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Photodynamic Therapy For Cutaneous Squamous Cell Carcinoma In-Situ & Mutation Burden Analysis

Yuemei Zhang

Photodynamic Therapy for Cutaneous Squamous Cell Carcinoma In-Situ
&
Mutation Burden Analysis.

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ABSTRACT

PHOTODYNAMIC THERAPY FOR CUTANEOUS SQUAMOUS CELL CARCINOMA IN-SITU & MUTATION BURDEN ANALYSIS. Yuemei (Amy) Zhang, Mei Zhong, Haifan Lin, and Sean Christensen. Department of Dermatology, Yale University School of Medicine, New Haven, CT.

SCC in-situ (SCCIS), or Bowen's disease, is a precursor to invasive squamous cell carcinoma (SCC) of the skin. Compared to other available treatment modalities, photodynamic therapy (PDT) may offer comparable efficacy with decreased morbidity and better cosmetic and functional outcomes. This retrospective study analyzes the effectiveness and outcomes of photodynamic therapy with aminolevulinic acid (ALA-PDT) treatment of cutaneous squamous cell carcinoma in-situ (SCCIS) with blue light. Data collection and statistical analysis was performed on the demographics, clinical history, and procedure details of patients who have biopsy-confirmed diagnoses of SCCIS treated initially with PDT. Treated lesions had a complete response rate of 55% (39/71) after initial PDT treatment. 83% (52/63) of lesions had a complete response with 1-2 cycles of PDT. Age and large size (>2 cm) were inversely correlated with complete initial response. The mean disease-free survival was 13.379 months (standard deviation of 2.0 months, C.I. [9.477, 17.280], 41 observations) for lesions receiving 1 PDT treatment, and 13.590 months (standard deviation of 2.3 months, C.I. [9.072, 18.107], 23 observations) for lesions receiving 2 treatments. There was no statistical difference between 1 and 2 PDT treatments in terms of disease-free survival function. Recurrence rates were 12% (4/33) following 1 PDT treatment and 32% (6/19) following 2 PDT treatments. Depending on clinical details, PDT may be an appropriate choice of treatment for SCCIS.

SCCIS and skin cancers arise when cancer-causing mutations develop and accumulate in keratinocytes. The majority of skin cancer cases are attributed to mutations caused by ultraviolet (UV) radiation. Specific UV-induced mutations in tumor-suppressor genes have been documented in normal skin, skin cancer precursors, and SCC lesions. This study aims to develop a system to quantify and characterize the mutations in *TP53, Hras, Nras, Kras, CDKN2a, Notch1, Notch2, Notch3, Fat1, Fgfr3, Knstrn*, and *Braf* in clinically and histologically normal skin, and relate this data to level of sun damage using next-generation sequencing. This project is still in process. At this point, primers have been designed to be used in concert with Illumina, Inc.'s extension-ligation system to target a total of 59,547bp within the aforementioned genes, and sequencing of an initial cohort of patient samples has been completed with a total sequence size of 1.54E11bp with 72.26% high-quality sequence. Ongoing work of sequence analysis will quantify the mutation burden in these samples and correlate these results to skin cancer history.

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INTRODUCTION

Skin Cancer

Skin cancer is the most commonly diagnosed cancer in the United States[1] and the most common malignancy worldwide[2, 3]. The incidence of both non-melanoma skin cancer (NMSC) and melanoma is increasing[1]. 99% of NMSC cases are basal cell carcinoma (BCC) or cutaneous squamous cell carcinoma (SCC).[4] One set of estimates is that the total number of NMSCs in the US population in 2012 at 5,434,193 and the total number of persons in the United States treated for NMSC at 3,315,554, with a BCC to SCC incidence ratio of approximately 1 based on the Medicare fee-for-service population.[5] In contrast, previous studies and multiple previous sources have reported that BCC is the most common type of NMSC and occurs at an approximately 4:1 ratio to SCC incidence. [6] A different sources states the average annual number of adults treated for skin cancer increased from 3.4 million in 2002-2006 to 4.9 million in 2007-2011 ($p<0.001$). During this period, the average annual total cost for skin cancer increased from $3.6 billion to $8.1 billion ($p=0.001$), representing an increase of 126.2%, while the average annual total cost for all other cancers increased by 25.1%. During 2007-2011, nearly 5 million adults were treated for skin cancer annually, with average treatment costs of $8.1 billion each year. [1]

Since risk of skin cancer correlates with increasing age, the aging baby boomer population may play a contributory role to changes in skin cancer rates.

Cutaneous squamous cell carcinoma (SCC) is the second most common type of skin cancer. Its incidence among the US Medicare fee-for-service population in 2012 is estimated to be 1,027,700, and while both BCC and SCC are increasing in incidence, SCC rates may be increasing at a faster rate than BCC.[5] A study of South Florida patients treated in 1996 for NMSC yielded a higher prevalence of SCC than BCC,[7] and a survey of Mohs fellowship directors showed an increasing ratio of SCC to BCC treated by Mohs.[8]

Environmental risk factors for skin cancer include exposure to ultraviolet (UV) radiation, chemical exposures, immunosuppression, immunocompromised conditions, chronic skin injury or irritation, and human papillomavirus (HPV) infection. Genus β human papillomavirus infection has been associated with SCC but not BCC.[9] NMSC is associated with chemical exposures to "pesticides, asphalt, tar and polycyclic aromatic hydrocarbons, and they typically result in SCC". [10] Arsenic is associated with both SCC and BCC. [11] Immunosuppression, such as in organ transplant recipients, and immunocompromised states, such as HIV infection, are also associated with increased risk of skin cancer, especially SCC, and the majority of SCCs in organ transplant recipients were found to contain HPV DNA[10]. Ionizing radiation has been implicated in SCC pathogenesis, which also means increased risk for airline pilots and other occupations involving increased exposure to ionizing radiation.[10]

UV radiation is the best known environmental risk factor for skin cancer, and in the northern hemisphere, decreasing latitude consistently correlates with increasing rates of skin cancer. [10] (Intensity of UV radiation generally increases as one gets closer to the Equator.) In SCC, there is a fairly straightforward correlation between cumulative

long-term UV dose and cancer risk, whereas BCC risk correlates more linearly with number of severe sunburns. [12] In Caucasians, skin cancer also tends to develop in areas with large amounts of sun exposure, with the head and neck as the most common sites of SCC[13, 14] and over 80% of NMSCs developing on the head, neck, and hands.[15] On the other hand, melanoma's most common anatomic sites vary by age group and gender, with melanoma occurring most commonly on the trunk in men and on the lower extremities followed by the trunk in women, but occurring most commonly on the head and neck in patients who were 80 years old or older. This may be related to cultural aspects of skin exposure that vary by age and gender.[15, 16] Furthermore, in the United States, where drivers sit on the left side of cars and therefore receive more UV radiation to the left side of their body, skin cancers are more likely to occur on the left than the right. [17]

Given the relationship between UV radiation and skin cancer, depletion of the ozone layer, an atmospheric layer that absorbs the majority of UV-B radiation from the sun, in recent history[18] may be contributory to the rise in skin cancer incidence.

Not only does UV radiation occur during typical outdoor activities, it can also occur during tanning lamp usage, therapeutic UV exposure, and occupational work like agriculture. Intentional tanning has been shown to increase the risk of both BCC and SCC [19]. Psoralen and ultraviolet A (PUVA) therapy is used to treat several skin conditions, such as atopic dermatitis, psoriasis, graft-versus-host disease, and vitiligo. Long-term PUVA therapy has been found to correlate with SCC risk in a dose-related manner. [20] Agricultural labor has also been associated with increased skin cancer risk. [10]

Humans have some natural protection against UV radiation in the form of melanin. Melanin is a naturally-occurring pigment in multiple organs and species, including human skin, and is produced by specialized cells called melanocytes which reside in the epidermis, among other places. It is produced and contained within organelles called melanosomes, which are then transported to nearby keratinocytes. [21] Epidermal melanin provides some protection against UV-induced DNA damage by partially filtering out UV radiation, and since darker skin tends to have more melanocyte activity and larger, more dispersed, and more melanized melanosomes than lighter skin, dark skin is more protective against UV-induced skin damage than paler skin. [22] Conversely, since UV-B rays are also necessary for vitamin D synthesis, darker-skinned people are more at risk for vitamin D deficiency than their lighter-skinned counterparts at the same latitude.[23]

Since susceptibility to UV-induced skin damage is dependent on intrinsic phenotypic characteristics, skin cancer, like many other cancers, has genetic risk factors as well. Light skin, red hair and to a lesser extent blonde hair, poor ability to tan, freckling, and by extension Caucasian race are well-known risk factors for both NMSC and melanoma.[24, 25] Certain *MC1R* alleles are associated with fair skin and red hair, which means they are also associated with increased melanoma and NMSC risk.[10] "Epidermal melanin in blacks filters twice as much ultraviolet B (UVB) radiation as does that in Caucasians,"[26] and a greater dose of UV radiation is required to produce erythema in black skin than in white.[21] In Hawaii, the incidence proportion of ethnic Japanese who develop NMSC is much lower than the incidence proportion for whites living in the same area. [27] Given the relationship between skin

color, tanning ability, and likelihood to develop sunburns, the Fitzpatrick skin type or phototype classification scheme was developed and adopted to characterize individuals' skin based on these characteristics, with lower numbers corresponding to generally lighter skin and greater likelihood of developing sunburns instead of tanning.[28]

Oculocutaneous albinism, an autosomal recessive condition which involves defective melanin synthesis, naturally also leads to an increased risk of melanoma and NMSC, especially SCC, compared to normal individuals.[10]

While skin pigment helps prevent DNA damage from UV rays, DNA repair mechanisms guard against skin cancer by repairing DNA after receiving UV-mediated damage.

UV radiation induces genetic mutations in the cells of sun-damaged skin primarily through the process of generating pyrimidine dimers in DNA, and to a lesser extent double-stranded breaks. Left uncorrected, these changes can be mutagenic or carcinogenic by leading to errors in DNA replication and transcription. However, numerous DNA repair mechanisms exist to combat this. Nucleotide excision repair is the main repair mechanism for UV-mediated damage involved in mammals, wherein thymine dimers are removed and then replaced on the DNA strand by polymerase and ligase.[29] In plants and some other organisms, photolyases can repair pyrimidine dimerization by harnessing the power of light to directly break intradimer bonds.[30]

However, in an autosomal recessive condition known as xeroderma pigmentosum, affected individuals have defective nucleotide excision repair enzymes. As a result, individuals under 20 years of age with xeroderma pigmentosum had over an 1000-fold

increase in rates of melanoma, BCC, and SCC compared to the general US population.[31]

Other genetic conditions that lead to an increased risk of skin cancer include dystrophic epidermolysis bullosa, which involves mutations in type VII collagen and is associated with SCC; Gorlin syndrome, also known as nevoid basal cell carcinoma syndrome, which involves a *PTCH* mutation and, as the name suggests, BCC; Bazex syndrome, which is associated with BCC; and Rombo syndrome, which is associated with BCC. Epidermodysplasia verruciformis is a rare inherited disorder that involves widespread cutaneous HPV colonization and an increased risk of SCC. [10]

Given their decreased susceptibility to UV-induced skin damage, the epidemiology of skin cancer in darker-skinned individuals is quite different. Instead of exposure to UV radiation, the most significant risk factor for SCC may in fact be chronic skin irritation and injury. [10] However, UV radiation still has an impact on skin cancer risk in individuals of color. Given differences in UV intensity and outdoor activities, people of Japanese ethnicity in Japan and Hawaii exhibit different rates of skin cancer.[32] UV radiation exposure has also been shown to have a significant effect on BCC risk in blacks. [26, 33]

Actinic keratosis and squamous cell carcinoma in-situ

Precursors to SCC include actinic keratosis and squamous cell carcinoma in-situ (SCCIS), also known as Bowen's disease (BD).

Actinic keratosis (AK) is a premalignant lesion containing atypical keratinocytes that are confined to the epidermis with no risk of metastasis, but it has the capacity to

transform into SCC.[10] The risk of AK evolving into SCC is estimated to be 0.075–0.096% per lesion per year.[10] Since the risk factors for AK are shared with risk factors for skin cancer, they can serve as markers for a high risk of developing SCC.[34] About 12% of Americans are estimated to have actinic keratoses.[35] Clinically, they tend to present as scaly erythematous papules varying in size.[10]

Squamous cell carcinoma in-situ (SCCIS), also known as Bowen's disease (BD), is a precursor to invasive SCC and refers to SCC that is confined to the epidermis.[36] Clinically, it can present as an erythematous plaque or patch with scale.[10, 36] SCCIS lesions are thought to have a 3-5% risk of progression to invasive SCC.[37] Treatment options for SCCIS include simple excisional surgery, Mohs surgery, cryotherapy, imiquimod, 5-fluorouracil, electrodessication and curettage, and photodynamic therapy (PDT).[36-38] Surgery has been reported to have low recurrence rates of 4.6%-8.3%.[39-42] Imiquimod, a topical immune response modifier, has been reported to have complete response rates of 93%[43] and 75%.[44] In the randomized controlled trial demonstrating 75% complete response, no recurrences occurred within the 9mo follow-up.[44] Fluorouracil, also known as 5-FU, is another topical treatment and has reported complete response rates of up to 92%[43] and 67%[45].

Tumorigenesis and Field Cancerization

During tumorigenesis, driver mutations in oncogenes accumulate, which leads to expansion of mutated clones and positive selection for clones with more malignant potential.[46, 47] These driver genes confer a selective advantage to the cells that have them.[46] In order for this model to work, the accumulation of random somatic mutations

in normal non-cancerous cells must be occurring in order for the accumulation of cancer-promoting mutations and malignant transformation to be possible.[48] Leukemia-associated somatic mutations have been shown to accumulate with age in blood cells in individuals without overt hematological disease.[49]

Specific mutations in tumor-suppressor genes have been documented in normal skin, actinic keratoses, and SCC lesions. Mutations in *TP53*, a tumor suppressor gene that plays a crucial role in the development of SCC, have been found in histologically and clinically normal skin[50, 51] and actinic keratosis,[52] a precursor of SCC. Up to 4% of normal skin contains keratinocytes with *TP53* mutations, and the mutation burden corresponds to actinic damage.[50] A high burden of *Notch1, FAT1, Kras, Nras,* and *Hras* mutations associated with skin cancer has also been found to exist in normal sun-exposed skin, and normal keratinocytes undergo pervasive positive selection for these mutations.[51] As part of the phenomenon known as "field cancerization,"[53] lesions of SCC are then more likely to develop in these "fields" of genetically-altered skin.[54]

Several mutations have been characterized in SCC, including functionally significant mutations in the tumor suppressor genes *TP53*, *Notch1*, *Notch2*, and *CDKN2A*.[55-57] SCC lesions have been reported to have mutation burdens of 63-95% in *TP53*,[56, 57] 82% in *Notch1* and/or *Notch2*,[57] and 28% in *CDKN2A*. [57]

TP53, for instance, is a tumor suppressor gene nicknamed "the guardian of the genome" which plays multiple critical roles inside the cell when DNA damage is incurred. [58] As a tumor suppressor gene, loss-of-function (LOF) mutations of *TP53* is generally associated with cancer, in contrast with oncogenes, where gain-of-function (GOF) mutations are associated with cancer.[59] It is located on the short arm of

chromosome 17 at 17p13.1 in humans. The gene is 19,149bp long with 11 exons, and is highly conserved across multiple species. [60, 61] p53, the protein encoded by *TP53*, is activated in response to DNA damage, oxidative stress, or other forms of cytotoxic stress.[62]

TP53 mutations have been implicated in multiple cancers, such as cutaneous SCC, BCC, melanoma, colon, lung, esophagus, breast, liver, brain, reticuloendothelial tissues, and hematopoietic malignancies.[63] Most functional *TP53* mutations occur in the DNA-binding domain, which affects p53's ability to activate downstream genes and induce cell cycle arrest or apoptosis.[62] Without proper p53 function, cellular mutations can rapidly accumulate without cell cycle arrest, which can ultimately lead to uncontrolled proliferation if the right set of mutations occur. This phenomenon occurs in Li-Fraumeni syndrome, a hereditary predisposition to cancer, including melanoma, involving autosomal dominant inheritance of a mutated *TP53* allele.[64]

Mutated keratinocytes in a field of damage appear to be precursors to SCC and SCC in situ. Many patients develop multiple primary lesions within the same field. Field treatment like PDT targets both the cancer and the surrounding field, so theoretically PDT may be more effective at preventing subsequent cancers than lesion-based therapy, such as surgery.

Although there is scientific awareness that field cancerization exists, laboratory-based methods of quantifying the mutation severity or the cancer risk of a field of skin, or of tracking response to therapy, do not exist for clinical use. Mutation burden analysis may be a useful clinical tool for these purposes.

Photodynamic Therapy (PDT)

Photodynamic therapy (PDT) is a treatment comprised of three necessary components: a photosensitizer, visible light, and oxygen, allowing for the generation of reactive oxygen species. The therapy involves two distinct stages: the first stage is application of the photosensitizer in the absence of light, and the second stage is the application of light. It has been utilized in a large number of dermatologic and oncologic conditions and in various anatomic sites, including skin conditions such as psoriasis, actinic keratosis, squamous cell carcinoma in-situ, basal cell carcinomas, as well as for cosmetic purposes; tumors of the oral cavity, pharynx, and larynx; Barrett's esophagus; cholangiocarcinoma; peritoneal carcinomatosis or sarcomatosis; prostate cancer; bladder cancer; and non-small cell lung cancer (NSCLC). [65, 66]

The use of light therapy dates back to the ancient Egyptians, who would treat vitiligo with sunlight and the oral ingestion of a plant that contained psoralen, a compound still used in modern medicine in combination with UV light to treat a variety of dermatologic conditions.[67] The history of contemporary phototherapy starts with Finsen using filtered light from a carbon-arc lamp to treat lupus vulgaris, a skin infection with *M. tuberculosis*, for which he was later awarded the Nobel Prize in Physiology or Medicine in 1903.[68] Shortly after, a German team discovered that acridine was a photosensitizer and coined the term "photodynamic effect," which they later used to treat solid tumors in one of the first applications of photodynamic therapy to cancer. [69-72]

Photodynamic therapy fell out of favor until Dougherty started clinical trials of PDT with hematoporphyrin derivative (HPD), a combination of porphyrins, as the photosensitizer in 25 patients with BCC, melanoma, chondrosarcoma, colon

adenocarcinoma, prostate cancer, mycosis fungoides, endometrial carcinoma, breast cancer, angiosarcoma, or SCC in 1978. The trial was successful and resulted in a commercially available photosensitizer. [69, 73] HPD was given the brand name Photofrin, and was "first given approval in 1993 by the Canadian health agency for use against bladder cancer."[74] Approval in more countries and for usage against more cancers quickly followed.[74] Even today, Photofrin is the most widely-used photosensitizer.[65]

The next major breakthrough in PDT research was the discovery that 5-aminolevulanic acid (ALA or 5-ALA) was a precursor of protoporphyrin IX (PPIX),[65] which opened the door for second-generation photosensitizers. In the heme biosynthetic pathway, ALA can be converted to PPIX and then ultimately heme. Normally, excess heme then provides negative feedback by inhibiting ALA synthase, the enzyme that synthesizes ALA, to prevent the accumulation of protoporphyrin and other intermediates. However, the addition of exogenous ALA bypasses this step, which then leads to an accumulation of PPIX within the cell because the final enzyme in the pathway, ferrochelatase, is rate-limiting.[66] In ALA-PDT, the pro-drug ALA is administered to patients and then converted to the photosensitizer PPIX by the patient's own cells.

Methyl aminolevulinate (MAL) is an esterified and therefore more lipophilic form of ALA, which allows topical MAL to penetrate skin more readily than ALA and achieve a higher intracellular concentration of PPIX.[75] It is used to treat similar conditions as ALA-PDT, and is commonly used in Europe, whereas ALA is used more commonly in the US.[66]

Photosensitizers such as PPIX are molecules that can absorb units of light energy, or photons, at specific wavelengths and then utilize this energy to undergo reactions with other molecules to eventually generate reactive oxygen species (ROS) and radicals, which then can inflict cellular damage. [74]

After absorption of a photon, the photosensitizer is excited to a singlet state, and then can rapidly transition directly to the ground state or drop to a less excited triplet state before dropping back to the ground state. Prior to returning to the ground state, the photosensitizer can generate phototoxicity through two different types of processes. In Type I processes, the photosensitizer, in its singlet or triplet state, interacts directly with a substrate via a reduction-oxidation (redox) reaction to generate a free radical (also known as "radical"), which can then interact with molecular oxygen to generate various reactive oxygen species (ROS). In Type II, the excited triplet photosensitizer directly undergoes a redox reaction with molecular oxygen to generate ROS. The Type II process predominates in the use of photodynamic therapy to generate cellular damage, although it is possible for both pathways to be at play in PDT. [74]

The ideal characteristics of a photosensitizer will vary depending on the context and therapeutic intent, but there are properties that are endorsed by multiple, although not necessarily all, sources:

1. Strong absorption in the visible or infra-red spectrum. Light at these wavelengths is readily available and has no tissue toxicity. Longer wavelengths in the 600-800 or 600-850nm range (red to deep or infra-red spectrum) may be superior for some applications.[65, 74] Light at these wavelengths can penetrate deeper into tissue,[74] but this feature is usually endorsed by sources referring to the usage of red

light for PDT, so it may be less relevant if another wavelength is being used and the main characteristic desired is for strong absorption in the relevant wavelength. However, light at longer wavelengths will likely not generate enough energy for the photochemical reaction.[65]

2. Have minimal dark toxicity. In other words, the ideal photosensitizer should not exhibit cytotoxicity in the absence of light.[65, 74] This allows the provider to have greater control over the effects of PDT by manipulating the amount and type of light provided.

3. Rapidly clear from the body. This should help decrease cytotoxicity. For instance, one of the most common adverse effects of PDT is photosensitivity in normal tissue, so rapid clearance could help reduce patient discomfort or inconvenience.[69]

4. For practical purposes in terms of production and clinical availability, ideally the photosensitizer would be a) a single well-characterized compound that b) has a simple or reliable means of production and c) a stable and reproducible formulation.[69, 74] Additionally, "The drug itself should be easily shipped and transported in a stable state and if needed to be reconstituted, this process should be possible by trained pharmacists without the requirement of specialized laboratories or tools. The administration of the drug itself should be possible in an outpatient setting."[69]

5. Selectively target or preferentially accumulate in diseased tissue over normal healthy tissue. [69, 74] During the topical application of ALA-PDT to treat neoplastic skin lesions, ALA may more readily penetrate tumor cells due to a disrupted stratum corneum, but this is not the main reason for its selective effect.[75, 76] Neoplastic cells are thought to have increased enzyme activity of porphobilinogen deaminase (PBGD),

which synthesizes a precursor of PPIX, and decreased ferrochelatase activity, which means PPIX accumulates more rapidly in diseased skin than normal skin.[75, 77, 78]

6. Be deliverable to the target tissue. One author suggests solubility in biological media for intravenous (IV) delivery,[74] while another suggests amphilicity- the dual properties of hydrophilicity for delivery through the bloodstream and lipophilicity to enter the tumor.[69] In the case of topical PDT for malignant or pre-malignant lesions, ALA's molecular size is small enough to allow penetration of the skin, and an esterified form of ALA, called methyl-aminolevulinate (MAL), has increased lipophilicity so it can penetrate deeper into the skin.[66]

7. Efficiently undergo the photodynamic reaction and efficiently generate ROS.[69, 74] Since the length of time spent in the triplet state affects the ability of the photosensitizer to generate ROS or interact with substrate, then longer durations of the triplet state are desirable.[74]

The majority of photosensitizers currently used in PDT have a cyclic tetrapyrrole or derivative structure. While other kinds of photosensitizers, such as methylene blue, acridine, and rose bengal, also exist, photosensitizers with a cyclic tetrapyrrole structure are used more predominantly in modern times because their structure is similar to endogenous porphyrins and therefore have low dark toxicity.[74] These second-generation photosensitizers include MAL, ALA, benzoporphyrin derivative monoacid ring A (BPD-MA), chlorins, bacteriochlorins, expanded porphyrins, and phthalocyanine derivatives. Further work is underway to develop third-generation photosensitizers through metalation or coupling with targeted biological molecules.[74]

The other important controllable component of the procedure is the light source. For electromagnetic radiation, which includes visible light, wavelength is inversely correlated with frequency, and since the Planck-Einstein relation states that the energy of a photon is directly correlated with its frequency, then photon energy is inversely related to wavelength. Above around 800nm, illumination is ineffective for PDT because it does not provide sufficient energy to excite the photosensitizer.[65] Since electrons can only absorb defined quantities of energy to reach an excited state, compounds will have absorption peaks at certain wavelengths. PPIX has a maximal absorption peak in the blue range at around 405nm[79] or 410nm[80] called the Soret band. It also has smaller absorption peaks in the visible range, called Q bands, at 505 or 510nm (green), 540nm, 575 or 580nm, and 635nm (red) wavelengths,[69, 80, 81] ROS are generated more rapidly at shorter wavelengths, which allows for shorter treatment times.[69] On the flip side, longer wavelengths of light are able to penetrate tissues more deeply, and the "optical window of tissue" is often considered 600nm-1200nm, which ranges from the red to near-infrared portions of the electromagnetic spectrum.[65] The skin's superficial nature is advantageous when it comes to PDT, since it means that light penetration is less of a concern than it may be for other bodily tissues and using shorter-wavelength higher-energy forms of light is feasible.

ALA-PDT and MAL-PDT have been used for the treatment of NMSC and other dermatological conditions. One of the earliest clinical trials involving ALA-PDT was a clinical trial in the early 1990s using 3-6hour incubation and 3.5 to 30min light exposure. Preliminary results from that clinical trial showed a complete response at the 2-3month follow-up in 72/80 (90%) BCC lesions, 6/6 lesions diagnosed either with early invasive

SCC or SCCIS, and 9/10 AKs. 2 SCC lesions that were raised ≥10mm above the surface of the skin were unresponsive to multiple treatments, and PDT had limited usefulness in treating the percutaneous nodules of 4 cases of metastatic breast cancer. [82] Another 1993 study using ALA-PDT with 4-8 hour incubation "observed a complete response after a single treatment for all 9 solar keratoses, 5 of 6 early invasive squamous cell carcinomas, and 36 of 37 superficial basal cell carcinomas. Only 1 of 10 nodulo-ulcerative basal cell carcinomas completely resolved. Eight cutaneous metastases of malignant melanoma were therapeutic failures."[83]

Both MAL-PDT and ALA-PDT are frequently used to treat actinic keratosis, and have been shown to have comparable results to other treatment modalities. Multiple randomized controlled trials have shown that both ALA-PDT and MAL-PDT have superior results compared to controls using a vehicle and light, with rates of complete response of greater than or equal to 70%.[84-86] Several trials also show that MAL-PDT can achieve similar treatment success rates as cryotherapy and 5-fluoruracil (5-FU) treatment. Depending on the study, PDT had complete response rates of 69-91% compared to complete response rates of 68-75% for cryotherapy, and when cosmetic outcomes and patient comfort were examined, PDT consistently performed superiorly to cryotherapy.[85, 87] One study that measured % reduction in lesional size instead of complete response rate had 70% reduction in lesional area for 5-FU treatment and 73% mean reduction in lesional area for PDT treatment.[88]

The results from a 1991-1992 phase I clinical trial using ALA-PDT to treat SCCIS, superficial BCC, and metastatic skin lesions from breast adenocarcinoma or SCC of the pinna were published by Cairnduff et al in 1994. This study had a complete

response rate of 89% in 36 cases of SCCIS, 50% complete response in superficial BCC, and poor treatment response in metastatic lesions.[89]

In the US, ALA, under the brand name Levulan, gained FDA approval for the treatment of AKs in Dec. 1999. [90]

PDT has also been used and studied in the treatment of BCC multiple times, and it can be an effective treatment against BCC.[91, 92] Compared to simple excision surgery, MAL-PDT does not have a statistically significant difference in complete response rates when treating superficial BCC, with complete response rates of 92.2% (118/128 lesions) after 1-2 treatments of PDT compared to 99.2% (117/118 lesions) for surgery. However, PDT performed significantly better in terms of cosmetic outcome.[93] In another study comparing PDT and surgery for nodular BCC, ALA-PDT and simple excision surgery had similar response rates of 94% (78/83) and 98% (86/88) respectively, but PDT had significantly higher recurrence rates within the first 3 years at 30.3% versus 2.3% respectively.[94] A 2012 study also found that ALA-PDT had statistically similar response rates and recurrence rates as surgery but superior cosmetic outcomes.[95] Compared to cryotherapy, PDT also has similar response rates, but it had 5% instead of 13% clinically obvious recurrences and performed significantly better in terms of cosmetic outcome.[96] In contrast, a different study found that for superficial BCC, imiquimod and 5-FU were more effective treatments than MAL-PDT.[97]

In contrast to BCC, PDT has been used much fewer times as a curative treatment for SCC, likely because SCC is more aggressive than BCC. Several cases have been documented of treating invasive SCC with PDT.[98, 99] Two studies have shown poor

response rates for SCC, one with 54% (19/35) complete response rate[100] and another with 25% (1/4) complete response rate.[81]

Since surgery performs as well or better than PDT in terms of efficacy when treating cutaneous malignancies, PDT may be preferable in cases where cosmetic outcome is particularly important, such as when the lesion size is large or the lesion is in an aesthetically sensitive area, in patients who are poor surgical candidates, when lesion size or location are contraindications to surgery, or when patient preference dictates that PDT is attempted first.

Treatment of Cutaneous Squamous Cell Carcinoma In-Situ with PDT

Although PDT is not FDA-approved for the treatment for SCCIS, several studies and case reports have been published about using PDT to treat SCCIS. Multiple treatment protocols exist, so studies and clinical treatments may use a variety of incubation times and light sources. A case report was published in 2000 in which ALA-PDT with 4-hour incubation with 20% ALA and red and near-infrared light successfully treated SCCIS on chronic radiation dermatitis and no recurrence was observed in 18 months of follow-up.[101] Then in 2001, a case series was published with 16 hour incubations with 2% ALA and "a newly designed light-emitting diode (LED) array with a peak wavelength of 630 nm," which is red light. The cases involved the treatment of SCCIS of the digit in 4 patients with chronic arsenicism, which resulted in 1 recurrence within the first 15-17mo following treatment.[102] The major risk factor for SCCIS in both these cases differs from the usual cause of SCCIS via sun exposure.

The largest randomized controlled trial of PDT for the treatment of SCCIS compared MAL-PDT to 5-fluorouracil treatment and cryotherapy, as well as placebo PDT. The light source used was 570-670 nm (red light), and incubation was 3 hours with 160mg/g MAL. "The clinically verified complete response rate of lesions 3 months after last treatment was 93% (103/111) in the methyl aminolevulinate PDT group, 21% (4/19) in the placebo PDT group, 86% (73/85) in the cryotherapy group, and 83% (24/29) in the fluorouracil group." Complete response rates at 12mo were not significantly different between PDT and 5-FU treatment, but cryotherapy performed significantly worse. "Lesion recurrence rates 12 months after the last treatment were 15% (15/103) in the methyl aminolevulinate PDT group, 50% (2/4) in the placebo PDT group, 21% (15/73) in the cryotherapy group, and 17% (4/24) in the fluorouracil group." "Cosmetic outcome (on-site evaluation) at 3 months was clearly superior with methyl aminolevulinate PDT compared with either cryotherapy or fluorouracil, with a good or excellent outcome in 94% (77/82) (95% CI, 86%-98%) of patients treated with methyl aminolevulinate PDT vs 66% (43/65) (95% CI, 53%-77%) treated with cryotherapy and 76% (16/21) (95% CI, 53%-92%) treated with fluorouracil; and this was maintained for 12 months."[103]

In 2007, a group in the Netherlands compared single illumination PDT, which is a typical single PDT treatment, against double-illumination PDT, wherein a lesion would be illuminated with 630 nm (red) light once at 4 hours after ALA application and once again at 6 hours after ALA application. Incubation utilized 20% ALA and lasted 4 hours. 25 lesions were assigned to each of the two treatment groups. Ultimately, the study found that complete response rate at 12mo was higher for the 2-fold illumination group at 88%

(22/25) as opposed to 80% (20/25), but this difference was not statistically significant.[104]

In 2008, a series of cases from Shanghai Skin Diseases & STD Hospital was published detailing their use of PDT for several conditions, including 13 cases of SCCIS. Their treatment procedures varied between cases, with 3-5 hour incubations with 20% or 10% ALA, 635nm light, and 3-5 treatments for all patients. 92.3% (12/13) of the SCCIS cases showed a complete response, and no SCCIS recurrences were reported in 12mo of follow-up.[99]

In 2009, a study was published looking at long-term follow-up in 19 cases of SCCIS. While the previous studies mentioned had follow-up lengths of around 18mo or less, this study followed patients for an impressive 60mo after treatment. Lesions were treated with 6hour 20% ALA incubation, 630nm (red) light, and 1 treatment session, and the therapy yielded 89.5% (17/19) tumor-free survival at 3mo and 53.3% (8/15) tumor-free survival at 60mo post-treatment.[105]

This was followed in 2011 by another study looking at long-term follow-up in PDT for SCCIS. 30 Caucasian patients with 43 lesions received 2 sessions of MAL-PDT 1 week apart, with 3 hour incubations with 160mg/g MAL and 635nm (red) light illumination. Lesions had 100% (43/43) complete response at the 6mo follow-up, and there was 11.6% (5/43) recurrence rate with a mean follow-up length of 50 months. Of the 5 recurrences, 2 of them occurred in immunosuppressed patients.[106]

In 2012, a case series of 51 SCCIS lesions treated by MAL-PDT was published. Incubation lasted 3 hours and used 160mg/g MAL, followed by illumination with 630nm (red) light. 76% (35/46) of treated lesions had a complete response, while 5 lesions were

not seen for a post-treatment evaluation. 12% (6/51) of treated lesions had a recurrence, with a mean follow-up time of 17 months from biopsy acquisition.[107]

A 2015 study included 31 SCCIS lesions treated with MAL-PDT. The treatment protocol involved incubation for at least 3 hours with 160mg/g MAL, illumination with 630nm (red) light, and 2 treatments spaced 1 week apart. At 3months post-treatment, 84% (26/31) treated SCCIS lesions exhibited a complete response. However, 53.8% (14/26) ended up having a recurrence within a median follow-up time of 43.5 months, with a 5-year estimated recurrence rate of 72%.[108]

In 2016, a retrospective study was published comparing the efficacy of MAL-PDT versus ALA-PDT for SCCIS, as well as BCC and AK. Incubation lasted 3 hours and either involved 20% ALA or 160mg/g MAL, followed by illumination with 630nm (red) light. If 2 PDT treatments were given, then the second treatment generally followed within a few weeks of the first. For SCCIS, 6 lesions received 1 session of ALA-PDT with a complete response rate of 83% (5/6), 3 received 2 sessions of ALA-PDT with a complete response rate of 100% (3/3), 4 received 1 session of MAL-PDT with a complete response rate of 75% (3/4), and 14 received 2 sessions of MAL-PDT with a complete response rate of 79% (11/14). Overall, complete response rate for SCCIS lesions treated with ALA-PDT was 89% (8/9) and complete response rate for SCCIS lesions treated with MAL-PDT was 78% (14/18). The difference between MAL-PDT and ALA-PDT was not statistically significant.[109]

All of these studies use red light illumination, even though MAL and ALA have multiple absorption peaks, and incubation times last at least 3 hours but can be overnight incubations in some cases. Most of the studies use ALA-PDT rather than MAL-PDT, and

the number of treatments varies. Reported complete response rates are good, ranging from 75% to 100%, although most studies had small sample sizes. However, long-term recurrence rates may also be high. The studies that looked specifically at long-term recurrence rates had average follow-up lengths of 43.5-60 months, with recurrence rates of 11.6%, 54%, and 57%.[105, 106, 108] The rest of the studies described had average follow-up lengths of 12-24 months. Cosmetic outcome appears to be very good with PDT, which could be a reason to choose it as initial therapy despite suboptimal response and recurrence rates.

STATEMENT OF PURPOSE

Photodynamic Therapy for Cutaneous Squamous Cell Carcinoma In-Situ
HYPOTHESIS

1) blue light ALA-PDT has a high complete response rate in the treatment of SCCIS

2) blue light ALA-PDT has a low recurrence rate or long disease-free survival interval in the treatment of SCCIS

SPECIFIC AIMS

1. Characterization of the clinical response rate of SCCIS lesions treated with blue light ALA-PDT and the relationship with factor variables including patient characteristics, lesion characteristics, and treatment procedure

2. Characterization of the recurrence rate of SCCIS lesions treated with blue light ALA-PDT

    a. Characterization of documented disease-free survival within the treatment field of SCCIS lesions treated with PDT

    b. Calculation of a Kaplan-Meier survival distribution function

    c. Comparison of disease-free survival between lesions receiving different numbers of PDT treatments

3. Determining the relationship between side-effects of PDT treatment and independent variables including patient characteristics, lesion characteristics, and treatment procedure

Mutation Burden Analysis

HYPOTHESIS

1) mutations in critical tumor-suppressor genes, namely *TP53, Hras, Nras, Kras, CDKN2a, Notch1, Notch2, Notch3, Fat1, Fgfr3, Knstrn, Braf*, exist in sun-damaged but clinically and histologically normal skin

2) the mutation burden corresponds with the level of sun damage and history of skin cancer.

SPECIFIC AIMS

1. Develop a sequencing strategy to detect rare somatic mutations in normal skin

    a. Detection of recurrent mutations in normal skin which correlate with previously described *TP53, Hras, Nras, Kras, CDKN2a, Notch1, Notch2, Notch3, Fat1, Fgfr3, Knstrn,* and *Braf* mutations in skin cancer

2. Comparison of mutation burden in *TP53, Hras, Nras, Kras, CDKN2a, Notch1, Notch2, Notch3, Fat1, Fgfr3, Knstrn,* and *Braf* with clinical degree of sun damage and other patient variables.

    a. Comparison of mutations in each individual gene and the composite mutation level in all 12 genes

    b. Analysis of mutation burden in relation to patient variables such as age, gender, biopsy site, and history of prior skin cancer (including both type and number of prior skin cancers).

METHODS

Photodynamic Therapy for Cutaneous Squamous Cell Carcinoma In-Situ

This was a retrospective study based on patients who had received PDT at Yale Dermatologic Surgery between 09/2013 to 03/2017 for a biopsy-confirmed diagnosis of SCCIS. Exclusion criteria included prior definitive treatment, such as Mohs surgery and 5-FU, for the same lesion; prior PDT for the same lesion; prior PDT in the same area as the lesion within the past year; and lack of any follow-up visits after PDT.

Prior to application of the photosensitizer, lesions were cleaned with acetone or isopropanol and any scale, crust, or superficial debris was removed. Crusted and keratotic areas were treated with curettage to the level of pinpoint bleeding. One tube of Levulan ® Kerastick® (20% aminolevulinic acid HCl) for topical solution) was applied to the area of each lesion. The area was then either wholly or semi-occluded with a Telfa® dressing, Glad® Press 'N' Seal® wrap and an opaque covering, or Tegaderm™ contact bandage and a Telfa® dressing, or left unoccluded. The medication was then allowed to incubate while protected from light. After the specified incubation time, any bandages were removed. Appropriate eye protection was used by the patient. The area was then treated with blue light at a wavelength of 417nm ±5 nm using the BLU-U® Blue Light Photodynamic Therapy Illuminator, with total fluence of 10 J/cm2 for 1,000 seconds or 16min and 40sec, apart from 1 patient who only received illumination for 4min due to side-effects. Patients were followed up as clinically appropriate. The treatments and follow-up visits were provided by Dr. Sean Christensen, Dr. Samuel Book, or Dr. David Leffell of the Yale Dermatologic Surgery practice.

We used the following search strategy to identify patients with SCC in situ treated with PDT: Patients of Yale Dermatologic Surgery (YMG) with a diagnosis code of SCC in-situ and a procedure code for PDT between the dates of 09/2013 and 03-2017 (inclusive) were initially collected by electronic database search Kasia Olszewski, an administrator in the Dermatology department. Chart review was subsequently performed by me and Dr. Christensen and only those patients with a record in Epic® of their diagnosis of SCC in-situ by a pathologist whose SCCIS lesion(s) were treated initially with ALA-PDT were included in the final study. A unique numerical identifier was assigned to each patient and lesion and associated with the clinical variables listed below.

I collected de-identified data on patient age, sex, visit dates, Fitzpatrick phototype; SCC in-situ characteristics: date of diagnosis, clinical history, lesion site/location, lesion size/depth, pathology report including pathologic subtype, prior treatments in same anatomic area, whether photographs were taken and appearance of the lesion in photos; treatment history and outcomes: including treatment types and dates, treatment protocols (incubation time, occlusion, illumination time), treatment side-effects, adverse effects post-treatment, results post-treatment (including lesion appearance post-treatment, tumor recurrence, or further treatments necessary and their types, dates, and results), follow-up dates and visit notes. For selected patients, photographic images in medical record that document tumor burden without identifying the patient were analyzed. Patient identifying information such as name, birthdate, medical record number or social security number, and address or phone number were not recorded as part of the study.

Statistical analysis was performed by me using Microsoft Excel®, XLSTAT®, and Stata®. Since patients may receive more PDT treatments long after their initial sequence due to a recurrence, for all analyses that required the number of PDT treatments, I used all PDT treatments that had been administered within a year of the previous PDT treatment, excluding PDT treatments that were administered after recurrence had occurred or after another form of treatment had been used, and referred to PDT treatments that fit these criteria with the phrase "consecutive treatments."

I used a contingency table and Fisher t-test to compare complete response rate between 1 consecutive PDT treatment and 2 consecutive PDT treatments. Since all lesions had to receive at least 1 PDT treatment according to our inclusion criteria, all lesions with information on response after first PDT treatment were included in the analysis of initial response to PDT treatment. Logistic regression was used for this analysis because the dependent variable was binary and the output of logistic regression is constrained to lie in the interval [0,1]. Lesions without follow-up data after the latest consecutive treatment were excluded as missing data. Missing variables were excluded from the regression instead of replaced with other values.

For disease-free survival analysis, patients were grouped according to number of consecutive treatments. Groups had mutually exclusive patients, and whether lesions had a complete or incomplete response to previous treatments did not matter for the survival analysis—only the lesion field's status after the latest treatment that met all above criteria did.

Disease recurrence or persistent disease were scored as event occurrences on the first date after all consecutive PDT treatments that disease was documented in the

medical record. Disease recurrence referred to documented disease after a documented complete response to treatment, whereas persistent disease did not involve a documented complete response to treatment. Since a lesion is only at risk for recurrence if it had a complete response, recurrence rate was defined as number of documented recurrences divided by the number of lesions that had had a complete response. If a lesion did not have a complete response to treatment, then for the survival analysis, it was noted as having an event occurrence on the date of its follow-up visit and disease-free during the time period between receiving PDT treatment and its follow-up visit. This holds true even if a lesion that had persistent disease after PDT treatment was then treated with another modality, such as liquid nitrogen or Mohs surgery, and exhibited a complete response to that treatment.

Since PDT treats an entire field and not just a specific lesion, an occurrence of new SCCIS within the same treatment field, even if it was not a recurrence of the previous SCCIS lesion, was marked as an event occurrence and equivalent to a recurrence for the survival analysis. Lesions that were disease-free and had no recurrence by the end of follow-up were labelled as censored on the last date of follow-up they were documented to be disease-free.

Mutation Burden Analysis

In order to study the mutation burden of actinically damaged skin, we will be using the techniques of punch biopsy, DNA extraction, PCR amplification, Illumina TruSeq Custom Amplicon® amplification, Illumina® next-generation sequencing, and DNA sequence analysis.

First, Dr. Christensen, Dr. Book, and Dr. Leffell collected discarded patient skin samples obtained during their surgical treatment of skin lesions. These discarded skin samples were obtained from clinically normal skin adjacent to a completely excised skin cancer. Each sample was categorized by Dr. Christensen as to the degree of clinical actinic damage.

Next, I took multiple punch biopsies ranging in size from 1mm to 4mm from the areas of normal skin in the discarded biopsy samples to compare DNA yields. Initially, 1mm punch biopsies were obtained, but based on DNA yield, subsequently 2mm punch biopsies were obtained.

I performed DNA extraction on the pooled punch biopsies via the Qiagen ® QIAamp® kit or phenol-chloroform extraction with ethanol precipitation, then performed PCR amplification on the extracted DNA samples for *TP53* and *HRAS* sequences to check DNA quality. We originally planned to use long-run PCR to amplify target genes, but this was unsuccessful.

The Illumina® TruSeq Custom Amplicon® system was used for library generation and sequencing. In this system, custom-designed oligo probes hybridize on the same strand to flanking regions of interest in unfragmented genomic DNA. Next, extension-ligation will occur between the custom probes across regions of interest. Unlike PCR, these amplicons would not serve as templates for subsequent rounds of amplification. PCR is then performed to add indices and sequencing primers to the ends of these amplicons, resulting in a tagged amplicon library ready for cluster generation and sequencing.

Dr. Christensen and I designed amplicons for the exons and 5' UTRs of *TP53, Hras, Nras, Kras, CDKN2a, Notch1, Notch2, Notch3, Fat1, Fgfr3, Knstrn,* and *Braf,* whose mutations are associated with skin cancer, and *VHL* as a control. We anticipate that we will be able to reliably detect mutations present at a frequency as low as 1% of the total input DNA based on the following calculation: Assuming 50% of reads are high-quality and able to be mapped to target regions, the Illumina HiSeq2500® sequencing machine has the capability to sequence 300 million reads at 150bp per read. Given our total target size of 59,547bp, this leads to a depth of sequencing of 23,616x per bp. The Illumina® HiSeqXTen® sequencing machine has even greater sequencing capability at 1 billion reads with 150bp per read, which leads to a calculated depth of sequencing of 78,719x.

Next-generation DNA sequencing via the Illumina® GA/HiSeq® system will be performed on the amplicons using the HiSeq2500® or HiSeqXTen®. In Illumina® sequencing, DNA molecules from the PCR products will be bound to primers on a slide and amplification will be performed to produce clusters of clonal DNA fragments, which are then linearized into single-stranded DNA templates.[110] These multiple clusters allow for the massively parallel process of sequencing by synthesis, wherein fluorescently-labeled nucleotides (ddATP, ddGTP, ddCTP, ddTTP) are added one at a time to the fragments and the fluorescent signatures are simultaneously recorded.[110] Mei Zhong is performing the library generation and the sequencing. Software for analysis then matches the sequence reads against known genomic sequences.[110] Computer-based analysis of the DNA sequencing results will eliminate irrelevant sequences, such as primer-dimers and other non-genomic DNA, and sequences of other genes. Resulting

sequence reads will be evaluated for the frequency of functionally significant mutations compared to silent mutations and wild-type sequences of these genes, which will then be analyzed for a correlation with the clinical degree of sun damage and number of prior skin cancers in patients from whom the biopsies were obtained. This analysis will be done by me and Dr. Sean Christensen.

RESULTS

Photodynamic Therapy for Cutaneous Squamous Cell Carcinoma In-Situ

62 patients, with a total of 74 unique lesions, who met our inclusion criteria were included in the study. Most patients with multiple PDT-treated lesions had them all treated during the same office visit, while 2 patients with multiple lesions had initiated PDT treatment for each lesion on separate dates. 51 patients had 1 lesion each, 10 patients had 2 lesions each, and 1 patient had 3 lesions. There were 31 male and 31 female patients, with an average age of 78 for both male and female patients, ranging from ages 52-98 years old for women and 50-94 years old for men. All patients with a documented Fitzpatrick phototype had one between 1-3, with 17 patients with phototype 1, 39 with phototype 2, 4 with phototype 3, and 2 patients whose phototypes were not recorded. (Table 1).

19 lesions were located on the scalp, 2 were on the ear, 42 were located somewhere on the face, 1 was on the neck, 1 was on the chest, 5 were located on the upper extremities, and 4 were located on the lower extremities (Table 2). 38 lesions were treated with only 1 cycle of PDT at this practice, 26 were treated with 2 cycles, and 10 lesions required treatment with more than 2 cycles in total (Table 3). However, many of the subsequent treatments occurred much later or were in response to recurrences. For instance, of the lesions that received 2 cycles of PDT, 2 of those received their second treatment after recurrence.

Number of patients:		62		Number of patients with separate lesions treated at the same time with PDT:			9
Number of lesions:		74		Number of patients with separate lesions treated at separate times with PDT:			2
Female	31			Male	31		
Age				Age			
50-59	3			50-59	4		
60-69	8			60-69	4		
70-79	3			70-79	7		
80-89	10			80-89	13		
>=90	7			>=90	3		
Age range	52-98			**Age range**	50-94		
Patients with 1 lesion:		51		Patients with 2 lesions:	10	Patients with 3 lesions:	1
Fitzpatrick phototypes:							
1	17						
2	39						
3	4						
unknown	2						

Table 1. Patient demographics

Scalp	19
Ear	2
Face	42
Neck	1
Chest or extremities	10

Table 2. Lesion sites

Lesions treated with 1 cycle of PDT (prior to any recurrence):	38
Lesions treated with 2 cycles of PDT:	26
Lesions treated with 3 or more cycles of PDT:	10
Lesions with complete response after 1 treatment:	39
Lesions with incomplete or partial response after 1 treatment:	28
Lesions with minimal or no response after 1 treatment:	4
Lesions without information on response after 1 treatment:	3
Lesions with recurrences:	9
Persistent lesions:	7
Unclear if persistent or recurrent:	1

Table 3. Treatment cycle numbers, responses, and recurrences

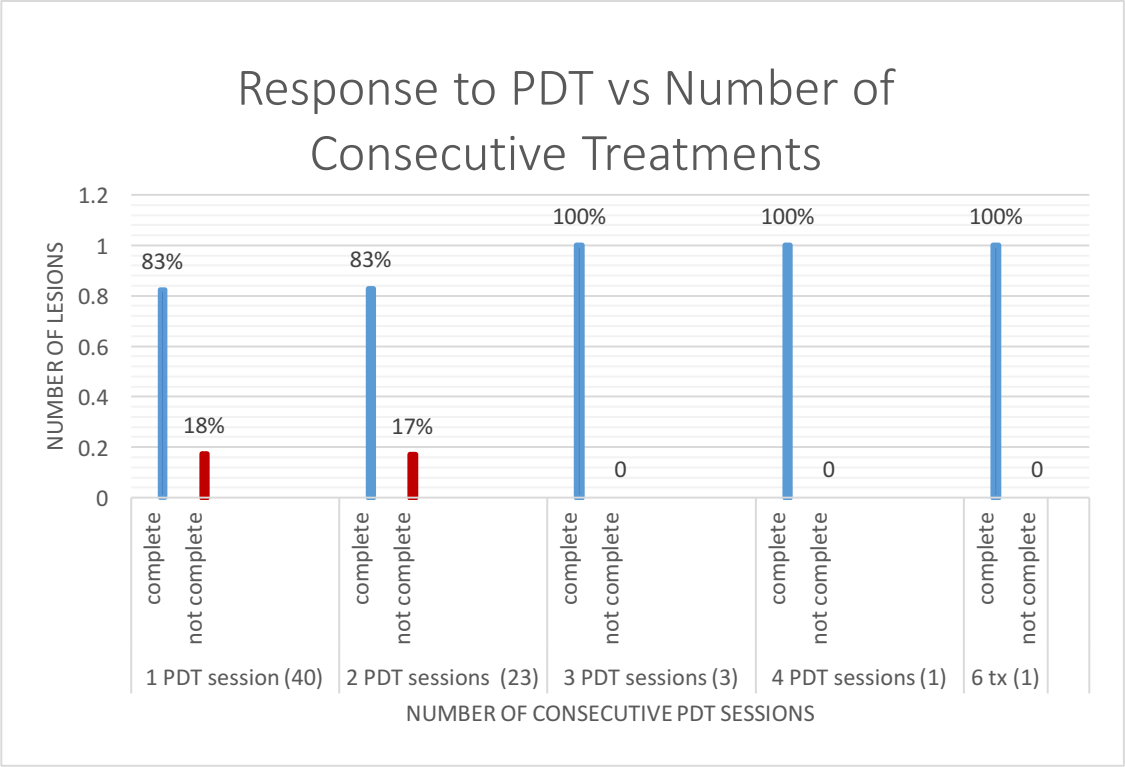

Figure 1. Responses to treatment after varying numbers of consecutive PDT sessions. Consecutive PDT sessions here refer to all PDT sessions occurring with a <1yr gap following the previous PDT session, prior to any recurrences that may have occurred. Only lesions with data on their responses to treatment are depicted.

Mean follow-up time for lesions was 12.4 months, ranging from 15 days to 36.9 months, with standard deviation of 11.7 months. Months are defined as lasting exactly 30 days for this work.

For convenience, the term consecutive will be used to refer to treatments that occurred within 1 year of the previous treatment and did not occur after a recurrence or other form of treatment. 41 lesions received 1 treatment, 28 received 2 treatments, 3 received 3 treatments 1 received 4 treatments, and 1 received 6 treatments (Figure 1). Of the 41 lesions that only received 1 treatment prior to any recurrence if a recurrence occurred, 83% (33/40) had a complete response to the treatment, 17% (7/40) did not have a complete response, and 1 lesion did not have documentation in the medical record of the type of response to treatment. For 28 lesions receiving 2 consecutive treatments of PDT, 83% (19/23) had a complete response, 17% (4/23) did not have a complete response, and 5 did not have a type of response documented. There is no statistically significant difference between complete response rates from 1 PDT treatment and 2 PDT treatments, with a p-value=1.0 according to Fisher's exact test with a 2x2 contingency table (Figure 2). The remainder are 3 lesions that received 3 consecutive treatments, 1 lesion that received 4 consecutive treatments, and 1 lesion that received 6 consecutive treatments. All of these had complete responses. Overall, 83% (52/63) of lesions had a documented complete response with 1-2 cycles of PDT. (Figure 1).

Of all the lesions that had documentation on their response to initial PDT treatment, 55% (39/71) of lesions had a complete response after 1 PDT cycle, 39.4% (28/71) had a partial response after 1 cycle, and 5.6% (4/71) lesions had minimal responses after 1 cycle (Figure 3). 5 lesions with a complete response after initial

treatment were treated with an additional PDT session despite not having a recurrence in the interim. 3 lesions in 3 different patients did not have sufficient information in their medical record for us to determine the response to initial PDT, but 2 of them received a second PDT treatment had had complete responses to that and the other patient returned 1 year later to receive Mohs surgery on this lesion site. Of the lesions that were documented to not have a complete response after 1 cycle of PDT, 1 lesion was treated with Mohs surgery, 3 have documentation suggesting that eventually the lesion resolved without further treatment, 2 received no further treatment or follow-up after the initial follow-up assessment, 1 had no further treatment and no further documentation regarding status of the lesion site (partly due to a patient request limiting the exam), and 25 patients then underwent a second round of PDT. 5 of these patients did not return for follow-up after the second PDT session, and 65% (13/20) of those who did had a complete response after 2 rounds of PDT. Of the 7 that did not have a complete response even after 2 rounds of PDT, 3 lesions were treated with more PDT and eventually had a complete response.

	Complete Response	Not Complete Response	
1 treatment	33	7	40
2 treatments	19	4	23
	52	11	63

Table 2. Contingency table for number of consecutive PDT treatments and response to treatment.

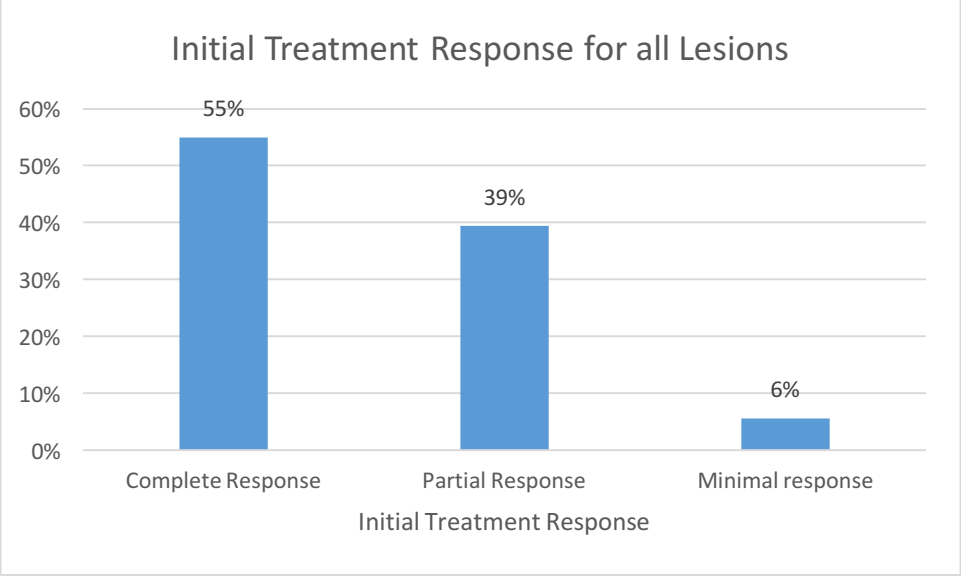

Figure 3. Initial treatment response for all lesions. % of all lesions with documented complete, partial, and minimal responses after first treatment.

A logistic regression model for complete response to initial PDT treatment analyzing various patient, lesion, and treatment factors was found to be statistically significant. The pseudo R^2, which measures goodness of fit and generally ranges from 0 to 1, was 0.5629 for the model. The factors that were significantly associated with a complete response after initial PDT treatment were age and large size. A lesion was considered large if it had any size measurements of at least 2cm, appeared to be larger than 2cm in clinical photos when measurements were not available, and/or was described as "large" in a note without specific measurements being given. The odds ratio of 0.0186913 for lesions of large size to have a complete initial response indicates that all else equal, lesions that are large have significantly worse odds to have a complete initial response than lesions that are not. The odds ratio of complete initial response and age is also less than 1, which means that increasing age is negatively correlated with likelihood of a complete response after initial PDT treatment. Other factor variables, such as sex, phototype, presence of clinical residual lesion during treatment, lesion site, occlusion, incubation time, presence of erythema, and presence of pain, were not found to be statistically significant, and the odds ratios of certain skin types and lesion sites were empty due to insufficient data points for those. By nature, one variable in each qualitative category needed to be designated a dummy variable and did not have calculations. (Table 4).

Likelihood of complete response after first PDT treatment			
Pseudo R^2 for overall regression model		0.5629	
P-value for overall regression model		<0.0001	
Independent variable	**Odds ratio**	**P-value**	**95% C.I.**
Male sex	1.21434	0.871	.1155304, 12.76392
Age	.9071233	0.038	.8271304, .9948524
Fitzpatrick phototype			
1	(dummy variable)		
2	1.73871	0.660	.147573, 20.48553
3	1 (empty)		
Presence of clinical residual lesion	1.534601	0.707	.1648968, 14.28167
Large size	.0186913	0.014	.0007888, .4429009
Lesion site			
Scalp	(dummy variable)		
Ear	1 (empty)		
Face	4.794044	0.224	.3827114, 60.0527
Neck	1 (empty)		
Chest or extremities	4.314282	0.498	.0626494, 297.0982
Occlusion during incubation	5.30028	0.264	.28346, 99.10734
Incubation time (hours)	3.338785	0.228	.4693498, 23.75091
Presence of erythema	2.909452	0.367	.2854477, 29.65485
Presence of pain	1.834726	0.635	.1502722, 22.40081

Table 4. Logistic regression model for complete response after 1 PDT treatment.

Patients' reports of side-effects of pain and erythema were significantly correlated with incubation time and with presence of erythema or pain, respectively. The logistic regression for pain as a dependent variable had a P-value of <0.0001 and pseudo R^2 of 0.5130 for the overall model (Table 5). The logistic regression for erythema as a dependent variable had a P-value of 0.0006 and pseudo R^2 of 0.3855 (Table 6). Incubation time in hours with ALA had a statistically significant relationship with both pain and erythema, with an odds ratio of 11.19503 with pain and of 4.110301 with erythema, meaning that longer incubation times are likely to cause increased pain and erythema. Pain and erythema also appears to correlate positively and significantly with one another, with presence of erythema as a factor variable having an odds ratio of 35.4397 with pain as the dependent variable, and presence of pain as a factor variable having an odds ratio of erythema of 8.384352. In the logistic regression model for pain as a dependent variable, age approaches near significance as a factor variable, with a p-value of 0.093 and odds ratio of .9252411. The factor variables of sex, age, phototype, presence of clinical residual lesion during treatment, large size, lesion site, and occlusion were not found to be clinically significant. (Tables 5 and 6).

Presence of pain as a side-effect			
Pseudo R^2 for overall regression model		0.5130	
P-value for overall regression model		<0.0001	
Independent variable	**Odds ratio**	**P-value**	**95% C.I.**
Male sex	.6731383	0.706	.0858131, 5.280255
Age	.9252411	0.093	.8449507, 1.013161
Fitzpatrick phototype			
1	(dummy variable)		
2	1.037243	0.977	.0862991, 12.46679
3	1 (empty)		
Presence of clinical residual lesion	10.00121	0.213	.3990067, 61.14648
Large size	.5972647	0.630	.073196, 4.873563
Lesion site			
Scalp	(dummy variable)		
Ear	1 (empty)		
Face	1.313394	0.805	.1512651, 11.40384
Neck	1 (empty)		
Chest or extremities	2.001625	0.725	.0421828, 94.97954
Occlusion during incubation	4.939418	0.112	.5137863, 565.6868
Incubation time (hours)	11.19503	0.032	1.230876, 101.8207
Presence of erythema	35.4397	0.040	

Table 5. Logistic regression model for pain

Presence of erythema as a side-effect			
Pseudo R^2 for overall regression model		0.3855	
P-value for overall regression model		0.0006	
Independent variable	**Odds ratio**	**P-value**	**95% C.I.**
Male sex	1.148807	0.886	.1724624, 7.652436
Age	1.016078	0.597	.9577616, 1.077945
Fitzpatrick phototype			
1	(dummy variable)		
2	.2336373	.2198271	.0369534, 1.477166
3	1 (empty)		
Presence of clinical residual lesion	.6247012	.5873517	.0989378, 3.944414
Large size	1.103538	0.914	.1843299, 6.606615
Lesion site			
Scalp	(dummy variable)		
Ear	1 (empty)		
Face	3.179493	0.211	.5185188, 19.49626
Neck	1 (empty)		
Chest or extremities	2.166933	0.587	.1330546, 35.29075
Occlusion during incubation	.8318299	0.853	.1194089, 5.79472
Incubation time (hours)	4.110301	0.033	1.120249, 15.08108
Presence of pain	8.384352	0.018	1.436711, 48.92936

Table 6. Logistic regression model for erythema

Survival analysis was limited by the fact that most patients did not have systematic follow up. Patients without evidence of disease were often discharged after a few months, since generally patients would follow-up with their primary dermatologists in the long-term rather than with a specialized dermatologic surgery clinic. However, patients with late recurrence were often referred back to Yale Dermatologic Surgery for evaluation. In this way, a large proportion of recurrences are likely to be detected in our study. Survival groups here refer to the number of PDT treatments that patients received within 1 year of previous treatment, before any recurrence or other treatment for persistent disease occurred, and with follow-up data.

Treated lesion fields had a mean disease-free survival time of 13.379 months (standard deviation of 2.0 months, C.I. [9.477, 17.280], 41 observations) if they only received 1 consecutive PDT treatment, mean disease-free survival time of 13.590 months (standard deviation of 2.3 months, C.I. [9.072, 18.107], 23 observations) if they received 2 PDT treatments meeting above criteria, and mean disease-free survival time of 0.933 months (insufficient data points to calculate standard deviation or C.I., 5 observations) for >2 consecutive PDT treatments. Once again, months here refer to 30-day intervals and not calendar months. The log-rank test revealed a statistically insignificant discrepancy between the survival curves for the 1 PDT treatment group and the 2 PDT treatments group, with a p-value of 0.801. One lesion that received 1 consecutive PDT treatment did not have data on initial treatment response but did have usable data for the survival function. (Figure 4).

Following 1 consecutive treatment, there were 12 failures total including 4 documented recurrences with a recurrence rate of 12% (4/33), and 29 censored entries;

for 2 consecutive treatments, there were 10 failures total including 6 documented recurrences with a recurrence rate of 32% (6/19), and 13 censored entries. None of the lesions receiving >2 consecutive PDT treatments were documented to have a recurrence, and apart from 2 lesions in this group with follow-up length of 33.6 months, the rest all had follow-up lengths of 1.4 months or less.

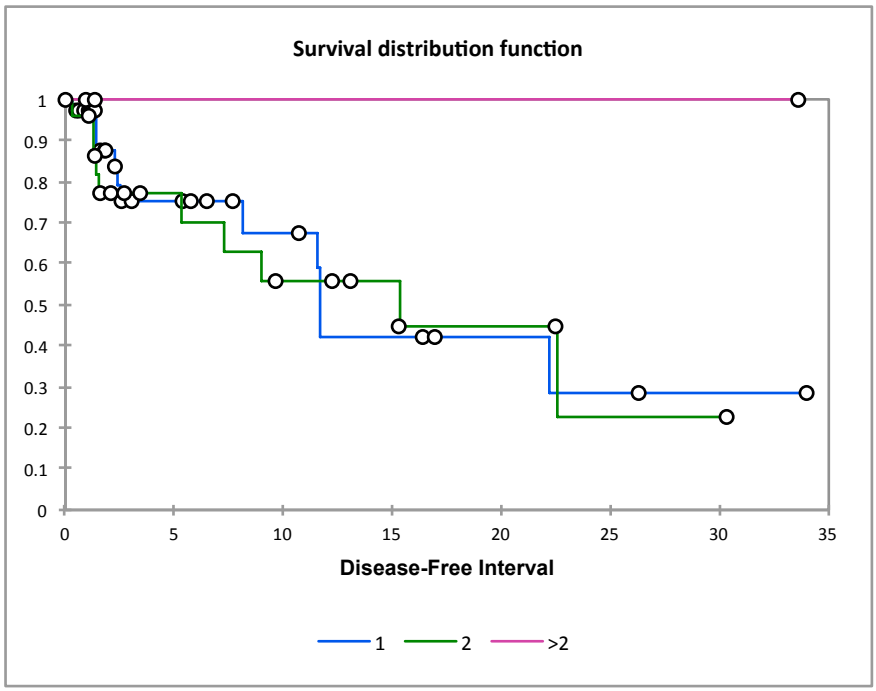

Figure 4. Kaplan-Meier survival functions for 1, 2, and >2 consecutive PDT treatments with follow-up data. Blue represents the group that received 1 treatment, green represents 2 PDT treatments, and red represents >2 consecutive PDT treatments. Only 5 lesions were included in the last group.

Mutation Burden Analysis

Since PDT treats the field surrounding a cancerous lesion as well as the lesion itself, it may have a clinical role in treating field cancerization and preventing further development of malignant cells. However, in the absence of visibly abnormal lesions, clinical observation of field cancerization may not be possible. In order to better quantify the risk posed by field cancerization in patients' skin, a method using sequencing and mutation burden analysis could prove useful.

A necessary step for mutation burden analysis would be collection of relevant DNA samples for testing. Punch biopsy is a method that is often used by dermatologists to collect skin samples and DNA can be extracted from these skin samples. DNA yield depends upon number and size of biopsies and DNA purification method. 1mm diameter punch biopsies fail to consistently yield sufficient DNA (≧1μg) for our study. Phenol-chloroform extraction led to higher DNA yields than the QIAamp® kit. Individual 2mm punch biopsies purified with phenol-chloroform extraction provided high DNA yields while minimizing required biopsy material. As expected, DNA yields correlated positively with punch biopsy size and the number of punch biopsies used in a single sample. However, even two 2mm punch biopsies when purified with the QIAamp® kit, with an average yield of 2.2μg and SD of 0.29 for 2 samples, yielded less DNA than one 2mm punch biopsy when using phenol-chloroform, with an average yield of 3.6μg and SD of 2.3 for 7 samples. Three 2mm punch biopsies when purified with phenol-chloroform had the highest average DNA yield of 6.5μg for 2 samples, with SD of 2.2. (Figure 5).

In order to sequence DNA samples for the mutation burden analysis, amplicons including target sequences must first be generated. Initially, long-range PCR was attempted but this proved unsuccessful. PCR results of more than 20 samples shows that PCR amplification can occur fairly reliably up to slightly over 1.5kb in amplicon size based on visual confirmation of gel electrophoresis, but PCR amplification of amplicon sizes larger than that are not consistently successful. With some variation between samples, PCR amplification of amplicon lengths around 1.5kb or less could sometimes be achieved with 30 cycles or fewer, but amplification at higher amplicon lengths did not yield visible bands at the right size even with 35 cycles or more. (Figure 6).

Subsequently, we attempted a different strategy using an extension-ligation system marketed by Illumina®. Using Illumina, Inc.'s Design Studio ® software, primers targeting the 5' UTR and CDS of *TP53, Hras, Nras, Kras, CDKN2a, Notch1, Notch2, Notch3, Fat1, Fgfr3, Knstrn*, *Braf,* and *VHL* (control gene), along with 10bp of intron padding around these sequences, was generated. Extra padding was also designed around important regions, such as regions with documented mutations in literature. These primers should promote the amplification of 250bp amplicons and cover both DNA strands of each target region utilizing dual-pool design. The cumulative target size was 59,547bp with 2 x 394 distinct amplicons. The design ultimately included 3 gaps totaling a length of 276bp.

The first sequencing run generated with 20 skin samples from 8 skin cancer patients yielded a total sequence of 1.54e11 bp with a quality score of 72.26%. Total high-quality sequence generated was 1.11e11 bp in length, and the average sequence per

sample was 5.57e9 bp. Dividing this last number by the total sequencing target size (59,547 bp)leads to an average of 93,500-fold coverage per sample.

The bioinformatic analysis of sequencing results is ongoing.

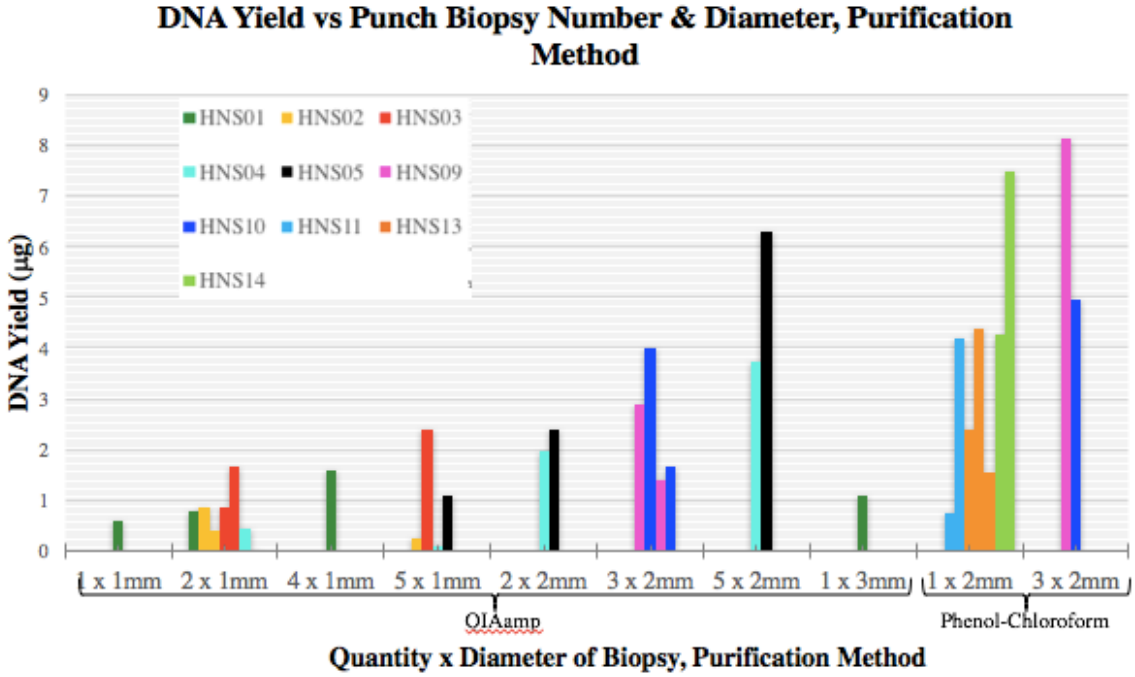

Figure 5. Comparison of DNA yield against punch biopsy number, diameter and purification method.

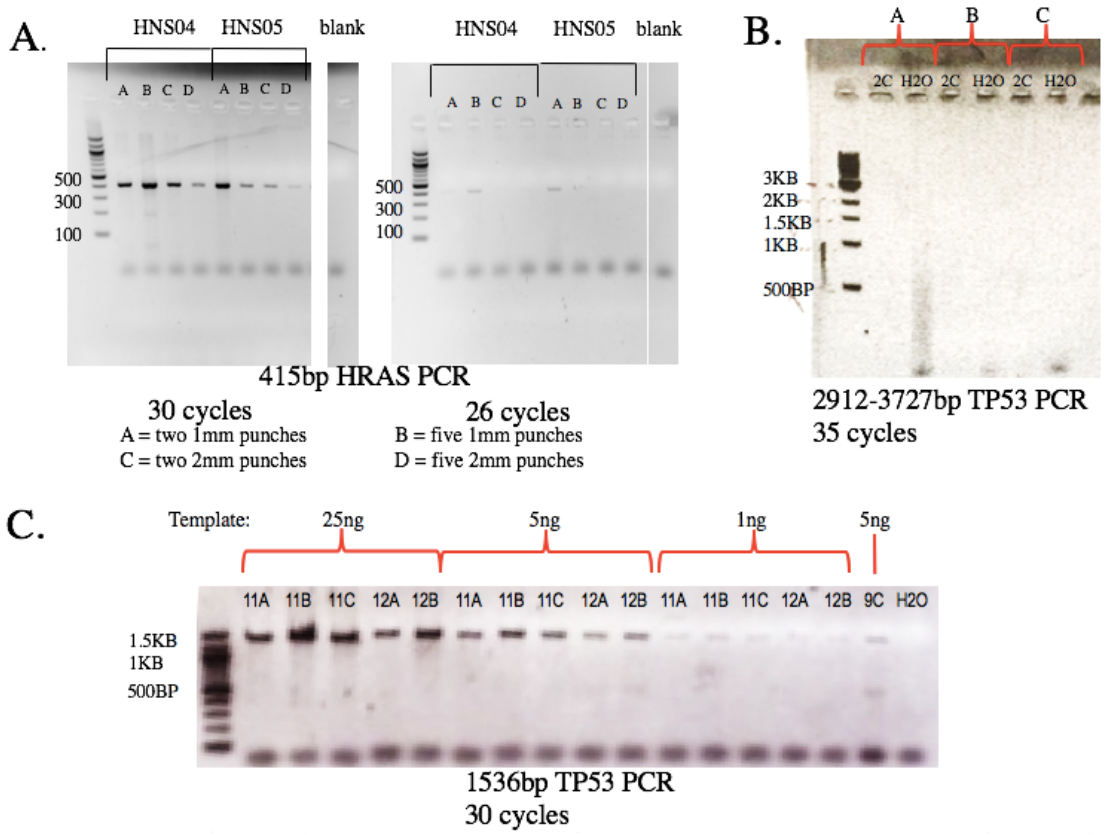

Figure 6. PCR results from purified genomic DNA extracted from human skin samples, as an indicator of DNA quality. PCR amplification occurs fairly reliably up to 1.5kb amplicon size, but did not work at >3kb amplicon sizes. **A.** Results of 30 and 26 cycles of PCR, Hras primer set with expected amplicon size of 415bp, and 5ng template DNA (except for 3.92ng for 4B and 1.5ng for 5A). **B.** PCR results with 35 cycles, TP53 primer set with expected band sizes of 3567bp (A), 3727bp (B), 2912bp (C), and 5ng template DNA. **C.** PCR results with 30 cycles, TP53 primer set with expected band size 1536bp, and 25ng, 5ng, and 1ng of template DNA.

DISCUSSION

While the complete response rate of 55% after 1 cycle of PDT may seem a bit low, 83% (52/63) of lesions had a documented complete response with 1-2 cycles of PDT. Following an incomplete initial response to PDT, only 65% (13/20) of lesions will exhibit a complete response to a second round of PDT, suggesting diminishing marginal returns with additional sessions of PDT. In contrast, 83% (19/23) of all patients with 2 consecutive PDT treatments had a complete response. This discrepancy is likely due to patients not being randomly assigned to receive 1 or 2 cycles of PDT, but rather many patients who received 2 cycles of PDT did so because their lesion did not completely resolve after receiving 1 cycle of PDT. Furthermore, the difference in complete response rates after 1 session of PDT versus 2 sessions of PDT was not statistically significant, and the response rates themselves were not different. Therefore, initially starting a protocol involving 2 PDT treatments may not be beneficial compared to initially starting a protocol involving 1 PDT treatment, and given contemporary concerns over healthcare costs as well as patient convenience, it may be worthwhile to initiate PDT with only 1 planned treatment and then decide whether to continue with more PDT sessions based on the follow-up assessment.

Most of the literature published on PDT treatment for SCCIS involves multiples PDT treatments, with reported complete response rates ranging from 75% to 100%. The most relevant comparison may be our 83% complete response rate following 1-2 sessions of PDT, which is within the range described by the literature. Complete response rate after initial treatment does not appear to be an appropriate comparison since most other studies did not limit themselves to 1 session of PDT if there was a clinical indication for

more. Complete response rate after 1-2 sessions of PDT appears comparable to imiquimod's reported 75-93%[43, 44] and 5-FU's 67-92% [43, 45]. Mohs surgery certainly exceeds PDT's complete response rate, since the surgery is designed to continue until reaching 100% complete response rate, so if PDT is chosen instead of surgery there is typically some reason other than response rate and considering this study did not find a different response rate, it should not impact that decision.

Clinically, it is unlikely that patients would receive more than 2 cycles of PDT for a persistent lesion unless there was some contraindication for definitive treatment such as surgery, so evaluation of the efficacy of >2 cycles of PDT is challenging. Usually the cycles would be spaced very far apart since they were not offered as part of the same treatment sequence, but rather for a recurrence later in time or for another lesion in a similar region. In our case, we had insufficient data on lesions receiving >2 cycles of PDT to draw substantial conclusions.

This study was fairly unique in the sense that patients were treated with blue light rather than red light, which has a shorter wavelength and in theory should more efficiently generate ROS and free radicals. The downside is that blue light is less able to penetrate deeply into tissue than red light, but the hope is that the superficial nature of SCCIS would lessen the potential negative impact.

The logistic regression models for response after first PDT treatment and PDT side-effects had relatively high pseudo R^2 values and low p-values, suggesting that the models fit the data fairly well and are likely significant. In contrast to linear regression models, where R^2 (without the "pseudo" in front) more directly indicates the amount of variation in results that the model can account for with factor variables, logistic models

do not obey the same assumptions regarding homogeneity of standard error values at different points in the model, and thus the interpretation of the pseudo R^2 and the appropriate method of calculating pseudo R^2 can be controversial. Furthermore, while R^2 is easier to interpret because its range is constrained between [0,1], under certain circumstances the lower bound of pseudo R^2 is above 0 and the upper bound is below 1.

Another complication of regression models is that data points missing any of the variables are completely excluded from the calculation of the model. While this may be more justifiable than the alternative method of systematically replacing missing data with certain values, it does mean that collecting more variables may lead to greater loss of data and precision, since the more variables are collected, the greater the likelihood that any data point would be missing some factor variable data.

In terms of precision and statistical significance, our study involved a fairly small sample size, especially in comparison to the number of variables that we were analyzing, which made statistical significance less likely. Given that we had some factor variables that were not statistically significant but close to statistical significance, further study with larger sample sizes may lead to more statistically significant findings.

Regression models also run the risk that strong correlation to an independent variable may actually indicate a relationship with a confounding factor left out of the model.

For complete response after initial PDT treatment, increasing age and large size were found to significantly correlate against complete response. Normally, if a treatment is less likely to be successful, the recommendation may be to preferentially pursue other types of treatment, but unfortunately increasing age and large size can also be

contraindications to surgery, which is often the reason why PDT is pursued in the first place. Since PDT has fewer side-effects than surgery, especially in this population, it may be worth pursuing as initial treatment anyway.

Side-effects of pain and erythema were significantly correlated with photosensitizer incubation time and with the presence of each other. While regression models indicate correlation and not causation, incubation times were chosen by the provider and possibly influenced by scheduling circumstances of the patient, but are otherwise unlikely to have been influenced by anything that could have served as a confounding variable for pain and erythema, and since the side-effects of pain and erythema strictly occur temporally after incubation time has already been fixed, it is likely that the relationship is one of causality and not merely correlation. Since incubation occurs because ALA needs time for absorption and metabolism into the photosensitizer PPIX, it makes theoretical sense for the side-effects of PDT to be caused by this step in the treatment procedure. The significant correlation between the presence of one side-effect and the other is not counterintuitive, since they are both occurring as a result of the same treatment. Although at first glance it might seem surprising that the odds ratios are not equal between that of pain as a factor variable and erythema as the dependent variable and vice versa, there are many cases of highly correlated conditions that would not have the same odds ratio as that of the converse case, such as with fair skin and skin cancer diagnosis or with pregnancy status and female sex.

Since the main goal of PDT treatment is clearance of SCCIS and prevention of recurrence, I do not think that incubation time should be adjusted with the purpose of avoiding or minimizing side-effects.

In terms of the disease-free survival analysis, the main difficulty is that the true recurrence rate is probably too low in relation to our follow-up lengths for us to reliably model the survival function. As a carcinoma in-situ, SCCIS is not as aggressive as invasive SCC, it may be less likely to recur and its recurrence may take longer. Previous studies that looked specifically at long-term recurrence rates had average follow-up lengths of 43.5-60 months, with recurrence rates of 11.6%, 54%, and 57% [105, 106, 108], which shows fairly large variation. Our recurrence rates of 12% (4/33) for 1 PDT treatment and 32% (6/19) for 2 PDT treatments are on the lower end of the reported range, but our follow-up time of 15 days to 37 months is also much shorter.

Even so, surgery has lower reported recurrence rates of 4.6%-8.3%.[39-42] From the perspective of preventing recurrences, surgery is clearly the superior treatment method, which means that if PDT is being utilized instead of surgery, it is for other reasons that outweigh the consideration of recurrence rate. This study highlights the fact that PDT treatment of SCCIS may have a recurrence rate as high as 1 in 3, and this must be considered when deciding between PDT and other forms of treatment such as surgery. Other than lesion size and patient age, this study does not identify specific factors correlated with PDT response, and the clinical considerations that ultimately lead to choosing PDT over surgery should remain unchanged.

Additionally, one challenge of this being a retrospective study is the lack of systematic follow up. This means that in practice, patients may only visit the clinic for procedures and short-term follow-up, and then resume follow-ups with their primary dermatologist, with referrals back to the dermatological surgery clinic in case of recurrence or a new lesion that requires specialty procedures. Therefore, the observed

disease-free survival intervals are likely shorter than the true disease-free survival intervals, since patients who remain disease-free may never return. Paradoxically, it is possible that some of the patients with the longest disease-free survival intervals may have the shortest documented disease-free survival lengths based on follow-up visits.

The survival curves that I obtained for 1 consecutive PDT treatment versus 2 consecutive PDT treatments were not statistically significant, had very similar mean disease-free survival times, and even appeared to overlap visually. Therefore, additional PDT treatments for the purpose of preventing future disease, or PDT treatments "just in case" after a complete response to initial treatment, are not clinically indicated and may lead to unnecessary discomfort and usage of healthcare resources.

To summarize, since 1 consecutive cycle of PDT and 2 consecutive cycles of PDT have similar complete response rates and disease-free survival functions, an additional cycle of PDT is not indicated unless complete response was not achieved following initial PDT treatment. 65% (13/20) of lesions that did not have a complete response to initial treatment displayed a complete response to a second round of PDT, suggesting there is some utility to continuing PDT after a failed treatment. However, it is between the patient and the doctor at that point to decide whether to continue PDT or pursue other treatment modalities. Since this study does not significantly change current knowledge of PDT's response and recurrence rates, decisions between PDT and surgery should depend on the existing decision-making variables of patient preference, patient-specific factors that make him a poor surgical candidate, and lesion-specific factors that contraindicate surgery such as large size and sensitive location. However, as this study has shown, some

of the factors such as increasing age and large lesion size that make surgery a less desirable choice may also decrease the effectiveness of PDT.

The project analyzing mutation burden in normal sun-damaged skin is still in process, and we have yet to perform the sequencing analysis. In the future, the results of this project could potentially be used to identify an individual patients' subsequent risk of developing skin cancer based on mutation burden, with prospective determination of mutation burden with clinical follow-up to assess cancer development. Clinical application of this information may also allow more appropriate screening and preventive treatment in high-risk patients. Punch biopsy, the method used to obtain DNA, is already commonly used in dermatology clinics and is therefore readily available. Mutation burden analysis in individual patients may also facilitate the monitoring of clinical response to photodynamic therapy (PDT) and other dermatological treatments.

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