Chapter one

1.1 Introduction:

Skin cancer is a type of disease where malignant cancer cells are to be found in the outer layer of skin. It divided into two general groups - malignant melanomas and non-melanoma cancers which are subdivided in to basal cell cancer (BCC) and squamous cell cancer (SCC) (Leiter, *et al*. 2014).

Non melanoma skin cancer (NMSC) is the most cancer affecting white-skinned individuals and the incidence is increasing worldwide. The incidence is varies widely with the highest rates in Australia (>1/100.000 person-years for BCC) and the lowest rates in Africa (<1/100.000 person-years for BCC) (Lomas, *et al*.2012).

In Sudan there are five hundred and thirty five patients with histological evidence of skin cancer during the period 1993- 2008. Squamous cell carcinoma (SCC) was the commonest skin malignancy accounting for 42.6% followed by basal cell carcinoma (BCC) which was seen in 32% of patients (Abdelsamie, *et al*.2012).

Exposure to ultraviolet B (UVB) radiation, depletion of stratospheric ozone, higher temperatures with actively tanned or used solariums/sunbeds, and exposure to high levels of arsenic in drinking water has increasing incidence of skin cancer (Gabriella, *et al*. 2010).

Any spots in the skin that are identified are suspicious to a cancer. Usually taken as a biopsy (incisional or excisional) to confirm the diagnosis, also blood tests and an imaging test such as a chest X-ray, computed tomography (CT) scan, magnetic resonance imaging (MRI), or positron emission tomography (PET) scan are used to check for the spread of skin cancer (Ainsley, 2014).

The five ways to eradicate skin cancers are burning, freezing, X-ray radiation, surgical excision, and excision by micrographic surgery (Arthur, 2001), also immunotherapy, cryosurgery, chemotherapy and photodynamic therapy are used (Ainsley, 2014).

Immunohistochemistry is a technique for identifying cellular or tissue constituents (antigens) by means of antigen-antibody interactions. The introduction of prognostic and predictive markers in immunohistochemistry has made a tremendous beneficial impact on patient diagnosis and management; many antibodies are now available to identify epitopes that survive the rigors of formalin fixation and processing to paraffin wax (Kim, *et al*.2013).

Bcl-2 is a member of a family of proteins that are involved in apoptosis. Bcl-2 is an integral inner mitochondrial membrane protein of 25 kD and has a wide tissue distribution. It is considered to act as an inhibitor of apoptosis (Neira, *et al*. 2008).

The androgen receptors (AR), also known as NR3C4 (nuclear receptor subfamily 3, group C, member 4), is a type of nuclear receptor that is activated by binding of either of the androgenic hormones testosterone or dihydrotestosterone in the cytoplasm and then translocating into the nucleus. The main function of the androgen receptor is as a DNA-binding transcription factor that regulates gene expression. Androgen regulated genes are critical for the development and maintenance of the male sexual phenotype (Antonio, *et al*. 2012).

Bcl-2 is a useful marker to differentiate cutaneous tumor as basal cell carcinoma (BCC) stains very strongly with bcl-2, this is negative in squamous cell carcinoma (SCC) and other trichoblastic tumours that mimic basal cell carcinoma on H and E staining (Eduardo, *et al*. 2012). The AR expression was present in approximately 78 % of BCC cases. However, no AR expression was observed in SCC (Yu-Fen, 2009).

1.2 Objectives:

1.2.1 General Objective:

To detect the expression of bcl-2 and androgen receptors in non-melanoma skin cancers among Sudanese patients.

1.2.2 Specific Objectives:

To detect the expression of bcl-2 and androgen receptors in non-melanoma skin cancers using immunohistochemical methods.

To correlate the expression of bcl-2 and androgen receptors with histological diagnosis.

Chapter two

2. Literature review

2.1 Scientific background:

Skin is a double-layered membrane covering the exterior of the body and consists of a stratified cellular epidermis and an underlying dermis of connective tissue. In adults, the skin weighs over 5 kg and covers a surface area approaching 2 m^2. The epidermis is mainly composed of keratinocytes and is typically 0.05–0.1 mm in thickness. The dermis contains collagen, elastic tissue and ground substance and is of variable thickness, from 0.5 mm on the eyelid or scrotum to more than 5 mm on the back (Eduardo, *et al*. 2012).

A key role of skin is to provide a mechanical barrier against the external environment. Keratinocyte-derived endogenous antibiotics (defensins and cathelicidins) provide an innate immune defense against bacteria, viruses and fungi. Melanin, which is mostly found in basal keratinocytes, provides some protection against DNA damage from ultraviolet radiation. An important function of skin is thermoregulation. Vasodilatation or vasoconstriction of the blood vessels in the deep or superficial plexuses helps regulate heat loss. Subcutaneous fat has important roles in cushioning trauma as well as providing insulation and a calorie reserve. Skin also has a key function in synthesizing various metabolic products, such as vitamin D (Marion, 2003).

2.2 Inflammatory disorders:

2.2.1 Psoriasis:

Psoriasis is thought to arise from an environmental trigger, on top of a genetic susceptibility. It is characterized by sharply demarcated, erythematous, papulosquamous plaques occur, mainly on the extensor surfaces (Caroline and Thomas, 1999).

2.2.2 Eczema:

Eczema, or dermatitis (these are interchangeable terms), is an inflammatory skin reaction, featuring itching, redness, scaling and clustered papulovesicles. Eczema can be endogenous (from within the body) or exogenous (from an external trigger) (Elaine, 2005).

2.2.3 Acne:

Acne is a chronic inflammatory disorder of the pilosebaceous units. A pilosebaceous unit consists of a hair follicle, erector pili muscle, sebaceous gland, and associated apocrine and eccrine sweat glands (Stephen and Joshua, 2012).

2.2.4 Rosacea:

Rosacea is a disorder characterized by frequent flushing, persistent erythema and telangiectasia, with episodes of inflammation, papules and pustules (Katharina, 2014).

2.2.5 Lichenoid disorders:

Lichenoid describes the clinical appearance of a flat-topped, shiny, papular rash. It also describes the histological appearance of a band-like inflammatory infiltrate in the superficial dermis, with liquefaction of the basal layer. A lichenoid eruption can occur due to lichen planus, drug eruption (mepacrine), graft versus host disease, pityriasis lichenoides, keratosis lichenoides chronica (Nekam's disease), lichen nitidus, lichen striatus, mycosis fungoides (Victoria and Andrew, 2006).

2.3 Tumors of skin:

2.3.1 Benign tumors:

2.3.1.1 Verruca vulgaris:

It is most likely seen on the fingers, hands and arms. It is small, raised, rough, clearly defined borders tumor. Also appear in clusters around a mother wart. It is caused by papilloma virus (Shangkuan and Lin, 2014).

2.3.1.2 Seborrheic keratosis:

It is frequently numerous and appear as sharply delineated, round or oval, flesh-colored or brown-black warty plaques with a rather greasy texture (Eduardo, *et al*. 2012).

2.3.1.3 Skin tags (acrochordons, cutaneous papilloma):

They are common benign tumors. The common locations are axilla, neck, and inguinal region. They begin as a tiny flesh-toned or brown lesion, millimeter increase to 1 cm in size (Wendy, 2010).

2.3.1.4 Actinic keratosis:

Actinic or solar keratoses are hyper-keratotic skin lesions occurring on sun-exposed skin. The common location includes scalp, temples and forehead. It appears slightly roughened area that often bleeds when excoriated (Steven, 2006).

2.3.1.5 Acanthomas:

Acanthomas are benign tumors of epidermal keratinocytes. The proliferating cells may show normal epidermoid keratinization or a wide range of aberrant keratinization (David, 2010).

2.3.1.6 Dermatofibromas:

Dermatofibromas are nodules derived from mesodermal and dermal cells. Which are fibrous reactions to minor trauma, insect bites, viral infections, ruptured cysts, or folliculitis (Mark, *et al*. 2003).

2.3.1.7 Melanocytic nevi:

They are hamartomas (abnormal collection of normal tissue constituents), which are a collection of nevomelanocytic cells, may be acquired or congenital (Hayder, 2008).

2.3.2 Malignant tumors:

2.3.2.1 Basal cell carcinoma:

Basal cell carcinomas (BCCs) are keratinocyte tumours of the epidermis. They are the most commonly diagnosed human cancer (Ervin, 2008).

It is divided into five main clinical subtypes, which to some extent correlate with their general growth pattern and treatment.

2.3.2.1.1 Superficial basal cell carcinoma:

This is presents with nodules and strands of basaloid cells that proliferate parallel to the epidermis and demonstrate slit-like retraction from the surrounding stroma (Goldenberg, *et al*. 2010).

2.3.2.1.2 Nodular basal cell carcinoma (NBCC):

This is presents with discrete, well-defined nodules and strands of basaloid cells in the papillary and reticular dermis, which may focally show a connection to the overlying epidermis (Vantuchov and Čuřík, 2006).

2.3.2.1.3 Morpheaform (sclerosing) basal cell carcinoma:

It is presents with thin strands of atypical basaloid cells in the dermis. These neoplastic strands are usually one-to-two strands thick and are enmeshed in a densely collagenized stroma with proplastic fibroblasts (Trakatelli, *et al.* 2013)

2.3.2.1.4 Fibroepithelioma of Pinkus:

Is a rare type of BCC that typically presents with elongated basaloid epithelial strands, which usually show multiple connection points to the overlying epidermis (Ghanima and Alfred, 2006).

2.3.2.1.5 Pigmented basal cell carcinoma:

The pigmentation may be present in both dendritic melanocytes and stromal macrophages. Its only significance lies in the clinical misinterpretation of the tumor as melanoma (Shashidhar, 2004).

2.3.2.2 Squamous cell carcinoma:

It is the second most common cancer of the skin, this tumor arises predominantly in sun exposed actinically damaged areas. It characterized by squamous cells with large nuclei and abundant eosinophilic cytoplasm. The cells exhibit prominent intracellular bridges and variable keratin formation, depending on the degree of differentiation (Jasim, 2012).

Several histologic variant of squamous cell carcinoma are identified, the following are the most reported variant:

2.3.2.2.1 Bowen's disease:

Also known as SCC in situ, it presents as a slow-growing, sharply demarcated erythematous scaly patch. They characterized histologically by hyperkeratosis, parakeratosis, and acanthosis with thickened and elongated rete ridges (Rinker, *et al*. 2001).

2.3.2.2.2 Verrucous squamous cell carcinoma:

It is a variant of well-differentiated squamous carcinoma, which shows exophytic nodular growth with acanthotic papillary processes (Shimizu, *et al*. 2006).

2.3.2.2.3 Acantholytic (adenoid) squamous cell carcinoma:

It is an uncommon variant of SSC characterized by acantholysis of tumor cells, creating pseudolumina and appearance of glandular differentiation (Zidar, *et al*. 2006).

2.3.2.2.4 Adenosquamous carcinoma:

It is an uncommon cutaneous malignant neoplasm with mixed glandular and squamous differentiation and a propensity for aggressive clinical behavior (Fu, *et al*. 2009).

2.3.2.2.5 Keratoacanthoma:

It is a rapidly growing skin tumor arising predominantly on the exposed surfaces of the body. This tumor is dome-shaped nodule with a central keratinous plug (Yazdain, *et al*. 2009).

2.3.2.2.6 Desmoplastic squamous cell carcinoma:

It is a rare but clinically significant variant (local recurrence and metastasis)**.** Histologically, characterized by tumor aggregates arranged in nests and strands surrounded by an intense desmoplastic stromal reaction (Kane, *et al*. 2004).

2.3.2.2.7 Spindle cell carcinoma:

It is a rare variant of SCC. Clinically, it may appear as an ulcerated mass on the sunexposed skin of elderly patients. Histologically, it is composed of atypical spindle cells arranged in a whorled pattern (Petter and Haustein, 2000).

2.3.2.2.8 Clear squamous cell carcinoma:

It is a rare variant of SCC of skin in which ultraviolet radiation has been suggested as possible etiology. They are usually resulting of glycogen accumulation, this can be due to hydropic degeneration (Ahmed, *et al*. 2013).

2.3.2.3 Malignant melanoma:

It is an aggressive, therapy-resistant malignancy of melanocytes, it may develop within a pre-existent benign melanocytic nevus, or other dermal dendrocytosis (Markovic, *et al*. 2007).

The four major subtypes of cutaneous melanoma are currently recognized are:

2.3.2.3.1 Acral lentiginous melanoma (ALM):

It is a major type of malignant melanoma, usually present as irregular, gradually enlarging, and variably pigmented macules. Most commonly found on the distal portions of the limbs—have usually reached more advanced stages than other types of melanoma when diagnosed (Pereda, *et al*. 2013).

2.3.2.3.2 Superficial spreading melanoma:

It is the most common variant characterized by large, irregular junctional melanocytic nests. The junctional nests varied in shape and size, showed focal tendency to confluence, and were often surrounded by a cuff of epidermal keratinocytes (Kutzner, *et al.* 2012).

2.3.2.3.3 Nodular melanoma (NM):

It is representing 15% to 30% of all melanomas, and it frequently lacks clinical features seen in other melanoma subtypes and has a faster growth rate (Kalkhoran, *et al.* 2010).

2.3.2.3.4 Lentigo maligna melanoma (LM):

It is a subtype of melanoma in situ that typically develops on sun-damaged skin. Histologic evaluation can be difficult due to the widespread atypical melanocytes that are present in the background of long-standing sun damage. Recurrence following standard therapies is common (McKenna, *et al.* 2006).

2.3.2.4 Kaposi sarcoma (KS):

It is a low-grade vascular tumor associated with Kaposi sarcoma herpesvirus/human herpesvirus 8 (KSHV/HHV8) infections. It predominantly presents at mucocutaneous sites, but may involve all organs and anatomic locations. Kaposi sarcoma lesions evolve from early (patch stage) macules into plaques that grow into larger nodules. Newer histologic variants include anaplastic, hyperkeratotic, lymphangioma-like, bullous, telangiectatic, ecchymotic, keloidal, pyogenic granuloma-like, micronodular, intravascular, glomeruloid and pigmented KS, as well as KS with sarcoidlike granulomas and KS with myoid nodules (Radu and Pantanowitz, 2013).

2.3.2.5 Cutaneous lymphomas:

They are a heterogenous group of lymphoproliferative disorders of the T- and B-lymphocytes with a low incidence and belong to the Non-Hodgkin lymphoma. The

skin is the second most abundant site of extranodal lymphoma formation (after the GI tract). The new classification of cutaneous T- and B-cell lymphomas provides a widely accepted nomenclature for primary cutaneous lymphomas and secondary based primarily on clinical, but also on histologic, cytologic and molecular features (Schad, *et al.* 2010).

2.4 Epidemiology of skin cancers:

Melanoma and nonmelanoma skin cancer (NMSC) are the most common types of cancer in white populations. Both tumor entities show an increasing incidence rate worldwide but a stable or decreasing mortality rate. The highest incidence rates have been reported from Queensland, Australia with 56 new cases per year per 100,000 for men and 43 for women (Lomas, *et al.*2012).

Also is the most prevalent cutaneous malignant neoplasm in the United States with an estimated incidence of 600 000–900 000 cases per year. The incidence of basal cell carcinoma was approximately 180/100 000 of the population, where as the incidence of cutaneous squamous cell carcinoma is, ranging from 81 to 136 for men and from 26 to 59 for women per 100 000 of the population (Eduardo, *et al.* 2012).

2.5 Risk factors of skin cancers:

2.5.1 Age:

The incidence of skin cancer increasing in older people, particularly in over the age of 70 years (David, 2010).

2.5.2 Sex:

Melanoma and nonmelanoma skin cancer (NMSC) is more common in men than in women (Leiter, *et al.* 2014).

2.5.3 Genetic factors:

Pigmentary traits such as red hair, fair skin, lack of tanning ability and propensity to freckle (the RHC phenotype) have been identified as genetic risk factors for both

melanoma and non-melanocytic skin cancers when combined with the environmental risk factor of high ultraviolet light exposure (Sturm, 2002).

2.5.4 Radiation:

Ultraviolet radiation (UV) from sun exposure is the most important causes. Sunburns and excessive exposures cause cumulative damage which induces cancer. Radiation therapy, phototherapy and psoralen and long-wave ultraviolet radiation (PUVA) can also predispose to skin cancers (Saladi and Persaud, 2005).

2.5.5 Immunosuppressive agents:

All immunosuppressive treatments have the potential to impair the skin immune system network of cells and cytokines, thus leading to an increased incidence of skin cancer (Gerlini, *et al*. 2005).

2.5.6 Human papillomavirus:

Cutaneous HPVs that belong to the beta genus may act as a co-carcinogen with UVR. E6 and E7 from certain cutaneous HPV types display transforming activities, further confirming their potential role in carcinogenesis (Accardi and Gheit, 2014).

2.5.7 Chemical carcinogens:

Chemical carcinogens are important in a small number of cases. In addition to arsenic a wide range of substances, especially hydrocarbons, have been incriminated (Eduardo, *et al*. 2012).

2.5.8 Other factors:

Some new potential risk factors were identified for consumption of certain food items, medication use and stress (Vries, *et al*. 2012).

2.6 Diagnosis of skin cancers:

2.6.1 Biopsy:

It include simple punch biopsies (handled akin to cores) and shave biopsies that should be mounted on edge in order to provide an adequate view of the epidermis, dermis and subcuticular substrates (Kim, *et al*. 2013).

2.6.2 Immunohistochemical techniques:

Immunohistochemistry has become an essential diagnostic tool in dermatopathology (Fuertes, *et al*. 2013).

2.6.3 Optical techniques:

These techniques include: optical coherence tomography, fluorescence spectrometry, reflectance spectrometry, Raman spectroscopy, and confocal microscopy (Calin, *et al*. 2013).

2.6.4 Multiphoton laser scanning microscopy (MPLSM):

This technique is based on the nonlinear process of 2-photon excitation of endogenous fluorophores, which can be used to acquire horizontal optical sectioning of intact biological tissue samples (Paoli, *et al*. 2009).

2.7 Treatment of skin cancers:

2.7.1 Surgery:

Excision surgical margin is the optimal treatment for skin lesions clinically diagnosed as basal cell or squamous cell carcinoma (Thomas, *et al*. 2003).

Mohs micrographic surgery (MMS) is a technique that aims to optimize control of the tumor margins. Under local anesthesia, the tumor, together with a small rim of clinically normal tissue, is excised and microscopically evaluated (Amjadi, *et al*. 2010).

Curettage and cautery (C and C) involves removing epidermis and dermis containing tumor tissue with a sharp ring curette instrument prior to charring of the base of the wound with electrocautery (Venura and Vishal, 2012).

2.7.2 Radiation therapy:

It is an important option for the treatment of skin cancer of deeper and extensive tumor and anatomic sites where it is difficult to obtain clear surgical margins (Wang, *et al*. 2009).

2.7.3 Other methods:

Other methods includes: cryosurgery, photodynamic therapy, immune response modifiers and topical chemotherapy (Julie, *et al*. 2007).

2.8 Bcl-2:

Bcl-2 is the prototype of the Bcl-2 family proteins, was the first defined molecule involved in apoptosis. It was initially cloned from the t(14;18) breakpoint in human follicular lymphoma. They inhibit apoptosis, autophagy, and proliferation. Genetic deletion causes major phenotypes in the lymphoid system, the kidney, the melanocytes, and other cells (Wen and Xiao, 2009).

A fundamental feature of carcinogenesis is deregulation of normal cellular genes termed proto-oncogenes. The products of activated proto-oncogenes and mutated tumor suppressor-genes act to cause increased cellular proliferation. They contribute to malignancy by inhibiting programmed cell death. In the cell death pathway, bcl-2 is the best understood gene, and it functions as a repressor of programmed cell death (Neira, *et al*. 2008).

Bcl-2 is useful marker to differentiate cutaneous tumor as basal cell carcinoma (BCC) stains very strongly with bcl-2, this is negative in squamous cell carcinoma (SCC) and other trichoblastic tumours that mimic basal cell carcinoma on H and E staining (Asher, 2008 , Eduardo, *et al*. 2012).

David (2003) who documented that basal cell carcinoma can usually be diagnosed based on morphology alone. However, there are other types of tumors such as adnexal tumors, trichoepithelioma, sebaceous epithelioma and squamous cell carcinomas that can resemble basal cell carcinoma. Basal cell carcinoma stain

diffusely positive with Bcl-2 whereas in trichoepithelioma only the periphery of the lobules stains.

2.9 Androgen receptors:

The androgen receptors (AR) belongs to the steroid receptor superfamily that function primarily as transcription factors to regulate the expression of target genes by binding to specific hormone-responsive elements. These steroid receptors may exist within the target cells in a nonactivated state (Cynthia and Chawnshang, 2002).

In the normal skin, the expression of androgen receptors (AR) could be identified in basal and differentiating sebocytes in sebaceous glands, pilosebaceous duct, keratinocytes, interfollicular epidermal keratinocytes, dermal fibroblasts, luminal epithelial cells of apocrine glands in genital skin, and in certain cells of the secretory coils of eccrine sweat glands in all body sites. Many benign and malignant skin tumors also expressed the staining of AR. The investigations stated that 78% of BCC showed positivity for AR immunoreactivity (Yu-Fen, 2009).

The AR expression was present in approximately 60 % of BCC cases. However, no AR expression was observed in SCC (David, 2003).

Chapter three

Materials and methods

3.1 Materials:

Formalin fixed histological biopsy that has been sent by surgeon to the histological laboratory and diagnosed as cutaneous squamous cell carcinoma and basal cell carcinoma were used in this study.

3.2 Methods:

3.2.1 Study design:

This is a descriptive retrospective study aimed at detecting expression of bcl-2 and androgen receptor among non-melanoma skin tumors by immunohistochemical methods.

3.2.2 Study area:

This study was conducted at Dr. Ahmed Ibrahim Shommo private laboratory-Khartoum and Sudan University of Sciences and Technology-College of medical laboratory sciences during the period from February 2015 to August 2015.

3.2.3 Study population:

Thirty five formalin fixed paraffin blocks (FFPB) were taken from Dr. Ahmed Ibrahim Shommo laboratory archive that includes H and E report. Patient's identification data and other information were obtained from patient's files.

3.2.4 Samples collection and preparations:

From each paraffin block two sections were cut into 3 μm, sections were floated into preheated floating water bath at 40°C, each sections were placed in slide coated with adhesive salinized glass slide, incubated overnight at room temperature.

3.2.5 Staining procedure:

Immunohistochemical staining procedure was carried out using monoclonal mouse anti human bcl-2, clone 100/D5, isotype: IgG1 / kappa (Thermo-Scientific) and

monoclonal mouse anti human androgen receptor, clone AR 441, isotype: IgG1 / kappa (Thermo-Scientific).

Both sections were dewaxed for 15 minute in hot plate oven and cleared in two changes of xylene for two minutes, then hydrated in descending concentrations of ethanol (100%, 90%, 70%, and 50%) and hydrated in water two minutes for each, then retrieved by water bath retrieval technique in citrate buffer at 95°C for 20 minutes, and then sections were cooled at room temperature. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase and methanol for 10 minutes, and then slides were incubated with 100-200 μm of primary antibodies for 20 minutes at room temperature in a moisture chamber (the primary antibodies for bcl-2 and androgen receptors (monoclonal) were ready to use). Sections were rinsed in 1% phosphate buffer for 3 minutes, and then enhancer solution (BioGenex) applied to sections for 20 minutes, then rinsed in tris buffer saline. Binding of antibodies were detected by incubating for 20 minutes with dextran labeled polymer (BioGenex-Super Sensitive TM Polymer-HRP Kits). The sections were washed in three changes of TBS, followed by adding 3,3 diaminobenzidine tetra hydrochloride (DAB) (BioGenex) as a chromogen to produce the characteristic brown stain for visualization of the antibody-enzyme complex for 7 minutes. Sections were counterstained with Mayer's hematoxylin for 3 minutes and bluing in running tape water for 10 minutes, then the sections dehydrated by rinsing in ascending concentration of ethanol (50%, 70%, 90%, and 100%). Finally the sections were clearing in xylene and mounting by DPX mounting media. For each run of staining, positive and negative control slides were also prepared. The positive controls were containing the antigen under investigation and the negative control slides were prepared from the same tissue block, but were incubated with TBS instead of the primary antibodies. Each slide was evaluated with investigator then the results were confirmed by consultant histopathologist.

Positive bcl-2 staining was identified in form of brown cytoplasmic reaction while the positive androgen receptors staining was identified in form of brown nuclear reaction.

3.2.6 Statistical analysis:

The obtained results and variables were arranged in standard master sheet, then were entered a computer program 11.5 SPSS. Frequencies, means and chi-square test values were analyzed.

3.2.7 Ethical consideration:

All samples were taken ethically after permission of administration of Dr. Ahmed Ibrahim Shommo laboratory.

Chapter four

Results

A total of 35 patients with skin cancer were investigated by immunohistochemistry to detect the expression of bcl-2 and androgen receptor. The patient's age ranged between 29-75 years with mean of age was 58(±13.7), 15 (43%) of patients were younger than 60 years, and 20 (57%) of patients were older than 60 years as indicated in table (4.1).

Out of 35 patients, 22 (63%) were males and 13 (37%) were females as indicated in table (4.2).

Among study subjects histopathological diagnosis of cancer revealed that 19 (54%) were basal cell carcinoma and 16 (46%) were squamous cell carcinoma as indicated in table (4.3).

The site of cancer in study population were showed that 23 (65.7%) of samples in face and neck, 8 (22.9%) of samples in chest and back and 4 (11.4%) of samples in thigh and legs as indicated in table (4.4).

Histopathological diagnosis and bcl-2 expression showed that positive in 16 (84.2%) samples and negative in 3 (15.8%) samples among basal cell carcinoma. Two (12.5%) positive samples and 14 (87.5%) negative samples among squamous cell carcinoma with significant statistical association (P value < 0.05) as indicated in table (4.5).

Histopathological diagnosis and androgen receptors expression showed that positive in 11 (57.9%) samples and negative in 8 (42.1%) samples among basal cell carcinoma. Two (12.5%) positive samples and 14 (87.5%) negative samples among squamous cell carcinoma with significant statistical association (P value < 0.05) as indicated in table (4.6).

Table (4.1): Distribution of age group among study population.

Age group	Frequency	Percentage (%)
Less than 60 years	15	43
More than 60 years	20	57
Total	35	100

Table (4.2): Distribution of sex among study population.

Sex	Frequency	Percentage (%)
Male	22	63
Female	13	37
Total	35	100

Table (4.3): Histopathological diagnosis of study population.

Carcinoma	Frequency	Percentage (%)
Basal cell carcinoma	19	54
Squamous cell carcinoma	16	46
Total	35	100

Table (4.4): Distribution of site of skin cancer among study population.

Site of cancer	Frequency	Percentage (%)
Face and neck	23	65.7
Chest and back	8	22.9
Thigh and legs	4	11.4
Total	35	100

Table (4.5): Relation between histopathological diagnosis and Bcl-2 result.

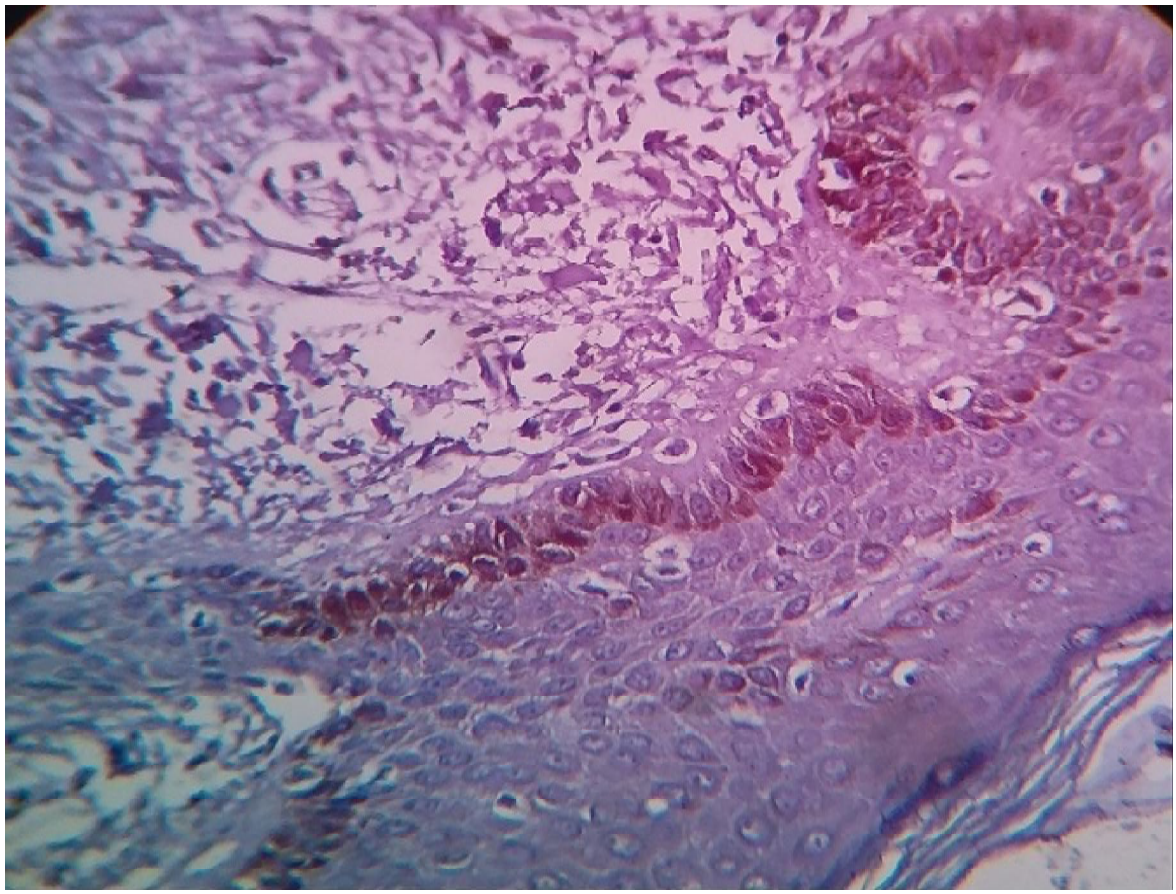
Diagnosis	Bcl-2		Total
	Positive	Negative	
Basal cell carcinoma	16 (84.2%)	3 (15.8%)	19 (100%)
Squamous cell carcinoma	2 (12.5%)	14 (87.5%)	16 (100%)
Total	18 (51.4%)	17 (48.6)	35 (100%)

P. value = 0.000

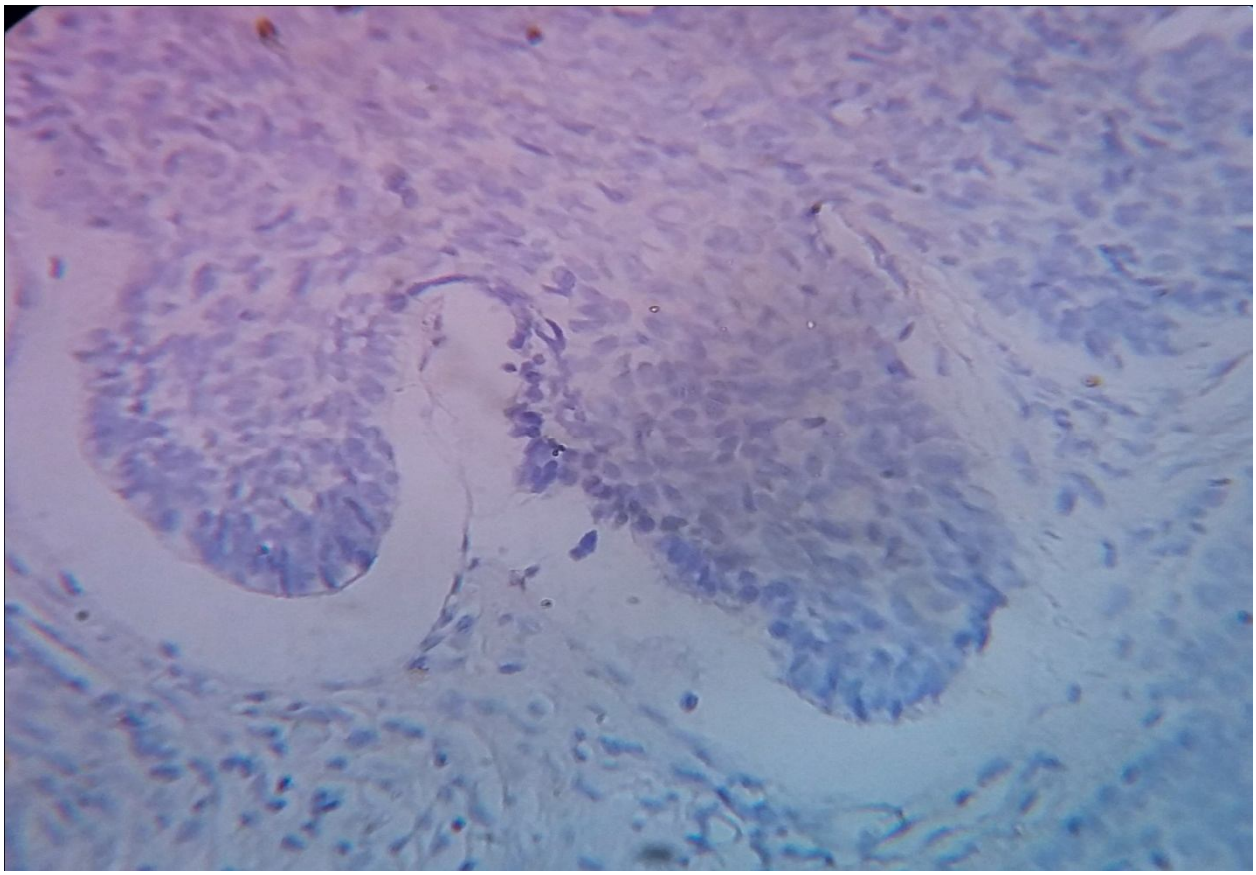
Table (4.6): Relation between histopathological diagnosis and androgen receptors result.

Diagnosis	Androgen receptor		Total
	Positive	Negative	
Basal cell carcinoma	11 (57.9%)	8 (42.1%)	19 (100%)
Squamous cell carcinoma	2 (12.5%)	14 (87.5%)	16 (100%)
Total	13 (37.1%)	22 (62.9%)	35 (100%)

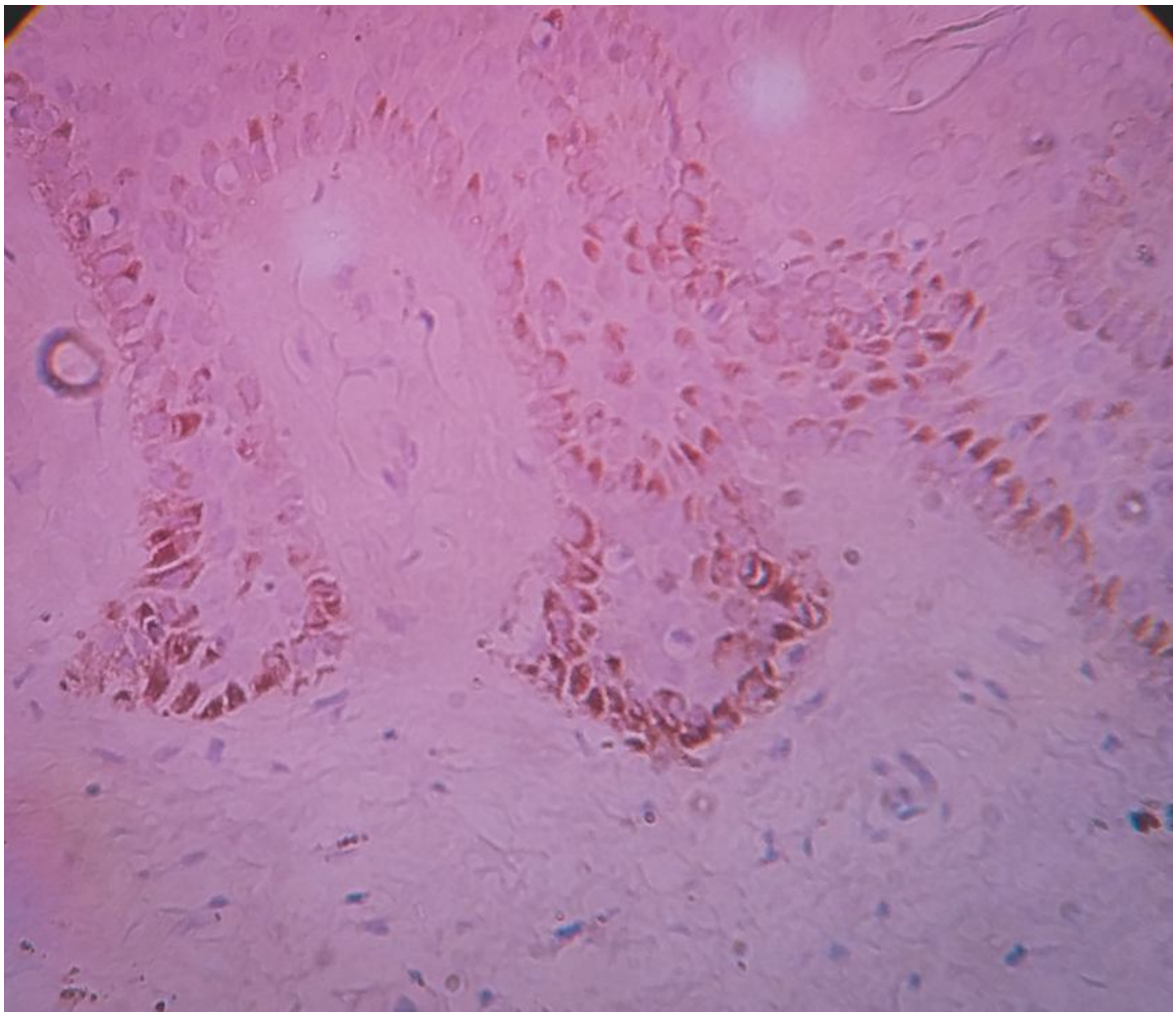
P. value = 0.006



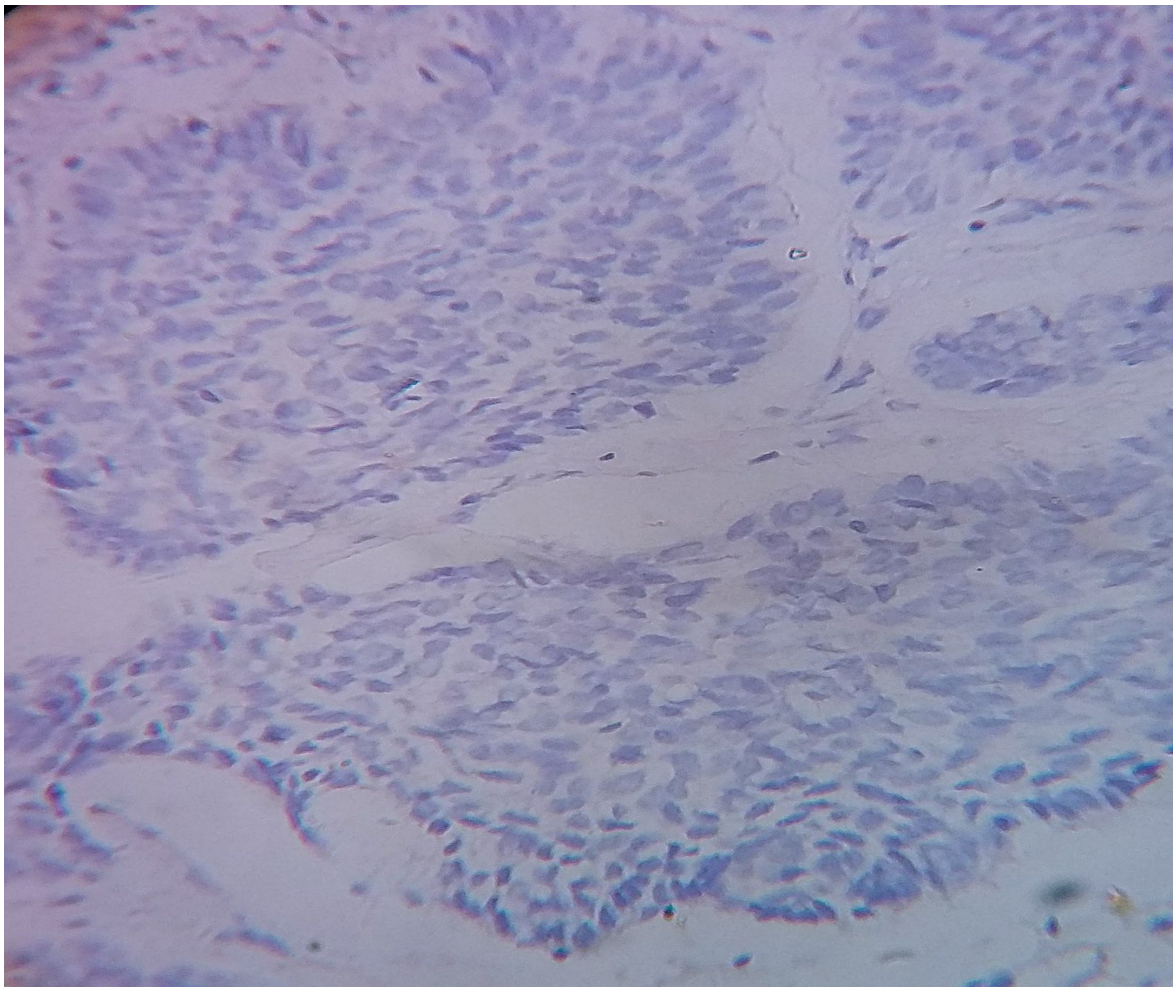
Micrograph (4.1): Basal cell carcinoma showing positive cytoplasmic expression of Bcl-2 (40x)



Micrograph (4.2): Squamous cell carcinoma showing negative cytoplasmic expression of Bcl-2 (40x)



Micrograph (4.3): Basal cell carcinoma showing positive nuclear expression of Androgen receptors (40x).



Micrograph (4.4): Squamous cell carcinoma showing negative nuclear expression of Androgen receptors (40x).

Chapter five

Discussion

Skin cancer is the most common type of cancer in fair-skinned populations in many parts of the world. The incidence, morbidity and mortality rates of skin cancers are increasing and, therefore, pose a significant public health concern (Narayanan, *et al*.2010).

The present study we found that most people affected with skin cancer are more than 60 years representive (57%) which is likely due to accumulated exposure to UV radiation and exposure to the sun for a longer time. This is compatible with the study of Natafji and Tidman (2015), they reported that most basal cell and squamous cell carcinomas typically appear after age of 50 years. However, the number of skin cancers in people aged 65 years or older has increased dramatically. This is agree with study of Etzkorn, *et al*. (2013), they reported that individual older than 50 years were more affected with skin cancer.

The study revealed that male are more affected than female because they spend more time under the sun, which agreed with study of Simanainen, *et al*. (2015), they reported that males are significantly more susceptible to non-melanoma skin cancers than females.

Basal cell carcinoma is the more frequent skin cancer than squamous cell carcinoma because it arises in the skin's basal cells, which line the deepest layer of the epidermis (the outermost layer of the skin) which is usually exposed to sun. This study is compatible with study of Abbas and Kalia (2015), they mentioned that frequency of basal cell carcinoma were two times more than squamous cell carcinoma.

The present study showed that face and neck are more affected area than the other part of the body skin; this is may be due to exposure to high levels of ultraviolet (UV) radiation. This is compatible with study of Abbas and Kalia (2015), they

reported that keratinocyte carcinomas were most commonly located on the head and neck, and increasing rates are occurring on the trunk. This is also agree with the study of Youl, *et al*. (2011), they reported that the highest densities for skin cancer were observed on chronically sun-exposed areas of the body including the face, the scalp, the neck and ears.

Bcl-2 is emerging as a crucial regulator of epidermal homeostasis and cell's fate in the stressed skin. Deregulation of Bcl-2 is also chiefly involved in skin carcinogenesis and response to cancer therapy (Nys and Agostinis, 2012).

In this study 84.2% samples of basal cell carcinoma were positive for Bcl-2 while 15.8% samples were negative and 12.5% samples of squamous cell carcinoma were positive for Bcl-2 while 87% samples were negative (P. value < 0.05). This finding showed that there was relation between expression of Bcl-2 and differential diagnosis of skin cancer. This is agree with study of Eduardo, *et al*.(2012), they evaluated the usefulness of Bcl-2 to differentiate cutaneous tumor, as basal cell carcinoma stains very strongly with bcl-2, while squamous cell carcinoma usually showed negative results. This study also compatible with the study of Asher (2008), who found that since basal cell carcinomas are typically diffusely positive for Bcl-2 marker, cutaneous squamous cell carcinomas are generally negative, but some with focal positivity.

Androgen receptor (AR) is widely distributed in the skin, suggesting a role for androgens acting via AR in skin carcinogenesis; AR inactivation modified some genes expression in the skin, suggesting possible molecular mechanism for the AR effect on skin (Simanainen, *et al*. 2015).

In the present study 57.9% samples of basal cell carcinoma were positive for androgen receptor while 42.1% samples were negative and 12.5% samples of squamous cell carcinoma were positive for androgen receptor while 87% samples were negative (P. value <0.05). This study agree with the study of Yu-Fen (2009),

who evaluated the usefulness of androgen receptor to differentiate cutaneous tumor as androgen receptor expression was present in approximately 78 % of basal cell carcinoma cases. However, no androgen receptor expression was observed in squamous cell carcinoma. This study also compatible with the study of David (2003), who reported that 60% of basal cell carcinoma showed positivity for AR immunoreactivity.

Chapter six

Conclusion and recommendations

6.1 Conclusion:

At the end of this study we conclude that:

- Most patients with Non-melanoma skin cancer were older than 60 years.
- Most affected patients are males.
- Basal cell carcinoma was the most common type of Non-melanoma skin cancer among Sudanese patients.
- Expression of Bcl-2 and androgen receptor among patients with skin cancer usually associated with basal cell carcinoma.

6.2 Recommendations:

At the end of this study we recommended that:

- Further studies should be done involving large sample size.
- Bcl-2 and androgen receptors should be used to differentiate basal cell carcinoma from squamous cell carcinoma.

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Appendix I:

Materials and instruments used for processing the specimens and staining include:

-Oven.

-Paraffin wax.

-Embedding machine.

-Moulds.

-Cassette.

-Rotary Microtome.

-Microtome Knifes.

-Frosted slides (75x25x2mm, coated with 0.01% Poly-L-lysine).

-Pencil.

-Water bath.

-Distilled water (DW).

-Coplin jars.

-Tap water.

-Citrate buffer (PH 6.8) composed of 72.7 ml from solution A + 22.8 ml from solution B. Solution A (0.2 M sodium di-hydrogen orthophosphate, 2.83g di-sodium hydrogen orthophosphate and 100 ml DW) and solution B(2.1g citric acid and 100 ml DW).

-Phosphate buffer (PH 7.4) composed of 9.5 ml from solution A + 40.5 ml from solution B. Solution A(0.2 M sodium di-hydrogen orthophosphate, 3.12g di-sodium hydrogen orthophosphate and 100 ml DW) and solution B(0.2 M sodium di-hydrogen orthophosphate, 2.83g di-sodium hydrogen orthophosphate and 100 ml DW).

-Peroxidase blocking solution: (0.25 % casein in PBS+ 0.015 mol/L sodium azide).

-Hydrogen peroxide.

-Monoclonal mouse anti human Bcl-2 and Androgen receptor.

-Enhancer.

-Dextran polymer.

-DAB (3.3 diaminobenzidine in chromogen solution).

-DAB substrate buffer (PH 7.5).

-Mayer’s Haematoxylin (1g Haematoxylin, 50g chloral hydrate, 50g potassium aluminum sulfate, 0.2g sodium iodate, 1g citric acid and 1000ml distilled water).

-DPX (distyrene plasticiser xylene) mounting media.

-Cover glass.

-Microscope.

Appendix II:

Staining protocol and Abs data sheets:

BioGenex
49026 Milmont Drive, Fremont, CA 94538
Tel : +1 (800) 421-4149 Fax: +1 (510) 824-1490,
support@biogenex.com

Super Sensitive™* Polymer-HRP Detection System
A Biotin-Free Detection System

Doc. No. 932-QDMAN-5X Rev. No.: M
Release Date : 13-Dec-2013

F. STAINING PROCEDURE

The tissue sections should not be allowed to dry out at any point during the rehydration and staining procedures.
The following protocol is applicable to both the manual and automated experiments.

1. **Peroxide Block** (optional).

Apply Peroxide Block to cover the specimen according to tissue size or autostaining slide parameters. Incubate for 5-10 minutes at room temperature. Drain and blot gently around the section.

2. **Power Block™**

Apply the Power Block to cover the section and incubate for 10 minutes at room temperature. Drain and blot gently (Note: do not wash the tissue section with washing buffer).
(Antibodies attach non-specifically to highly charged sites. This non-specific binding can be minimized by the use of a proteinaceous blocking reagent such as the Power Block).

3. **Application of Primary Antibody:**

NOTE: Ensure that the primary antibody is at the proper dilution. BioGenex ready-to-use Super Sensitive antibodies have been optimally diluted for use with these reagents and should not require further dilution

The detection system must be matched to the species of the primary antibody.

Blot slides around sections. Add appropriate volume of Primary Antibody to cover specimen according to tissue size or autostaining slide parameters. Likewise, add negative control serum to the negative control slide. Incubate the slides for the recommended time period and at the recommended temperatures. Rinse well with buffer.

4. **Application of Super Enhancer™ Reagent:**

Blot slides around the sections. Add appropriate volume of Super Enhancer™ Reagent to cover the specimen according to tissue size or autostaining slide parameters. Incubate for 20 minutes at room temperature. Rinse well with buffer.

5. **Application of Polymer-HRP Reagent:**

Blot slides around the sections. Add appropriate volume of Polymer-HRP Reagent to cover the specimen according to tissue size or autostaining slide parameters. Incubate for 30 minutes at Room Temperature. Rinse thoroughly with Buffer at least thrice.

6. **Application of Substrate Solution:**

Blot slides around the sections. Add appropriate volume of Substrate solution to cover the specimen according to tissue size or autostaining slide parameters. Incubate for 10 minutes at room temperature or until acceptable color intensity has been reached. Rinse well with deionized water or rinse buffer

7. Counterstaining Procedures:

Immerse the slides in a bath of Mayer's hematoxylin for 1-10 minutes, depending on the strength of hematoxylin used. Rinse slides with tap water. Optional: immerse in ammonia water for 10 seconds, then rinse with tap water. (See Appendix, Section X).

8. Mounting Procedures:

Aqueous Mounting: While slides are still wet, mount coverslip using 1-2 drops of aqueous mounting medium available from BioGenex. (See Appendix, Section X).

Thermo SCIENTIFIC

IVD DATA SHEET
Rev 032910B

BCL-2alpha Ab-1

Catalog # MS-123-P0, -P1, or -P (0.1ml, 0.5ml, or 1.0ml at 200ug/ml)
Catalog # MS-123-R7 (7.0ml)
Catalog # MS-123-PCS

Please note this data sheet has been changed effective March, 29 2010

INTENDED USE:

- ***For In Vitro Diagnostic Use:*** This product is intended for qualitative immunohistochemistry with normal and neoplastic formalin-fixed, paraffin-embedded tissue sections, to be viewed by light microscopy.
- ***Description:*** Expression of Bcl-2alpha oncoprotein inhibits the programmed cell death (apoptosis). In most follicular lymphomas, neoplastic germinal centers express high levels of Bcl-2alpha protein, whereas the normal or hyperplastic germinal centers are negative.
- ***Expected Staining Pattern:*** Cytoplasm
- ***Positive Control:*** Raji Cells. Tonsil or follicular lymphomas.

MATERIALS PROVIDED:

BCL-2alpha Ab-1 (refer to catalog number):

- #MS-123-P (or -P0, -P1): 200ug/ml of antibody purified from ascites. Prepared in 10mM PBS, pH 7.4, with 0.2% BSA and 0.09% sodium azide.

or

- #MS-123-R7: (7.0ml) of antibody prediluted in 0.05mol/L Tris-HCl, pH 7.6 containing stabilizing protein and 0.015mol/L sodium azide.

or

- #MS-123-PCS: 5 positive control slides.

- ***Antibody Concentration:*** 200ug/ml
- ***Host:*** Mouse
- ***Mol. Wt. of Antigen:*** 25-26kDa
- ***Epitope:*** aa 41-54
- ***Species Reactivity:*** Human. Does not react with mouse and rat. Others-not known.
- ***Clone Designation:*** 100/D5
- ***Ig Isotype / Light Chain:*** IgG1 / kappa
- ***Immunogen:*** A synthetic peptide, aa 41-54 (GAAPAPGIFSSQPG-Cys) of human Bcl-2 protein.
- ***Microbiological State:*** This product is not sterile.

MATERIALS REQUIRED, BUT NOT PROVIDED:

- ***Antibody Diluent:*** For concentrated antibodies, the antibody must be diluted before using. Use Lab Vision Antibody Diluent (catalog # TA-125-UD). Refer to diluent product instructions for use.
- ***Negative Control Reagent:*** Refer to the "General Protocol" instructions.
- ***Visualization System:*** Refer to the "General Protocol" instructions.

METHODS AND PROCEDURES:

Using UltraVision LP detecton systems	
Specimen Preparation	Refer to the "General Protocol" instructions.
Dilution of Concentrated Antibody	1:50-1:100 in antibody diluent
Tissue Section Pretreatment	Staining of formalin-fixed tissue sections requires treating the tissue sections in boiling 10mM citrate buffer, pH 6.0 (Lab Vision catalog # AP-9003), for 10-20 minutes followed by cooling at room temperature for 20 min.
Primary Antibody Incubation Time	20 minutes at Room Temperature
Visualization	To detect antibody, follow the instructions provided with the visualization system.

Thermo Fisher Scientific
Anatomical Pathology
46360 Fremont Blvd.,
Fremont, CA 94538, USA
Tel: 1-510-771-1560
Fax: 1-510-771-1570
http://www.thermo.com/labvision

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Manufactured by:
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For
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Anatomical Pathology
93-96 Chadwick Road, Astmoor
Runcorn, Cheshire WA7 1PR, UK
Tel: 44-1928-562600
Fax: 44-1928-562627
Labvision.uk@thermofisher.com

Thermo SCIENTIFIC

DATA SHEET
Rev 121508L

Androgen Receptor Ab-1 (Clone AR 441)

Mouse Monoclonal Antibody

Cat. #MS-443-P0, -P1, or -P (0.1ml, 0.5ml, or 1.0ml at 200µg/ml) (Purified Ab with BSA and Azide)
Cat. #MS-443-P1ABX or -PABX (0.1ml or 0.2ml at 1.0mg/ml) (Purified Ab without BSA and Azide)
Cat. #MS-443-B0, -B1, or -B (0.1ml, 0.5ml, or 1.0ml at 200µg/ml) (Biotin-labeled Ab with BSA and Azide)
Cat. #MS-443-R7 (7.0ml) (Ready-to-Use for Immunohistochemical Staining)
Cat. #MS-443-PCS (5 Slides) (Positive Control for Histology)
Cat. #MS-443-PCL (0.1ml) (Positive Control for Western Blot)

Please note this data sheet has been changed effective Dec 15, 2008.

Description: The expression of AR is reportedly inversely correlated with histologic grade i.e. well differentiated prostate tumors show higher expression than the poorly differentiated tumors. In prostate cancer, AR has been proposed as a marker of hormone-responsiveness.

Comments: Ab-1 is excellent for staining of formalin/paraffin tissues.

Mol. Wt. of Antigen: 110kDa

Epitope: aa 299-315

Species Reactivity: Human and Dog. Does not react with mouse. Others-not tested.

Clone Designation: AR 441

Ig Isotype: IgG_1

Immunogen: A synthetic peptide from human AR.

Applications and Suggested Dilutions:

- Gel Supershift (Order Ab at 1mg/ml)
- Immunofluorescence
- Immunoprecipitation (Denatured verified) (Use Protein G; Ab 2µg/mg protein lysate)
- Western Blotting (Ab 1µg/ml for 2hrs at RT)
- Immunohistology (Formalin/paraffin) (Ab 1:25 for 20 min at RT using Lab Vision's UltraVision LP Systems)

* [Staining of formalin-fixed tissues requires boiling tissue sections in 10mM citrate buffer, pH 6.0 for 10-20 min followed by cooling at RT for 20 min.]

* Requires detection with a high-sensitivity detection system such as UltraVision LP (Cat# TL-015-HD).

The optimal dilution for a specific application should be determined by the investigator.

Positive Control: LnCap cells or prostate carcinoma

Cellular Localization: Nuclear

Supplied As:

200µg/ml of antibody purified from ascites fluid by Protein G chromatography. Prepared in 10mM PBS, pH 7.4, with 0.2% BSA and 0.09% sodium azide. Also available without BSA and azide at 1mg/ml,

or

Prediluted antibody which is ready-to-use for staining of formalin-fixed, paraffin-embedded tissues.

Storage and Stability:

Ab with sodium azide is stable for 24 months when stored at 2-8°C. Antibody WITHOUT sodium azide is stable for 36 months when stored at below 0°C.

Suggested References:

1. Ruizeveld de Winter J A, et al. (1991) J Histochem Cytochem 39: 927-936.
2. Chodak G w, et al. (1992) J Urol 147: 798-803.

Limitations and Warranty:

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Thermo Fisher Scientific
Anatomical Pathology
46360 Fremont Blvd.
Fremont, CA 94538, USA
Tel: 1-510-771-1560
Fax: 1-510-771-1570
http://www.thermo.com/labvision

Manufactured by:
NeoMarkers
For
Lab Vision Corporation

EC REP
Thermo Fisher Scientific
Anatomical Pathology
93-96 Chadwick Road, Astmoor
Runcorn, Cheshire WA7 1PR, UK
Tel: 44-1928-562600
Fax: 44-1928-562627
Labvision.uk@thermofisher.com