EXPRESSION LEVELS OF ALDH1A2 and ALDH1A3 ARE ALTERED IN HUMAN TISSUE BIOPSIES IN INVASIVE SQUAMOUS CELL CARCINOMA

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DEDICATION

To my children, Brianna, Malcolm, and Blake, who inspire me to look beyond the obstacles in life.

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ABSTRACT

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EXPRESSION LEVELS OF ALDH1A2 and ALDH1A3 ARE ALTERED
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The regulation of gene expression by retinoids has been shown to be profoundly altered in various types of precancerous and cancerous lesions, including cutaneous squamous cell carcinoma (cSCC). Aldehyde dehydrogenase family 1, subfamily members A2 (ALDH1A2), and A3 (ALDH1A3) participate in the oxidation of retinal to retinoic acid and their expression has been implicated in various types of cancers. The purpose of this study was to compare the intensity and localization of ALDH1A2 and ALDH1A3 in various stages of cSCC. Forty-two diseased biopsies and 32 healthy controls were used in this study. Immunohistochemistry was used to detect the immunoreactivity and localization of ALDH1A2 and ALDH1A3. Results showed percent ALDH1A2 positive cells were significantly higher in the tumor and stroma of actinic keratosis and invasive SCC groups than other groups. ALDH1A3 expression levels were significantly higher in tumors of all stages of cSCC compared to the epidermis of the control group.

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CHAPTER I

INTRODUCTION

Squamous cells are flat cells that make up most of the cells in the outer part of the epidermis.[1] These cells shed continuously as new cells form. When the cells are damaged, for example via ultraviolet (UV) radiation, the unrepaired DNA triggers mutations. These changes in the DNA cause oncogenes to stay activated and tumor suppressor genes to shut off, causing an unsuppressed growth leading to cutaneous squamous cell carcinoma (cSCC).[2] Although squamous cells are located throughout the human body, these cancers commonly appear on sun-exposed areas of the body.[3] Initially, lesions can present as an innocuous plaque-like or verrucous tumor then can develop into a large, necrotic, and infected lesion.[4] Of all non-melanoma skin cancer, cSCC makes up 20%.[5] Most concerning to patients and clinicians is the ability for this type of cancer to metastasize to any organ in the body. Metastatic cSCC is deadly with some larger studies finding a mortality rate of >70%.[4] Staging systems have been developed to aid with assessing the prognosis of cSCC.[6] These systems also assist with determining high-risk variants and ultimately, increase survival rates in patients with cSCC. Although recent changes have been made to increase stratification of cSCC, research shows the new guidelines have done little to improve survival rates and provides no information on

micrometastasis or extracapsular nodal extension, which can assist in determining the prognosis of patients.[6]

Retinoid compounds are involved in epithelial cell growth, differentiation, and maintenance.[7] Specifically, retinoic acid (RA) directs the differentiation of immature skin cells into mature epidermal cells.[8] The regulation of gene expression by retinoids has been shown to be profoundly altered in various types of precancerous and cancerous lesions, including cSCC.[9] The aldehyde dehydrogenase 1 (ALDH1) family consists of enzymes that produce RA via the oxidation of retinal.[7] The ALDH1A family is comprised of three members, ALDH1A1, ALDH1A2, and ALDH1A3. ALDH1A1 participates in the oxidation of retinal and acetaldehyde metabolism. In contrast, ALDH1A2 and ALDH1A3 are the key enzymes in the oxidation of retinal to RA.[7] Despite the similar structure and function of these isoenzymes, findings from multiple studies suggest that these enzymes perform different roles in cancer progression.[7,10-16] Expression of ALDH1A2 has been implicated in tumor suppression of prostate cancer, and head and neck squamous cell carcinoma (HNSCC), with high expression correlating with an improved prognosis.[15,16] The mRNA expression of ALDH1A3 has been linked to a poorer prognosis in pancreatic cancer and glioblastoma. In non-small cell lung carcinoma (NSCLC) and non-muscle invasive bladder cancer (NMIBC), suppressed ALDH1A3 expression was associated with impaired colony forming ability and growth, suggesting ALDH1A3 suppression as a possible treatment option.[17-20] Increasing the understanding of the expression of

ALDH1A2 and ALDH1A3 in cSCC can contribute to effective prognosis and treatment.

PROBLEM STATEMENT

The role of ALDH in aldehyde oxidation, minimization of ROS production, and mediation of RA signaling cascades allow ALDHs to play a significant role in cellular differentiation, proliferation, and tumorigenesis.[21] Accumulating evidence indicates ALDHs can be used for prognosis and treatment in various forms of cancer.[7,10-16] Further research needs to be done to determine which isoenzyme of the ALDH family can be used as a prognostic tool in cSCC. Based on research in mouse tissues in our lab, it is hypothesized that ALDH1A2 and/or ALDH1A3 will be altered in human tissue samples of cSCC, indicating a poor prognosis. Indication of this could provide further evidence of the role of ALDHs in cancer prognosis.

HYPOTHESIS

Previously in our laboratory, ALDH1A2 immunoreactivity decreased in the tumor and increased in the stroma, while ALDH1A3 increased in the tumor in SKH-1 mice during the progression to cSCC. The purpose of the current study is to compare the intensity and localization of ALDH1A2 and ALDH1A3 in human skin biopsies from patients with various stages of cSCC and healthy controls. The central hypothesis is that ALDH1A2 and ALDH1A3 intensity and/or localization will be altered in tissue samples of invasive cSCC, indicating a poor prognosis. The aims of this study are to:

AIM1: Identify changes in ALDH1A2 intensity and localization during the progression to cSCC in humans by immunohistochemistry.

AIM2: Identify changes in ALDH1A3 intensity and localization during the progression to cSCC in humans by immunohistochemistry.

DEFINITIONS

National Comprehensive Cancer Network – A not-for-profit partnership of 31 leading cancer centers committed to patient care, research, and education.

Dermal solar elastosis – A progressive condition of elastic tissue in the dermis cause by prolonged exposure to UV rays. This condition presents as yellow, thickened, coarsely wrinkled skin.

Extracapsular nodal extension – Tumor that has metastasized to the lymph nodes. This is associated with aggressive behavior of a tumor and a major risk factor for a poor prognosis.

Micrometastasis – A small collection of cancer cells, less than or equal to 2 mm, that has migrated from the original tumor and spread to another party of the body via the lymphovascular system.

ASSUMPTIONS AND LIMITATIONS

This study assumes the samples used are representative of the disease progression in cSCC and the size of the sample is sufficient to detect significant differences, if they exist. In addition, the reliability of the instruments used are consistent and valid. Limitations of this study include the lack of diversity in male

and female tissue samples and the contrasting alterations of the epidermis/dermis in varying disease states.

SIGNIFICANCE AND RATIONALE

Approximately 9500 people are diagnosed in the US with skin cancer every day. Every hour more than two people die of the disease.[22,23] Current research indicates 15-35 per 100 000 people are diagnosed with cSCC per year.[24] Diagnosed cases are on the rise and expected to increase 2-4% per year.[24] Consequently, research in cSCC is essential to not only improve preventative measures but expand curable treatment options. The study of the localization of ALDH1A2 and ALDH1A3 in skin cell tissue can provide pathologists with meaningful clinical biomarkers in the prediction of prognosis and therapeutic monitoring of cSCC and possibly contribute to early detection. In addition, a retrospective study of organ transplant recipients, who are at an increased risk for cSCC, found that low doses of the systemic retinoids Acitretin reduced cSCC recurrence for the first 3 years of treatment.[25] However, not all patients benefited from retinoid treatment.

The purpose of this present study is to detect the localization of ALDH1A2 and ALDH1A3 in SCCs. If differences in the localization of AlDH1A2 and ALDH1A3 are identified, this may lead to the development of biomarkers to assist clinicians in distinguishing between indolent and aggressive tumors. These biomarkers could aid in the selection of treatment and monitoring, especially the decision to use or not use synthetic retinoids.

CHAPTER II

REVIEW OF THE LITERATURE

CUTANEOUS SQUAMOUS CELL CARCINOMA

There exists a wide diversity of cSCC. Many having differing histopathology and clinical behaviors. Cutaneous squamous cell carcinomas can vary from indolent tumors with limited potential to metastasize to aggressive tumors with a high invasive potential.[26-29] The major characteristic of cSCC is the malignant transformation of normal epidermal keratinocytes.[30] The main pathogenic event involved in this transformation is the development of apoptotic resistance through the functional loss of tumor protein p53 (*TP53*), a tumor suppressor gene. The mutation of *TP53* is observed in over 90% of skin cancers diagnosed in the US, including precursor skin lesions, which implies that the loss of *TP53* is an initial incident in the growth of cSCC.[30] UV radiation causes damage to DNA by creating pyrimidine dimers, which are known to cause the genetic mutation of *TP53*. Continued exposure to UV radiation causes keratinocytes to experience clonal expansion, causing continual genetic defects and ultimately leading to invasive cSCC.[30] Mutations of B cell leukemia/lymphoma 2 (BCL2) and mitogen-activated protein kinase kinase kinase (MAP3K4) are also assumed to contribute to the pathogenesis of cSCC. In addition, changes in transcellular signal transduction pathways, such as cyclo-oxygenase (COX) and the epidermal growth factor receptor (EGFR) have been

indicated in the development of cSCC.[31] Causes of cSCC include UV radiation exposure, exposure to ionizing radiation or chemical carcinogens, immunosuppression, and human papillomavirus infection (HPV).[31] Cutaneous squamous cell carcinoma types discussed in this review are actinic keratosis (AK) and squamous cell carcinoma in situ (SCCIS), common precursors to SCC formation, and SCC and invasive squamous cell carcinoma (SCCI), whose tumors emerge from the invasive progression of the AK and SCCIS.[32]

AK lesions serve as precursors to SCC formation. These lesions develop from disproportionate UV damage on surfaces of the body commonly exposed to sun.[27-29] Immunosuppression can contribute to tumor development as well. As a result, patients receiving immunosuppression therapy are at a high-risk for the development of AK and SCC.[33] Clinical outcomes for AKs include spontaneous regression, long-term benign AK, or development into SCCI. A majority of SCCs are found to have evolved from AKs; however, only 5-10% progress to SCCI over time.[34] Characteristics of AKs include dysplasia of the keratinocytes in the basal layers of the epidermis or pleomorphic keratinocytes with nuclear atypia.[35] Hyperkeratosis and parakeratosis can often be observed, as well as a thinning granular layer. In addition, buds of atypical epidermis can extend towards the papillary dermis and inflammation may develop. Dermal solar elastosis in the dermis is almost always associated with AK.[36,37]

SCCIS lesions are characterized as superficial growths of cancerous cells located on the skin’s outer layer. The most common cause is excessive sun

exposure.[35] SCCIS is not considered a severe condition but the capability of development into SCCI ranges from 3-5%.[38] Metastatic rates in SCCI tumors are approximately 20%.[39] SCCIS is also called intraepidermal carcinoma, carcinoma in situ, or Bowen disease after John T. Bowen, an American dermatologist who first recognized the condition in 1912.[40-42] Histopathologically, SCCIS presents with prominent dyskeratosis and aberrant mitosis within all levels of the epidermis combined with distinct parakeratosis. Keratinocytes within SCCIS exhibit intense mitotic activity, pleomorphism, and enlarged nuclei. In contrast to AK, the basal epidermal membrane remains intact.[35]

SCC is a form of keratinocytic skin cancer. It often begins with AK or SCCIS.[43] Common SCC presents microscopically with nests and nodules of abnormal squamous epithelium which begin in the epidermis and invade the dermis.[44] SCC have eosinophilic, dyskeratotic or clear cytoplasm, and large nuclei. Keratinocytes in SCC are smaller and contain more basophilic nuclei at the edge of tumor nests.[44] Keratinocytes increase in size towards the center of the nests, where cell keratinization can be observed and leads to the production of parakeratotic keratin which produces eosinophilic pearls that provide differentiation.[44] These tumors can invade the subcutaneous fat, muscles, cartilage, fascia, and bones. Undifferentiated tumors may infiltrate the dermis without forming nests.[44]

Invasive SCC (SCCI) are often described as conventional SCC. A majority (97%) of SCCIs develop from the malignant progression of AKs.[35] As a result,

these lesions are considered to be different points on the same spectrum of disease.[45] Histopathologically, AKs and SCCIs resemble each other. However, in SCCI tumors, cell transmigration though the basement membrane to the dermis is often observed.[26,46] This migration tends to develop in the later stages of SCCI; therefore, early indicators to assist in diagnosis include a thickness of epidermal atypia and the participation of hair follicles.[47] The establishment of nests of atypical tumor cells in the dermis are observed in the advanced stages of invasion.[35] The majority of SCCIs are well-differentiated and consist of slightly enlarged, hyperchromatic nuclei keratin.[35] This results in the formation of extra cellular keratin pearls and intracellular bridges. It is important to note that these types of tumors have a low-malignant potential of approximately 0.5%.[48] Poorly differentiated SCCI with greatly enlarged nuclei and reduced keratin production also occur. This specific subtype of SCCI occurs less commonly and usually derived from AKs located on the ear and lip.[49] These subtypes are significantly more aggressive with an increased rate of metastasis.[50] A third subtype of SCCIs is found to be moderately differentiated. This subtype exhibits features found in both well-differentiated and poorly differentiated SCCI tumors.[35]

STAGING SYSTEMS

Staging systems have been created to assist with the prognosis of cSCC and identifying high-risk variants. The American Joint Committee on Cancer (AJCC) updated the *Cancer Staging Manual* in 2010 to include high-risk factors for primary tumor designation, although these updates excluded

immunosuppression and tumor recurrence (see Table 1). Critical changes made in the seventh edition of the *Cancer Staging Manual*, include allowances for stratification to the tumor stage category. High-risk factors added were a poorly differentiated tumor, tumor depth greater than 2 mm, Clark level of IV (see Table 2) or more, perineural involvement, and specific site locations such as, ear and non-hair bearing lip.[6] Even with these improvements, there is still a failure to identify key critical risk factors, such as immunosuppression and tumor recurrence, leading to an inability to stratify poor outcomes. Consequently, an alternative system, Brigham and Women's (BWH) tumor (T) staging system was created and allowed for the division of the T2 stage into separate groups, T2a and T2b.[51] (see Table 3) The goal of this new system was the ability to place patients with poorer prognosis in a higher T stage category. Risk factors for the BWH staging system include a tumor diameter of 2 cm or greater, poorly differentiated tumors, tumor invasion beyond fat, and perineural invasion of 1 mm or greater.[52] The AJCC guidelines have been updated to incorporate stratification of the regional lymph node designation to include, the number of nodes affected, dimensions of the tumor, and if the metastatic spread is ipsilateral or contralateral.[6] Studies are still finding inherent faults in the AJCC guidelines. One prospective study found the stratification of lymph nodes increased the complexity of the diagnosis but did not provide any benefit in prognosis.[53] In addition, survival rates did not increase with increases in nodal stage group, which is the purpose of the cancer staging systems.[53]

Table 1. American Joint Committee on Cancer (AJCC) tumor staging system.[72]

Designation	Description
T1	Tumor ≤ 2 cm in greatest dimension with fewer than two high-risk features
T2	Tumor ≥ 2 cm in dimension or tumor any size with two or more high-risk features
T3	Tumor with invasion of the maxilla, mandible, orbit, or temporal bone
T4	Tumor with invasion of skeleton or (axial or appendicular) or perineural invasion of skull base

Table 2. Clark's Levels of Skin Cancer[72]

	Anatomic Depth
Level 1	Melanoma confined to epidermis (in situ)
Level 2	Invasion into the papillary dermis
Level 3	Invasion to the junction of the papillary and reticular dermis
Level 4	Invasion into the reticular dermis
Level 5	Invasion into the subcutaneous fat

Table 3. Brigham and Women's Hospital (BWH) tumor staging system.[72]

Designation	Description
T1	0 high-risk factors
T2a	1 high-risk factor
T2b	2 to 3 high-risk factors
T3	4 or more high-risk factors or bone invasion

High-risk factors in cSCC include sub-clinical metastasis, cSCC that is staged as N0, and tumors that extend beyond the basement membrane.[4] A cSCC lesion with these risk factors brings the capacity to metastasize at a rate of up to 39%.[54] Tumors that extend to greater depths have a higher capacity to metastasize. Brantsch et al 2008 divided metastatic potential in three subcategories: ≤ 2 mm, no detectable risk; 2.1-6.0 mm, low-risk; > 6.0 mm, high-risk.[58] Others have established different subcategory values; however, most agree that cSCC < 2 mm are at minimal risk for metastasis. Histologic features are also important for prognosis as they can determine the aggressiveness of the cSCC tumor in evaluating prognosis. Broders first described histologic grading.[55] Grading consists of four grades ranging from I (one-fourth of the cell is differentiated) to IV (cells do not tend to differentiate within the tumor)[56] (see Table 4). The less differentiated the tumor, the poorer the prognosis and cure rate.[51,57] Anatomic location of the cSCC can play a key role in prognosis as well. Tumors located on the face, pre/post auricular, genitalia, hands, ear, and feet are all at a higher risk of metastasis. A recent prospective study of 615 patients identified the ear as a statistically significant risk factor for metastasis (P = 0.004).[58] Tumors, such as ear and lower vermillion lip, are considered high risk due to their proximity to lymphovascular structures or the thinness of the skin.[59] Other studies found tumors around the eyelid or periocular region to act more aggressively.[60] The findings of these studies have led the National Comprehensive Cancer Network (NCCN) to stratify anatomic risk level for

metastasis. Another clinical feature taken into consideration when studying the ability of the tumor to metastasize is horizontal size. Horizontal tumor size of > 2 cm suggests an increased ability for metastasis. AJCC guidelines requires a changing in the stage of the tumor from T1 to T2 when the horizontal size exceeds 2 cm.[61] Other risk factors that can alter tumor staging include, perineural and lymphovascular involvement, tumor recurrence, incomplete excision, and multiple tumors.[58] With incidences of non-melanoma skin cancer on the rise, cSCC warrants particular attention due to its ability to metastasize. Research into associated biochemical pathways and therapies targeted at these pathways hold a great potential for the future in decreasing risk of metastatic cSCC.

Table 4. Broders' Classification[72]

Parameter	Characteristics
Grade 1	0-25% undifferentiated cells
Grade II	25-50% undifferentiated cells
Grade III	50-75% undifferentiated cells
Grade IV	75-100% undifferentiated cells

VITAMIN A METABOLISM

Vitamin A is a term used to encompass a variety of biologically active fat-soluble substances derived from retinols.[62] The term retinoid is used to encompass all the natural and synthetic forms of the substance. These include, retinol, retinal, RA, and retinyl esters.[62] Similar in structure, the derivatives possess a β-ionone ring, a hydrocarbon side chain containing four double bonds

and a side group with an alcohol group (retinol), an aldehyde group (retinal), a carboxylic acid group (RA), or an ester group (retinyl ester).[63] Each one of these biologically active derivatives possess a specific function and are referred to as retinoids, which consist of all natural and synthetic forms of vitamin A.[43]

Vitamin A is primarily stored in the liver; however, extrahepatic storage occurs in the skin and other sites.[64] Chylomicrons deliver retinyl esters to the liver's parenchymal cells where retinyl ester hydrolase releases the fatty acids from the retinyl ester producing retinol.[64] Retinol binds with cellular retinoid binding protein (CRBP) and is subsequently esterified via Lecithin:retinol acyltransferase (LRAT) producing a retinyl ester that can then be stored in the stellate cells of the liver.[64] When vitamin A is needed, the retinyl esters can be converted back to retinol and combined with retinol binding protein (RBP) to produced holo-retinol-RBP.[64] This complex, combined with transthyretin, can transport retinol in the blood system. The holo-retinol-RBP-transthyretin complex has a half-life of 11 to 15 hours.[62]

Once the complex reaches the target cells, retinoic acid 6 (STRA6) protein transporter mediates the uptake into the cell.[65] In the skin, a majority of the retinol entering the keratinocyte is stored as a retinyl ester.[66] In the cytosol, retinol has different fates. Retinol can bind to CRBP to be esterified for storage or oxidized to form retinal.[62] Retinal can be oxidized via retinal dehydrogenases using zinc and nicotinamide adenine dinucleotide (NAD) producing RA.[64] RA can combine with CRABP2 and move into the nucleus where binds its transcription factor

receptor and regulates gene expression.[62] Retinol is a 20-carbon molecule and is the circulating form of vitamin A.[67] Retinol can be converted, via an oxidation reaction, to an aldehyde form known as retinal (retinaldehyde), which is critical to the function of vision (see Figure 1).[67] In this rate-limiting reaction, the alcohol group from the retinol molecule is replaced with an aldehyde group.[67] It is important to note that the cells can interconvert this reaction, reducing retinal back to retinol. Retinal is oxidized to produce RA, the biologically active form of vitamin A and a key signaling molecule that plays a role in differentiation of many types of cells.[67,68] RA can bind to the retinoic acid receptors RARα, RARβ, RARγ, that form heterodimers with retinoid X receptors, RXRα, RXRβ, RXRγ. These transcription factors facilitate the majority of RA actions and mediate gene expression for growth and development.[69]

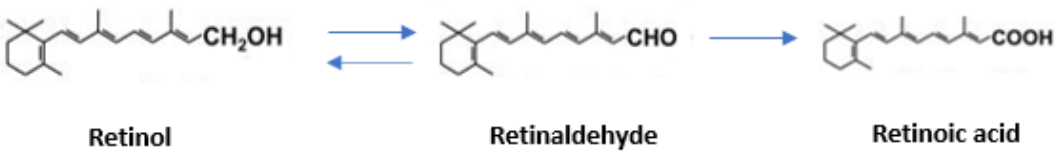

Figure 1. Retinol conversion *Redrawn based on information obtained from PubChem®

Several enzymes are involved in the biosynthesis of RA. In the first reversible reaction, retinol is oxidized to form retinal via retinol dehydrogenases, RDH1, RDH10, and DHRS9.[64] Retinal is further oxidized to form RA. This reaction is known to be catalyzed by ALDH1A1, ALDH1A2, and ALDH1A3, members of a superfamily of 19 NAD-dependent ALDHs each with specific

biological functions.[64,70] The conversion of retinal to RA is irreversible; therefore, these RA synthesizing enzymes play a critical role in RA activity.

CLINICAL USE OF RETINOIDS IN CSCC

Retinoid signaling is often disrupted early in the development of cSCC.[71] Retinoids' ability to induce differentiation and arrest proliferation makes them a potential candidate to treat cancers. Current treatment for cSCC is focused on surgery, radiation therapy (RT), chemotherapy or any combination of the these.[72] Although treatment can be effective, 91% of patients experience recurrence within 10 years.[72] This threat is increased for high-risk patients. Research has shown retinoids can play a critical role in the treatment of cSCC.[43] Both dietary and oral synthetic retinoids have shown promising results in the prevention and treatment of cSCC, but use of oral synthetic retinoids is limited due to their side effects.[73,74] A current prospective cohort study found high dietary vitamin A linked with a reduction in risk of cSCC after 23 years of follow up; however, at a 10-14 year follow up there was no significance.[75,76] In addition, the amounts of vitamin A and carotenoids consumed were several times greater than the recommended dietary allowances (RDA). These levels indicated excessive dietary vitamin A is preventative. Harwood et al found low-dose retinoids (0.2 to 0.4 mg/kg per day) significantly reduced cSCC in organ transplant patients in the first three years post-surgery.[25] This effect was sustained for approximately 8 years with a tolerable level of side effects, which included dry eyes, headaches, epistaxis, nail fragility, and pseudoporphyria.[25] Disadvantages of retinoids include adverse side

effects and the delivery method. Patients may experience mucocutaneous reactions, liver toxicity and abnormal serum lipid profiles, increasing risk of coronary heart disease, and increased fracture risk. Particularly concerning, is the teratogenic effect of all retinoids and possible skeletal abnormalities in long term use.[77] The delivery method can be challenging due to the rapid metabolism of some retinoids and changes within the cells can render retinoids resistant.[71] Therefore, retinoid research into prevention and treatment of cancer is critical.

FUNCTIONS OF ALDHS

Humans possess 19 known ALDH proteins that are found throughout the body.[7] ALDHs catalyze oxidation of a wide range or endogenous and exogenous aldehydes, provide cellular detoxification, and protect from reactive oxygen species (ROS; see Figure 2).[70] Although the prime function of ALDH enzymes is NAD(P)+ -dependent oxidation, studies have identified additional functions of this family of enzymes. ALDH1A1, ALDH2, ALDH3A1, and ALDH4A1 catalyze ester hydrolysis.[78] ALDH2 may possess nitrate reductase activity in the production of CGMP and vasorelaxation.[79] ALDH2 has been identified as an acetaminophen binding protein, which could weaken the biological activity of ALDH.[80] ALDH1A1 is a bound flavopyridol-binding protein in non-small cell lung carcinomas.[80] Endogenously, antioxidants and oxidative stress induces some ALDH genes to trigger a better response to environmental chemicals and drugs.[81] Increase ALDH expression in tumor cells can lead to degradation of chemotherapeutic

agents (cyclophosphamide and other oxazaphosphorines), which can produce negative clinical outcomes in treatment of cancer patients.[78]

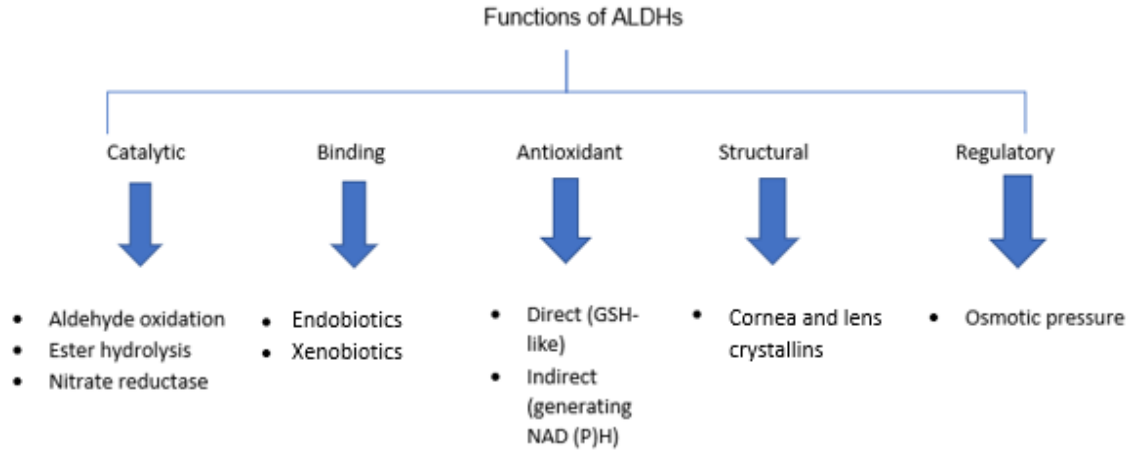

Figure 2. Various functions of ALDHs. Adapted from *Analysis and update of the human aldehyde dehydrogenase (ALDH) gene family* by V. Vasiliou and D. Nebert, 2005 Henry Stewart Publications.

The ALDH1A family consists of enzymes that produce RA via the oxidation of retinal, which is mainly involved in the biological functions of cell differentiation, cell cycle arrest, and eventually, apoptosis.[7] The ALDH1A family is comprised of three members, ALDH1A, ALDH1A2, and ALDH1A3. ALDH1A1 participates in the oxidation of retinal and acetaldehyde metabolism, and as previously mentioned, the detoxification of cyclophosphamide.[82] In contrast, ALDH1A2 and ALDH1A3 are the key enzymes in the oxidation of retinal to RA.[7] Despite their similar structure and function of these isoenzymes, multiple findings of studies suggest that these enzymes perform different roles in cancer progression.[7,10-16]

ALDH ALTERATION IN CANCER PROGNOSIS

ALDH expression is altered in various forms of cancer.[15,16,18,20,83,84] Elevated activity of ALDH1A2, ALDH1A3, ALDH1A7, ALDH2, ALDH3A1, ALDH4A1, ALDH5A1, ALDH6, and ALDH9A1 were seen in both normal and cancer stem cells.[85] As a result, these proteins may be both biomarkers for stem cells and critical regulators of stem cell functions.[82]

ALDH activity in cancer stem cells is used as a prognostic biomarker in clinical evaluation.[7] ALDH activity was found to play a crucial role in the biotransformation of many drugs that generate aldehydes.[7] Aldehydes can have both beneficial and detrimental effects on cancers.[7]

ALDH1A1 AND CANCER

Past research in ALDH activity has focused on ALDH1A1 protein expression and clinicopathologic considerations.[86] This includes the prognosis of patients with tumors. In colorectal carcinoma, head and neck cancer, gastric cancer, esophageal SCC, breast cancer, and bladder cancer, a high expression of ALDH1A1 correlated with the progression of the tumor, metastasis, and a poor prognosis.[87-96] Other studies showed ALDH1A1 to be a better prognostic marker in primary glioblastoma than age and *O*^{6}-methylguanine-DNA methyltransferase (MGMT) promoter methylation, two known prognostic markers for overall survival.[97]

ALDH1A2 AND CANCER

Expression levels of ALDH1A2 have been linked to prognosis markers in different types of cancers. Research by Seidensaal et al indicated an improved prognosis for patients with OPSCC with high expression of ALDH1A2 in the presence of CRABP2.[16] In addition, a high ALDH1A2 and CRABP2 immunoreactivity pattern acted as predictor for reduced progression and overall survival. In this same study, it was shown that inhibition of ALDH1A2-RAR signaling caused the loss of cell adhesion and formed a mesenchymal-like phenotype. Immunohistochemistry (IHC) was performed on tissue microarrays of sample OPSCC tumors. Immunoreactivity of RA transporter CRABP2 was performed, and expression patterns were evaluated by positive tumor cells and immunoreactivity intensity. Univariate Kaplan-Meier analysis showed a significant better overall survival for high CRABP2 compared to low CRABP2. Further analysis showed the prognosis for high ALDH1A2 tumors with high CRABP2 expression was associated with an increased probability for an improved overall survival. Tumors with high ALDH1A2 combined with the absence of CRABP2 dictated a poorer prognosis as did low ALDH1A2 tumors. Multivariate Cox progression models confirmed these results, showing the correlation of positive progression free survival and overall survival prognosis for OPSCC patients with high ALDH1A2 and high CRABP2 tumors. In search of a mode of action, FaDu cells were cultured with an ALDH1A inhibitor, WIN18.446 or an RAR inhibitor BMS493. Control cells formed well-defined cell clusters with tight cell-cell

junctions. Within 3 days, inhibitor treated cells displayed a loss of cell-cell junctions and a scattered phenotype. Within 6 days, FaDu cells detached and developed spheroid-like structures. Inhibitor treated cells showed a significant up-regulation of vimentin, Twist, and N-cadherin transcription levels as seen in mesenchymal-like morphology. In addition, this phenotype is associated with higher cell motility. To confirm, a scratch wounding assay with control was perform and as predicted, a clear trend towards accelerated migration was found in the inhibitor treated cells when compared to control. Combined, these studies suggest a correlation between low ALDH1A2 expression and poor prognosis by increasing metastasis.

In prostate cancer, ALDH1A2 has been implicated as a candidate tumor suppressor gene and retinoids could be utilized in prevention or treatment. An early molecular event in the development of prostate cancer is aberrant DNA methylation.[15] In a 2005 prostate cancer study by Kim et al ALDH1A2 expression was found to be silenced by aberrant DNA methylation.[15] ALDH1A2 was seen in normal prostate epithelium, hypermethylated in prostate cancer, and ALDH1A2 re-expression suppressed colony formation. To identify which genes were silenced by aberrant DNA methylation, researchers treated four different cell lines with the DNA methyltransferase inhibitor, 5-aza-dC. RNA from the treated and controlled cells were compared via microarray hybridization to complementary DNA (cDNA) microarrays of approximately 19 600 different genes. Of the 271 cells identified as low in expression and cross-referenced by a

gene list of other downregulated genes in prostate tumors, 25 genes were identified. Of these 25 genes, 19 were shown to be significantly negatively correlated with recurrence-free survival. Of those 19, one gene, ALDH1A2 was induced 6-fold by 5-aza-dC. This result was validated by semi-quantitative RT-PCR, which showed a significantly reduced expression in prostate tumors and this decreased expression was linked with a reduction in time the patient was recurrence free (P = 0.03). To examine the re-expression of ALDH1A2 in 5-aza-dC treated prostate cancer cells, researchers utilized IHC on normal and prostate cancer cells with an antibody specific for ALDH1A2. They found ALDH1A2 expression in the epithelial of normal prostate tissue but not in cancer tissue. It is important to note that this expression pattern could be affected by the limited stromal content of the tumor specimens. Researchers found the ALDH1A2 promoter was hypermethylated in prostate cancer cells using bisulfite sequencing. Transcript levels of ALDH1A2 were inversely correlated with DNA methylation levels. With ALDH1A2 responsible for the second and irreversible step in RA synthesis, researchers hypothesized a pathogenic connection between the decreased expression of ALDH1A2 and tumor development. To investigate this theory, researchers cultured DU145 cells with all-*trans* RA or 5-aza-dC, which induces ALDH1A2. Seeded at low density, both cells formed both smaller and fewer colonies and flatter, less-refractile morphology when compared to control cells. These discoveries taken together, support the implication that

ALDH1A2, as a tumor suppressor gene, could support the role of retinoids in the prevention of cancer and be utilized as a treatment in prostate cancer.

In an ovarian cancer study, an unfavorable prognosis, including a shorter disease-free time and overall survival, was associated with a low expression of ALDH1A2. Choi et al evaluated the expression of ALDH isoenzymes-encoding genes and investigated the role of ALDH1A2, a prominent downregulated gene in ovarian cancer.[83] Using six ovarian cancer cell lines and four human ovarian surface epithelial (HOSE) cell lines, researchers used an Illumina microarray to identify differentially regulated genes. Fifteen ALDH isoforms showed differential expression patterns and three isoforms (ALDH1A2, ALDH1B1, and ALDH9A1) were downregulated. ALDH1A2 was the most prominent downregulated isoform. Compared to normal cells, its expression was approximately 50-fold lower in ovarian cancer cells. This result was further validated by a real-time PCR and immunoblotting of ovarian cancer cell line and HOSE cell, which showed decreased ALDH1A2 expression levels. To determine methylation levels of ALDH1A2 in ovarian cancer cells lines, researchers employed several methods of validation. Conventional methylation-specific PCR analysis (MSP) indicated the methylation of ALDH1A2 was distinctly higher in ovarian cancer cell lines compared to HOSE cells. A real-time qRT-PCR revealed low levels of ALDH1A2 were reversed when treated with the demethylation agent 5-aza-CdR. Methylation status of ALDH1A2 was additionally confirmed in Methylation and Expression database of Normal and Tumor tissue (MENT). Researchers found

the hypermethylation of ALDH1A2 was significantly higher in ovarian cancer cells compared to normal ovarian tissue, confirming the downregulation of ALDH1A2 in ovarian cancer. This downregulation was found to correlate with poor prognosis in ovarian cancer patients. Utilizing IHC, researchers compare benign, borderline, and malignant ovarian tumor tissue to normal ovarian epithelial tissues. A decrease with tumor progression was observed in ALDH1A2 expression. A relationship was observed between ALDH1A2 immunoreactivity and early tumor state and serous cell type. Kaplan Meier plots showed patients with lower ALDH1A2 expression and those with advanced tumor state had a shorter disease-free and overall survival. For disease-free survival, multivariate analysis showed ALDH1A2 expression and tumor grade were independent prognostic factors. For overall survival, Cox proportional hazards model showed high ALDH1A2 expression, high tumor stage, serous cell type and old age were independent factors. Taken together, these results indicate that in ovarian cancer ALDH1A2 is linked with tumorigenesis, patient survival rate, and disease recurrence rate.

Similar results were identified in a 2016 study by Ma and Zhao. Most ovarian malignancies are epithelial in origin.[98,86] They are further characterized as, mucinous, clear cell, serous, endometroid, transitional cell tumors, and others. Despite ALDH1's activity being used as a stem cell marker in ovarian cancer, which isoenzymes are responsible remained obscure. Ma and Zhao found ALDH1A2 and ALDH1A3 expression associated with overall survival in

ovarian cancer patients.[86] They also determine the use of ALDH1A2 and ALDH1A3 as prognostic markers in specific types of ovarian cancer patients. Survival curves were plotted for all patients, serous cancer patients, and endometrioid cancer patients. Results determined no correlation between overall survival and high ALDH1A2 expression for all ovarian cancer patients followed for 20 years. In *TP53* wild-type ovarian cancer, high ALDH1A2 mRNA expression was significantly associated with a worse overall survival rate, HR 2.86 (1.56-5.08), $P = 0.00036$. Expression of ALDH1A3 below and above the median expression did not show a correlation to prognosis for all ovarian cancer patients. In contrast, high ALDH1A3 expression was associated with a decreased overall survival in grade II ovarian cancer patients, HR 1.53 (1.14-2.07), $P = 0.005$. In addition, high ALDH1A3 expression was significantly associated with an improved overall survival in *TP53* wild-type ovarian cancer patients, HR 0.56 (0.32-1.00), $P = 0.04$. Additional studies of ALDH1A2 and ALDH1A3 expression in certain types of ovarian cancer could be critical in the design of treatment and assessment of prognosis.

In summary, previous studies have shown ALDH1A2 expression linked to prognosis in cancer patients. In most of these studies, high expression of ALDH1A2 indicated an improved outcome and/or low ALDH1A2 expression indicated a worse outcome. In addition, ALDH1A2 was epigenetically silenced in both prostate and ovarian cancer. Treating both prostate and ovarian cancer cells with a methylation inhibitor increase ALDH1A2 expression and reduced

tumor cell growth. Patients presenting with OPSCC showed an improved prognosis with a high expression of ALDH1A2 only in the presence of CRABP2.[16] In contrast, increased ALDH1A2 expression indicated a worse overall survival rate when *TP53* was not mutated.[86] In cSCC, most patients have mutated *TP53*,[30] predicting that high ALDH1A2 expression in the tumor may be better. However, stratifying by *TP53* genotype may produce better results. Previous research in our laboratory with SKH1 mice revealed a reduction of ALDH1A2 in the tumor and an increase of ALDH1A2 in the stroma during the progression of SCC. This current study expects to see similar results in the human tissue during the progression of cSCC reduced ALDH1A2 expression in the SCCI tumor and increased ALDH1A2 in the stroma.

ALDH1A3 IN CANCER

Research has shown that the expression of ALDH1A3 varies with cancer locations and can be linked to the progression of cancer in patients. ALDH activity plays an important role in the resistance of drugs and in the disease progression of tumors, specifically melanoma.[99] Samson et al found that ALDH1A1 and ALDH1A3 had higher and broader expression in melanoma patients and ALDH1A3 correlated with a better overall survival in metastatic melanoma, specifically metastatic v-Raf murine sarcoma viral oncogene homolog B1 (BRAF)-mutant melanoma.[99] In addition, upregulation of glycolysis, hypoxia and angiogenesis was discovered in high ALDH1A3 cohorts of gene set enrichment analysis (GSEA). This would indicate BRAF/MEK inhibitor sensitivity

in these patients. A high ALDH1A3 expression was found in pre-treatment patients before BRAF/MEK inhibitor treatment, predicting a more positive treatment response in patients with BRAF-mutant melanoma. In determining these results, researchers analyzed expression levels of 19 ALDH enzymes from 244 patients with metastatic melanomas using The Cancer Genome Atlas (TCGA) database. Identifying ALDH1A3 as highly expressive, further research showed a correlation of high ALDH1A3 expression with a positive overall survival, $P = 0.023$. Stratified further by driver mutations, Kaplan-Meier survival analysis showed a favorable overall survival rate in BRAF-hotspot patients displaying a high ALDH1A3 expression. Stratification of the BRAF WT cohort rat sarcoma (RAS)-hotspot mutation, neurofibromatosis type 1 (NF1)-mutation, and triple wild type (TWT) revealed a favorable prognosis of RAS-hotspot and ALDH1A3 expression. Using a Cox PH model, researchers adjusted for potential age and sex bias, and confirmed better overall survival correlated with ALDH1A3 in all metastatic and BRAF-mutant patients. To explain the underlying reasons that contribute to better prognosis in high expressions of ALDH1A3 in BRAF-mutated melanoma patients, researchers compared high and low expression groups using the GSEA. High ALDH1A3 cohorts displayed an upregulation of pathways associated to a proliferative state. These pathways have been known to display an increased sensitivity to BRAF inhibitors. These pathways included epithelial mesenchymal transition, hypoxia, glycolysis, and angiogenesis. Research has shown cancer cells dependent on the production of ATP through

glycolysis are sensitive to BRAF inhibitors.[100] Also, inhibition of angiogenesis and hypoxia has been demonstrated through BRAF treatments nullifying their effect in melanoma progression.[101]

In non-small lung cancers (NSCLC), ALDH1A3 is a principal ALDH isoenzyme responsible for tumorigenicity in most NSCLCs and the inhibition of ALDH1A3 is a potential therapeutic approach. Several different populations of cancer stem cells have been identified in NSCLC. They include CD133, CD44, and ALDH.[102-105] In a genome-wide gene expression analysis, Shao et al identified genes differentially expressed in ALDH+ and ALDH- cells.[18] Researchers found that ALDH1A3 was the predominant isozyme responsible in most NSCLCs for ALDH activity, and inhibiting ALDH1A3 could potentially eliminate the ALDH+ subpopulation in NSCLCs. To expand on previous studies that demonstrated tumorigenicity and self-renewal capabilities of ALDH+ cells in NSCLC lines, researchers contrasted global gene microarray expression studies on ALDH+ and ALDH- cells from the same NSCLCs to find common gene expression differences in tumor cell subpopulations. Using Aldefluor assay, researchers separated ALDH+ and ALDH- cells from the cell lines. Researchers used anchorage-dependent and independent colony formation assays to confirm the colony-forming capabilities of ALDH+ cells. Gene expression differences were noted. The primary differences in the ALDH+ and ALDH- cells were the upregulated expression of ALDH1A3 in ALDH+ cells, leading researchers to hypothesize that ALDH1A3 is the chief ALDH isozyme in NSCLC. To analyze

ALDH1A3 protein expression, researchers employed IHC in 455 NSCLC specimens. Kaplan-Meier survival analysis revealed ALDH1A3 high expression correlated with better overall survival but not recurrence-free survival. In addition, a Western blot analysis confirmed that ALDH+ subpopulations were comprised of significantly more ALDH1A3 compared to the ALDH- cells. Researchers performed a knock down of ALDH1A3 using siRNAs in NSCLC cells and found this significantly impaired liquid colony-forming ability in all but one NSCLC lines. Researchers also noted that a shALDH1A3 knockdown of ALDH1A3 reduced its transcription expression by approximately 3 to 5-fold in two cell lines suggesting ALDH1A3 is the key isozyme for maintaining NSCLC ALDH+ cells and clonogenic growth *in vitro*. This research has shown that ALDH1A3 isozyme is a robust marker for a subpopulation of clonogenic subpopulation of NSCLC cells. These findings can provide critical information in developing treatment for the ALDH1A3 subset of lung cancer cells.

Studies have shown that breast cancer cells displaying high ALDH activity are tumorigenic.[84] Marcato et al used microarray gene expression analysis and immunohistological analysis of breast cancer tissues to show a correlation between ALDH1A3 levels and metastatic progression.[84] Researchers compared aldefluor activity and ALDH activity in seven breast cancer cell lines. To determine if expression level of any ALDH isoform correlated with the activity, mRNA was isolated from each cell line and researchers performed a qPCR with isoform-specific primers. Of the 19 isoforms, only levels of ALDH1A3 correlated

with the aldefluor activity in the cell lines. Researchers postulated that if aldefluor activity is a sign of cancer's potential to metastasize, then ALDH1A3 should be common in higher grade/stage breast cancer.[84] To verify this, researchers ran IHC on 47 breast cancer samples and found ALDH1A3 expression was the best predictor of the aggressiveness of the disease and its presence correlated significantly with higher grade tumors, higher cancer stages, and proximal metastasis.

In non-muscle invasive bladder cancer (NMIBC), ALDH1A3 may be an independent methylation marker, which may be sued for assessing the recurrence and progression of NMIBC tumors in patients.[108] Patients with NMIBC frequently relapse after initial treatment.[106, 107] It is a challenge for clinicians to develop monitoring procedures for patients at low risk and create more established methods to identify high-risk resistant cancers before they progress. DNA methylation patterns are emerging as a new method of identifying the development and prognosis of cancer. Due to this, researchers identified methylation markers to predict patient outcomes using microarray analysis of DNA methylation and RNA expression patterns in tissues from long-term follow-up patients with NMIBC.[108] Researchers identified methylated and expressed genes in NMIBC using an independent set of Infinium microarray methylation date from a western population. Pyrosequencing (PSQ) analysis using bisulfite-modified genomic DNA from 187 human bladder specimens was performed on four of the six candidate genes, Homeobox A9 (HOXA9), ISL LIM homeobox 1

(ISL1), ALDH1A3, and Eomesodermin (EOMES) previously identified. To test the reliability, the values obtain from the Infinium array and PQS were compared. The Pearson correlation coefficient of 0.715 to 0.940 identified the correlation in the acceptable range. ALDH1A3 showed significant inverse correlations to its methylation and expression levels. Methylation levels were compared to prognostic factors such as the number of tumors, tumor size, grade, and stage. Increases in the number, size, grade, and stage of tumors were significantly associated with the increased methylation values for ALDH1A3. Univariate and multivariate Cox regression analysis demonstrated that methylation markers in ALDH1A3 were significantly related to progression and recurrence and were independent predictors of disease recurrence. The findings of this study suggest that methylation markers of ALDH1A3 can be used to assist the assessment of disease relapse and progression in NMIBC patients as well as assist in the clinical process regarding therapy.

In summary, researchers have been able to link ALDH1A3 expression to the progression of cancer in patients. In metastatic BRAF-mutant melanoma, researchers found a correlation between high levels of ALDH1A3 and positive overall survival rate.[99] Similar results were found in ovarian cancer patients with normal (wild type) *TP53*. Results also indicated higher levels of ALDH1A3 correlated with a better overall survival rate in NSCLC.[18] Similarly, a significant correlation was found between low ALDH1A3 expression in NMIBC and increased tumor progression and recurrence.[108] In contrast to these studies,

ALDH1A3 high expression indicated a higher-grade tumor and increase cancer stage in breast cancer and worse overall survival in grade II ovarian cancer patients.[84,86] In a previous mice study in our laboratory, elevated levels of ALDH1A3 expression were observed in all stages of cancer progression compared to adjacent skin. This study is expected to see similar results in human cSCC tissue.

CHAPTER III

METHODS

RESEARCH DESIGN

This retrospective, blinded, controlled study employed qualitative and quantitative research design techniques. The focus of this study was to determine the intensity of expression and the localization of ALDH1A2 and ALDH1A3 in cSCC and surrounding tissues. IHC was utilized to determine varying levels of the protein in tissue. An excellent method for protein detection, IHC uses antibodies to detect proteins that have altered expression in abnormal cells.[109] By identifying specific markers, IHC provides invaluable information of protein localization and expression in tissue.[109] Identical methods for collecting data will be used for both aims discussed in this research.

SAMPLES

The study consisted of five groups of tissue samples. These groups were comprised of human skin biopsies from patients with cSCC, SCCIS, SCCI, precursor AK, and normal skin not exposed to sun control, collected at Ohio State University (OSU) with informed consent. A total of 74 Formalin-Fixed Paraffin-Embedded (FFPE) tissue sections (37 for each protein) were cut at OSU and mailed to Dr. Everts at Texas Woman's University (TWU). This study was approved by the Institutional Review Board (IRB) at both OSU and TWU. IRB approval was obtained on January 13, 2020.

IMMUNOHISTOCHEMISTRY

Indirect IHC was used to test the localization and immunoreactivity of the proteins of interest. FFPE tissue samples were rehydrated and de-waxed using Xylene and ethanol baths. After applying a heat-induced antigen retrieval, tissue sections were treated with 3% hydrogen peroxide, blocked with 3% bovine serum albumin (BSA) plus 2% normal goat serum (NGS), and a streptavidin and biotin blocking kit (Vector, Burlingame, CA). The samples were incubated overnight with affinity-purified rabbit polyclonal antibodies at 4 ˚C. To amplify the signal, multiple antibodies with high specificity were used, such as biotinylated anti-rabbit secondary and horseradish peroxidase-conjugated anti-biotin tertiary antibody. Chromogenic substrate 3-amino-9-ethylcarbazole (AEC+; Dako, Carpinteria, California) was applied, followed by counterstaining with Gils Hematoxylin III and mounted in aqueous mounting fluid. Hydrogen peroxide and BSA were obtained from Fischer Scientific (Pittsburg, PA). Normal goat serum and the secondary antibody were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The tertiary antibody was obtained from Bethyl Laboratories (Montgomery, TX). ALDH1A2 and ALDH1A3 antibodies were produced and validated in Dr. Ong's laboratory.[110,111]

SEMI-QUANTITATION OF IHC

Pictures were taken of each section of the epidermis and dermis of each tissue using a Nikon Eclipse 80i microscope for ALDH1A2 and ALDH1A3. Each slide was sectioned into a range of 9 to 16 pictures. The pictures of each section

were then scored blinded using NIS-Elements software. RGB (red, green, blue) thresholding values were set at: red (74-165), green (56-117), blue (66-109). Utilizing the Simple ROI Editor, sections were selected for scoring. Values for each section scored were exported into a spreadsheet and averaged for an overall score in both the epidermis and dermis of each tissue. Averaged scores were input into SPSS Statistics software (IBM) version 25.

Manual scoring was also performed for tissue samples. Tissues were scored blinded on a 4-point scale indicating the approximate percentage of positive cells present: 0.5 = 0-5%, 1 = 6-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100% and intensity of immunoreactivity: 1 = weak, 2 = moderate, 3 = strong, 4= very strong. Only cells exhibiting immunoreactivity of 3 and 4 are presented in the results as percent positive cells.

STATISTICS

Homogeneity of variance was tested using Levene's test of equality of error variances. When not significant (variances are equal), a 2 x 4 (gender x stage) multivariate analysis of variance (ANOVA) was conducted followed by a Tukey's post -hoc analysis. When there was an interaction between gender and stage then an interaction variable was created and one-way ANOVA with Tukey's post-hoc test was performed on this interaction variable. When Levene's test was significant (unequal variances), a Kruskal-Wallis one-way analysis of variance was used, followed by Mann Whitney U tests.

CHAPTER IV

RESULTS

ALDH1A2 ANALYSIS

IHC was performed to examine the expression and localization patterns of ALDH1A2 in AK, SCCIS, SCC, and SCCI compared to control, no-sun exposed skin. After scoring the epidermis of the control group and tumors for each stage of cSCC, using the Nikon Basic Research, results were analyzed using SPSS. For ALDH1A2, the mean for percent positive in the groups were as follows: Control (n = 16): 0.5367 (± 0.63515); AK (n = 3): 2.5372 (±1.4996); SCCIS (n = 6): 0.2573 (±0.33675); SCC (n = 7): 0.3417 (±0.66551); SCCI (n = 5): 2.5139 (±3.06190; see Figure 3). When analyzing well-differentiated and poorly differentiated SCCI tumors, the percent positive in well-differentiated tumors was 9.028565 (± 3.29292) and 0.18373333 (± 0.153472) in poorly differentiated tumors. Levene's test revealed that the variances were unequal; therefore, a Kruskal Wallis was used, followed by Mann-Whitney tests. Each tumor stage group and control group were compared to one another. Results indicated the percent positive in the AK group was significantly higher than the control, no sun group ($p < 0.025$), the SCCIS group ($p < 0.020$), and SCC group ($p < 0.030$). The percent positive in the SCCI group was significantly higher than the SCC group ($p < 0.028$; see Figure 3).

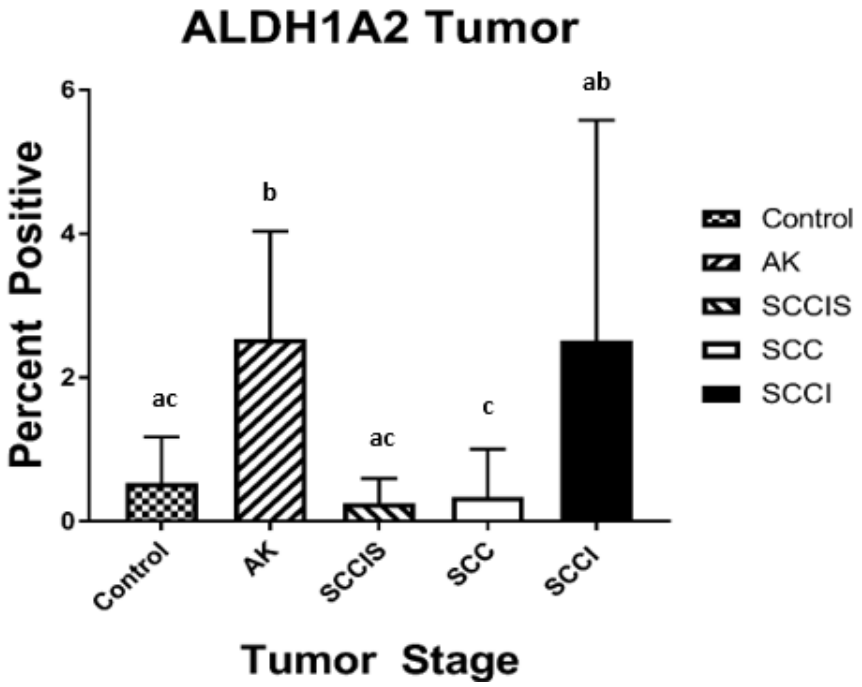

Figure 3. ALDH1A2 expression in tumor stages. ALDH1A2 percent positive expression levels of the control group and all tumor stages in human tissue. Immunohistochemistry was performed with antibodies against ALDH1A2, and tissues were scored using Nikon Basic Research and analyzed using SPSS. Results with different letters are significantly different from each other ($p < 0.05$). AK and SCCI lesions are significantly higher than SCC tumors.

Analysis was performed on the dermis of the control group compared to the stroma of all stages of tumor progression. Slides were scored manually on a 4-point scale for percent positive as 0.5 = 1-5%, 1 = 6-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100%. Levene's test revealed that the variances were unequal, therefore a Kruskal Wallis was used, followed by Mann-Whitney tests. There was a gradual increase in the percent of ALDH1A2 positive cells in the stroma as tumors progressed from SCCIS to SCC to SCCI (see Figure 4). A percentage of ALDH1A2 positive cells in the SCC and SCCI groups were significantly greater

than the control, no-sun group ($p < 0.005$); and the SCCI group was significantly greater than SCC ($p < 0.05$). The AK group was also significantly greater than the control, no-sun group and the SCCIS group ($p < 0.05$). Figure 5 shows the tumor images.

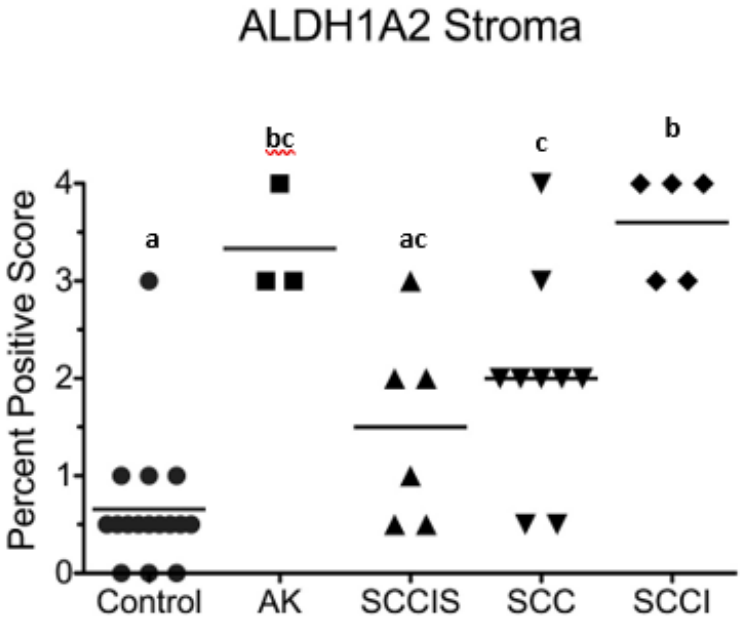

Figure 4. ALDH1A2 percent positive expression levels in the dermis of the control group and the stroma of all stages of tumor progression were plotted by group. Immunohistochemistry was performed with antibodies against ALDH1A2, and tissues were scored manually using a 4-point scale of percent positive cells from 0.5 = focal to 4 being 75-100% of the cells and analyzed using SPSS. Note the increase in expression from control tissue through SCCIS, SCC, and SCCI tumor stages. Results with different letters are significantly different from each other ($p < 0.05$).

ALDH1A2 Tumor Progression Stages

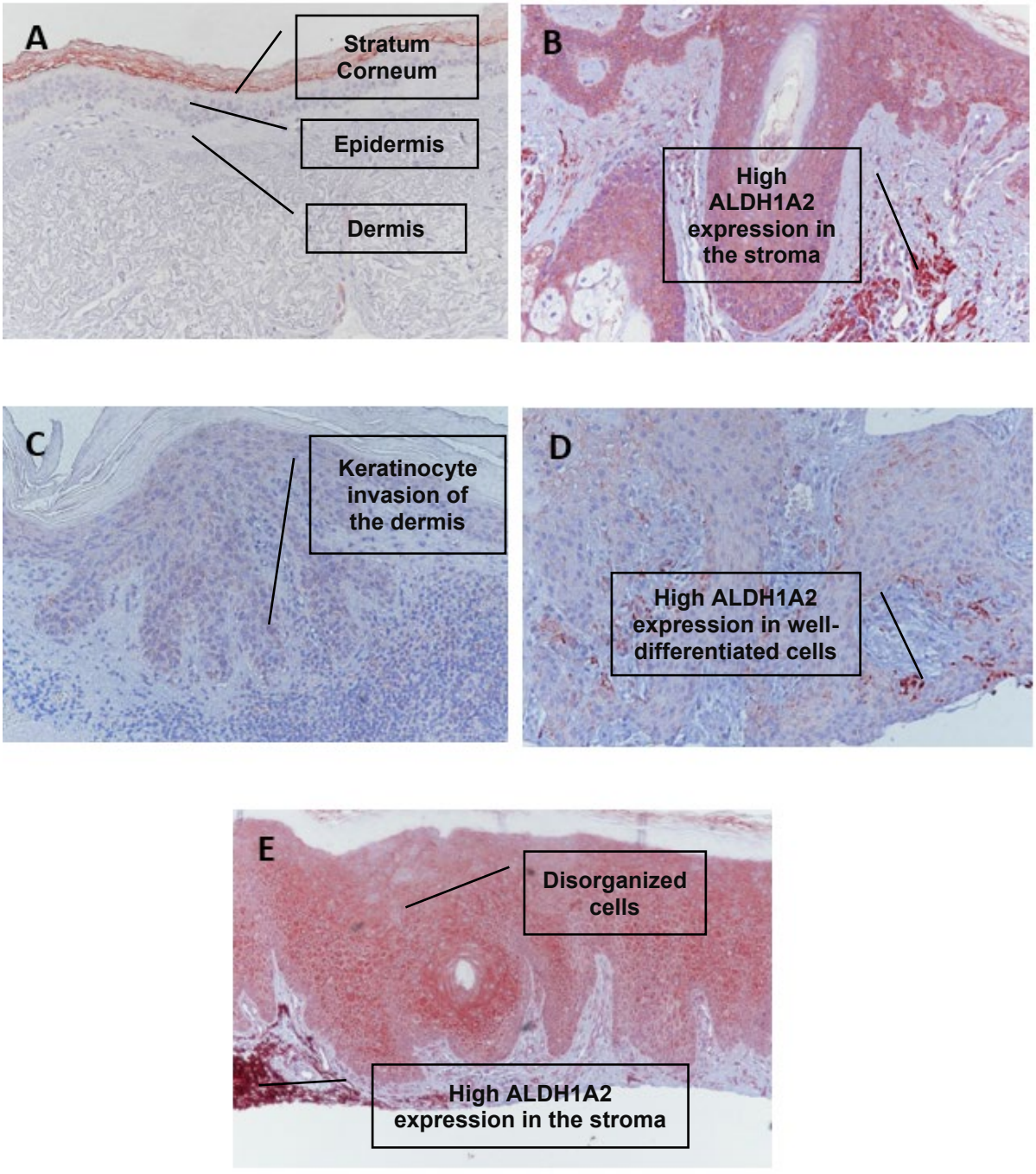

Figure 5. Expression of ALDH1A2 tumor progression stages in cSCC. Immunohistochemistry was performed with antibodies against ALDH1A2 using a red chromogen. A) Control group/No Sun B) Bowened AK C) SCCIS D) SCC E) SCCI. 10x objective x 10x eyepiece = 100x magnification.

ALDH1A3 ANALYSIS

IHC was performed to examine the expression and localization patterns of ALDH1A3 in AK, SCCIS, SCC, and SCCI compared to non-sun exposed skin biopsies. After scoring the epidermis of the control group and tumors for each stage of cSCC using the Nikon Basic Research software, results were analyzed using SPSS. For ALDH1A3, an ANOVA was run, followed by Tukey's post hoc test. The mean for percent positive in the groups were as follows: Control (n = 16): 1.1455 (± 0.90635); AK (n = 3): 4.2815 (±4.09982); SCCIS (n = 5): 1.2882 (±.2.52858); SCC (n = 7): 1.6521 (± 3.57307); SCCI (n = 5): .2886 (± .25239; see Figure 6). Tukey's post hoc test on all tumors showed no significant differences in the control group compared to the various stages of tumor progression. However, manual scoring using a 4-point scale of 0.5 = 1-5%, 1 = 6-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100% positive cells showed significant effects when analyzed by Kruskal Wallis followed by Mann-Whitney tests. The control, no sun group (n = 16) was significantly lower than the AK group (n = 3, $p < 0.006$), the SCCIS group (n = 5, $p < 0.05$), the SCC group (n = 7, $p < 0.05$), and the SCCI group (n = 5, $p < 0.05$; see Figure 6). The AK group was also significantly greater than the SCCIS group ($p < 0.05$), but there were no other differences between the tumor stages.

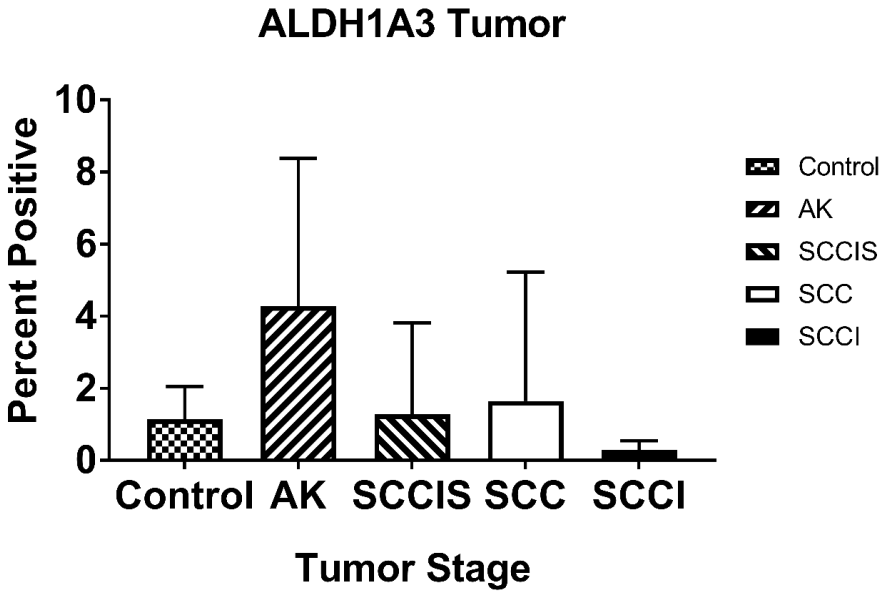

Figure 6. ALDH1A3 expression in all tumor stages. ALDH1A3 percent positive expression levels of the control group and all tumor stages in human tissue. Immunohistochemistry was performed with antibodies against ALDH1A2, and tissues were scored using Nikon Basic Research and analyzed using SPSS.

Analysis of the stroma within tumor progression stages compared to the dermis of the control tissues was performed. Manual scoring of the tissue was performed using the same scale mentioned above. There were no significant differences in the dermis of the control group compared to the stroma of the various stages of tumor progression (see Figure 7). Figure 8 shows the images of the tumors.

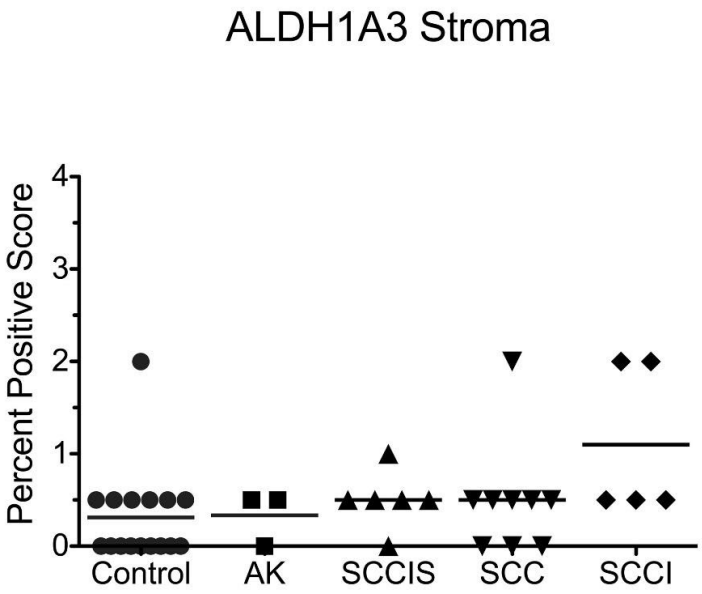

Figure 7. ALDH1A3 percent positive expression levels in the dermis of the control group and the stroma of all stages of tumor progression were plotted by group. Immunohistochemistry was performed with antibodies against ALDH1A3, and tissues were scored manually on a 4-point scale and analyzed using SPSS.

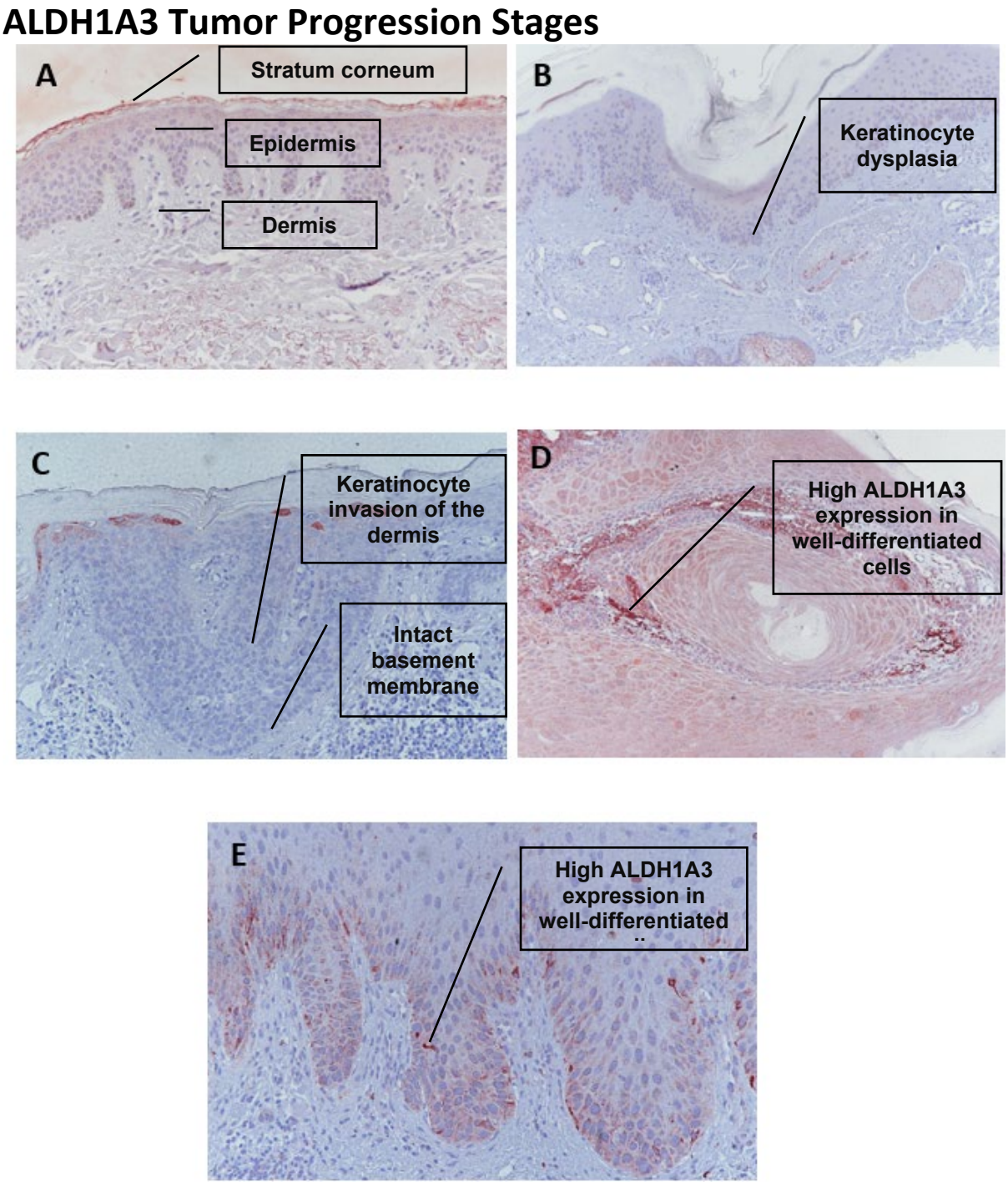

Figure 8. Expression of ALDH1A3 tumor progression stages in cSCC. Immunohistochemistry was performed with antibodies against ALDH1A3 using a red chromogen. A) Control/No Sun group B) Ak C) SCCIS D) SCC E) SCCI. 10x x 10x = 100x. magnification.

CHAPTER V

DISCUSSION

A number of previous studies have focused on the relationship between ALDH expression and the progression and prognosis for patients with various cancers. The role of ALDH expression as an anti-tumor agent is context dependent as results of these studies have shown varying levels of expression can indicate different prognosis depending on the cancer type.[15,16,18,83,84,86,99,108] In most cancer types high ALDH1A2 expression had a beneficial effect, however high ALDH1A3 had more mixed results. The present study investigated the expression and localization patterns of ALDH1A2 and ALDH1A3 in various tumor stages of cSCCs and hypothesized IHC immunoreactivity would demonstrate an altered intensity and/or localization of ALDH1A2 and ALDH1A3 in human biopsies of SCCI. Results of this study clearly demonstrated an increased expression of ALDH1A2 in the tumor and stroma of SCCI tissue samples and increased ALDH1A3 expression in the tumor of all cSCC tissue samples. A progressive increase in the percentage of ALDH1A2 positive cells in the stroma was observed as the tumor progressed through all stages and SCCI tumors expressed significantly higher ALDH1A2 positive cells than the SCC group. In ALDH1A3, significant differences were noted in manual scoring of the control (no sun) epidermis compared to the SCCI tumors, however there were no differences

between tumor stages. In addition, no significant changes were detected in the stroma of any SCC compared to the dermis of the control, no sun group.

Human skin is composed of two major compartments, the epidermis, and the dermis. Keratinocytes are the major cell type found the in the epidermis.[112] Initially located at the basal layer of the epidermis, keratinocyte cells divide and move upward through suprabasal layers.[109] Here the cells undergo terminal differentiation, which forms the protective barrier from the environment known as the stratum corneum.[112] The epidermis and dermis are separated by the basement membrane. Breakdown of the basement membrane, along with extra cellular matrix (ECM) remodeling, are hallmark properties associated with tumor progression and invasion.[113]

Well-differentiated epithelial cells possess extensive junctional networks. These networks and their functions act to restrict motility, preserve the integrity of the tissue, and allow cells to function cohesively as a unit.[114] As a carcinoma progresses, advanced tumor cells experience a down-regulation in epithelial cell markers resulting in increased cell motility and an expression of mesenchymal genes, known as epithelial to mesenchymal transition (EMT). EMT promotes common features of SCC, which include altered growth control, increased invasiveness, and loss of contact inhibition, which inhibits healthy cells from proliferating and growing.[114]

Analysis of the expression levels of ALDH1A2 in the epidermis compared to the tumors showed a significant increased expression in the tumor of the SCCI

group compared to the SCC group, suggesting more advanced tumors express more ALDH1A2. Similar results were found in a study of OPSCC patients where tumors with high ALDH1A2 expression levels in the absence of CRABP2 showed a poorer prognosis.[16] High ALDH1A2 also lead to worse outcomes in ovarian cancer patients with normal (wild type) *TP53*.[86] In contrast, lower levels of ALDH1A2 was associated with poorer prognosis in patients with prostate and ovarian cancers.[15] These differences may be more related to the differentiation level of the tumor, as well-differentiated SCCI tumors expressed higher percent positive ALDH1A2 cells when compared to poorly differentiated SCCI tumors; however, there were not enough samples to run statistics on this difference. RA regulates this differentiation primarily by binding to RARs. This initiates a cascade of changes in chromatin structure.[68] These changes initiate differentiation and promote stable epigenetic changes. In cancer cells, these changes can promote transformations that are less anaplastic. Tumors producing higher levels of ALDH1A2 and ALDH1A3 have the ability to produce more biologically active retinoids to promote this process.[68] In addition, the AK group and the SCCI group showed nearly identical percent positive expression levels. These similarities can be attributed to their close histopathological resemblance, which can be distinguished by SCCI tumor cell infiltration into the basement membrane.[26]

In the stroma, this study found the expression of ALDH1A2 in the SCC and SCCI groups was significantly higher than in the dermis of the control group.

Further analysis of the dermis compared to the stroma expression levels of ALDH1A2 showed a progressive increase from SCCIS to SCC to SCCI. The increased expression levels indicate that a high ALDH1A2 level in the stroma may lead to a poorer prognosis for patients. The stroma is crucial for the healthy maintenance and homeostasis of the epithelial tissues. Changes in the epithelial tissue inevitably induce changes in the stroma. This is especially true in the progression of tumors. Morphological changes seen in the stroma are cancer-associated fibroblasts (CAFs; desmoplasia) and ECM; angiogenesis, by way of newly formed blood vessels and lymph vessels; inflammation and immune response as seen in the development of lymphocytes, macrophages, and dendritic cells (DCs).[116] Tissue damage signals boost DCs maturation that promotes their capacity to regulate T cell responses including the control of regulatory T cells (Tregs).[117] Tregs are specialized T cells that suppress immune responses. All these actions play a role in tumor development and progression.

CAFs are activated fibroblasts located in the tumor microenvironment (TME).[118] Approximately, 60-70% of tumor tissue is composed of desmoplastic stroma which consists of collagen deposition and CAFs.[118] In breast, liver, lung, and kidney carcinomas, CAFs have differentiated via EMT.[199,120] CAFs not only provide a physical support for tumors, but also assist with tumor progression, metastasis, and therapeutic resistance. Guan et al found that RA showed therapeutic effects through the modulation of CAFs in the TME.[121] In pancreatic ductal adenocarcinoma, CAFs are found in the TME. Researchers treated CAFs

with RA and found the cells to become static and saw a reduction in ECM, indicating possible future therapeutic uses of RA in the treatment of CAFs.[121]

Growth of a vascular network is required for tumor growth and metastasis.[122] New blood vessels are required to deliver the needed nutrients and oxygen for growth. This is accomplished via angiogenesis. In a study by Muthukkaruppan et al tumor without a blood supply only grew to 1-2 mm^3, then stopped.[122] Tumors located in an area where angiogenesis was possible grew beyond 2 mm^3.[122] Thus, research for new therapeutic options like anti-angiogenesis are increasing in interest. In patients with poorly differentiated carcinomas, without the sodium-iodine say porter (NIS) or the TSH-receptor, are limited in therapeutic regimes such as radioiodine therapy and TSH-suppressive L-thyroxine therapy. Hoffman et al found an anti-proliferation effect of RA in thyroid cancer cell lines.[123] RA decreased secretion of vascular endothelial growth factor, VEGF, an autocrine regulator of angiogenesis in the thyroid gland. These results were more pronounced in poorly differentiated cells. All of this suggests an anti-angiogenic effect of RA and a negative effect on endothelial cell proliferation.[123]

The TME is comprised of a variety of different cell types and ECM components. The development of tumors depends on the interactions between the cells and the ECM.[124, 125] Immune cells, such as DCs and macrophages serve a dual function of both suppressing and promoting tumor growth.[125] DCs producing ALDH1A2 play an important role in gut immunity.[126] Dendritic cells in

the gut producing ALDH1A2 increase FOXP3-positive Tregs, which eliminate self-reactive lymphocytes,[126] and inhibit Th17 cell development that play an important role in the pathogenesis of immune mediated diseases.[127,128] DCs in the dermis function similarly to DCs in the gut.[128] Both the gut and the skin both function as major barriers to the outside world and are exposed to a multitude of environmental antigens. A study by Guilliams et al indicated mouse ear dermal DCs showed ALDH activity and induced FOXP3-positive Tregs.[129] In addition, ALDH1A2 localized to dermal DCs in C3H/HeJ mice.[128] These studies taken together suggest that RA synthesis in the skin and gut DCs regulate immune response. Macrophages play an important role in the regulating the immune function and the repair of damaged tissues and other processes in the development of cancer. Research has shown retinoids play a pivotal role in the anti-tumor process through differentiation, recruitment, and polarization of macrophages.[130] Tumor-associated macrophages (TAMs) are the majority of non-cancer cells within tumors.[131] In sarcoma, research by Devalaraja et al has shown monocytes within the tumor differentiate to immunosuppressive TAMs rather than immunostimulatory DCs, although the mechanism remains unclear.[132] Genetic and pharmacologic inhibition of RA production in tumor cells synergize the immune checkpoint blockade by increasing antigen producing cells (APCs) and anti-tumor immune responses.[132] In HNSCC, TAM infiltration is conducive to angiogenesis, therefore treatment to block the development of TAMs into the TME are utilized as antiangiogenetic therapies.[133,134]

Previous melanoma research has indicated that a high ALDH1A3 expression correlates with better overall survival rate in all metastatic types of melanomas and BRAF-mutant patients.[99] Similar results were found in NSCLC tumors with high expression of ALDH1A3 correlating with a better prognosis.[18] Contrasting these studies, a NMIBC study found increased methylation values of ALDH1A3 significantly related to tumor progression and recurrence.[17] Marcato et al found high levels of ALDH1A3 expression the best predictor of the aggressiveness of breast cancer tumors and correlated its presence significantly with higher grade tumors, cancer stages, and proximal metastasis.[84] Similar results were found in this study. Scoring of tissues indicated a significantly increased ALDH1A3 expression in AK, SCCIS, SCC, and SCCI groups compared to the control group (no sun).

In summary, this study found ALDH1A2 expression levels in the tumors of the SCCI group were significantly greater than the SCC group. Similar results were found in the stroma with a significant increase in the SCC and SCCI groups compared to the control group (no sun). ALDH1A3 expression was significantly higher in the AK, SCCIS, SCC, and SCCI groups compared to the control group (no sun). These results indicate specific patterns in the expression of these proteins through the different stages of tumor progression, which could provide prognostic information for treatment of cSCC. Although there appeared to be differences between well-differentiated and poorly differentiated tumors, this study did not obtain enough samples to determine with this certainty. Future

studies should focus on expanding the number of samples in each category, including an expanded sample size of well and poorly differentiated cells. In addition, prognosis/recurrence information for tissue samples would allow researchers to determine what combination(s) of tumor stages and protein expressions are at a higher risk for a lower survival/higher recurrence rate. This research can be expanded to studies with Aldh1A inhibitors and Aldh1A2 and Aldh1A3-defecient mice to determine beneficial or detrimental effects of these expression patterns and identify the mechanisms involved.

REFERENCES

1. Kurn H, Daly DT. Histology, epithelial cell. [Updated 2021 May 10] In: *StatPearls*. StatPearls Publishing; 2021 Jan.
2. Fabbri M. MicroRNAs and cancer epigenetics. *Curr Opin Investig Drugs*. 2008;9:583-590.
3. El-Deiry, Wafik S. Targeting mutant p53 shows promise for sunscreens and skin cancer. *J Clin Invest*. 2007;117(12):3658-3660.
4. Burton KA, Ashack KA. Khachemoune A. Cutaneous squamous cell carcinoma: a review of high-risk and metastatic disease. *Am J Clin Dermatol*. 2016;17:491-508. doi:10.1007/s40257-016-0207-3
5. Surdu S. Non-melanoma skin cancer: occupational risk from UV light and arsenic exposure. *Rev Environ Health*. 2014;29(3):255-264.
6. Warner CL, Cockerell CJ. The new seventh edition American Joint Committee on Cancer staging of cutaneous non-melanoma skin cancer: a critical review. *Am J Clin Dermatol*. 2011;12(3):147-154.
7. Vasiliou V, Nebert DW. Analysis and update of the human aldehyde dehydrogenase (ALDH) gene family. *Hum Genomics*. 2005 Jun;2(2):138-143. doi:10.1186/1479-7364-2-2-138
8. Mezquita B, Mezquita C. Two opposing faces of retinoic acid: induction of stemness or induction of differentiation depending on cell-type. *Biomolecules*. 2019;9(10):567. doi:10.3390/biom9100567
9. Xu N, Shao MM, Zhang HT, et al. Aldehyde dehydrogenase 1 (ALDH1) expression is associated with a poor prognosis of bladder cancer. *Cancer Epidemiol*. 2015 Jun;39(3):375-381.
10. Morgan CA, Hurley TD. Characterization of two distinct structural classes of selective aldehyde dehydrogenase 1A1 inhibitors. *J Med Chem.* 2015;58:1964-1975. doi:10.1021/jm501900s
11. Chute JP, Muramoto GG, Whitesides J, et al. Inhibition of aldehyde dehydrogenase and retinoid signaling induces the expansion of human hematopoietic stem cells. *Proc Natl Acad Sci USA.* 2006;103:11707-11712. doi:10.1073/pnas.0603806103
12. Corti S, Locatelli F, Papadimitriou D, et al. Transplanted ALDHhiSSClo neural stem cells generate motor neurons and delay disease progression of nmd mice, an animal model of SMARD1. *Hum Mol Genet.* 2006;15:167-187. doi:10.1093/hmg/ddi446
13. Condello S, Morgan C, Nagdas S, et al. β-Catenin-regulated ALDH1A1 is a target in ovarian cancer spheroids. *Oncogene.* 2015;34:2297. doi:10.1038/onc.2014.178
14. Moreb JS, Mohuczy D, Ostmark B, Zucali JR. RNAi-mediated knockdown of aldehyde dehydrogenase class-1A1 and class-3A1 is specific and reveals that each contributes equally to the resistance against 4-hydroperoxycyclophosphamide. *Cancer Chemother Pharmacol.* 2007;59:127-136. doi:10.1007/s00280-006-0233-6

15. Kim H, Lapointe J, Kaygusu, G, et al. The retinoic acid synthesis gene ALDH1a2 is a candidate tumor suppressor in prostate cancer. *Cancer Res.* 2005;65:8118-81124. doi:10.1158/0008-5472.CAN-04-4562
16. Seidensaal K, Nollert A, Feige AH, et al. Impaired aldehyde dehydrogenase 1 subfamily member 2A-dependent retinoic acid signaling is related with a mesenchymal-like phenotype and an unfavorable prognosis of head and neck squamous cell carcinoma. *Mol Cancer.* 2015;14:204. doi:10.1186/s12943-015-0476-0
17. Kim YJ, Yoon HY, Kim JS, et al. HOXA9, ISL1 and ALDH1A3 methylation patterns as prognostic markers for nonmuscle invasive bladder cancer: array-based DNA methylation and expression profiling. *Int J Cancer*. 2013 Sep 1;133(5):1135-1142. doi: 10.1002/ijc.28121
18. Shao C, Sullivan JP, Girard L, et al. Essential role of aldehyde dehydrogenase 1A3 for the maintenance of non-small cell lung cancer stem cells is associated with the STAT3 pathway. *Clin Cancer Res.* 2014 Aug 1;20(15):4154-4166. doi:10.1158/1078-0432.CCR-13-3292
19. Kong B, Wu W, Cheng T*,* et al. A subset of metastatic pancreatic ductal adenocarcinomas depends quantitatively on oncogenic Kras/Mek/Erk-induced hyperactive mTOR signaling. *Gut*. 2016;65:647-657.
20. Zhang W, Yan W, You G. Genome-wide DNA methylation profiling identifies ALDH1A3 promoter methylation as a prognostic predictor in G-CIMP− primary glioblastoma. *Cancer Lett*. 2013;328(1):120-125. doi:10.1016/j.canlet.2012.08.033
21. Singh S, Brocker C, Koppaka V. Aldehyde dehydrogenases in cellular responses to oxidative/electrophilic stress. *Free Radic Biol Med*. 2013;56:89-101. doi:10.1016/j.freeradbiomed.2012.11.010
22. Rogers HW, Weinstock MA, Feldman SR, Coldiron BM. Incidence estimate of nonmelanoma skin cancer (keratinocyte carcinomas) in the US population, 2012. *JAMA Dermatol.* 2015;151(10):1081-1086.
23. Mansouri B, Housewright C. The treatment of actinic keratoses-the rule rather than the exception. *J Am Acad Dermatol*. 2017;153(11):1200. doi:10.1001/jamadermatol.2017.3395
24. Lomas A, Leonardi-Bee J, Bath-Hextall F. A systematic review of worldwide incidence of nonmelanoma skin cancer. *Br J Dermatol*. 2012;166(5):1069-1080.
25. Harwood CA, Leedham-Green M, Leigh IM. Proby, CM. Low-dose retinoids in the prevention of cutaneous squamous cell carcinomas in organ transplant recipients. *Arch Dermatol.* 2005;4:456-464.
26. Lohmann CM, Solomon AR. Clinicopathologic variants of cutaneous squamous cell carcinoma. *Adv in Anat Path.* 2001;8(1):27-36.
27. Cassarino DS, Linden KG, Barr RJ. Cutaneous keratocyst arising independently of the nevoid basal cell carcinoma syndrome. *Am J Dermatopath.* 2005;27(2):177-178.

28. Cassarino DS, DeRienzo DP, Barr RJ. Cutaneous squamous cell carcinoma: a comprehensive clinicopathologic classification—part two. *J Cutan Path.* 2006;33(4):261-279.
29. Cassarino DS, DeRienzo DP, Barr RJ. Cutaneous squamous cell carcinoma: a comprehensive clinicopathologic classification—part one. *J Cutan Path.* 2006;33(3):191-206.
30. Brash DE. Roles of the transcription factor p53 in keratinocyte carcinomas. *Br J Dermatol*. 2006 May.154(suppl 1)8-10.
31. Hanneman KK, Cooper KD, Baron ED. Ultraviolet immunosuppression: Mechanisms and consequences. *Dermatol Clin*. 2006;24(1):19-25.
32. Charifa A, Volner K, Oakley A. Intraepidermal Carcinoma. [Updated 2020 Nov 10].*StatPearls*. StatPearls Publishing; 2021 Jan.
33. Zamoiski RD, Yanik E, Gibson TM, et al. Risk of second malignancies in solid organ transplant recipients who develop keratinocyte cancers. *Cancer Res*. 2017;77(15):4196-4203. doi:10.1158/0008-5472.CAN-16-3291
34. Rossi R, Mori M, Lotti T. Actinic keratosis. *Int J Dermatol.* 2007;46(9):895-904.
35. Yanofsky VR, Mercer SE, Phelps RG. Histopathological variants of cutaneous squamous cell carcinoma: a review. *J Skin Cancer.* 2011; 2011:210813. doi: 10.1155/2011/210813
36. Cockerell CJ, Wharton JR. New histopathological classification of actinic keratosis (incipient intraepidermal squamous cell carcinoma) *J Drugs Dermatol.* 2005;4(4):462-467.
37. Billano RA, Little WP. Hypertrophic actinic keratosis. *J Am Acad Dermatol.* 1982;7(4):484-489.
38. Cox NH, Eedy DJ, Morton CA. Guidelines for management of Bowen's disease: 2006 update. *Br J Dermatol.* 2007;156(1):11-21.
39. Kao GF. Carcinoma arising in Bowen's disease. *Arch Dermatol.* 1986;122(10):1124-1126.
40. Scalvenzi M, Villani A, Mazzella C, Fabbrocini G, Costa C. Cutaneous Bowen's disease: an analysis of 182 cases according to age, sex, and anatomical site from an Italian center. *J Med Sci*. 2019 Feb 28;7(4):696-697.
41. Fernández-Sánchez M, Charli-Joseph Y, Domínguez-Cherit J, et al. Acral and multicentric pigmented Bowen's disease in HIV-positive patients: report on two unusual cases. *Indian J Dermatol*. 2018 Nov-Dec;63(6):506-508.
42. Wozniak-Rito AM, Rudnicka L. Bowen's disease in dermoscopy. *Acta Dermatovenerol Croat*. 2018 Jun;26(2):157-161.
43. Everts HB, Akuailou EN. Retinoids in cutaneous squamous Cell carcinoma. *Nutrients*. 2021 Jan 5;13(1):153.
44. Schmitz L, Oster-Schmidt C, Stockfleth E. Nonmelanoma skin cancer – from actinic keratosis to cutaneous squamous cell carcinoma. *J Dtsch Dermatol Ges.* 2018;16:1002-1013.

45. Freeman RG. History of the American society of dermatopathology. *Am J Dermatopathol.* 1984;6(1): 25-33.
46. Anwar J, Wrone DA, Kimyai-Asadi A, Alam M. The development of actinic keratosis into invasive squamous cell carcinoma: evidence and evolving classification schemes. *Clin Dermatol.* 2004;22(3):189-196.
47. Ulrich M, Stockfleth E, Roewert-Huber J, Astner S. Noninvansive diagnostic tools for nonmelanoma skin cancer. *Bri J Dermatol*, 2007;157(2):56-58.
48. Alam M, Ratner D. Cutaneous squamous-cell carcinoma. *New Engl J Med.* 2001;344(13):975-983.
49. Lee D, Nash M, Har-El G. Regional spread of auricular and periauricular cutaneous malignancies. *Laryngoscope.* 1996;106(8):998-1001.
50. Rowe DE, Carroll RJ, Day CL. Prognostic factors for local recurrence, metastasis, and survival rates in squamous cell carcinoma of the skin, ear, and lip: implications for treatment modality selection. *J Am Acad Dermatol.* 1992;26(6):976-990.
51. O'Hara J, Ferlito A, Takes RP, et al. Cutaneous squamous cell carcinoma of the head and neck metastasizing to the parotid gland: a review of current recommendations. *Head Neck*. 2011;33(12):1789-1795.
52. Karia PS, Jambusaria-Pahlajani A, Harrington DP, et al. Evaluation of American Joint Committee on Cancer, International Union against Cancer, and Brigham and Women's Hospital tumor staging for cutaneous squamous cell carcinoma*. J Clin Oncol*. 2014;32(4):327-334.
53. Clark JR, Rumcheva P, Veness MJ. Analysis and comparison of the 7th edition American Joint Committee on Cancer (AJCC) nodal staging system for metastatic cutaneous squamous cell carcinoma of the head and neck. *Ann Surg Oncol*. 2012;19(13):4252-4258.
54. Gore SM, Shaw D, Martin RC, et al. Prospective study of sentinel node biopsy for high-risk cutaneous squamous cell carcinoma of the head and neck. *Head Neck*. 2015;38(1):884-889.
55. Broders AC. Squamous-cell epithelioma of the skin: a study of 256 cases. *Ann Surg*. 1921;73(2):141-160.
56. Weinberg AS, Ogle CA, Shim EK. Metastatic cutaneous squamous cell carcinoma: an update. *Dermatol Surg.* 2007;33(8):885-899.
57. Samarasinghe V, Madan V, Lear JT. Management of high-risk squamous cell carcinoma of the skin. *Expert Rev Anticancer Ther*. 2011;11(5):763-769.
58. Brantsch KD, Meisner C, Scho¨nfisch B, et al. Analysis of risk factors determining prognosis of cutaneous squamous-cell carcinoma: a prospective study. *Lancet Oncol*. 2008;9(8):713-720.
59. Breuninger H, Eigentler T, Bootz F, et al. Brief S2k guidelines—cutaneous squamous cell carcinoma. *J Dtsch Dermatol Ges*. 2013;11(Suppl 3):37-47.
60. Thosani MK, Schneck G, Jones EC. Periocular squamous cell carcinoma. *Dermatol Surg.* 2008;34(5):585-599.
61. Compton C, Byrd D, Garcia-Aguilar J, et al. Cutaneous squamous cell carcinoma and other cutaneous carcinomas. In: Compton CC, Byrd DR,

Garcia-Aguilar J, Kurtzman SH, Olawaiye A. Washington MK, eds. *AJCC cancer staging atlas.* Springer-Verlag New York. 2012:357-370.
62. Green, AS, Fascetti, AJ. Meeting the vitamin A requirement: The efficacy and importance of β-carotene in animal species, *Sci World J*. 2016 Oct 19. doi:10.115/2016/7393620
63. Takenouchi-Ohkubo N, Asano M, Chihaya H, Chung-Hsuing WU, Ishikasa K, Moro I. Retinoic acid enhances the gene expression of human polymeric immunoglobulin receptor (pIgR) by TNF-alpha. *Clin Exp Immunol.* 2004;135(3):448-454. doi:10.1111/j.1365-2249.2004.02398
64. Napoli JL. Physiological insights into all-trans-retinoic acid biosynthesis. *Biochim Biophys Acta Mol Cell Biol Lipids BBA-Mol Cell Biol L*. 2012;1821(1):152-167.
65. Kawaguchi R, Yu J, Honda J, et al. A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A. *Science.* 2007;315:820-825.
66. O'Byrne SM, Blaner WS. Retinol and retinyl esters: biochemistry and physiology. *J Lipid Res*. 2013;54(7):1731-1743. doi:10.1194/jlr.R037648
67. Kedishvili NY. Retinoic acid synthesis and degradation. *Subcell Biochem*. 2016;81:127-161. doi:10.1007/978-94-024-0945-1_5
68. Gudas LJ. Emerging roles for retinoids in regeneration and differentiation in normal and disease states. *Biochim Biophys Acta.* 2012 Jan;1821(1):213-221. doi:10.1016/j.bbalip.2011.08.002
69. Niederreither K, Fraulob V, Garnier JM, et al. Differential expression of retinoic acid-synthesizing (RALDH) enzymes during fetal development and organ differentiation in the mouse. *Mech Dev.* 2002;110:165-171. doi:10.1016/S0925-4773(01)00561-5
70. Clark DW, Palle K. Aldehyde dehydrogenases in cancer stem cells: potential as therapeutic targets. *Ann Trans Med*. 2016; Dec;4(24):518. doi:10.21037/atm.2016.11.82
71. Tang XH, Gudas LJ. Retinoids, retinoic acid receptors, and cancer. *Annu Rev Pathol*. 2011;6(1):345-364.
72. Burton KA, Ashack KA, Khachemoune A. Cutaneous squamous cell carcinoma: a review of high-risk and metastatic disease. *Am J Clin Dermatol.* 2016;17:491-508. doi:10.1007/s40257-016-0207-3
73. Tzimas G, Nau H. The role of metabolism and toxicokinetics in retinoid teratogenesis. *Curr Pharm Des.* 2001;7:803-831. doi:10.2174/138161201339770.
74. Lens M, Medenica L. Systemic retinoids in chemoprevention of non-melanoma skin cancer. *Expert Opi Pharmacother.* 2008;9:1363-1374. doi:10.1517/14656566.9.8.1363
75. Fung TT, Spiegelman D, Egan KM, et al. Vitamin and carotenoid intake and risk of squamous cell carcinoma of the skin. *Int J Cancer.* 2003;103:110-115. doi:10.1002/ijc.10798

76. Kim J, Park MK, Li W, et al. Association of vitamin A intake with cutaneous squamous cell carcinoma risk in the United States. *JAMA Dermatol*. 2019; doi:10.1001/jamadermatol.2019.1937
77. De Graaf YG, Euvrard S, Bouwes Bavinck JN. Systemic and topical retinoids in the management of skin cancer in organ transplant recipients. *Dermatol Surg.* 2004;30:656-661.
78. Sladek NE. Human aldehyde dehydrogenases: potential pathological, pharmacological, and toxicological impact. *J Biochem Mol Toxicol*. 2003;17 (7):23. doi:10.1002/jbt.10057
79. Sydow K, Daiber A, Oelze M, et al. Central role of mitochondrial aldehyde dehydrogenase and reactive oxygen species in nitroglycerin tolerance and cross-tolerance. *J Clin Invest.* 2004;113:482-489.
80. Vasiliou V, Pappa A, Petersen DR. Role of aldehyde dehydrogenases in endogenous and xenobiotic metabolism. *Chem Biol Interact*. 2000;129:1-19. doi:10.1016/S0009-2797(00)00211-8
81. Pappa A, Chen C, Koutalos Y, et al. Aldh3a1 protects human corneal epithelial cells from ultraviolet- and 4-hydroxy-2-nonenalinduced oxidative damage. *Free Radic Biol Med*. 2003;34:1178-1189. doi:10.1016/S0891-5849(03)00070-4
82. Vassalli G. Aldehyde dehydrogenases: not just markers, but functional regulators of stem cells. *Stem Cells Int*. 2019;2019:3904645. doi:10.1155/2019/3904645
83. Choi J-A, Kwon H, Cho H, et al. ALDH1A2 Is a candidate tumor suppressor gene in ovarian cancer. *Cancers*. 2019;11(10):1553. doi:10.3390/cancers11101553.
84. Marcato P, Dean CA, Pan D, et al. Aldehyde dehydrogenase activity of breast cancer stem cells is primarily due to isoform ALDH1A3 and its expression is predictive of metastasis. *Stem Cells*. 2011;Jan;29(1):32-45. doi:10.1002/stem.563.
85. Muzio G, Maggiora M, Paiuzzi E, Oraldi M, Canuto RA. Aldehyde dehydrogenases and cell proliferation. *Free Radic Biol Med*. 2012 Feb 15;52(4):735-746. doi:10.1016/j.freeradbiomed.2011.11.033
86. Ma YM, Zhao S. Prognostic values of aldehyde dehydrogenase 1 isoenzymes in ovarian cancer. *Onco Targets Ther.* 2016 Apr 4;9:1981-1988. doi:10.2147/OTT.S101063
87. Jiang F, Qiu Q, Khanna A, et al. Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer. *Mol Cancer Res*. 2009;7:330-338.
88. Xu SL, Zeng DZ, Dong WG, et al. Distinct patterns of ALDH1A1 expression predict metastasis and poor outcome of colorectal carcinoma. *Int J Clin Exp Pathol.* 2014;7:2976-2986.
89. Wang K, Chen X, Zhan Y, et al. Increased expression of ALDH1A1 protein is associated with poor prognosis in clear cell renal cell carcinoma. *Med Oncol.* 2013;30:574.

90. Yang L, Ren Y, Yu X, et al. ALDH1A1 defines invasive cancer stem-like cells and predicts poor prognosis in patients with esophageal squamous cell carcinoma. *Mod Pathol.* 2014;27:775-783.
91. Ginestier C, Hur MH, Charafe-Jauffret E, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell.* 2007;1:555-567.
92. Mieog JS, de KEM, Bastiaannet E, et al. Age determines the prognostic role of the cancer stem cell marker aldehyde dehydrogenase-1 in breast cancer. *BMC Cancer*. 2012;12:42.
93. Neumeister V, Agarwal S, Bordeaux J, Camp RL, Rimm DL. In situ identification of putative cancer stem cells by multiplexing ALDH1, CD44, and cytokeratin identifies breast cancer patients with poor prognosis. *Am J Pathol*. 2010;176:2131-2138.
94. Li XS, Xu Q, Fu XY, Luo WS. ALDH1A1 overexpression is associated with the progression and prognosis in gastric cancer. *BMC Cancer*. 2014;14:705.
95. Xu J, Muller S, Nannapaneni S, et al. Comparison of quantum dot technology with conventional immunohistochemistry in examining aldehyde dehydrogenase 1A1 as a potential biomarker for lymph node metastasis of head and neck cancer. *Eur J Cancer*. 2012;48:1682-1691.
96. Keymoosi H, Gheytanchi E, Asgari M, Shariftabrizi A, Madjd Z. ALDH1 in combination with CD44 as putative cancer stem cell markers are correlated with poor prognosis in urothelial carcinoma of the urinary bladder. *Asian Pac J Cancer Prev*. 2014;15:2013-2020.
97. Adam SA, Schnell O, Poschl J, et al. ALDH1A1 is a marker of astrocytic differentiation during brain development and correlates with better survival in glioblastoma patients. *Brain Pathol*. 2012;22:788-797.
98. Leung PC, Choi JH. Endocrine signaling in ovarian surface epithelium and cancer. *Hum Reprod Update.* 2007;13:143-162.
99. Samson JM, Ravindran Menon D, Smith DE, et al. Clinical implications of ALDH1A1 and ALDH1A3 mRNA expression in melanoma subtypes. *Chem Biol Interact.* 2019 Dec 1;314:108822. doi:10.1016/j.cbi.2019.108822
100. Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. *Proc Natl Acad Sci USA* 2004;101:781-786.
101. Clay MR, Tabor M, Owen JH, et al. Single-marker identification of head and neck squamous cell carcinoma cancer stem cells with aldehyde dehydrogenase. *Head Neck* 2010;32:1195-1201.
102. Bertolini G, Roz L, Perego P, et al. Highly tumorigenic lung cancer CD133+ cells display stem-like features and are spared by cisplatin treatment. *Proc Natl Acad Sci USA.* 2009;106:16281-16286.
103. Ho MM, Ng AV, Lam S, Hung JY. Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. *Cancer Res.* 2007;67:4827-4833.

104. Jiang F, Qiu Q, Khanna A, et al. Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer. *Mol Cancer Res.* 2009;7:330-338.
105. Levina V, Marrangoni AM, DeMarco R, et al. Drug-selected human lung cancer stem cells: cytokine network, tumorigenic and metastatic properties. *PLoS One.* 2008;3:e3077.
106. Babjuk M, Oosterlinck W, Sylvester R, et al. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder. *Eur Urol.* 2008;54:303-314.
107. Hall MC, Chang SS, Dalbagni G, et al. Guideline for the management of nonmuscle invasive bladder cancer (stages Ta, T1, and Tis): 2007 update. *J Urol.* 2007;178:2314-2330.
108. Kim YJ, Yoon HY, Kim JS, et al. HOXA9, ISL1 and ALDH1A3 methylation patterns as prognostic markers for nonmuscle invasive bladder cancer: array-based DNA methylation and expression profiling. *Int J Cancer*. 2013 Sep 1;133(5):1135-1142. doi:10.1002/ijc.28121
109. Hawes D, Shi SR, Dabbs DJ, et al. Immunohistochemistry. *Mod Surg Pathol*. 2009;48-70. doi:10.1016/B978-1-4160-3966-2.00016-3
110. Everts HB, King LE Jr, Sundberg JP, Ong DE. Hair cycle-specific immunolocalization of retinoic acid synthesizing enzymes Aldh1a2 and Aldh1a3 indicate complex regulation. *J Invest Dermatol.* 2004 Aug;123(2):258-263.
111. Everts HB, Sundberg JP, Ong DE. Immunolocalization of retinoic acid biosynthesis systems in selected sites in rat. *Exp Cell Res*. 2005 Aug 15;308(2):309-319.
112. Fisher, GJ., Voorhees, JJ., Molecular mechanisms of retinoid actions in skin. *The FASEB Jour*. 1996;10(9);1002-1013.
113. Commandeur S, Ho SH, de Gruijl FR, et al. Functional characterization of cancer-associated fibroblasts of human cutaneous squamous cell carcinoma. *Exp Dermatol*. 2011;20(9):737-742.
114. Christiansen JJ, Rajasekaran AK. Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. *Cancer Res*. 2006;66(17):8319-8326.
115. Williams SJ, Cvetkovic D, Hamilton TC. Vitamin A metabolism is impaired in human ovarian cancer. *Gynecol Oncol.* 2009;112(3):637-645. doi:10.1016/j.ygyno.2008.11.015
116. De Wever O, Mareel M. Role of tissue stroma in cancer cell invasion. *J Pathol* 2003;200:429-447.
117. Maldonado RA, von Andrian UH. How tolerogenic dendritic cells induce regulatory T cells. *Adv Immunol*. 2010;108:111-165. doi:10.1016/B978-0-12-380995-7.00004-5
118. Erkan M, Hausmann S, Michalski CW, et al. The role of stroma in pancreatic cancer: diagnostic and therapeutic implications. *Nat Rev Gastroenterol Hepatol*. 2012;9(8):454-467.

119. Iwano M, Plieth D, Danoff TM, et al. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest*. 2002;110(3):341-350. doi:10.1172/JCI0215518
120. Radisky DC, Kenny PA, Bissell MJ. Fibrosis and cancer: do myofibroblasts come also from epithelial cells via EMT? *J Cell Biochem*. 2007;101(4):830-839. doi:10.1002/jcb.21186
121. Guan J, Zhang H, Wen Z, et al. Retinoic acid inhibits pancreatic cancer cell migration and EMT through the downregulation of IL-6 in cancer associated fibroblast cells. *Cancer Lett*. 2014;345(1):132-139.
122. Muthukkaruppan VR, Kubai L, Auerbach R. Tumor-induced neovascularization in the mouse eye. *J Natl Cancer Inst.*1982; Sep;69(3):699-708.
123. Hoffmann S, Rockenstein A, Ramaswamy A, et al. Retinoic acid inhibits angiogenesis and tumor growth of thyroid cancer cells. *Mol Cell Endocrinol*. 2007;Jan 29;264(1-2):74-81.
124. Song N, Li T, Zhang XM. Immune cells in tumor microenvironment. *Prog Biochem Biophys.* 2014;41:1075-1084.
125. Domingues P, Gonzalez-Tablas M, Otero A, et al. Tumor infiltrating immune cells in gliomas and meningiomas. *Brain Behav Immun*. 2016;53:1-15.
126. Mercer F, Unutmaz D. The biology of FoxP3: a key player in immune suppression during infections, autoimmune diseases, and cancer. *Adv Exp Med Biol*. 2009;665:47-59.
127. Tesmer LA, Lundy SK, Sarkar S, Fox DA. Th17 cells in human disease. *Immunol Rev*. 2008;223:87-113. doi:10.1111/j.1600-065X.2008.00628
128. Duncan J, Silva K, Johnson C, et al. Endogenous retinoids in the pathogenesis of alopecia areata. *Journ of Invest Derm*. 2013;133:334-343.
129. Guilliams M, Crozat K, Henri S, et al. Skin-draining lymph nodes contain dermis-derived CD103 dendritic cells that constitutively produce retinoid acid and induce Foxp3 regulatory T cells. *Blood.* 2010;115(10):1958-1968.
130. Jiang L, Dong R, Ying M, et al. Immune cells in the tumour: new routes of retinoids for chemoprevention and chemotherapeutics. *Br J Pharmacol*. 2018;175(23):4285-4294.
131. Liu Y, Cao XT. The origin and function of tumor-associated macrophages. *Cell Mol Immunol*. 2015;12:1-4.
132. Devalaraja S, To T, Folkert I, et al. Tumor-derived retinoic acid regulates intratumoral monocyte differentiation to promote immune suppression. *Cell*. 2020;181:1098-1114.
133. Polverini PJ, Leibovich SJ. Effect of macrophage depletion on growth and neovascularization of hamster buccal pouch carcinomas. *J Oral Pathol*. 1987;16:436-441.
134. Liss C, Fekete MJ, Hasina R, et al. Paracrine angiogenic loop between head-and-neck squamous-cell carcinomas and macrophages. *Int J Cancer.* 2001;93:781-785.