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Desensitization Of Basal Cell Carcinoma To The Anti-Tumoral Effect Of Vitamin D

Rawia Khasawneh
Wayne State University,

DESENSITIZATION OF BASAL CELL CARCINOMA TO THE ANTI-TUMORAL EFFECT OF VITAMIN D

by

RAWIA KHASAWNEH

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Approved by:

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Advisor Date

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DEDICATION

This manuscript is dedicated in honor of my loving family

(My parents, husband, siblings, and my two lovely kids)

whose encouragement and unlimited support

is the hidden reason for my achievement

ACKNOWLEDGEMENT

The first acknowledgement and the greatest Thanks go to my advisor, Dr.Heydari. During the years I spent in the lab, his caring supervision, patience, and kindness were provided with unlimited support. I learned from him not only the basics of research and result interpretation, but also the concepts of team-work, and leadership by setting a positive example. My big thanks are to my committee members Drs. Robert Arking, Diane Cabelof, and Kequan Zhou. Their valuable ideas and suggestions enriched my research experience and helped sharpening my skills.

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CHAPTER 1: BACKGROUND AND SIGNIFICANCE

Epidemiology and Etiology of Basal Cell carcinoma:

Skin Cancer is the most common cancer in humans. Cancer statistics reveal that incidence of skin cancer increases annually, making this cancer a growing public concern [1]. The two main types of skin cancer are Melanoma, which is the most aggressive and lethal form and accounts for about 4% of skin cancers, and Non melanoma skin cancer (NMSC). NMSC consists of many subtypes. The two major subtypes are squamous cell carcinoma SCC (16%) and Basal cell carcinoma BCC (80%)[2]. BCC is rarely fatal and metastatic, less than 0.5% of BCC were reported to metastasize in distal locations. However, the economic burden of BCC is one of the highest due to its high prevalence with more than one million case reported in the US. In spite of the relatively low mortality and metastasis rate, the disease has been considered to be a public health issue due to its high frequency and recurrence which increases morbidity caused by this disease [1, 3].

NMSCs are predominantly diseases of the Caucasian population as they are especially prevalent in this ethnic group, mainly in areas of higher sun exposure[1]. Despite its relatively low incidence in Black, Hispanic and Asian groups, BCC is still the most common skin cancer in these populations. Besides, there are differences between Caucasian and non-Caucasian populations in the anatomical location of the tumors, in their recognized etiologic factors and in prognosis [4].

Despite the increasing incidence of the disease in the younger population, BCC is considered to be the disease of the elderly as the majority of patients

diagnosed with this type of cancer are over 65 years of age. [1]. Skin cancer is predominantly found in light-skinned Caucasians especially Type1and type 2 skin, according to Fitzpatrick photo-typing [5]. Men were found to have higher incidence and death rate of BCC comparing to women [1]. In general, the low socioeconomic groups have less incidence of skin cancer than the higher socioeconomic groups, but with lower survival rates and poor outcome [6], probably related to late diagnosis due to lower accessibility of health care. Also, it was shown that incidence of BCC is related to lack of UV protection during sun exposure, which may not be a priority for lower socioeconomic population due to lack of awareness or financial resources. Interestingly, an increase in prevalence of BCC among girls is emerging, especially among high socioeconomic group. This increase is linked to frequent intentional tanning. This procedure requires high exposure to UV light, which was recognized as a major risk factor for BCC [7].

ALL types of NMSC, including BCC and SCC, are more common in immune-compromised patients. This includes patients who are taking immune suppressant drugs such as organ transplant patients, and is unfortunately associated with a more aggressive growth, higher risk of metastases, and poor outcome expectancy [2]. Despite high recurrence rate in BCC, multiple primary BCC lesions can be caused by genetic syndromes. For instance, Gorlin-Goltz syndrome is usually characterized by multiple basal cell carcinoma and keratocystic tumors along with other skeletal abnormalities [8].

Pathology and Pathophysiology of Basal Cell Carcinoma:

The vast majority of sporadic basal cell carcinoma lesions appear in areas of the body that are frequently exposed to sun. The risk of developing BCC is not only directly associated with UV exposure during adulthood, but it is also linked to appearance of freckles and frequent incidence of sunburns during childhood[5].

In literature, BCC has been associated with some genetic factors. For instance, the majority of BCC cases are associated with mutations in hedge-hog pathway. Some of these mutations were reported to be in sonic hedgehog gene, others were found in PTCH or SMO receptors [9]. Other genetic mutations that were typically found in BCC are in P53 tumor suppressor gene, Ras oncogene, and RUNX3 transcription factor encoding gene [5, 10]. Syndromes that include BCC were related to genetic polymorphisms, especially when multiple tumors form simultaneously in different areas including the trunk that is not highly exposed to sun. Some of these polymorphisms that were associated with BCC were found in glutathione S-transferase, NADPH and cytochrome P-450. In other cases, trisomy of chromosome 6 was associated with genetic predisposition to BCC and was associated with more aggressive tumor growth[5].

Early stages of BCC tumor appear like a small flat or raised sore of the skin that never heals and grows slowly. It can look translucent or pearly, usually surrounded by superficial vessels, and can bleed easily[5]. In medical literature there are six subtypes of BCC that are well defined and classified. Subtypes of BCC

include nodular, superficial, pigmented, morpheaform, cystic and fibroepithelioma[11]. These subtypes differ in their appearance and aggressiveness. The superficial and the nodular are considered to be less aggressive subtypes, while higher aggressiveness is usually associated with the infiltrative, the micronodular and the morphoeic subtypes of BCCs.

Histologically, BCC tissue appears to have a hyper-chromatic nucleus surrounded by little cytoplasm with no variation in size and staining density. The intercellular bridges are not well defined and they can be hardly recognized by regular microscopes. BCC mass is lined by a fibromyxoid stroma with a lot of collagen fibers and air bubbles [11]. The prognosis of basal cell carcinoma is dependent on early detection and availability of treatments. However, survival rate is 100% in patients with un-metastasizing BCC. Once it metastasizes the prognosis becomes very poor.

The risk of metastasis in BCC is related to the size of the tumor. Among BCC tumors that are at least 3 cm in diameter, only 2% metastasize. while 50% of BCC tumors that are greater than 10 cm form metastasis [5]. However, when BCC tumor does not metastasize, it practices local invasion to surrounding tissue. When not diagnosed at early stages, BCC can cause severe deformity which may leads to morbidity or impact the patient's quality of life after the treatment [5].

Vitamin D and its metabolism:

Vitamin D is a fat soluble vitamin. It is the only vitamin that can be obtained from diet or can be biosynthesized in skin keratinocytes from precursors in response to

sun exposure. The term refers to a group of antirachitic steroid derivatives with similar biochemical activity. It is present in the diet from animal or plant sources as vitamin D_2 (ergocalciferol). Additionally, it is synthesized in the skin as vitamin D_3 (cholecalciferol) from 7-dehydrocholesterol, which is present in abundance, by the action of 290-320 nm UVB radiation.

Vitamin D3 is regulated in the body through different steps of activation and inactivation that maintain adequate levels of the active form in blood and. In the first step of activation, Vitamin D3 precursors, Cholecalciferol is hydroxylated in the liver to form hydroxycholecalciferol or 25 hydroxyvitamin D3 [12, 13], or calcidiol. This step is carried out by VD3 25-hydroxylase (CYP27A1) enzyme which catalyzes the reaction of binding a hydroxyl group to vitamin D precursor at carbon number 25 producing 25(OH) VD3. This form of vitamin D is the major form of vitamin D in blood stream and majority of vitamin D reservoir in the body is in that form. Even though this form of vitamin D has some activity at the bone and intestine level, it accounts for only 1% of the potency of the final biologically active hormone [12]. The hormonal form of vitamin D is 1, 25-dihydroxycholecalciferol, or calcitriol, which is formed after further hydroxylation in the kidney by the renal enzyme 25 OH VD31α-hydroxylase (CYP27B1) [12, 13].

Interestingly, In addition to liver and kidney, keratinocytes express VD3 25 hydroxylase and 25 OH VD3 1α-hydroxylase. This indicates the presence of both vitamin D biosynthesis and activation pathways in skin, showing the skin's ability to supply its own 1,25 hydroxyvitamin D when exposed to UV light [14]. Hence, skin is

of unique importance in the synthesis, storage, and release of vitamin D into the circulation [11]. Exposure to sunlight a few times a week can reduce the risk of osteoporosis, osteomalacia, muscle weakness and fractures [15].

The hormonal active form of vitamin D, 1,25 (OH)2 vitamin D (calcitriol), is a potent seco-steroid that acts by binding to an intranuclear receptor, the vitamin D receptor (VDR) present in target tissues of most cells and tissues in the body [16-18]. The abundance of the active form of vitamin D is regulated not only by activation of cacidiol, but also by inactivation by calcitriol. This inactivation is mediated by another renal enzyme called 25-Hydroxyvitamin D-24 hydroxylase (24OHase), and it is also a cytochrome P450 enzyme (CYP24A1). Besides the renal tissue, this enzyme was found to be produced in keratinocyte. 24OHase can regulate vitamin D activity by initiating inactivation pathways for both calcidiol and calcitriol. This enzyme can be induced by high levels of the active form of vitamin D (1,25(OH)2D3), so it protects the body from excessive production of the hormonal form of vitamin D[19].

Vitamin D and cancer:

There is a theory of that the severity of cancer could be reduced by oral supplementation of vitamin D [20]. Vitamin D may play a role in the development and progression of a wide spectrum of cancers [21, 22]. Prostate cancer [19,16], non-small cell lung cancer [18], colorectal cancer [23, 24], breast and ovarian cancer [20], [25, 26] are some of the cancers associated with vitamin D metabolism.

Nevertheless, although skin cancer is the most common cancer in humans, the relationship between skin cancer and vitamin D is not clear [4]. Exposure to solar radiation is the main risk factor for the development of NMSC. Ultraviolet radiation B (UVB) acts as the carcinogenic agent inducing DNA damage [3].

On the other hand, Vitamin D biosynthesis in the skin and the DNA damage are both induced by the same spectrum of UVB radiation [3]. Vitamin D was found to possess anti-proliferating and pro-differentiating effect [27]. A beneficial role of vitamin D in cancer prevention has been reported. This fact led to the theory that vitamin D biosynthesis in keratinocytes upon UVB exposure is potentially a defense mechanism against cancer [2, 3].

However, Epidemiological studies failed to provide strong consistent evidence for the anticipated benefit of vitamin D supplementation. Some studies suggested a protective effect of vitamin D [28]. At the same time, other studies concluded a positive correlation between pre-diagnostic serum levels of vitamin D and the incidence of BCC [3]. For instance, a cohort study was conducted by Henry ford hospital epidemiology group. Participants were 3223 NMSC patients who were diagnosed between 1997 and 2009. Results suggested a positive correlation between serum levels of Vitamin D and developing NMSC, and the observed association was stronger with BCC than with SCC [29]. Many other studies reported no association between vitamin D intake and the development of skin cancer including the Nurses' Health Study [27]. Besides, a prospective case-control study assessing the relation between pre-diagnostic serum levels of 1,25-(OH)2 D3 and

subsequent development of malignant melanoma (MM), failed to show significant differences between cases and controls [30]. Another case-control study investigated the relation between MM and dietary factors. Results showed no association between melanoma risk and total vitamin D intake, calorie-adjusted vitamin D intake, consumption of milk or vitamin D supplementation [31]. Furthermore, a cohort study investigated the association between BCC and diet including dietary fat and vitamins (A, C, D and E), and showed no association between these nutrients intake and BCC [32].

Several Laboratories investigated the effects of calcitriol (1, 25 (OH) 2 D3) in cellular physiology. Calcitriol was shown to be involved in multiple cellular mechanisms that include calcitriol or was shown to be involved in multiple cellular mechanisms that include cellular growth, differentiation [33, 34], malignant cellular invasion, apoptosis [16, 35], including UV induced apoptosis [34] and immune modulation. In fact, calcitriol is one of the most potent regulators of cellular growth in both normal and cancer cells [17]. The local production of calcitriol is postulated to be important for regulating cell growth and therefore possibly preventing the cell from becoming autonomous and developing into a unregulated cancer cell [17].

Additionally, in vitro studies have shown increased expression of vitamin D receptor (VDR) in BCC [36], SCC [16] and MM [35] as well as increased expression of mRNA of the main metabolites and enzymes involved in calcitriol metabolism in BCC [36, 37] and SCC [16, 37]. However, the function of the VDR in SCCs is unknown [16]. Furthermore, combination of 1,25 hydroxyvitamin D and retinoids has

been reported to be effective in the treatment and prevention of cancerous and precancerous skin lesions, such as actinic keratosis, SCCs, cutaneous T-cell lymphomas and BCCs [16]. Studies done on Vitamin D receptor (VDR) null mouse models show increased SCC's when exposed to UVB, portraying a protective role of VDR [38]. Additional studies show that Vitamin D and VDR are protective against UVB induced skin cancer by altering signaling pathways and promoting DNA damage response [38].

A large number of analogues of 1,25 hydroxyvitamin D with potent anti-proliferative and pro-differentiating effects on cancer cells in vitro have been developed and tested in animal models and a small number of human studies. Calcipotriol, an analogue has been evaluated for topical treatment of advanced breast cancer. A phase I clinical trial in patients with advanced breast and colon cancer has been completed in the United Kingdom without evidence of clear antitumor effects. Phase I/II clinical trials are currently underway in patients with breast, pancreatic, hepatocellular carcinoma and myelodisplasia [13]. Therefore, the exact role played by Vitamin D in skin cancer is still inconclusive which calls for more direct approach to determine the biological role of Vitamin D in the development of several types of cancers including colon cancer and skin cancer [39, 40] .

The Institute of Medicine recommended that the daily allowance for vitamin D should be 15 µg (600 IU). However, the RDA for seniors above 70 years old was increased to 20 µg (800 IU), with the safe upper limit set at 100 µg (4,000 IU) for all

adults [41]. However, it has been largely accepted in clinical practice that administrating large doses of vitamin D to skin cancer patients is recommended as part of any treatment and/or prevention regimen

Vitamin D and mTOR pathway:

The mammalian target of rapamycin (mTOR) is an important cell signaling protein and it plays a role in many important cellular functions. mTOR impact the regulation of cell growth, cell cycle, nutrient status, as well as many transcription and translation factors in the cell [42]. mTOR is considered serine/threonine protein kinase that belongs to the (PIKK) family. This protein is large and consists of multiple domains that forms two distinct complexes called TORC1 and TORC2. mTOR is highly conserved among many species, that we can find similarity in the structures and the functions between mTOR in humans, rats, mice and TOR in plants, drosophila and even fungi [43, 44]. The tow TOR complexes are composed of different domains and hence possess different functions and roles. For instance, Only TORC1 can bind to Rapamycin but not TORC2.

mTOR regulates different cellular functions through interacting with different pathways. For example, it impacts translation of some proteins related to cellular growth in response to amino acids, especially the branched amino acids, through interaction with PI 3K signaling. On the other hand, mTOR regulates the translation of other proteins through the phosphorylation of pS6K and 4E-BP1, which result in activation of the first one and inhibition of the later [44].

Many mechanistic studies suggested that Vitamin D can exert differentiation and inhibits proliferation of cancer cells by blocking mTOR through increasing REDD1, which is mTOR inhibitor [45-47].

mTORC1 is part of a vital regulatory pathway in the cell which is Ras-PI3K-AKT-mTOR pathway. mTOR responses are initiated by PI3K impacts mTOR pathway through Akt which is serine/threonine kinase. AKT is activated by Phosphorylation in two locations serine 473/threonine 308 which in turn phosphorylates and inhibits the activity of TSC1/2 (tuberous sclerosis protein complex). TSC complex inhibition results in suppression of Rheb protein (Ras homolog enriched in brain), which increases the activity of mTOR complex [47]. mTOR itself is a protein kinase that influence cell cycle regulation, cellular growth, metabolism, and autophagy by phosphorylation of two essential proteins. The first one is 4E-binding protein (4E-BP) that is involved in initiation of ribosome biogenesis. The second protein that gets phosphorylated by mTOR is ribosomal protein S6 kinase (p70S6K) impacting protein synthesis [45, 47]. mTOR phosphorylates TIF4E-binding protein (4EBP), impairing its ability to bind to TIF4E protein. P-4EBP and p-S6K can be used as indicators of mTOR signaling [48] .

VDR is known to negatively regulate mTOR through up-regulating REDD1 (regulated in development and DNA damage response 1) protein. The gene encoding REDD1 protein is VDR target gene that contains VDRE in its promoter [45]. Up-regulation of this gene is known to block the activity of mTOR gene through promoting the activation of TSC1/2 complexes, which in turn inhibits Rheb resulting

in suppression of mTOR activity [47] . VDR effect on its target gene transcription regulation was shown to be impaired when some proteins called VDREBP (vitamin D response elements binding proteins) are up-regulated as they compete with VDR over the binding sites of its response elements in the promoter of the target gene Protein analysis shows that REDD1 is successfully up-regulated in cancer tissue despite the elevation in VDREBP.

Active AKT is known to block the assembly and activation of TSC1/2 complex which opposes with the effect of VDR. Up-regulation of PI3K-AKT-mTOR pathway was shown in many other cancers such as breast cancer, and it was anticipated to alter the cancer response to endocrine treatments [48, 49]. Upstream of this pathway there is Ras gene which is an oncogene and was reported to have gain of function mutations which result in up-regulating two main pathways, PI3K-AKT-mTOR and Ras-Raf-MEK-ERK pathway [50]. Ras point mutations are common, and were repeatedly reported in many types of cancers including, but not limited to, colon, skin, and breast cancers [51].

Hh-Gli pathway and basal cell carcinoma:

PTCH receptor is a plasma membrane receptor that is integrated in the phospholipids bi-layer of the membrane. The normal function of PTCH is to bind and inhibit another plasma membrane receptor called smoothened (SMO). This binding, and hence the blocking of SMO is released after PTCH receptor binds to its ligand (Hedgehog) protein [46]. Over expression of this pathway may result from over expression of the Hh ligand causing continuous suppression of PTCH receptor, or

from truncation of the PTCH receptor resulting in failure to bind to SMO, and sometimes from a gain of function mutation in SMO itself [52]. SMO, when unbound, can activate a signal transduction pathway that result in over expression of downstream glioblastoma Gli1 and Gli2 signaling proteins [52, 53], and switching the Gli code to the transcription activation mode [54].

Gli code system involves three transcription factors Gli1, Gli2, and Gli3. These three proteins cooperate to regulate cellular function in response to upstream signals [54]. Gli1 is the main transcription activator, while Gli3 is a transcription repressor. Gli2 can operate as either activator or repressor, depending on the level of C-terminal cleavage [53]. Gli2 and Gli3 possess a transcription repression function only in the cleaved form. Gli2 turns to a transcription activator when it is not cleaved, and non-cleaved Gli3 is inactive [54]. Hedge-hog signaling pathway controls the expression of Gli1 and the cleavage of Gli proteins, resulting in switching the Gli-code between the transcription inhibition and the activation modes [53]. In presence of Hh protein, SMO is active. Consequently, Gli1 is transcriptionally expressed and the cleavage is inhibited, rendering Gli-code in the transcription activation mode [9, 54]. On the other hand, when SMO is inhibited by PTCH, Gli1 becomes transcriptionally inactive. Also, Gli2 and Gli3 will be expressed and cleaved. In this case, Gli-code is in the transcription inhibition mode [53, 54]

Gli1 target genes are critical for cellular regulation and growth control. For instance, up-regulation of Gli1 increases the expression of transforming growth factor-β (TGF-β) and epidermal growth factor (EGF) [53, 55]. Over expression of

Gli1 is common in many types of cancers as it facilitates the rapid growth of the cancer [54]. Up-regulation of un-cleaved Gli2 results in over expression of the anti-apoptotic protein bcl-2[52]. Elevated levels of bcl-2 can block the intrinsic apoptosis pathway [35].

Hedge-hog pathway mutations characterize basal cell carcinoma, resulting in excessive signaling of the pathway [46]. Over-expression of Hh-Gli pathway results in modifying the Gli-code, and affects tumor progression and recurrence [53, 56]. Some studies discussed vitamin D ability to inhibit Hh-Gli pathway signaling and promote differentiation [46], and facilitate alterations in the Gli-code [46, 53]. Interestingly, down-regulation of Hh-Gli signaling by vitamin D is independent from its nuclear receptor VDR [57]. In fact, PTCH utilizes the active form of vitamin D and its derivatives for SMO inhibition [58]. What is more, vitamin D supplementation to a hedgehog mutant mouse was shown to reduce Hh-Gli signaling [56]. These results support the expected benefit from supplementing BCC patients with high doses of vitamin D.

Hypothesis and Specific Aims:

Cancer in general is an increasing problem. Basal cell carcinoma has the highest incidence and recurrence, and thus the greatest economic burden [1, 3]. Many factors were shown to impact the risk for developing BCC such as age, gender, ethnicity, socioeconomic status, and sun exposure [1]. Vitamin D can be obtained from diet or biosynthesized in keratinocytes. Vitamin D3 synthesis from its precursor (7-dehydrocholesterol) is initiated by exposure to UVB radiation of wave

length between 290-320 nm [3]. The correlation between this exact range of radiation with onset of skin cancer raised many controversies related to the benefit of sun exposure [3, 7] .

Vitamin D is known to have an anti-proliferative and pro-differentiating effect through impacting some signaling pathways in the cell [28, 29]. Vitamin D3 activates its nuclear receptor VDR which hetero-dimerize with RXR and operates as a transcription factor[59]. Upon binding to a specific response element in the gene's promoter and recruiting the required co-activators, VDR initiates the transcription of the target gene [60].

It was shown that Up-regulation of VDR target gene REDD1 results in negative regulation of mTOR via activating TSC1/2 complexes [45]. Down-regulation of mTOR pathway signaling results in reduced 4EBP and S6K phosphorylation, which consequently reduces cellular growth and proliferation [45, 47].

Vitamin D was also shown to have anti-proliferation activity by down-regulating hedgehog-Gli pathway signaling in a VDR independent fashion [57]. Binding of Hh protein ligand to PTCH receptor blocks its ability to inhibit SMO receptor. Continuous activity of SMO leads to up-regulating Gli1 and shifting the Gli code to the transcription activation mode [53]. Research illustrated that PTCH inhibits SMO through vitamin D3 and its derivatives [58]. Supplementing a hedgehog mutant mouse model with vitamin D was shown to reduce Hh-Gli signaling [56]. Hedgehog mutation is very common in basal cell carcinoma [47, 56].

Due to the anticipated benefit of vitamin D, current recommendations include high dosage supplementation of vitamin D as a part of skin cancer treatment regimen[41]. However, the correlation between vitamin D and BCC is not clear due to the shortage of mechanistic studies and the inconsistency of the data obtained from the epidemiological studies [3, 27]. Interestingly, many epidemiological studies question effectiveness of Vitamin D supplementations on BCC treatment including a study performed in southeast Michigan[29]. Besides, many practitioners have continuously observed ineffectiveness of the Vitamin D supplementation on BCC treatment (personal communication).

On the other hand, Vitamin D is known to be an immune suppressant [61, 62] . It was reported that compromising the immune function is associated with increasing incidence of NMSC. Organ transplant patients who are taking immune suppressant medications have significantly higher risk of developing NMSC [1].

The purpose of this study is to investigate the sensitivity of BCC tissue to the anti-proliferative effect of Vitamin D at the molecular level. Hence, predicting possible advantages/disadvantages of vitamin D supplementation for BCC patients. Based on our preliminary data, practitioner's observations, and epidemiological studies we hypothesize that the anti-proliferative impact of vitamin D is compromised in BCC thus rendering Vitamin D supplementation ineffective and potentially detrimental. To test our hypothesis, we will investigate the following aims:

1. Specific Aim1: To determine Vitamin D metabolism pathway including Vitamin D activation genes, Vitamin D receptor (VDR), and VRD co-activator in all

specimens from BCC patients (cancer, proximal, Distal tissues) as compared to the controls specimens.

2. Specific Aim2: To determine genes and pathways up/down stream of VDR that impact proliferation and/or differentiation in all specimens from BCC patients (cancer, proximal, distal tissues) as compared to the controls specimens.

Figure 1.1: *Vitamin D structure and activation:* a) vitamin D3 is formed from 7-hydroxycholesteril in keratynocytes or obtained from diet. b) calcidiol is the major form of vitamin D in blood stream. It is formed from vitamin D3 by hydroxylation, mediated by the hepatic enzyme (VD3 25-hydroxylase). c) calcitriol is the active hormonal form of vitamin D. it is formed from calcidiol by a second hydroxylation reaction mediated by the renal enzyme (25(OH) VD3 1α-hydroxylase). d) Vitamin D is inactivated by further hydroxylation the renal enzyme (1α,25(OH)2 VD3 24-hydroxylase).

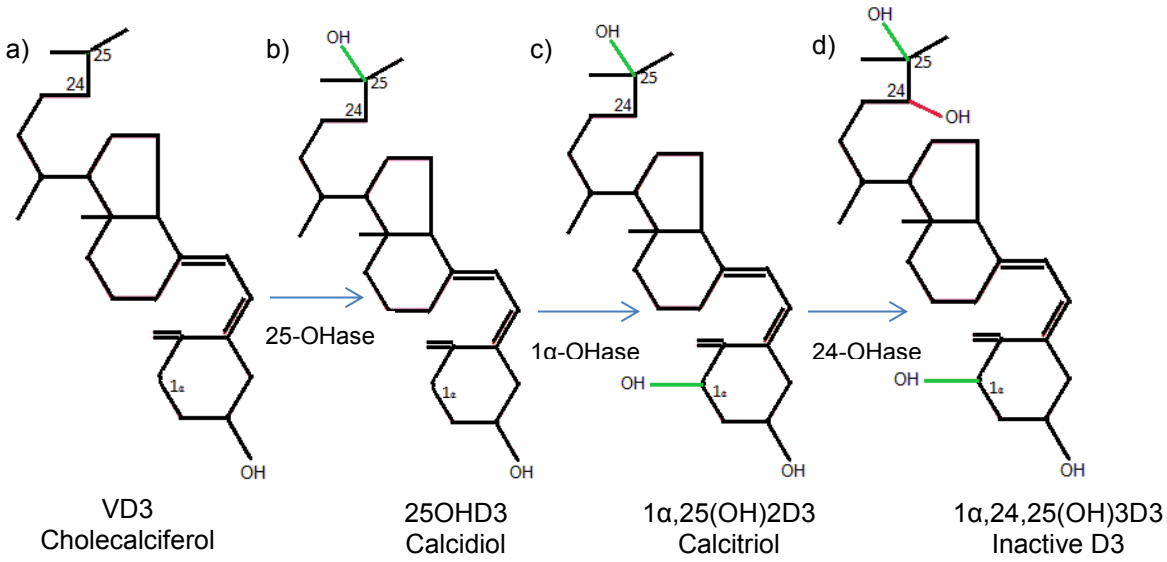
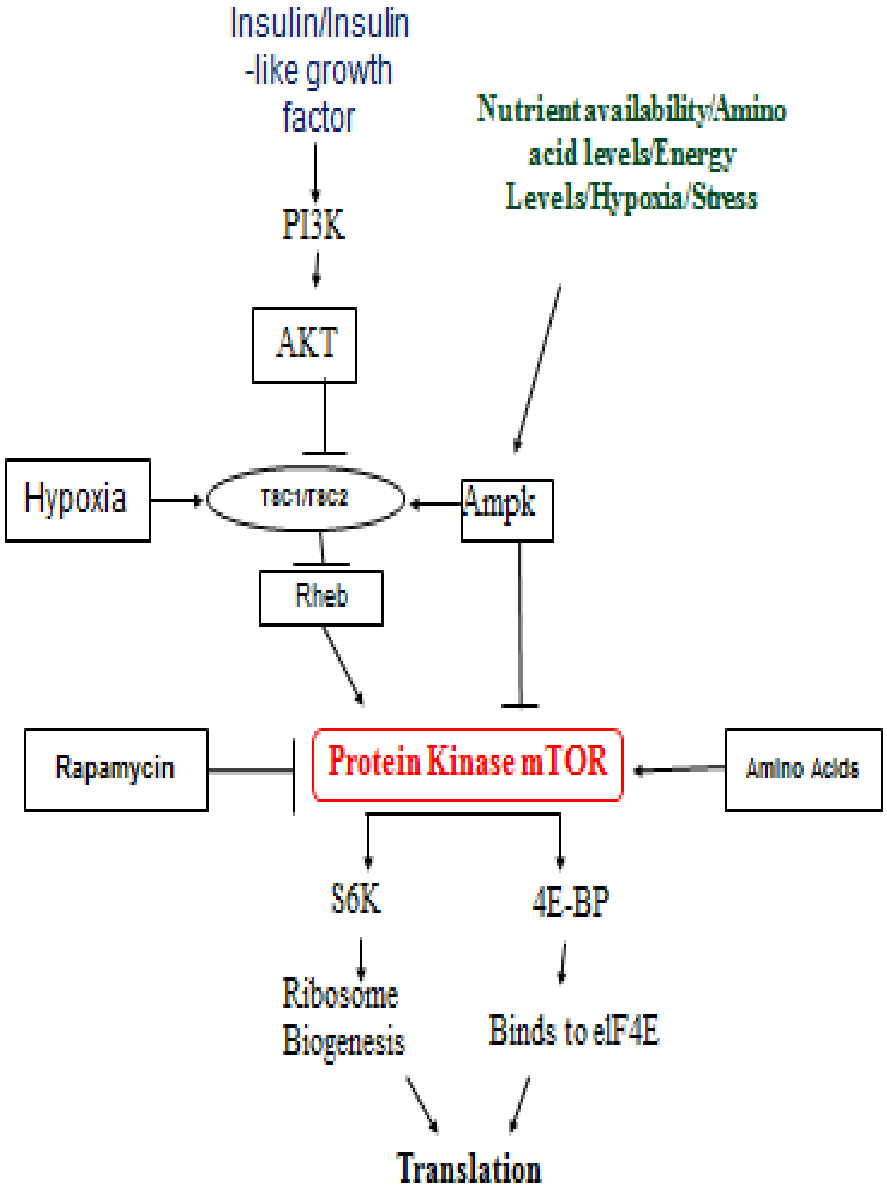

a)
b)
c)
d)
OH
25
24
1α
25-OHase
1α-OHase
24-OHase
VD3
Cholecalciferol
25OHD3
Calcidiol
1α,25(OH)2D3
Calcitriol
1α,24,25(OH)3D3
Inactive D3

Figure 1.2: mTOR pathway: The Mammalian Target of Rapamycin protein kinase responds to environmental factors, which impact its activity in various ways. Manipulation of TOR activity can be initiated through AKT, AMPK, TSC, or inhibition through direct binding to mTOR complexes such as rapamycin. mTOR impacts important cellular functions and growth through S6K and 4EBP phosphorylation which control the translation process.


Insulin/Insulin
-like growth
factor
Nutrient availability/Amino
acid levels/Energy
Levels/Hypoxia/Stress
PI3K
AKT
Hypoxia
TSC1/TSC2
Ampk
Rheb
Rapamycin
Protein Kinase mTOR
Amino Acids
S6K
4E-BP
Ribosome
Biogenesis
Binds to eIF4E
Translation

CHAPTER 2: RESEARCH DESIGN AND METHODS

This case control study is a human study with mechanistic approach. Our samples are tissue specimens excised from cases and controls in Henry Ford Hospital. Subjects were recruited by dermatology research group after obtaining the required protocols approved by Henry ford health system IRB (institutional review board).

Participant Selection:

Participants are either basal cell carcinoma patients (Cases), or non-cancer patients (Controls). Subject's eligibility for the study was determined according to the inclusion and exclusion criteria in the protocol. Approval of the protocol was obtained prior to starting the recruitment process.

BCC Patient Selection (Cases):

Basal cell carcinoma participants are 20 patients from south east Michigan from both genders. Subjects' age ranges between 46 and 89 years with average 74 years old. All patients were diagnosed with BCC in the head and nick area. In details, locations include nose, ear, cheeks, eye canthus, lips, chin, neck, temple, and forehead.

Inclusion Criteria: For inclusion, the subject must:

1. Be at least 18 years old at time of participation.
2. Have a basal-cell carcinoma and accept to have it excised.
3. Agree to abide by the investigator's guidelines regarding skin cancer screening;

4. Be able to understand the requirements of the study and the risks involved, and to sign the informed consent form.

5. Agree to follow and undergo all study-related procedures (blood sample).

Exclusion Criteria: Subjects will be excluded if any of the following apply:

1. Women who are lactating, pregnant, or planning to become pregnant.

2. Patients with a recent history of serious systemic disease.

3. Patients with history of intestinal malabsorption, resection, or bypass, which are known to affect vitamin D nutrition.

4. Patients receiving pharmacologic vitamin D replacement (i.e., 50,000 IU/ week or more), as well as systemic or topical agents involving vitamin analogs or derivatives (such as Dovonex) in the last 4 months.

5. Patients undergoing PUVA (Psoralen Ultraviolet A) therapy.

6. Patients using medications known to affect vitamin D metabolism such as Dilantin, Phenobarbital, and Cholestyramine.

7. Patients on immunosuppressive therapy.

8. Any reason the investigator feels the patient should not participate in the study.

Control selection:

Control subjects are six healthy individuals whose age is higher than 50 old. Cases and controls are coming from the same population and geographic area of south east Michigan. Controls were never diagnosed with skin cancer. All controls were scheduled for eye-lifting procedure, and agreed to donate their excised facial tissue for this study. This assured comparable location of the skin specimens, and

therefore the accessibility of sun light among specimens. All inclusion, exclusion, legal documentation, and recruiting procedures were similar for cases and controls with the exception of BCC diagnosis. While it is an inclusion criterion for the cases; cancer diagnosis is considered an exclusion criterion for the controls.

Recruitment Process:

Patients with non-melanoma skin cancer coming to Henry Ford Hospital for treatment were the candidates for this study. Medical records of eligible candidates were reviewed first to determine if any exclusion criteria applied. Candidates that were still eligible after record review were contacted by an investigator, co-investigator, or collaborative member. Invitations to participate were delivered to candidates via mail or phone. Eligible subjects who showed interest in participating in this study were scheduled for a screening visit.

Screening visit:

The study involved attending Henry Ford outpatient facility for one screening visit prior to the actual tumor excision and blood draw appointment. Failure to attend the screening visit resulted in exclusion of the subject from study. Many critical procedures were performed during this visit. First, authorized members of the study team confirmed inclusion and exclusion criteria from the medical record and the interview. Also, participant's skin was examined, typed, and assessed for skin cancer risk. Finally, the interviewer provided educational consent to the subject about the study, and obtained the required legal permissions.

1. Inclusion/Exclusion Criteria determination, Informed Consent and HIPAA Authorization: Patients were assessed for inclusion and exclusion criteria. If the patient was found eligible to participate in the study, written informed consent and written authorization to disclose protected health information under HIPAA regulations were obtained from the patient. This was completed prior to performing any procedure related to the study by study staff as listed in the IRB application. Sufficient time was given to answer subjects' questions, and patients were not forced to participate by any means.

2. Demographic Information: Demographic data that were collected at the screening visit, including gender, age and skin photo-type. All our participants, cases and controls, were Caucasian. BCC patients were 6 males and 14 females. Descriptive statistics was performed on age data for cases from both genders. Age of patients at time of diagnosis was tested for normal distribution.

3. Pertinent Medical History and risk factors: At the visit, information related to the current or past medical history of skin cancer was obtained. This included date of onset, anatomical areas affected, disease course, and result of previous biopsies, previous treatments, and treatment responses. Presence of risk factors for skin cancer (such as previous history, family history, degree of exposure to UV radiation, history of several previous severe sunburns especially in childhood and adolescence) was documented. Other relevant information consisting of, but not limited to, complete past medical history, including the presence of systemic disorders and conditions causing an immune-compromised state would be

acknowledged. Also, Interviewer obtained a list of all systemic and topical medications taken by the subject at the time recruitment.

4. Dermatological Examination: A focused dermatologic examination was performed for all patients. It included skin photo-typing and skin cancer screening for suspicious lesions. All participating cases were diagnosed with basal cell carcinoma lesion in the neck and head area. Exact distribution of cancer locations is shown in table (2.1).

Excision of tissue specimens from BCC patients:

Dermato-surgeons at Henry Ford Hospital excised tissue specimens from the enrolled patients. Specimens were collected from each recruited BCC patient from three different locations; the cancer mass itself, normal tissue surrounding the cancer and directly adjacent to it (proximal), and normal tissue relatively far from the cancer location (distal). Distal location is at least one inch far from the proximal (figure 2.2). Proximal tissue was separated from the cancer mass under the microscope by a specialized pathologist to avoid contamination of proximal tissue with cancer cells. Collected specimens will be snap-frozen in liquid nitrogen containers. Tissue specimens were then transported to our lab, and kept in liquid nitrogen for further processing and analysis.

Experimental Procedures:

1. Gene Expression Analysis:

a) Tissue processing and total RNA extraction: Snap-frozen tissue specimens were kept in liquid nitrogen until the time of processing. Tissue

specimens obtained from the same patient (Cancer, proximal and distal) were handled and processed at the same time. A piece that weighs approximately 50 mg from each tissue specimen (From BCC patients and controls) was used for RNA extraction. Frozen tissue was homogenized with 1000 ml of the lysis buffer using the polytron homogenizer. RNA was then isolated by RNeasy spin column in accordance to the protocol provided with the RNeasy RNA kit (Qiagen, Valencia, CA). Extracted RNA was stored at (-80 ℃).

b) ***Nucleic acid concentration assessment:*** Nano-drop spectrophotometer technology was used to measure the concentration of RNA and cDNA in our experiment. The sample's absorbance was measurements in sample volumes of 1μL, and analyzed by a computer linked to the instrument. The Nucleic acid concentration will be calculated by the software.

c) ***RNA Gel Electrophoresis:*** Quality of the isolated RNA was assessed using the denaturing agarose gel electrophoresis. 1.5% agarose gel was prepared with 10X mops buffer (10 ml) and distilled water (72ml), and then mixed with 18ml of formaldehyde and solidified in a gel casket. 25μL of sample buffer and 1μL of ethidium bromide were added to 5μL of RNA sample before being loaded on the gel. Electrophoresis was run at 150 volt at room temperature for 1 hour. The formed bands were visualized under UV light in the Trans-illuminator. This method enables both quantitative and qualitative detection of the RNA, as the fluorescence density in the bands indicates the quantity.

d) ***cDNA preparation:*** cDNA was prepared 1µg of RNA. The first strand was synthesized by the random primers according to the protocol provided with ImProm-II reverse transcriptase system from (Promega, Madison, WI). cDNA was then purified in a Qiaquick spin columns provided with the QIAquick PCR Purification Kit in accordance to the provided protocol by the manufacturer (Qiagen, Valencia, CA). Nano-drop spectrophotometer technology was also used to quantify the concentration of the cDNA preparations before they were stored at -20◦C.

e) ***Quantitative Real-Time PCR:*** Gene expression profile in the cancer, proximal, distal tissue from the BCC patients as well as from the controls was analyzed using the quantitative real-time PCR assay. The real-time PCR reactions were prepared according to the protocol provided with the LightCycler 480 SYBR Green I Master kit from (Roche, Mannheim, Germany). The studied genes were amplified in a hot-start reaction by performing a pre-incubation step at 95℃ for 5 min before 40 amplification cycles. Each cycle consisted of 10 seconds at 95℃ followed by 30 seconds at 60℃ with an end point measurement of cyber green fluorescence. Dissociation curve was generated in the third step by heating the mixture to 95℃ for10 seconds followed by cooling down to 60℃ for 30 seconds and heating to 95℃ again for 30 seconds with all points measurements of the dye fluorescence.

We analyzed the expression of 17 genes of interest in the collected tissue samples. The expression of each gene was studied in all samples, obtained from cancer patients (cancer tissue, normal proximal, and normal distal tissue) and from controls, in the same 69 multiwall plate to be loaded in the thermo-cycler at the

same time. A different set of primers was used in each run. Each set of primers was specifically designed to amplify one of the studied genes. Our studied genes are: VDR, 1αOHase, 25OHase, 24OHase, RPL13, PARP-1, src, DRIP, P53, Bcl-2, Apex-1, VEGF, MDM2, Gli1, Ras, GADD45A, and Caspace-3. In this study, 3 µL of template cDNA was added to each reaction of a total volume of 25 µL. Primers for VDR, 1αOHase, 25OHase, 24OHase, SRC, DRIP were designed in our lab using the (Beacon Designer, PREMIER Biosoft International) program. Gli1and Ras primers sequences were taken from articles [51, 55] respectively. Sequences of primers used to amplify these genes are shown in table (1). Primer sets that were used to study other genes were purchased from (Real time Primers, LLC; Elkins Park, PA). They were designed and synthesized by the selling company.

Protein Expression Analysis:

a) Protein extraction: Whole cell protein extract was prepared from 100 mg of the skin tissue using RIPA buffer (Igepal CA-630, sodium deoxycholate, SDS 0.1%, and 1X PBS) with protease inhibitors added just before use (β-Mercapto-ethanol 10mM, PMSF 10µg/ml, pepstatin A 1ug/ml, Triton X-100 15µl/ml, Protease inhibitor 1X). Skin tissue was first crushed with the buffer manually using porcelain mortar and pestle, and then was fully homogenized using the Teflon homogenizer. Samples were left on ice before they were centrifuged for 10 minutes at 14000 rcf and 4℃. Protein concentration in the supernatant was quantified by performing Bradford assay using Protein Assay Kit I (Bio-Rad, Hercules, CA, USA). Whole cell extracts were stored in -80℃.

b) Western Blot Assay: Protein expression was analyzed by performing western blot assay with 100 µg of the whole cell extract. Protein extract was run on 10% SDS-PAGE gel to perform the western blot assay. Protein bands were transferred to a nitro-cellulose membrane, which was then blotted by incubation with manufacturer-recommended dilutions of protein- specific primary antisera developed against our target protein (s), followed by incubation in secondary anti-body. Bands were then incubated in SuperSignal West Pico chemiluminescence substrate (Pierce Biotechnology), and visualized and quantified using a Chemimager system (AlphaInnotech, San Leandro, CA, USA). Protein quantity was measured as (IDV) of the band per microgram of protein. The remaining gel after transfer was stained using gel code blue (Pierce Biotechnology) to ensure equal loading and uniform transfer.

The primary antisera used in this study were specifically against: VDR (D-6, Santa Cruz Biotechnology, Santa Cruz, CA, USA), REDD1 (poly Ab, proteintech group inc, Chicago, IL, USA), 4E-BP1 (T37/46, Cell Signaling Technology, Beverly, MA, USA), VDREBP (hnRNP, Santa Cruz Biotechnology, Santa Cruz, CA, USA), P-AKT (S473, Cell Signaling Technology, Beverly, MA, USA), P-AKT(T308, Cell Signaling Technology, Beverly, MA, USA). The secondary anti-bodies that were used are: Goat-Anti rabbit IgG-HRP linked (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Goat-Anti mouse IgG-HRP linked (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Results are shown as integrated density value (IDV) per microgram of protein.

2. Detection of KRas Mutation:

a) ASB-PCR Assay: KRas mutation was detected in the tissue samples from BCC patients (cancer, proximal, and distal) as well as from controls using the ASB-PCR (Allele-Specific PCR with Blocker). The technique was applied as described in literature [51]. Each mutant-specific forward primer was designed to detect a specific mutant (ras) genotype. Amplification of the wild type sequence is prohibited by adding a blocking agent, which is complementary to the wild type sequence with a phosphorylated 3` end rendering it un-extendable (Figure 2.1). Mutant allele specific primers and the wild type blocker were designed as forward primers. All mutant specific, wild type, and blocker primers were used with the same reverse primer.

b) Primers used in Detecting KRas point mutation in BCC tissue specimens: Four different mutant specific primers were used. Each primer was designed to detect a different single point mutation, and has the mutated base located in the 3' end of the primer sequence. The mutations that are detected in this assay are on the Guanine bases in the locations 215 and 216 of the primer. The mutant specific primer 2.1 contains the substitution of G in the location 216 with T. Mutant specific primer 4.1 has G 215 substituted with A. Mutant specific primer 5.1 contains the substitution of G 215 with C. Finally, mutant specific primer 7.1 has G 216 substituted with C. The blocker is complementary of the wild type, and has the wild type form of the mutated bases G 215 and G 216 located in the center of the blocker sequence with a phosphorylated 3' end. The sequences of the primers used

in the assay, and the associated mutations detected by each primer are shown in Table 2.2. Melting temperatures are also distinct between the mutant specific primers and the wild type specific blocker. Melting temperature of the blocker is equivalent to the PCR extension temperature, while Tm of the mutant allele specific primer is about 10 ℃ below that. The last difference between the two types of primers is the concentration used in the reaction. To effectively block the amplification of the wild type allele in the PCR reaction, the concentration of the blocker should be four times that of the mutant specific primer in the reaction.

3. Data Analysis and Testing For Statistical Significance:

Gene expression in all samples was measured as fold increase of the controls average. Fold change values for samples from tissue different groups were compared to each other. An array of three housekeeping genes (RPL13A, B.actin, and GAPDH) was used as an internal control to normalize real-time PCR data. B.actin protein level was used as an internal control for all western-blot protein analysis assays. ANOVAs analysis was performed between the four specimen groups (cancer, proximal, distal, control) to test for significant difference between the means, followed by Bonferroni's multiple comparison's test between each two groups. In all tests, a P value ≤0.05 was considered significant. Fold change values were calculated from the real-time (Ct.) Values using the PCR array analysis software. Real time data results are illustrated in two forms, column graph representing only groups' averages and SEMs, and scatter plots for individual data points. Protein expression analysis is shown in a column graph with averages and

SEMs. Individual samples in protein assays were shown in a picture of the western-blot membrane image. Groups with similar letters in the graphs are not significantly different from each other. *P*value≤0.05 is considered significant.

Age data for participating BCC patients, was tested for normal distribution through *Kolmogorov-Smirnov* normality test using SPSS software. The number of BCC patients in each age group was plotted against the center of its age range; a trend-line was drawn using the Microsoft Excel program.

Table 2.1: *Distribution of BCC locations among participants:* All of the 20 cases recruited in our study were diagnosed with basal cell carcinoma in the head and nick area. On the other hand, Normal tissue from the control participants was excised during eyelid-left surgery, assuring comparable sun exposure among different tissue groups.

Cancer Location	Number of Cases
Nose	4
Cheek	1
Eye canthus	1
Ear	3
Neck	2
Temple	4
Forehead	3
Chin	1
Lip	1
Total Cases	20

Table 2.2: *The Real-Time primers sequences:* Real time gene specific primers were used to detect the message of the target genes. Sequences of both sense and antisense primers used in our study are shown in the table below.

Gene	Sense Primer Sequence	Antisense Primer Sequence
VDR	5′-GTCGTCCATGGTGAAGGA-3′	5′-CCAGTTCGTGTGAATGATGG-3′
1αOH	5′-CCGGGAGAGCTCATACAG-3′	5′-TGTTTGCATTTGCTCAGA-3′
25OH	5′-AGCAAATAGCTTCCAAGG- 3′	5′-GGCAAGTACCCAGTACGG-3′
SRC	5′- TGAAAGTGGAAAAGAAAGAA CAGATG-3′	5′-GTCAAGGTCAGCTGTAAA CTGGC-3′
DRIP	5′-GGAGCATCACAGTGGTAG TCAGG-3′	5′-CCGCTTTGGAGGCTTATC TTTGC-3′
Gli1	5′-CTCCCGAAGGACAGGTAT GTAAC-3′	5′-CCCTACTCTTTAGGCACT AGAGTTG-3′

Table 2.3: *Sequences of the primers used in detection of KRas gain of function mutations:* ASB-PCR technique was employed in the detection of point mutation of KRas oncogene in BCC tissue samples. It involves the usage of Mutant allele-specific primers (ASP) accompanied with wild-type allele specific Blocker. 6 different ASPs were used to detect 6 possible point mutations, with the mutated base located at the 3` end of the primer. The wild-type blocker is a primer that complements the wild-type sequence with the possibly mutated base located at the center of the primer sequence. The blocker has a phosphorylated 3` end to block the extension of the wild-type allele.

	PRIMER SEQUENCE (FP)	Nucleotide Substitution
WT	AAACTTGTGGTAGTTGGAGCT**GG**	
MUT 2.1	TGTGGTAGTTGGAGCTG**A**	G216A
MUT 4.1	AACTTGTGGTAGTTGGAGCT**A**	G215A
MUT 5.1	ACTTGTGGTAGTTGGAGCT**T**	G215T
MUT 7.1	TGTGGTAGTTGGAGCTG**C**	G216C
BLOCKER	TTGGAGCT**GG**TGGCGTAGG-**PO4**	
REVERSE PRIMER	TGATTCTGAATTAGCTGTATCGTCAA	

Figure 2.1*: Illustration of tissue excision protocol:* three tissue specimens were excised from each recruited basal cell carcinoma patient. The first specimen is the BCC mass itself and is named (Cancer). The second tissue specimen is the normal tissue adjacent to the BCC mass and directly surrounding it. This piece of tissue was carefully separated from the cancer mass under the microscope by a specialized trained pathologist to insure uniformity of the cell type and avoid contamination of the tissue specimen with cancer cells. This specimen is named (Proximal) to distinguish it from the other normal tissue specimen obtained from the same patient. The third specimen is a normal tissue excised from a location that is relatively far from the cancer mass, and was named (Distal).

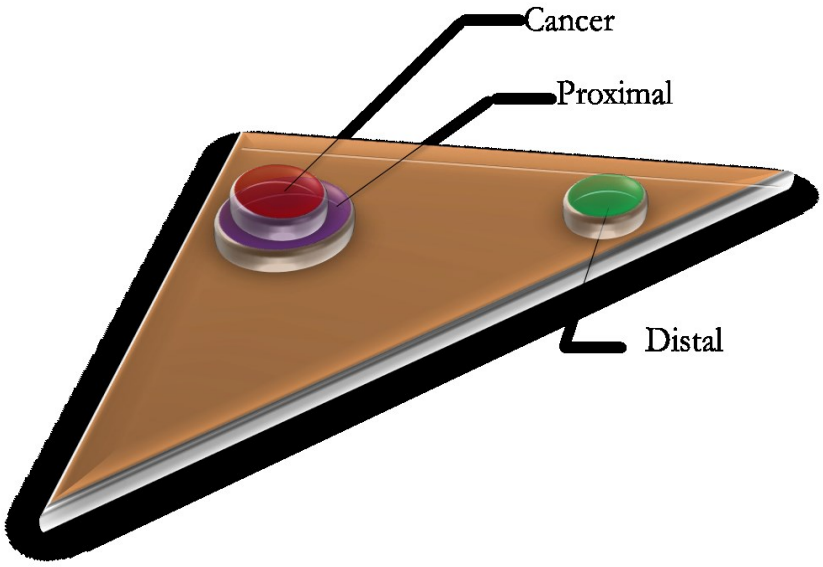
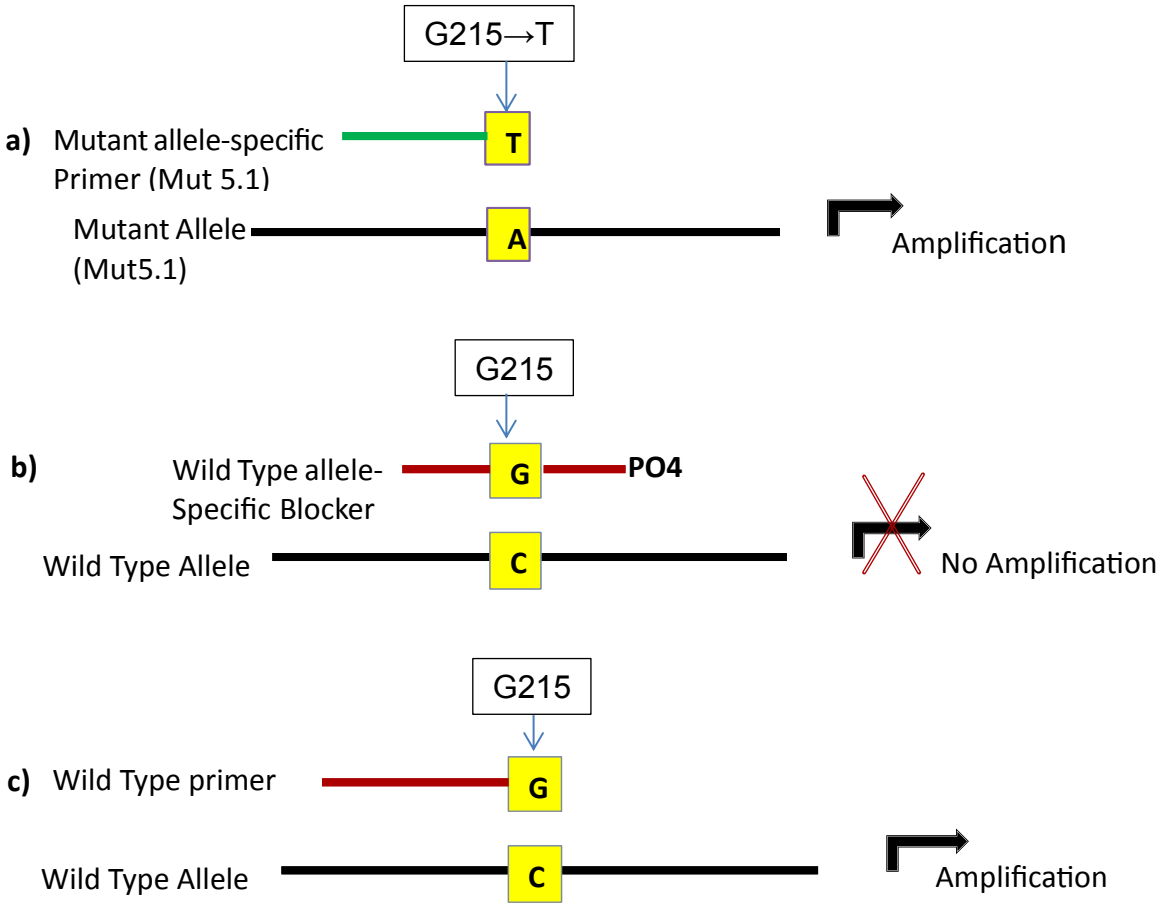

Cancer
Proximal
Distal

Figure 2.3*: Illustration of ASB-PCR technique that was used to detect KRas point mutation in BCC tissue specimens:* Shown example represents KRas mut 5.1 point mutation, the mutation that was successfully detected in our BCC patients. The mutation involves the conversion of G215 to T. a) The (mut 5.1) mutant allele specific primer is anchored at the 3` end with the T base that base pairs with the A. When the primer is annealed to the mutated sequence in the cDNA, perfect matching causes the amplification of the mutant allele. b) The wild-type specific blocker is a primer that is complementary to the wild-type allele sequence. Therefore, it perfectly base pairs with wild-type allele in the cDNA sample. The 3` end of the blocker is phosphorylated rendering the wild-type allele unamplified. C) shows the similarity between the wild-type allele primer and the wild-type allele blocker.


G215→T
a)
Mutant allele-specific
Primer (Mut 5.1)
T
Mutant Allele
(Mut5.1)
A
Amplification
G215
b)
Wild Type allele-
Specific Blocker
G
PO4
Wild Type Allele
C
No Amplification
G215
c)
Wild Type primer
G
Wild Type Allele
C
Amplification

CHAPTER 3: RESULTS AND ANALYSIS

This case-control study aims to explore Vitamin D metabolism in basal cell carcinoma tissue and in normal tissue from both BCC patients and controls. Hence, predicting the sensitivity of different tissue groups to the anti-proliferative effect of vitamin D. We collected three tissue specimens from each BCC patient; cancer tissue, normal tissue proximal to the cancer, and normal tissue distal to the cancer. Eyelid tissue from cancer-free subjects was used as a control.

Analysis of cases and controls demography:

Cases and controls were recruited in Henry Ford Hospital after the approval of our protocol. Twenty BCC patients were recruited in this study. Out of which, 14 were males and 6 were females. Patients' ages ranged between 46 and 89 years. All patients were diagnosed with BCC in the head and neck area. Controls were 6 cancer-free individuals whose age was greater than 50 years at the time of participation. Cases and controls were from south east Michigan. Controls were pre-scheduled for eyelid lifting procedure, and have agreed to donate their excised tissue for this study. Obtaining all specimens from sun-exposed locations reduces the chance that observed differences among tissue groups are related to variations in sun exposure. All participants, cases and controls, were Caucasian individuals, which eliminated race-related differences. However, all controls were females as no male participants could be successfully recruited.

Analysis of age distribution among BCC patients: Cases ages at the time of diagnoses ranged between (43-89 years old). Their average age was 74 years, and

both the mode and the median age were 80 years old. BCC cases age data failed to pass the KS normality test indicating that they were not normally distributed around the mean age, at least in our sample (Table 3.1).

To illustrate the abnormal distribution of patients' age, they were grouped based on their ages at time of diagnosis. These groups were ≤45, 46-55, 56-65, 66-75, 76-85, and 86-90 years old. The number of patients within each age group was plotted at the middle age range covered in that group (figure 3.1). The trend line indicated that the number of cases diagnosed at each age was increasing gradually as the age increase until 75 years of age. A peak in the number of cases was formed between 75 and 85 years, followed by a rapid decline after 85 years of age. The trend line appears skewed, which is compatible with the result of the normality test.

Total-RNA yield from BCC patients' tissue specimens:

Specimens obtained from the same person (cancer, proximal, distal) were handled at the same time throughout the process of RNA extraction and purification. Cancer tissue showed tendency to yield more RNA concentration than the normal tissue from same person. Figure (3.2) shows an image of denaturing RNA gel electrophoresis for RNA extracts. The three specimens were taken from the same person, at the same time, and had the same weight before processing. In agreement with the picture, RNA nano-drop spectrophotometer quantification revealed that cancer tissue yielded about 2-2.5 times as much RNA as the normal tissue of the same patient.

In order to neutralize this factor and avoid any impact on the accuracy of our results, gene expression analysis was normalized to total-RNA quantity. RNA extract volumes used for cDNA preparation were adjusted to contain equal amounts of RNA (1µg of RNA). Furthermore, cDNA concentrations were quantified once more before they were used for gene expression analysis. Finally three housekeeping genes (RPL13, GAPDH, and β-actin) were used as internal controls to normalize the data using the PCR array analysis program.

Analysis of the expression of vitamin D metabolism key genes:

Studying the differences in vitamin D metabolism key genes among tissue groups is a main objective of this study. We analyzed the expression of the key genes in metabolizing vitamin D 25OHase, 1αOHase and 24OHase genes[63]. Quantitative Real-Time was employed for that purpose. cDNA was constructed from 1µg of purified RNA and was used to quantify the message of the target genes. An array of three housekeeping genes (RPL13A, GAPDH, β.actin) was used as an internal control to normalize the data using the PCR array analysis software. Real-Time Ct. values were converted to fold changes of a reference point, which was the average Ct. of the control group. Data was analyzed using the one-way ANOVA analysis, with subsequent Bonferroni multi group comparison. Groups with similar letters were not significantly different from each other. *P*value<0.05 was considered significant.

Vitamin D3 25-hydroxylase (CYP27A1) gene expression was analyzed in tissue specimens. Gene expression data for VD3 25-OHase is shown in (Figure 3.3)

and it suggest that the expression of this gene is significantly down-regulated in the cancer tissue comparing to the non-cancerous tissue groups obtained from BCC patients as well as from the controls. Both proximal and distal tissue groups are not significantly different from each other. Despite the visual elevation of VD3 25-Ohase message in the proximal and distal tissue groups comparing to the controls, the differences were not statistically significant.

25-OH D_3 1α-hydroxylase enzyme catalyzed the formation of 1α, 25 (OH)2 VD3, which is the hormonal form of vitamin D. this enzyme was shown to be regulated at the transcription level [64]. Expression analysis of the gene encoding this enzyme shows that the message is significantly up-regulated in the cancer and the proximal tissue groups. The distal tissue specimens show a tendency to be higher than the control group, but without a statistical significance (Figure 3.4).

Inactivation of the hormonal form of vitamin D3 occurs through a further hydroxylation by the catabolism enzyme 1α, 25 (OH) 2 VD3 24-hydroxylase (CYP24A1). Our data indicate that this gene is significantly down-regulated in the distal tissue group comparing to other tissue groups. Cancer and proximal and control tissue groups seem to retain similar message levels of this gene (Figure 3.5).

Analysis of vitamin D receptor (VDR) and its co-activators:

Vitamin D exerts its hormonal effect through activating a nuclear receptor (VDR) which hetero-dimerizes with RXR. VDR-RXR heterodimer binds to specific sequences of the DNA in the promoter region of the target genes called vitamin D

response elements. Once that happens, two types of co-activator complexes (SRC and DRIP) bind to VDR promoting the transcription initiation of the target gene [59].

Vitamin D receptor was studied at the gene message level and the protein level in all tissue groups. Expression analysis of VDR gene shows that the cancer tissue group has a significant up-regulation of VDR gene comparing to both the proximal and the control tissue. VDR gene expression in the distal tissue group shows tendency to be higher than the control, but lower than the cancer. Statistically, the distal tissue group is not significantly different from any other tissue group (Figure 3.6). VDR protein analysis in different tissue groups is shown in (Figure 3.7). It reveals that VDR protein level in the cancer group is significantly higher than other tissue groups. Proximal and distal are not significantly different from each other or from the control group. In conclusion, both gene expression and protein analysis indicate that BCC tissue contains higher levels of vitamin D receptors than the cancer free tissue of the patients.

SRC is a protein complex that operates as a co-activator of VDR. It binds to the receptor subsequent to its binding to the VDRE in the target gene's promoter. SRC possesses a histone-acetylase activity. Chromatin remodeling caused by SRC makes the promoter region more accessible to other transcription factors. This action is followed by the binding of DRIP complex which recruits components of the transcription machinery in place[65]. We assessed the expression of the genes encoding SRC and DRIP co-activator in BCC patients and the controls specimens. Only the cancer tissue showed significant increase in the expression of SRC as

compared to other tissue groups. SRC gene expression in the proximal and the distal groups mimic the control group (Figure 3.8). Similarly, DRIP gene expression in the cancer tissue was significantly higher than other tissue groups. No significant differences were found in DRIP gene expression among proximal, distal, and control tissue groups (Figure 3.9).

Studying the impact of VRD over-expression in BCC on mTOR pathway:

VDR is known to negatively regulate mTOR through impacting REDD1. The latter gene is a VDR target gene that contains VDRE in its promoter. Up-regulation of this gene is known to inhibit the activity of mTOR. VDR effect on its target gene transcription regulation was shown to be impaired when VDREBP are up-regulated. These proteins compete with VDR over the binding sites in the target genes promoters [45]. We investigated whether the observed up-regulation of VDR in cancer tissue is translated into reduction of mTOR signaling. For that purpose, we studied some important proteins upstream and downstream of mTOR.

VDREBP protein levels analysis in all tissue groups is shown in Figure 3.11. Our data shows that VDREBP protein levels are significantly elevated in the cancer tissue comparing to the other groups. In contrast, the proximal tissue contains significantly lower amounts of this protein than the other groups. The distal and the control groups are not normal significantly different from each other.

REDD1 protein levels (Figure 3.10) reveal that the level of this protein in the cancer group is significantly higher than in other tissue groups. The proximal tissue tends to have a lower REDD1protein level than the distal and the control groups.

However, the three groups (proximal, distal, and control) are not significantly different from each other.

mTOR phosphorylates TIF4E-binding protein (4EBP), impairing its ability to bind to TIF4E protein [43]. Level of 4EBP phosphorylation has been used as an indicator for mTOR activity. Therefore, P-4EBP protein levels were assessed in cancer and normal tissue of BCC patients and compared to the control group (Figure 3.12). Results show that p-4EBP levels are significantly elevated in the cancer tissue comparing to the control group. On the other hand, the proximal tissue contains significantly lower amounts of this protein than the control. The distal tissue is not significantly different from the control group.

Analysis of key genes up-stream of mTOR in (Ras- IP13-AKT-mTOR) pathway:

IP13-AKT-mTOR is one of the pathways that were reported to be up-regulated in many cancers[66]. AKT is phosphorylated in two sites (S473 and Th 308). Both AKT phosphorylation sites are required to up-regulate mTOR activity. Ras is an important oncogene upstream of this pathway and is recurrently reported to have a gain of function mutation in many types of cancers [49].

Protein analysis shows that levels of AKT protein that are phosphorylated at the location (S473) are significantly elevated in both cancer and proximal tissue. In contrast, p-AKT (S473) levels in the distal and the control tissue groups are not significantly different from each other (Figure 3.13). P-AKT (Th308) protein levels were also detected in our tissue groups. Quantification of the exposed bands

indicated that levels of P-AKT phosphorylated at the location (Th308) follows the same rend of p-AKT (S473) as shown in Figure 3.14.

The expression of KRas gene was also assessed in our tissue groups. KRas mRNA was quantified using real-time PCR with KRas wild-type allele primers. Our data suggest that KRas gene is up-regulated in all tissue groups obtained from BCC patients comparing to the control group. However, we noticed that the expression decreases as the location of the tissue specimen gets farther from the cancer location. Statistically, KRas gene expression in the cancer and the proximal groups is significantly higher than the control group but not than the distal tissue. The distal tissue group is not significantly different from any other group including the control. (Figure 3.15)

Detection of KRas point mutation in BCC issue samples using the ASB-PCR technique:

Mutant allele specific real-time PCR with blocker ASB-PCR was used to detect the presence of point mutations in KRas gene [51]. Specifically designed primers were used to detect different possible point mutation that may occur in cancer cells. Each mutant allele specific primer was used to detect the presence of its corresponding point mutation in our tissue samples. Each ASB-PCR experiment was repeated with and without the addition of the wild-type allele specific blocker to minimize the non-specific amplification of the wild-type allele.

Tissue samples were inspected first for the presence of the point mutation (mut 2.1) in KRas gene using (mut 2.1) allele specific forward primer(Figure 3.16).

Results show that the message obtained using (mut 2.1) primer follows the same trend obtained from the wild-type primer. The only significant difference observed was in the cancer group in absence of the blocker. Upon adding the wild-type specific blocker to the PCR reaction, the previously noted significant increase was vanished. Our results suggest that the observed message is a non-specific amplification of the wild-type allele, and we concluded that our samples do not have (mut 2.1) Kras point mutation.

KRas point mutation (mut 4.1) was also tested in BCC tissue groups using mutant allele specific (mut 4.1) primer. The analysis was performed with and without adding the wild-type blocker. The message obtained using (mut 4.1) primer followed the same previously described trend, without showing any statistical significant among the groups neither with nor without the wild-type blocker. The results suggest that the observed message is a non-specific amplification. In conclusion, no Kras point mutation was detected by this mutant allele specific primer in our tissue specimens (Figure 3.17).

The third KRas point mutation allele specific primer that was tested with our samples was (mut 7.1) primer. The message generated by his primer also followed the same trend obtained by the wild-type primer. No statistical significance was found among the groups with or without the wild-type specific blocker. This indicated that our samples are negative for the mutation detected by his mutant allele specific primer (Figure 3.19).

Finally, tissue specimens were tested using KRas mutant allele specific (mut 5.1) primer. The resulting message from this primer without adding the wild-type specific blocker follows the same trend as the wild-type primer with no statistical significance among tissue groups. Interestingly, upon adding the wild-type allele specific blocker to the PCR reaction, all tissue groups from the BCC patients (cancer, proximal, and distal) showed significantly higher message levels comparing to the control group. Our data suggest the presence of this Kras point mutation in cancer, proximal and distal tissue specimens excised from BCC patients (Figure 3.18).

Gli1 gene expression analysis as an indicator of Hedge-hog pathway activity:

Expression of Gli1 gene was quantified in tissue specimens as an indicator of Hh-Gli pathway signaling. Our data clearly revealed that cancer tissue has a significantly higher expression of Gli1 gene compared to the other groups. This result indicates higher activity of Hh-Gli pathway in the cancer tissue, despite the higher vitamin D receptor and activation genes in that tissue groups. Proximal and distal tissue groups showed Gli1 gene expression that mimics the control group (Figure 3.20).

Analysis of P53-MDM2 pathway:

P53 is an important tumor suppressor gene in the cell. It is known to be regulated through MDM2. Elevated level of p53 protein promotes up-regulation of MDM2, which in turn binds and inactivates p53. There is emerging evidence in literature suggesting VDR involvement in this regulation [67]. VDR gene was found

to contain a p53 response element in its promoter. On the other hand, MDM2 promoter contains both VDR and P53 response elements [67, 68].

Expression of P53 gene was quantified and compared among different tissue groups. Our data shows that cancer tissue has a significantly higher message of P53 gene comparing to all other groups. The Proximal and the distal groups are not significantly different from each other or from the control in regards to p53 gene expression (Figure 3.21).

Similarly, MDM2 gene expression analysis (Figure 3.22) proposes that the cancer tissue show significantly higher expression comparing to other tissues. Proximal and distal groups show slightly lower MDM2 expression than the control group, but the differences are not statistically significant.

Analysis of VEGF gene expression in BCC tissue specimens:

Vascular endothelial growth factor (VEGF) gene is VDR target gene, and it contains VDRE in its promoter [69, 70]. Up-regulation of this protein causes vascular growth which triggers angiogenesis around the cancer, which promotes tumor growth [70].

Expression Analysis of VEGF gene was carried out in BCC tissue groups as well as the control group (Figure 3.23). Our results propose that tissue specimens from the cases generally up-regulate VEGF gene comparing to the controls. The elevation in VEGF gene expression is statistically significant only in the proximal tissue group despite the visual trend of higher message of VEGF in the cancer tissue as well.

Gene expression analysis of important cell regulators:

Caspase-3, apoptosis-related cysteine peptidase-3, is a key enzyme in controlling the programmed cell death, and it is involved in both intrinsic and extrinsic apoptosis pathways[71]. Expression of caspase-3 gene was analyzed in BCC tissue groups (Figure 3.25). Data analysis discloses a significant up-regulation of caspase-3 gene message in the cancer tissue comparing to all other tissue groups. We observed a trend of gradual decrease among the tissue specimens from the cases, inferring an inverse relationship between caspase-3 message and the distance from the cancer location. In other words, Caspase-3 message in the proximal tissue is significantly lower than in the cancer, and significantly higher than in the distal tissue. Yet, the proximal and the distal groups are not statistically significant from the control group.

Bcl-2 protein is an anti-apoptotic protein that halts the intrinsic (mitochondrial) apoptosis pathway through blocking the release of cytochrome C from the mitochondria [72]. Gene expression analysis of our groups proposes a dramatic elevation in the Bcl2 message in the cancer tissue comparing to the other groups. Proximal and distal tissue specimens are slightly higher than the control, but not significantly different from it (Figure 3.24).

PARP-1 is a chromatin associated protein, and is important for cell survival and growth. PARP-1 plays an important role in DNA repair pathways especially the BER, and function as a transcription regulator for some genes [73]. The expression and the cleavage of PARP-1 were both found to be down-regulated by vitamin D3

[34, 69, 73, 74]. The message of PARP-1 gene was quantified in our tissue specimens (Figure 3.26). Data clearly reveals that the cancer tissue has significantly higher expression of PARP-1 gene comparing to the other tissue groups. Proximal and distal tissue has PARP-1 gene expression similar to that in the control group.

Apex-1 gene expression was also analyzed as another gene involved in DNA repair. Our results indicate that Apex-1 gene is significantly down-regulated in the proximal and distal comparing to the cancer and the control. Interestingly, the expression of this gene in the cancer tissue group mimics that in the control group (Figure 3.27).

Expression analysis of GADD45A gene (growth arrest and DNA-damage-inducible protein alpha) also indicates higher message in the cancer tissue than all other groups. Data analysis discloses a general up-regulation of GADD45A gene in all tissue specimens obtained from BCC patients compared to the control. This up-regulation is greater in the cancer tissue, with a trend of a gradual decline with the increasing distance from the cancer. GADD45A message levels in the cancer and the proximal tissue are significantly higher than the control. The distal group message level falls between the proximal group and the control, but it is not significantly different from any of them (Figure 3.28).

Table 3.1: *Demographic data analysis for BCC Patients:* participants are 20 Caucasian BCC patients from southeast Michigan. Patients are 14 men and 6 women of age range between (43-89 years old). Average age is 74 years; median age and mode are both at 80 years. Ages of men and women are not significantly different from each other. Participants' age data failed to pass the KS normality test.

Subjects Ages	Males	Females	All BCC
Sample size (N)	14	6	20
Mean age	74.357	74.667	74.45
Standard deviation (SD)	12.555	11.518	11.949
Std. error of mean(SEM)	3.356	4.944	2.741
Lower 95% conf. limit	67.109	61.622	68.858
Upper 95% conf. limit	81.605	87.045	80.042
Minimum age	43	56	43
Median age (50th percentile)	80	77.5	80
Maximum age	89	86	89
Normality test KS	0.3163		
Normality test P value	0.0005		
Passed normality test?	No		
Participants Race	All participants are Caucasian		

Figure 3.1: *Ages of BCC patients at time of diagnosis is not normally distributed:* BCC patients were grouped based on their ages at time of diagnosis. Each group covers 10 years within the patient's age range as the following: ≤45, 46-55, 56-65, 66-75, 76-85, and 86-90. The number of patients in each group was plotted at the middle of group. The trend line shows a peak at (76-85 Y/O) group with a gradual increase before 75 Y/o and a sharp decline after 85 Y/o causing the skewed distribution.

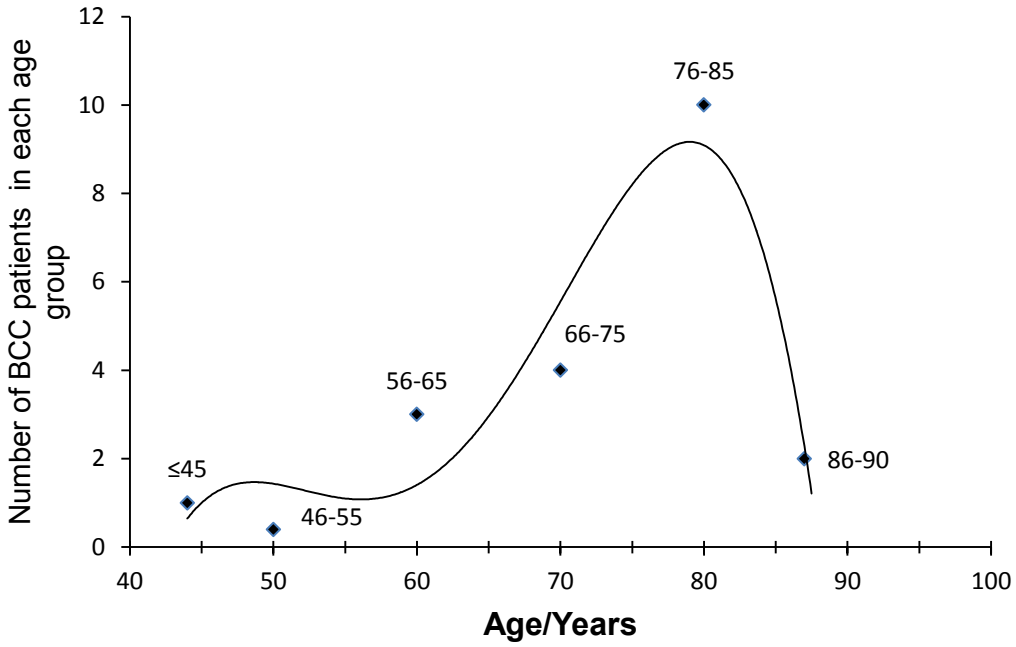

Number of BCC patients in each age group
12
10
8
6
4
2
0
40
50
60
70
80
90
100
Age/Years
≤45
46-55
56-65
66-75
76-85
86-90

Figure 3.2: *RNA integrity gel*: denaturing RNA gel electrophoresis image for cancer, proximal and distal samples taken from the same patient. Cancer tissue yielded 2.5 times as much RNA as the same weight of the normal tissue excised from the same person at the same time. Density of the bands is a quantity indicator.

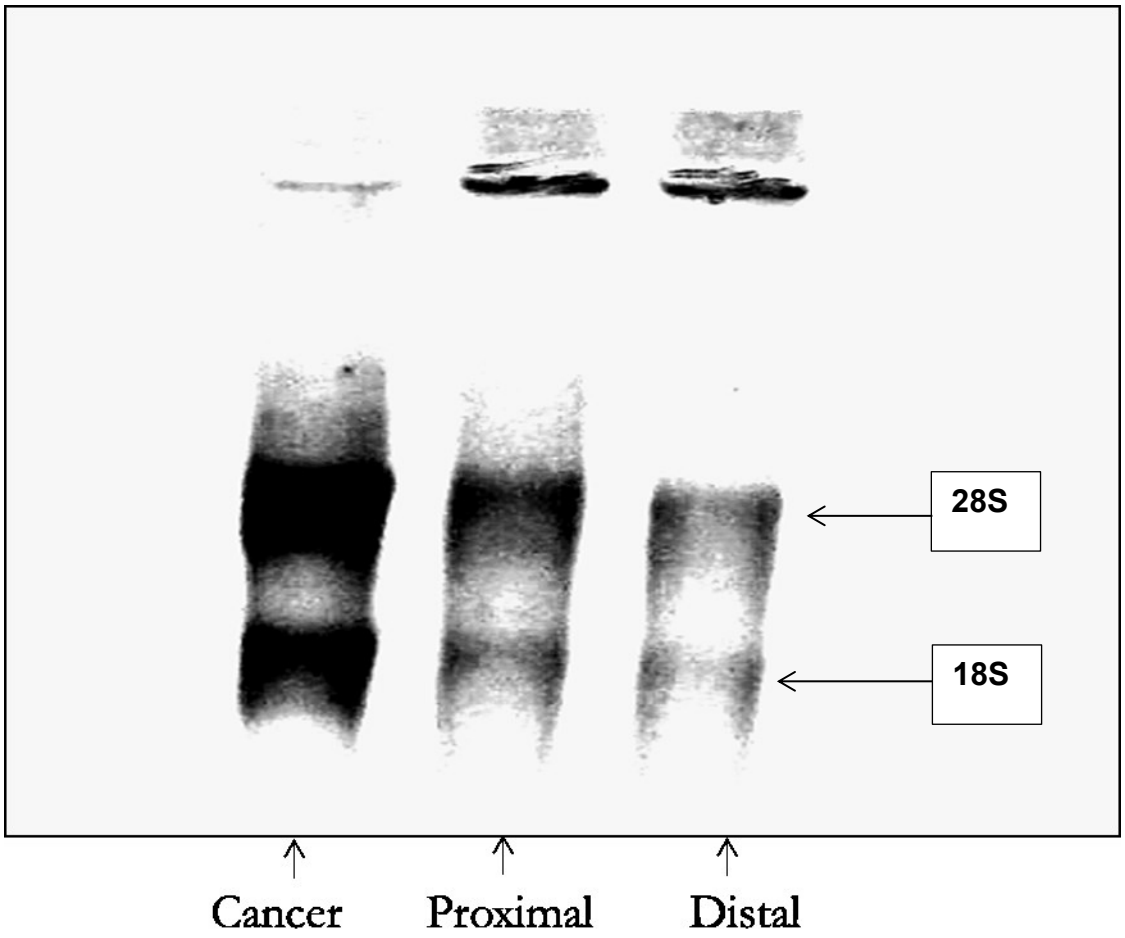
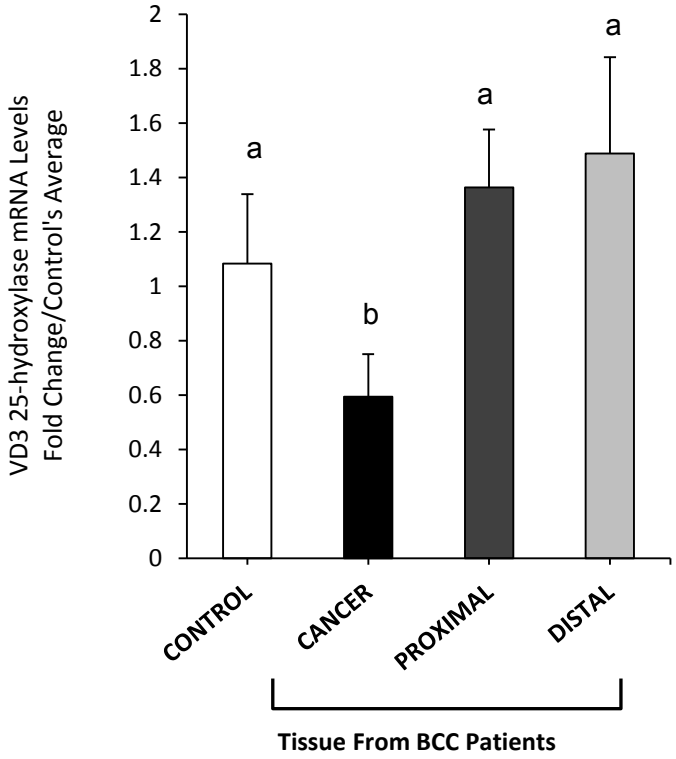

28S
18S
Cancer
Proximal
Distal

Figure 3.3: *Vitamin D activation gene Vitamin D3 25-hydroxylase (CYP27A1) expression analysis in BCC Patients' tissue:* Data suggests that expression of VD3 25-OHase is significantly down regulated in basal cell carcinoma tissue comparing to normal tissue obtained from BCC patients as well as from the controls. Both proximal and distal tissue groups are not significantly different from each other. Despite the visual elevation of VD3 25-Ohase message in the normal tissue of the patients comparing to the controls, the difference was not statistically significant. cDNA was constructed from 1µg of purified RNA and used to quantify the message of VD3 25-OHase gene which performs the first step of VD activation yielding 25(OH)VD3. mRNA was quantified by real-time PCR and expressed as fold change of the controls group average. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant.

a)


VD3 25-hydroxylase mRNA Levels
Fold Change/Control's Average
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
a
a
CONTROL
CANCER
PROXIMAL
DISTAL
Tissue From BCC Patients

b)

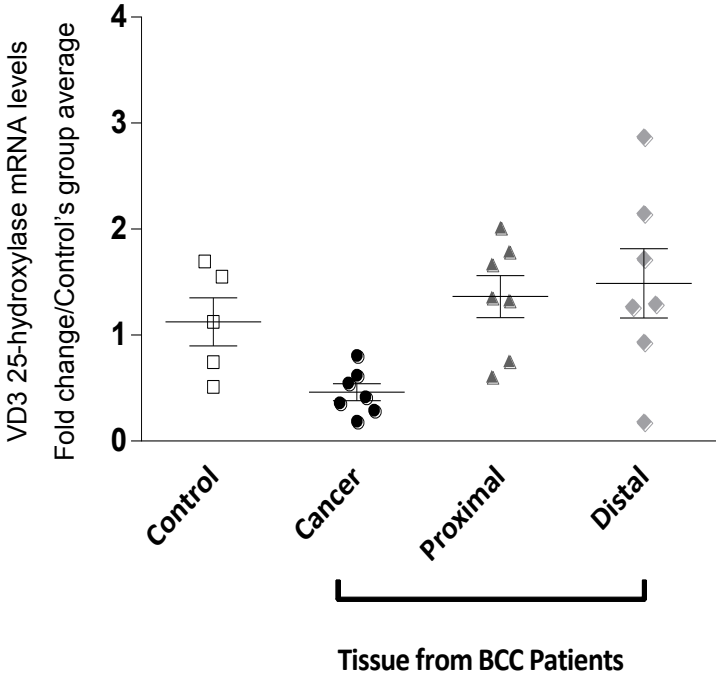
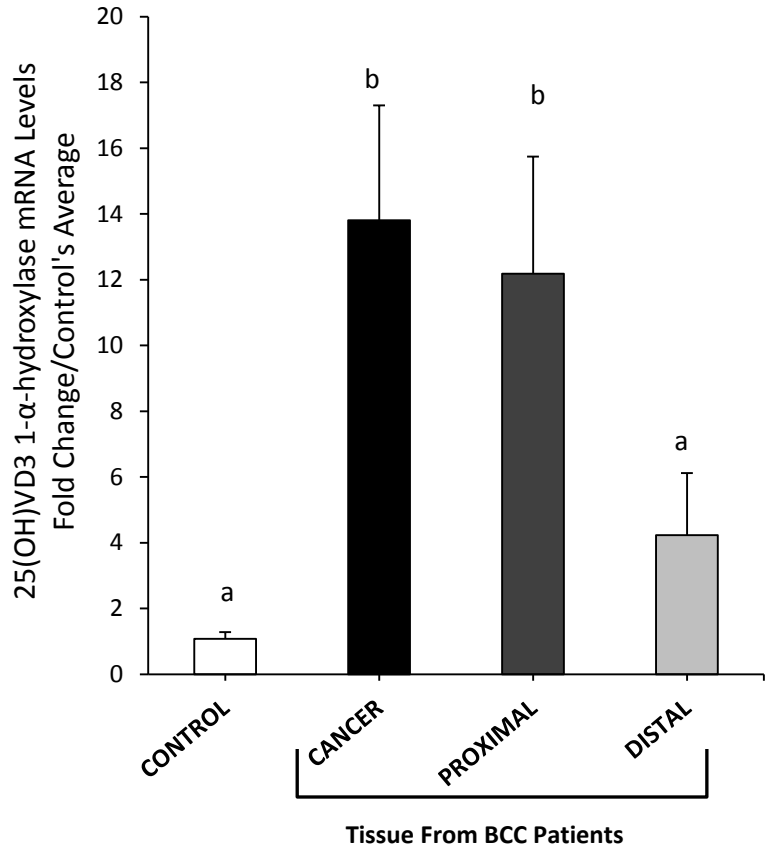

VD3 25-hydroxylase mRNA levels
Fold change/Control's group average
4
3
2
1
0
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.4: *25-Hydroxyvitamin D_3 1-alpha-hydroxylase (CYP27B1) expression analysis in all tissue groups:* cDNA was constructed from 1µg of purified RNA and used to quantify the message of the vitamin D activation gene (VD3 1α-hydroxylase). mRNA was quantified by real-time PCR expressed as fold changes of the controls group average. Data shows that the message of VD3 1α-hydroxylase which performs the final step of activation of vitamin D and forming the hormonal form of it is significantly elevated in both the cancer and the proximal tissue groups comparing to the proximal and the control groups. The proximal tissue specimens show a clear tendency to be higher than the controls with no statistical significance. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant.

a)


25(OH)VD3 1-α-hydroxylase mRNA Levels
Fold Change/Control's Average
20
18
16
14
12
10
8
6
4
2
0
a
b
b
a
CONTROL
CANCER
PROXIMAL
DISTAL
Tissue From BCC Patients

b)

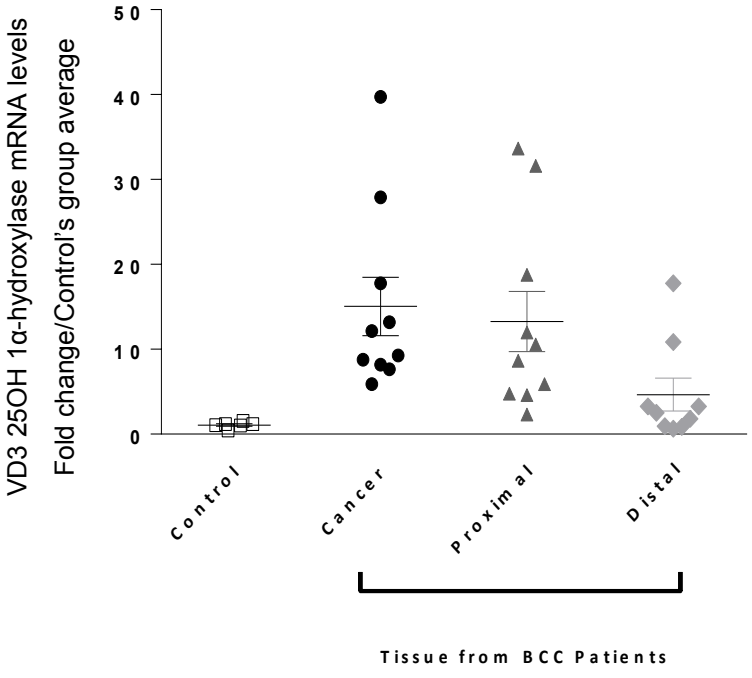
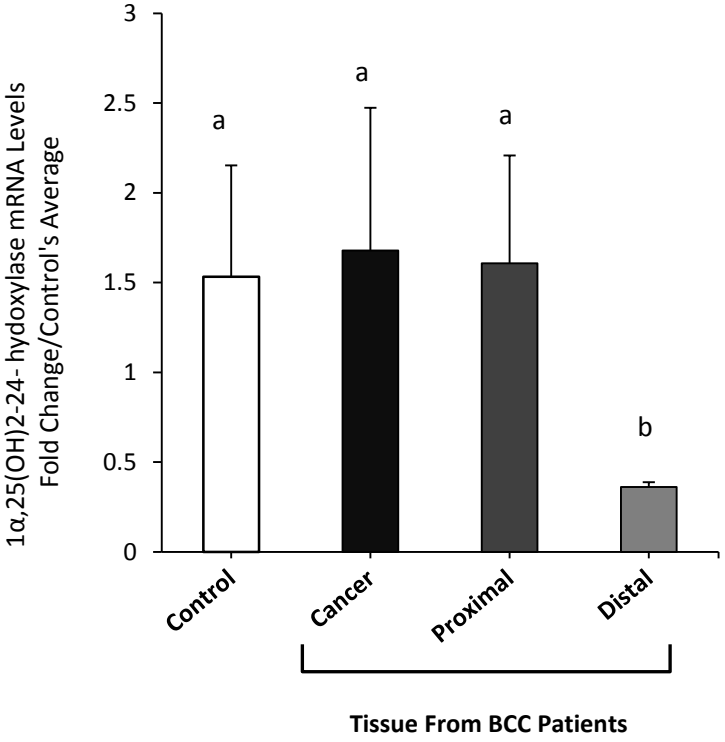

VD3 25OH 1α-hydroxylase mRNA levels
Fold change/Control's group average
50
40
30
20
10
0
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.5: *Vitamin D3 catabolism gene 1α, 25 (OH)2 VD3 24-hydroxylase (CYP24A1) expression analysis in BCC patients tissue groups:* 1µg of purified RNA from different tissue specimens were used to construct cDNA, which was in turn used to quantify the message of the gene. mRNA of 1α,25(OH)2VD3 24-OHase gene was quantified in tissue specimens using real-time PCR and was expressed as fold changes of the controls group average. Our data clearly reveals that the distal tissue group obtained from BCC patients significantly down-regulates VD3 catabolism gene comparing to other tissue groups. Cancer tissue itself along with the proximal tissue retains normal VD3 catabolism gene activity, showing no significant difference from the control group. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant.

a)


1α,25(OH)2-24- hydoxylase mRNA Levels
Fold Change/Control's Average
3
2.5
2
1.5
1
0.5
0
a
a
a
b
Control
Cancer
Proximal
Distal
Tissue From BCC Patients

b)

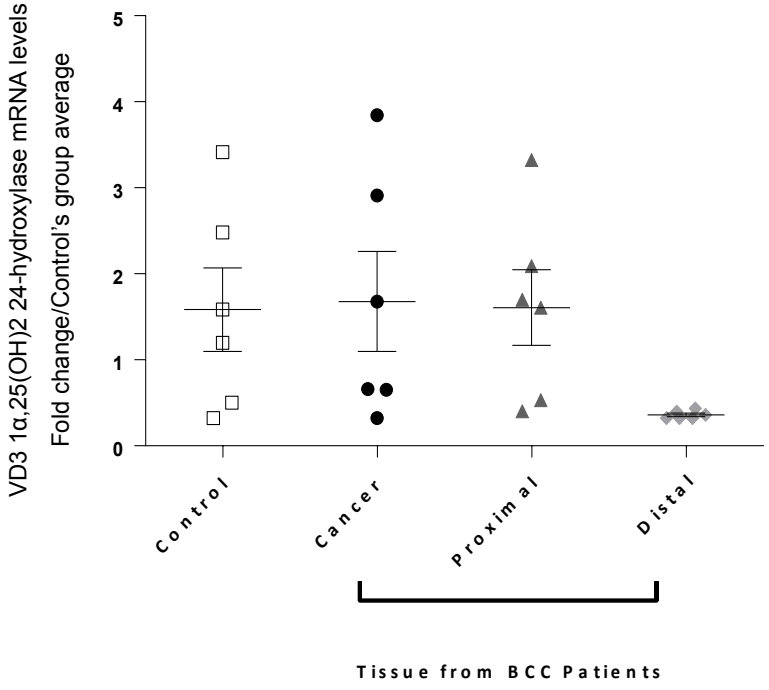
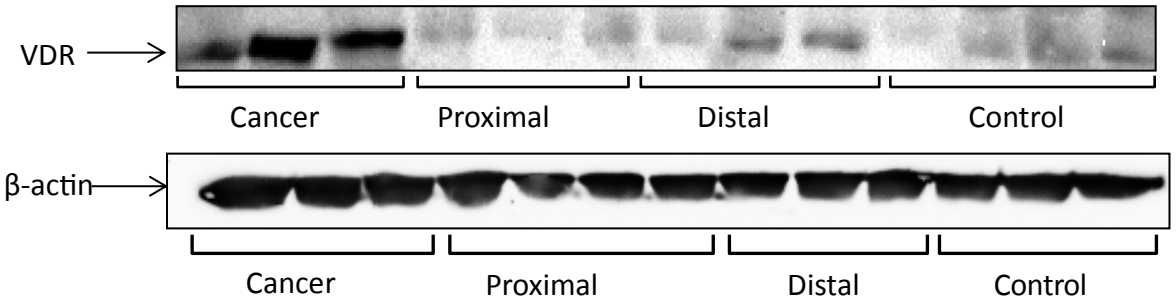

VD3 1α,25(OH)2 24-hydroxylase mRNA levels
Fold change/Control's group average
5
4
3
2
1
0
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.6: *Vitamin D receptor (VDR) protein analysis in tissue groups:* Whole cell extract was prepared using RIPA buffer, and then used to quantify VDR protein levels in tissue samples. Western blot was performed by running all groups on 10% SDS-PAGE before targeted bands were plotted by specific anti-bodies. β.actin protein level was used as an internal control. Quantifying the exposed bands reveals that VDR protein level in the cancer group is significantly higher than those in other tissue groups. Proximal and distal are not significantly different from each other or from the control group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. a) Image of the quantified western blot bands for VDR and β.actin. b) Results shown in column representing the average and the SEM for each tissue group. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant

a)


VDR
Cancer
Proximal
Distal
Control
β-actin
Cancer
Proximal
Distal
Control

b)

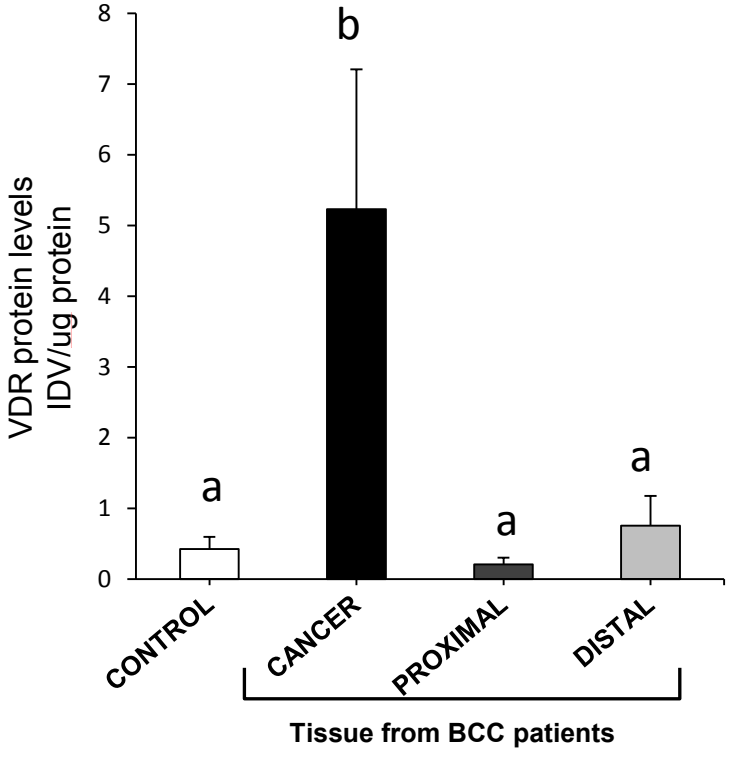
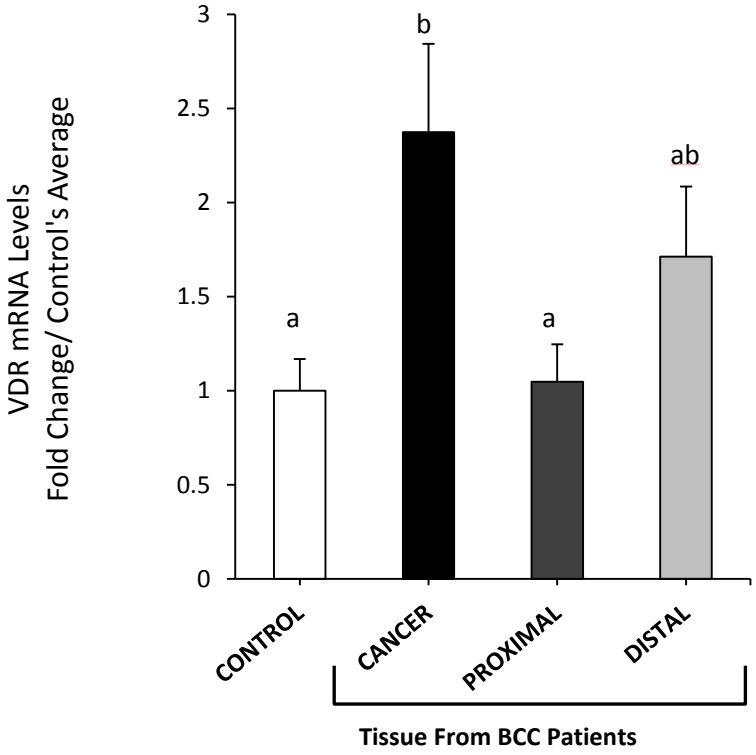

VDR protein levels
IDV/ug protein
8
7
6
5
4
3
2
1
0
a
b
a
a
CONTROL
CANCER
PROXIMAL
DISTAL
Tissue from BCC patients

Figure 3.7: V*itamin D receptor VDR gene expression analysis in tissue groups: expression analysis in all tissue groups:* cDNA was constructed from 1µg of purified RNA and used to quantify the message of vitamin D receptor (VDR) gene. mRNA of VDR was quantified by real-time PCR and expressed as fold change of the controls group average. Data analysis shows that the cancer tissue group has a significant up-regulation of VDR gene comparing to both the proximal and the control tissue. In contrast to the proximal tissue, the distal tissue group shows tendency to be higher than the normal, but lower than the cancer. The distal tissue group is not statistically significantly different from any other tissue group. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant.

a)


VDR mRNA Levels
Fold Change/ Control's Average
3
2.5
2
1.5
1
0.5
0
a
b
a
ab
CONTROL
CANCER
PROXIMAL
DISTAL
Tissue From BCC Patients

b)

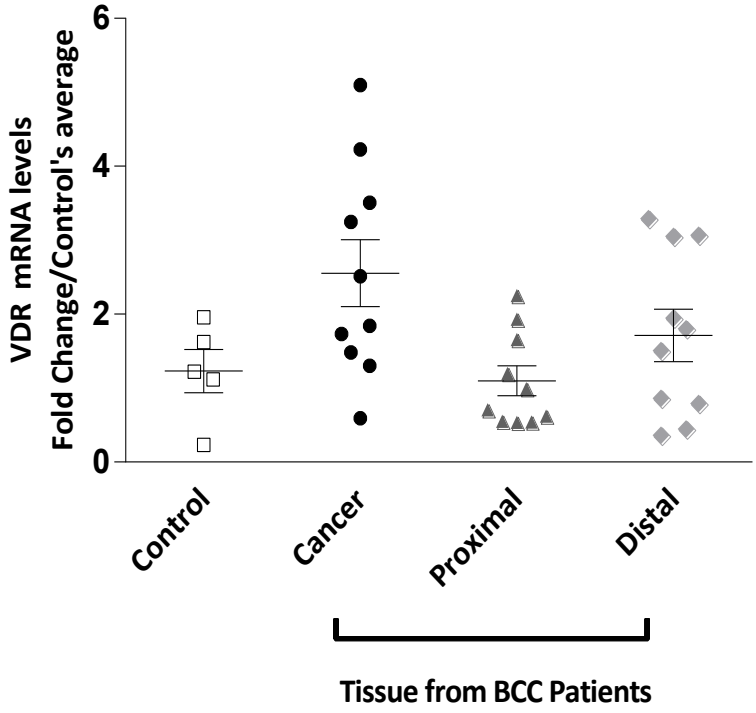
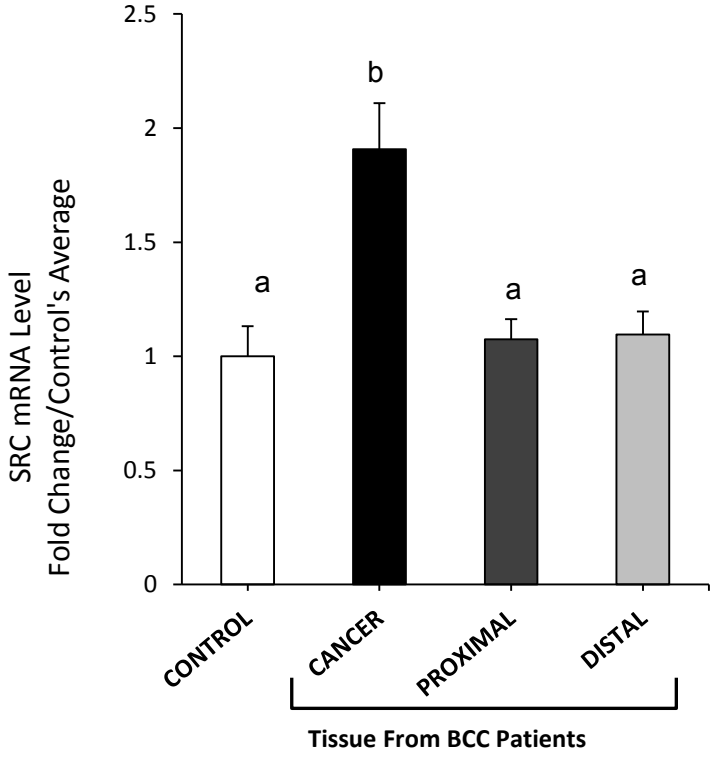

VDR mRNA levels
Fold Change/Control's average
6
4
2
0
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.8: *Comparison of VDR Co-activator (SRC) gene expression among tissue groups:* cDNA was constructed from 1µg of purified RNA and used to quantify the message of VDR co-activator SRC gene, which was then quantified by real-time PCR and expressed as fold change of the controls average. Only the Basal cell carcinoma tissue shows significantly elevated expression of SRC comparing to the normal tissue from BCC patients and from the controls. Proximal and distal tissue groups have normal SRC expression that mimics the control group. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant.

a)


2.5
2
1.5
1
0.5
0
SRC mRNA Level
Fold Change/Control's Average
a
b
a
a
CONTROL
CANCER
PROXIMAL
DISTAL
Tissue From BCC Patients

b)

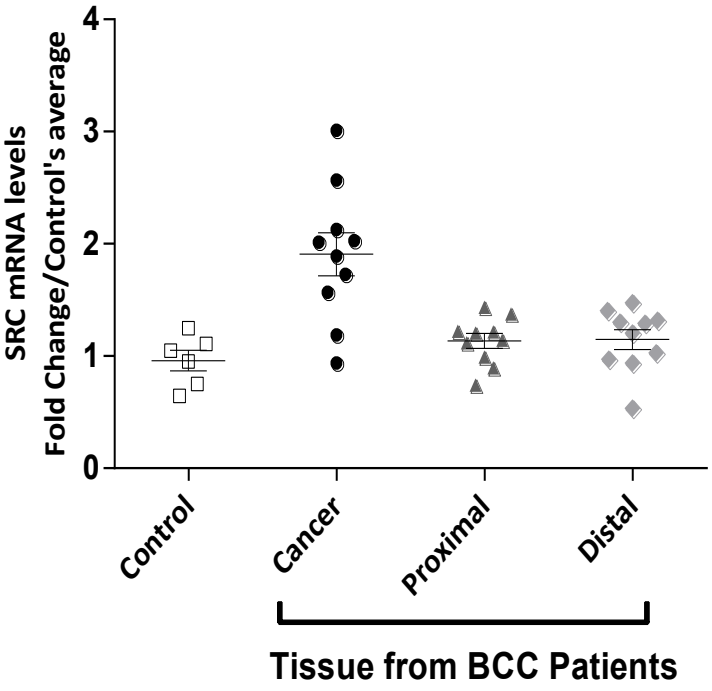
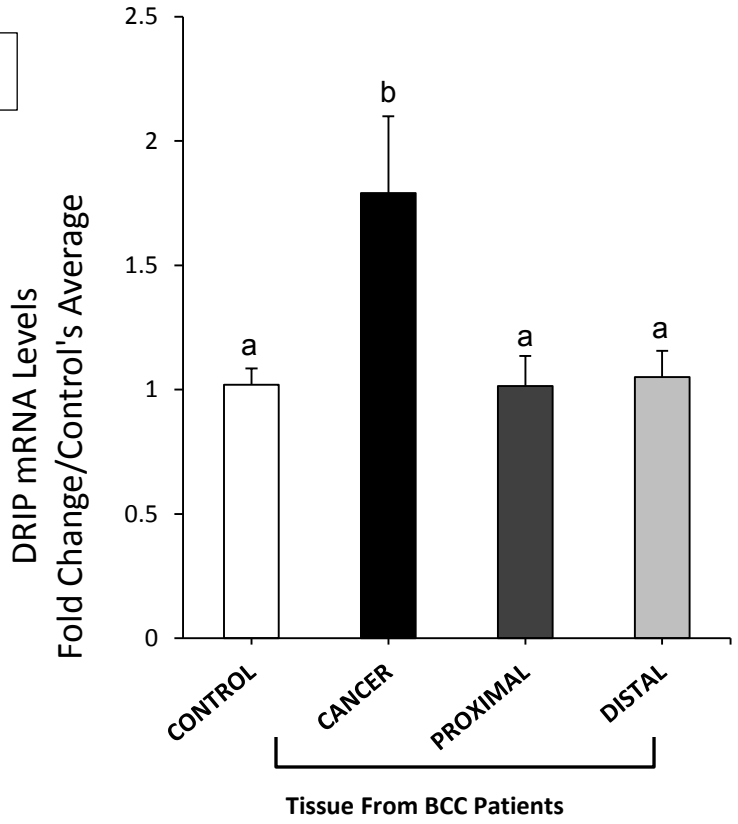

4
3
2
1
0
SRC mRNA levels
Fold Change/Control's average
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.9: *Analysis of DRIP (VDR Co-activator) gene expression among tissue groups:* 1µg of purified RNA from different tissue specimens were used to construct cDNA, which was in turn used to quantify the message of the gene. mRNA of VDR co-activator DRIP gene was quantified in tissue specimens using real-time PCR and was expressed as fold changes of the controls group average. Our data clearly reveals that BCC patients' cancer tissue shows significantly higher expression of DRIP comparing to the normal tissue from BCC patients and from the controls. Proximal and distal tissue groups have normal DRIP expression that mimics the control group. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant.

a)


2.5
2
1.5
1
0.5
0
DRIP mRNA Levels
Fold Change/Control's Average
a
b
a
a
CONTROL
CANCER
PROXIMAL
DISTAL
Tissue From BCC Patients

b)

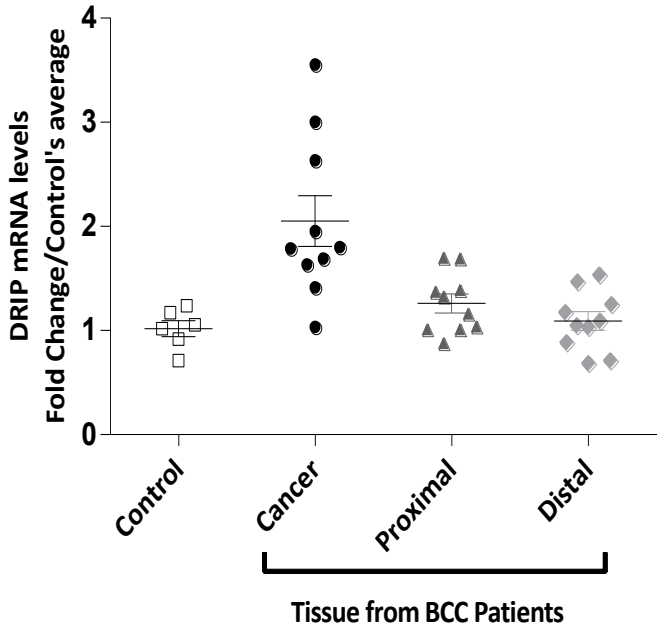
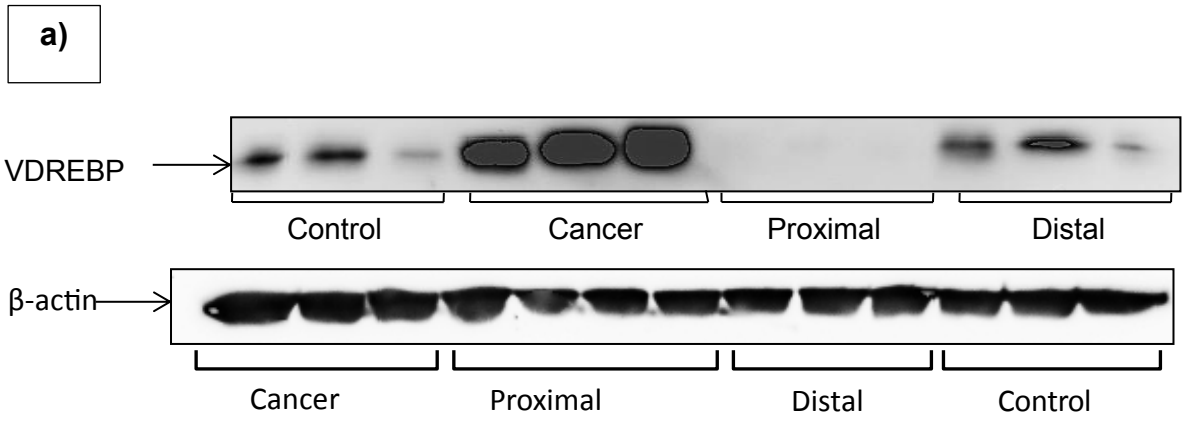

4
3
2
1
0
DRIP mRNA levels
Fold Change/Control's average
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.10: *Protein Levels of vitamin D response element binding protein VDREBP:* Whole cell extract was prepared using RIPA buffer, and then used to quantify VDREBP protein levels in tissue samples. Western blot was performed by running all groups on 10% SDS-PAGE before targeted bands were plotted by specific anti-bodies. β.actin protein level was used as an internal control. Results show that VDREBP protein levels are significantly elevated in the basal cell carcinoma tissue. In contrast to the cancer tissue, the normal tissue proximal to the cancer location contains extremely low amounts of this protein. The normal tissue that is distal to the cancer shows normal levels of this protein that is not significantly different from the control group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. a) Image of the quantified western blot bands for VDREBP and β.actin. b) Results shown in column representing the average and the SEM for each tissue group. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant


a)
VDREBP
Control
Cancer
Proximal
Distal
β-actin
Cancer
Proximal
Distal
Control

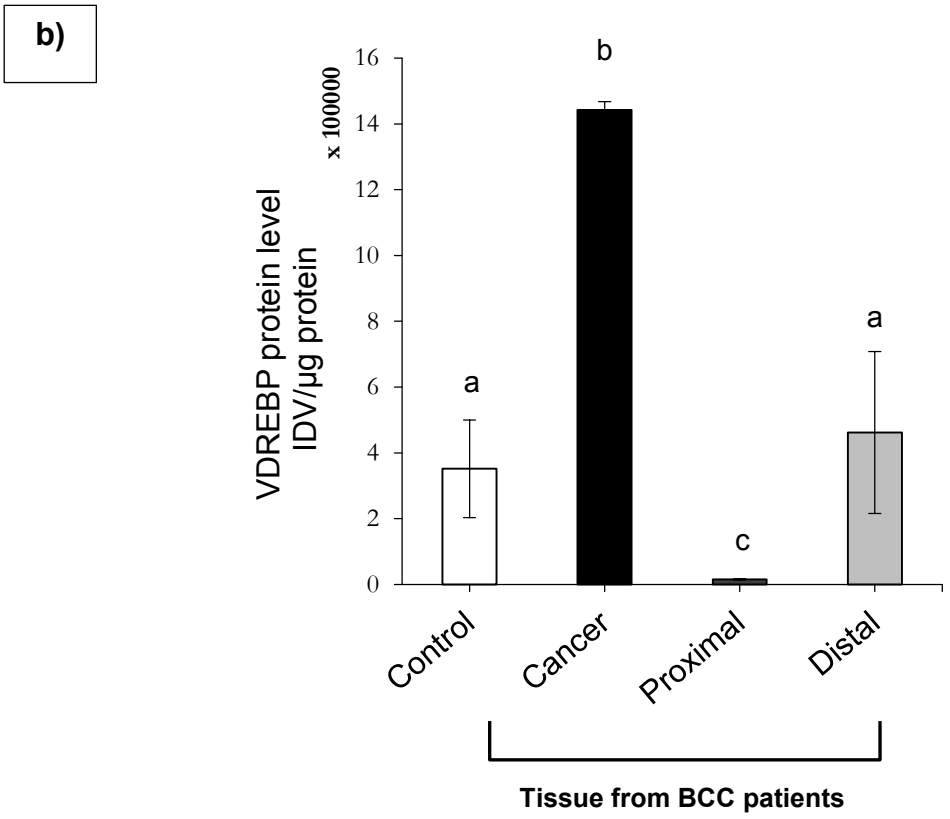
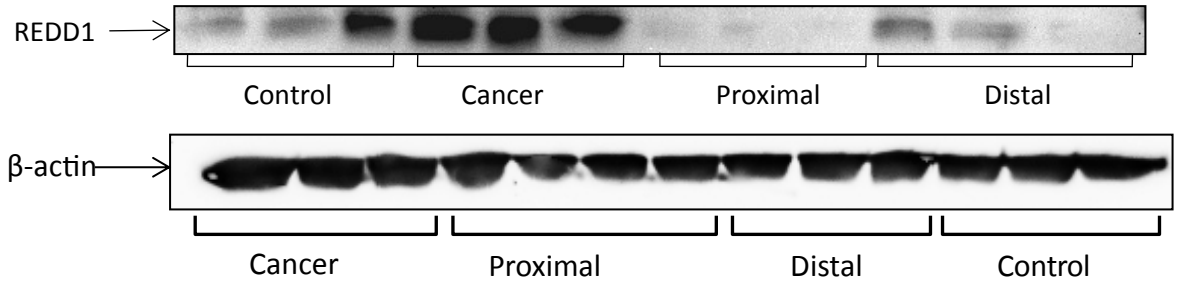

b)
VDREBP protein level
IDV/µg protein
x 100000
16
14
12
10
8
6
4
2
0
a
b
c
a
Control
Cancer
Proximal
Distal
Tissue from BCC patients

Figure 3.11: *Assessment of REDD1 (mTOR blocker) protein levels in BCC patients' tissue:* Whole cell extract was prepared using RIPA buffer, and then used to quantify REDD1 protein levels in tissue samples. Western blot was performed by running all groups on 10% SDS-PAGE before targeted bands were plotted by specific anti-bodies. β.actin protein level was used as an internal control. Quantifying the exposed bands reveals that REDD1 protein level in the cancer group is significantly higher than those in other tissue groups. Proximal tissue group shows a visible trend to have lower protein levels of REDD1 than the distal and the control groups. However, statistical analysis did not confirm any significant differences between the normal tissue from BCC patients and the controls. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. β.actin protein level was used as an internal control. . a) Image of the quantified western blot bands for REDD1 and β.actin. b) Results shown in column representing the average and the SEM for each tissue group. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant

a)


REDD1
Control
Cancer
Proximal
Distal
β-actin
Cancer
Proximal
Distal
Control

b)

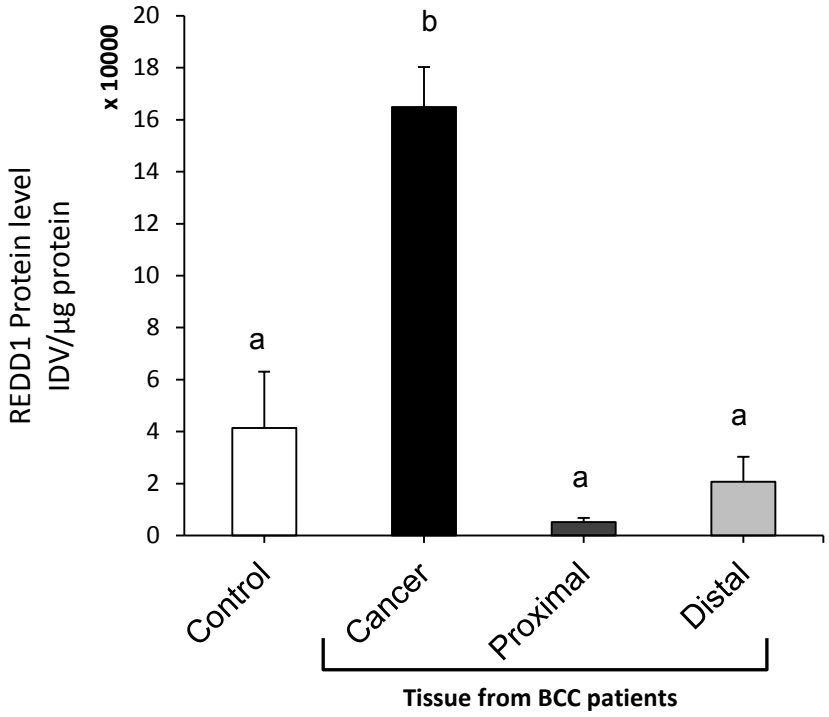
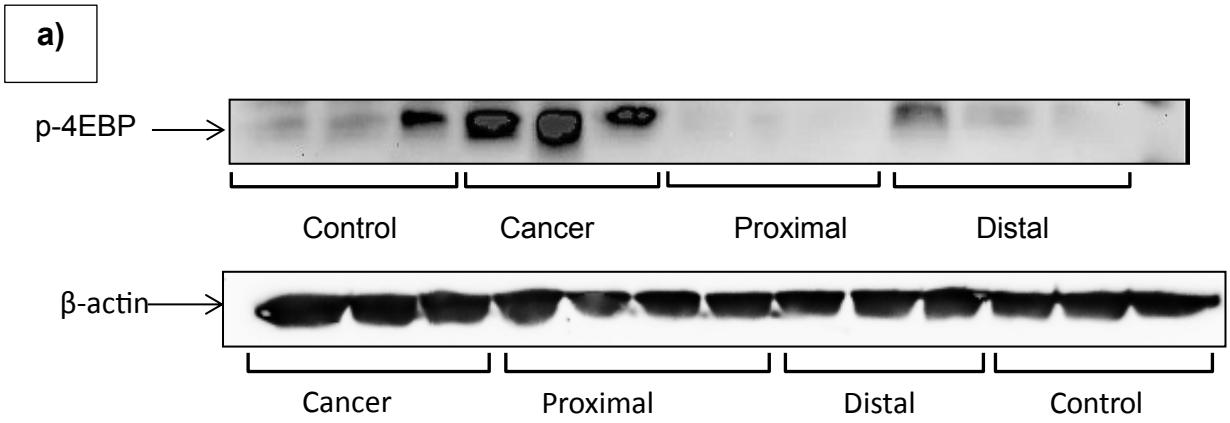

REDD1 Protein level
IDV/µg protein
x 10000
20
18
16
14
12
10
8
6
4
2
0
a
b
a
a
Control
Cancer
Proximal
Distal
Tissue from BCC patients

Figure 3.12: *Assessment of p-4EBP Protein Levels in cancer and normal tissue of BCC patients as compared to the controls:* Whole cell extract was prepared using RIPA buffer, and then used to quantify p-4EBP protein levels in tissue samples. Western blot was performed by running all groups on 10% SDS-PAGE before targeted bands were plotted by specific anti-bodies. β.actin protein level was used as an internal control. Results show that p-4EBP protein levels are significantly elevated in the cancer tissue comparing to the control group. On the other hand, the normal tissue obtained from BCC patients that is proximal to the cancer location contains extremely low amounts of this protein. The normal tissue that is distal to the cancer shows normal levels of this protein that is not significantly different from the control group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. a) Image of the quantified western blot bands for p-4EBP and β.actin. b) Results shown in column representing the average and the SEM for each tissue group. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant


a)
p-4EBP
Control
Cancer
Proximal
Distal
β-actin
Cancer
Proximal
Distal
Control

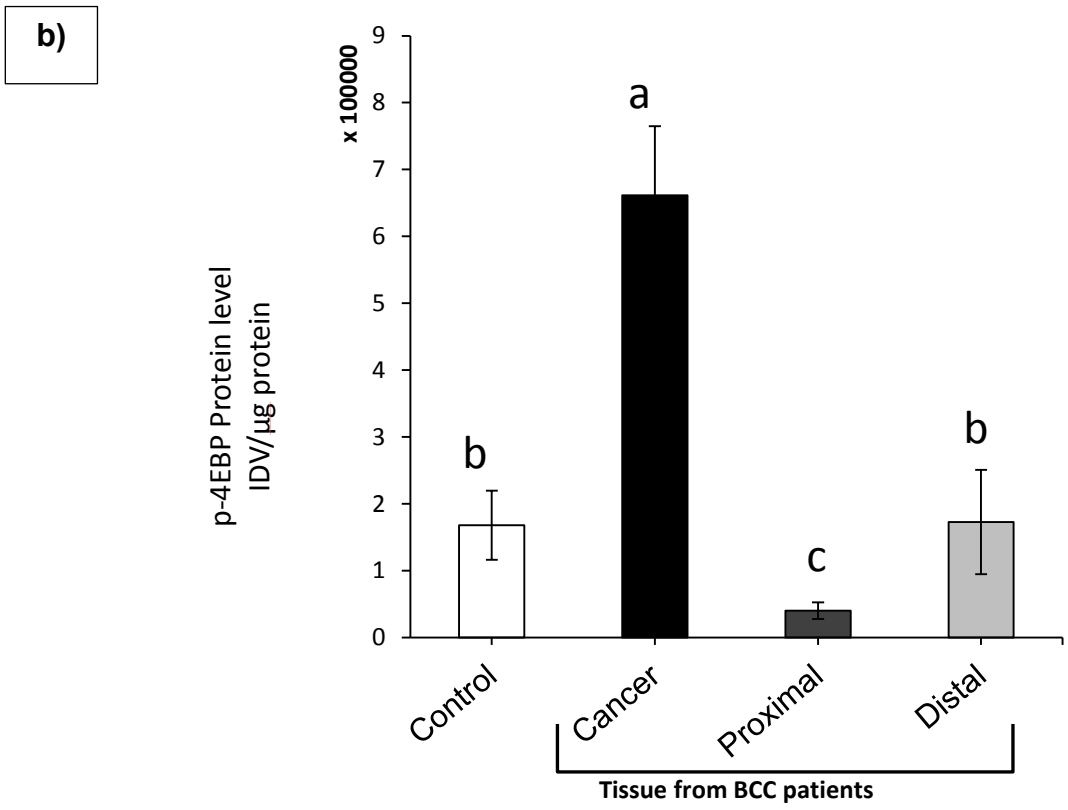
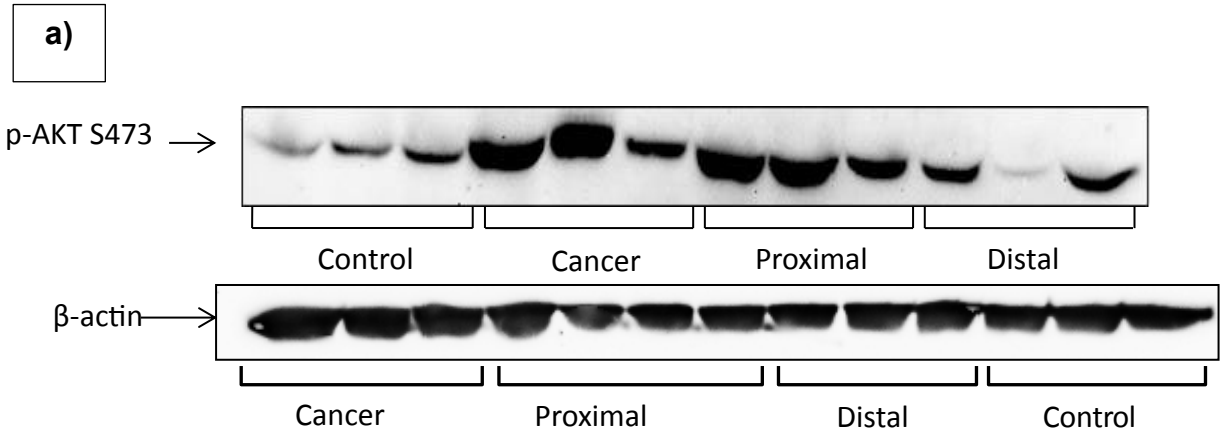

b)
p-4EBP Protein level
IDV/µg protein
x 100000
9
8
7
6
5
4
3
2
1
0
a
b
c
b
Control
Cancer
Proximal
Distal
Tissue from BCC patients

Figure 3.13: *p-AKT (S473) protein analysis in all tissue groups:* Whole cell extract was prepared using RIPA buffer, and then used to quantify p-AKT (S473) protein levels in different tissue samples. Western blot was performed by running all groups on 10% SDS-PAGE before targeted bands were plotted by specific anti-bodies. β.actin protein level was used as an internal control. Protein analysis on tissue specimens obtained from BCC patients shows that levels of AKT protein phosphorylated at the location (S473) are significantly elevated in both cancer tissue and normal tissue adjacent to the cancer as compared to the control group. In contrast, p-AKT (S473) levels in the normal distal tissue from BCC patients and the normal tissue obtained from the controls are not significantly different from each other. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. a) Image of the quantified western blot bands for p-AKT(S473) and β.actin. b) Results shown in column representing the average and the SEM for each tissue group. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant


a)
p-AKT S473
Control
Cancer
Proximal
Distal
β-actin
Cancer
Proximal
Distal
Control

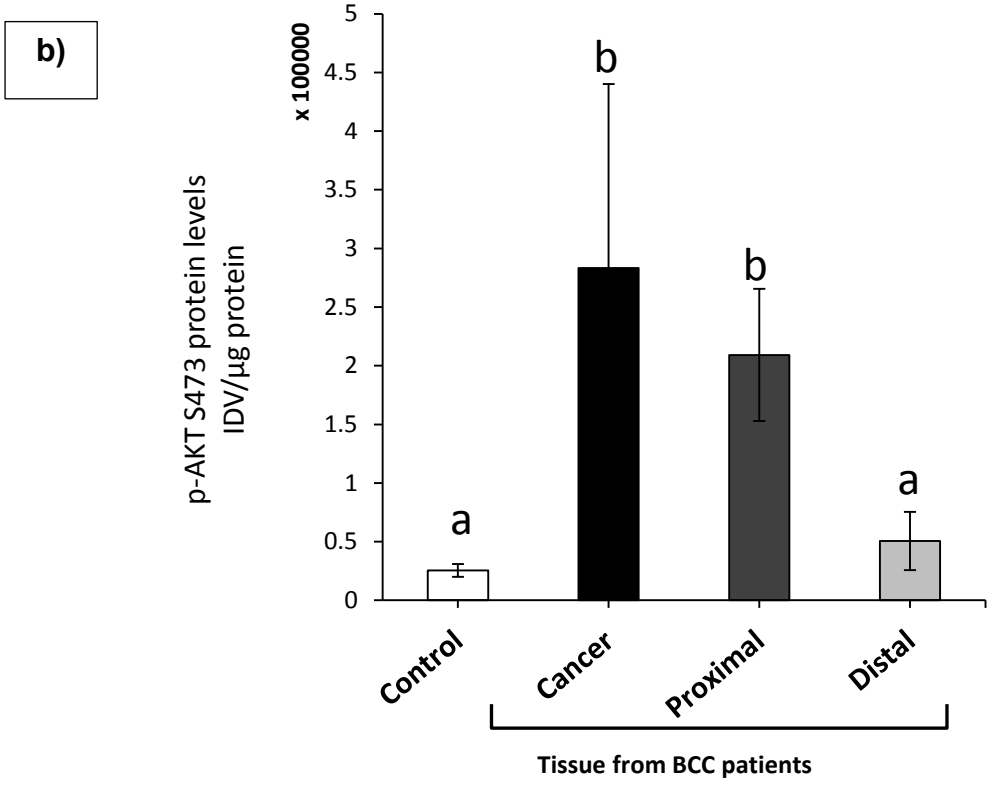
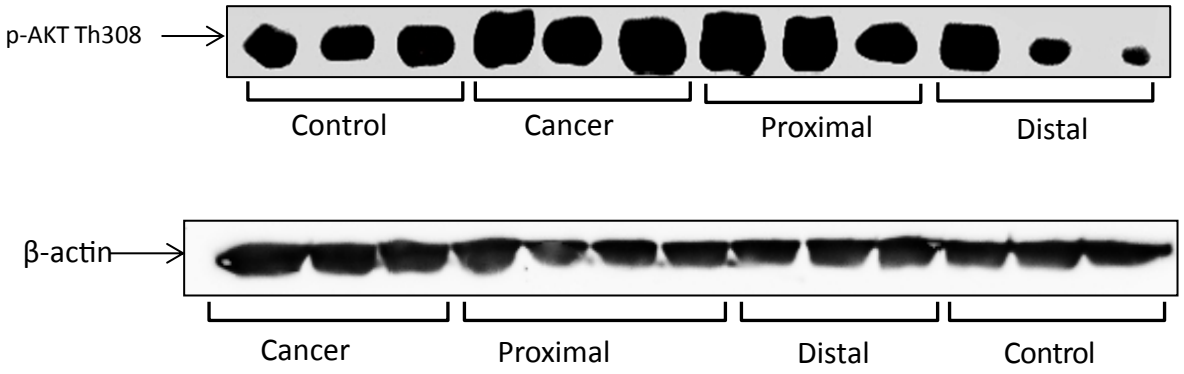

b)
x 100000
p-AKT S473 protein levels
IDV/µg protein
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
b
a
Control
Cancer
Proximal
Distal
Tissue from BCC patients

Figure 3.14: *p-AKT (Th308) protein levels in BCC tissue groups and the control group:* Whole cell extract was prepared using RIPA buffer, and then used to quantify p-AKT (Th308) protein levels in tissue specimens. Western blot was performed by running all groups on 10% SDS-PAGE before targeted bands were plotted by specific anti-bodies. β.actin protein level was used as an internal control. Quantification of the exposed bands indicates that levels of AKT protein phosphorylated at the location (Th308) follows the same rend of p-AKT (S473). Statistical analysis of the data suggests that levels of p-AKT (Th308) are significantly elevated in cancer tissue and proximal normal tissue comparing to the controls. In contrast, levels of this protein in the normal distal tissue and the control group are not significantly different from each other. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. a) Image of the quantified western blot bands for p-AKT (Th308) and β.actin. b) Results shown in column representing the average and the SEM for each tissue group. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant

a)


p-AKT Th308
Control
Cancer
Proximal
Distal
β-actin
Cancer
Proximal
Distal
Control

b)

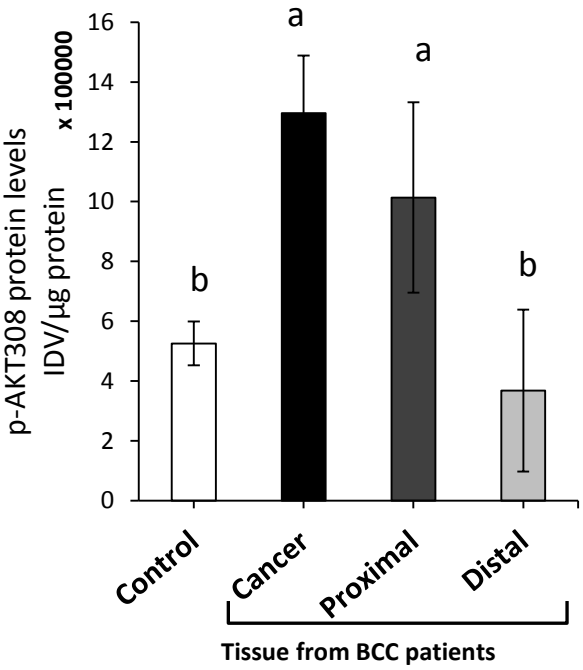
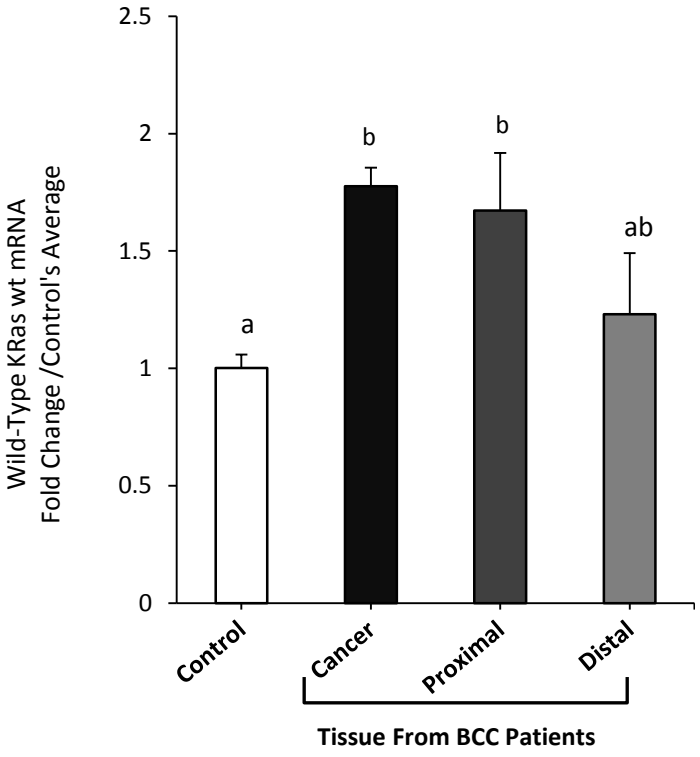

p-AKT308 protein levels
IDV/µg protein
x 100000
16
14
12
10
8
6
4
2
0
a
a
b
b
Control
Cancer
Proximal
Distal
Tissue from BCC patients

Figure 3.15: *KRas wild type gene expression in different tissue groups:* cDNA was constructed from 1µg of purified RNA and used to quantify the message of vitamin D receptor KRas gene. mRNA was quantified using real-time PCR assay with KRas wild-type allele primers, and results were expressed as fold changes of the controls group average. Analysis of the wild-type KRas gene expression reveals that KRas is up-regulated in all tissue groups obtained from BCC patients. The observed elevation gradually decreases in relation to the distance of the tissue specimen from the cancer. Statistically, KRas expression is significantly higher in cancer and proximal tissue groups than the control group but not than the distal tissue. The later tissue group is not statistically significantly different from any other group including the control. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant.

a)


Wild-Type KRas wt mRNA
Fold Change /Control's Average
2.5
2
1.5
1
0.5
0
a
b
b
ab
Control
Cancer
Proximal
Distal
Tissue From BCC Patients

b)

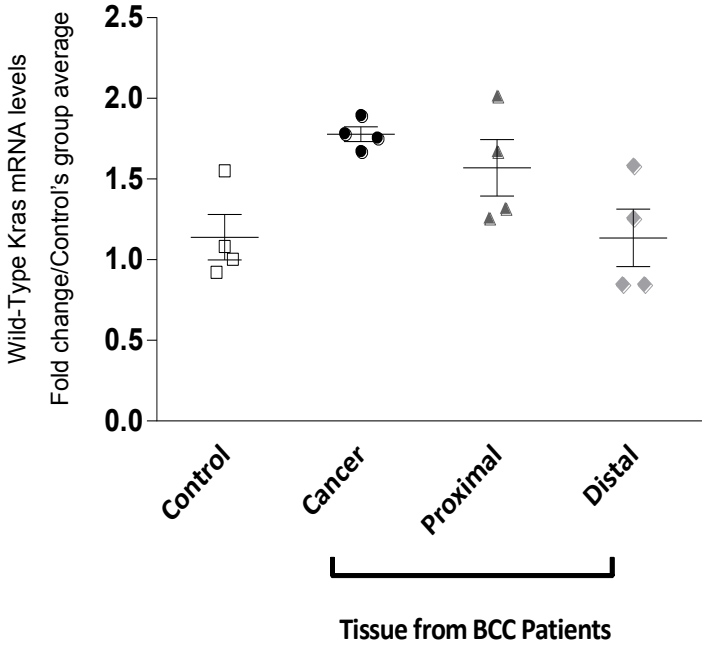

Wild-Type Kras mRNA levels
Fold change/Control's group average
2.5
2.0
1.5
1.0
0.5
0.0
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.16*: Detection of (mut 2.1) KRas point mutation in BCC tissue groups:* mutant allele specific real-time PCR with/without blocker was used to detect the presence of (mut 2.1) mutation in all tissue groups. cDNA was constructed from 1µg of purified RNA and used to quantify the message of KRas mutation using allele specific (mut 2.1) PCR forward primer and the wild-type reverse primer. The analysis was performed for each tissue group with and without adding wild-type allele blocker to the PCR reaction for comparison. mRNA was quantified expressed as fold change of the controls group average. Results shows that the message obtained using (mut 2.1) primer follows the same trend obtained by the wild-type primer with the absence of the significance except for the cancer group which shows significant increase in the message level without using the wild type blocker. Upon adding the wild-type allele blocker to the PCR reaction the previously mentioned significance disappeared suggesting that the observed message is non-specific amplification of the wild-type allele and no (mut 2.1) KRas point mutation was detected in BCC tissue specimens. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. Pvalue≤0.05 is considered significant.

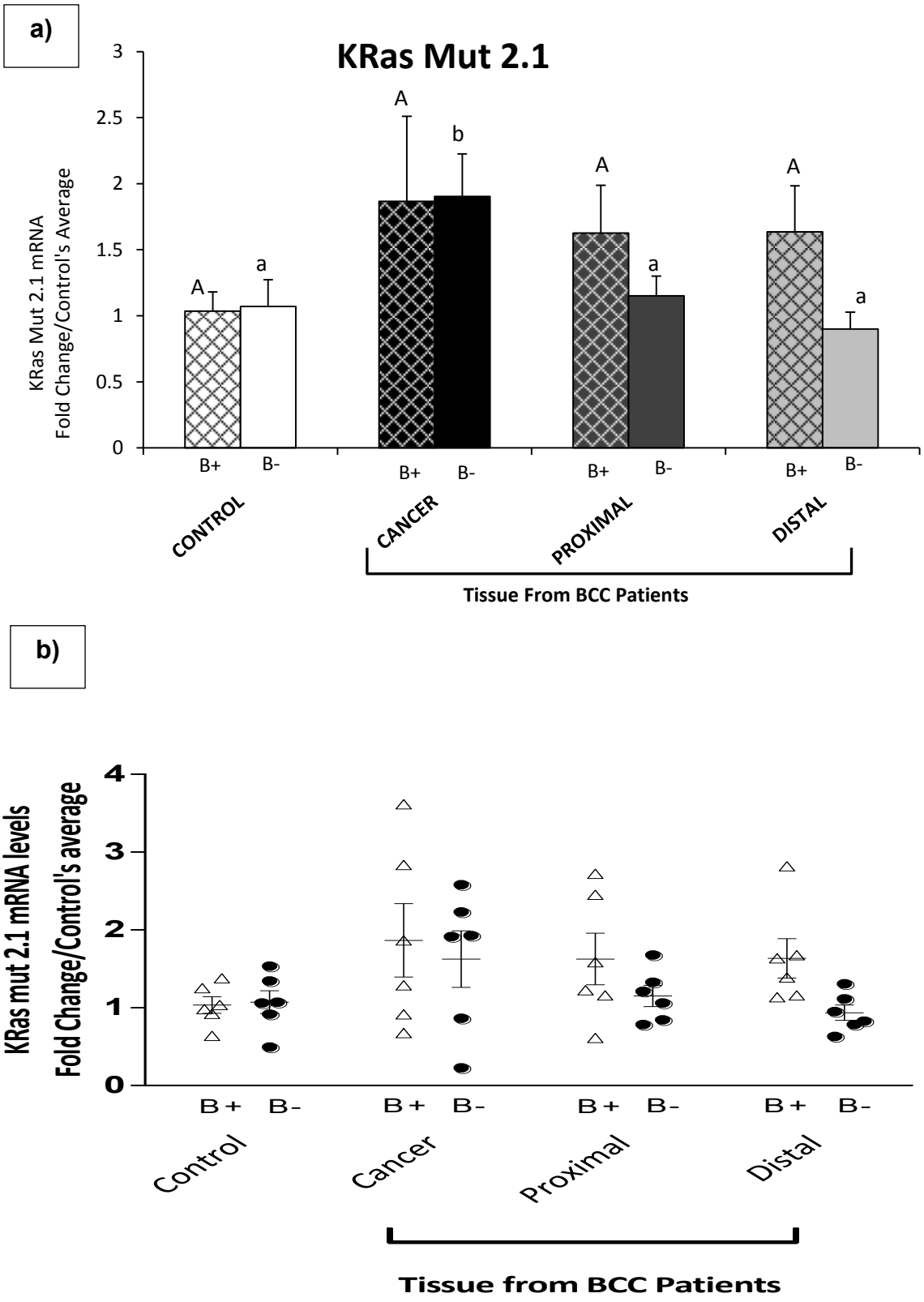
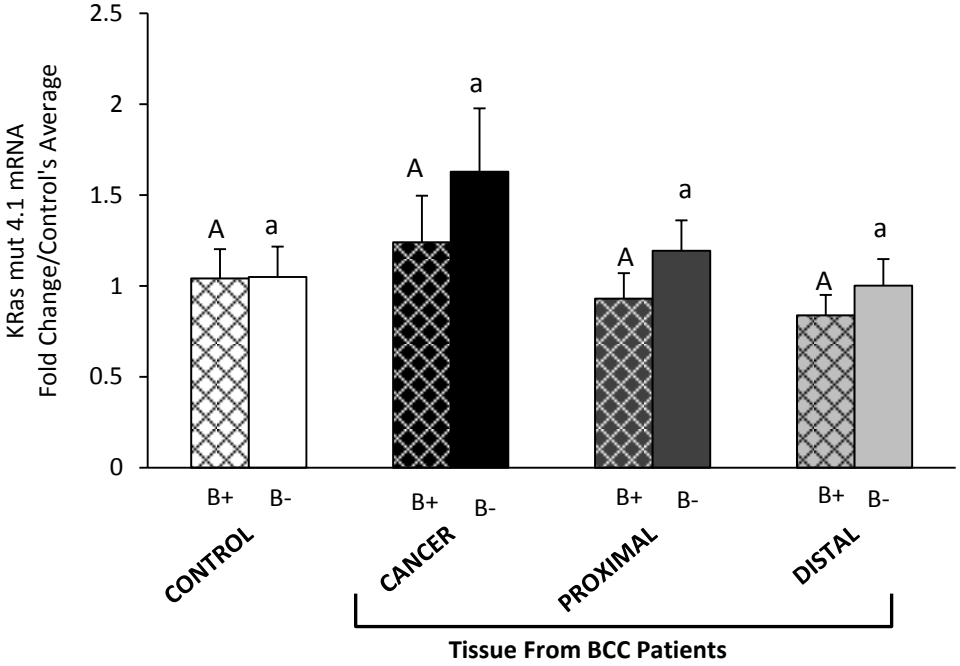

a)
KRas Mut 2.1
KRas Mut 2.1 mRNA
Fold Change/Control's Average
3
2.5
2
1.5
1
0.5
0
A
a
A
b
A
a
A
a
B+
B-
B+
B-
B+
B-
B+
B-
CONTROL
CANCER
PROXIMAL
DISTAL
Tissue From BCC Patients
b)
KRas mut 2.1 mRNA levels
Fold Change/Control's average
4
3
2
1
0
B+
B-
B+
B-
B+
B-
B+
B-
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.17: *Detection of (mut 4.1) KRas point mutation in BCC tissue groups:* mutant allele specific real-time PCR with/without blocker was used to detect the presence of (mut 4.1) mutation in all tissue groups. cDNA was constructed from 1µg of purified RNA and used to quantify the message of KRas mutation using allele specific (mut 4.1) PCR forward primer and the wild-type reverse primer. The analysis was performed for each tissue group with and without adding wild-type allele blocker to the PCR reaction for comparison. mRNA was quantified expressed as fold change of the controls group average. The message obtained using (mut 4.1) primer follows the same trend obtained the wild-type primer without showing any statistical significant among the groups neither with nor without the wild-type specific blocker. The results suggest that the observed message is non-specific amplification of the wild-type allele and no (mut 4.1) KRas point mutation was detected in BCC tissue specimens. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. Pvalue≤0.05 is considered significant.

a)


KRas Mut 4.1
KRas mut 4.1 mRNA
Fold Change/Control's Average
2.5
2
1.5
1
0.5
0
A
a
A
a
A
a
A
a
B+
B-
B+
B-
B+
B-
B+
B-
CONTROL
CANCER
PROXIMAL
DISTAL
Tissue From BCC Patients

b)

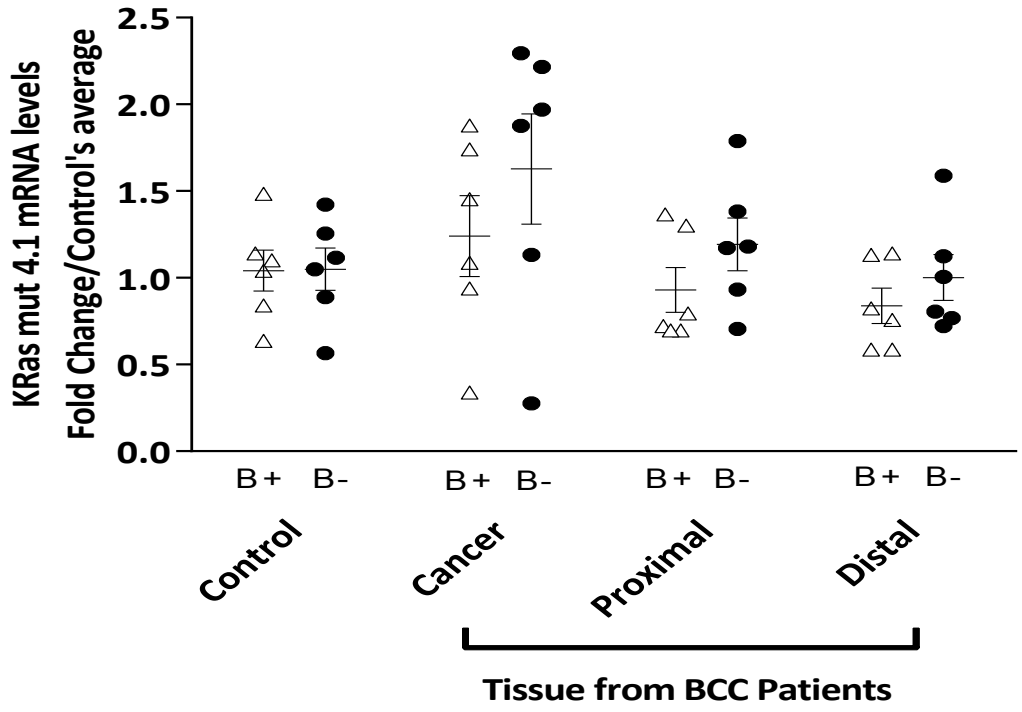
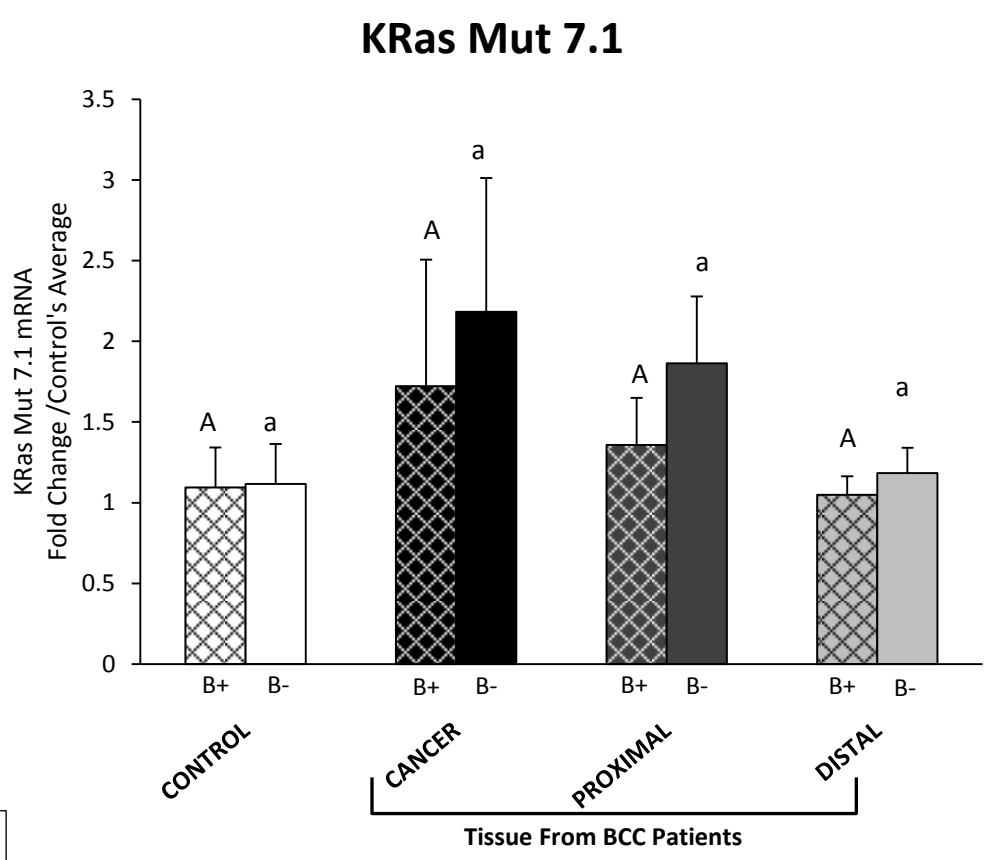

KRas mut 4.1 mRNA levels
Fold Change/Control's average
2.5
2.0
1.5
1.0
0.5
0.0
B+
B-
B+
B-
B+
B-
B+
B-
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.18: *Detection of (mut 7.1) KRas point mutation in BCC tissue groups:* mutant allele specific real-time PCR with/without blocker was used to detect the presence of (mut 7.1) mutation in all tissue groups. cDNA was constructed from 1µg of purified RNA and used to quantify the message of KRas mutation using allele specific (mut 7.1) PCR forward primer and the wild-type reverse primer. The analysis was performed for each tissue group with and without adding wild-type allele blocker to the PCR reaction for comparison. mRNA was quantified expressed as fold change of the controls group average. The message obtained using (mut 7.1) primer follows the same trend obtained by the wild-type primer without showing any statistical significant among the groups neither with nor without the wild-type specific blocker. The results suggest that the observed message is non-specific amplification of the wild-type allele and no (mut 7.1) KRas point mutation was detected in BCC tissue specimens. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. Pvalue≤0.05 is considered significant.

a)


KRas Mut 7.1
KRas Mut 7.1 mRNA
Fold Change /Control's Average
3.5
3
2.5
2
1.5
1
0.5
0
A
a
A
a
A
a
A
a
B+
B-
B+
B-
B+
B-
B+
B-
CONTROL
CANCER
PROXIMAL
DISTAL
Tissue From BCC Patients

b)

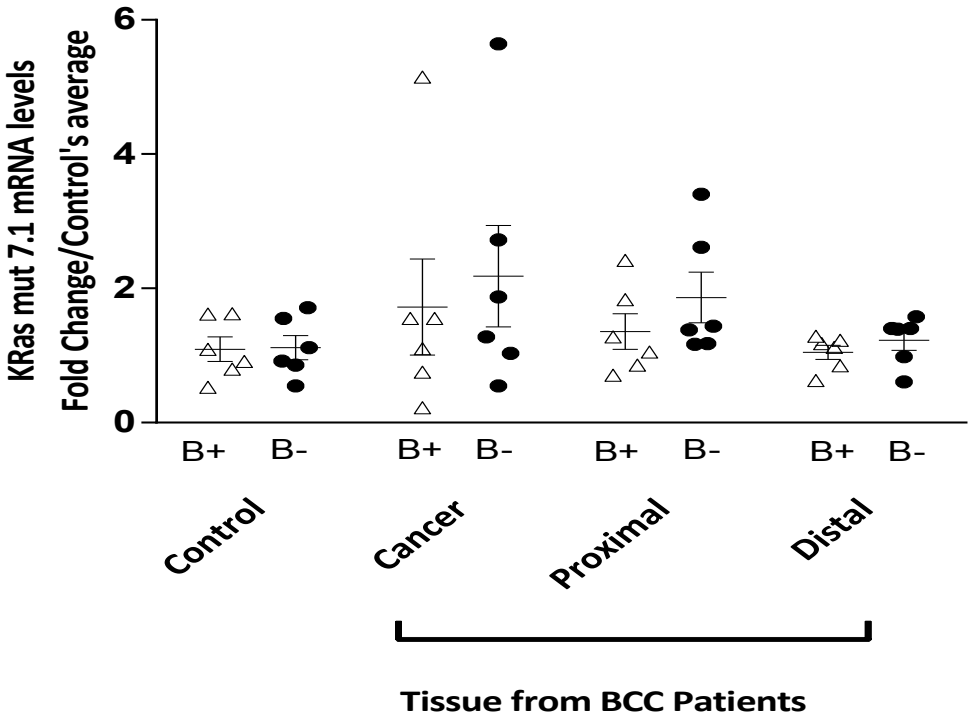
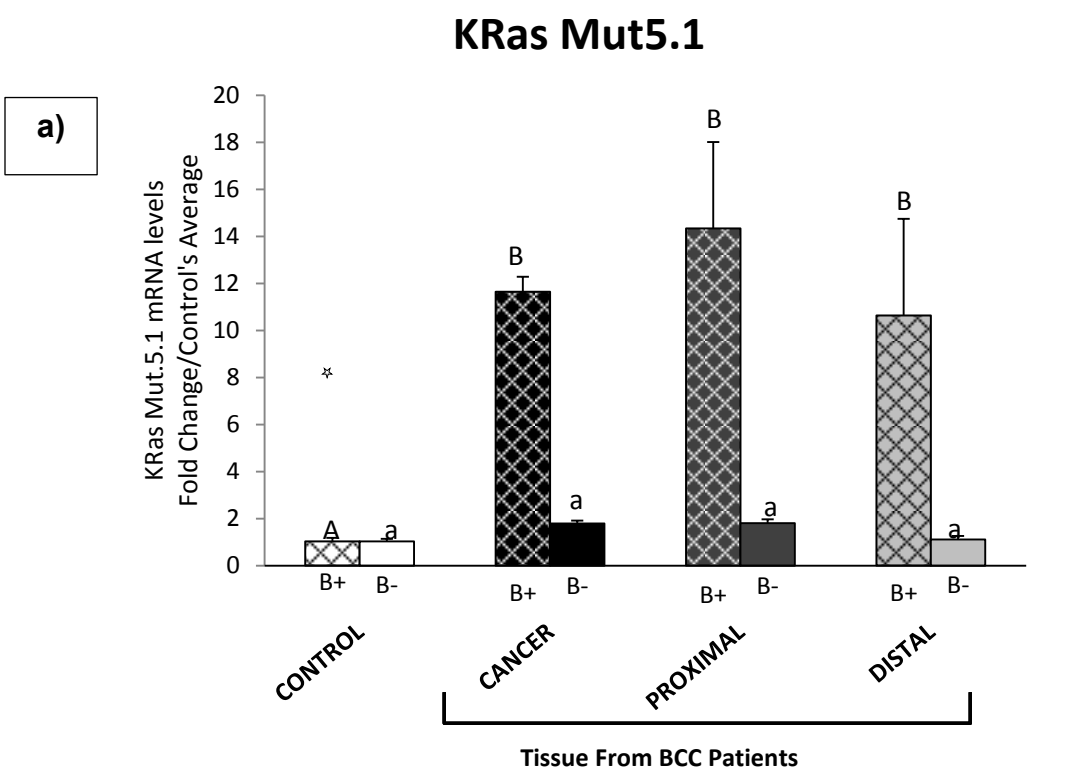

KRas mut 7.1 mRNA levels
Fold Change/Control's average
6
4
2
0
B+
B-
B+
B-
B+
B-
B+
B-
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.19: *Detection of (mut 5.1) KRas point mutation in BCC tissue groups:* mutant allele specific real-time PCR with/without blocker was used to detect the presence of (mut 5.1) mutation in all tissue groups. cDNA was constructed from 1µg of purified RNA and used to quantify the message of KRas mutation using allele specific (mut 5.1) PCR forward primer and the wild-type reverse primer. The analysis was performed for each tissue group with and without adding wild-type allele blocker to the PCR reaction for comparison. mRNA was quantified expressed as fold change of the controls group average. The message obtained using (mut 5.1) primer without wild-type specific blocker follows the same trend as the wild-type primer with no statistical significance among the tissue groups. Upon adding the wt. blocker to the PCR reaction, all tissue groups of the BCC patients show significant increase in the message of (mut 5.1) mutant allele comparing to the control group. Results suggest the presence of KRas point mutation (mut 5.1) in cancer, proximal and distal tissue specimens excised from BCC patients. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. Pvalue≤0.05 is considered significant.


KRas Mut5.1
a)
KRas Mut.5.1 mRNA levels
Fold Change/Control's Average
20
18
16
14
12
10
8
6
4
2
0
A
a
B
a
B
a
B
a
B+
B-
B+
B-
B+
B-
B+
B-
CONTROL
CANCER
PROXIMAL
DISTAL
Tissue From BCC Patients

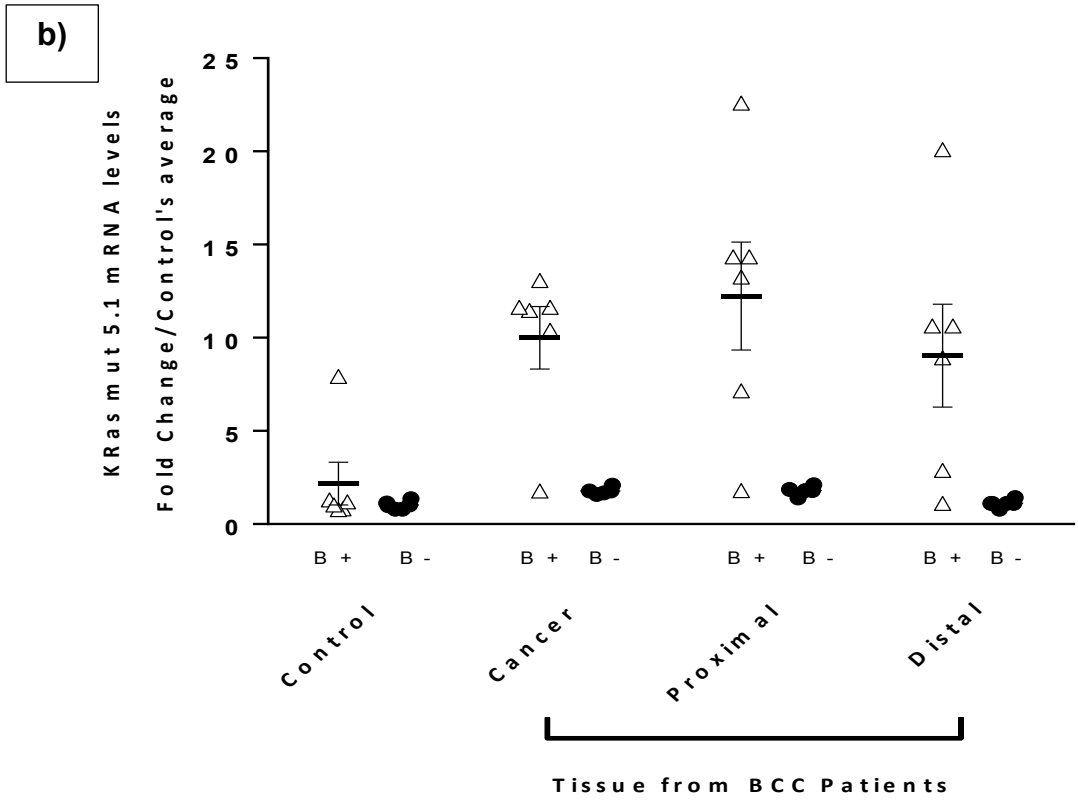
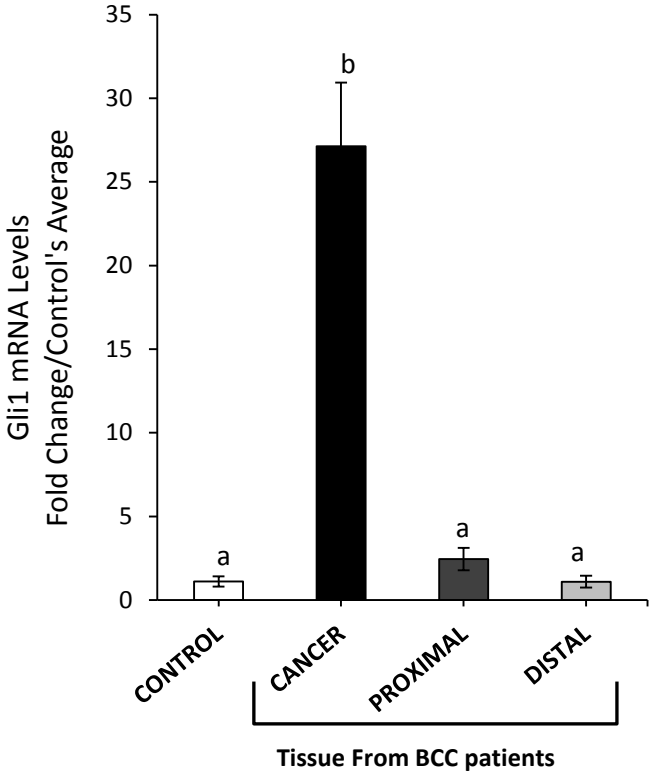

b)
KRas mut5.1 mRNA levels
Fold Change/Control's average
25
20
15
10
5
0
B +
B -
B +
B -
B +
B -
B +
B -
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.20: *Analysis of Gli1 gene expression among tissue groups:* 1µg of purified RNA from different tissue specimens were used to construct cDNA, which was in turn used to quantify the message of the gene. mRNA of Gli1 gene was quantified in tissue specimens using real-time PCR and was expressed as fold changes of the controls group average. Our data clearly reveals that BCC patients' cancer tissue shows significantly higher expression of Gli1 comparing to the normal tissue from BCC patients and from the controls indicating higher activity of hedgehog pathway in the cancer tissue. Proximal and distal tissue groups have normal Gli1 expression that mimics the control group. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant.

a)


Gli1 mRNA Levels
Fold Change/Control's Average
35
30
25
20
15
10
5
0
a
b
a
a
CONTROL
CANCER
PROXIMAL
DISTAL
Tissue From BCC patients

b)

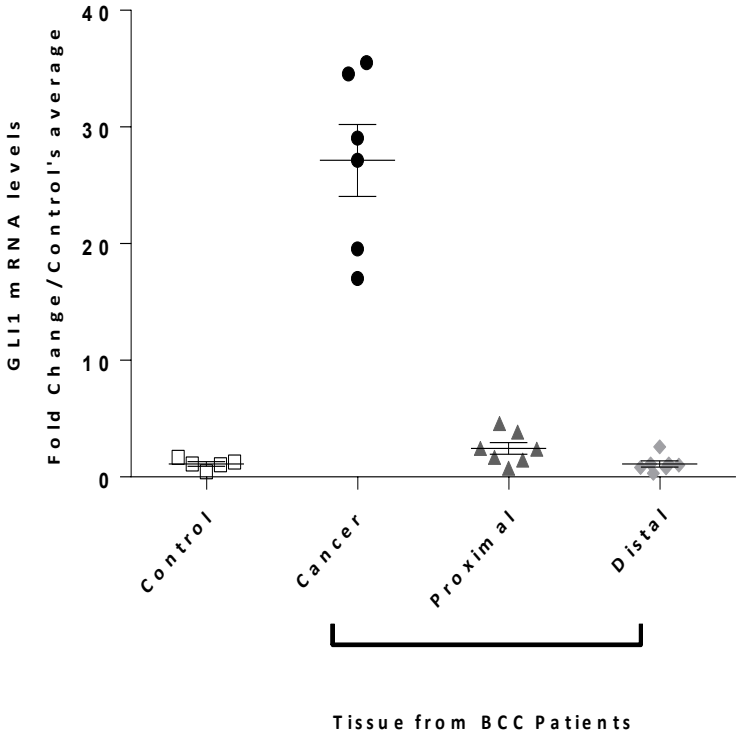
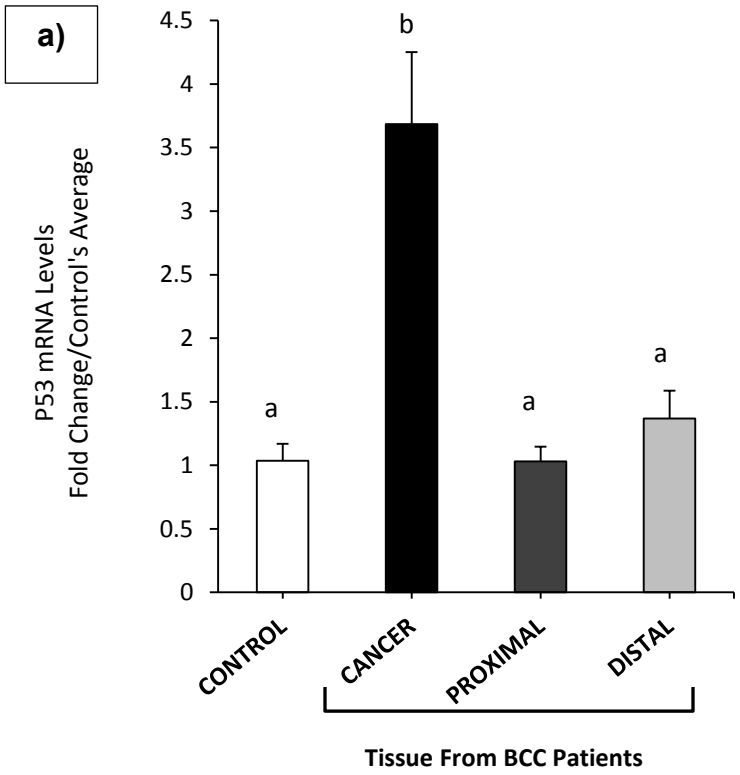

GLI1 mRNA levels
Fold Change/Control's average
40
30
20
10
0
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.21*: Expression Analysis of tumor suppressor gene P53 in BCC tissue groups:* mRNA of P53 gene was quantified in tissue specimens using real-time PCR. 1µg of purified RNA from different tissue specimens were used to construct cDNA, which was in turn used to quantify the message of the gene. Data was expressed as fold changes of the controls group average. Our data clearly reveals that BCC patients' cancer tissue shows significantly higher expression of P53 comparing to the normal tissue from BCC patients as well as to the controls. Proximal and distal tissue groups have P53 expression is not significantly different from the control tissue group. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant.


a)
P53 mRNA Levels
Fold Change/Control's Average
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
a
a
CONTROL
CANCER
PROXIMAL
DISTAL
Tissue From BCC Patients

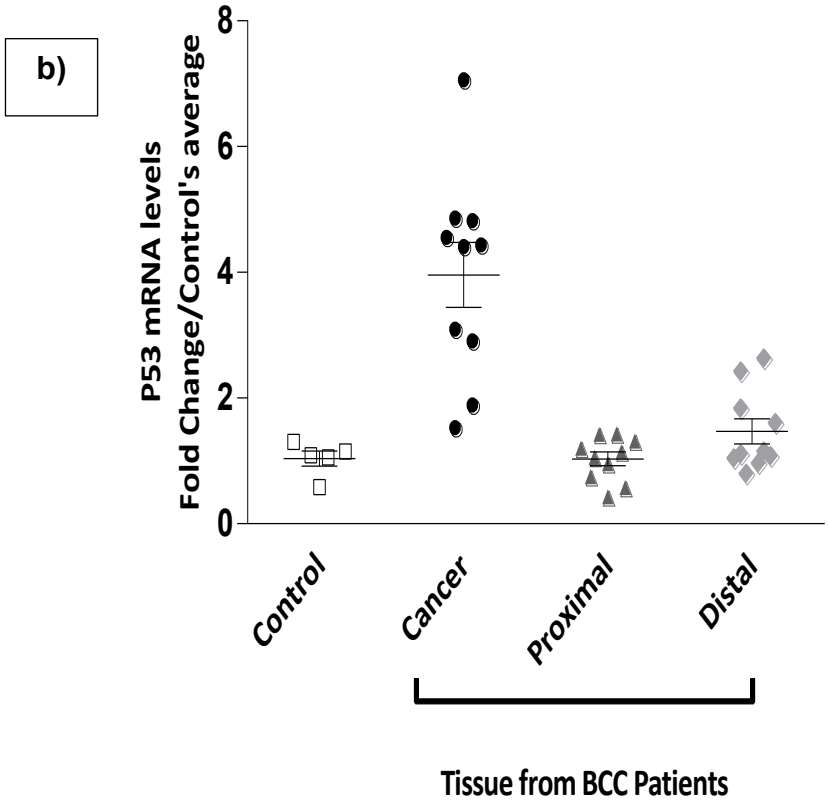
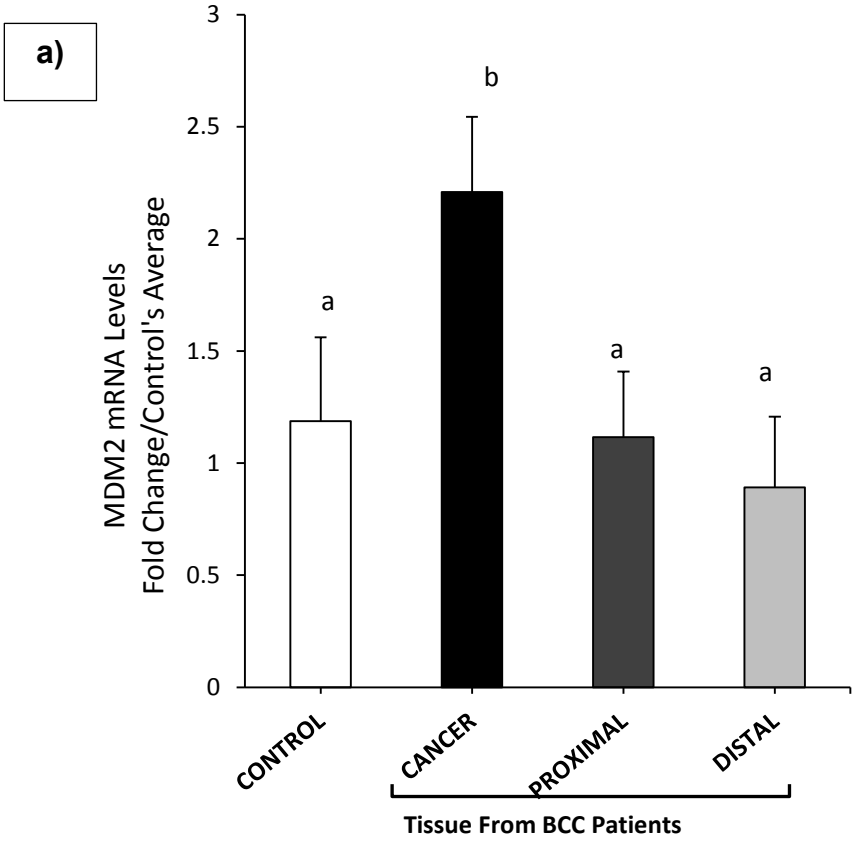

b)
P53 mRNA levels
Fold Change/Control's average
8
6
4
2
0
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.22: *MDM2 gene expression Analysis in experimental tissue groups:* mRNA of MDM2 gene was quantified in tissue specimens using real-time PCR. 1µg of purified RNA from different tissue specimens were used to construct cDNA, which was used to quantify the message of the gene. Data was expressed as fold changes of the controls group average. Results analysis proposes that basal cell carcinoma tissue obtained from our cases show significantly higher expression of MDM2 comparing to the normal tissue from BCC patients as well as to the controls. Proximal and distal tissue groups show a slight decrease in MDM2 expression but it is not statistically significantly from the control group. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant.


a)
MDM2 mRNA Levels
Fold Change/Control's Average
3
2.5
2
1.5
1
0.5
0
a
b
a
a
CONTROL
CANCER
PROXIMAL
DISTAL
Tissue From BCC Patients

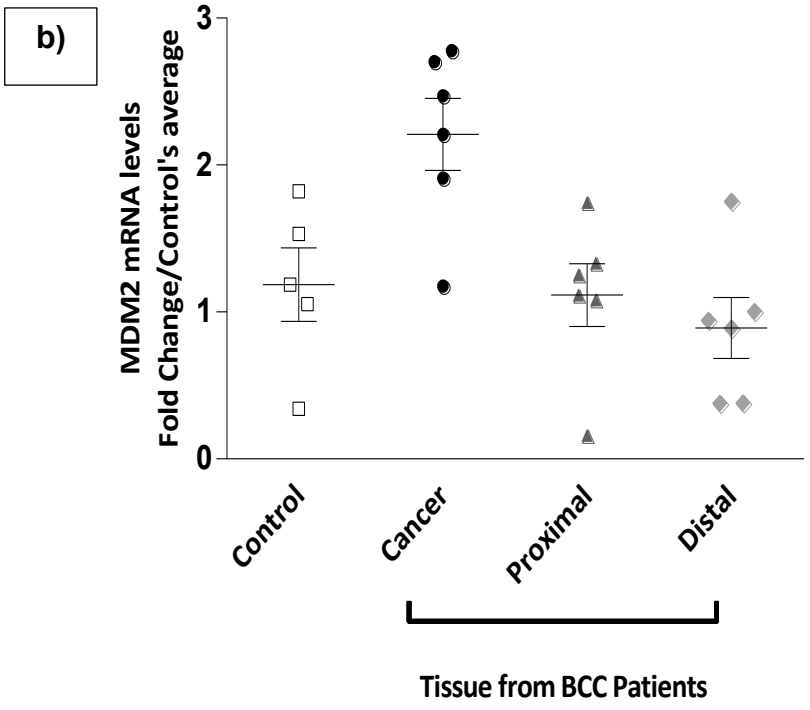
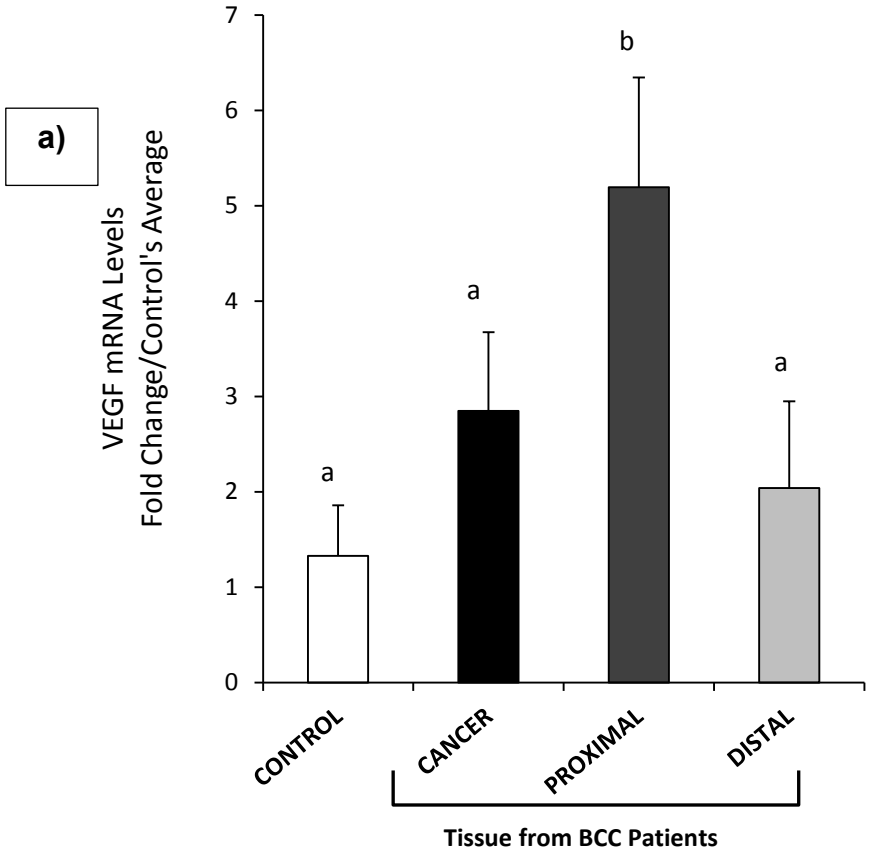

b)
MDM2 mRNA levels
Fold Change/Control's average
3
2
1
0
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.23: *Vascular endothelial growth factor (VEGF) gene expression Analysis in BCC tissue groups:* mRNA of VEGF gene was quantified in tissue specimens using real-time PCR. cDNA was constructed from 1µg of purified RNA from different tissue specimens, and was used to quantify the message of the gene. Data was expressed as fold changes of the controls group average. Results propose that basal cell carcinoma tissue obtained from our cases show up-regulation of VEGF gene expression comparing to the controls. The elevation in VEGF expression is statistically significant only in the proximal tissue group despite the visual trend of higher message of VEGF in the cancer tissue group a) Results shown in column representing group's averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant.


a)
VEGF mRNA Levels
Fold Change/Control's Average
7
6
5
4
3
2
1
0
a
a
b
a
CONTROL
CANCER
PROXIMAL
DISTAL
Tissue from BCC Patients

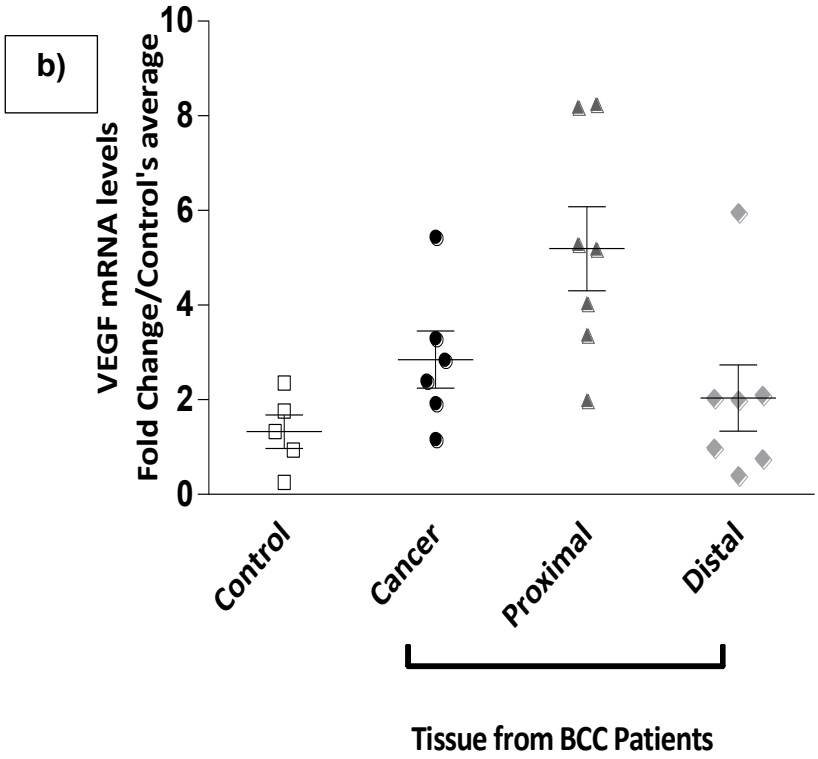
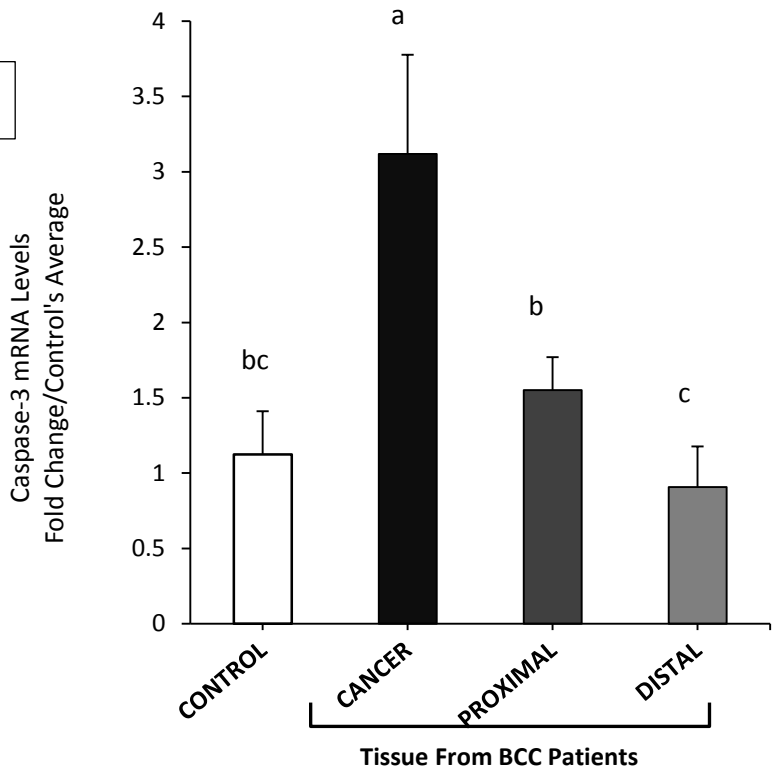

b)
VEGF mRNA levels
Fold Change/Control's average
10
8
6
4
2
0
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.24*: Gene expression analysis of caspase 3, apoptosis-related cysteine peptidase in BCC tissue groups:* cDNA was constructed from 1µg of purified RNA and used to quantify the message of caspase-3 gene. mRNA of caspase-3 was quantified by real-time PCR and expressed as fold change of the controls group average. Data analysis discloses a significant up-regulation of caspase-3 gene message level in the cancer tissue comparing to all other tissue groups. A trend of gradual decrease was observed in the normal tissue of the patients in relation to the distance of the specimen from the cancer location. Caspase-3 message level in the proximal tissue is significantly lower than in the cancer, and the level in the distal tissue is significantly lower than in the proximal. Despite the significant difference between the proximal and the distal groups, they both are not statistically significant from the control group. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant.

a)


Caspase-3 mRNA Levels
Fold Change/Control's Average
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
bc
c
CONTROL
CANCER
PROXIMAL
DISTAL
Tissue From BCC Patients

b)

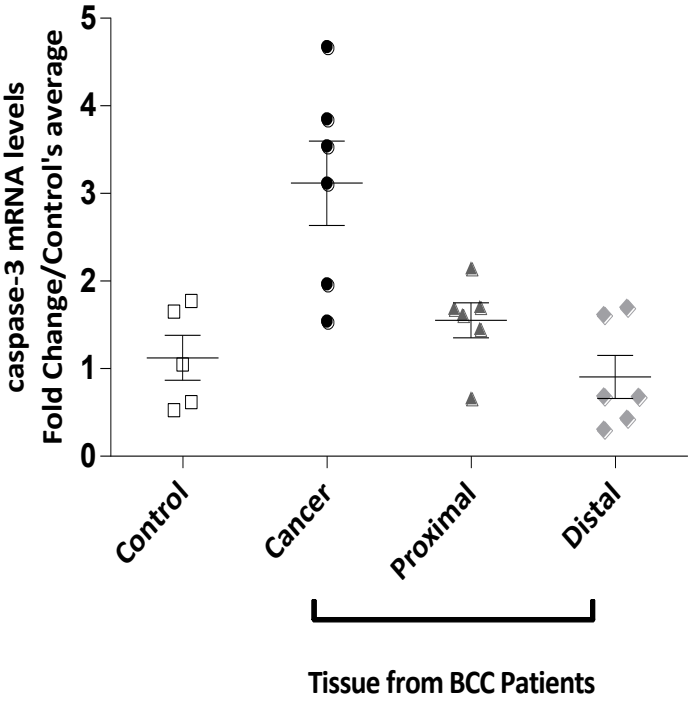
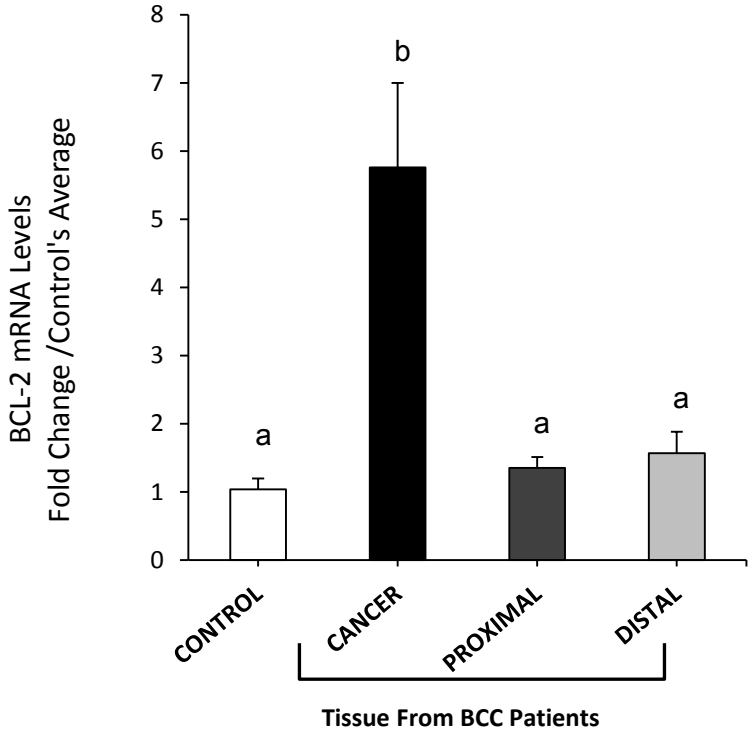

caspase-3 mRNA levels
Fold Change/Control's average
5
4
3
2
1
0
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.25: *Expression analysis of the anti-apoptotic gene Bcl2 in BCC tissue groups:* cDNA was constructed from 1µg of purified RNA and used to quantify the message of Bcl2 gene. mRNA of VDR was quantified by real-time PCR and expressed as fold change of the controls group average. Data proposes a dramatic elevation in the anti-apoptotic gene Bcl2 message level in the cancer tissue comparing to all other tissue groups. The normal tissue excised from BCC patients is slightly higher, but not statistically significant, than the control tissue. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant.

a)


BCL-2 mRNA Levels
Fold Change /Control's Average
8
7
6
5
4
3
2
1
0
a
b
a
a
CONTROL
CANCER
PROXIMAL
DISTAL
Tissue From BCC Patients

b)

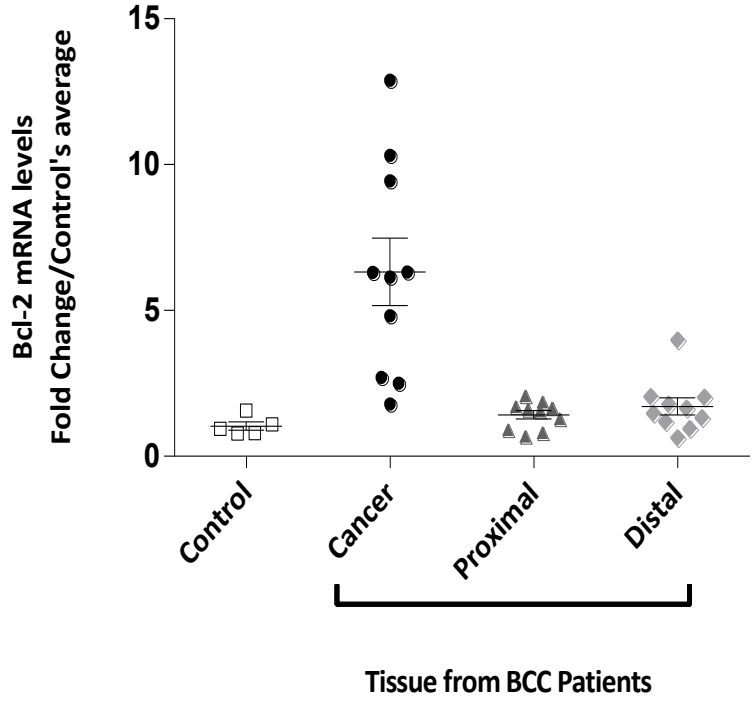
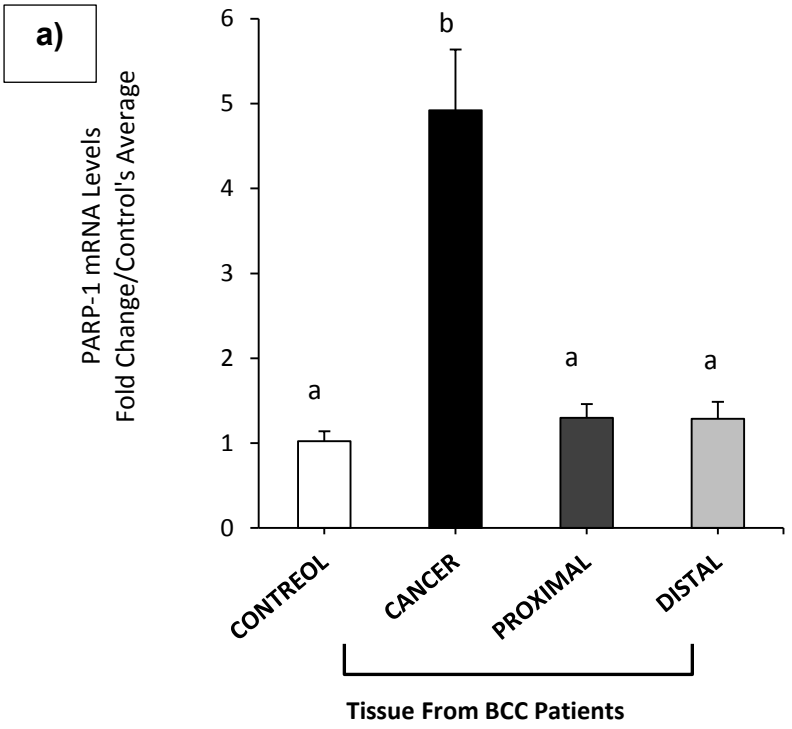

Bcl-2 mRNA levels
Fold Change/Control's average
15
10
5
0
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.26: Expression analysis PARP-1 *gene in BCC tissue groups:* 1µg of purified RNA from different tissue specimens were used to construct cDNA, which was in turn used to quantify the message of the gene. mRNA of PARP-1 gene was quantified in tissue specimens using real-time PCR and was expressed as fold changes of the controls group average. Our data clearly reveals that BCC patients' cancer tissue shows significantly higher expression of PARP-1 comparing to the normal tissue from BCC patients and from the controls indicating higher DNA repair activity in the cancer tissue. Proximal and distal tissue groups have normal PARP-1 expression that mimics the control group. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant.


a)
PARP-1 mRNA Levels
Fold Change/Control's Average
6
5
4
3
2
1
0
a
b
a
a
CONTREOL
CANCER
PROXIMAL
DISTAL
Tissue From BCC Patients

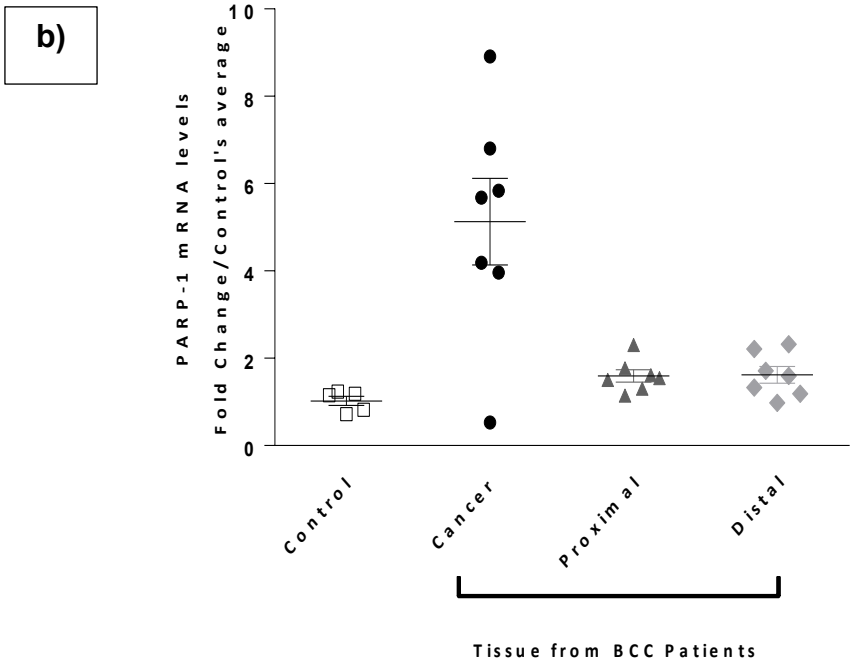
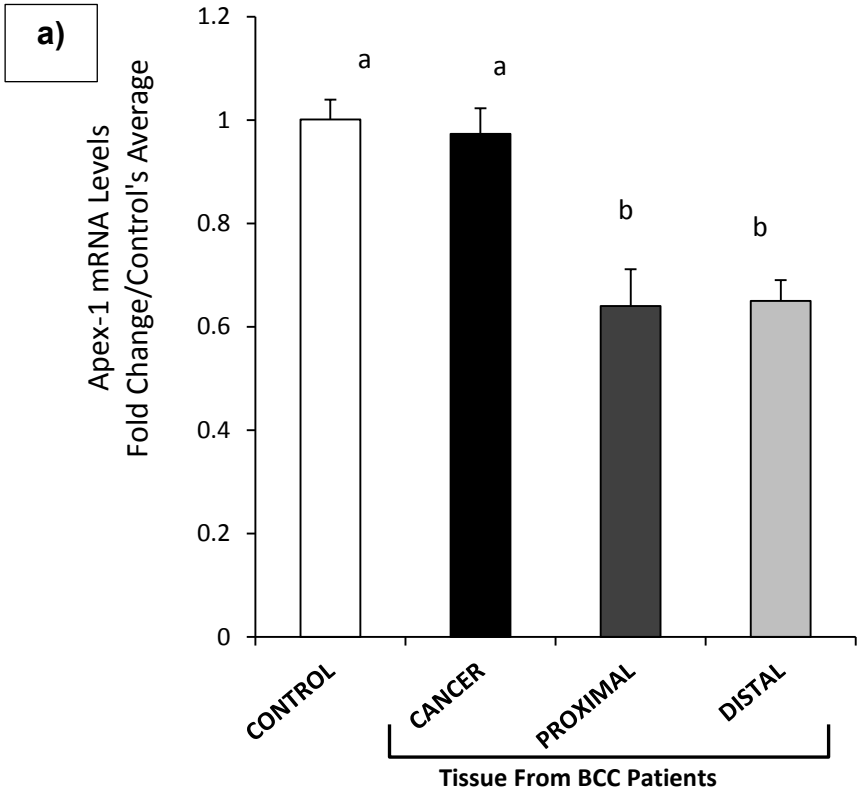

b)
PARP-1 mRNA levels
Fold Change/Control's average
10
8
6
4
2
0
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.27: Apex-1 gene expression analysis in BCC tissue groups**:** Expression analysis Apex-1 gene in was measure using Real-time PCR. 1µg of purified RNA from different tissue specimens were used to construct cDNA, which was in turn used to quantify the message of the gene. mRNA of Apex-1 gene was quantified expressed as fold changes of the controls group average. Data clearly shows that Apex-1 gene is significantly down-regulated in normal tissue obtained from BCC patients. The expression of this gene is, interestingly, equally reduced in the proximal and distal locations. Cancer tissue itself maintains normal expression of this gene as the message levels in the cancer group mimic those of the controls. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. Pvalue≤0.05 is considered significant.


a)
Apex-1 mRNA Levels
Fold Change/Control's Average
1.2
1
0.8
0.6
0.4
0.2
0
a
a
b
b
CONTROL
CANCER
PROXIMAL
DISTAL
Tissue From BCC Patients

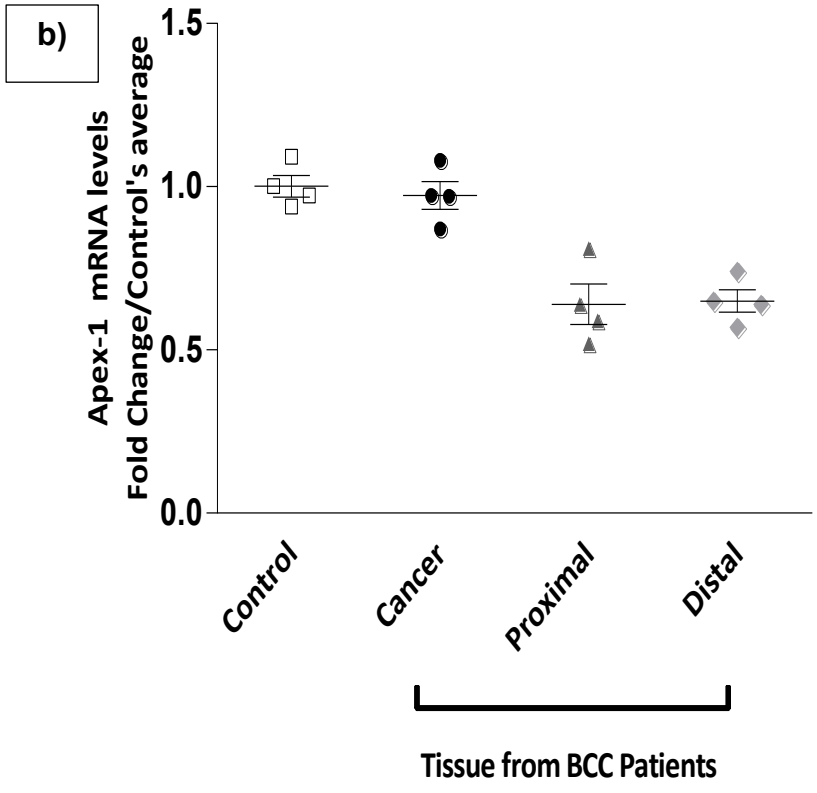
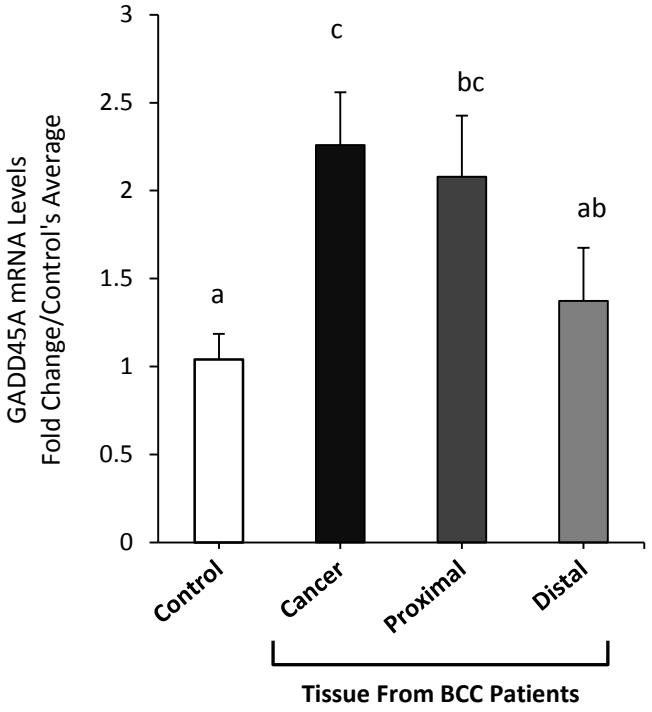
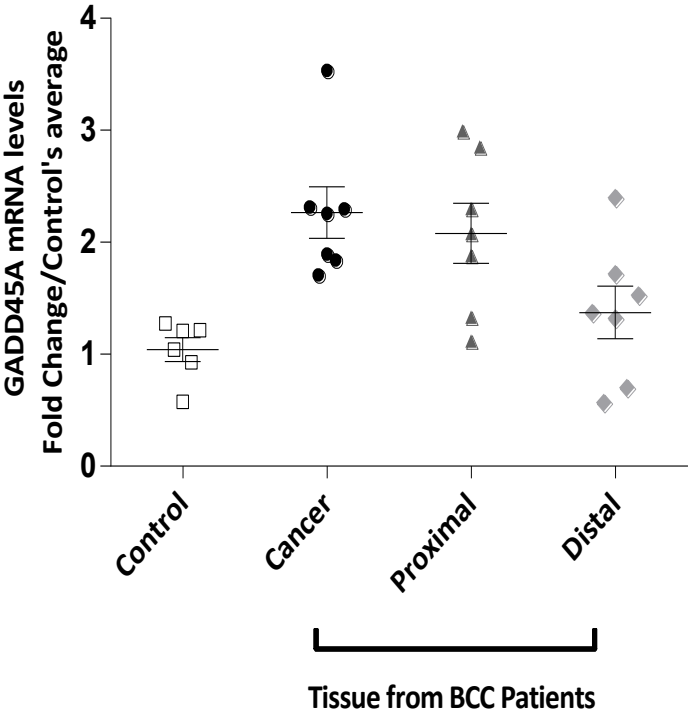

b)
Apex-1 mRNA levels
Fold Change/Control's average
1.5
1.0
0.5
0.0
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

Figure 3.28: *Expression analysis of the growth arrest and DNA-damage-inducible protein alpha GADD45A:* cDNA was constructed from 1µg of purified RNA and used to quantify the message of GADD45A gene. mRNA was quantified by real-time PCR and expressed as fold change of the controls group average. Data analysis discloses a general up-regulation of GADD45A gene message level in BCC patients' tissue groups comparing to the controls. The up-regulation is the greatest in the cancer tissue group with a trend of gradual decline in the message level in the tissue with the increasing distance from the cancer. GADD45A message level in the cancer and the proximal tissue is significantly higher than the control. The distal group message level falls between the proximal and control making it statistically not significantly different from any of the two groups. a) Results shown in column representing groups averages and SEMs. b) Individual data points presented as scatter plots, error bars show the mean and the SEM for each tissue group. Data was analyzed by one-way ANOVAs analysis with subsequent Bonferroni's multi group comparison. Groups with similar letters are not significantly different from each other. *P*value≤0.05 is considered significant.

a)


GADD45A mRNA Levels
Fold Change/Control's Average
3
2.5
2
1.5
1
0.5
0
a
c
bc
ab
Control
Cancer
Proximal
Distal
Tissue From BCC Patients

b)


GADD45A mRNA levels
Fold Change/Control's average
4
3
2
1
0
Control
Cancer
Proximal
Distal
Tissue from BCC Patients

CHAPTER 4: DISCUSSION AND CONCLUSION

The relation between Vitamin D status and the onset and progression of non-melanoma skin cancer-NMSC is not clear. Most of the published human studies about vitamin D and NMSC are epidemiological, correlational, and controversial; thus they do not address causality. For example, some studies showed beneficial influence of vitamin D supplementation on onset and progression of NMSC while others suggested adverse impact of vitamin D, and sometimes lack of association was concluded [28, 29, 32].

Mechanistic studies on BCC are few, and mostly performed using cell-lines or animal models. These approaches give rise to problems related to generalizing the findings to other experimental models. This study is unique for its mechanistic approach while using human tissues taken from NMSC patients and control individuals. Also, comparing three groups of normal tissue- proximal, distal, and control- is a novel approach.

Cases and controls were recruited in Henry Ford Hospital after obtaining the required approvals. Cases are 20 basal cell carcinoma patients from south east Michigan, controls are 6 individuals who were never diagnosed with cancer from the same geographic area. This reduces the differences between the two groups in exposure to environmental factors. Cases are 14 Caucasian males and 6 Caucasian females, and their age ranges between 46 and 89 years with average age of 74. Cases are 6 women who were pre-scheduled for eyelid-lift surgery, and age more than 50 years. Age of the control subjects ranges between 50 and 69. Ages of the

cases and controls are not an exact match, but it was the closest we could achieve. Unfortunately, our group could not successfully recruit male subjects among the controls despite the attempts. All patients were diagnosed with BCC in the head and nick area. Therefore, all cases and controls specimens were excised from locations that are exposed to sun. Also, With the exception of BCC diagnoses, all inclusion and exclusion criteria were similarly applied in recruiting both cases and controls. Interestingly, while the race of the subjects were not pre-determined. However, this was an advantage design as it eliminated variations among participants due to race differences.

The trend line for age distribution at time of diagnosis shows gradual increase of number of cases diagnosed with the increasing age after 55 years of age, and rapid decline after 85 years of age. About 50% of the cases are between 75-85 years old. We suggest that this can be partially due to the drop in the population after that age. The skewed peak is harmonious with the result of the normality test. KS test result suggested that age data is not normally distributed.

Upon isolating total RNA from tissue samples, we observed higher yield of RNA per milligram of tissue from the cancer tissue comparing to normal tissue from BCC patients as well as tissue from controls. This can possibly be related to the histology of BCC tissue. This type of cancer cells has hyper-chromatic nucleus surrounded by a small amount of cytoplasm [11] implying higher activity in the nucleus and larger proportion of nuclei to the cell volume.

To understand metabolism of vitamin D in BCC tumors and normal tissue from cases as well as from control subjects, we analyzed the expression of the key genes in vitamin D metabolism. The first step of activating Vitamin D3 precursors is carried out by VD3 25-hydroxylase (CYP27A1) forming 25(OH) VD3, which is the main form of vitamin D in the body [75]. The hormonal form of vitamin D is 1α, 25 (OH) 2 VD3, and its formation is catalyzed by 25-Hydroxyvitamin D_3 1-alpha-hydroxylase (CYP27B1) gene. 25(OH) VD3 1α-OHase is considered a renal enzyme, but it is also expressed in other tissues including keratinocytes [26].

Interestingly, in cancer tissue, the expression of VD3 25-hydroxylase is significantly down regulated, while VD3 1α-OHase, which performs the final step of activation, is over expressed. Cells adjacent to the cancer up regulate both activation enzymes. Distal tissue shows significant increases only in VD3 25-OHase expression. Over expression of VD3 1α-OHase in and around the cancer location is not a surprising. This gene was shown to be up regulated in keratinocytes by factors like TNF-α and INF-γ even in conditions other than cancer [76]. We propose the that normal tissue of the BCC patients increases the local availability of 1α-OHase substrate , while the cancer tissue and its feeding cells increase the activation of the hormonal vitamin D. Studies have provided evidence that 1α-OHase is transcriptionally regulated. For instance, MCF-7 cells overexpressing 1α-OHase gene could locally activate vitamin D analogs and exert the transcription effect of VDR [26]. Besides, others have demonstrated that renal 1α-OHase is being regulated by different positive and negative regulators at the transcription level[64].

Further hydroxylation by the catabolism enzyme VD3 24-hydroxylase (CYP24A1) inactivates VD3 [39, 77]. Analyses of this gene suggest that distal tissue maintains lower expression of this enzyme as compared to other tissue groups.

Ligand-bound VDR, is a transcription factor that regulates the transcription of several target genes [59, 78]. Upon hetero-dimer zing with RXR[65], VDR binds to vitamin D responsive elements VDRE region in the target gene's promoter [79, 80]. Sequentially, two types of co-activator complexes (SRC then DRIP) bind to VDR and facilitate the transcription initiation of the target gene [59]. SRC, due to its histone acyl-transferase activity [81], makes the promoter region more accessible to other factors [65]. Consecutively, DRIP complex binds and recruits the transcription machinery components [60, 65]. Cancer tissue significantly over-expresses VDR at the message and the protein levels, coupled with higher gene expression of both co-activators comparing to other tissue groups.

Our results suggest higher activation of Vitamin D and VDR in BCC tissue comparing to other tissue groups. According to literature, this may be advantageous for the patient as it can possibly negatively impact the cancer growth. Vitamin D has been anticipated to regulate proliferative and differentiating of keratinocytes. This idea gave rise to the belief that vitamin D supplementation may be beneficial for skin cancer patients [65, 82, 83].

Some possible mechanism by which vitamin D may exert its anti-proliferative effect were assessed in tissue samples. The main objective was to investigate whether our findings are associated with the anticipated impact on BCC tissue. VDR

is known to negatively regulate mTOR signaling pathway through REDD1. The gene encoding REDD1 is a VDR target gene [45]. up-regulating this protein promotes the activation of TSC1/2 complexes, which in turn inhibits Rheb resulting in suppression of mTOR activity [47]. VDR effect on its target gene was shown to be impaired when VDREBP are up-regulated as they compete with VDR over the binding sites on the responsive elements [45]. P-4EBP and p-S6K proteins can be used as indicators of mTOR activity [48] . Despite the elevated level of VDREBP protein in the cancer tissue, Protein analysis shows that REDD1 is significantly up-regulated in that tissue group. However, P-4ebp is also significantly high in that tissue group as compared to other groups. This interestingly suggests that the expected negative regulation to mTOR signaling by VDR through REDD1 is compromised in the cancer tissue.

Up-stream of TSC1/2 complex, protein analysis shows that the levels of phosphorylated AKT, in both S473 and Th308 locations, are significantly elevated in cancer and proximal tissue comparing to the controls. Active AKT is known to block the assembly and activation of TSC1/2 complex which opposes with the effect of VDR.

Ras gain of function mutation result mainly in up-regulating two pathways, PI3K-AKT-mTOR and Ras-Raf-MEK-ERK pathways [50]. KRas point mutation is common, and it was implicated with many types of cancers including colon, skin, and breast cancers [51]. Over expression of KRas gene in all tissue groups of the BCC patients comparing to the control was concluded from gene expression analysis. This can possibly result from a gain of function mutation of this gene.

The presence of a KRas point mutation in BCC tissue specimens was investigated using ASB-PCR. Among the tested point mutations, only KRas (mut 5.1) Gain of function mutation was successfully detected in our BCC patients specimens. This mutation was surprisingly detected in all tissue groups from the cases including the non-cancerous tissue (proximal and distal). In our assay the (mut 5.1) mutant allele specific primer is anchored at the 3` end with the T base, which base pairs with the discriminating base in the mutant allele. When the primer is annealed to the mutated sequence in the cDNA, perfect matching causes the amplification of the mutant allele. The wild-type specific blocker is a primer that is complementary of the wild-type allele sequence. Therefore, it perfectly base pairs with wild-type allele in the cDNA sample. The 3` end of the blocker is phosphorylated rendering the wild-type allele unamplified.

We suggest that the lost mTOR regulation by VDR is possibly related to the presence of KRas gain of function mutation. It is possible that the mutation results in over activation of Ras-PI3K-AKT-mTOR signaling pathway, and hence, overcome the inhibition effect of vitamin D. However, confirmation of KRas mutation causality in eliminating vitamin D regulation of mTOR needs further investigation. Up-regulation of PI3K-AKT-mTOR pathway was reported in other cancers such as breast cancer, and it was shown to alter the cancer’s response to endocrine treatments [48, 49].

Hedge-hog mutation is characteristic of BCC resulting in excessive signaling of the pathway [46]. Over expression of Hh-Gli pathway, results in modifying the Gli code, which affects tumor progression and recurrence [53, 56]. Interestingly, Vitamin D has the ability to inhibit Hh-Gli pathway and alter the Gli code to promote differentiation [46, 53]. PTCH was found to block SMO through vitamin D3 and its derivatives [9, 56]. Studies have shown that providing the active form of vitamin D to PTCH mutant mouse model reduced Hh-Gli signaling and reduced the incidence of spontaneous skin cancer in that model [9, 56]. In addition to the direct impact of calcitriol, VDR seems to have a role in regulating Hh-Gli signaling [9, 46]. Knocking out VDR resulted in Hh protein ligand over expression, and hence up regulated Hh-Gli signaling [9]. Vitamin D supplementation was projected to be beneficial for prevention and treatment of skin cancer [84].

Despite the up-regulation of both vitamin D activation gene 1α-OHase and VDR in cancer tissue specimens, Gli 1 gene is significantly overexpressed in that tissue group. The expected impact of higher activation of vitamin D and its receptor on inhibiting Hh-Gli signaling was not observed in our tissue samples. Other investigators revealed a cross talk between mTOR/S6K1 pathway and Hh-Gli pathway. Their results suggest that up regulation of mTOR pathway can cause SMO independent activation of Gli1 [85]. Therefore, we expect that the up regulation of mTOR pathway that we have observed in BCC samples may contribute to Gli1. Concomitantly, we propose that the up regulation of mTOR in our BCC samples opposes the effect of vitamin D on Hh-Gl1 signaling. Both vitamin D and VDR

impact PTCH capacity to block SMO, while mTOR/S6K1 up-regulates Gli1 downstream of SMO [57, 85].

Up regulation of Hh-Gli pathway was shown to overexpress the anti-apoptotic gene Bcl2, and Gli2 protein seems to be responsible for that [52]. This is compatible with our results that show significant over expression of Bcl2 in the cancer tissue. On the other hand, other publications reported that pathology of basal cell carcinoma reveals the presence of a large number of apoptotic bodies in BCC tissue [71, 86]. Also, analysis of mitotic and apoptotic indexes showed that BCC has high proliferation associated, with high apoptosis which was suggested to contribute to the benign nature of this tumor [63]. In harmony with that, our data shows that caspase-3 gene expression is up regulated in BCC cancer tissue comparing to all other tissue groups, which may predict high apoptotic activity. This finding doesn't contradict with our previously observation of elevated Bcl2 expression. Bcl2 can solely impact the intrinsic apoptosis pathway. Besides, Bcl2 effect on apoptosis is dependent on BAX/Bcl2 protein ratio [72]. Bax is a pro apoptotic protein from Bcl2 family, which competes with Bcl2. When in excess, Bax counters Bcl2 activity and releases Cytochrome C from the mitochondria, and thus activates caspase-3 [72].

Active P53 protein up-regulates MDM2 transcription, which in turn, binds and deactivates P53 as a feedback inhibition mechanism [87, 88]. Recent research revealed interactions between VDR and p53/MDM2 [68, 89]. VDR promoter region contains P53 responsive element. MDM2 promoter contains both P53 and VDR responsive elements. Both responsive elements were shown to cooperate in MDM2

transcription activation [67, 68]. Also, MDM2 is phosphorylated by S6K1, which inactivate MDM2 and inhibit its ability to bind P53. This means that the normal down regulation of mTOR pathway by VDR would result in reduced MDM2 phosphorylation. Consequently, this is expected to yield more MDM2 in the nucleus to inactivate p53. In our cancer tissue samples, gene analysis revealed that P53 gene is overexpressed. MDM2 gene was also found to be overexpressed in that tissue group in spite of the elevated levels of VDREBP. However, our earlier results suggest that the down regulation of mTOR/S6K1 by VDR is diminished in this tissue group. Thus VDR overexpression in cancer tissue is not expected to reduce MDM2 phosphorylation and inactivation. However, further steps to investigate MDM2 phosphorylation are required to confirm that conclusion.

Recently, the relation between P53 and VDR was shown to be more complicated than it was thought. P53 is a tumor suppressor gene that may get inactivated due to a loss of function mutation in many types of cancers[88]. However, it was revealed that some mutant P53 acquire oncogenic activity [10]. P53 gain of function mutations were reported in many types of cancer including skin cancer [10].

Further interrelationship was found between GOF mutantP53 and vitamin D. Mutant P53 was found to up-regulate VDR, increase its nuclear localization, and facilitate its binding to VDRE [89]. In contrast mutant P53 inhibited VDR down regulation of the genes that have inverse VDRE in their promoter. What is more, mutant P53 was shown to manipulate VDR transcriptional activity, converting

vitamin D from a pro-apoptotic to an anti-apoptotic agent. Consequently, it was concluded that P53 status can manipulate the impact of vitamin D on tumor cells [89]. Investigating whether the overexpressed P53 in our tissue specimen is a wild type or mutant protein is an important future direction. In case P53 is mutated in our BCC specimens, testing for possible impact on VDR activity will be the second logical step.

Vascular endothelial growth factor (VEGF) is a vitamin D target gene, which contains VDRE in its promoter [70]. Our gene expression analysis of VEGF indicated that this gene is over expressed in both cancer and proximal tissue. These are the same tissue groups that are overexpressing 1αOHase. However, VEGF up regulation is greater and statistically significant in the proximal tissue, which is sometimes referred to as “feeding cells”. VEGF protein binds to the VEGFR2 receptor on the plasma membrane of nearby vascular endothelial cells promoting angiogenesis and providing blood supply to the new tumor growth[90]. VEGF expression is also up regulated by Ras-MEK-ERK and by PI3-AKT[91]. In our study, we have shown that both Ras and AKT are up-regulated in the proximal tissue samples. Besides the over expression of both (1αOHase, and 25OHase) vitamin D activating enzymes, VDREBP protein that competes with VDR over the response elements in the target genes promoter is extremely low in this tissue groups. We suggest that these factors may contribute to the observed up regulation of VEGF in the proximal tissue group.

We tested the expression of three genes related to DNA damage repair in our tissue groups. APEX-1 (apurinic/ apyrimidinic endonuclease 1/redox factor1) is a key enzyme in Base excision repair; it facilitates the removal of AP sites from the damaged DNA region [92]. Apex-1 gene expression is lower in the non-cancerous specimens from patients as compared to the cancer tissue. Both proximal and distal locations have equal expression of this gene. This may indicate lower BER activity in the non-cancerous tissue of BCC patients than in the cancer location.

GADD45A is another DNA repair gene that was tested in this study. This protein regulates nucleotide excision repair, especially when damage is caused by UV radiation [92] . It interacts with the chromatin and aids in recruiting the nucleotides and other NER factors [93]. GADD45A expression is the highest in the cancer tissue and it shows gradual decrease in the normal tissue. All our tissue specimens are excised from locations that are exposed to UV light, specifically head and nick, thus UV induced DNA damage is predicted. GADD45 A was shown to be directly regulated by VDR at the transcription level as well as the post translational modification level [78]. Also, GADD45A is known to be regulated by P53 [92]. Both VDR and P53 were shown previously to be over expressed in our cancer tissue group. GADD45 was also proposed to contribute to the BER pathway. It was suggested that it facilitates Apex-1 interaction with PCNA [92].

PARP-1 Protein is involved in many important cellular functions; mainly DNA single-strand and double-strand breaks repair, chromatin remodeling, transcription regulation, and cell death. Post translational modification can favor one function of

PARP-1 over the other [73]. We have seen over expression of PARP-1 gene in the cancer tissue over all other tissue groups from cases and controls. Vitamin D is known as PARP-1 inhibitor, which contributes to Vitamin D anti-inflammatory property[94]. Vitamin D mediated PARP-1inhibition can occur at the expression level[74], or via post translational modification [94]. VDR was also shown to inhibit PARP-1 cleavage by caspase-3 [34]. Our data is inconclusive in regards to PARP-1 activity due to its regulation by post-translational modifications. However, taking in consideration the expression of the three genes, we predict higher repair activity in cancer tissue comparing to other non-cancerous tissue of BCC patients and controls.

Figure 4.1: *Negative regulation of mTOR by vitamin D is lost in BCC tissue:* results suggest higher activation of Vitamin D and VDR in BCC tissue. Protein analysis shows that REDD1 is successfully up-regulated in cancer tissue despite the elevation in VDREBP. P-4EPB protein analysis reveals that it the expected negative regulation to mTOR signaling by VDR is absent in the cancer tissue. Point mutation in Kras gene was detected in tissue specimens obtained from BCC patients. Our results suggest that the lost mTOR regulation by VDR is possibly related to the presence of KRas gain of function mutation which results in over activation of Ras-PI3K-AKT-mTOR signaling pathway, and hence, overcome the inhibition effect of vitamin D.

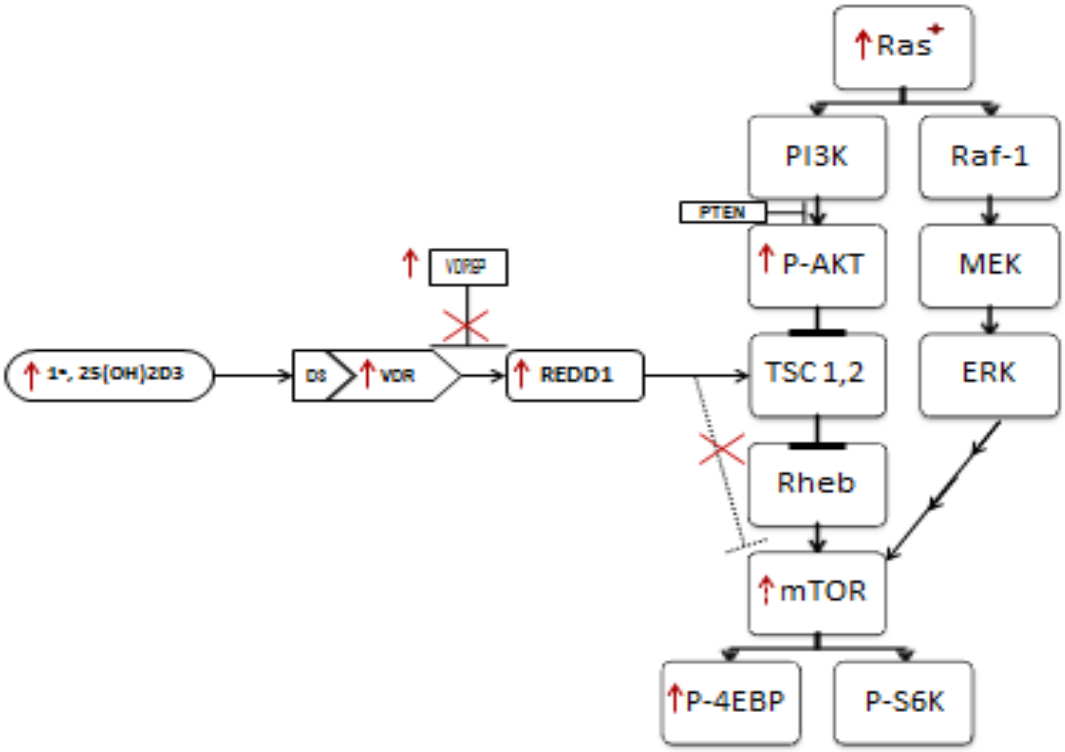

Ras
PI3K
Raf-1
PTEN
P-AKT
MEK
VDRIP
1α, 25(OH)2D3
D3
VDR
REDD1
TSC 1,2
ERK
Rheb
mTOR
P-4EBP
P-S6K

Figure 4.2: *Vitamin D over-activation in BCC tissue is not resulting in reduction of Hh-Gli pathway signaling:* despite the upregulation of vitamin D avtivation gene 1α-OHase in BCC tissue samples, Gli 1 expression analysis showed significant up-regulation in the cancer tissue specimens of BCC patients. The predicted impact of vitamin D in reducing Hh-Gli signaling and blocking Gli1 expression was not found in our tissue samples. Others showed that a cross talk between PI3K-AKT-mTOR pathway and Hh-Gli pathway may be implicated with SMO independent activation of the later pathway.

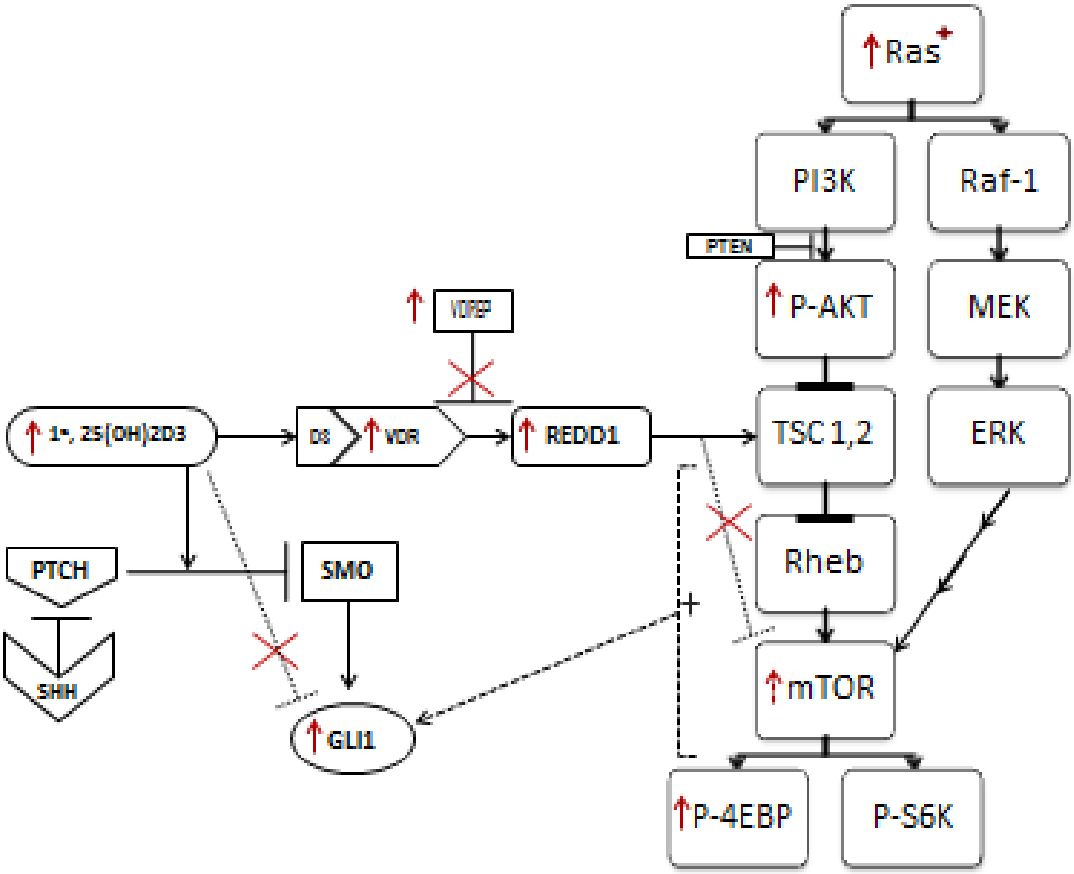
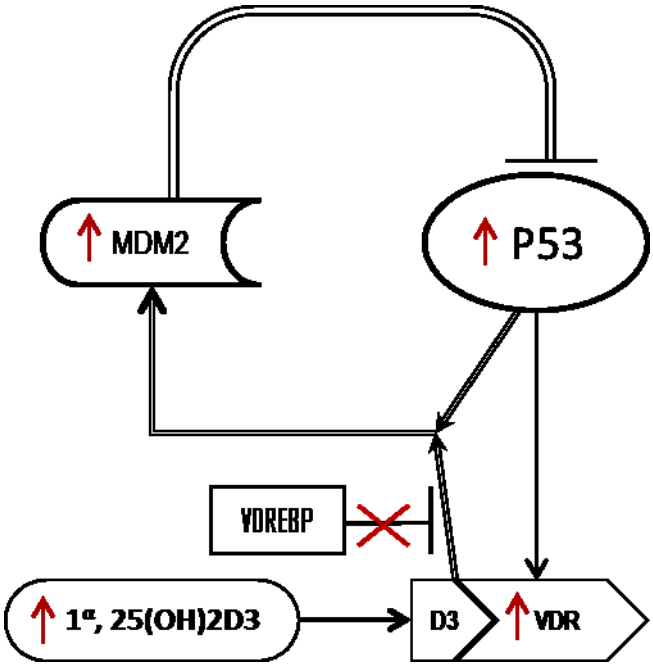

Ras
PI3K
Raf-1
PTEN
P-AKT
MEK
VDRIP
1α, 25(OH)2D3
D3
VDR
REDD1
TSC 1,2
ERK
Rheb
PTCH
SMO
SHH
GLI1
mTOR
P-4EBP
P-S6K

Figure 4.3: *VDR role in P53-MDM2 regulation:* Active P53 protein up-regulates the transcription of MDM2 protein which in turn binds and deactivates P53 as feedback inhibition mechanism. Recent publications revealed VDR involvement in this mechanism in their experimental model. VDR promoter region contains P53 responsive element, and MDM2 promoter contains both P53 and VDR responsive elements which were shown to cooperate in MDM2 transcription activation. In our BCC tissue specimens, we found that BCC cancer tissue that up regulate VDR also show over expression of MDM2 in spite of the elevated levels of VDREBP. Also, P53 gene expression was found to be elevated as well.


MDM2
P53
VDREBP
1α, 25(OH)2D3
D3
VDR

CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS:

The main goal for this study is to interpret the impact of vitamin D status on the onset and progression and possibly treatment of basal cell carcinoma. Most of human studies in literature in regards to vitamin D and NMSC are epidemiological. Mechanistic studies about BCC are few, and mostly are animal models or cell cultures.

The unique approach for this study involves human tissue samples taken from cancer patients and cancer-free individuals from southeast Michigan. This study design exclusively distinguishes between the behavior of non-cancerous tissue from cancer patient and tissue from cancer-free patients. We are also establishing the alterations in the genetic profile of patient's non-cancerous tissue according to their location in relation to the cancer. This idea has a great significance in understanding factors that may influence the onset and progression of the disease, as well as the risk for future recurrence.

In conclusion, we believe that the anticipated role of vitamin D is not conserved in basal cell carcinoma cells. We have observed that Vitamin D ability to negatively regulate mTOR and Hedgehog-Gli signaling pathways was diminished. In addition, BCC cancer tissue and its feeding cells seem to up-regulate vitamin D activation enzymes, vitamin D receptor along with its co-activators proposing higher local activity of vitamin D in the cancer tissue.

In our samples, BCC cancer tissue specimens have acquired a gain of function mutation in KRAS gene, with up-regulation in PI3k-AKT-mTOR pathway. Interestingly, Kras mutation was also observed in the cancer-free specimens from BCC patients. We suggested that this finding possibly contribute to the observed lack of sensitivity of BCC tissue to Vitamin D effect. However, proving causality needs further investigation.

This will be the first planned future direction related to this study. This goal can be approached using cell cultures of BCC cells that carry a KRAS mutation similar to the one we identified in our tissue samples. Our goal will be to show that silencing the mutated KRAS may result in down regulation of mTOR pathway in response to adding vitamin D to the media.

P53 was also found to be up regulated in our cancer tissue samples. It unknown whether the expressed P53 in these samples is a wild type or a mutant form. P53 mutations are common in many types of cancers including skin cancers. It was suggested in literature that P53 status can manipulate the impact of vitamin D [89]. It was shown that this impact is related to binding of mutant P53 to VDRE [89].

Investigating P53 and its possible interaction with vitamin D in BCC is important to understand and interpret our results. Thus, it will be a future direction for this study. P53 mutation can be possibly detected

by allele specific primer and blocker Real-time PCR. If P53 is successfully detected in BCC tissue samples, a mobility shift assay may determine binding of P53 to vitamin D response elements in our experimental model. Cell cultures can then be used to inspect whether silencing the mutant P53 will result in alterations in the cells response to vitamin D.

Our data propose privation of the benefits expected from supplementing BCC patients with high doses of vitamin D. However, it does not suggest a detrimental effect of vitamin D for those patients. Vitamin D possesses a well-characterized immune suppression effect [61, 62]. BCC cancer statistics shows an association between compromised immune function and high incidence of BCC with worse prognosis, especially among people who takes immune response weakening medications such as organ transplant patients [2]. Up regulation of VD3 1α-OHase was observed in alveolar macrophages isolated from lung cancer patients [95]. It was suggested that local activation of vitamin D around the cancer may be beneficial for the cancer. It possibly prevents possible immune system attacks to the cancer cells [95].

Studying the impact of vitamin D supplementation on the immune response of BCC patients and its possible effect on the disease outcome makes our second future direction. Achieving this goal can start with

isolating immune cells from the cancer and proximal tissue. Isolated cells can be studied for 1α-OHase expression, degree of maturation. Actual binding of VDR to the inverse response elements in the promoters of the target genes that which may deteriorate the maturation, may be detected through mobility shift assay.

Skin cancer is mainly classified as melanoma and non-melanoma skin cancer. Non-melanoma skin cancer is classified, in turn, to many subtypes including but not limited to basal cell carcinoma, squamous cell carcinoma. Many health care professionals tend to generalize their findings regarding skin cancer to include all non-melanoma skin cancer types.

We tried to investigate whether our conclusions applies to all NMSC. Tissue specimens were collected from three SCC patients. Vitamin D metabolism genes were analyzed in comparison to BCC specimens. We concluded that BCC and SCC are acquire different behavior in regards to vitamin D metabolism, thus generalizing our conclusions to NMSC would have been a scientific mistake. However, Full assessment of vitamin D possible effect on other types of skin cancer such as SCC and MM is another possible future direction for our research group.

New experiments need to be designed and established for that purpose. Subject recruitment and sample collection followed by

repeating the procedures that were used in studying BCC will be repeated with other types of NMSC.

In conclusion, we suggest that supplementation of Vitamin D to BCC patients has no expected benefit. We also propose that the outcome of future steps of this this study will have a greater biologically significance if we prove a detrimental effect of Vitamin D with respect to progression of BCC. If we find that cancer progression and the risk of recurrence is modifiable in individuals by selecting the best regimen for maintaining adequate levels of vitamin D, the current requirement, and/or supplementation of vitamin D recommended by health care providers may become outdated.

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ABSTRACT

DESENSITIZATION OF BASAL CELL CARCINOMA TO THE ANTI-TUMORAL EFFECT OF VITAMIN D

by

RAWIA KHASAWNEH

December 2013

Advisor: Dr. Ahmad R. Heydari

Major: Nutrition and Food Science

Degree: Doctor of Philosophy

The relationship between Vitamin D and NMSC is not very clear. Most of human studies in literature in regards to vitamin D and NMSC are epidemiological which are inconsistent and inconclusive. Mechanistic studies about BCC are few, and mostly are animal models or cell cultures.

The main goal for this study is to interpret the impact of vitamin D status on the onset and progression and possibly treatment of basal cell carcinoma. Three tissue samples were collected from BCC patients (Cancer, Proximal, and Distal), and from cancer-free individuals from southeast Michigan. This study design helps identifying alterations in the genetic profile of patient's non-cancerous tissue based on their location in relation to the cancer. This idea has a great significance in understanding factors that may influence the onset and progression of the disease, as well as the risk for future recurrence.

We have observed that Vitamin D ability to negatively regulate mTOR and Hedgehog-Gli signaling pathways was diminished. In addition, BCC cancer tissue and its feeding cells seem to up-regulate vitamin D activation enzymes, vitamin D receptor along with its co-activators proposing higher local activity of vitamin D in the cancer tissue.

In conclusion, we believe that the anticipated role of vitamin D is not conserved in basal cell carcinoma cells.

AUTOBIOGRAPHICAL STATEMENT

I received my Bachelor's degree in 2000 majoring in food science and technology from Damascus University. During My undergraduate years, I received four presidential awards based on academic achievement in four consecutive years. Upon graduation as the first student in my class, I started working in Damascus University as a lecturer. I taught several undergraduate courses for three years. In 2001 I earned my first graduate degree from Damascus University, the graduate diploma in Food Science (equivalent to non-thesis masters). In 2005 I began my graduate work as a Master's student at Wayne State University in the Department of Nutrition and Food Science and I earned my master's degree in 2008. Then, I status working on my PhD requirements under the mentorship of Dr. Ahmad Heydari studying the relationship between vitamin D status and non-melanoma skin cancer. In 2012 I joined the WSU coordinated program in dietetics aiming to be a registered dietitian in 2014.