 2B**). Specifically, male cSCC presented more mesenchymal, spindle features, compared with female cSCC. Importantly, only males presented metastatic lung cSCC deposits (**Fig. 2B** and **C**). Taken together, these data show male mice developed more aggressive primaries and metastatic cSCC than females exposed to the same dose of carcinogen.

DNA damage accumulates equally in male and female animal skin and cSCC

Increased mutation burden underpins more aggressive forms of epithelial cancer (20), and human male epithelial tumors have a higher mutation burden (5–9). Therefore, we examined whether the more aggressive male cSCCs in our animals are due to greater mutation accumulation, or less DNA repair, leading to higher mutation burden. For this, we compared the tumor mutation burden (TMB) in DMBA/TPA-induced mouse cSCCs (DT-cSCC), and found deep targeted exome sequencing of DT-cSCC revealed the number of total mutations increased with increasing histologic grade, but did not differ by sex (**Fig. 2D** and **E**). Furthermore, we found no differences in the pattern and types of mutations by sex (Supplementary Table S2; Supplementary Fig. S1A).

Mouse tumors display a range of morphologic features within the same histologic grade, and cancer-driving mutations can associate specific histologic features (21). Therefore, to ensure specimens were exactly comparable between males and females, we focused on mutations accrued in clinically and histologically normal DMBA/TPA-treated skin (DT-skin). We found males and females accumulate the same amount of genetic damage in DT-skin (**Fig. 2D**), and similar to DT-cSCCs, there was no sex disparity in the number, pattern, and types of mutations (Supplementary Fig. S1B; Supplementary Table S2). These data indicate carcinogens damage male and female DNA similarly, and the more aggressive phenotype of male cSCC is independent of mutation burden.

Unique transcriptomic and immune changes by sex in mice

Male animals develop more aggressive cSCC independently of the mutation spectrum. Therefore, we explored whether the transcriptomic response to carcinogen exposure differed by sex. To reduce the gene expression variability due to histologic differences between

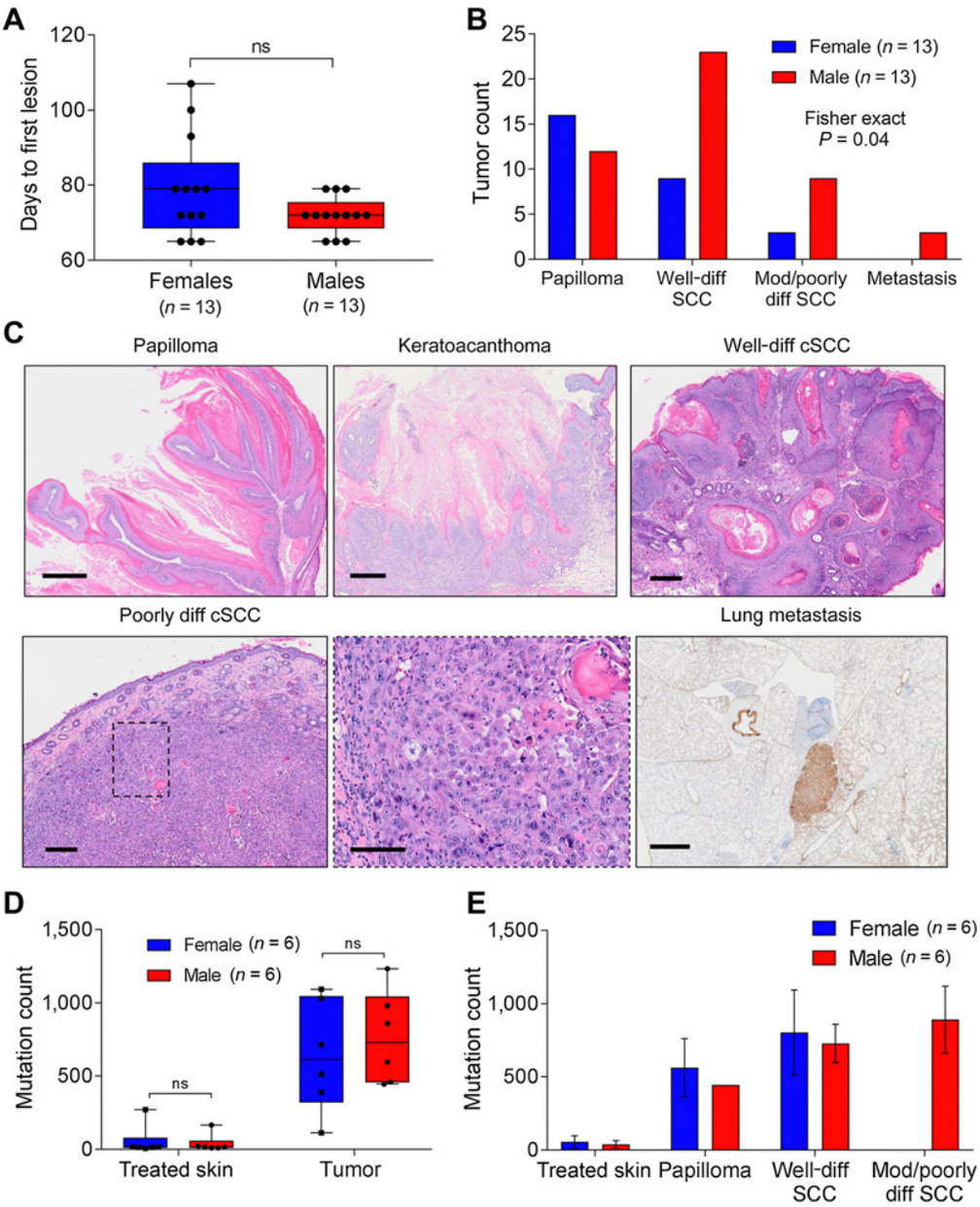

Figure 2.

Male animals are more susceptible to chemically induced aggressive cSCC. **A,** Days to first lesion in male and female skin exposed to DMBA/TPA. n.s, not significant Mann–Whitney test, $P = 0.1$. **B,** Histologic grade of cSCC tumors by sex (Fisher exact test, $P = 0.04$). **C,** Representative H&E of papilloma (scale bar: 400 μm), keratoacanthoma (scale bar: 500 μm), well-differentiated cSCC (scale bar: 500 μm), poorly differentiated cSCC (scale bar: 500 μm) and insert (box, scale bar: 100 μm) and lung metastasis stain for pan-cytokeratin (brown, scale bar: 1 mm). **D,** Tumor mutation burden (TMB) in DMBA/TPA-exposed skin (treated skin) and DMBA/TPA-induced cSCC (tumor) by sex, Mann–Whitney test, $P = 0.7$, $P = 0.8$ (**E**) TMB in DMBA/TPA lesions by histologic grade and sex. Error bars represent SEM (bar).

tumors, we compared the histologically normal, carcinogen exposed DT-skin of males and females. We first investigated the autosomal gene expression changes in DT-skin and normal skin, and then studied whether DT-skin gene expression varied by sex. We found the most significant changes in DT-skin involved critical cancer immune regulatory pathways including the IFN gamma (IFNG) and IFNα response pathways; as well as the inflammation-related and allograft rejection genes (**Fig. 3A**; Supplementary Table S3A). In contrast, normal, untreated skin expressed genes involved in RAS signaling (**Fig. 3A** and **B**). We then investigated sex-specific changes in DT-skin, and found female DT-skin presented more transcriptomic changes than male skin overall, including higher expression of critical genes involved in cancer immunity, compared with males. We noted specifically the cytokine IFN gamma (*Ifng*), which is known to drive

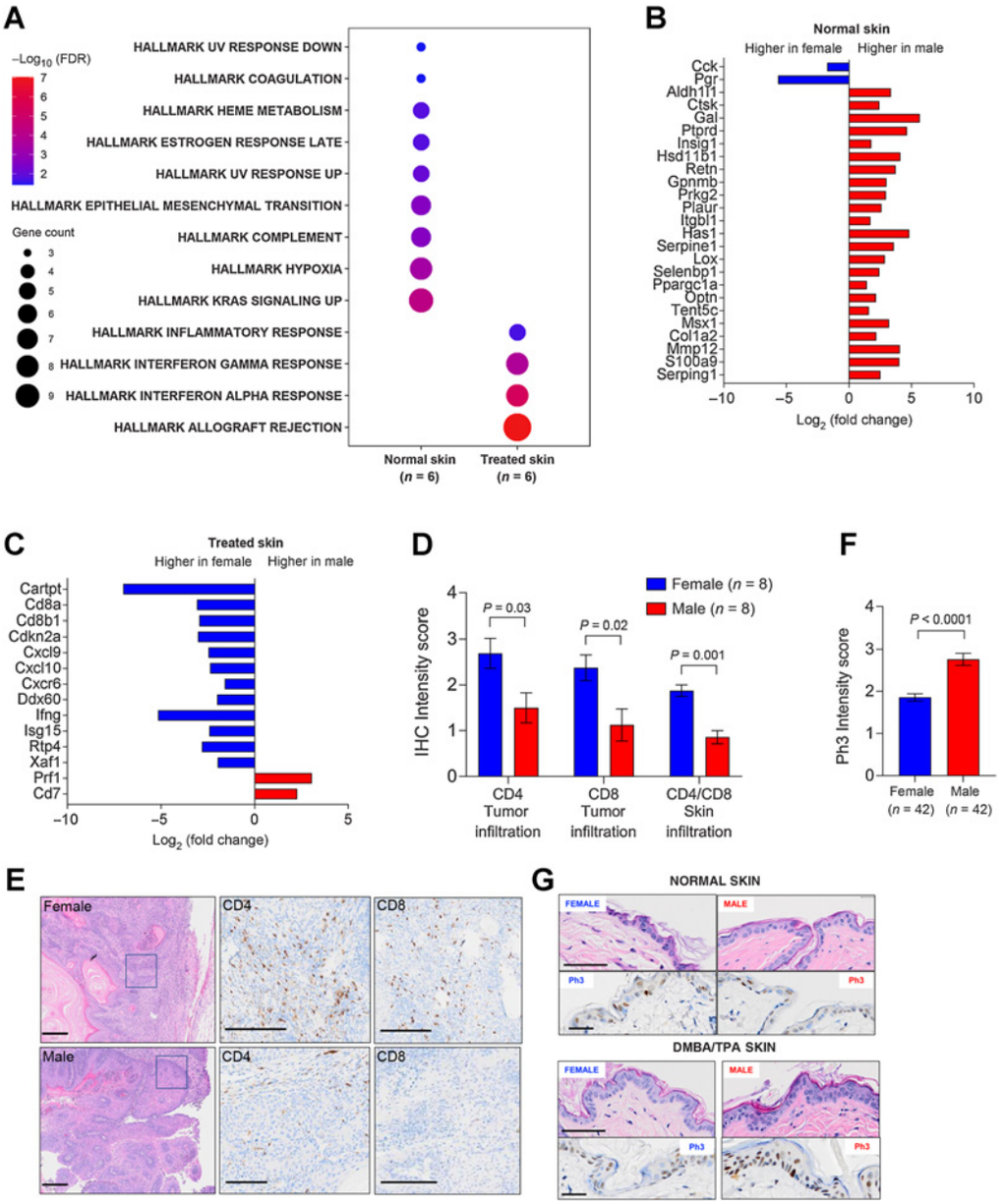

Figure 3.

Female mouse skin upregulates immune cancer pathways and cells in response to chemical carcinogenesis. **A,** Gene pathways differentially expressed by sex in normal skin (left) and in DMBA/TPA-exposed skin (treated skin, right). Size of spheres represent numbers of enriched genes in pathway, color represents significance of enrichment (red most significant). **B,** Differentially expressed genes enriched in pathways in normal skin and treated skin (**C**). Negative fold change (blue) represents genes expressed higher in female skin, positive fold change (red) represents genes expressed higher in male skin. **D,** Quantification of CD4 and CD8 in tumors and DMBA/TPA tumors and treated skin by sex, Mann–Whitney test. **E,** Representative images of H&E and CD4 and CD8 T-cell IHC in male and female cSCC (scale bar: 200 μm). **F,** Quantification of mitotic cell marker phospho-histone 3 (Ph3) IHC intensity score low (1), moderate (2), high (3), and very high (4), Mann–Whitney test. **G,** Photomicrographs of H&E (scale bar: 50 μm) and mitotic cells (Ph3, scale bar 15 μm) IHC by sex in normal and DMBA/TPA-skin.

potent antitumor activity (22–24), to be increased in female carcinogen-treated skin. Intriguingly, we additionally observed female upregulation of the G_1- to S-phase tumor suppressor, senescence-inducing cell-cycle regulator *Cdkn2a* (**Fig. 3C**; Supplementary Table S3B). Cdkn2a can exert ample antitumor effects (25, 26), and when expressed in keratinocytes restricts proliferation, increases senescence and differentiation, and reduces tumor growth (27), consistent with its tumor-suppressive roles. Furthermore, recent work shows natural and immune checkpoint cancer immune control is achieved via Cdkn2a-dependent signaling, and IFNs directly activate *Cdkn2a* (28). We therefore examined cSCC incidence, histologic grade, and metastasis in an available model of spontaneous carcinogenesis in *Cdkn2a*$^{fl/fl}$ animals (29), and found that although the study was not powered to detect sex differences, *Cdkn2a*$^{fl/fl}$ female mice presented more spindle,

aggressive primary tumor and metastatic cSCC than *Cdkn2a*^{wt} females (two-way ANOVA $P = 0.0796$; Supplementary Fig. S1C; Supplementary Table S4).

We next investigated whether, in addition to immune-gene expression changes, female animals present a unique immune cell landscape compared with males. For this, we studied the proportion and subtypes of adaptive and innate immune cells by gene expression, which revealed a trend in female skin for increased antigen-presenting CD1 cells and CD4/CD8 tumor lymphocytes. In contrast, male animal skin tended to be enriched for macrophages (Supplementary Fig. S1D) although these were not significant ($P \geq 0.5$, Mann–Whitney test). We explored gene expression by IHC staining of CD4$^+$ and CD8$^+$ T cells in DT-cSCC and DT-skin by sex, and confirmed female tumors and female DT-skin were more infiltrated with immune cells than male DT-cSCC and DT-skin (**Fig. 3D** and **E**). To confirm the immune response to the DT challenge is sex specific, we compared the early inflammatory and CD4$^+$ cell infiltration in immunocompetent and immunosuppressed mice. We confirmed that DT-skin from immunocompetent females presented a denser immune cell (Supplementary Fig. S1E and S1F) and CD4$^+$ infiltration (Supplementary Fig. S1G and S1H) across all the layers of the skin, compared with males. However, immunosuppressed females and males, treated with oral prednisolone in addition to DMBA/TPA, had an equally reduced inflammatory cell response and CD4$^+$ infiltration (Supplementary Fig. S1E–S1I). These data indicate the early inflammatory response to carcinogen in immunocompetent animals differs by sex, and this difference is lost in immunosuppressed animals.

Tumors in animals arise weeks after being exposed to the DMBA carcinogen, but the total number of mutations per cSCC rises progressively in tumors of increasing histologic grade (**Fig. 2E**). We hypothesized the selection and growth of the more mutated clones leading to more aggressive cSCC occurs due to increased cell division and a proliferation advantage of these clones. As males present more aggressive disease, and female epidermis expresses higher levels of the cell-cycle regulator *Cdkn2a*, we investigated whether a higher epidermal proliferation rate in the male epidermis could underpin speedier clonal selection and disease progression of male disease. For this, we compared the rate of the mitosis-specific marker phospho-histone 3 (phospho-H3) in male and female epidermis and observed that DT-skin, compared with animal-matched non-DT skin was thickened, presenting increased number of layers, independently of sex (Supplementary Fig. S1J, $P < 0.0001$). Next, we compared male to female DT-epidermis and found that phosphoH3 in male epidermis was increased in males (Supplementary Fig. S3F and 3G; $P < 0.0001$). We compared the expression of phospho-H3 in a subset of cSCCs, and again observed a trend for higher expression in males (Supplementary Fig. S1K and S1L). Thus, male and female mouse epidermis modulate the rate of epidermal proliferation differently following exposure to a chemical carcinogen.

Taken together, these findings indicate the transcriptome and immune cell response of male and female mice differs at the earliest stage of carcinogenesis, independently of DNA damage. Males present higher rates of epidermal proliferation following chemical carcinogen exposure compared with females. In contrast, females strongly upregulate immune responses and cancer-linked lymphocytes, implicating immunity in delayed female tumorigenesis.

Human female skin upregulates immune-related cancer defense pathways

To validate the role of immune-related expression changes and immune cells modulating carcinogenesis in females, we compared the sex-specific changes in single-cell RNA of normal male and female human keratinocytes obtained from cSCC-adjacent skin (19). Human cSCC is driven by UV light and arise in fields of severely sun-damaged skin, so we reasoned cSCC-adjacent keratinocytes, from sun-exposed anatomic sites of adults, will accumulate significant carcinogen exposure. We found that similar to animal DT-skin, the most prominently upregulated pathways in human keratinocytes overlapped with mouse skin. Strikingly, the most prominent pathway expression changes in all female keratinocyte subtypes were upregulation of IFNG, IFNα and allograft rejection pathway genes (**Fig. 4A**; Supplementary Table S5). In further agreement with the mouse experiment, we found female human sun-exposed epidermis and cSCCs had a trend to higher counts of CD4, CD8 T cells, and CD1C dendritic cells, whereas male skin and cSCC showed a trend for more macrophages (Mann-Whitney $P = 0.38$; Supplementary Fig. S2A and S2B).

The use of the human cSCC single-cell RNA-seq data allows expression patterns to be isolated to specific cell types, and this reveals that in human skin and cSCC, *IFNG* is almost exclusively expressed by T cells. Cytokines *CXCL10* and *CXCL9*, which can chemoattract T cells, are expressed from multiple cell types including keratinocytes and other immune cells such as Langerhans cells. The immune pathway enrichment of human female keratinocytes (**Fig. 4A**) is particularly enriched for genes that play a role in antigen presentation such as HLA genes and *TAP1*, suggesting a complex multicellular immune response in human skin including alterations in cytokine production, T-cell recruitment, and IFNG production, and increased antigen presentation of keratinocytes for immune recognition in female skin after UV damage (Supplementary Fig. S2C and S2D). Overall, these data indicate that the biological response to chemical and UV light carcinogen exposure in keratinocytes varies by sex in mice and humans.

Female immunity inhibits aggressive cSCC

Because we found immunity to be critical to the response to carcinogen exposure, and immunocompetent women have less aggressive forms of cSCC (**Fig. 1B**), we reasoned women with compromised immune systems would present more aggressive cSCC, comparable with immunocompetent men. To test this hypothesis, we investigated sex, age, and histologic grade in cSCC specimens excised from a cohort of immunosuppressed patients ($n = 160$, men $= 106$ and women $n = 54$; Supplementary Tables S1 and S6). Strikingly, we discovered cSCC appeared at a younger age in both immunosuppressed men and women, supporting the critical role of immunity in delaying cancer in both sexes (median age men $= 72$, women $= 71$, $P = 0.25$; **Fig. 4B**). We then compared the histologic grade in immunosuppressed and immunocompetent women, which revealed immunosuppressed women present more histologically aggressive disease ($P = 0.029$; **Fig. 4C**), of equivalent grade to men. In contrast, men present with aggressive disease regardless of their underlying immune status (**Fig. 4C**).

Next, we restricted our study by sex to the more aggressive, risky variants of cSCC in the immunosuppressed population, and found, as expected, that aggressive disease occurred earlier in life in both sexes. However, the lower age at diagnosis particularly occurred in women, suggesting the loss of immunity is more damaging to females (median age men $= 73$, median age women $= 70$, $P = 0.04$; **Fig. 4D**). Taken together, these data show the immune system is critical to contain carcinogenesis, and validate the immune response underpins less aggressive disease in females.

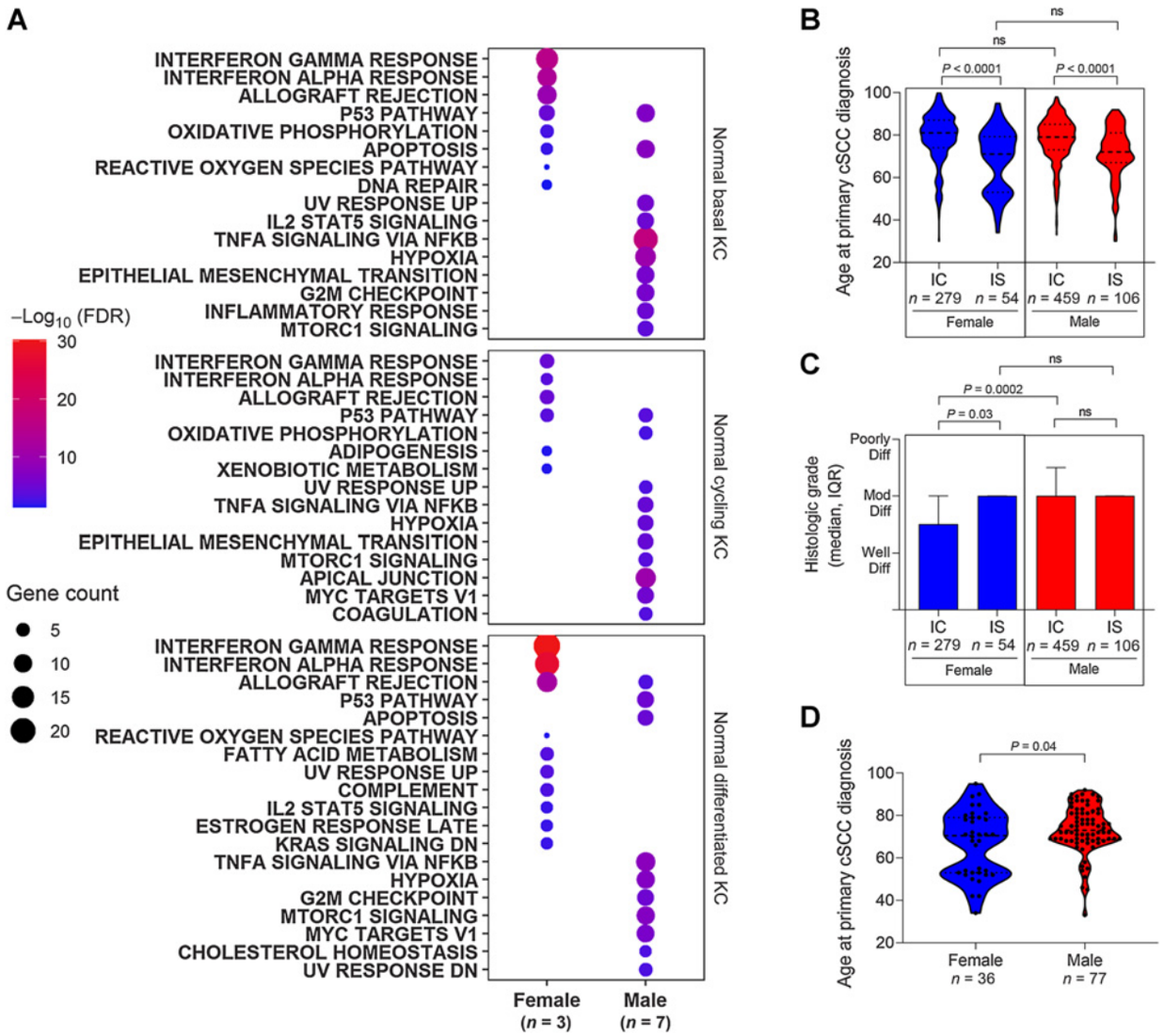

Figure 4.

cSCC histologic grade of human cSCC is tied to female immunity. **A,** Pathways enriched for genes differentially expressed by sex in human adult keratinocytes from UVR-exposed, tumor-adjacent skin by sex. Female pathways (left) represent gene pathways expressed higher in female keratinocytes. Male pathways (right) represent gene pathways expressed higher in male keratinocytes. Keratinocytes grouped into basal, cycling, and differentiated cells. Size of spheres represent numbers of enriched genes in pathway, color represents significance ($-\log_{10}$(FDR) of enrichment (red most significant). **B,** Age comparison of immunocompetent (IC) and immunosuppressed (IS) patients diagnosed with primary cSCC by sex, Mann–Whitney. **C,** Histologic grade comparison of primary cSCC by sex in immunocompetent and immunosuppressed patients [median, interquartile range (IQR), Mann–Whitney]. **D,** Age comparison of immunocompetent and immunosuppressed women and men diagnosed with moderately and poorly differentiated primary cSCC, Mann–Whitney test.

Discussion

The molecular landscape of cancer differs by sex, and men are more frequently diagnosed with cancer and die more often from cancer than women. Men traditionally are exposed to more carcinogens due to professional hazards and behavior, but in risk-adjusted epidemiologic studies, there is still an unexplained greater proportion of men with cancer compared with women (2–4). Furthermore, there is significant variation in the DNA alterations and RNA landscape by sex in most cancers, suggesting there are sex-specific susceptibilities and biological processes driving female and male cancer (5–9). However, comparing human tumors from different sexes is a limited approach as human tumors are not matched for age, histologic grade, underlying susceptibility to cancer, and dose of carcinogen.

In this study, we show immunocompetent women have less aggressive cSCC than men. We compared the development and the molecular hallmarks of cSCC in male and female animals using a well-established mouse model of DMBA/TPA carcinogenesis, adjusted for carcinogen exposure (18), showing that similar to other studies using UVB as the carcinogenic challenge (15, 16), males present histologically more aggressive cSCC and metastasis.

We show that the rate of DNA damage accumulation is equal in male and female mice exposed to equal doses of DMBA. This indicates

both sexes repair damage similarly following exposure to the chemical carcinogen. Intriguingly, we observe the overall TMB increases as tumors become more aggressive, or with increasing histologic grade in both sexes despite animals not receiving additional exposures to carcinogen. This suggests that in DMBA-driven cSCC, there is a selection for clones with more mutations as cell division progresses and tumors advance. Previous work comparing mutation burden in adjacent areas of early dysplasia, intermediate dysplasia, and primary cutaneous melanoma reveal increasing mutation burden with oncogenic stage (30), raising the hypothesis that areas with early damage must receive additional carcinogenic exposure to drive cancer progression. However, our study indicates carcinogenic damage conferring full oncogenic potential may occur at the early stage and will depend on successful expansion of clones with higher TMB, as we discontinued carcinogen exposure before cSCC onset.

Intriguingly, we find the transcriptional response to carcinogen exposure that differs between the sexes. At the earliest stage of disease, female animals increase cancer immune-related responses and recruit an immune cell landscape involved in cancer defense, which is reversed in animals on prednisolone with a compromised immune system. Male animals, in contrast, have more macrophages, which are linked to poor prognosis in cancer and comparatively show few immune-related gene expression changes. The macrophage male sex bias, although from a small cohort, is mirrored in the human immune cell cSCC landscape as well as in a mouse hepatocellular carcinogenesis model driven by diethylnitrosamine (31), indicating they may be specifically involved in the male response to extrinsic damage.

One critical gene expression difference we found between male and female animals is the upregulation of *Cdkn2a* in females, a fundamental cell-cycle regulator that exerts profound influence in both cell proliferation (25, 26) and cancer-immune responses (27, 28). Intriguingly, our preliminary data show the histologic grade and rate of metastasis is more advanced in $Cdkn2a^{fl/fl}$ than in *Cdkn2a* wild-type females, suggesting *Cdnkn2a* expression may play a critical role restricting female cSCC carcinogenesis. In further support for a central role of *Cdnkn2a*, we found the mitotic rate of carcinogen-exposed epithelium was higher in male epidermis, indicating greater proliferation, than female skin. In addition, epidemiologic studies indicate that *CDKN2A* germline loss-of-function carriers, who are at high risk for cSCC and melanoma, are particularly susceptible to tobacco-induced lung cancers (32). This further supports a central role for CDKN2A in the response to environmental damage beyond skin carcinogenesis.

We validated the relevance of the *in vivo* findings in human female and male epidermal keratinocytes, which originated from a sun-exposed site (19), and in human cSCCs. Keratinocytes from women mirror the response observed in female mouse epidermis, upregulating critical cancer-immune pathways, genes, and immune cells following UVR, compared with men. The use of human cSCC single-cell RNA-seq data allows expression patterns to be isolated to specific cell types, indicating the cytokine differences are expressed by multiple cell types, including keratinocytes and immune cells, in keeping with a complex multicellular immune response in human skin, which requires further study.

Similar to the mouse model, although cSCC arises at a similar age in immunocompetent men and women, men have significantly more aggressive disease, and the aggressive tumors arise at a younger age in men. Restricting our analysis to immunosuppressed patients, we show cSCC arises in younger men and women. Importantly, cSCC in immunosuppressed women are significantly more aggressive, similar to male disease, while male disease is aggressive regardless of immune status. Although our study includes a broad and heterogeneous range of diagnoses and medication driving immune suppression in our patients, these data show the immune response plays a critical role constraining cSCC progression at the earliest stage of disease in both sexes, as the age of incidence drops in both men and women. However, the data indicate the mechanisms restricting tumor progression are more robust in females. Thus, this study, adjusted for risk factors, implicates distinct biology driving male and female cancer and shows differences in immune responses between the sexes. Future studies should be conducted to identify the potential regulators of sex-linked SCC dimorphism, to understand whether the sex bias in cSCC onset and outcome is due to chromosomal content or due to hormonal causes, and if our findings extend to other epithelial and nonepithelial cancers with a strong male bias.

These results are strongly aligned with clinical observations revealing higher incidence of excess immunity-linked disease in females and unique immune responses to infectious disease by sex (33). Personalized medicine approaches stratify patients with cancer by genotype; however, to date, the potential for cancer stratification and therapy by sex has not been explored. Further work will be necessary to identify the potential regulators of sex-linked cSCC dimorphism.

Authors' Disclosures

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Authors' Contributions

T. Budden: Data curation, software, formal analysis, validation, visualization, methodology, writing–original draft. **C. Gaudy-Marqueste:** Resources, data curation. **S. Craig:** Data curation, methodology. **Y. Hu:** Data curation. **C.H. Earnshaw:** Data curation. **S. Gurung:** Data curation. **A. Ra:** Data curation. **V. Akhras:** Data curation. **P. Shenjere:** Data curation. **R. Green:** Data curation. **L. Jamieson:** Data curation. **J. Lear:** Data curation. **L. Motta:** Data curation, methodology. **C. Caulín:** Resources, data curation, formal analysis. **D. Oudit:** Data curation. **S.J. Furney:** Data curation, software, formal analysis, methodology. **A. Virós:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing.

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References

1. Sampathkumar NK, Bravo JI, Chen Y, Danthi PS, Donahue EK, Lai RW, et al. Widespread sex dimorphism in aging and age-related diseases. Hum Genet 2020; 139:333–56.
2. Clocchiatti A, Cora E, Zhang Y, Dotto GP. Sexual dimorphism in cancer. Nat Rev Cancer 2016;16:330–9.
3. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018: cancer statistics, 2018. CA Cancer J Clin 2018;68:7–30.
4. Pearce MS, Parker L. Childhood cancer registrations in the developing world: still more boys than girls. Int J Cancer 2001;91:402–6.
5. Dunford A, Weinstock DM, Savova V, Schumacher SE, Cleary JP, Yoda A, et al. Tumor-suppressor genes that escape from X-inactivation contribute to cancer sex bias. Nat Genet 2017;49:10–6.
6. Yuan Y, Liu L, Chen H, Wang Y, Xu Y, Mao H, et al. Comprehensive characterization of molecular differences in cancer between male and female patients. Cancer Cell 2016;29:711–22.
7. Li CH, Haider S, Shiah YJ, Thai K, Boutros PC. Sex differences in cancer driver genes and biomarkers. Cancer Res 2018;78:5527–37.
8. Lopes-Ramos CM, Kuijjer ML, Ogino S, Fuchs CS, DeMeo DL, Glass K, et al. Gene regulatory network analysis identifies sex-linked differences in colon cancer drug metabolism. Cancer Res 2018;78:5538–47.
9. Lotz M, Budden T, Furney SJ, Virós A. Molecular subtype, biological sex and age shape melanoma tumour evolution. Br J Dermatol 2020;184:328–37.
10. Venables ZC, Autier P, Nijsten T, Wong KF, Langan SM, Rous B, et al. Nationwide incidence of metastatic cutaneous squamous cell carcinoma in England. JAMA Dermatol 2019;155:298–306.
11. Karia PS, Han J, Schmults CD. Cutaneous squamous cell carcinoma: estimated incidence of disease, nodal metastasis, and deaths from disease in the United States, 2012. J Am Acad Dermatol 2013;68:957–66.
12. Schmults CD, Karia PS, Carter JB, Han J, Qureshi AA. Factors predictive of recurrence and death from cutaneous squamous cell carcinoma: a 10-year, single-institution cohort study. JAMA Dermatol 2013;149:541–7.
13. Stang A, Khil L, Kajüter H, Pandeya N, Schmults CD, Ruiz ES, et al. Incidence and mortality for cutaneous squamous cell carcinoma: comparison across three continents. J Eur Acad Dermatol Venereol 2019;33:6–10.
14. Manyam B V, Garsa AA, Chin R-I, Reddy CA, Gastman B, Thorstad W, et al. A multi-institutional comparison of outcomes of immunosuppressed and immunocompetent patients treated with surgery and radiation therapy for cutaneous squamous cell carcinoma of the head and neck. Cancer 2017;123: 2054–60.
15. Dehelean CA, Soica C, Pinzaru I, Coricovac D, Danciu C, Pavel I, et al. Sex differences and pathology status correlated to the toxicity of some common carcinogens in experimental skin carcinoma. Food Chem Toxicol 2016;95: 149–58.
16. Thomas-Ahner JM, Wulff BC, Tober KL, Kusewitt DF, Riggenbach JA, Oberyszyn TM. Gender differences in UVB-induced skin carcinogenesis, inflammation, and DNA damage. Cancer Res 2007;67:3468–74.
17. Abel EL, Angel JM, Kiguchi K, DiGiovanni J. Multi-stage chemical carcinogenesis in mouse skin: fundamentals and applications. Nat Protoc 2009;4:1350–62.
18. Nassar D, Latil M, Boeckx B, Lambrechts D, Blanpain C. Genomic landscape of carcinogen-induced and genetically induced mouse skin squamous cell carcinoma. Nat Med 2015;21:946–54.
19. Ji AL, Rubin AJ, Thrane K, Jiang S, Reynolds DL, Meyers RM, et al. Multimodal analysis of composition and spatial architecture in human squamous cell carcinoma. Cell 2020;182:497–514.
20. Frese KK, Tuveson DA. Maximizing mouse cancer models. Nat Rev Cancer 2007; 7:654–8.
21. Fu Y, Jung AW, Torne RV, Gonzalez S, Vöhringer H, Shmatko A, et al. Pan-cancer computational histopathology reveals mutations, tumor composition and prognosis. Nat Cancer 2020;1:800–10.
22. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 2001;410:1107–11.
23. Kaplan DH, Shankaran V, Dighe AS, Stockert E, Aguet M, Old LJ, et al. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. Proc Natl Acad Sci U S A 1998;95:7556–61.
24. Zaidi MR, Merlino G. The two faces of interferon-γ in cancer. Clin cancer Res 2011;17:6118–24.
25. Lee S, Schmitt CA. The dynamic nature of senescence in cancer. Nat Cell Biol 2019;21:94–101.
26. Pérez-Mancera PA, Young ARJ, Narita M. Inside and out: the activities of senescence in cancer. Nat Rev Cancer 2014;14:547–58.
27. Azazmeh N, Assouline B, Winter E, Ruppo S, Nevo Y, Maly A, et al. Chronic expression of p16(INK4a) in the epidermis induces Wnt-mediated hyperplasia and promotes tumor initiation. Nat Commun 2020;11:2711.
28. Brenner E, Schörg BF, Ahmetlić F, Wieder T, Hilke FJ, Simon N, et al. Cancer immune control needs senescence induction by interferon-dependent cell cycle regulator pathways in tumours. Nat Commun 2020;11:1335.
29. Li Z, Gonzalez CL, Wang B, Zhang Y, Mejia O, Katsonis P, et al. Cdkn2a suppresses metastasis in squamous cell carcinomas induced by the gain-of-function mutant p53(R172H). J Pathol 2016;240:224–34.
30. Shain AH, Yeh I, Kovalyshyn I, Sriharan A, Talevich E, Gagnon A, et al. The genetic evolution of melanoma from precursor lesions. N Engl J Med 2015;373: 1926–36.
31. Romualdo GR, Prata GB, da Silva TC, Fernandes AAH, Moreno FS, Cogliati B, et al. Fibrosis-associated hepatocarcinogenesis revisited: establishing standard medium-term chemically-induced male and female models. PLoS One 2018;13: e0203879.
32. Helgadottir H, Höiom V, Jönsson G, Tuominen R, Ingvar C, Borg A, et al. High risk of tobacco-related cancers in CDKN2A mutation-positive melanoma families. J Med Genet 2014;51:545–52.
33. Klein SL, Flanagan KL. Sex differences in immune responses. Nat Rev Immunol 2016;16:626–38.