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Role of the immune-inflammatory microenvironment in the development of cutaneous squamous cell carcinoma

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ABSTRACT

Cutaneous squamous cell carcinoma (cSCC) is the second most common non-melanoma skin cancer and represents an increasing public health burden due to its rising incidence, biological heterogeneity, and potential for aggressive clinical behavior. A significant proportion of cSCCs develop from actinic keratosis (AK) within a broader context of field cancerization (CC), a condition characterized by genetically altered but clinically inconspicuous skin chronically exposed to ultraviolet radiation. Although genetic alterations in keratinocytes are central to cSCC pathogenesis, growing evidence indicates that tumor progression is critically influenced by the inflammatory and immune components of the tumor microenvironment.

This doctoral thesis explores the transcriptional profiles of the immune microenvironment underlying the progression from field cancerization and AK to invasive cSCC, with the aim of delineating immune-related mechanisms associated with malignant transformation. Using next generation targeted immune RNA sequencing, gene expression patterns were analyzed in paired samples of CC, AK, and cSCC obtained from patients.

Multivariate and differential expression analyses revealed a progressive, condition-specific remodeling of the immune microenvironment along the AK–cSCC continuum. Invasive cSCC was characterized by the upregulation of inflammatory and cytotoxic-related transcriptional programs, together with increased macrophage-related and regulatory immune signatures, indicating a shift toward an immunoregulatory and myeloid-enriched milieu. In contrast, pre-neoplastic lesions such as AK exhibited a distinct immune profile, with relative enrichment of antigen presentation, dendritic cell–associated pathways, and selected T cell–related signatures. These findings indicate a qualitative reprogramming of immune regulation during malignant progression, rather than a uniform amplification of immune activity.

Overall, this work provides the immunological framework for cSCC development, highlighting the pivotal role of the tumor microenvironment in disease evolution. The results support the concept that early immune dysregulation contributes to carcinogenesis and may represent a window for therapeutic intervention aimed at preventing progression to invasive disease.

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1. INTRODUCTION

1.1 Definition of Cutaneous Squamous Cell Carcinoma, AK and Cancerization field

Cutaneous squamous cell carcinoma (cSCC) is a keratinocyte cancer arising from the uncontrolled proliferation of squamous keratinocytes and/or keratinocytes in cutaneous appendages. cSCC can develop de novo in sun-exposed areas of the skin or on pre-existing skin alterations, such as scars, chronic ulcers, sunburns, and chronic inflamed skin, or it may arise from precancerous lesions such as actinic keratoses (AK) (AIOM – Italian Association of Medical Oncology, 2024).

cSCC can be intraepithelial (i.e. in situ), also known as Bowen's disease, if it remains confined to the epidermis; at this stage, the tumor is generally easier to treat. While the majority of cSCCs are indolent and amenable to surgical excision, a subset demonstrates aggressive behavior with local invasion, in fact up to 5% of in situ cSCC may progress to invasive cSCC (Fania et al., 2021). The cSCC is considered invasive when it penetrates the underlying dermis and subcutaneous tissue, with a risk of developing lymph node and visceral metastases. Metastatic cSCC, although uncommon, carries a poor prognosis, with reported five-year survival rates of 20–50% depending on the extent and location of metastases (Corchado-Cobos et al., 2020). The cSCC can be further also classified as “low risk” or “high-risk” based on the likelihood of recurrence. European guidelines define low risk cSCCs as a tumor $\leq$ 20 mm in diameter, tumor thickness $\leq$ 4 mm, tumor invasion into dermis only, no lymph vascular or perineural invasion identified in the specimen and only of a well-differentiated or moderately differentiated histology (Stratigos et al., 2023). High-risk cSCC refers to invasive cutaneous squamous cell carcinoma without locoregional or distant metastases (N0, M0), but with features associated with an increased risk of local recurrence and metastasis; it remains potentially curable with surgery or radiotherapy (Stratigos et al., 2023). Advanced cSCC is divided into locally advanced cSCC (la-cSCC) and metastatic cSCC (m-cSCC). La-cSCC is a non-metastatic disease that is not amenable to curative surgery or radiotherapy because of factors such as multiple recurrences, large tumor size, deep invasion (including bone, muscle, or nerves), or unacceptable morbidity from surgery, corresponding to unresectable T3/T4 tumors. Metastatic cSCC includes disease with locoregional (in-transit or nodal) or distant metastases and typically requires systemic therapy (Stratigos et al., 2023).

Actinic keratosis (AK) is a common keratinocyte dysplasia characterized by atypical epidermal keratinocyte proliferation. It has been described by various terms in the literature and is considered a precancerous lesion that may progress to invasive cSCC. Importantly, it is currently not possible to predict which individual AK lesions will progress to invasive cSCC (Kandolf et al., 2024).

Field cancerization refers to an area of clinically sun-damaged skin surrounding or adjacent to actinic keratoses that show subclinical genetic alterations similar to AK lesions. Clinically, it is defined as a sun-damaged anatomical area characterized by features such as telangiectasia, atrophy, pigmentation changes, and a rough, sandpaper-like texture, though it remains unclear whether a visible AK lesion is required for its diagnosis (Kandolf et al., 2024).

1.2 cSCC

1.2.1 Epidemiology of cSCC

The cSCC is a prevalent malignancy of epidermis and represents the second most frequent form of non-melanoma skin cancer (NMSC) worldwide, second only to basal cell carcinoma (BCC).. The average age at diagnosis of cSCC is approximately 65 years; however, cases of early onset between 20 and 30 years of age have been reported, particularly in geographic areas characterized by high levels of solar exposure such as Australia, Florida, New Zealand, and Southern California (Joseph R. Kallini, 2015).

The steady rise in incidence, together with the substantial mortality associated with advanced disease stages, has positioned cSCC as an emerging public health concern. Over the past three decades, the number of reported cases has increased by 50% to 300%, and projections suggest that, by 2030, the incidence in European countries may double compared with current levels. Lifetime risk estimates for developing cSCC range from 7% to 11% in Caucasian individuals, with a higher prevalence observed in men (9–14%) compared with women (4–9%) (Corchado-Cobos et al., 2020).

Although it has traditionally been assumed that approximately 80% of NMSC are BCCs and 20% are cSCCs, corresponding to a 4:1 ratio, evidence published in 2020 demonstrated that this proportion varies markedly according to sex, age, race, and geographic location, approaching a 1:1 ratio in individuals older than 65 years (Lukowiak et al., 2020).

Higher incidence rates of cSCC are reported in countries such as Australia, the United States, and New Zealand, where intense ultraviolet radiation exposure combined with a high prevalence of fair skin phototypes substantially increases disease risk. In Australia, age-standardized incidence rates for both sexes ranged from 467 cases per 100,000 person-years between 2011 and 2014, with rates of 573 per 100,000 person-years in men and 371 per 100,000 person-years in women.

In the United States, cSCC is not included in national cancer registries, complicating the accurate estimation of incidence and mortality. Nevertheless, age-standardized estimate suggested incidence rates of 497 cases per 100,000 person-years in men and 296 in women. Incidence rates were comparable between sexes up to the age of 50, but after 65 years, incidence in men was nearly twice that observed in women (Stang et al., 2019). Regional differences have also been noted, with higher incidence in southern and central areas of the country.. Currently, approximately 1.8 million new cases of cSCC are estimated to be diagnosed annually in the United States (Stang et al., 2019).

Across Europe, the lowest incidence rates have been reported in northwestern countries such as the United Kingdom. Between 2013 and 2015, age-standardized incidence rates based on the European standard population were 77 cases per 100,000 person-years overall, with 111 cases per 100,000 person-years in men and 42 in women. The highest rates within this region were documented in Northern Ireland (Nanz et al., 2024).

In Italy, comprehensive national data on the incidence of NMSC are lacking. However, estimates from the Trentino regional Skin Cancer Registry reported an incidence of 29 cases per 100,000 inhabitants between 1993 and 1998. According to data from the Italian Association of Cancer Registries (AIRTUM), approximately 83,000 new cases of non-melanoma skin cancer were diagnosed in 2018, including about 19,000 cases of cSCC. Incidence was higher among men,

particularly those over 65 years of age, and exhibited a clear North–South gradient (Queirolo et al., 2024).

Annual deaths attributable to cSCC are estimated to range from 3,932 to 8,791, accounting for roughly 75% of deaths associated with non-melanoma skin cancers (Ciuciulete et al., 2022). Mortality figures are likely underestimated due to delayed diagnoses and disparities in access to healthcare. A recent analysis based on data from the Global Burden of Disease (GBD) study, encompassing 204 countries and territories, revealed a marked increase in cSCC-related mortality, with a 151% rise in the number of deaths between 1990 and 2021 (Liu et al., 2025).

1.2.2 Risk Factors of cSCC

The etiology of cSCC is multifactorial and results from the interplay of environmental, immunological, and genetic factors. Among environmental determinants, cumulative lifetime exposure to ultraviolet (UV) radiation, represents the primary risk factor in cSCC pathogenesis. More than 70% of tumors arise on chronically sun-exposed areas, with the head and neck involved in approximately 55% of cases, the extensor surfaces of the hands and forearms in 18%, and the lower limbs in 13%. These areas are subjected to repeated and prolonged solar irradiation over decades, leading to cumulative DNA damage and field cancerization. Early exposure to tanning beds, particularly before the age of 25, has been strongly associated with a significantly increased risk of developing cSCC.

Chronic cutaneous inflammation resulting from wounds, scars, burns, ulcers, or fistulous tracts has also been implicated in cSCC carcinogenesis. Indeed, cSCC represents the most frequent malignancy arising in burn scars, typically developing after a prolonged latency period of approximately 30 years in individuals who sustained burns earlier in life (Wunderlich et al., 2024). Additional, less common but well-established environmental risk factors include exposure to chemical carcinogens such as pesticides, herbicides, polycyclic aromatic hydrocarbons (found in industrial oils, lubricants, and coal tar), arsenic, and ionizing radiation. Notably, ionizing radiation is often associated with more aggressive forms of cSCC, characterized by high recurrence rates and metastatic rates ranging from 10% to 30% (Que et al., 2018).

Individual host-related factors, including advanced age, male sex, and fair skin phototypes particularly Fitzpatrick phototypes I, II, also play a significant role in increasing cSCC risk. These phototypes offer reduced protection against UV radiation due to lower melanin content, accounting for the higher incidence of cSCC observed in Caucasian populations. From an epidemiological standpoint, lifetime risk estimates for cSCC range between 7% and 11% in fair-skinned populations, although substantial regional variability exists. Such variability is influenced by latitude, ambient UV index, occupational and recreational sun exposure, and cultural behaviors related to tanning and photoprotection (Corchado-Cobos et al., 2020). For example, outdoor workers—including farmers, construction workers, and fishermen—exhibit significantly higher incidence rates compared with indoor workers, highlighting the role of occupational exposure as an independent risk factor.

Immunosuppressed patients, such as organ transplant recipients, have a markedly increased risk of developing cSCC due to immunosuppressive therapy. Solid organ transplant recipients exhibit

a 65- to 250-fold higher prevalence of cSCC compared with the general population, along with an increased metastatic rate, rising from 3–5% in immunocompetent individuals to 7–13% in this group. Similarly, patients with AIDS, non-Hodgkin lymphoma, chronic lymphocytic leukemia, or those who have undergone hematopoietic stem cell transplantation face a substantially elevated risk of cSCC. In chronic lymphocytic leukemia, this risk may be increased by 8- to 10-fold. Moreover, the use of immunosuppressive therapies for autoimmune conditions such as rheumatoid arthritis or inflammatory bowel disease further contributes to cSCC susceptibility (Fania et al., 2021). Moreover, drugs like azathioprine, cyclosporine, and tacrolimus also have direct carcinogenic effects, increasing UV sensitivity and promoting AK progression to cSCC (Reinehr & Bakos, 2019)

Inherited disorders associated with photosensitivity or defective DNA repair mechanisms may predispose individuals, particularly at younger ages, to cSCC. Among these, xeroderma pigmentosum is an autosomal recessive condition characterized by extreme UV sensitivity due to mutations affecting nucleotide excision repair pathways, leading to an impaired ability to correct UV-induced DNA damage. As a result, affected individuals exhibit an exceptionally high risk of cSCC, reaching up to a 10,000-fold increase in patients under 20 years of age (Hosseini et al., 2024).

In summary, cSCC is an increasingly prevalent malignancy driven by demographic aging, cumulative UV exposure, and environmental and behavioral factors. Its epidemiology reflects complex interactions between host susceptibility, environmental carcinogenesis, and healthcare practices. Understanding these patterns is essential for developing effective prevention strategies, optimizing surveillance programs, and allocating healthcare resources appropriately.

1.2.3 Clinico-Pathological aspects of cSCC

Regarding clinical and morphological features, in the early non-invasive phase, cSCC may appear as a small flesh-colored plaque or papule, sometimes with a scaly or hyperkeratotic surface. At this stage, it can be difficult to distinguish from actinic keratosis. Over time, the lesion tends to enlarge and develop crusts and ulcerations, presenting a firm consistency upon palpation. In some cases, the lesion may also be pigmented, with colors ranging from light brown to dark brown (Stratigos et al., 2020).

Several histological variants of cSCC have been described. Among these, Bowen's disease is characterized by atypical keratinocytes throughout all layers of the epidermis. Key histological features include parakeratosis, hyperkeratosis, nuclear pleomorphism, and atypical mitoses (Stratigos et al., 2020).

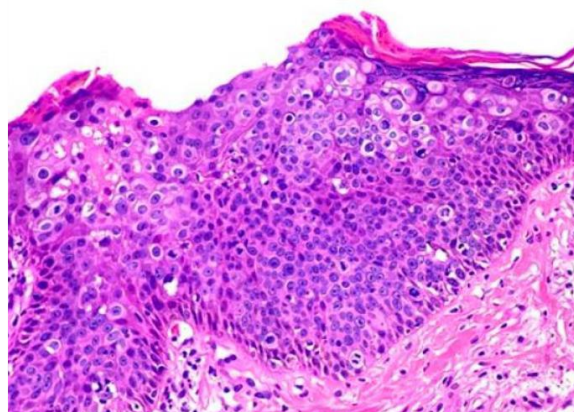


Figure 1: Histological image (Hematoxylin/Eosin staining) of the epidermis with Bowen's disease. Parakeratosis, hyperkeratosis, and full-thickness dysplasia are evident. Furthermore, the dermo epidermal junction is intact (Fania et al., 2021).

Keratoacanthoma is considered by some authors as a cSCC variant characterized by rapid growth and a tendency toward spontaneous resolution. It consists of a symmetrical and circumscribed proliferation of keratinocytes, forming a round, firm nodular lesion with raised margins and a central depression containing keratinous material (Fania et al., 2021).

Other histological variants of cSCC include:

- Desmoplastic cSCC, characterized by fibrous stroma involving at least 30% of the dermis, often associated with a higher likelihood of metastasis.
- Spindle cell cSCC, typically developing on post-traumatic scars, with desmoplastic stroma <30%, a myxoid component, and pleomorphic cells.
- Acantholytic (or adenoacanthoma) cSCC, mainly observed in the head and neck region of elderly patients, with an adenoid pattern and dyskeratotic cells.
- Pseudovascular (adenoid) cSCC, showing glandular and pseudovascular structures.
- Verrucous cSCC (Buschke-Löwenstein tumor), occurring in genital areas, characterized by pronounced papillomatosis and low atypia.
- Epithelioid cuniculatum, mainly developing on the foot, sometimes following a plantar wart, with hyperkeratosis, acanthosis, and well-differentiated squamous epithelium.
- Adenosquamous cSCC, showing mixed glandular and squamous differentiation, associated with more aggressive behavior.
- Neurotropic cSCC, notable for perineural spread.

These histological variants underscore the heterogeneity of cSCC, which can vary significantly in morphology, clinical behavior, and treatment response (Fania et al., 2021).

1.2.4 Molecular characteristics of cSCC

Driver genes implicated in cSCC pathogenesis

The molecular pathogenesis of cSCC is characterized by the progressive accumulation of somatic genetic alterations in epidermal keratinocytes, ultimately leading to uncontrolled proliferation, defective terminal differentiation, genomic instability, and resistance to apoptosis. These alterations arise predominantly as a consequence of chronic ultraviolet (UV) radiation exposure, which induces direct DNA damage and overwhelms cellular repair mechanisms (Brash DE., 2015; Chang, D., 2021).

Among the most frequently altered driver genes in cSCC, *TP53* plays a central role. Loss-of-function mutations in *TP53* impair the DNA damage response, allowing keratinocytes harboring UV-induced mutations to escape cell cycle arrest and apoptosis, thereby facilitating clonal expansion (Purdie et al., 2009; South et al., 2014). Similarly, inactivation of *CDKN2A*, which encodes the tumor suppressors p16INK4a and p14ARF, disrupts cell cycle control and senescence

pathways, further promoting malignant transformation. Mutations in *NOTCH1* and *NOTCH2*, which normally regulate keratinocyte differentiation and exert tumor-suppressive functions in the epidermis, lead to impaired differentiation and enhanced proliferative capacity, reinforcing the oncogenic phenotype (Wang et al., 2011; Pickering et al., 2014). Tumors harboring disruptive mutations in *TP53* and *NOTCH* genes, together with a high tumor mutation burden and a strong UV mutational signature, are often associated with more aggressive clinical phenotypes. These tumors demonstrate increased rates of local recurrence, regional lymph node involvement, and distant metastasis (Cho et al., 2018; Inman et al., 2018).

In parallel, activation of key oncogenic signaling cascades—including the RAS/MAPK and PI3K/AKT pathways—contributes to tumor initiation, survival, and progression. These pathways promote cell growth, metabolic reprogramming, and resistance to apoptotic stimuli. Overexpression and constitutive activation of epidermal growth factor receptor (EGFR), frequently observed in cSCC, further amplify downstream signaling and are associated with increased tumor aggressiveness (Xie et al., 2019) (Figure 2).

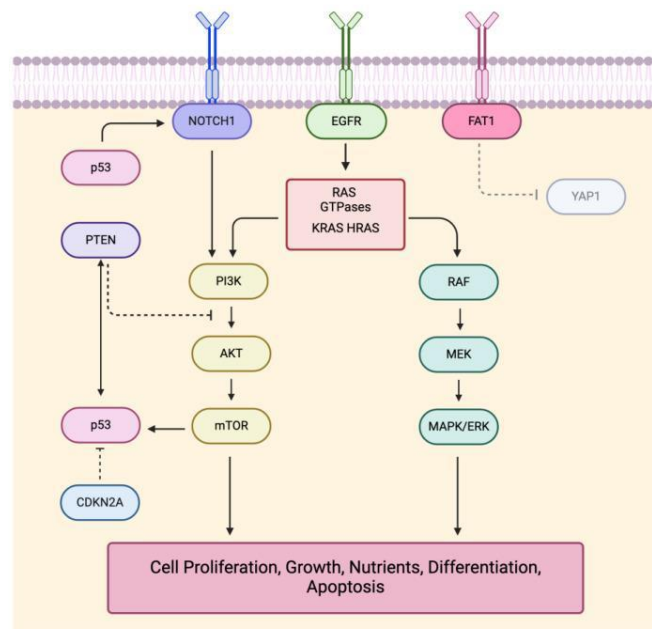


Figure 2: Altered driver genes and pathways involved in the progression from AK to cSCC (Hosseini et al., 2024).

Mutations in RAS genes (HRAS, KRAS, NRAS) and EGFR are also common in cSCC. These alterations activate signaling pathways that enhance proliferation and survival. HRAS is the most frequently mutated RAS gene in cSCC, followed by KRAS. EGFR, crucial for proliferation, apoptosis, differentiation, and tissue homeostasis, frequently shows gain-of-function mutations and aberrant overexpression. Some studies link EGFR mutations to worse prognosis and increased metastasis (Hosseini et al., 2024).

A defining molecular hallmark of cSCC is the presence of UV signature mutations, particularly cytosine-to-thymine (C>T) transitions at dipyrimidine sites, which provide compelling evidence for UV radiation as the dominant mutagenic driver (Brash et al., 1991; Alexandrov et al., 2013). As a result, cSCC exhibits one of the highest tumor mutation burdens among solid malignancies, a feature with significant biological and therapeutic implications.

Recent advances in genomic and transcriptomic profiling have led to efforts to classify cSCC into molecular subtypes based on mutation burden, UV mutational signatures, immune infiltration patterns, and stromal composition. This emerging molecular classification framework not only enhances understanding of tumor biology but also lays the foundation for refined prognostic stratification and the identification of clinically actionable molecular targets (Keohane et al., 2021). cSCCs characterized by EGFR overexpression or activation of the PI3K/AKT pathway may derive benefit from targeted therapies, whereas tumors with high mutation burden and an immune-inflamed microenvironment appear particularly susceptible to immune checkpoint inhibition (Migden et al., 2018; Stratigos et al., 2020).

Key molecular alterations in metastatic cSCC

The transition from in situ cSCC to invasive and metastatic disease is driven by the accumulation of additional genetic alterations and profound remodeling of the tumor microenvironment. Activation of transcription factors such as STAT3 and NF- κ B plays a pivotal role in this process by promoting proliferation, angiogenesis, immune evasion, and resistance to cell death (Kim et al., 2009; Karin M., 2006). These pathways also facilitate epithelial–mesenchymal transition (EMT), a key biological program that endows tumor cells with enhanced migratory and invasive capabilities.

Loss or downregulation of cell adhesion molecules, particularly E-cadherin, disrupts epithelial integrity and promotes detachment of tumor cells from the primary lesion, facilitating invasion into surrounding tissues and dissemination via lymphatic and hematogenous routes (Wheelock et al., 2008). Concurrently, alterations in extracellular matrix composition and increased activity of matrix metalloproteinases further support invasive behavior (Egeblad et al., 2010).

The tumor microenvironment (TME) plays an equally critical role in cSCC metastatic progression. Tumor-associated macrophages, neutrophils, and myeloid-derived suppressor cells contribute to a pro-tumorigenic milieu through the secretion of cytokines, chemokines, and growth factors such as IL-6, TNF- α , and VEGF (Ruffell et al., 2012; Gabrilovich DI, 2017). These mediators promote angiogenesis, suppress antitumor immune responses, and enhance tumor cell survival.

1.3 AK and Cancerization field

1.3.1 Actinic Keratosis

AK consists of abnormal proliferation of atypical keratinocytes in the basal layer of the epidermis in areas of skin chronically damaged by UV exposure. Over time, these dysplastic keratinocytes extend toward the upper layers of the epidermis (Kandolf et al., 2024).

Actinic keratosis (AK) is a precancerous skin lesion that primarily develops as a result of chronic UV-B-induced damage and is considered the main precursor of cSCC. It is estimated that approximately 70% of cSCC cases arise from AK, making it crucial to understand the mechanisms underlying the progression from AK to cSCC, as this knowledge could inform early therapeutic interventions before malignant transformation occurs (Li et al., 2025). The rate of progression from actinic keratosis to cSCC is variable, ranging from 0% to 0.075% per lesion per year. This

rate increases to 0.53% if the patient has cSCC at another site and up to 2.57% in elderly patients with multiple AKs four years after diagnosis. However, not all AKs progress to cSCC; some remain stable or may regress spontaneously, with reported regression rates ranging from 15% to 53% per year. It is important to note that regressed lesions often recur, with long-term recurrence rates exceeding 50% (Malvey et al., 2024). The prevalence of AK ranges from 11% to 60% in Caucasian individuals over 40 years old, primarily affecting fair-skinned individuals in sun-exposed areas such as the face, bald scalp, and dorsal hands (Reinehr & Bakos, 2019).

Clinically, AK appears as an erythematous plaque ranging from a few millimeters to over 1 cm in diameter and can be single or multiple. Lesions may vary in color from white, pink, red, to brown, with a rough surface, adherent scaling, or superficial crusts. Margins are often poorly defined, and varying degrees of hyperkeratosis may be present. Early lesions are often more palpable than visible. Most AKs are asymptomatic, although some patients report burning, tenderness, occasional bleeding, or pruritus (Reinehr & Bakos, 2019).

Currently, several clinical and histological classification systems exist for AK lesions. Olsen et al. (1991) proposed a clinical classification based on lesion thickness. Grade 1 lesions are slightly palpable, grade 2 lesions are moderately thick, and grade 3 lesions are thick and hyperkeratotic; however, the Olsen classification has no predictive value (Figure 3).



Figure 3: Clinical classification of actinic keratosis (AK) into poorly palpable (grade 1), moderately palpable (grade 2) and thickened and hyperkeratotic lesion (grade 3) (Cavicchini Stefano, 2015).

Subsequently, Roewert-Huber et al. (2007) introduced a histological classification currently used for AK lesions. This system is based on the extent of atypical keratinocytes within the epidermis. In AK grade I (mild) (Figure 4A), atypical keratinocytes are limited to the basal and suprabasal layers, comprising the lower third of the epidermis. In AK grade II (moderate) (Figure 4B), atypical keratinocytes extend through the lower two-thirds of the epidermis, and in AK grade III (severe) (Figure 4C), atypia is observed throughout the full thickness of the epidermis.

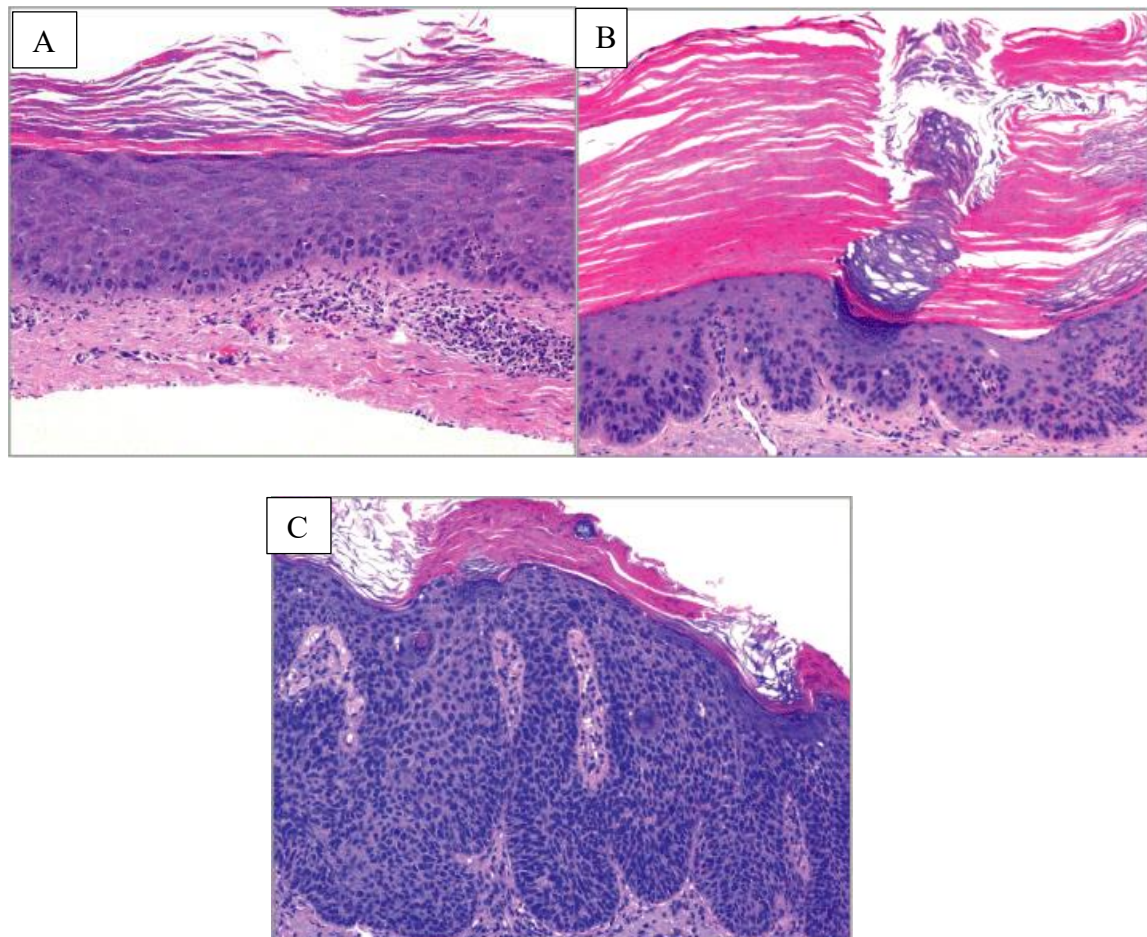


Figure 4: Histological classification of actinic keratosis (AK). A) AK grade I, mild: atypical keratinocytes in the basal and suprabasal layers of the epidermis; B) AK grade II (moderate): atypical keratinocytes extend to the lower two-thirds of the epidermis, alternating with areas of normal epidermis; alternating orthokeratosis and parakeratosis are observed; C) AK grade III (severe): atypical keratinocytes throughout the entire thickness of the epidermis with complete loss of maturation; mild epidermal hyperplasia is observed (Röwert-Huber et al., 2007).

However, a study by Schmitz et al. (2016) showed that the clinical classification of AK lesions according to the Olsen system does not reliably correspond to their histological classification using the Röwert-Huber system. Consequently, it is recommended to treat all AK lesions and the entire field of cancerization, as clinical assessment alone does not accurately reflect histological features, nor can it predict which lesions may progress to cSCC (Schmitz et al., 2016).

This is because AK progression to cSCC can occur via two main pathways: the classical or differentiated pathway (Figure 5). In the classical pathway, progression begins with cellular atypia in the basal layer (AK I), extending to the lower two-thirds of the epidermis (AK II), and eventually involving the full thickness (AK III). Only after complete epidermal transformation cells acquire the ability to invade the underlying dermis. In the differentiated pathway, dermal invasion can occur directly from an AK grade I lesion without full-thickness epidermal involvement, meaning progression occurs independently of lesion grade (Fernandez Figueras, 2017).

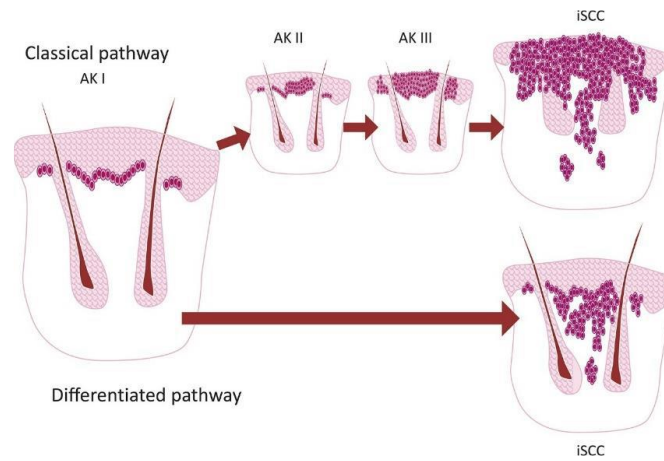


Figure 5: Progression from AK to cSCC: the classical and differentiated pathway (Fernandez Figueras, 2017).

Based on histopathological features, several AK variants have been described, including hypertrophic, atrophic, bowenoid, acantholytic, epidermolytic, lichenoid, and pigmented types. Hypertrophic AK is characterized by marked thickening of the stratum corneum (hyperkeratosis), appearing as a raised lesion. The atrophic variant shows epidermal thinning, with lesions appearing thinner than other variants. Bowenoid AK resembles Bowen’s disease, with marked cellular atypia throughout the epidermis. Acantholytic AK exhibits loss of cohesion between epidermal cells (acantholysis), leading to intraepidermal spaces. Epidermolytic AK is associated with epidermal cell degeneration, vacuolization, and cytoplasmic alterations. Lichenoid AK presents as pink papules or plaques, often on the upper extremities and upper trunk, with a band-like inflammatory infiltrate in the upper dermis, making differentiation from BCC challenging. Pigmented AK is characterized by melanin accumulation in epidermal cells, resulting in lesions ranging from brown to yellow black, with a smooth, verrucous, or slightly scaly surface, which can be difficult to distinguish clinically from solar lentigo, lentigo maligna, seborrheic keratosis, or pigmented basal cell carcinoma (Reinehr & Bakos, 2019).

1.3.2 Concept of “Field Cancerization”

The concept of “field cancerization” (CC) was first introduced in 1953 by Slaughter et al. in the normal tissue surrounding oropharyngeal carcinomas (Slaughter et al., 1953). Multiple new secondary tumors were found to subsequently arise within this field that were clinically suggestive of tumor recurrence but were in fact second independent primary tumors (Slaughter et al., 1953). Since this initial study, field cancerization has been applied to numerous other epithelial tissues. CC has been widely recognized in various cancers, including vulvar, head and neck, cervical, breast, and colon cancers. Skin tissue is particularly susceptible to the CC concept and formation, due to chronic UV exposure in sun-exposed areas. including skin. (Willenbrink et al., 2020).

The term “field cancerization” refers to an area of subclinical alterations at the periphery of clinically visible AKs, showing genetic changes similar to those in AK lesions but without histological atypia. At the cellular level, CC represents the expansion of a mutant clone that creates a field of cells predisposed to subsequent tumor development (Thamm et al., 2024).

The initial alterations known as basal atypia are often found both in the case of subclinical field cancerization and AKs and include the presence of hyperchromatic and pleomorphic nuclei with the alteration of the nuclear-cytoplasmic ratio as well as loss of polarity (Fernandez Figueras,

2017). Actinic elastosis is also met in both cases, but the presence of hyperkeratosis and lymphocytic infiltrate differentiate AK from subclinically damaged skin (Fernandez Figueras, 2017). Microscopically, AK can be differentiated from CC by hyperkeratosis, often containing parakeratotic areas indicating abnormal keratinization, and lymphocytic infiltrate, which may play a role in disease progression (Fernandez Figueras, 2017).

Therefore, it is important to treat not only visible lesions but also the surrounding skin to reduce the risk of AK recurrence and the likelihood of cSCC development (Willenbrink et al., 2020).

1.3.3 Pathogenetic Mechanisms of AK

The pathogenesis of AK is complex, involving a combination of molecular, biological, environmental, and immunological factors. Chronic UV exposure is the main pathogenic factor, as in cSCC. UV-A light causes indirect damage through reactive oxygen species (ROS) production, while UV-B rays directly damage DNA, contributing to lesion development. Other mechanisms involved include inflammation, oxidative stress, immunosuppression, impaired apoptosis, dysregulated cell proliferation, and tissue remodeling (Reinehr & Bakos, 2019).

UV radiation is absorbed by DNA, membrane phospholipids, and trans-urocanic acid (UCA), producing mediators such as arachidonic acid and platelet-activating factor (PAF). Inflammation is mediated via the arachidonic acid pathway and pro-inflammatory cytokine production in keratinocytes, including IL-1, IL-6, IL-8, interferon gamma (IFN- γ), granulocyte colony-stimulating factor (G-CSF), macrophage inflammatory protein beta (MIP- β), and tumor necrosis factor alpha (TNF- α), which activate the immune system and promote abnormal keratinocyte proliferation (Reinehr & Bakos, 2019). ROS generated during UV-A exposure contribute to oxidative stress, damaging DNA and altering signaling pathways such as c-Jun N-terminal kinases (JNK) and mitogen-activated protein kinases (MAPK), promoting proliferation, inflammation, and apoptosis. The inflammatory state activates transcription factors such as NF- κ B, responsible for cytokine production and promoting cell proliferation (Wang et al., 2024). Inflammation is often accompanied by immunosuppression, hindering the immune response against mutated cells and allowing lesion progression. PAF release and the conversion of trans-UCA to cis-UCA further enhance immunosuppression, compromise DNA integrity, inhibit nucleotide excision repair, and promote 8-oxo-deoxyguanosine formation, creating a tumor-promoting environment (Wang et al., 2024).

A crucial mechanism in AK pathogenesis is apoptotic dysfunction, primarily due to TP53 mutations—an early event in cutaneous tumorigenesis—that suppress programmed cell death, allowing damaged cells to survive. Loss of p53 function and reduced activity of other apoptotic factors, including Fas, Fas-ligand, and Bcl-2, prevent clearance of UV-mutated cells, favoring proliferation. UV-induced dysregulation of the cell cycle further contributes to keratinocyte expansion and AK formation (Reinehr & Bakos, 2019).

1.4 Biological mechanism of cSCC development from AK

1.4.1 Molecular Alterations

The transition from AK to cSCC is a multifaceted process shaped by genetic, cellular, and microenvironmental modifications. The immune response also plays a critical role in this progression.

cSCC exhibits the highest tumor mutational burden (TMB) of all cancers, with an average of 45.2 mutations per megabase. This exceptionally high mutational load complicates the identification of specific genetic factors capable of predicting malignant conversion from AK to cSCC (Li et al., 2025). Moreover, molecular genetic studies show that AK and cSCC share many of the same genetic alterations, which are also detectable in UV-exposed normal skin (Hosseini et al., 2024). These overlaps make it difficult to distinguish driver mutations from passenger mutations.

Although TP53 plays a central role in cSCC tumorigenesis, its mutation alone does not determine malignancy, as similar alterations are found in normal skin and AK lesions that do not always progress to cSCC.

After TP53, the second most mutated driver family comprises the NOTCH genes, particularly NOTCH1, NOTCH2, and NOTCH3. NOTCH1 expression is regulated by p53, and its mutations are believed to occur early in cSCC carcinogenesis (Hosseini et al., 2024). Genomic profiling of AK has shown frequent NOTCH1 and NOTCH2 mutations, with mutation rates increasing in parallel with UV exposure. NOTCH1 mRNA is upregulated in AK compared with normal skin, suggesting an early oncogenic role.

The CDKN2A gene, encoding the cell-cycle inhibitors p16INK4a and p14ARF, is also implicated in AK–cSCC progression. CDKN2A loss-of-function mutations arise early in cSCC development and drive uncontrolled cellular proliferation. These mutations are more frequent in cSCC (30.8%) than in AK (11.1%), while being absent in normal skin, suggesting a “gatekeeper” role in the transition to malignancy.

1.5 Tumor Microenvironment and Immune System

The development of cSCC from AK is not solely dependent on the acquisition of driver mutations in keratinocytes but is also strongly influenced by the dynamic interactions between neoplastic cells and components of TME, including those of the immune system. The increased incidence of cSCC in immunocompromised individuals underscores the fundamental role of immunosurveillance in skin carcinogenesis. However, a marked infiltration of specific inflammatory populations, such as neutrophils, macrophages, and T lymphocytes, paradoxically correlates with greater tumor aggressiveness and a higher metastatic potential. This apparent contradiction is attributable to the ability of tumor cells to remodel the surrounding microenvironment, transforming it into a niche permissive to tumor growth and capable of suppressing antitumor immune responses (Winge et al., 2023).

The cSCC TME consists of normal and malignant keratinocytes, endothelial cells, CAFs, DCs, Langerhans cells, tumor-associated macrophages (TAMs), MDSCs, NK cells, CD4+ and CD8+ T cells, and T regulatory cells (Tregs). The abundance of these populations varies between patients, indicating that the TME is dynamic and shaped by host-related factors. Their interactions can either suppress or promote tumor progression (Winge et al., 2023). A recent study defined the cellular composition and architecture of cSCC using single-cell RNA sequencing and spatial transcriptomics. This study identified four tumor keratinocyte subpopulations in cSCC. Three of these exhibit the expression of specific genes shared with normal tissues and correspond to: basal cells (characterized by the expression of the alpha chain of type XVII collagen), proliferating cells (distinguished by the expression of the proliferation marker MKI67), and differentiating cells (expressing keratin 1, KRT1). Although tumor keratinocyte subpopulations share some marker genes with their normal counterparts, they also display significant differences. Tumor basal

keratinocytes show increased expression of genes associated with type I interferon responses, glycolysis, and hypoxia, whereas tumor differentiating cells exhibit reduced expression of apoptotic genes and those involved in terminal differentiation. A fourth subpopulation was also identified that showed no similarity to counterparts in normal tissues and accounted for 2.7% to 13.8% of tumor cells; this population was termed tumor-specific keratinocytes (TSKs). These cells express genes associated with epithelial–mesenchymal transition (EMT), such as vimentin (VIM) and integrin alpha 5 (ITGA5), which promote cell migration and tumor invasiveness (Ji et al., 2020). Spatial transcriptomics revealed that TSKs reside in a fibrovascular niche at the tumor's leading edge, expressing genes involved in tumor–stroma communication, including factors linked to cancer associated fibroblasts (CAFs), melanocytes, endothelial cells, dendritic cells (DCs), Langerhans cells, myeloid-derived suppressor cells (MDSCs), macrophages, NK cells, and T-cell clusters. Crosstalk between stromal and tumor cells promotes tumor proliferation and immune evasion (Ji et al., 2020). Key tumor-promoting components in this niche include TGF- β , VEGF, IL-6, IL-10, and the chemokine CXCL12, which enhance angiogenesis and recruit immunosuppressive cells (Winge et al., 2023).

Stromal cells and the extracellular matrix (ECM) play central roles in cSCC development. Beyond structural support, the ECM regulates growth-factor secretion and contributes to neovascularization. During cSCC progression, ECM remodeling is mediated by matrix metalloproteinases (MMPs). MMP-13 correlates tumor invasiveness; MMP-9 and MMP-11 facilitate tumor migration; MMP-10 supports both ECM remodeling and angiogenesis; MMP-7 is enriched at the invasive front, implicating it in tumor progression (Hosseini et al., 2024).

CAFs significantly influence the TME by promoting inflammation, immunosuppression, and angiogenesis. They secrete VEGF and immune-modulating cytokines such as IL-6, IL-10, TGF- β , and CXCL13, which recruit suppressive immune cells and drive tumor progression. In cSCC, Martínez-Nieto et al. reported upregulation of integrin $\alpha 11 \beta 1$ in CAFs. Mouse models demonstrate that loss of $\alpha 11$ impairs CAF differentiation and function, reducing tumor growth and burden—highlighting $\alpha 11$ as a key mediator of malignant progression (Martínez-Nieto et al., 2022; Winge et al., 2023).

MDSCs are heterogeneous myeloid-derived cells with potent immunosuppressive activity. Rare in healthy individuals, they expand in cancer and chronic inflammation. In cSCC, MDSCs promote immunosuppression by producing nitric oxide (NO), which suppresses E-selectin expression on tumor vasculature, limiting T-cell infiltration (Saeidi et al., 2023).

TAMs contribute to all stages of cutaneous carcinogenesis. cSCC displays increased TAM infiltration compared with normal skin. Three types have been described: M1-like, M2-like, and polyactivated TAMs expressing both signatures. M2-like TAMs are predominant, driven by low IFN- α and TNF- β levels and activation by IL-4 from Th2 cells (Bottomley et al., 2019). These macrophages secrete IL-10 and MMPs and produce VEGF-C, promoting lymph angiogenesis and facilitating metastasis. Elevated lymphatic vessel density correlates with metastatic risk in cancers such as oral cSCC and melanoma (Saeidi et al., 2023).

The membrane glycoprotein CD200 is overexpressed in cSCC, particularly on endothelial cells, and suppresses macrophage M1 activation while promoting M2 polarization and reducing macrophage adhesion and migration (Saeidi et al., 2023).

DCs, essential antigen-presenting cells, orchestrate immune responses by presenting tumor antigens to T cells in regional lymph nodes. Langerhans cells, located in the epidermis, are the first to encounter tumor antigens and can elicit strong CD8⁺ T-cell responses. However, their numbers are significantly reduced in cSCC, and their function is impaired by the TME. UV radiation also weakens DC function through apoptosis and impaired migration, shifting immune responses toward less effective Th2 profiles and promoting Treg activity. Cytokines such as IL-10, TGF- β , and VEGF-A further suppress DC differentiation and function (Saeidi et al., 2023). Conversely, plasmacytoid DCs (pDCs), abundant in cSCC, produce large quantities of type I IFN and can cross-present antigens to CD8⁺ T cells (Saeidi et al., 2023).

Tumor-associated neutrophils (TANs) are present in both precancerous and cancerous lesions. Mouse models confirm that TAN frequency correlates with tumor size. In early stages, TANs contribute to ECM remodeling and angiogenesis; in advanced disease, they suppress immunity by inhibiting CD8⁺ T-cell function and upregulating PD-L1. TANs can polarize into antitumor N1 or protumor N2 phenotypes, with TGF- β driving N2 polarization. Clinically, elevated circulating neutrophils correlate with high-risk cSCC and poor prognosis (Chiang et al., 2023). TAN-mediated immunosuppression involves arginine depletion and PD-L1/PD-1 signaling. Neutrophil depletion *in vivo* enhances CD8⁺ T-cell proliferation and IFN- γ levels, underscoring their immunosuppressive role (Hosseini et al., 2024).

NK cells also suppress cSCC, but their cytotoxicity can be impaired by the TME. Reduced NK-cell function or numbers increase cSCC risk. Mouse studies demonstrate that NK deficiency accelerates tumor formation, whereas NK-cell therapy reduces tumor burden, although NK cells isolated from tumors show diminished cytotoxicity and IFN- γ production (Hosseini et al., 2024).

T-cell infiltration is a hallmark of cSCC, yet their antitumor activity is compromised. T regulatory cells (Tregs), marked by FOXP3, represent over 50% of tumor-infiltrating T cells—far more than in healthy skin—and strongly contribute to immune evasion. Their numbers are higher in moderately and poorly differentiated cSCC and correlate with worse prognosis (Chiang et al., 2023). A 2017 study by de Oliveira et al. showed that Treg recruitment into cSCC depends on CCR5, which binds CCL3, CCL4, and CCL5 produced by infiltrating MDSCs. CCR5-deficient mice develop fewer tumors and exhibit a Th1-skewed immune response. Conversely, adoptive transfer of CCR5⁺ Tregs increases cSCC formation and reduces NK-cell infiltration (de Oliveira et al., 2017). Cytotoxic CD8⁺ T cells are essential for tumor elimination through IFN- γ production but tend to accumulate in peritumoral rather than intratumoral regions, limiting their efficacy.

To further clarify the contribution of TME and the immune system to the transition from AK to cSCC, a recent study by Zou et al. used single-cell RNA sequencing (scRNA-seq) to map the progression from normal skin to AK, cSCC *in situ*, and ultimately invasive cSCC (Zou et al., 2023). This high-resolution approach allowed the authors to identify key mechanisms involved in carcinogenesis by deeply characterizing the TME and its interactions with keratinocytes. The study identified eleven lymphocyte subpopulations, including CD4⁺ T cells (CD4T), regulatory T cells (Treg), naïve CD4⁺ T cells (CD4T_{naïve}), naïve CD8⁺ T cells (CD8T_{naïve}), effector CD8⁺ T cells (CD8T_{eff}), exhausted CD8⁺ T cells (CD8T_{ex}), natural killer T cells (NKT), proliferating T cells, double-negative naïve T cells (DNT), B cells, and plasma cells. In poorly differentiated cSCC—the most aggressive form—the CD8T_{ex} subset, which is characterized by limited or absent effector activity, was significantly enriched. Their abundance suggests that the TME of highly aggressive

tumors may promote an immunosuppressive state, weakening the ability of the immune system to control tumor growth. Conversely, the observed reduction of naïve CD4 T cells—a helper T-cell population that has not yet encountered its antigen—as malignancy increases indicates a diminishing capacity to generate effective antitumor immune responses. This contributes to a progressively impaired immune landscape that facilitates tumor progression (Zou et al., 2023).

An important component of the study focused on intercellular communication within the TME, which enabled the identification of key pathways involved in tumor progression. The interactions between TME cells and keratinocytes—particularly basal keratinocytes and Langerhans cells—were significantly stronger than in normal skin. This enhanced communication suggests that these cellular populations are central mediators of TME signaling in cSCC.

Finally, the study identified several dysregulated signaling pathways in invasive cSCC, including MHC-II, laminin, and Tumor Necrosis Factor (TNF), all potentially implicated in tumor development. Basal tumor keratinocytes displayed aberrant expression of MHC-II molecules, promoting Treg differentiation and contributing to immune evasion. Laminin, a key component of the extracellular matrix and basement membrane, regulates keratinocyte morphology and motility; its hyperactivation in cSCC keratinocytes enhances invasiveness and metastatic potential. TNF plays a dual role in cancer, as it can support antitumor immunity but also promote tumor progression by inducing inflammatory responses, stimulating cell proliferation, and regulating the development of Treg and myeloid-derived suppressor cells (Zou et al., 2023).

2. RATIONALE AND AIM

The cSCC represents one of the most common NMSC and constitutes a growing public health challenge due to its increasing incidence, biological heterogeneity, and potential for aggressive clinical behavior. Although the accumulation of ultraviolet (UV)-induced genetic alterations in epidermal keratinocytes is a well-established driver of cSCC initiation, genetic damage alone is insufficient to fully explain disease progression, invasion, and metastatic dissemination.

Increasing evidence indicates that TME, and its immune and inflammatory components, plays a central and active role in shaping the trajectory of cutaneous carcinogenesis. Chronic UV exposure not only induces DNA damage but also profoundly alters local immune surveillance, promoting a state of persistent inflammation and immunological dysregulation. This condition is particularly evident within the so-called *cancerization field*, a clinically inconspicuous yet genetically and biologically altered skin area that provides fertile ground for the development of AKs and their progression toward invasive cSCC. While AK is widely recognized as the principal precursor lesion of cSCC, the molecular and immunological mechanisms governing the transition from field cancerization to AK and ultimately to invasive carcinoma remain incompletely defined. Recent advances in transcriptomic technologies have revealed that the progression along the AK–cSCC is accompanied by dynamic remodeling of the immune microenvironment rather than by a linear accumulation of genetic events. Shifts in immune cell composition, activation states, cytokine signaling, and immune regulatory pathways appear to critically influence whether premalignant lesions remain stable, regress, or progress to invasive disease. Among these pathways, inflammatory and interferon-related programs, macrophage polarization, immune checkpoint signaling, and T-cell functional states have emerged as key determinants of tumor behavior.

On the basis of this rationale, the present doctoral thesis aims to investigate the progressive, condition-specific immune-inflammatory microenvironment during the pathogenesis of cSCC from precursor lesions. Understanding these immune dynamics is essential for identifying early events associated with malignant transformation and for uncovering potential biomarkers and therapeutic targets aimed at intercepting disease progression at a pre-invasive stage.

The primary objective of this study is to characterize the immune-inflammatory microenvironment underlying the progression from field cancerization (CC) and AK to cSCC. The expected outcome is to define the transcriptional immune landscape associated with CC, AK and cSCC by RNA sequencing and identify differentially expressed immune-related genes and pathways that distinguish pre-neoplastic lesions from cSCC, with particular emphasis on inflammatory, interferon-related, cytotoxic, antigen-presentation, and immune checkpoint-associated signatures

3. MATERIALS AND METHODS

3.1 Patient Recruitment

Patients with cSCC were recruited from the Dermato-Oncology Unit of the University of L'Aquila between November 2022 and May 2024. Eligibility criteria included a clinical and histological diagnosis of cSCC and consent to participate in the study. For each patient, three fresh biopsies or FFPE samples were collected from the CC, AK, and cSCC and preserved in RNA later (Invitrogen). Ethical approval for the study was obtained from the IRB Ethics Committee, and the research was conducted in accordance with the relevant guidelines and regulations outlined in the Declaration of Helsinki.

3.2 RNA Extraction and Sequencing

Total RNA was extracted from tissue using the AllPrep DNA/RNA/miRNA Universal Kit (QIAGEN) following a homogenization cycle with Precellys 24 (Bertin). RNA was reverse transcribed into cDNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) and subsequently sequenced using NGS techniques. Sequencing was performed using Ion Torrent™ NGS technology with the Ion Oncomine Immune Response Research Assay on the GeneStudio S5™ System platform, Thermo Fisher Scientific. This approach generates DNA sequences, referred to as “reads,” which are then mapped to the reference genome or transcriptome to identify the genes expressed in each sample under investigation. In this study, the amplicon libraries were prepared using IonChef (Thermo Fisher). The Ion Oncomine Immune Response Research Assay panel allow the amplification and sequencing of 395 genes related to immune system (see Supplementary material, page 37).

3.3 Bioinformatic and Statistical Analysis

Reads were aligned to the human genome (Ensembl GRCh38) using Torrent Suite™ 5.8.1 software. The Coverage Analysis plugin was used to generate amplicon counts, which were imported into the Transcriptome Analysis Console (TAC) software, applying a cut-off > 65%. Differentially expressed genes between “cSCC vs AK” and “AK vs CC” were determined based on adjusted p-values ($p < 0.05$) and log2 fold change (≥ 1). PCA analysis and Volcano plots were generated using TAC (Thermo Fisher) after loading the CHP files of each analyzed sample. Two

types of analyses were performed: gene family-based analyses and cell-specific signature analyses. All analyses were conducted using RStudio.

The analyses of the data obtained from the sequencing of fresh samples and those obtained from FFPE samples were analyzed separately and subsequently compared to verify that the same results were obtained regardless of specimen.

4. RESULTS

For this study, a total of 26 patients with a diagnosis of cutaneous squamous cell carcinoma (cSCC) were recruited (Table 1). The study population consisted of 17 male and 9 female patients. The mean age at diagnosis was 83,46 years (range 64–94 years).

Overall, 26 samples of CC (21 fresh biopsies and 5 FFPE specimens), 19 samples of AK (14 fresh biopsies and 5 FFPE samples) and 26 cSCC (21 fresh biopsies and 5 FFPE samples) were collected from the same patients (Table 1).

Table 1: Demographic characteristics of the study cohort. The table summarizes patient ID, sex, age at diagnosis, and the availability of tissue samples.

Patient ID	Sex	Age at diagnosis	CC sample	AK sample	cSCC samples	Type of sample
JSCC02	F	91	✓		✓	FB
JSCC03	M	84	✓		✓	FB
JSCC04	M	90	✓		✓	FB
JSCC05	M	85	✓		✓	FB
JSCC06	M	69	✓		✓	FB
JSCC12	F	86	✓		✓	FB
JSCC16	M	91	✓		✓	FB
JSCC17	M	64	✓	✓	✓	FB
JSCC21	F	81	✓	✓	✓	FB
JSCC27	M	88	✓	✓	✓	FB
JSCC28	M	89	✓	✓	✓	FB
JSCC29	M	94	✓	✓	✓	FB
JSCC30	M	73	✓	✓	✓	FB
JSCC31	M	74	✓	✓	✓	FB
JSCC32	M	89	✓	✓	✓	FB
JSCC33	M	75	✓	✓	✓	FB
JSCC34	M	81	✓	✓	✓	FB
JSCC35	F	86	✓	✓	✓	FB
JSCC36	F	85	✓	✓	✓	FB
JSCC37	M	87	✓	✓	✓	FB
JSCC38	F	81	✓	✓	✓	FB
FFPE 1	F	78	✓	✓	✓	FFPE
FFPE 2	M	88	✓	✓	✓	FFPE
FFPE 3	F	83	✓	✓	✓	FFPE
FFPE 4	M	90	✓	✓	✓	FFPE
FFPE 5	F	88	✓	✓	✓	FFPE

AK, Actinic keratosis; CC, Cancerization Field; cSCC, cutaneous squamous cell carcinoma; FB, fresh biopsy; FFPE, formalin fixed samples.

In this Results Section, findings derived from biopsy specimens and FFPE samples are presented separately. Specifically, all results obtained from biopsy samples are reported first, followed by those derived from FFPE specimens.

Overall, 56 fresh samples were sequenced, including 21 CC, 14 AK, and 21 cSCC.

Principal Component Analysis (PCA) showed the distribution of the samples based on the expression of genes analyzed, demonstrating a clear separation between AK, CC, and cSCC (Figure 6).

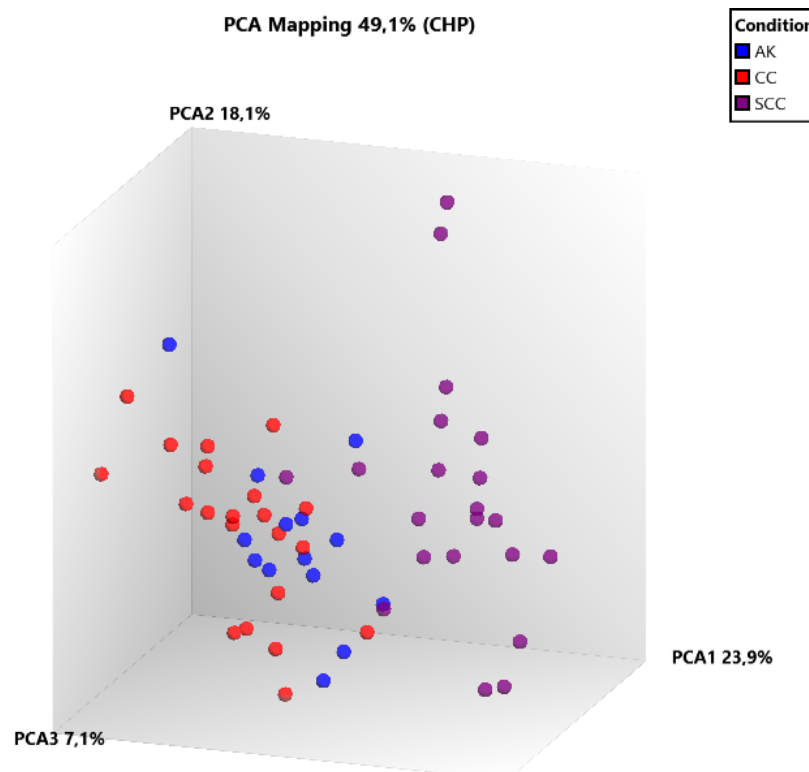


Figure 6: Three-dimensional graph resulting from the PCA analysis of fresh samples showing the spatial distribution of the three conditions: AK, CC and cSCC.

The first three principal components accounted for 49.1% of the total variance (PCA1: 23.9%, PCA2: 18.1%, PCA3: 7.1%). cSCC samples formed a clearly separated cluster, primarily along PCA1, whereas AK and CC samples were distributed closer together with partial overlap. This spatial arrangement indicates a greater similarity between AK and CC profiles, while cSCC exhibits a markedly different multivariate pattern.

Differential expression analysis allowed the identification of significantly deregulated genes. To further characterize the magnitude and statistical significance of the observed transcriptional changes, differential expression results were visualized using a Volcano plot made by a fold change versus p-value distribution (Figure 7).

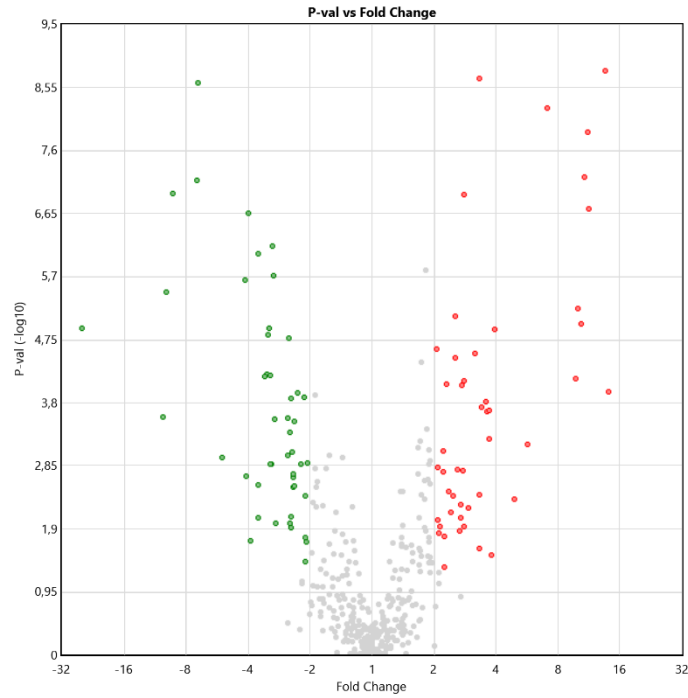


Figure 7: Volcano Plot of fresh derived samples, cSCC vs AK: Significantly up-regulated genes with a log2 fold change ≥ 1 are highlighted in red, while down-regulated genes with a log2 fold change ≤ -1 are highlighted in green. Gray dots represent genes with no significant changes in expression. Dots located at the top of the graph correspond to higher P-values, indicating stronger statistical evidence of expression differences compared to those located at the bottom.

The fold change versus p-value plot revealed a clear stratification of differentially expressed genes according to both effect size and statistical significance. A distinct subset of genes exhibited significant upregulation, characterized by positive fold change values and high $-\log_{10}(\text{p-value})$ scores, indicating strong statistical support. Conversely, a separate group of genes showed significant downregulation, with negative fold change values and similarly elevated significance levels. The majority of transcripts clustered around low fold change values and low $-\log_{10}(\text{p-value})$ scores, indicating limited or non-significant differential expressions. Overall, this distribution highlights the presence of a defined subset of genes with robust and statistically significant expression changes, contrasted against a broader background of minimally altered transcripts.

In line with the separation observed in the PCA analysis, differential gene expression profiling further quantified the extent of transcriptional divergence among the three conditions (Figure 8). The comparison between AK and CC identified a limited number of significant differentially expressed genes (DEGs), with a total of 10 genes, including 6 upregulated and 4 downregulated transcripts. In contrast, a substantially higher number of DEGs was observed when comparing cSCC vs AK, with 94 genes identified, evenly distributed between upregulated (47) and downregulated (47) genes. The largest number of DEGs was detected in the CC versus cSCC comparison, comprising 134 genes, of which 70 were upregulated and 64 downregulated.

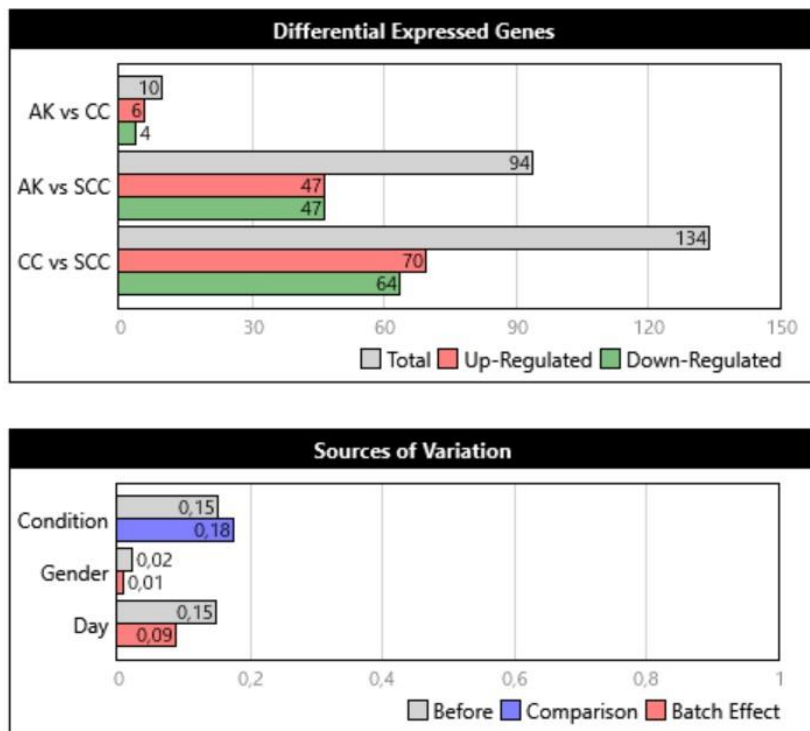


Figure 8: Differentially expressed genes in the three conditions in fresh samples: CC, AK and cSCC.

The top ten most upregulated and the top ten most downregulated genes are presented in the following tables (Table 2, Table 3). For each gene, fold change, p-value, FDR-adjusted p-value, and a functional description are reported. Positive and negative fold change values indicate gene upregulation and downregulation, respectively.

Table 2: Top 10 upregulated genes identified by differential expression analysis in cSCC vs AK, and their function in the immune inflammatory microenvironment

GENE	FUNCTION	FOLD CHANGE	P-VALUE	FDR P-VAL
<i>CXCL8</i>	Encodes a CXC chemokine that plays a key role in mediating the inflammatory response.	25,59	$1,18 \times 10^{-5}$	$2,00 \times 10^{-4}$
<i>IL6</i>	Encodes a cytokine involved in inflammatory processes and B-cell maturation.	10,36	$3,00 \times 10^{-4}$	$2,20 \times 10^{-3}$
<i>CXCL1</i>	Encodes a CXC chemokine with antimicrobial activity involved in inflammation and neutrophil chemoattraction.	9,98	$3,42 \times 10^{-6}$	$8,01 \times 10^{-5}$
<i>TDO2</i>	Encodes a heme enzyme that catalyzes the first and rate-limiting step of tryptophan metabolism via the kynurenine pathway.	9,32	$1,10 \times 10^{-7}$	$4,99 \times 10^{-6}$
<i>CCL3</i>	Encodes a small inducible cytokine, also known as macrophage inflammatory protein 1 alpha, involved in inflammatory responses.	7,1	$7,04 \times 10^{-8}$	$4,00 \times 10^{-6}$
<i>ISG15</i>	Encodes a ubiquitin-like protein that is conjugated to intracellular targets in response to interferon-alpha and interferon-beta activation.	6,97	$2,37 \times 10^{-9}$	$3,14 \times 10^{-7}$
<i>CCL18</i>	Encodes a cytokine with chemotactic activity for naïve, CD4+ and CD8+ T cells and nonactivated lymphocytes, but not for monocytes or granulocytes.	5,35	$1,00 \times 10^{-3}$	$6,70 \times 10^{-3}$
<i>CCL4</i>	Encodes a mitogen-inducible monokine that acts as a major HIV-suppressive factor produced by CD8+ T cells.	4,13	$2,21 \times 10^{-6}$	$5,50 \times 10^{-5}$
<i>CXCL13</i>	Encodes a CXC chemokine that selectively attracts B-1 and B-2 cells through interaction with the CXCR5 receptor.	4,11	$2,00 \times 10^{-3}$	$1,03 \times 10^{-2}$
<i>IFI6</i>	Encodes a protein that may play a critical role in the regulation of apoptosis.	3,97	$2,19 \times 10^{-7}$	$7,91 \times 10^{-6}$

Table 3: Top ten downregulated genes identified by differential expression analysis in cSCC vs AK, and their function in the immune inflammatory microenvironment.

GENE	FUNCTION	FOLD CHANGE	P-VALUE	FDR P-VAL
<i>ALOX15B</i>	Encodes a tumor suppressor whose downregulation promotes tumor growth, invasion, and therapy resistance, partly through regulation of ferroptosis.	-14,09	1,00x10 ⁻⁴	1,20x10 ⁻³
<i>DGAT2</i>	Encodes a regulator of lipid metabolism and lipid droplet formation with context-dependent roles in cancer, promoting or inhibiting tumor growth and therapy resistance.	-13,6	1,57x10 ⁻⁹	3,14x10 ⁻⁷
<i>KRT7</i>	Encodes a protein that promotes tumor progression, metastasis, and chemoresistance by regulating cell structure, signaling pathways, and the tumor immune microenvironment.	-11,32	1,92x10 ⁻⁷	7,63x10 ⁻⁶
<i>RORC</i>	Encodes the transcription factor ROR γ , which regulates Th17 cell differentiation and immune responses, with roles in circadian regulation and disease.	-11,22	1,33x10 ⁻⁸	1,06x10 ⁻⁶
<i>EGR2</i>	Encodes a transcription factor with context-dependent roles in cancer, acting as a tumor suppressor or promoter by regulating apoptosis, immune cell function, and cell differentiation.	-10,76	6,22x10 ⁻⁸	4,00x10 ⁻⁶
<i>VTCN1</i>	Encodes a B7 family costimulatory protein expressed on antigen-presenting cells that interacts with T-cell receptors and is associated with tumor progression.	-10,39	1,02x10 ⁻⁵	2,00x10 ⁻⁴
<i>CA4</i>	Encodes a carbonic anhydrase that functions as a tumor suppressor, inhibiting cell proliferation and cancer progression, partly through modulation of Wnt/ β -catenin signaling.	-10,05	5,97x10 ⁻⁶	1,00x10 ⁻⁴
<i>ARG1</i>	Encodes an enzyme that modulates immune responses by regulating arginine availability, T-cell activity, and inflammation.	-9,78	6,77x10 ⁻⁵	8,00x10 ⁻⁴
<i>EGR3</i>	Encodes an EGR family C2H2-type zinc-finger transcriptional regulator involved in muscle, lymphocyte, endothelial, and neuronal development.	-7,11	5,72x10 ⁻⁹	5,70x10 ⁻⁷
<i>MLANA</i>	Encodes a protein localized to the endoplasmic reticulum membrane, melanosomes, and the trans-Golgi network.	-5,66	7,00x10 ⁻⁴	4,90x10 ⁻³

Further, we performed the gene family's enrichment analysis. Immune gene families refer to groups of genes that share related biological functions or participate in common immunological processes, such as antigen presentation, cytotoxic activity, or interferon signaling. These families

are defined based on functional annotation and pathway involvement, and their aggregated expression provides insight into the activity of specific immune mechanisms within a tissue.

Immune gene family scores were compared between cSCC and AK to characterize changes in the immune microenvironment associated with disease progression. Comparative analysis of immune-related families of genes revealed distinct transcriptional profiles between cSCC and AK samples (Figure 9). Antigen presentation–related signatures were significantly higher in AK compared with cSCC ($p = 0.0108$), indicating a relative enrichment of antigen-presenting pathways in pre-neoplastic lesions. Furthermore, T cell–associated gene families were significantly higher in AK compared with cSCC ($p = 0.0055$), indicating a relative enrichment of T cell–related immune activity in pre-neoplastic lesions.

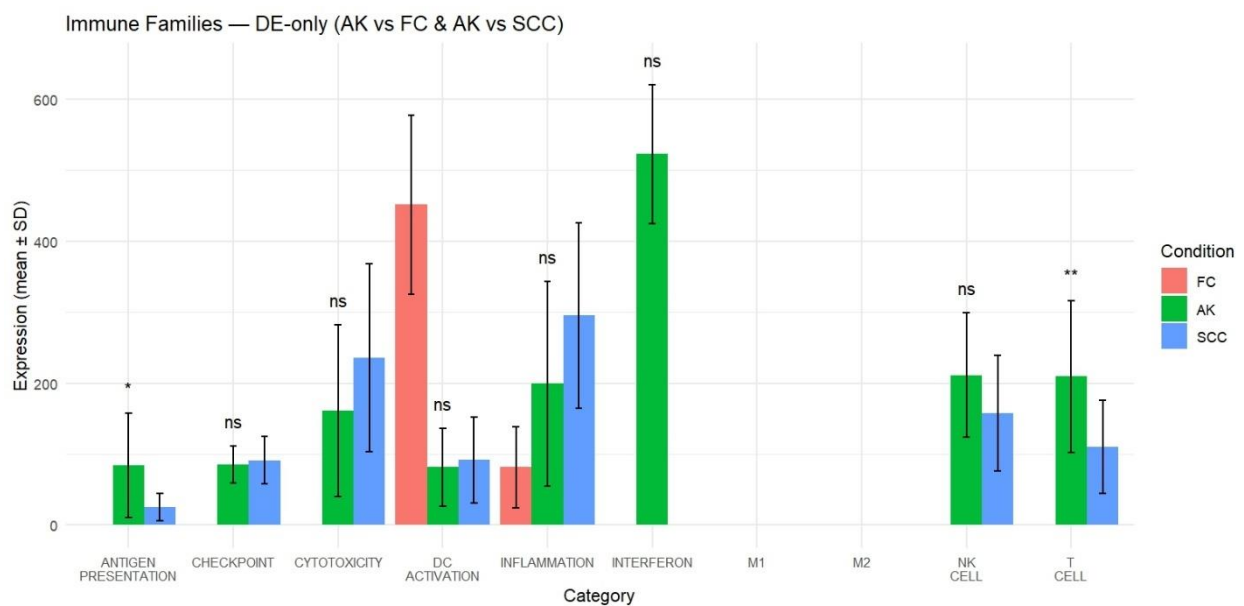


Figure 9: Immune gene families scores in cancerization field (CC), actinic keratosis (AK) and squamous cell carcinoma (cSCC) derived from fresh samples.

Other immune pathways displayed non-significant trends between conditions. Cytotoxicity-associated gene family showed higher mean expression in cSCC compared with AK, approaching but not reaching statistical significance ($p = 0.095$). Similarly, NK cell–related signatures tended to be higher in AK than in cSCC ($p = 0.081$) but not reaching statistical significance, while inflammatory pathway–associated scores were modestly elevated in cSCC ($p = 0.0549$). No statistically significant differences were observed for interferon-related gene families ($p = 0.473$) or immune checkpoint–associated ($p = 0.535$), indicating comparable expression levels between AK and cSCC for these pathways. Moreover, analysis of myeloid cell–associated signatures revealed no significant differences in dendritic cell activation ($p = 0.614$), nor in macrophage polarization signatures, including M1 ($p = 0.491$) and M2 ($p = 0.288$), between the two conditions. Overall, these results indicate selective immune remodeling during progression from AK to cSCC, characterized by a significant reduction in antigen presentation and a concomitant decrease in T cell–associated gene family, while other immune-related pathways exhibit trends without reaching statistical significance. Immune gene family scores were also compared between CC and AK to identify early immune alterations associated with pre-neoplastic transformation (Figure 9). Dendritic cell activation–related signatures were significantly increased in CC compared with AK

($p = 0.00042$), indicating a marked reduction in dendritic cell–associated immune activity already at the AK stage.

In contrast, inflammatory pathway–associated scores tended to be higher in AK relative to CC; although this difference did not reach statistical significance ($p = 0.0639$), suggesting an early but variable increase in inflammatory signaling during lesion development.

No other immune gene family categories showed significant differences between CC and AK, indicating that most immune-related alterations become more pronounced at later stages of disease progression.

After characterizing the gene expression behavior of immune gene families in cSCC vs AK, we further evaluated immune cell-specific pathways within cSCC vs AK comparison. Immune signatures or immune cell-specific pathways represent composite expression patterns derived from a predefined set of genes whose coordinated expression characterizes a particular immune state, cell population, or functional response. Immune signatures are often empirically defined and may integrate genes from multiple functional families, serving as higher-level biomarkers of immune infiltration, activation, or suppression.

Immune cell-specific pathways scores were compared between cSCC and AK to assess changes in adaptive and innate immune programs associated with malignant progression. Among T cell–related pathways, T cell–associated signatures were significantly higher in AK compared with cSCC ($p = 0.0055$), indicating a relative enrichment of T cell–related immune activity in pre-neoplastic lesions. In parallel, regulatory T cell (Treg) pathways were significantly higher in cSCC ($p = 0.0016$), suggesting increased immunoregulatory mechanisms in advanced disease (Figure 10).

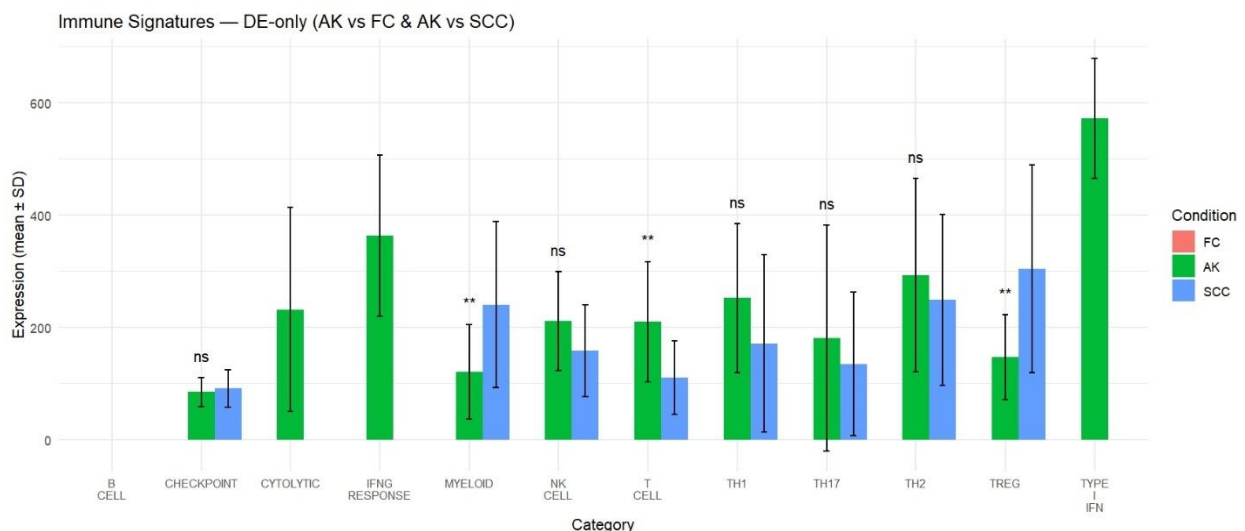


Figure 10: Immune cell–specific pathways in cancerization field (CC), actinic keratosis (AK) and squamous cell carcinoma (cSCC) derived from fresh samples.

Myeloid-associated signatures showed significantly higher expression in cSCC ($p = 0.0046$), indicating an overall amplification of myeloid cell–related immune components. Other immune pathways exhibited non-significant differences between conditions. For examples, NK cell–associated signatures tended to be higher in AK compared with cSCC, although this difference did

not reach statistical significance ($p = 0.081$). Type I interferon signatures also showed a trend toward higher expression in AK ($p = 0.0928$), while IFN γ response-associated signatures did not differ significantly between groups ($p = 0.166$). Helper T cell polarization signatures, including Th1, Th2, and Th17, showed no statistically significant differences between AK and cSCC ($p = 0.112$, $p = 0.444$, and $p = 0.452$, respectively). Likewise, immune checkpoint-associated signatures were comparable between conditions ($p = 0.535$).

Overall, these results indicate that progression from AK to cSCC is associated with a selective remodeling of immune cell-specific pathways, characterized by a reduction in T cell-associated signatures and a concomitant increase in regulatory T cell and myeloid-associated signatures in cSCC; while interferon-related and helper T cell polarization pathways remain largely unchanged or show non-significant trends.

Following the expansion of the cohort through the inclusion of FFPE-derived samples, PCA was performed to assess whether sample type influenced the overall transcriptional clustering across conditions (Figure 11).

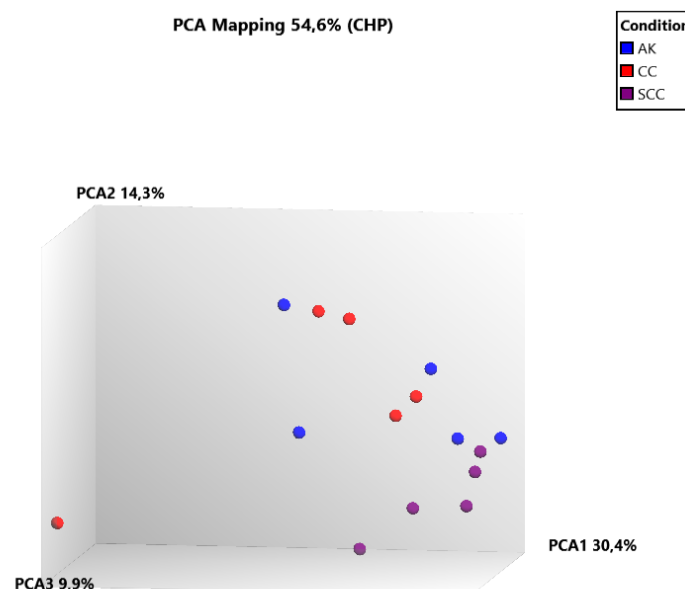


Figure 11: Three-dimensional graph resulting from the PCA analysis of the FFPE-derived samples showing the spatial distribution of the three conditions: AK, CC and cSCC.

PCA of the expanded dataset showed that the first three principal components accounted for 54.6% of the total variance (PCA1: 30.4%, PCA2: 14.3%, PCA3: 9.9%) (Figure 11). Samples continued to display condition-dependent clustering, with cSCC samples grouping separately from AK and CC along PCA1. AK and CC samples were distributed closer to each other in the multivariate space, showing partial overlaps across PCA2 and PCA3. Importantly, the FFPE-derived samples did not disrupt the overall separation pattern observed between conditions, indicating that the primary variance structure was driven by biological differences rather than only sample processing.

Notably, the clustering pattern observed in the FFPE-expanded cohort closely mirrored that of the fresh biopsy dataset, indicating consistency in condition-driven variance across sample types.

To evaluate the distribution of effect size and statistical significance within the FFPE-derived dataset, differential expression results were further visualized using a Volcano plot made by a fold change versus p-value plot (Figure 12).

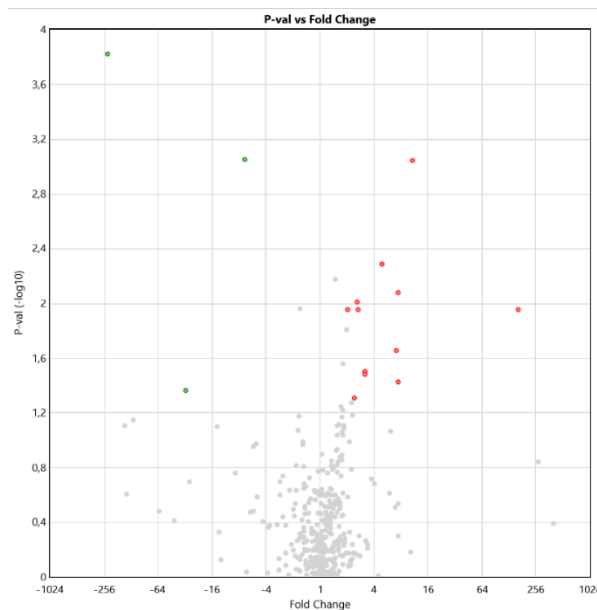


Figure 12: Volcano Plot of FFPE-derived samples, cSCC vs AK: Significantly up-regulated genes with a log2 fold change ≥ 1 are highlighted in red, while down-regulated genes with a log2 fold change ≤ -1 are highlighted in green. Gray dots represent genes with no significant changes in expression. Dots located at the top of the graph correspond to higher P-values, indicating stronger statistical evidence of expression differences compared to those located at the bottom.

The fold change versus p-value distribution in the FFPE-derived cohort showed a limited number of genes exhibiting both substantial fold changes and high statistical significance. A small subset of transcripts displayed significant upregulation, characterized by positive fold change values and elevated $-\log_{10}(\text{p-value})$ scores, while very few genes showed significant downregulation with negative fold change values (Figure 12). Many genes clustered around low fold change values and low significance levels, indicating minimal differential expression. Overall, this pattern reflects a reduced dynamic range and lower sensitivity in the FFPE-derived dataset compared with fresh biopsy samples, while still preserving a defined subset of statistically supported transcriptional changes.

Compared with the fresh biopsy dataset, the FFPE derived volcano plot showed a reduced number of genes reaching high statistical significance (Figure 12); however, the overall distribution of fold change and significance remained consistent between the two datasets.

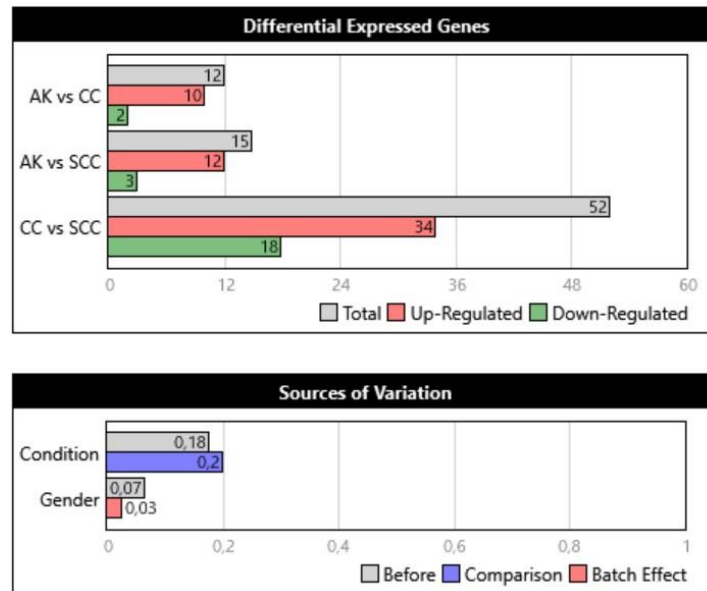


Figure 13: Differentially expressed genes in the three conditions of FFPE-derived samples: CS, AK and cSCC

Differential expression analysis of the FFPE-derived cohort identified a limited number of differentially expressed genes across all pairwise comparisons. The comparison between AK and CC yielded 12 differentially expressed genes, the majority of which were significantly upregulated (10 upregulated and 2 downregulated) (Figure 13). Similarly, the cSCC vs AK comparison identified 15 differentially expressed genes, including 12 upregulated and 3 downregulated transcripts. The largest number of differentially expressed genes was observed in the CC versus cSCC comparison, with a total of 52 genes, comprising 34 upregulated and 18 downregulated genes.

Although the absolute number of differentially expressed genes was reduced in the FFPE-derived cohort, differential expression in the two comparisons remained consistent with that observed in fresh biopsy specimens alone.

5. DISCUSSION

The present doctoral work provides an immune characterization of the transition from AK to cSCC, focusing on the dynamic transcriptional changes in immune-related genes that accompany early malignant progression. Together, these findings reinforce the concept that progression from AK to cSCC is an active, biologically regulated process, driven not only by genetic alterations but also by a profound reorganization of the immune–inflammatory microenvironment, as reflected by the extensive reprogramming of inflammatory and immune-related gene expression.

The comparative transcriptomic analysis revealed a robust upregulation of chemokines and cytokines, including *CXCL8*, *CXCL1*, *CXCL13*, *IL6*, *CCL3*, *CCL4*, and *CCL18*, highlighting the establishment of a strongly pro-inflammatory microenvironment in cSCC relative to AK. These chemokines and cytokines represent central regulators of the inflammatory cascade, orchestrating leukocyte trafficking (*CXCL8*, *CXCL1*, *CCL3*, *CCL4*), B-cell recruitment and organization within tertiary lymphoid structures (*CXCL13*), macrophage polarization and tissue remodeling (*CCL18*), and the amplification of pro-tumorigenic inflammatory and survival pathways (*IL-6*), collectively fostering a microenvironment permissive to cSCC development and progression.

Their coordinated overexpression suggests that inflammation plays a permissive and potentially driving role in malignant transformation, supporting the concept of inflammation-associated carcinogenesis in chronically sun-damaged skin. This interpretation is consistent with previous evidence demonstrating enhanced lymph angiogenesis and inflammatory signaling within the cSCC microenvironment (Moussai et al., 2011).

In parallel, the upregulation of genes involved in interferon signaling and innate immune responses, such as *ISG15* and *IFI6*, further indicates sustained immune activation during tumor progression. *ISG15* encodes a ubiquitin-like modifier that is rapidly induced in response to interferon signaling and plays a central role in antiviral defense, modulation of protein stability, and regulation of innate immune responses (Perng & Lenschow, 2018). Similarly, *IFI6* is an interferon-stimulated gene involved in the regulation of apoptosis and cellular stress responses and has been associated with interferon-mediated cell survival mechanisms, particularly in epithelial and cancer cells exposed to chronic inflammatory stimuli (Cheriyath et al., 2007). However, this inflammatory milieu appears to be functionally ambivalent. While initially reflecting immune recognition of dysplastic and malignant keratinocytes, it may ultimately favor tumor growth by promoting tissue remodeling, immune cell polarization, and the release of protumorigenic mediators. Importantly, the upregulation of *ISG15* and *IFI6* does not necessarily imply an intrinsically ambivalent immune response. Rather, their expression likely reflects a persistent interferon-rich inflammatory environment, indicative of ongoing immune activation. Chronic activation of interferon signaling has been shown to exert context-dependent effects, potentially contributing to immune exhaustion, altered immune cell polarization, and selection of tumor cell populations with increased resistance to immune-mediated clearance (Benci et al., 2016; Snell et al., 2017). In this context, the observed upregulation of *TDO2* in our study, a key enzyme of the kynurenine pathway, is particularly relevant. By modulating tryptophan metabolism, *TDO2* has been implicated in immune tolerance and suppression of antitumor T-cell responses (Platten et al., 2019). This suggests a metabolic mechanism through which cSCC may evade immune surveillance despite the presence of an inflammatory microenvironment.

Equally informative are the genes found to be downregulated during the transition from AK to cSCC. The reduced expression of transcription factors such as *EGR2* and *EGR3*, as well as immune-regulatory genes including *RORC* and *ARG1*, points to a progressive loss of immune homeostasis and regulatory control. Notably, *RORC* plays a critical role in Th17 cell differentiation and immune balance, while *ARG1* is involved in arginine metabolism and immune modulation. *EGR2* and *EGR3* are zinc-finger transcription factors known to be critically involved in the maintenance of immune tolerance, particularly through the regulation of T-cell anergy, regulatory T-cell function, and anti-inflammatory signaling pathways. Their reduced expression has been associated with impaired negative feedback mechanisms in T-cell activation and a breakdown of peripheral immune regulation, potentially contributing to chronic inflammation and tumor-promoting immune escape. Their downregulation may reflect a shift toward an immune contexture that favors tumor persistence rather than effective antitumor immunity. Moreover, the decreased expression of *VTCN1*, a member of the B7 family involved in T-cell costimulation, further underscores the complex immune dysregulation characterizing early cSCC. These findings align with immunohistochemical evidence showing progressive immune checkpoint activation and immune exhaustion during the evolution from AK to invasive disease (Stravodimou et al., 2021).

Taken together, these transcriptional changes delineate a tumor microenvironment that is simultaneously inflamed and immunologically compromised. This duality is consistent with previous histopathological, immunological, and spatial transcriptomic studies indicating that immune activation and immune escape coexist toggle concurrently during cutaneous squamous carcinogenesis. In particular, spatial transcriptomic analyses have demonstrated that invasive cSCC is characterized by distinctive gene expression programs not only within tumor cells but also across immune and stromal compartments, highlighting the critical role of microenvironmental remodeling in early invasion (Gim et al., 2025). Importantly, the present work extends these observations by demonstrating that immune dysregulation is already molecularly established at the early invasive stage, reinforcing the notion that immune escape mechanisms are activated early in cSCC development.

Several immunohistochemical and transcriptomic studies have characterized the immune cell composition of the cSCC microenvironment, consistently describing an enrichment of innate immune cells and immunosuppressive myeloid populations alongside dysfunctional adaptive immunity. In particular, tumor-associated macrophages (TAMs) represent one of the most abundant leukocyte populations in cSCC, often displaying heterogeneous activation states and contributing to extracellular matrix remodeling, angiogenesis, and immune suppression (Pettersen et al., 2011; Bottomley et al., 2019). The prominent upregulation of macrophage-associated chemokines observed in our transcriptomic analysis, including *CCL3*, *CCL4*, and *CCL18*, is highly consistent with these observations. Notably, *CCL18* has been associated with alternatively activated macrophages and tissue remodeling functions in cSCC, supporting a macrophage-driven inflammatory microenvironment already at early invasive stages (Pettersen et al., 2011).

Neutrophil recruitment and activation have also been implicated in cSCC progression, with *CXCL8* and *CXCL1* acting as key mediators of neutrophil chemotaxis and amplification of inflammatory signaling (Bottomley et al., 2019; Saeidi et al., 2023). The strong upregulation of these chemokines in our dataset suggests an active contribution of neutrophil-associated

inflammatory pathways, in line with histopathological evidence of neutrophil infiltration in invasive lesions.

In addition to myeloid cells, cSCC has been shown to harbor functionally impaired antigen-presenting cells. Although dendritic cells are present within and around tumor nests, they often exhibit reduced T-cell stimulatory capacity and a tolerogenic phenotype, contributing to ineffective antitumor immunity (Bluth et al., 2009; Bottomley et al., 2019). The inflammatory yet immunosuppressive transcriptional profile observed in our study is therefore consistent with a tumor microenvironment in which immune activation coexists with immune dysfunction.

Another key contribution of this study lies in its support of the “field cancerization” concept at the molecular level. The differential expression patterns identified between AK and CC indicate that malignant progression is driven by broader alterations in the surrounding tissue environment. Chronic ultraviolet exposure appears to prime the epidermis and dermis for carcinogenesis by inducing persistent inflammatory signaling, stromal activation, and immune remodeling. Within this altered field, specific molecular events may then tip the balance toward invasion and malignant behavior, in line with microenvironment-focused models of cSCC progression described in the literature (Gim et al., 2025; Moussai et al., 2011).

Substantial evidence indicates that AK is not merely a genetically altered precursor lesion, but an immunologically “visible” state in which dysplastic keratinocytes can still elicit an active immune surveillance response. Reviews of cSCC immunobiology describe AK and early keratinocytic neoplasia as settings in which antigen-presenting cells and effector T cells contribute to recognition and, in a subset of cases, immune-mediated regression of premalignant clones (Bottomley et al., 2019; Saeidi et al., 2023).

Consistent with this framework, our immune gene family analysis shows that antigen presentation–related signatures are significantly higher in AK than in cSCC ($p = 0.0108$), together with a significant enrichment of T cell–associated gene families in AK ($p = 0.0055$). These findings support a model in which pre-neoplastic lesions retain a microenvironment compatible with antigen processing/presentation and T-cell engagement, whereas progression to invasive disease is accompanied by loss of these immune functions. Mechanistically, dendritic cell (DC) dysfunction is a recurring theme in established cSCC microenvironments: tumor-associated myeloid DCs may be present but exhibit impaired ability to stimulate T-cell proliferation, in association with local immunosuppressive mediators (e.g., TGF- β , IL-10, VEGF), ultimately limiting effective antitumor T-cell priming and reinforcement (Bluth et al., 2009; Bottomley et al., 2019).

At the level of immune cell–specific pathways, this “surveillance-to-dysregulation” transition is further supported by the divergence between effector and regulatory programs. While T cell–associated pathways remain significantly higher in AK, cSCC shows a marked increase in regulatory T-cell (Treg) pathways ($p = 0.0016$), together with a significant increase in myeloid-associated signatures ($p = 0.0046$). This pattern is coherent with the recognized evolution of cSCC toward a myeloid-enriched, immunoregulatory niche, where macrophages and other myeloid populations contribute to remodeling, suppression of antitumor immunity, and reduced T-cell effectiveness (Bottomley et al., 2019; Saeidi et al., 2023).

Importantly, several pathways showed informative *trends* that align with published models even if they did not reach statistical significance in our cohort. For example, NK-associated signatures tended to be higher in AK ($p = 0.081$), fitting with the notion that innate cytotoxic surveillance can be comparatively more represented in premalignant contexts, whereas the invasive tumor milieu can subvert innate effector functions (Bottomley et al., 2019). In parallel, type I interferon signatures showed a trend toward higher expression in AK ($p = 0.0928$), while IFN- γ response signatures were not significantly different ($p = 0.166$), suggesting that interferon-related activation may be present across stages but not necessarily discriminative in bulk transcriptomic profiles of early invasive lesions (Bottomley et al., 2019; Saeidi et al., 2023).

Finally, emerging translational data in AK further supports the plausibility of immune-mediated clearance at the premalignant stage. Recent work has highlighted that AK keratinocytes can induce immune-recruiting signals (e.g., via TSLP) that promote selective accumulation of CD4⁺ Th2 cells and downstream keratinocyte killing pathways in prevention settings, underscoring that AK lesions can be immunologically “actionable” and capable of mounting coordinated immune programs under appropriate conditions (Vesely & Christensen, 2025).

Taken together, integrating our signatures with the literature suggests that AK is characterized by a comparatively preserved antigen-presentation and T cell–associated immune landscape, whereas progression to cSCC is associated with a shift toward myeloid enrichment and increased immunoregulatory (Treg) programs, consistent with established concepts of immune evasion and immunoediting in cutaneous squamous carcinogenesis (Bottomley et al., 2019; Saeidi et al., 2023).

From a translational perspective, the identification of a consistent inflammatory and immunomodulatory gene signature distinguishing AK from cSCC has potential clinical implications. These transcriptional changes may serve as candidate biomarkers for risk stratification, helping to identify lesions or fields of cancerization with a higher likelihood of progression. Furthermore, the involvement of pathways related to immune regulation and metabolism suggests that therapeutic strategies targeting the tumor microenvironment—rather than tumor cells alone—may be particularly effective in preventing or treating early cSCC. In this regard, the results provide a molecular rationale for the increasing interest in immunomodulatory approaches and support current evidence highlighting the clinical relevance of immune checkpoint pathways in cutaneous squamous carcinoma (Stravodimou et al., 2021).

Despite its strengths, this study has certain limitations that should be noted. The sample size, while adequate for exploratory transcriptomic analysis, may limit the generalizability of the findings. Additionally, bulk RNA sequencing does not allow for precise attribution of gene expression changes to specific cell populations within the tumor microenvironment. Nevertheless, the strong concordance between the observed transcriptional patterns in our fresh bioptic samples and FFPE specimens and with existing spatial transcriptomic and immunohistochemical data supports the biological relevance of the results (Gim et al., 2025; Moussai et al., 2011).

In conclusion, this doctoral work demonstrates that the progression from AK to cSCC is characterized by a coordinated reorganization of inflammatory and immune pathways within the tumor microenvironment. Early cSCC emerges within a biologically active and dynamically regulated microenvironment that both reflects and promotes malignant transformation. By elucidating these molecular events, this study contributes to a deeper understanding of cutaneous

squamous carcinogenesis and lays the groundwork for future research aimed at improving early detection, risk assessment, and therapeutic strategies in cSCC.

6. SUPPLEMENTARY MATERIAL

Table 4: List of 395 genes included in the Oncomine Immune Response Research Assay, Thermo Fisher Scientific

Gene	Target	NCBI_NAME	NCBI_ACCESSION	GENE_FUNCTION
ABCF1	ABCF1_11741283	ATP_binding_cassette_subfamily_F_member_1	NM_001025091	Housekeeping
ADGRE5	ADGRE5_26552764	adhesion_G_protein-coupled_receptor_E5	NM_078481	Adhesion,migration
ADORA2A	ADORA2A_771873	adenosine_A2a_receptor	NM_000675	Checkpoint_pathway
AIF1	AIF1_385491	allograft_inflammatory_factor_1	NM_001623	Macrophage
AKT1	AKT1_358467	AKT_serine_threonine_kinase_1	NM_001014431	Tumor_marker
ALOX15B	ALOX15B_13391445	arachidonate_15-lipoxygenase_type_B	NM_001141	Macrophage
ARG1	ARG1_174278	arginase_1	NM_000045	Myeloid_marker
AXL	AXL_24872596	AXL_receptor_tyrosine_kinase	NM_021913	Innate Immune response
B3GAT1	B3GAT1_170280	beta-13-glucuronyltransferase_1	NM_018644	NK_activation
BAGE	BAGE_153290	B_melanoma_antigen	NM_182482	Tumor_antigen
BATF	BATF_237345	basic_leucine_zipper_ATF-like_transcription_factor	NM_006399	Helper_T_cells
BCL2	BCL2_10401144	B-cell_CLL_lymphoma_2	NM_000633	Apoptosis
BCL2L11	BCL2L11_620729	BCL2_like_11	NM_138621	Apoptosis
BCL6	BCL6_21502257	B-cell_CLL_lymphoma_6	NM_001706	Type_II_interferon_signaling
BRCA1	BRCA1_42344338	BRCA1_DNA_repair_associated	NM_007300	Tumor_marker
BRCA2	BRCA2_98179922	BRCA2_DNA_repair_associated	NM_000059	Tumor_marker
BST2	BST2_218322	bone_marrow_stromal_cell_antigen_2	NM_004335	Type_I_interferon_signaling
BTLA	BTLA_227334	B_and_T_lymphocyte_associated	NM_181780	Checkpoint_pathway
BUB1	BUB1_701803	BUB1_mitotic_checkpoint_serine_threonine_kinase	NM_004336	Proliferation
C10orf54	C10orf54_562672	chromosome_10_open_reading_frame_54	NM_022153	Checkpoint_pathway
C1QA	C1QA_67171	complement_component_1_q_subcomponent_A_chain	NM_015991	Innate Immune response
C1QB	C1QB_111199	complement_component_1_q_subcomponent_B_chain	NM_000491	Innate Immune response
CA4	CA4_420530	carbonic_anhydrase_4	NM_000717	Neutrophil
CBLB	CBLB_296407	Cbl_proto-oncogene_B	NM_170662	T_cell_receptor_signaling
CCL17	CCL17_288394	C-C_motif_chemokine_ligand_17	NM_002987	Chemokine_signaling
CCL18	CCL18_198296	C-C_motif_chemokine_ligand_18	NM_002988	Lymphocyte_infiltrate
CCL2	CCL2_242346	C-C_motif_chemokine_ligand_2	NM_002982	Lymphocyte_infiltrate
CCL20	CCL20_297403	C-C_motif_chemokine_ligand_20	NM_004591	Chemokine_signaling
CCL21	CCL21_432539	C-C_motif_chemokine_ligand_21	NM_002989	Lymphocyte_infiltrate
CCL22	CCL22_79185	C-C_motif_chemokine_ligand_22	NM_002990	Chemokine_signaling
CCL3	CCL3_238343	C-C_motif_chemokine_ligand_3	NM_002983	Lymphocyte_infiltrate
CCL4	CCL4_121220	C-C_motif_chemokine_ligand_4	NM_002984	Lymphocyte_infiltrate
CCL5	CCL5_110219	C-C_motif_chemokine_ligand_5	NM_002985	Lymphocyte_infiltrate
CCNB2	CCNB2_9861095	cyclin_B2	NM_004701	Proliferation
CCR1	CCR1_54149	C-C_motif_chemokine_receptor_1	NM_001295	Cytokine_signaling
CCR2	CCR2_385483	C-C_motif_chemokine_receptor_2	NM_001123396	Helper_T_cells
CCR4	CCR4_76182	C-C_motif_chemokine_receptor_4	NM_005508	Chemokine_signaling
CCR5	CCR5_85193	C-C_motif_chemokine_receptor_5_gene_pseudogene	NM_001100168	Lymphocyte_infiltrate
CCR6	CCR6_271363	C-C_motif_chemokine_receptor_6	NM_004367	Chemokine_signaling
CCR7	CCR7_133237	C-C_motif_chemokine_receptor_7	NM_001838	TCR_coexpression
CD14	CD14_329435	CD14_molecule	NM_000591	Dendritic_cell,macrophage
CD160	CD160_194300	CD160_molecule	NM_007053	Checkpoint_pathway

CD163	CD163_21422245	CD163_molecule	NM_004244	Macrophage
CD19	CD19_12011317	CD19_molecule	NM_001178098	B_cell_marker
CD1C	CD1C_12531357	CD1c_molecule	NM_001765	Antigen_presentation
CD1D	CD1D_10661173	CD1d_molecule	NM_001766	Antigen_presentation
CD2	CD2_350452	CD2_molecule	NM_001767	Lymphocyte_infiltrate
CD209	CD209_9921101	CD209_molecule	NM_021155	Dendritic_cell,macrophage
CD22	CD22_10251132	CD22_molecule	NM_001771	B_cell_marker
CD226	CD226_9021006	CD226_molecule	NM_006566	Adhesion,migration

Gene	Target	NCBI_NAME	NCBI_ACCESSION	GENE_FUNCTION
CD244	CD244_868973	CD244_molecule	NM_001166663	Checkpoint_pathway
CD247	CD247_152252	CD247_molecule	NM_198053	TCR_coexpression
CD27	CD27_409517	CD27_molecule	NM_001242	Drug_target
CD274	CD274_461569	CD274_molecule	NM_014143	Checkpoint_pathway
CD276	CD276_12011310	CD276_molecule	NM_001024736	Checkpoint_pathway
CD28	CD28_601704	CD28_molecule	NM_006139	Checkpoint_pathway
CD33	CD33_387493	CD33_molecule	NM_001772	Myeloid_marker
CD37	CD37_496605	CD37_molecule	NM_001774	Lymphocyte_infiltrate
CD38	CD38_519628	CD38_molecule	NM_001775	Adhesion,migration
CD3D	CD3D_400503	CD3d_molecule	NM_000732	TCR_coexpression
CD3E	CD3E_677780	CD3e_molecule	NM_000733	TCR_coexpression
CD3G	CD3G_616717	CD3g_molecule	NM_000073	TCR_coexpression
CD4	CD4_147255	CD4_molecule	NM_000616	Helper_T_cells
CD40	CD40_300407	CD40_molecule	NM_001250	Drug_target
CD40LG	CD40LG_479579	CD40_ligand	NM_000074	T_cell_receptor_signaling
CD44	CD44_10151123	CD44_molecule_Indian_blood_group	NM_000610	Adhesion,migration
CD47	CD47_826931	CD47_molecule	NM_001777	Adhesion,migration
CD48	CD48_695798	CD48_molecule	NM_001778	Checkpoint_pathway
CD52	CD52_70175	CD52_molecule	NM_001803	Lymphocyte_infiltrate
CD53	CD53_28133	CD53_molecule	NM_001040033	Adhesion,migration
CD6	CD6_17771886	CD6_molecule	NM_006725	TCR_coexpression
CD63	CD63_692800	CD63_molecule	NM_001780	Lymphocyte_infiltrate
CD68	CD68_172280	CD68_molecule	NM_001251	Macrophage
CD69	CD69_195303	CD69_molecule	NM_001781	Checkpoint_pathway
CD70	CD70_259363	CD70_molecule	NM_001252	Drug_target
CD74	CD74_787896	CD74_molecule	NM_001025159	Antigen_processing
CD79A	CD79A_184290	CD79a_molecule	NM_001783	B_cell_receptor_signaling
CD79B	CD79B_484590	CD79b_molecule	NM_001039933	B_cell_receptor_signaling
CD80	CD80_457557	CD80_molecule	NM_005191	Checkpoint_pathway
CD83	CD83_480580	CD83_molecule	NM_004233	Antigen_presentation
CD86	CD86_169267	CD86_molecule	NM_175862	Checkpoint_pathway
CD8A	CD8A_14271531	CD8a_molecule	NM_171827	TCR_coexpression
CD8B	CD8B_403510	CD8b_molecule	NM_172213	TCR_coexpression
CDK1	CDK1_837939	cyclin-dependent_kinase_1	NM_001786	Proliferation
CDKN2A	CDKN2A_696829	cyclin-dependent_kinase_inhibitor_2A	NM_000077	Tumor_marker
CDKN3	CDKN3_434534	cyclin-dependent_kinase_inhibitor_3	NM_005192	Proliferation
CEACAM1	CEACAM1_10471155	carcinoembryonic_antigen_related_cell_adhesion_molecule_1	NM_001712	Checkpoint_pathway
CEACAM8	CEACAM8_745847	carcinoembryonic_antigen_related_cell_adhesion_molecule_8	NM_001816	Myeloid_marker
CIITA	CIITA_294397	class_II_major_histocompatibility_complex_transactivator	NM_000246	Type_II_interferon_signaling
CLEC4C	CLEC4C_322429	C-type_lectin_domain_family_4_member_C	NM_130441	Dendritic_cell
CMKLR1	CMKLR1_36143	chemerin_chemokine-like_receptor_1	NM_001142345	Dendritic_cell,macrophage
CORO1A	CORO1A_457563	coronin_1A	NM_007074	Lymphocyte_infiltrate

CRTAM	CRTAM_312417	cytotoxic_and_regulatory_T-cell_molecule	NM_019604	TCR_coexpression
CSF1R	CSF1R_19612067	colony_stimulating_factor_1_receptor	NM_005211	Cytokine_signaling
CSF2RB	CSF2RB_11971301	colony_stimulating_factor_2_receptor_beta_common_subunit	NM_000395	Cytokine_signaling
CTAG1B	CTAG1B_276373	cancer_testis_antigen_1B	NM_001327	Tumor_antigen
CTAG2	CTAG2_407763	cancer_testis_antigen_2	ENST00000369585	Tumor_antigen
CTLA4	CTLA4_584689	cytotoxic_T-lymphocyte_associated_protein_4	NM_005214	Drug_target
CTSS	CTSS_356460	cathepsin_S	NM_004079	Lymphocyte_infiltrate
CX3CL1	CX3CL1_190298	C-X3-C_motif_chemokine_ligand_1	NM_002996	Type_II_interferon_signaling

Gene	Target	NCBI_NAME	NCBI_ACCESSION	GENE_FUNCTION
CX3CR1	CX3CR1_35133	C-X3-C_motif_chemokine_receptor_1	NM_001337	Lymphocyte_infiltrate
CX3CR1	CX3CR1_147243	C-X3-C_motif_chemokine_receptor_1	NM_001171172	Lymphocyte_infiltrate
CX3CR1	CX3CR1_207292	C-X3-C_motif_chemokine_receptor_1	NM_001171171	Lymphocyte_infiltrate
CX3CR1	CX3CR1_58187	C-X3-C_motif_chemokine_receptor_1	NM_001171174	Lymphocyte_infiltrate
CXCL1	CXCL1_357459	C-X-C_motif_chemokine_ligand_1	NM_001511	Chemokine_signaling
CXCL10	CXCL10_354459	C-X-C_motif_chemokine_ligand_10	NM_001565	Type_II_interferon_signaling
CXCL11	CXCL11_261361	C-X-C_motif_chemokine_ligand_11	NM_005409	Type_II_interferon_signaling
CXCL13	CXCL13_202307	C-X-C_motif_chemokine_ligand_13	NM_006419	Type_II_interferon_signaling
CXCL8	CXCL8_197302	C-X-C_motif_chemokine_ligand_8	NM_000584	Cytokine_signaling
CXCL9	CXCL9_149250	C-X-C_motif_chemokine_ligand_9	NM_002416	Type_II_interferon_signaling
CXCR2	CXCR2_379486	C-X-C_motif_chemokine_receptor_2	NM_001557	Chemokine_signaling
CXCR3	CXCR3_11138	C-X-C_motif_chemokine_receptor_3	NM_001504	Chemokine_signaling
CXCR4	CXCR4_100208	C-X-C_motif_chemokine_receptor_4	NM_003467	Lymphocyte_infiltrate
CXCR5	CXCR5_153252	C-X-C_motif_chemokine_receptor_5	NM_001716	Type_II_interferon_signaling
CXCR6	CXCR6_48153	C-X-C_motif_chemokine_receptor_6	NM_006564	Lymphocyte_infiltrate
CYBB	CYBB_14221529	cytochrome_b-245_beta_chain	NM_000397	Type_II_interferon_signaling
DDX58	DDX58_540643	DEXD_H-box_helicase_58	NM_014314	Interferon_signaling
DGAT2	DGAT2_439548	diacylglycerol_O-acyltransferase_2	NM_032564	Neutrophil
DMBT1	DMBT1_64696575	deleted_in_malignant_brain_tumors_1	NM_007329	Innate_immune_response
EBI3	EBI3_527636	Epstein-Barr_virus_induced_3	NM_005755	T_cell_regulation
EFNA4	EFNA4_548649	ephrin_A4	NM_005227	Tumor_marker
EGFR	EGFR_31583261	epidermal_growth_factor_receptor	NM_005228	Tumor_marker
EGR2	EGR2_454559	early_growth_response_2	NM_000399	T_cell_differentiation
EGR3	EGR3_411516	early_growth_response_3	NM_004430	Tumor_marker
EIF2AK2	EIF2AK2_257357	eukaryotic_translation_initiation_factor_2_alpha_kinase_2	NM_001135651	Type_II_interferon_signaling
ENTPD1	ENTPD1_708815	ectonucleoside_triphosphate_diphosphohydrolase_1	NM_001098175	Checkpoint_pathway
EOMES	EOMES_9391044	eomesodermin	NM_005442	Checkpoint_pathway
FAS	FAS_473575	Fas_cell_surface_death_receptor	NM_000043	B_cell_receptor_signaling
FASLG	FASLG_417521	Fas_ligand	NM_000639	Type_II_interferon_signaling
FCER1G	FCER1G_31138	Fc_fragment_of_IgE_receptor_Ig	NM_004106	Lymphocyte_infiltrate
FCGR1A	FCGR1A_547652	Fc_fragment_of_IgG_receptor_Ia	NM_000566	B_cell_marker
FCGR2B	FCGR2B_792903	Fc_fragment_of_IgG_receptor_IIb	NM_004001	B_cell_marker
FCGR3A	FCGR3A_575682	Fc_fragment_of_IgG_receptor_IIIa	NM_000569	Macrophage
FCGR3B	FCGR3B_620722	Fc_fragment_of_IgG_receptor_IIIb	NM_000570	NK_activation
FCRLA	FCRLA_710815	Fc_receptor_like_A	NM_001184866	B_cell_marker
FOXM1	FOXM1_10551166	forkhead_box_M1	NM_021953	Proliferation
FOXO1	FOXO1_9761081	forkhead_box_O1	NM_002015	PD-1_signaling,tumor_marker
FOXP3	FOXP3_865975	forkhead_box_P3	NM_014009	T_cell_regulation
FUT4	FUT4_31443252	fucosyltransferase_4	NM_002033	Myeloid_marker,stem_cell
FYB	FYB_12891393	FYN_binding_protein	NM_001465	Lymphocyte_infiltrate
G6PD	G6PD_10841194	glucose-6-phosphate_dehydrogenase	NM_000402	Housekeeping
GADD45GIP1	GADD45GIP1_329429	GADD45G_interacting_protein_1	NM_052850	Apoptosis
GAGE1,GAGE12I,GAGE12F	GAGE1_144260	G_antigen_1	NM_001468,NM_001477,NM	Tumor_antigen
GAGE10	GAGE10_137250	G_antigen_10	NM_001098413	Tumor_antigen
GAGE12J	GAGE12J_144260	G_antigen_12J	NM_001098406	Tumor_antigen
GAGE13	GAGE13_116232	G_antigen_13	NM_001098412	Tumor_antigen
GAGE2C,GAGE2A,GAGE2E	GAGE2C_104217	G_antigen_2C	NM_001472,NM_001127212	Tumor_antigen
GATA3	GATA3_14061514	GATA_binding_protein_3	NM_001002295	Helper_T_cells
GBP1	GBP1_771872	guanylate_binding_protein_1	NM_002053	Type_II_interferon_signaling
GNLY	GNLY_251357	granulysin	NM_006433	NK_activation

Gene	Target	NCBI_NAME	NCBI_ACCESSION	GENE_FUNCTION
GPR18	GPR18_238340	G_protein-coupled_receptor_18	NM_001098200	TCR_coexpression
GRAP2	GRAP2_283387	GRB2-related_adaptor_protein_2	NM_004810	TCR_coexpression
GUSB	GUSB_17671872	glucuronidase_beta	NM_000181	Housekeeping
GZMA	GZMA_165265	granzyme_A	NM_006144	Lymphocyte_infiltrate

GZMB	GZMB_581688	granzyme_B	NM_004131	Lymphocyte_infiltrate
GZMH	GZMH_233339	granzyme_H	NM_033423	Lymphocyte_infiltrate
GZMK	GZMK_178286	granzyme_K	NM_002104	Lymphocyte_infiltrate
HAVCR2	HAVCR2_594705	hepatitis_A_virus_cellular_receptor_2	NM_032782	Checkpoint_pathway
HERC6	HERC6_17021806	HECT_and_RLD_domain_containing_E3_ubiquitin_protein_ligase_family_member_6	NM_017912	Dendritic_cell
HGF	HGF_14151518	hepatocyte_growth_factor	NM_000601	Cytokine_signaling
HIF1A	HIF1A_13371439	hypoxia_inducible_factor_1_alpha_subunit	NM_001530	PD-1_signaling,tumor_marker
HLA-A	HLA-A_345445	major_histocompatibility_complex_class_I_A	NM_002116	Antigen_processing
HLA-B	HLA-B_639746	major_histocompatibility_complex_class_I_B	NM_005514	Antigen_processing
HLA-C	HLA-C_9191023	major_histocompatibility_complex_class_I_C	NM_002117	Antigen_processing
HLA-DMA	HLA-DMA_764869	major_histocompatibility_complex_class_II_DM_alpha	NM_006120	Antigen_processing
HLA-DMB	HLA-DMB_854962	major_histocompatibility_complex_class_II_DM_beta	NM_002118	Antigen_processing
HLA-DOA	HLA-DOA_55163	major_histocompatibility_complex_class_II_DQ_alpha	NM_002119	Antigen_processing
HLA-DOB	HLA-DOB_182290	major_histocompatibility_complex_class_II_DO_beta	NM_002120	Antigen_processing
HLA-DPA1	HLA-DPA1_129237	major_histocompatibility_complex_class_II_DP_alpha_1	NM_033554	Antigen_processing
HLA-DPB1	HLA-DPB1_175280	major_histocompatibility_complex_class_II_DP_beta_1	NM_002121	Antigen_processing
HLA-DQA1	HLA-DQA1_587686	major_histocompatibility_complex_class_II_DQ_alpha_1	NM_002122	Antigen_processing
HLA-DQA2	HLA-DQA2_840937	major_histocompatibility_complex_class_II_DQ_alpha_2	NM_020056	Antigen_processing
HLA-DQB2	HLA-DQB2_142243	major_histocompatibility_complex_class_II_DQ_beta_2	NM_001198858	Antigen_processing
HLA-DRA	HLA-DRA_186294	major_histocompatibility_complex_class_II_DR_alpha	NM_019111	Antigen_processing
HLA-DRB1	HLA-DRB1_877982	major_histocompatibility_complex_class_II_DR_beta_1	NM_002124	Antigen_processing
HLA-E	HLA-E_639747	major_histocompatibility_complex_class_I_E	NM_005516	Antigen_processing
HLA-F	HLA-F_101204	major_histocompatibility_complex_class_I_F	NM_001098479	Antigen_processing
HLA-F-AS1	HLA-F-AS1_726834	HLA-F_antisense_RNA_1	NR_026972	Antigen_processing
HLA-G	HLA-G_483585	major_histocompatibility_complex_class_I_G	NM_002127	Antigen_processing
HMBS	HMBS_796904	hydroxymethylbilane_synthase	NM_000190	Housekeeping
ICAM1	ICAM1_605711	intercellular_adhesion_molecule_1	NM_000201	Type_II_interferon_signaling
ICOS	ICOS_382487	inducible_T-cell_costimulator	NM_012092	Checkpoint_pathway
ICOSLG	ICOSLG_483588	inducible_T-cell_costimulator_ligand	NM_015259	Checkpoint_pathway
ID2	ID2_579679	inhibitor_of_DNA_binding_2_HLH_protein	NM_002166	T_cell_regulation
ID3	ID3_572678	inhibitor_of_DNA_binding_3_HLH_protein	NM_002167	T_cell_regulation
IDO1	IDO1_268369	indoleamine_23-dioxygenase_1	NM_002164	Drug_target
IDO2	IDO2_458562	indoleamine_23-dioxygenase_2	NM_194294	Checkpoint_pathway
IFI27	IFI27_37143	interferon_alpha_inducible_protein_27	NM_005532	Type_I_interferon_signaling
IFI35	IFI35_419526	interferon_induced_protein_35	NM_005533	Interferon_signaling
IFI44L	IFI44L_12771376	interferon_induced_protein_44_like	NM_006820	Interferon_signaling
IFI6	IFI6_47156	interferon_alpha_inducible_protein_6	NM_022873	Interferon_signaling
IFIH1	IFIH1_20172123	interferon_induced_with_helicase_C_domain_1	NM_022168	Innate_immune_response
IFIT1	IFIT1_158259	interferon_induced_protein_with_tetratricopeptide_repeats_1	NM_001548	Type_I_interferon_signaling
IFIT2	IFIT2_124224	interferon_induced_protein_with_tetratricopeptide_repeats_2	NM_001547	Cytokine_signaling
IFIT3	IFIT3_72174	interferon_induced_protein_with_tetratricopeptide_repeats_3	NM_001031683	Type_I_interferon_signaling
IFITM1	IFITM1_359459	interferon_induced_transmembrane_protein_1	NM_003641	Type_I_interferon_signaling
IFITM2	IFITM2_316422	interferon_induced_transmembrane_protein_2	NM_006435	Type_I_interferon_signaling
IFNA17	IFNA17_691788	interferon_alpha_17	NM_021268	T_cell_receptor_signaling
IFNB1	IFNB1_579686	interferon_beta_1	NM_002176	Type_II_interferon_signaling
IFNG	IFNG_400500	interferon_gamma	NM_000619	Type_II_interferon_signaling

Gene	Target	NCBI_NAME	NCBI_ACCESSION	GENE_FUNCTION
IGF1R	IGF1R_12291338	insulin_like_growth_factor_1_receptor	NM_000875	Adhesion,migration
IGSF6	IGSF6_411515	immunoglobulin_superfamily_member_6	NM_005849	Lymphocyte_infiltrate
IKZF1	IKZF1_189301	IKAROS_family_zinc_finger_1	NM_006060	Lymphocyte_development
IKZF2	IKZF2_537639	IKAROS_family_zinc_finger_2	NM_016260	Lymphocyte_development
IKZF3	IKZF3_427534	IKAROS_family_zinc_finger_3	NM_012481	TCR_coexpression
IKZF4	IKZF4_746852	IKAROS_family_zinc_finger_4	NM_022465	Lymphocyte_development
IL10	IL10_491598	interleukin_10	NM_000572	Drug_target
IL10RA	IL10RA_192296	interleukin_10_receptor_subunit_alpha	NM_001558	Lymphocyte_infiltrate
IL12A	IL12A_739842	interleukin_12A	NM_000882	Drug_target
IL12B	IL12B_314422	interleukin_12B	NM_002187	Drug_target
IL13	IL13_268378	interleukin_13	NM_002188	Cytokine_signaling
IL15	IL15_49155	interleukin_15	NM_000585	T_cell_regulation
IL17A	IL17A_233320	interleukin_17A	NM_002190	Helper_T_cells
IL17F	IL17F_35139	interleukin_17F	NM_052872	Dendritic_cell,macrophage
IL18	IL18_140246	interleukin_18	NM_001562	T_cell_regulation
IL1A	IL1A_14431546	interleukin_1_alpha	NM_000575	Cytokine_signaling
IL1B	IL1B_659766	interleukin_1_beta	NM_000576	Type_II_interferon_signaling
IL2	IL2_366451	interleukin_2	NM_000586	Drug_target
IL21	IL21_368450	interleukin_21	NM_021803	Cytokine_signaling
IL22	IL22_263349	interleukin_22	NM_020525	T_cell_regulation
IL23A	IL23A_386491	interleukin_23_subunit_alpha	NM_016584	Dendritic_cell,macrophage
IL2RA	IL2RA_240338	interleukin_2_receptor_subunit_alpha	NM_000417	Cytokine_signaling

IL2RB	IL2RB_9991107	interleukin_2_receptor_subunit_beta	NM_000878	TCR_coexpression
IL2RG	IL2RG_884988	interleukin_2_receptor_subunit_gamma	NM_000206	Lymphocyte_infiltrate
IL3RA	IL3RA_465565	interleukin_3_receptor_subunit_alpha	NM_002183	Dendritic_cell
IL4	IL4_380477	interleukin_4	NM_000589	Cytokine_signaling
IL6	IL6_521626	interleukin_6	NM_000600	Cytokine_signaling
IL7	IL7_545652	interleukin_7	NM_000880	Cytokine_signaling
IL7R	IL7R_551658	interleukin_7_receptor	NM_002185	TCR_coexpression
IRF1	IRF1_752861	interferon_regulatory_factor_1	NM_002198	Type_II_interferon_signaling
IRF4	IRF4_786895	interferon_regulatory_factor_4	NM_002460	Interferon_signaling
IRF9	IRF9_12191322	interferon_regulatory_factor_9	NM_006084	Type_II_interferon_signaling
IRS1	IRS1_37283828	insulin_receptor_substrate_1	NM_005544	Tumor_marker
ISG15	ISG15_66173	ISG15_ubiquitin-like_modifier	NM_005101	Type_I_interferon_signaling
ISG20	ISG20_642750	interferon_stimulated_exonuclease_gene_20	NM_002201	Type_I_interferon_signaling
ITGA1	ITGA1_814914	integrin_subunit_alpha_1	NM_181501	Adhesion,migration
ITGAE	ITGAE_33753481	integrin_subunit_alpha_E	NM_002208	Adhesion,migration
ITGAL	ITGAL_535638	integrin_subunit_alpha_L	NM_002209	Leukocyte_migration
ITGAM	ITGAM_31033209	integrin_subunit_alpha_M	NM_001145808	Leukocyte_migration
ITGAX	ITGAX_20482151	integrin_subunit_alpha_X	NM_000887	Dendritic_cell
ITGB1	ITGB1_679777	integrin_subunit_beta_1	NM_002211	Adhesion,migration
ITGB2	ITGB2_15421648	integrin_subunit_beta_2	NM_000211	Lymphocyte_infiltrate
ITGB7	ITGB7_24342544	integrin_subunit_beta_7	NM_000889	Leukocyte_migration
ITK	ITK_563669	IL2_inducible_T-cell_kinase	NM_005546	TCR_coexpression
JAML	JAML_78188	junction_adhesion_molecule_like	NM_001098526	Lymphocyte_infiltrate
JCHAIN	JCHAIN_134238	joining_chain_of_multimeric_IgA_and_IgM	NM_144646	B_cell_marker
KIAA0101	KIAA0101_319426	KIAA0101	NM_014736	Proliferation
KIR2DL1	KIR2DL1_9142	killer_cell_immunoglobulin_like_receptor_two_ig_domains_and_long_cytoplasmic_tail_1	NM_014218	Drug_target
KIR2DL2	KIR2DL2_27126	killer_cell_immunoglobulin_like_receptor_two_ig_domains_and_long_cytoplasmic_tail_2	NM_014218	NK_cell_marker
KIR2DL3	KIR2DL3_654757	killer_cell_immunoglobulin_like_receptor_two_ig_domains_and_long_cytoplasmic_tail_3	NM_015868	NK_cell_marker

Gene	Target	NCBI_NAME	NCBI_ACCESSION	GENE_FUNCTION
KLF2	KLF2_9111017	Kruppel_like_factor_2	NM_016270	T_cell_regulation,trafficking
KLRB1	KLRB1_177284	killer_cell_lectin_like_receptor_B1	NM_002258	NK_activation
KLRD1	KLRD1_515619	killer_cell_lectin_like_receptor_D1	NM_002262	Drug_target
KLRF1	KLRF1_188285	killer_cell_lectin_like_receptor_F1	NM_016523	NK_activation
KLRG1	KLRG1_410518	killer_cell_lectin_like_receptor_G1	NM_005810	NK_activation
KLRK1	KLRK1_698804	killer_cell_lectin_like_receptor_K1	NM_007360	NK_activation
KREMEN1	KREMEN1_12861389	kringle_containing_transmembrane_protein_1	NM_032045	Neutrophil
KRT5	KRT5_10631165	keratin_5	NM_000424	Tumor_marker
KRT7	KRT7_440543	keratin_7	NM_005556	Tumor_marker
LAG3	LAG3_13111419	lymphocyte_activating_3	NM_002286	Drug_target
LAMP1	LAMP1_374485	lysosomal_associated_membrane_protein_1	NM_005561	Lymphocyte_infiltrate
LAMP3	LAMP3_12361344	lysosomal_associated_membrane_protein_3	NM_014398	TCR_coexpression
LAPTM5	LAPTM5_555664	lysosomal_protein_transmembrane_5	NM_006762	Lymphocyte_infiltrate
LCK	LCK_89192	LCK_proto-oncogene_Src_family_tyrosine_kinase	NM_001042771	TCR_coexpression
LCN2	LCN2_153259	lipocalin_2	NM_005564	Innate_immune_response
LEXM	LEXM_10551156	lymphocyte_expansion_molecule	NM_001110533	T_cell_differentiation
LILRB1	LILRB1_15681666	leukocyte_immunoglobulin_like_receptor_B1	NM_001081637	Leukocyte_inhibition
LILRB2	LILRB2_891994	leukocyte_immunoglobulin_like_receptor_B2	NM_001080978	Lymphocyte_infiltrate
LMNA	LMNA_588696	lamin_A_C	NM_170707	Housekeeping
LRG1	LRG1_24131	leucine_rich_alpha-2-glycoprotein_1	NM_052972	Neutrophil
LRP1	LRP1_25642670	LDL_receptor_related_protein_1	NM_002332	Housekeeping
LST1	LST1_22120	leukocyte_specific_transcript_1	NM_007161	Leukocyte_inhibition
LY9	LY9_13751476	lymphocyte_antigen_9	NM_002348	Lymphocyte_infiltrate
LYZ	LYZ_120222	lysozyme	NM_000239	Innate_immune_response
M6PR	M6PR_229336	mannose-6-phosphate_receptor_cation_dependent	NM_002355	T_cell_regulation
MAD2L1	MAD2L1_115221	MAD2_mitotic_arrest_deficient-like_1_yeast	NM_002358	Proliferation
MADCAM1	MADCAM1_9291024	mucosal_vascular_addressin_cell_adhesion_molecule_1	NM_130760	Adhesion,migration
MAGEA1	MAGEA1_22129	MAGE_family_member_A1	NM_004988	Tumor_antigen
MAGEA10	MAGEA10_241320	MAGE_family_member_A10	NM_021048	Tumor_antigen
MAGEA12	MAGEA12_30130	MAGE_family_member_A12	NM_005367	Tumor_antigen
MAGEA3	MAGEA3_20121	MAGE_family_member_A3	NM_005362,NM_005363	Tumor_antigen
MAGEA4	MAGEA4_28131	MAGE_family_member_A4	NM_001011548	Tumor_antigen
MAGEC2	MAGEC2_249358	MAGE_family_member_C2	NM_016249	Tumor_antigen
MAPK1	MAPK1_13241425	mitogen-activated_protein_kinase_1	NM_002745	Tumor_marker
MAPK14	MAPK14_14031511	mitogen-activated_protein_kinase_14	NM_139012	Innate_immune_response
MELK	MELK_300401	maternal_embryonic_leucine_zipper_kinase	NM_014791	Proliferation
MIF	MIF_157270	macrophage_migration_inhibitory_factor_glycosylation-inhibiting_factor	NM_002415	Innate_immune_response
MKI67	MKI67_581686	marker_of_proliferation_Ki-67	NM_002417	Proliferation
MLANA	MLANA_159265	melan-A	NM_005511	Tumor_antigen
MMP2	MMP2_11051213	matrix_metallopeptidase_2	NM_004530	Tumor_marker
MMP9	MMP9_11741283	matrix_metallopeptidase_9	NM_004994	Tumor_marker

MPO	MPO_15121620	myeloperoxidase	NM_000250	Myeloid_marker
MRC1	MRC1_529634	mannose_receptor_C_type_1	NM_002438	Dendritic_cell,macrophage
MS4A1	MS4A1_127232	membrane_spanning_4-domains_A1	NM_021950	Drug_target
MTOR	MTOR_62366338	mechanistic_target_of_rapamycin	NM_004958	PD-1_signaling,tumor_marker
MX1	MX1_232336	MX_dynamin_like_GTPase_1	NM_001178046	Interferon_signaling
MYC	MYC_510619	v-myc_avian_myelocytomatosis_viral_oncogene_homolog	NM_002467	Tumor_marker
NCAM1	NCAM1_26012709	neural_cell_adhesion_molecule_1	NM_181351	Adhesion,migration
NCF1	NCF1_509617	neutrophil_cytosolic_factor_1	NM_000265	Chemokine_signaling
NCR1	NCR1_754861	natural_cytotoxicity_triggering_receptor_1	NM_004829	NK_cell_marker

Gene	Target	NCBI_NAME	NCBI_ACCESSION	GENE_FUNCTION
NCR3	NCR3_234342	natural_cytotoxicity_triggering_receptor_3	NM_147130	NK_cell_marker
NECTIN2	NECTIN2_13881496	nectin_cell_adhesion_molecule_2	NM_001042724	Adhesion,migration
NFATC1	NFATC1_27682875	nuclear_factor_of_activated_T-cells_1	NM_172387	PD-1_signaling
NFKBIA	NFKBIA_734840	NFKB_inhibitor_alpha	NM_020529	T_cell_receptor_signaling
NKG7	NKG7_557664	natural_killer_cell_granule_protein_7	NM_005601	Lymphocyte_infiltrate
NOS2	NOS2_91195	nitric_oxide_synthase_2	NM_000625	Innate_immune_response
NOTCH3	NOTCH3_738847	notch_3	NM_000435	Tumor_marker
NRP1	NRP1_14831588	neuropilin_1	NM_003873	Dendritic_cell
NT5E	NT5E_12531355	5-nucleotidase_ecto	NM_002526	Checkpoint_pathway
NTN3	NTN3_12821377	netrin_3	NM_006181	B_cell_marker
OAS1	OAS1_757865	2-5-oligoadenylate_synthetase_1	NM_016816	Type_II_interferon_signaling
OAS2	OAS2_15981707	2-5-oligoadenylate_synthetase_2	NM_016817	Interferon_signaling
OAS3	OAS3_667776	2-5-oligoadenylate_synthetase_3	NM_006187	Interferon_signaling
PDCD1	PDCD1_143244	programmed_cell_death_1	NM_005018	Drug_target
PDCD1LG2	PDCD1LG2_315423	programmed_cell_death_1_ligand_2	NM_025239	Checkpoint_pathway
PECAM1	PECAM1_22132	platelet_and_endothelial_cell_adhesion_molecule_1	NM_000442	Adhesion,migration
PGF	PGF_742842	placental_growth_factor	NM_002632	Tumor_marker
PIK3CA	PIK3CA_12781384	phosphatidylinositol-45-bisphosphate_3-kinase_catalytic_subunit_alpha	NM_006218	PD-1_signaling,tumor_marker
PIK3CD	PIK3CD_19842095	phosphatidylinositol-45-bisphosphate_3-kinase_catalytic_subunit_delta	NM_005026	PD-1_signaling,tumor_marker
PMEL	PMEL_233339	premelanosome_protein	NM_006928	Drug_target
POLR2A	POLR2A_42334334	polymerase_RNA_II_subunit_A	NM_000937	Housekeeping
POU2AF1	POU2AF1_230337	POU_class_2_associating_factor_1	NM_006235	B_cell_marker
PRDM1	PRDM1_484588	PR_domain_1	NM_001198	PD-1_signaling
PRF1	PRF1_672778	perforin_1	NM_005041	NK_activation
PSMB9	PSMB9_384491	proteasome_subunit_beta_9	NM_002800	Type_II_interferon_signaling
PTEN	PTEN_19732078	phosphatase_and_tensin_homolog	NM_000314	PD-1_signaling,tumor_marker
PTGS2	PTGS2_14761583	prostaglandin-endoperoxide_synthase_2	NM_000963	Tumor_marker
PTK7	PTK7_15161625	protein_tyrosine_kinase_7_inactive	NM_002821	Tumor_marker
PTPN11	PTPN11_14261533	protein_tyrosine_phosphatase_non-receptor_type_11	NM_002834	PD-1_signaling,tumor_marker
PTPN6	PTPN6_17741884	protein_tyrosine_phosphatase_non-receptor_type_6	NM_080548	T_cell_receptor_signaling
PTPN7	PTPN7_471581	protein_tyrosine_phosphatase_non-receptor_type_7	NM_001199797	Lymphocyte_infiltrate
PTPRC	PTPRC_710817	protein_tyrosine_phosphatase_receptor_type_C	NM_002838	Lymphocyte_infiltrate
PTPRCAP	PTPRCAP_383516	protein_tyrosine_phosphatase_receptor_type_C_associated_protein	NM_005608	TCR_coexpression
PVR	PVR_9571064	poliovirus_receptor	NM_006505	Checkpoint_pathway
PYGL	PYGL_586697	phosphorylase_glycogen_liver	NM_002863	Neutrophil
RB1	RB1_24592560	RB_transcriptional_corepressor_1	NM_000321	Tumor_marker
RORC	RORC_12011307	RAR_related_orphan_receptor_C	NM_005060	Helper_T_cells
RPS6	RPS6_124229	ribosomal_protein_S6	NM_001010	Tumor_marker
S100A8	S100A8_239334	S100_calcium_binding_protein_A8	NM_002964	Myeloid_marker,MDSC
S100A9	S100A9_174280	S100_calcium_binding_protein_A9	NM_002965	Myeloid_marker,MDSC
SAMHD1	SAMHD1_19002006	SAM_and_HD_domain_containing_deoxynucleoside_triphosphate_triphosphohydrolase_1	NM_015474	Lymphocyte_infiltrate
SDHA	SDHA_16361735	succinate_dehydrogenase_complex_flavoprotein_subunit_A	NM_004168	Housekeeping
SELL	SELL_139245	selectin_L	NM_000655	Leukocyte_migration
SH2D1A	SH2D1A_540642	SH2_domain_containing_1A	NM_002351	Lymphocyte_activation
SH2D1B	SH2D1B_173275	SH2_domain_containing_1B	NM_053282	Lymphocyte_activation
SIT1	SIT1_148254	signaling_threshold_regulating_transmembrane_adaptor_1	NM_014450	Lymphocyte_infiltrate
SKAP2	SKAP2_809912	src_kinase_associated_phosphoprotein_2	NM_003930	B_cell_marker
SLAMF7	SLAMF7_9161020	SLAM_family_member_7	NM_021181	Drug_target
SLAMF8	SLAMF8_9071016	SLAM_family_member_8	NM_020125	Lymphocyte_infiltrate
SNAI1	SNAI1_138246	snail_family_transcriptional_repressor_1	NM_005985	Tumor_marker,stemness

Gene	Target	NCBI_NAME	NCBI_ACCESSION	GENE_FUNCTION
SNAI2	SNAI2_722828	snail_family_transcriptional_repressor_2	NM_003068	Tumor_marker,stemness
SRGN	SRGN_230328	serglycin	NM_002727	Lymphocyte_infiltrate
SSX2	SSX2_233349	SSX_family_member_2	NM_003147	Tumor_antigen

STAT1	STAT1_18871996	signal_transducer_and_activator_of_transcription_1	NM_007315	Type_II_interferon_signaling
STAT3	STAT3_17511856	signal_transducer_and_activator_of_transcription_3	NM_139276	Drug_target
STAT4	STAT4_245352	signal_transducer_and_activator_of_transcription_4	NM_003151	Helper_T_cells
STAT5A	STAT5A_18501954	signal_transducer_and_activator_of_transcription_5A	NM_003152	Cytokine_signaling
STAT6	STAT6_231339	signal_transducer_and_activator_of_transcription_6	NM_003153	Helper_T_cells
TAGAP	TAGAP_10531160	T-cell_activation_RhoGTPase_activating_protein	NM_054114	Lymphocyte_infiltrate
TAP1	TAP1_15701675	transporter_1_ATP-binding_cassette_sub-family_B_MDR_TAP	NM_000593	Type_II_interferon_signaling
TARP	TARP_416517	TCR_gamma_alternate_reading_frame_protein	NM_001003806	Lymphocyte_infiltrate
TBP	TBP_259367	TATA-box_binding_protein	NM_003194	Housekeeping
TBX21	TBX21_9251036	T-box_21	NM_013351	Type_II_interferon_signaling
TCF7	TCF7_677799	transcription_factor_7_T-cell_specific_HMG-box	NM_003202	Tumor_marker
TD02	TD02_55162	tryptophan_23-dioxygenase	NM_005651	Checkpoint_pathway
TFRC	TFRC_10891196	transferrin_receptor	NM_001128148	Housekeeping
TGFB1	TGFB1_13681474	transforming_growth_factor_beta_1	NM_000660	Checkpoint_pathway
TIGIT	TIGIT_383491	T-cell_immunoreceptor_with_lg_and_ITIM_domains	NM_173799	TCR_coexpression
TLR3	TLR3_24932595	toll_like_receptor_3	NM_003265	Dendritic_cell
TLR7	TLR7_99206	toll_like_receptor_7	NM_016562	Innate_immune_response
TLR8	TLR8_36137	toll_like_receptor_8	NM_138636	Lymphocyte_infiltrate
TLR9	TLR9_619727	toll_like_receptor_9	NM_017442	Drug_target
TNF	TNF_450555	tumor_necrosis_factor	NM_000594	Checkpoint_pathway
TNFAIP8	TNFAIP8_188295	TNF_alpha_induced_protein_8	NM_014350	Lymphocyte_infiltrate
TNFRSF14	TNFRSF14_846950	tumor_necrosis_factor_receptor_superfamily_member_14	NM_003820	Checkpoint_pathway
TNFRSF17	TNFRSF17_254359	tumor_necrosis_factor_receptor_superfamily_member_17	NM_001192	B_cell_marker
TNFRSF18	TNFRSF18_348456	tumor_necrosis_factor_receptor_superfamily_member_18	NM_004195	Drug_target
TNFRSF4	TNFRSF4_304389	tumor_necrosis_factor_receptor_superfamily_member_4	NM_003327	Drug_target
TNFRSF9	TNFRSF9_894998	tumor_necrosis_factor_receptor_superfamily_member_9	NM_001561	Drug_target
TNFSF10	TNFSF10_251357	tumor_necrosis_factor_superfamily_member_10	NM_003810	Apoptosis
TNFSF13B	TNFSF13B_774879	tumor_necrosis_factor_superfamily_member_13b	NM_006573	B_cell_marker
TNFSF14	TNFSF14_224334	tumor_necrosis_factor_superfamily_member_14	NM_003807	Checkpoint_pathway
TNFSF18	TNFSF18_128228	tumor_necrosis_factor_superfamily_member_18	NM_005092	Checkpoint_pathway
TNFSF4	TNFSF4_347439	tumor_necrosis_factor_superfamily_member_4	NM_003326	Checkpoint_pathway
TNFSF9	TNFSF9_325412	tumor_necrosis_factor_superfamily_member_9	NM_003811	Cytokine_signaling
TOP2A	TOP2A_27522855	topoisomerase_DNA_II_alpha	NM_001067	Proliferation
TP63	TP63_143246	tumor_protein_p63	NM_003722	Tumor_marker
TRIM29	TRIM29_882990	tripartite_motif_containing_29	NM_012101	Tumor_marker
TUBB	TUBB_369479	tubulin_beta_class_I	NM_178014	Housekeeping
TWIST1	TWIST1_9291033	twist_family_bHLH_transcription_factor_1	NM_000474	Tumor_marker,stemness
TYROBP	TYROBP_350455	TYRO_protein_tyrosine_kinase_binding_protein	NM_198125	Lymphocyte_infiltrate
VCAM1	VCAM1_829933	vascular_cell_adhesion_molecule_1	NM_001078	Leukocyte_migration
VEGFA	VEGFA_17121817	vascular_endothelial_growth_factor_A	NM_001171623	Chemokine_signaling
VTCN1	VTCN1_49157	V-set_domain_containing_T_cell_activation_inhibitor_1	NM_024626	Checkpoint_pathway
XAGE1B	XAGE1B_469547	X_antigen_family_member_1B	NM_001097594	Tumor_antigen
ZAP70	ZAP70_673781	zeta_chain_of_T_cell_receptor_associated_protein_kinase_70kDa	NM_001079	T_cell_receptor_signaling
ZBTB46	ZBTB46_13361440	zinc_finger_and_BTBD_domain_containing_46	NM_025224	Dendritic_cell
ZEB1	ZEB1_74175	zinc_finger_E-box_binding_homeobox_1	NM_001174093	Tumor_marker,stemness

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