METASTASIS SUPPRESSOR GENES AND PROTEINS IN NON-MELANOMA SKIN CANCERS

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By

Önder Bozdoğan

August, 2014

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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ABSTRACT

METASTASIS SUPPRESSOR GENES AND PROTEINS

IN NON-MELANOMA SKIN CANCERS

Önder Bozdoğan

Ph.D in Molecular Biology and Genetics

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August 2014

Skin cancers are the most common cancer in human population. They are practically divided into two major group; melanoma and non-melanoma skin cancer (NMSC). NMSC often refers to two common neoplasms; cutaneous squamous cell carcinoma (cSCC) and basal cell carcinoma (BCC). BCCs are slow growing, malignant, significantly invasive but rarely metastasizing carcinomas. cSCCs are the malignant tumor of keratinocytes with significant squamous differentiation. In contrast to BCCs, SCCs have significant metastatic capacity. Metastasis is a complex multistep process and strictly positively or negatively controlled by tens of genes or proteins. Besides supporting genes, a group of gene, called metastasis suppressor genes (MSG), slow or inhibit metastasis without significantly affecting tumorigenicity.

The aim of this study was to find out distribution and importance of the seven selected metastasis suppressor gene/proteins including NM23-H1, NDRG1, E-cadherin, RHOGDI2 (ARHGDIB), CD82/KAI1, MKK4, and AKAP12 in NMSC.

Ninety six BCCs, 32 cSCCs, 6 in-situ SCCs, two cell lines (HaCaT, A-431) were included for immunohistochemistry study. Eleven BCCs, 8 normal skin adjacent to the BCCs, 3 normal skin frozen tissue, and, two cell lines were inserted for qRT-PCR studies. Promoter methylations of *CD82/KAI1* and *MKK4* genes were analyzed in 7 tumors

and 5 normal tissue samples by bisulfite sequencing method.

In immunohistochemistry study, NM23-H1 was protected in NMSC. Similarly, relatively preserved cytoplasmic expressions of NDRG1 were also detected. AKAP12 and RHOGDI2 were decreased in both tumor groups. However, CD82/KAI1 downregulation was only detected in BCCs. E-Cadherin was relatively protected in BCCs but significant lost was seen in cSCCs. Cytoplasmic positivity of MKK4 was more pronounced in cSCC when compared to BCCs. Immunohistochemical study of cell lines showed similar finding as in seen cSCC. In qRT-PCR study, we found significant upregulation of *NM23-H1* (1.4 fold; p=0.032) and downregulation of *AKAP12* (-1.2 fold; p=0.006) when BCC was compared to normal skin. *NDRG1* showed significantly higher levels (2.2 fold, p=0.001) in BCC when compared to the skin adjacent to the BCC. *MKK4* (-2.1-fold, P=0.001), ARHGDIB *(RHOGDI2)* (-4.7-fold, P=0.001), *CD82/KAI1* (-2.4-fold, P=0.001) and *AKAP12* (-9.7-fold, P=0.001) were downregulated but *NDRG1* (34.4-fold, p=0.001) was upregulated in A-431 cell line when compared to HaCaT. CD82/KAI and MKK4 promoters were heavily unmethylated in BCCs and normal skin.

In conclusion, we have demonstrated differential expression patterns for the seven MSPs in NMSCs. In SCCs, the MSG expression signature is similar but not identical to BCCs. The preserved levels of NM23-H1 and NDRG1 may contribute to the non-metastatic features of NMSC.

Key Words: Metastasis suppressor gene, skin cancer, metastasis, NM23-H1, NDRG1, E-cadherin, RHOGDI2, CD82/KAI1, MKK4, AKAP12

ÖZET

MELANOM DIŞI DERİ KANSERLERİNDE

METASTAZ BASKILAYICI GENLER VE PROTEİNLER

Önder Bozdoğan

Moleküler Biyoloji ve Genetik Doktorası

Tez Yöneticisi: Doç.Dr. Işık G. Yuluğ

Ağustos 2014

Deri kanserleri insanlarda en sık görülen kanserlerdir. Pratik olarak melanoma ve melanoma dışı deri kanserleri (MDDK) olmak üzere iki alt gruba ayrılabilir. MDDK sıklıkla bazal hücreli karsinom (BHK) ve deri kökenli skuamoz hücreli karsinomu (dSHK) tanımlar. BHK'lar yavaş büyüyen, malign, invaziv ancak nadiren metastaz yapan tümörlerdir. dSHK'lar ise belirgin skuamoz differansiyasyon gösteren keratinositlerin malign neoplazileridir. BHK'lardan farklı olarak dSHK belirgin metastaz kapasitesine sahiptirler. Metastaz, katı olarak pozitif veya negatif olarak onlarca gen ve proteinle kontrol edilen karmaşık basamaklı bir sürectir. Metastazı destekleyici genlerin yanı sıra metastaz baskılayıcı genler (MBG) adı verilen bir grup gen tümorojeniteyi etkilemeden metastazı yavaşlatır veya durdurur.

Bu çalışmanın amacı NM23-H1, NDRG1, E-cadherin, RHOGDI2 (ARHGDIB), CD82/KAI1, MKK4 ve AKAP12'nin dahil olduğu yedi seçilmiş metastaz baskılayıcı genin/proteinin MDDK' daki önemini araştırmaktır.

İmmunhistokimyasal çalışma için 96 BHK, 32 dSHK, 6 in-situ SHK, iki hücre hattı (HaCaT, A-431) dahil edildi. 11 BHK, 8 tümör komşuluğunda normal deri, 3 normal deri donuk dokuları ve hücre hatları qRT-PCR çalışmasına katıldı. Ayrıca 7 BHK ve 5 normal dokuda *CD82/KAI1* ve *MKK4* genlerine ait promoter metilasyonları bisülfit

sekanslama yöntemiyle analiz edildi.

İmmunhistokimyasal çalışmada, MDDK'larda NM23-H1'in korunduğu izlendi. Göreceli olarak sitoplazmik NDRG1 ekspresyonunun da korunduğu saptandı. Her iki tümor grubunda da AKAP12 ve RHOGDI2 ekspresyonlarının azaldığı görüldü. CD82/KAI düzeylerinin azalması sadece BHK'da saptandı. E-cadherin düzeyi BHK'da göreceli olarak korunurken, belirgin düşme dSHK'da saptandı. MKK4 sitoplazmik ekspresyonu dSHK'da BHK'a göre daha belirgindi. Hücre hatlarını immunhistokimyasal çalışması dSHK'dakine benzer bulgular verdi. Kantitatif eş zamanlı PCR çalışmasında BHK'da normal deri dokusuna göre *NM23-H1* 'de artış (1,4 kat; p=0.032), *AKAP12*'de azalma (-1.2 kat; p=0.006) bulduk. *NDRG1*'de komşuluktaki deriye göre BHK'da artış (2.2 kat, p=0.001) saptandı. HaCaT hücre hattına göre A-431'de *MKK4* (-2.1 kat, P=0.001), ARHGDIB *(RHOGDI2)* (-4.7 kat, P=0.001), *CD82/KAI1* (-2.4 kat, P=0.001) ve *AKAP12*'de (-9.7 kat, P=0.001) azalma, *NDRG1*'de ise (34.4 kat, p=0.001) artış bulundu. Promotor metilasyon araştırmasında *CD82/KAI1* ve *MKK4* genlerinde metilasyon saptanmadı.

Sonuç olarak çalışılan yedi MBP/G ile MDDK'da farklı ekspresyon örüntüleri saptadık. SHK'da MBG ekspresyonu BHK'a benzemekle birlikte, farklılıklar da göstermektedir. NM23-H1 ve NDRG1 ekspresyonlarının korunması, MDDK'da metastazın önlenmesinde katkısı olabilir.

Anahtar sözcükler: Metastaz baskılayıcı genler, deri kanseri, metastaz, NM23-H1, NDRG1, E-cadherin, RHOGDI2, CD82/KAI1, MKK4, AKAP12

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ABBREVIATIONS

A-BCC	Aggressive Basal Cell Carcinoma
AK	Actinic Keratoses
AKAP12	A Kinase (PRKA) Anchor Protein 12, Gravin
bp	Base Pairs
BCC	Basal Cell Carcinoma
BCNS	Basal Cell Nevus Syndrome
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
cSCC	Cutaneous Squamous Cell Carcinoma
-cyt	Cytoplasmic Staining
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial–Mesenchymal Transition
FBS	Fetal Bovine Serum
Fig.	Figure
HPV	Human Papilloma Virus
HSCORE	Immunohistochemical Histological Score
IS-SCC	In-situ Cutaneous Squamous Cell Carcinoma
KAI1	Kangai 1
kg	Kilogram
m	Meter
MEK4/ MKK4	Mitogen-Activated Protein Kinase Kinase 4
mg	Milligram
min	Minute
ml	Milliliter
mRNA	Messenger Ribonucleic Acid
MSG	Metastasis Suppressor Gene
MSP	Metastasis Suppressor Protein
µg	Microgram
µl	Microliter
µm	Micrometer
N	Normal Non-Lesional Skin
NE- BCC	Normal Epidermis Adjacent To Basal Cell Carcinoma
NE-SCC	Normal Epidermis Adjacent To Squamous Cell Carcinoma
NA-BCC	Non-aggressive Basal Cell Carcinoma

NDRG1	N-Myc Downstream Regulated 1
NM23-H1	Nucleoside Diphosphate Kinase 1
NMSC	Non-melanoma Skin Cancer
-nuc	Nuclear Staining
Oligo(dT)	Oligodeoxythymidylic Acid
PCR	Polymerase Chain Reaction
PTCH	Patched Homolog
PUVA	Photochemotherapy
qRT-PCR	Quantitative Real Time Reverse Transcription Polymerase Chain Reaction
RHOGDI2	Rho GDP Dissociation Inhibitor Beta
RNA	Ribonucleic Acid
Rpm	Revolutions Per Minute
RT PCR	Reverse Transcription Polymerase Chain Reaction
Str.	Stratum
SUFU	*Suppressor of fused*
TAM	Tumor-associated Macrophages
UV	Ultra Violet

CHAPTER 1- INTRODUCTION

1.1. Skin, Function and Histology.

Skin is the largest organ of the human body covering the exterior of the whole human body [1]. It weights approximately 3-5 kg and approaches 2 m^2 in an adult human [1, 2]. Main function of the skin is to provide a barrier for environment. However, it has also important roles in thermoregulation, synthesizing important products (vitamin D), cushioning the trauma and physiological and sociological wellness [1, 3].

Skin is composed of three histologically and functionally different layers; *epidermis, dermis and subcutaneous tissue* **(Fig.1.1)** [3]. Epidermis is a stratified squamous epithelium and the main cell type is called keratinocyte. However, other cells types, melanocytes, Langerhans cells, Merkel cells, and free nerve axons are also found in the epidermis [3]. Histologically four well defined layers of epidermis can be determined.

- Basal cell layer **(stratum basale)**
- Prickle cell or squamous layer **(stratum spinosum)**
- Granular cell layer **(stratum granulosum)**
- Keratin or cornified layer **(stratum corneum)** [1].

Basal cell layer is composed of cuboidal or columnar cells with basophilic cytoplasms [3]. This layer is often mitotically active and contains also melanocytes and Merkel cells [1, 2]. The cells in the prickle layer are polygonal with wide abundant eosinophilic cytoplasms and have oval nucleus and conspicuous nucleoli.

Langerhans cells are located at the mid and at the upper parts of this layer [3]. Granular layer is composed of 3-5 layer of flattened keratinocytes with basophilic granular cytoplasms consists of keratohyaline protein [2]. Stratum corneum is the uppermost layer of epidermis and composed of anucleated eosinophilic keratinocytes [3]. An eosinophilic acellular keratinous layer (stratum lucidum) may be recognized between str. granulosum and str. corneum in palm and soles [1].

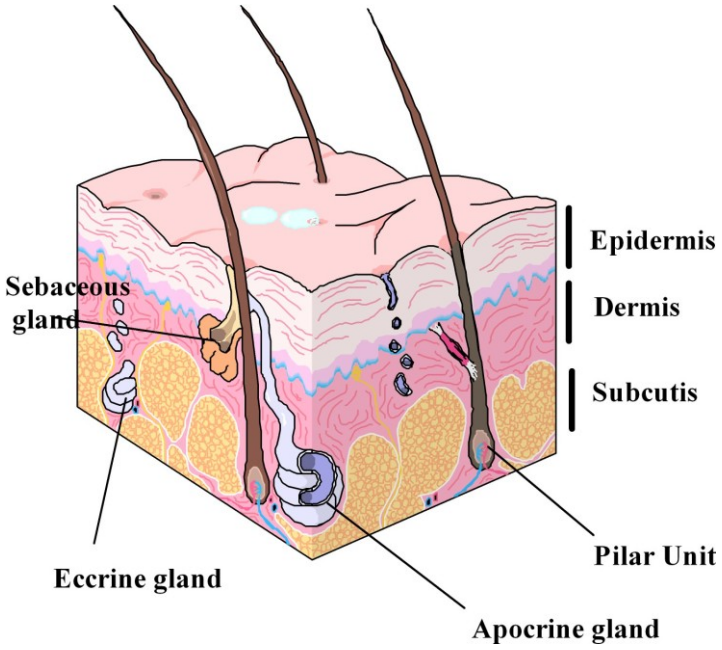


Fig. 1. 1. Microanatomy of the normal skin. **The figure is created by the author helping by the references 1-5**

There are different types of skin adnexa or appendages, distribute in connective tissue of the dermis or subcutis, include pilosebaceous unit and sweet glands [4]. Sweet glands in the human skin are generally divided into two major types; Eccrine and apocrine glands [2]. Eccrine glands are simple coiled glands distributes in many areas of the skin and they are mainly responsible for the thermoregulation of the human body [1]. Apocrine glands are generally limited to axillae, groin, external auditory canal, eyelid and areola of the breasts [2]. Apocrine glands secrete their

products by decapitation; simply apical cytoplasms fell off into lumen [4]. Apocrine glands are connected to pilosebaceous unit and open into the infundibulum of the hair follicle [3, 4]. The main function of apocrine glands is not known in the human, but they are responsible for production of the body scent and probably help sexual attraction in other mammals [1].

Pilosebaceous unit includes hair, hair follicle, sebaceous gland and piloerector muscle [3]. These units are distributed whole skin except palms and soles and a part of genital skin [4]. The hair follicle divided into three different segments; *infundibulum, isthmus, and the inferior segment* [3]. Infundibulum is an area between opening of the follicle and sebaceous gland opening, and isthmus is between sebaceous gland opening and piloerector muscle insertion [3]. The inferior segment includes papilla which is responsible for hair growth [4]. Sebaceous glands are holocrine glands open to pilar follicle and empty their secretion. However, a group of sebaceous glands opens directly to surface located at areola, eyelids and vermilion border of lips [4].

Dermis mainly composed of connective tissue, blood vessels, nerves and skin adnexa. Dermal connective tissue has significant amount of collagen and elastic fibers which are responsible tensile strength of the skin [5]. Dermis can be divided two different zones*; papillary dermis and reticular dermis* [3]. Papillary dermis is below the dermoepidermal junction and composed of lose thin connective tissue network of collagen I and III [3, 4]. The papillary dermis forms conic structures called dermal papilla which interdigitate with epidermal rete ridges. [4] Different

from papillary dermis reticular dermis has more thick compact bundles of collagen fibers basically composed of collagen I [3].

Subcutaneous tissue is located under the dermis and composed of mature fat tissues which are divided into lobules with vascular connective tissue septa [1]. Subcutaneous tissue has important functions including thermo regulation, insulation and cushioning the mechanical injuries [3].

1.2. Skin Carcinomas

Malignant skin tumors are the most common malign human neoplasms and an important part of daily medical practice [6-9]. Because of their frequency and increasing incidence, these neoplasms pose important medical, economical, and social problems of healthcare services worldwide [6, 8, 10]. Despite established detailed classification schemas for skin cancers, practically they are separated as two different groups, melanoma and non-melanoma skin cancer (NMSC) [11]. Although there are other types of NMSC including skin adnexal tumors, soft tissue tumors and lymphomas, this term commonly refers to two common neoplasms; cutaneous squamous cell carcinoma (cSCC) and basal cell carcinoma (BCC) [7]. BCCs are more commonly seen and are at least 70% of diagnosed of NMSC [11]. The incidence of NMSC changes due to geographic localization and race. The incidence is estimated more than 1000/100 000 person-per year in Australia, however it shows more very lower rates in some part of Africa less than 1/100 000 [6]. In Germany, NMSC age standardized incidence rates were 100.2 and 72.6 in 100 000 men and women, respectively [12]. Similar data were came from Italy with an

incidence rate of 87.9 for BCC and 28.9 for SCC per 100 000 people [13]. Based on the data of Turkish Health Minister Reports (2005), skin carcinoma is the third common carcinoma and the incidence of is 18.91/100 000 person per year [14]. The incidence in Turkey is probably higher when unregistered patients are taken into account.

1.2.1. Basal Cell Carcinoma

1.2.1.1. Clinical Features

Basal cell carcinomas (BCCs) are slow growing, malignant, but rarely metastasizing carcinomas and usually seen on sun exposed areas particularly head and neck of the elderly persons [15, 16]. In large published series, the mean age of the patients is sixth or seventh decade. [17, 18] Although BCCSs are usually seen at elderly, the age range is very wide; between second to ninth decade [17, 18]. Males are slightly more affected than women [1, 17, 18]. Besides detected on sun exposed skin areas, rarely BCCs may be seen on non-sun exposed area including vulva [19].

The clinical appearances of BCCs are closely related to histopathological subtype. Clinically, the lesions may show nodular and/or ulcerative, diffuse, superficial (multifocal) and pigmented appearances [1]. Nodular BCCs represent well defined slow growing waxy nodules or papules sometimes with telangiectasias and ulceration [20, 21]. Superficial BCCs are seen as an erythematous elevated plaque or macule different color or hue from surrounding skin [21]. Superficial BCCs have a predilection to trunk than the other subtypes. Infiltrative types represent as a

plaque with ill–defined borders [20]. This type sometimes looks like a scar tissue and clinical diagnosis may be difficult [21]. BCCs are usually asymptomatic but pain may be rarely only symptom [16].

1.2.1.1. Etiology and Pathogenesis

The etiology of BCCs is shown to be related to multiple factors [22]. Ultra violet (UV) radiation is a well known environmental factor contributes to the pathogenesis of BCCs [8, 23]. UV radiation causes characteristic covalent bonds between adjacent pyrimidines and generates cyclopyrimidine dimers (TT) and/or pyrimidine-pyrimidine (6-4) adducts [8]. UVB is probably the major participant and more mutagenic than UVA [8, 22]. Besides UV radiation, a group of etiological factors are described for BCCs including; Human papilloma virus (HPV), immunosuppression, non-Hodgkin lymphoma, PUVA therapy, photosensitizing drugs, ionizing radiation, occupational factors, arsenic, burns and scars [8, 22].

BCCs may be related to a group of familial inherited syndromes. One of well known, **Basal Cell Nevus Syndrome (BCNS)**, also named as **Gorlin Syndrome** or **Gorlin–Goltz Syndrome** is characterized by multiple BCCs in early ages [24, 25]. Besides early and multiple onset of BCCs; keratocysts of jaw, palmoplantar pits, skeletal anomalies, medulloblastomas, fibromas and calcification of falx cerebri may be seen [26, 27]. Basal cell nevus syndrome (BCNS) is a relatively common autosomal-dominant condition, and caused by several mutations in the sonic hedgehog pathway [28]. The Hedgehog pathway includes several molecular components including, ligands (sonic, Indian, desert, hedgehog proteins), receptor

(PTCH1, PTCH2), signal transducer (smoothened), and transcription factors (Gli proteins) [29]. This complex pathway is activated when ligands bind to PTCH receptor. PTCH receptor releases bounded SMO to signal downstream. Eventually Gli proteins act as a transcription factor for activating related genes **(Fig. 1.2)** [29]. The most common affected gene/protein in BCNS is *PTCH1* (9q22.3) [28]. The others are *PTCH2* and *SUFU* in this pathway [30, 31]. *PTCH* genes act as a tumor suppressor and have some important regressive roles in cell growth and differentiation [32]. The other responsible gene *Suppressor of fused (SUFU)* codes a negative regulator of the Sonic Hedgehog pathway [33]. It has been showed that significant numbers of sporadic BCCs share the same irregularities as seen in BCNS [34, 35]. After the molecular mechanism background of BCCs was established, the new therapy strategies opened, such as SMO inhibitors [29].

Besides BCNS, the other syndromes related to BCCs are Rombo syndrome, Bazex–Dupre–Christol syndrome, Multiple Hereditary Infundibulocystic Basal Cell Carcinoma syndrome, and Xeroderma Pigmentosum [25, 36-38]. Furthermore, BCCs are also an ancillary feature in other different cutaneous syndromes [27].

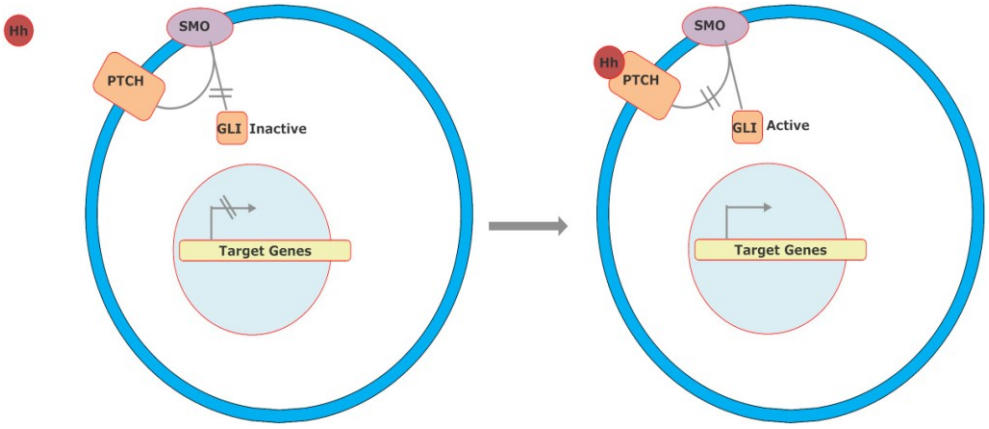


Fig. 1.2. Simplified Hedgehog signal pathway. Without ligand PTCH inhibits SMO. After ligand binds to PTCH, It releases SMO and GLI activates. GLI translocates into the nucleus and induces target gene transcription. **The figure is created by the author helping by the references 28, 29, 32.**

1.2.1.3. Histopathology

Histopathologically, these tumors are classified into several distinct morphological types but a significant percentage of mixed morphology BCCs may be seen in daily practice [39]. Classically, BCCs are classified as superficial, nodular, infiltrative (with or without sclerotic-morpheiform stroma), and micronodular subtypes **(Table 1.1)** [15, 40]. Basosquamous cell carcinoma and metatypical BCCs are controversial issues and it has been generally thought that these tumors are somewhere between BCCs and SCCs [1, 40]. All subtypes are basically formed of small cells with scant cytoplasm and hyperchromatic nucleus **(Fig. 1.3)** [40]. Besides the subtypes described above; there are also rare variants including divergent adnexal differentiation [1, 41].

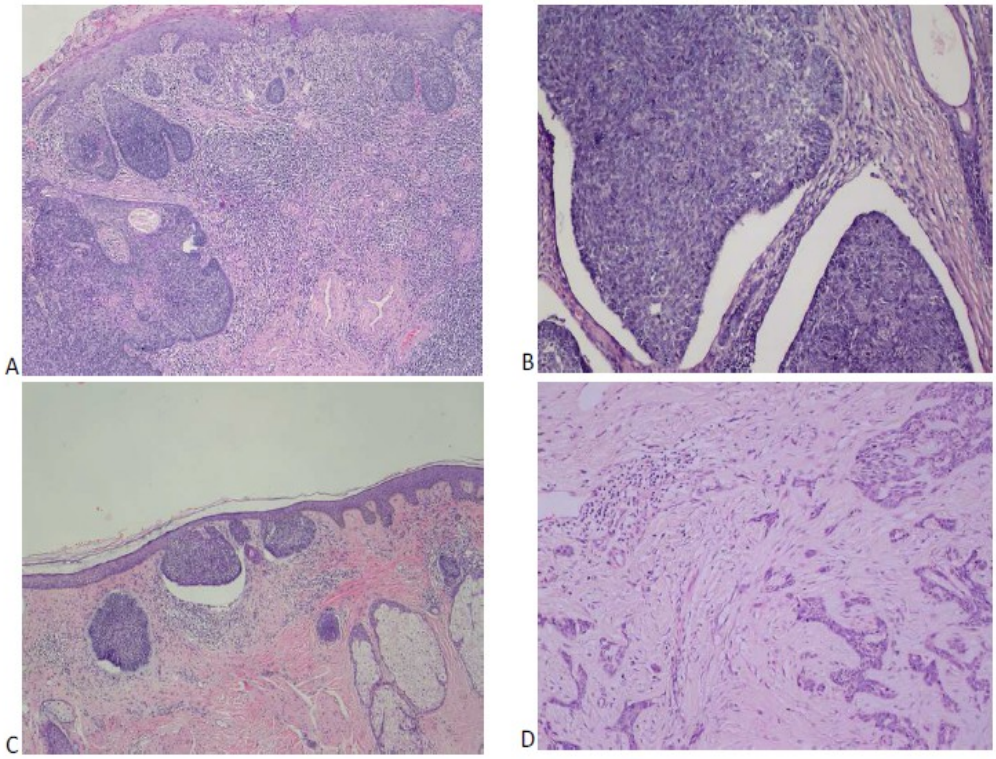


Fig. 1.3. Microscopic pictures of different types of basal cell carcinoma. Typical nodules of basaloid cells are scattered in skin tissue in nodular type BCCs (A; B).An important diagnostic feature of separation artifact is clearly seen (B). Multifocal, small basaloid nodules are attached to epidermis in superficial BCC (C). Infiltrated basaloid neoplastic cells groups are separated by collageneous stroma in infiltrative type (D). (A, C, D, x40; B, x100)

Nodular BCCs are generally thought to be the most common subtype. Histologically, they are composed of different size basaloid nodules with peripheral palisading **(Fig. 1.3.A, B)**. There are also clefts between the stroma and the tumor **(Fig.1.3.B)**. The stroma often contains mucin and is stained blue-grey by H&E [1, 15, 40].

Superficial BCCs are more commonly seen on the trunk and the extremities than the other subtypes, however at least 40% of them are seen on the head and neck area.[40] Histopathological examination reveals small multiple buds and nodules, composed of small basaloid cells, which are attached to the atrophic epidermis **(Fig. 1.3.C)** [15, 40]. Superficial BCCs usually stay in the papillary dermis for a long time and usually do not invade the reticular dermis [15].

Infiltrative BCC consists of invasive cords and strands of basaloid cells with a different type of stroma. A group of infiltrative BCCs, that show significant collagen deposition, is called morpheiform, sclerotic or fibrosing BCC **(Fig. 1.3.D)** [15].

Micronodular BCCs are a relatively new recognized variant of BCC [42]. Although tumor nodules are seen as in the nodular type, they are very small, approximately near the size of hair bulb, and peripheral palisading is less obvious [40, 43]. There is usually no connection to the epidermis [43].

Table 1.1. Classification of Basal Cell carcinoma according to WHO (World Health Organization) classification [44].

• Superficial basal cell carcinoma
• Nodular (solid) basal cell carcinoma
• Micronodular basal cell carcinoma
• Infiltrating basal cell carcinoma
• Fibroepithelial basal cell carcinoma
• Basal cell carcinoma with adnexal differentiation
• Basosquamous carcinoma
• Keratotic basal cell carcinoma
• Other variants

1.1.1.4. Aggressive-Non-aggressive Basal Cell Carcinoma

BCCs have significant invasion capacity but rarely metastase [45]. The estimated metastasis incidence is very low, between 0.0028% and 0.55% [45, 46]. However, there is no adequate hypothesis to explain why this carcinoma cannot metastasize. Since metastasis is so rare, the clinically important point of morbidity is the recurrence of the tumor. The recurrence rate is not easily estimated due to the various factors including the surgical margin, the type of surgery (Mohs surgery or classical excision), nonsurgical therapies, morphology and the subtype of BCCs. The recurrence rate of primer BCCs after surgical excision is estimated to be less than 5% [47]. Following Mohs micrographic surgery, the recurrence rates in the five-year period are reported to be between zero and 6.5% for a primary tumor, and between 2.9% and 12% for incomplete excised BCCs [48, 49].

From the clinicopathological point of view, BCCs may be practically separated into two groups including high risk (aggressive) and low risk (non-aggressive) [50-52]. Clinically possible aggressive features are large tumors (2 cm <); facial location, especially the midline of the face, periocular area, nose, and ears; and neglected tumors [15, 53]. Histopathological subtype of BCCs, perineural and vascular space invasion, and positivity of the surgical margins are also important for recurrence of the tumor [15, 54, 55] Infiltrative, micronodular and basosquamous types could be classified as aggressive BCCs with a significant recurrence rate [15, 53]. Although nodular and superficial BCCs are generally located in the low-risk group, finding the exact surgical margin in surgical specimens may be difficult for superficial BCCs [15, 20].

1.2.2. Squamous Cell Carcinoma

1.2.2.1. Clinical Features

Cutaneous squamous cell carcinoma is the malignant tumor of keratinocytes with significant squamous differentiation [11]. Squamous cell carcinoma (cSCC) is the second most common skin cancer after BCC [56]. cSCCs are generally seen in the elderly but they may also be detected in the younger age group [40]. Similar to BCCs, cSCCs develop with several inherited conditions including Xeroderma pigmentosa, Albinism, Dystrophic Epidermolysis Bullosa, Rothmund–Thomson syndrome and Epidermodysplasia Verruciformis [1, 57]. In contrast to BCCs, SCCs have significant metastatic capacity [56]. The percentage of metastasis is described

between 1 and 9.9% in the literature [58, 59]. These varying rates are probably due to data from different medical clinics or the inclusion of special locations such as the lip, anal and vulval area into the case series [56]. It is well established that a group of clinical and pathological features are associated with high risk [60, 61]. Thick SCCs, localization, tumor size, tumor differentiation, histological subtypes, perineural invasion, immunosuppression, DNA ploidy or aneuploidy, and high proliferation antigen expression are thought to be important risk factors [56, 60].

There are two distinct clinicopathological types of precancerous or preinvasive lesions; ***actinic keratoses and in-situ carcinoma (Bowen disease).*** Actinic keratoses (AKs) are common skin lesions, which are generally accepted as a precancerous lesion for cSCCs [62]. AKs present as flesh-colored scaly macules and plaques, sometimes with hyperkeratosis. Erythematous and pigmented lesions may occur [63]. The transformation rates of AK to cSCCs are reported as 0.075% and 20% per one-year period [62, 64-66].

In-situ SCCs (IS-SCC) are seen in the skin as in other mucosal areas. Although Bowen disease is often used as synonym of in situ SCCs, the usage of the last term is more suitable [67]. IS-SCCs are generally seen on all skin areas but have a predilection for sun-exposed areas of the head and neck, and the hands They are often characterized as well-delineated, erythematous macules or plaques. They may sometimes be pigmented, especially at genital areas [1, 68].

Classical invasive cSCCs show a spectrum of clinical findings. Early invasive cSCCs are flat or elevated, usually skin colored and scaly lesions similar to actinic keratoses [69]. However, well-developed cSCCs represent well-delineated erythematous

papules, nodules or plaques; and sometimes have a verrucous appearance [69, 70]. Advanced lesions may show significant hyperkeratosis, central ulceration, and bleeding [69].

1.2.2.2. Etiology and Pathogenesis

cSCCs share nearly the same etiological factors as described for BCCs above[8]. Tobacco usage has been described as a risk factor for only SCC and not BCC, but this is not supported with new epidemiological studies when lip SCCs are excluded [71, 72].

Although it is not clear that cSCCs shows similar multistep carcinogenesis as in cervical carcinomas, there are important clues [73]. It is generally accepted that tumor suppressor p53 inactivation mutation is probably the first step of the carcinogenesis [74, 75]. It has been reported that p53 mutations were detected with high percentage (74%) in sun-exposed normal skin when compared to the mutation rate (5%) in non-sun exposed skin [76]. p53 mutations are also detected with high percentages in actinic keratoses and in cSCCs [74]. In cutaneous carcinogenesis beside inactivation mutations, p53 levels may also be regulated by activation or upregulation of several tyrosine kinases including EGFR [73, 77]. These kinases down-regulate p53 by a c-JUN-dependent mechanism [73]. Similar to p53, p14 and p16 (CDKN2A locus) genes are downregulated in cSCCs by mutation or epigenetic mechanisms [78, 79]. There are also important clues for a role of the RAS activating pathway in the oncogenesis of cSCCs. However, the activating RAS

mutation rate is not high (21%) in human cSCCs [73, 80]. RAS activation probably takes place by indirect mechanisms such as the EGFR-related pathway [81].

1.2.2.3. Histopathology

AKs are separated into various clinicopathological subtypes including *Acantholytic, Pigmented, Bowenoid, Atrophic, and Hypertrophic AKs* [62]. All types of AKs except Bowenoid AKs include atypical keratinocytes, mainly at the basal epidermal levels, with orto, hyper and parakeratosis [82]. Bowenoid AKs show full thickness keratinocytic atypia. However, they usually have less significant atypia and cellular crowding than IS-SCCs [62, 68]. The acantholytic subtype represents discohesion of atypical keratinocytes at different levels of the epidermis [62].

IS-SCC represents hyperkeratosis, parakeratosis, acanthosis, and full thickness atypical keratinocytes with mitoses, and loss of maturation and polarity **(Fig.1.4.A, B)** [68, 69]. The basal epidermal layer is usually spared and they show more significant atypia than in Bowenoid actinic keratoses [68].

Invasive cSSCs can have various histopathology appearances due to the invasion level, grade and subtype. Early invasive cSCCs are similar in morphology to AK or IS-SCC with focal invasive areas. Well-differentiated cSCCs consist of squamous nests and islands with significant keratotic areas named “keratin pearls” **(Fig. 1.4.C)** [62]. In moderately differentiated cSCC, keratin is also detectable but cells show more hyperchromasia and mitosis **(Fig. 1.4.D)** [62, 69]. Poorly differentiated cSCCs show scant keratinization, significant atypia and are generally more deeply invasive [69].

Besides classical cSCCs, several different morphological variants have been described. However, most of cSCC variants demonstrate little significance for prognosis and therapy **(Table 1.2)** [68].

Acantholytic squamous cell carcinoma is a rare variant of cSCCs, and characterized by acantholysis and pseudoglandular appearance. Though controversial, this subtype is usually considered as an aggressive variant of cSCCs **(Fig. 1.4. E)** [83]. Verrucous squamous cell carcinoma is a low-grade specific variant of SCC most commonly seen skin, mucosal surfaces and genitalia. They consist of verrucous architecture of well-differentiated squamous cells with little atypia [84]. Spindle cell cSCCs are composed of spindle or pleomorphic cells usually with no keratinization, similar to spindle cell sarcomas, and immunohistochemistry may be needed to differentiate them **(Fig. 1.4.F)** [85].

Adenosquamous carcinoma, as the name implies, consists of squamous and adenocarcinoma areas in the same tumor.[88] It is often considered to be a high-risk cSCC [86].

Besides the subtypes classified by WHO, other types of morphological variants have been described [86, 87].

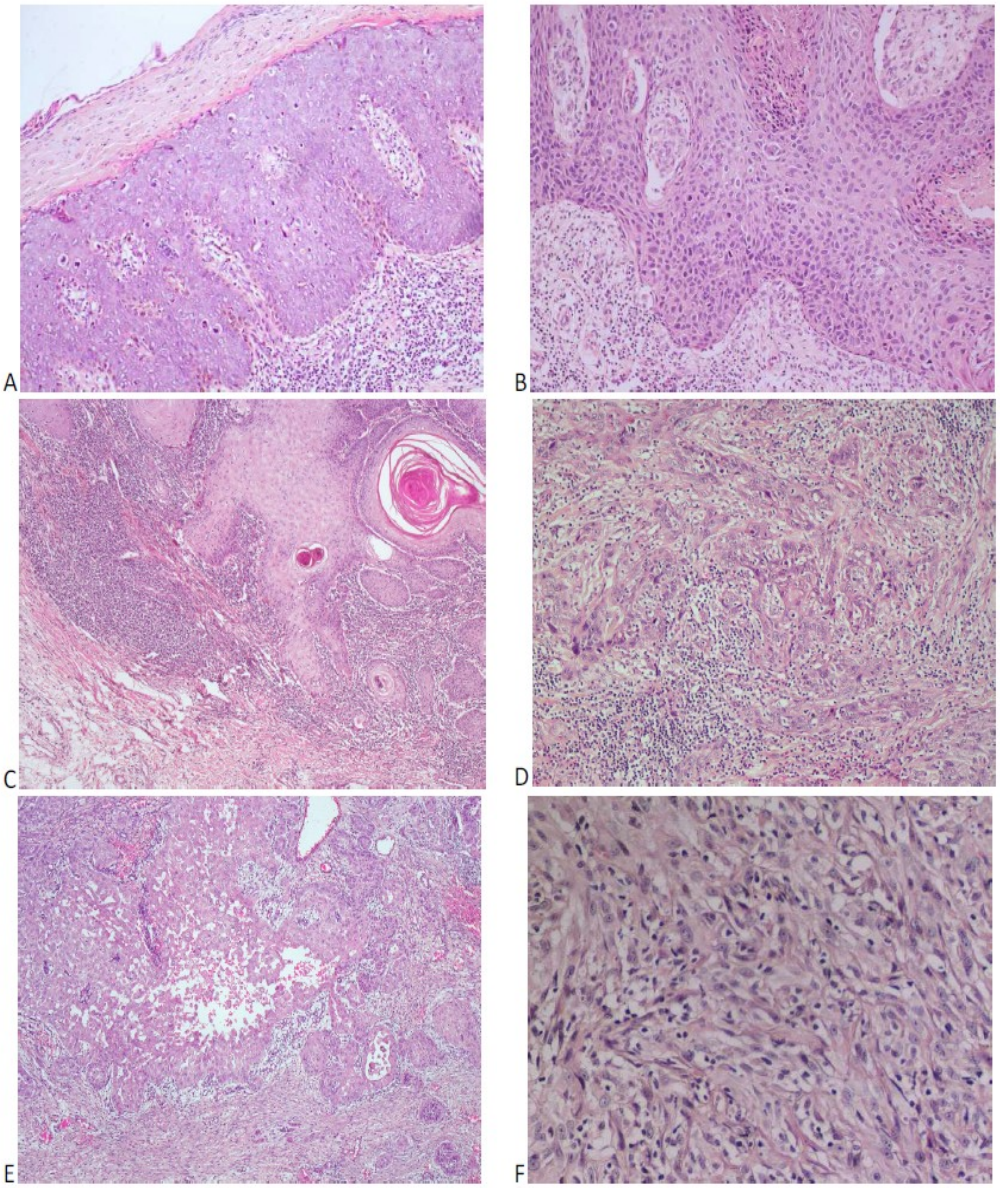


Fig 1.4. Histopathologic appearances of the cutaneous squamous cell carcinoma (cSCC). In-situ SCCs show lost of maturation, significant atypia and mitoses (A, B). Classical well differentiated squamous cell carcinoma is composed of invasive squamous cell islands with keratin pearls (C).However moderately differentiated cSCC is more invasive and less differentiated (D). Acantholytic cSCC shows pseudoglandular features and acantholysis (E). Spindle cell tumor with no significant differentiated morphologic features. This tumor is immunohistochemically positive for cytokeratins (Spindle cell cSCC) (F). (A, C, D, E, x40; B, D, x100, F, x200)

Table 1.2. Classification of squamous cell carcinoma according to WHO (World Health Organization) classification and as described by Cassarino et al.[44, 86, 87]

WHO	Cassarino et al.
• Acantholytic squamous cell carcinoma • Spindle-cell squamous cell carcinoma • Verrucous squamous cell carcinoma • Pseudovascular squamous cell carcinoma • Adenosquamous carcinoma	• Clear cell squamous cell carcinoma • Acantholytic (adenoid) squamous cell carcinoma • Signet ring cell squamous cell carcinoma • Papillary squamous cell carcinoma • Pigmented squamous cell carcinoma • Follicular squamous cell carcinoma • Squamous cell carcinoma arising from adnexal cysts • Squamoid eccrine ductal carcinoma • Invasive Bowen's disease • Malignant proliferating pilar tumor • Desmoplastic squamous cell carcinoma • Squamous cell carcinoma arising in chronic conditions • Radiation-induced squamous cell carcinoma • Lymphoepithelioma-like carcinoma • Squamous cell carcinoma arising in actinic Keratosis • Tricholemmal carcinoma

Classically, cSCCs are graded by Broders' system: However, this system is complicated and not easily used. The classical textbook ***McKee's Pathology of the Skin*** offers a simple three-tiered grading system: Well-differentiated, moderately differentiated, and poorly differentiated squamous cell carcinoma. The fourth group which includes anaplastic or indifferantiated carcinoma may be added [1]. This last grading system is more commonly used in daily practice.

1.3. Metastasis

Metastasis is a complex multistep process briefly describes as spread of a disease (commonly a tumor) to discontinuous nearby tissues or distant organ/tissue [89, 90]

In the metastasis process, spread of tumor may occur through several pathways; *direct seeding of body cavities, lymphatic spread, and hematogenous spread* [91]. Historically, the first accepted hypothesis about metastasis was emphasized by Paget S. (1889). He described in this hypothesis that cancer cells (seed) migrate and grow in a suitable biochemical and biological environment (soil) [92]. After Paget's description of "seed and soil hypothesis", there was an extraordinary effort to control metastasis in the basic and clinical science area [90, 92]. Today, we learn that metastasis is a very complex and multistage process [93]. Furthermore this process is very important in determining for prognosis of an oncology patient. Despite better therapy options in controlling local cancer, investigators focus on systemic metastatic disease because of its fatal progress [89].

1.3.1. Multistep Metastasizing Process

The data from experimental and clinical studies support that metastasis is a multistep process [89]. The tumor cells need a group of genetic and epigenetic changes to regulate this complex multistep process **(Fig. 1.5)** [91]. The main stages of metastasis are categorized briefly as:

- Detachment from main tumor.

- Invasion

- Intravasation

- Transport by blood or lymph

- Arrest

- Extravasation

- Metastatic colonization

- Proliferation.

- Micrometastasis

- Macrometastasis [56, 94].

The first step in the metastatic cascade is **detachment of tumor cells** from primary tumor mass. The tumor cells then invade the extracellular matrix, called the **invasion** step [91]. The invasion and detachment steps need significant changes in tumor cell morphology and biology [90]. These steps are regulated by an important and complex process called **Epithelial–mesenchymal transition (EMT)** [95]. EMT was initially described for fetal development and wound healing but now it has been thought that it is also a very important mechanism for tumor progress and spread [96]. It is well known that epithelial cells usually have polarized organization and significant junctions to other cells and matrix proteins [90]. During the EMT process, epithelial cancer cells lose their polarity and cell-cell adhesion and acquire mesenchymal characteristics which are required for detachment, invasion and metastasis [96, 97]. In the invasion step, the cancer cells change their morphology, gain spindle cell morphology, and look like fibroblasts [90].

There are important clues that the downregulation of E-Cadherin, an important metastasis suppressor protein (MSP), by several pathways, triggers the EMT process. Promoter methylation of the gene or by E-cadherin repressors including SNAIL and SLUG are probably the reasons of E-cadherin downregulation at the early steps of the tumor progression [98, 99]. Loss of E-cadherin expression provides a significant chance for tumor cells to dissociate from the primary tumor mass [91].

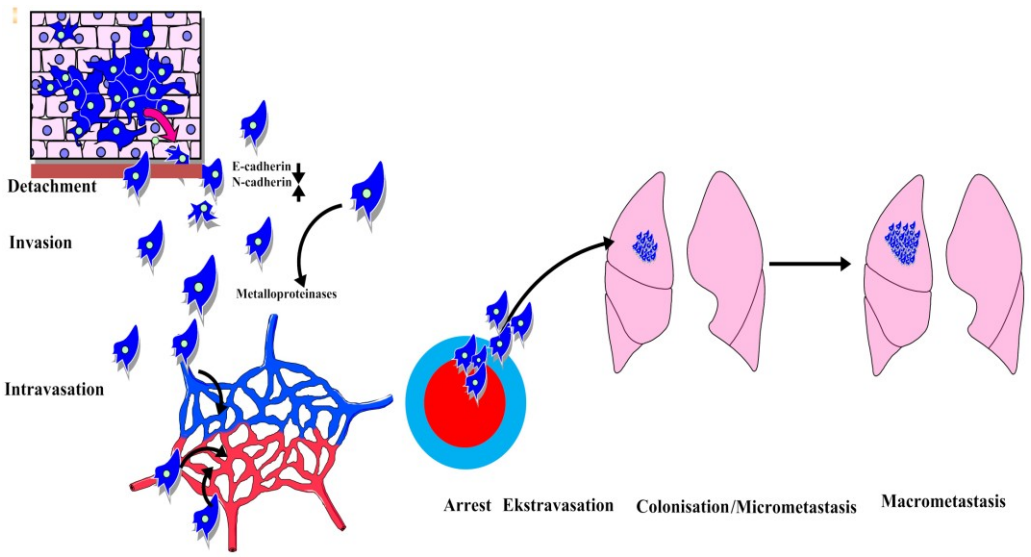


Fig. 1.5. Steps of classical metastasis process.

Other adhesion molecules, such as NCAM, DCC, CEACAM1, Mel-CAM, are also down regulated in specific tumor types [90]. Despite downregulation of E-cadherin and other adhesion molecules, there are important clues that another cadherin, N-cadherin, is upregulated and positively controls the EMT process [100, 101]. Besides N-cadherin, vimentin is also upregulated and it is thought to be a marker of EMT [96]. After the dissociation process, tumor cells release proteolitic enzymes and also induce stromal cells for secreting [91]. At this point, tumor-associated macrophages (TAM) and other inflammatory cells have important roles for supporting the invasion step [102, 103]. One of the supportive roles of TAMs is to secrete proteolitic enzymes [104]. These enzymes such as matrix metalloproteinases may degrade the extracellular matrix and create a way through [91]. Cell motility is generally realized by polymerization and depolymerization of the actin cytoskeleton [90].

When malignant tumor cells approach the intravascular area, the circulating surviving tumor cells may show a tendency for tropism to some tissues [105]. Though millions of tumor cells enter the bloodstream, only a very small fraction can survive due to mechanical trauma and immune cells [106]. Organ tropism is well defined for metastatic human tumors but the exact mechanism is not very clear [107]. Probably intrinsic features of the tumor cells and the microenvironmental factors of target tissue determine the organ specific metastasis [108]. When the metastatic tumor cells reach the target tissue extravasation occurs. Two types of tumor arrest can be described. In nonspecific arrest, tumor cells stick in the capillaries because of their size. The other arrest type is specific to the interaction between tumor membrane protein (Selectins) and the target organ capillaries [106]. The tumor cells lose their mesenchymal characters and gain epithelial features similar to the primary tumor (mesenchymal epithelial transmission). As a result, a new colony is established [91, 97]. Colonization is regulated by close interactions of tumor cells and the microenvironment [106].

1.3.1. New Approaches

Besides classical multistep sequential approach, some observation pointed out that all metastasis is not differentiated and not similar to primary tumor [109]. It has been postulated that there are two major types of metastasis (Brabletz); *Type 1 plasticity metastasis, and Type 2 genetic type metastasis* [97]. Type one metastasis shows differentiated morphology. This type metastasis is probably regulated by reversible genetic alternation (Epigenetic mechanisms). However, type 2 metastasis

has undifferentiated phenotype and is usually related of fixed accumulated genetic alternations [97, 109].

From classic point of view, metastasis is a late event in oncogenesis and tumor cells need to acquire a group of genetic and epigenetic changes with time needed for surviving and proliferating at distant size. This classical hypothesis is now called **linear model of metastasis** [110]. However, there are also clues that cancer cells disseminate at very early stages of tumor progression even at precancerous lesions and proliferate parallel to primary tumor. This fact is called **parallel model of metastasis** [110, 111]. Probably both of the models are reliable [111].

1.4. Metastasis Related Genes.

The multistep and complex metastasis process is strictly positively and negatively controlled by tens of genes or proteins [56, 93, 112]. The genes and proteins supporting metastasis are well known and have been studied extensively [112, 113]. According to Nguyen et al, the metastasis related genes are divided into three groups; metastasis initiation, metastasis progression, and metastasis virulence genes [113].

Metastasis initiation genes support and modulate basically invasion step [114]. EMT related genes *TWiST1, SNAi1* and *SluG,* and other genes modulate invasion and angiogenesis are thought to be in this group [107, 115].

Metastasis progression genes code proteins for primer tumor growth and also modulate extravasation, survival and re-initiation and colonization at distant sites [113]. *PTGS2, EREG, MMP1, LOX, ANGPTL4, CCL5* may be given examples [115].

Metastasis virulence genes express at specific metastasis sites, e.g. bone, and help survival of the cancer cells at a specific microenvironment [113]. One special gene coded parathyroid hormone-related protein (pTHRp) helps to establish osteolytic metastasis in bone [115].

A group of proteins specifically inhibits metastasis is called as **metastasis suppressor proteins**. Literally, a **metastasis suppressor** is a protein that acts to slow or prevent metastases from spreading in the body of an organism with cancer [116, 117]. However, these proteins are different from ones that act to suppress tumor growth and they suppress development of metastasis without significantly affecting tumorigenicity [117, 118].

Metastasis suppressor genes or proteins open a new approach and a study area at metastasis research, and give hope to clinical therapy. NM23-H1 is first described in 1988, and a prototype of MSGs [119, 120]. Nowadays, approximately, thirty genes/proteins are described as MSG, however numbers are not exact and different from one review to another [105, 121].

Pure MSGs would suppress metastases but have no effect on tumorigenicity (proliferation) according to their definition [116, 121]. However, in the real world, MSPs have also other important roles in cell functions.[122] MSG-coded proteins have a diverse range of biomedical activities [121, 122]. They play various roles in cell functions for instance: cell surface signaling (CD82/KAI1), cellular signaling

(MKK4), transcriptional regulation (BRMS1). MSGs also affect different metastasis steps. They inhibit tumor cell motility and invasion, effects extravasation at the secondary site or function at tumor dormancy [121].

1.4.1. Metastasis Suppressor Proteins/Genes Studied

In this study, we selected seven important genes/proteins which includes nearly all steps of metastasis. These selected genes/proteins were summarized in table 1.3.

1.4.1.1. N-Myc Downstream Regulated 1. (NDRG1)

NDRG1, also called Cap43, is a member of NDRG family proteins which includes other proteins NDRG2, NDRG3, NDRG4 and it has been showed that this protein reduces metastases in colon, breast and prostate neoplasms [123-126]. Although absolute function of this protein is not well known, NDRG1 has various functions on stress (hypoxic) response, nerve myelination, cell differentiation, interaction to heavy metals, and hormones, recycling of E-cadherin, DNA damage response and mast cell maturation [127-129]. Congenital NDRG1 mutation has been detected in an autosomal recessive demyelinating polyneuropathy; Charcot-Marie-Tooth disease type 4D (CMT4D) [130]. NDRG1 has also a role in mouse keratinocyte differentiation [131, 132]. Though, the metastasis/tumor suppressor features of NDRG1 is well documented, a group of reports have been speculated that NDRG1 acts as an oncogene and it is upregulated in a group of human neoplasm [128, 133-136]. Probably, MSP function of NDRG1 is type of cancer dependent. The

mechanism of metastasis suppressor function by NDRG1 is not clear; however there are some clues of interaction of NDRG1 with WNT signaling and E-cadherin [137-139].

Table 1.3: Metastasis Suppressor Genes Studied

Gene	Abbrevation-Synonyms	Accesion Numbers
N-Myc Downstream Regulated 1	**NDRG1, CAP43 DRG-1, RTP**	**HGNC:7679, Entrez Gene: 10397, Ensembl: ENSG00000104419, UniProtKB: Q92597.**
NME/NM23 Nucleoside Diphosphate Kinase 1	**nm23-H1, NM23-H1, NME1, NM23, NM23A, GAAD.**	**HGNC: 7849, Entrez Gene: 4830, Ensembl: ENSG00000239672, UniProtKB: P15531**
Rho GDP Dissociation Inhibitor (GDI) Beta	**Rho GDI 2, ARHGDIB , GDID4, RhoGDI2 GDIA2, RAP1GN1, D4**	**HGNC: 679, Entrez Gene: 397, Ensembl: ENSG00000111348, UniProtKB: P52566.**
Cadherin 1, Type 1, E-Cadherin (Epithelial)	**CDH1, CDHE, CAM 120/80, ECAD, LCAM, CD324.**	**HGNC: 1748, Entrez Gene: 999, Ensembl: ENSG00000039068, UniProtKB: P12830.**
CD82 Molecule	**KAI1, ST6, CD82, SAR2, IA4, TSPAN27, tetraspanin-27, Tspan-27.**	**HGNC: 6210, Entrez Gene: 3732, Ensembl: ENSG00000085117, UniProtKB: P27701.**
Mitogen-Activated Protein Kinase Kinase 4	**MKK4, MAP2K4, SERK1, JNKK1, PRKMK4, JNKK, MEK4, SAPKK-1, MAP2K4**	**HGNC: 6844, Entrez Gene: 6416, Ensembl: ENSG00000065559, UniProtKB: P45985**
A Kinase (PRKA) Anchor Protein 12	**AKAP12, AKAP250, gravin, SSeCKS, AKAP-12**	**HGNC: 370, Entrez Gene: 9590, Ensembl: ENSG00000131016, UniProtKB: Q02952**

1.4.1.2. Rho GDP Dissociation Inhibitor Beta (RHOGDI2, LY-GDI, D4‑GDI)

RHO family GTPases regulate several important cellular mechanism including adhesion, migration and cell proliferation **(Fig. 1.6)** [140]. RHOGDIs are a small group of proteins mainly control Rho GTPases [141]. However the role RHOGDIs in

cellular area is more complex, and besides RHOGTPase inhibitor, they are work as a chaperons and transport RHOGTPases [140]. RHODGI family includes three proteins: *RHOGDI1, RHOGDI2, and RHOGDI3* [141]. RHOGDI1 is the well known and prototype of the family and ubiquitously expressed in various human tissues [142]. However the other member of the family, RHOGDI3, is expressed only a limited number of the organs with low levels, including pancreas, brain, lung, testis, and kidney [140, 142]. Initially, RHOGDI2 is thought to be limited to hematopoietic cells, but now, its expression has been shown in various tissues [143, 144]. The role of RHOGDI2 is complex and type of tumor dependent [140]. RHOGDI2 acts as a metastasis suppressor protein in bladder tumors and its expression is closely related to prognosis of the patient [144, 145]. Probably it acts as an MSP in the other types of epithelial tumors [144, 146, 147]. Despite generally accepted as an MSP, it has been shown that this protein has a more complex dual role in breast cancer [148-150]. Furthermore, RHOGDI2 supports invasion in pancreatic carcinoma cells [151]. RHOGDI2 may have cancer specific functions in tumor suppression and metastasis.

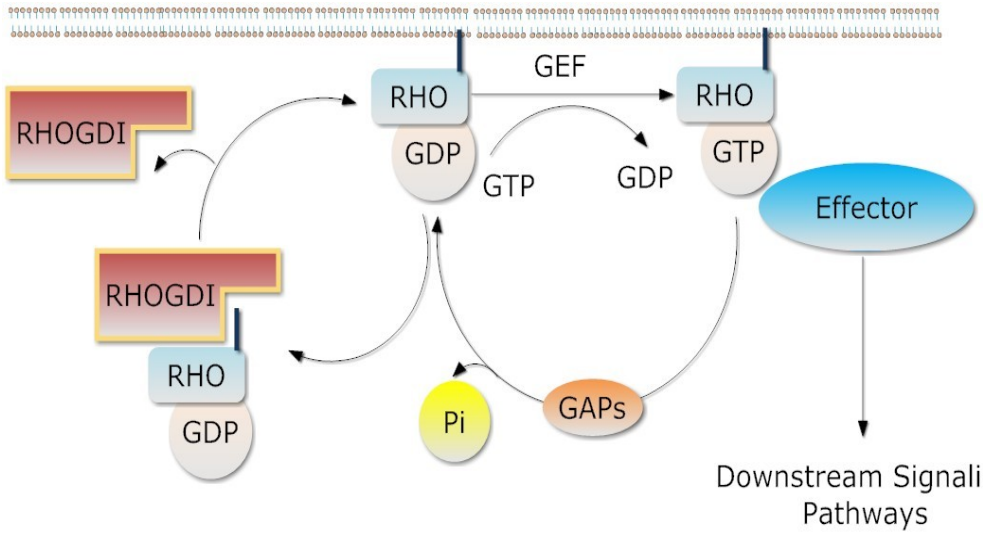


Fig 1.6. RhoGTPase pathway. **The figure is created by the author helping by the references 141-142.**

1.4.1.3. E-Cadherin

Cadherins are big superfamily of proteins and classically separated as three groups, classical cadherins (Type I), non-classical cadherins and protocadherins [152]. E-Cadherin is a member of the classical cadherins, a well-known member of cell-cell adhesion proteins, and loss of its expression plays an important role in tumor invasion and metastasis [152-155]. E-Cadherin is a transmembrane protein. The extracellular part contains five elements that interact with other molecules on the neighboring cells and the internal part of the molecule forms complexes with β-catenin, gp-120 catenin and α-catenin.[156] Besides adhesion, E-cadherin also functions as a negative regulator of the canonical WNT signaling pathway [154, 156].

E- Cadherin is an extensively investigated protein and has been studied in human tumors [157-160]. The main role of E-cadherin in the metastasis cascade is at the epithelial mesenchymal transmission (EMT) step. Loss of E-cadherin expression triggers EMT and invasiveness of the carcinoma cells [161]. Downregulation of E-cadherin is most commonly regulated by promoter methylation or transcription repressors (e.g. SNAIL, SLUG, SIP1, ZEB1).[156] E-cadherin is also important in the differential diagnosis of breast cancer in daily practice; the downregulation or loss of E-cadherin is a specific point in the diagnosis of lobular type breast carcinoma [162, 163].

1.4.1.4. CD82/KAI1

CD82/KAI1 protein, also called TSPAN27, is a member of big 4-span transmembrane tetra-spantin superfamily (TMSF4) which has important roles in adhesion, motility and also tumor progression [164-166]. In the human genome, 33 genes code tetra-spantin proteins [165-166]. Main function of tetra-spantins is to organize other transmembrane molecules including, growth receptors, integrins, and they form tetraspantin-enriched microdomains (TEMs) on the cell surface [165-167]. CD82/KAI1 was first described experimentally in prostate carcinoma cell line AT6.1 as a MSG [168]. The importance of this protein was also demonstrated in breast cancer [169, 170]. However, CD82 expression is very complex in breast carcinoma. CD82/KAI downregulation is mainly seen ER-positive breast cancer while CD82 is retained in ER-negative breast cancer [170, 171]. The prognostic significance and reduced levels of CD82 have been shown in prostate and breast carcinoma, squamous carcinoma of the penis, oral region and larynx, non-small cell lung carcinoma, papillary carcinoma of the thyroid, gastric carcinoma, transitional cell carcinoma, endometrial carcinoma, and cervical carcinoma [172-179].

CD82 is closely associated with EGFR, and its ectopic expression suppresses EGFR-mediated cell migration.[180] New data have also shown that *CD82/KAI1* is a hypoxia target gene regulated by HIF1α [181].

CD82/KAI1 is suggested as a specific immunohistochemical marker for human chromophobe renal cell carcinoma [182].

1.4.1.5. Mitogen-Activated Protein Kinase Kinase 4 (MKK4, MEK4)

MAP kinases (MAPK) are important intracellular enzymes which phosphorylate effector proteins [183]. MAPKs are triggered by external or internal various stimuli and convert the stimuli to different cellular responses including differentiation, proliferation, survival and death **(Fig. 1.7)** [183]. MKK4 is an important component and a key protein of the MAP kinase in stress activated protein kinase signaling [184, 185]. MKK4 particularly activates and phosphorylates both Jun N-terminal kinase (JNK) and p38 which have roles in tumor suppression [183, 185, 186]. Recently, it has been shown that the tumor suppressor function of the MKK4 may be related to inducing replicative senescence [187]. It has been shown that MKK4 inhibits metastases of prostatic and ovarian cancers experimentally in mice [188, 189]. Furthermore immunohistochemical expression of MKK4 is decreased in prostate and ovarian tumors [188, 190]. Although tumor or metastasis suppressor function of MKK4 is generally accepted, there are some clues MKK4 has a pro-oncogenic roles at least a group of human tumors [185, 191, 192]. Some experimental data which were come from breast cancer and pancreatic cell line studies are shown pro-oncogenic role of MKK4 [193]. Furthermore, MKK4 were closely related to proliferation (Ki67 index) in pancreatic adenocarcinomas [192].

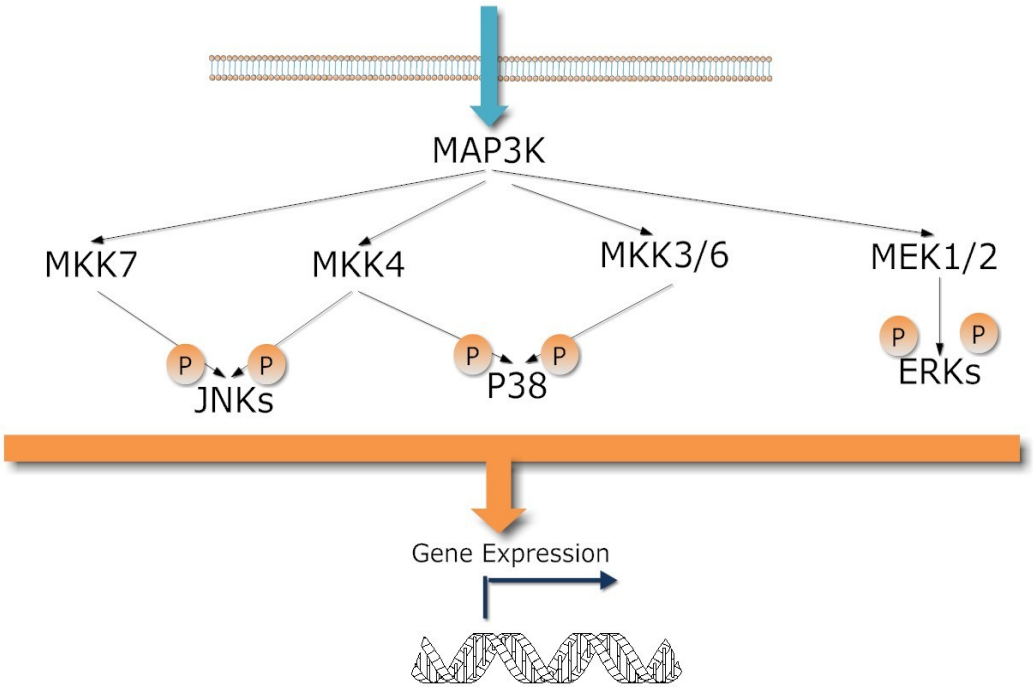


Fig. 1.7: MAP kinase pathway. **The figure is created by the author helping by the references [183]**

1.4.1.6. Nucleoside Diphosphate Kinase 1 (NM23-H1)

NM23-H1 is the first described and prototype of MSPs [119]. *NM23-H1* gene encodes a nucleoside diphosphate kinase A (NDPK-A). Although, the metastasis suppressor mechanism of NM23-H1 is not clear, its interaction with kinase suppressors of RAS (KSR) and, as a result, alteration of the MAPK signaling pathway is a probable mechanism [121]. It has also recently been suggested that it suppresses metastasis by inhibiting the expression of EDG2 (lysophosphatidic acid receptor) [194]. NM23-H1 has the 3'-5' exonuclease activity, and it has been postulated that this property is required for metastasis suppressor properties [195]. It has been shown that downregulation of NM23 is closely related to aggressive behavior and metastasis in a group of human tumor [196-200].

1.4.1.7. A Kinase (PRKA) Anchor Protein 12 (AKAP12, Gravin, AKAP250)

A-kinase Anchoring Proteins (AKAPs) are a group of scaffold proteins which have specific sites especially for protein kinase A and C, and also for phosphoprotein phosphatases [201]. AKAP12, also called SSeCKS/Gravin, was first described as a minor prognostic autoantigen in myestenia gravis [202, 203]. As similar to other AKAPs its role is as a binding partner of protein kinase C (PKC) and A (PKA), calmodulin, F-actin, cyclins, Src, and phospholipids.[204] Significant clinical and experimental evidence has shown that AKAP12 is an important tumor and metastasis suppressor [204]. AKAP12 expression is downregulated in various solid human cancers and leukemias [204, 205]. The downregulation AKAP12 is generally controlled by epigenetic mechanism [206]. It has been shown that AKAP12 promoter methylation is widespread detected and significantly correlated with Gleason score in human prostate carcinoma [207]. Similar to prostate carcinoma, AKAP12 gene is significantly methylated and its expression is downregulated in hepatocellular carcinoma [208]. Hypermethylation of AKAP12 promoter is also documented in skin carcinoma, gastric carcinoma, and pancreatic cell lines [209-211].

1.4.2. Other Metastasis Suppressor Proteins

Many proteins have some roles in negative regulation of metastasis. However, as a definition, MSP should have no or minimal effect on tumor growth or proliferation [121]. Nowadays, more than 30 proteins are generally accepted as MSP with more expected [212]. The list of well-known and generally expected MSPs is presented in **table 1.4.**

1.5. OBJECTIVES AND RATIONALE

1.5.1 Aim of the Study

As described above, metastasis is a complex multistep process and very important in determining for prognosis of an oncology patient. There has been an extraordinary effort to prevent and retard metastasis in the basic and clinical science area. Metastasis suppressor proteins give a hope that new therapy strategy for metastasis will be found. Non-melanoma skin cancers differ from internal organ cancers in that they have low metastatic rates and good prognosis. Thus, NMSCs are interesting biological model for metastasis suppressor research. The main aim of this study was to analyze differentially expressed genes and proteins which may contribute to inhibit metastasis pathway in Non-Melanoma skin cancer. We also established the association between these proteins and clinicopathological parameters.

1.5.2 Rationale and Strategy

We collected fresh normal and pathological assessment of NMSC and paired normal tissue samples. Parafin embedding biopsies were also selected from archival specimens. Clinicopathological data for BCCs and cSCCs were collected by using the described conventional important clinicopathological parameters for BCCs and cSCCs. The gene expression profiles of target genes in tumor and paired-normal skin tissues, and cell lines were determined by qRT-PCR studies. The protein

expression profiles were analysed semi-quantitavely by immunohistochemistry studies. The schema of the study design is demonstrated in **Figure 1.8**

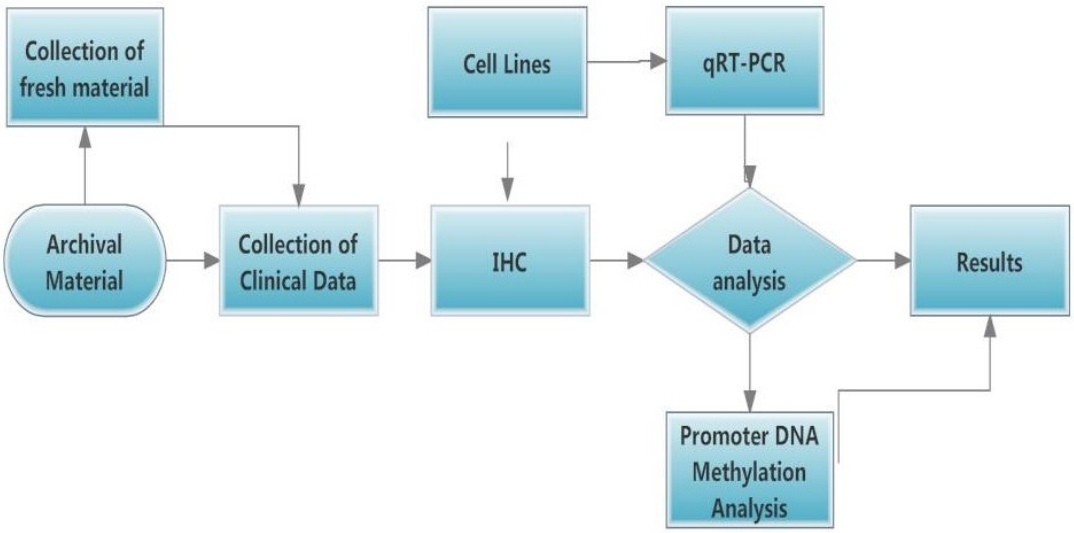


Fig. 1.8. The study design.

Table1.4. Metastasis Suppressor Proteins described in the literature.

Metastasis Suppressor Proteins	

• Nonmetastatic 23 (Nm23)
• Kai1/Cd82
• Mitogen Activated Protein Kinase Kinase
  ▪ MKK4
  ▪ MKK6
  ▪ MKK7
  ▪ P38
• Rho Gdi-Dissociation Factor 2 (RHOGDI2)
• N-Myc Downstream Regulated Gene 1 (NDRG1)
• E-Cadherin
• Src-Suppressed Protein Kinase C Substrate (SSECKS) (Akap12)
• Breast Cancer Metastasis Suppressor 1 (BRMS1)
• Gelsolin
• Kiss1
• Deleted In Liver Cancer 1 (DLC-1)
• Cd44
• Cell Adhesion Molecule 1 (CADM1)
• Mdm2 Binding Protein (MTBP)
• Caspase 8
	• Collapsin Response Mediator Protein 4 (CRMP4)
	• Deleted In Colorectal Cancer (DCC)
	• Farnesoid X Receptor (FXR)
	• Growth Arrest-Specific 1 (Gas1) Leukemia Inhibitory Factor Receptor Alpha (LIFRA)
	• Raf Kinase Inhibitory Protein (Rkip)
	• Ribonucleotide Reductase Subunit M1 (RRM1)
	• Stefin A
	• Lysine-Specific Demethylase 1 (LSD1)
	• Hormonally Regulated Neu-Associated Kinase (HUNK)
	• Tissue Inhibitor of Metalloproteases (TIMPS)
	  ▪ Timp1
	  ▪ Timp2
	  ▪ Timp3
	  ▪ Timp4
	• Kruppel-Like Factor (Klf) 17
	• Caveolin-1
	• Ovarian Cancer G Protein-Coupled Receptor 1 (OGR1)
	• Lysine-Specific Demethylase 1 (LSD1)
	• Lass2/Tmsg1
	• Metastamir and Non-Coding RNA

*Adapted from references [121, 212-217]

CHAPTER 2- MATERIALS AND METHODS

2.1 Study Groups.

The study groups are summarized at **table2.**1 and the detailed clinicopathologic features of cases are demonstrated in **appendix A**.

Table 2.1. Study groups

Immunohistochemistry		
Normal skin	**BCC**	**SCC**
n=10	**n=96 (92 patients)**	**n= 38 (37 patients)**
4M/6F	**47M/45F**	**26M/11F**
42.7± 14.6	**Mean age =66.3±13.4**	**Mean age =69,6± 11.7**
	Two groups:	• **32 cSCC**
	• **NA-BCC (n=68)**	• **6 IS-SCC**
	• **A-BCC (n=28)**	
qRT-PCR		
n= 11 BCC (9 patients , 6M/3F), Mean Age: 69,1 ±17,7		
3 normal non-lesional skin samples.		
8 normal skin samples adjacent to the BCCs		
Cell Lines		
Normal immortalized keratinocyte cell line (HaCaT)		
Squamous carcinoma cell line (A-431)		

2.1.1. Basal Cell Carcinoma Group.

For this study, a total of 96 BCCs from 92 patients (47M/45F) were included. All patients were Caucasian and the mean age was 66.3 ±13.4 years. All lesions were excised from the head and neck area except for five lesions from the trunk. The subclassifying BCCs is confused, because of their wide and heterogeneous morphological spectrum, we used the criteria summarized by Carr RA. *et al.* for

classifying our group [15]. Two major tumor groups were created and immunohistochemically scored to establish the differential expression patterns and contribution of the proteins:

1. Morphologically **non-aggressive BCC (NA-BCC)** types including nodular, adenoid, superficial and also mixed carcinomas with less than 50% infiltrative pattern (n=68).
2. Morphologically **aggressive BCC (A-BCC)** types including pure infiltrative BCCs with/without desmoplasia and mixed carcinomas with more than 50% infiltrative pattern (n=28).

2.1.2 Squamous Cell Carcinoma Group

32 squamous cell carcinoma of the skin, 6 in-situ carcinoma tissue total 38 SCCs from 37 patients (26M/11F) were included in this study. Thirty-two of 38 SCCs were from head and neck area including lips, 3 were from trunk and 3 were from extremities. All patients are Caucasians and mean age was 69.6± 11.7 and age range was between 40-89 years.

2.1.3. Normal Skin Control Group

Ten normal skin tissues (4M/6F) from reconstructive operations, confirmed as a normal by microscopy, were included as normal tissue group. Mean age was 42.7± 14.6 and the age range was 26-72. The normal epidermis adjacent to BCCS and SCCs were also studied after histopathologically confirmed as a normal.

2.1.4. Quantitative Real-Time PCR Study Group

Unfixed fresh tissues of 11 BCC, 3 normal non-lesional skin samples, and 8 normal skin samples adjacent to the BCCs were included for qRT-PCR studies. All tissues were histopathologically re-confirmed by frozen sections before RNA isolation.

2.1.5. Clinicopathological Features

Clinicopathological data for BCCs were collected by using the described conventional important clinicopathological parameters for BCCs. The maximum diameter of the tumor, invasion depth, perineural invasion, anatomical invasion (Clark's) level and local recurrences were investigated. Tumor-associated inflammation was graded as previously described by Kaur P. *et al.* [218]. Data for the SCCs were collected by using CAP (The College of American Pathologists) protocols for squamous carcinoma of the skin [219].

2.1.6. Cell Lines

Normal immortalized keratinocyte cell line **(HaCaT)**, and squamous carcinoma cell line **(A-431)** were included for immunohistochemistry and RT-PCR studies in this study.

2.2. Cell Culture

A-431 cell lines were cultured in essential medium (DMEM-low glucose) (Hyclone-Thermo Scientific, Waltham, MA, USA) and HaCaT was grown in high glucose essential medium (DMEM-high glucose) (Hyclone), supplemented with 10% fetal bovine serum (FBS), 50 mg/ml penicillin/streptomycin at 37°C and in 5% carbon dioxide. Before the cells were reaching confluence, they were passaged. The medium was aspirated and then the cells were washed with 1X PBS. After adding Trypsin/EDTA solution to the flasks, the detached cells were mixed with fresh medium gently and they were transferred to new flasks.

For further studies and cryopreservation, exponentially growing cells were harvested by trypsinization at passage four or five. After neutralizing by adding fresh growth medium, the cells were counted and centrifuged at 1500 rpm for 5 minutes. For cryopreservation, the cells (approximately $4x10^6$ cells/ml for one vial) were remixed with freezing medium (90%FBS/10%DMSO) and stored at -80^o for short term or liquid nitrogen vapor for long term. Besides cryopreservation, the cell pellets were fixed in 10% buffered formalin for immunohistochemistry studies, and they also were stored at -80^o for qRT-PCR studies.

2.3. Immunohistochemistry Studies and Evaluation

2.3.1. Immunohistochemistry

All tissues were fixed in buffered formalin (%10) overnight. After standard tissue processing, all biopsies were embedded into paraffin. 5 µ thin sections were cut by

rotary microtome and taken on adhesive slides. After drying the sections at 37^{o} in the incubator overnight, de-paraffinized and rehydrated slides by xylene and alcohol steps were pre-treated in a Thermo Lab Vision® PT Module (Waltham, MA, USA) in different antigen retrieval buffers (Citrate pH=6 or EDTA pH=9) for 23 minutes (min) at 98^{o} C. After waiting for cooling period, the sections were placed in 3% H_2O_2 (5 min) for stopping endogenous peroxidases activity. After applying Ultra V Block (Thermo Scientific, Waltham, MA, USA) to reduce nonspecific background staining, all primary antibodies at appropriate dilutions were incubated overnight in a moist environment and then stained by the labelled streptavidin-biotin immuno-enzymatic antigen detection system (UltraVision-Thermo Scientific; Waltham, MA, USA) with DAB chromogen. Afterwards, the slides were counterstained with Mayer's hematoxylin for two minutes and closed with coverglass. All staining steps were carried out by specific capillary cover-plate system in Thermo-Shandon Sequenza® manual staining station (Waltham, MA, USA) for standardization except antigen retrieval steps. Skipping the primary antibody step was used as the negative control. The companies, blocking time, dilutions and positive controls and incubation time are demonstrated in **table 2.2**.

2.3.2. Immunohistochemical Analyses-HSCORE

All immunohistochemical stained slides were evaluated by external and internal controls. Stained slides were semiquantitatively evaluated by using a specific immunohistochemical histological score (HSCORE) technique described before, which covers both the intensity and proportional distribution of

immunohistochemical staining at the different intensity levels. This specific calculation method was first described by McCarty et al. Briefly the HSCORE has been formulated as HS=Σ (Pi x i /100) by the authors [220]. Pi points out the relative percentage of stained cells (0-100%) at each intensity, and i shows the intensity of staining and ranges from 0 (No staining) -3 (Strong brown staining).[220] After evaluating the whole slide for specific staining, a minimum of 6 randomly selected areas at medium power magnification (20X) were analyzed for HSCORE in normal and neoplastic tissue. All analyses and countings were made at Olympus BX50 microscope by the author. Nuclear ($_{nuc}$) and cytoplasmic ($_{cyt}$) expressions were evaluated separately for NM23-H1, MKK4, RHOGDI2 and NDRG1. Only membranocytoplasmic staining of E-cadherin, CD82 and AKAP12 were accepted as positive. Mathematical calculations were performed by a simple Excel® macro.program by the author. The Excel ® macro is demonstrated in **Fig. 2.1.**

Immunohistochemistry staining of cell culture were established by the same technique. However, slides were evaluated for intensity (0-3) and homogeneity.

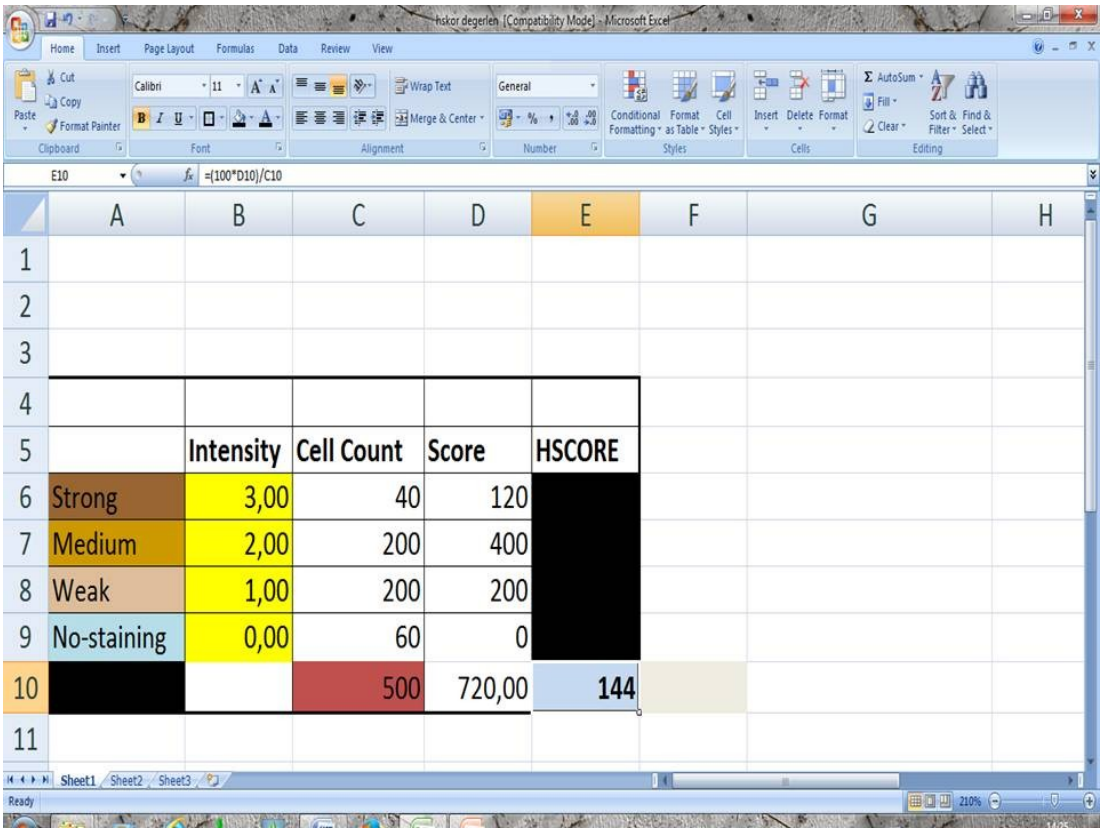


Fig. 2.1. The print screen of the simple Excel® macro. In this example, 500 cell count with different intensity. The macro normalizes the Scores by percentage (%) and calculates the HSCORE.

2.4. qRT-PCR Studies and Analysis

2.4.1. PCR Primer Design.

The gene specific primers were designed from exon-exon boundaries which includes large intron sequence by web based **Primer 3** (http://bioinfo.ut.ee/primer3-0.4.0) or primer.exe programs. Four sequences were designed before by other authors in the literature **(Table2.3)**. The sequences of the primers were also analyzed by the blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for specificity. All designed primers were purchased from Iontek Inc. (Istanbul, Turkey). Designed and used primers are documented in **table 2.3.**

Table2.2. Primer antibodies used in this study.

Antibody	Company	Dilution	Antigen Retrivieal	Incubation	Control Tissue
RHOGDI2	Abcam	1/100	Citrat; pH=6	Overnight	Tonsil
NM23-H1	Abcam	1/200	No	Overnight	Ductal carcinoma,Breast
MKK4	Novocastra; Leica	1/20	Citrat; pH=6	Overnight	Ductal carcinoma, Breast
CD82	Novocastra; Leica	1/20	Citrat; pH=6	Overnight	Tonsil
AKAP12	Atlas	1/100	Citrat; pH=6	Overnight	Testis
NDRG1	Santa Cruz	1/100	EDTA; pH=9	Overnight	Placenta
E-CADHERIN	Cell Signaling	1/100	Citrat; pH=6	Overnight	Adenocarcinoma, colon
P53	Thermo	1/100	Citrat; pH=6	Overnight	Adenocarcinoma, colon

Table 2.3. qRT-PCR primer sequences used in this study

GENE	F	R
GAPDH	5'-AGGTGAAGGTCGGAGTCAAC-3' *	5'-GGGTCATTGATGGCAACA-3'
HPRT1*	5'-GCTGACCTGCTGGATTACAT-3'	5'-CCCTGTTGACTGGTCATTAC-3'
KAI1/CD82	5'-AGCAGAACCCGCAGAGTCCT-3'	5'-CTTCCACGAAACCAGTGCAG-3'
MAP2K4*	5'-AGTGGACAGCTTGTGGACTCT-3	5'-AACTCCAGACATCAGAGCGGA- 3'
NM23 (NME1)	5'-CCTGAAGGACCGTCCATTCT-3'	5'-CCGTCTTCACCACATTCAGC-3'
ECADHERIN(CDH1)	5'-GTCCTGGGCAGAGTGAATTT-3'	5'-TCTGTGCCCACTTTGAATCG-3'
AKAP12	5'-TCACAGAGGTTGGACAGAGA-3'	5'-GTGAACAACCGCTGACTTAG-3'
RHOGDI2(ARHGDIB)	5'-CCTCCACCACAGAAGTCCCT-3'	5'-GCTTTCGGATCTGTCACCAC-3'
NDRG1	5'-CAAGATCTCAGGATGGACC-3'	5'-GACCACTTCCACGTTACTC-3'

* Mol Cancer. 2010; 9: 226, ** Designed by Dedeoğlu BG, PhD. ***Gynecol Oncol. 2007 ; 105: 312–320.

2.4.2. Amplification Efficiencies

Human placental tissue cDNA was selected for the detection of the amplification efficiencies because of high expression of MSGs in this tissue. The PCR efficiencies

were calculated by 5 or 10-fold dilution series of cDNAs, due to relative expression of the gene level in the normal human placental tissue **(Table2.4)**.

2.4.3. qRT-PCR Studies

For skin tissues, total RNA was isolated using commercial RNA extraction kit for frozen tissue (Qiagen, Fibrous Tissue kit) in accordance with the manufacturer's instructions. NucleoSpin RNA kit (Macherey-Nagel, Düren Germany) was used for cell culture pellets. The concentration of the isolated RNA was measured with the NanoDrop ND-1000 spectrophotometer (Montchanin, DE, USA) at 260 nm/280 nm absorbance ratio was also measured for quality of RNA samples.

Table 2.4. Amplification efficiencies of the used primer pairs.

Gene	Efficiency	Efficiency %
GAPDH	1,93	93
HPRT1	1,86	86
CDH1	1,91	91
MAP2K4	1,96	96
RHOGDI2(ARHGDIB)	1,91	91
CD82	1,9	90
AKAP	2	100
NDRG1	1,93	93
NME1(NM23-H1)	2	100

500 ng of total RNA was used for cDNA synthesis. RNA was reverse-transcribed using oligo-DT primers and all conversions were established with First Strand cDNA Synthesis Kit (Fermantas, Thermo, Waltham, MA, USA). All qRT-PCR experiments were performed using the SYBR® Green chemistry, in 96-well reaction plates with specific optical caps (Bioplastics; Landgraaf, Netherland) in a MX3005P

thermocycler (Strategene®, Agilent, Santa Clara, California, USA). GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) and HPRT1 (Hypoxanthine phosphoribosyl transferase 1) genes were selected as reference genes. The qPCR reaction includes 10 µl 2x SYBR Green PCR Master Mix (Finnzyme-Thermo, Waltham, MA, USA), forward and reverse gen specific primers at optimized concentrations of 300 nM except NDRG1 primers (150nM), 2 µl cDNA template (500 ng/ml), and PCR grade water up to a final volume of 20 µl. 20 µl mineral oil was also added to prevent evaporation in PCR cycling.

The PCR cycling conditions were as follows: an initial denaturation of 95°C for 5 min and then 40 repeated cycles of denaturation (95°C; 30 s), annealing (58-60°C for different primers, 30 s), extension (72°C, 30 s) steps, and final extension of 72°C of 5 min. The fluorescence data were also investigated by melt-curve analysis (55°C to 95°C in 80 cycles) for the confirmation of one single specific product. All qPCR examinations were done twice.

2.4.4. qRT-PCR Data Analysis

The ampication plots, melt curves, Ct values were collected from the original program, Mx.Pro, MX3005p v.4.10 (Strategene®, Agilent, Santa Clara, California, USA) **(Fig. 2.2).** Data analysis was performed by REST© 2009 (Qiagen, Hilden, Germany) software [221]. This software uses the classic formulation Ratio = $(E_{\text{target}})^{\Delta CPtarget\ (control - sample)} / (E_{\text{ref}})^{\Delta CPref\ (control - sample)}$ and shows both fold changes and standard errors between the controls and the samples. It has been generally thought that the advantage of the software is that it gives reliable results in small

study groups due to randomizations.

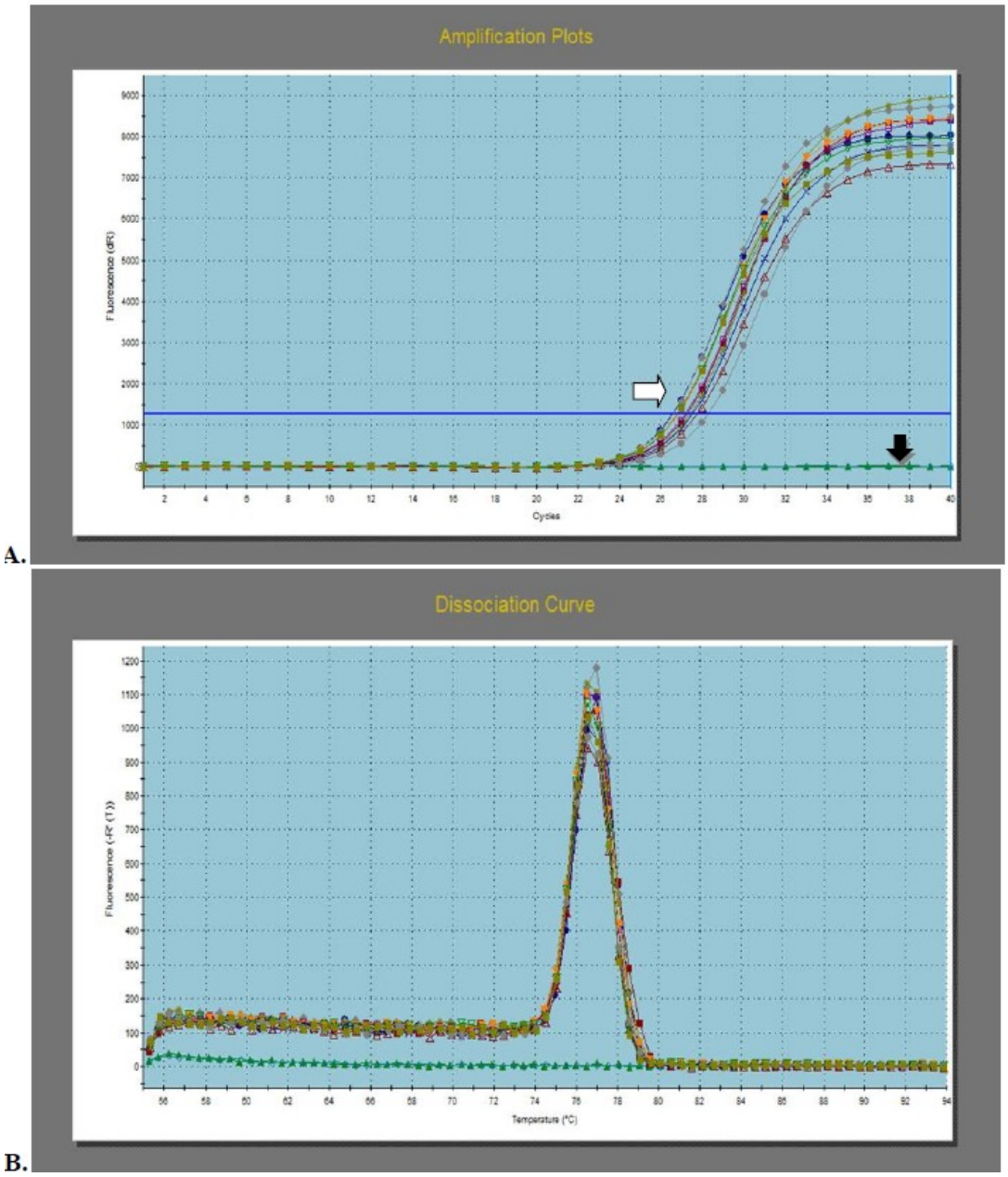


Fig. 2.2. Amplification curve and melt curve graph. Amplification plots (A) and melt (Dissociation) curve graps (B) for qRT-PCR reaction of reference *HPRT1* gene for control skin tissues to detect specificity of primer sequences. When fluorescent signal reaches to detectable level by the sensor of the qRT-PCR program, it is showed as an amplification curve (A). The no-template control gives no fluorescent signal (A-Black arrow) . The point at which the amplification curve reaches to the threshold level is named as the Ct value (A-White arrow). The control samples give single peak at the same temperature in the melt curve which shows single specific product (B).

2.5. Promoter DNA Methylation Analysis

For a preliminary study, seven basal cell carcinoma and 3 normal skin tissues

adjacent to BCC and two normal non-lesional skin tissues as controls were included in promoter DNA methylation analysis.

2.5.1 Bisulfite Modification of DNA, Sequencing and Analysis

DNA extraction was carried out by QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with minor modifications. Before DNA extraction, H&E stained frozen sections of all skin tissues were evaluated and they were macrodissected if required. After DNA extraction, 1 ug of DNA was measured and used in bisulfite conversion using Epitect Bisulfite kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Primers for bisulfite modified genomic DNA of MKK4 were specially designed using Methyl Primer Express v1.0 software, and specific nested primers for CD82 adapted from a previous study [222]. Bisulfite sequencing PCR (BSP) primers sequences were listed in **table 2.5.** Epitect Methylated and Unmethylated Bisulfite Treated DNAs were used as positive control of the BSP reaction. Following BSP from tissue DNA, PCR products were purified from the 2% agarose gel (Qiaquick Gel Extraction Kit - Qiagen, Hilden, Germany), ligated into pGEM-T Easy Vector System (Promega) and then were transformed into competent E. coli DH5α, and mini-prep plasmid isolation was carried out from overnight grown cultures of selected single colonies (Ampicillin and blue-white selection), using the MN-Nucleospin Plasmid kit (Düren, Germany), following the user protocol. Quantification of plasmid concentration was done with NanoDrop ND-1000 spectrophotometer. PCR-confirmed plasmids were sent for sequencing,

with SP6 primers. Sequencing results were analyzed using free QUMA (http://quma.cdb.riken.jp/) software [223].

Table2.5. BSP primers

	F	R
MKK4	GGGYGGTTTTGTAGTTTAGTAT	CCTTACCCTACATACTACTAACRACC
CD82 $_{\text{first round}}$*	AGGGTAGGGTAGGATTAGGAA	CTCCTTTTCACCCACCAACTACT
CD82 $_{\text{nested}}$*	AGGTTGGTTGGGGTAYGGTTAT	AAAACXAAAACTAAAACTAACTTTACC

* Oncol Rep. 2009 ;21(1):159-64.

2.6. Statistical Analysis

Statistical analyses were performed by using the PASW® Statistics 18 software (Chicago, IL, USA). The acquired data and differences between the HSCOREs of the groups were first studied with the non-parametric Kruskal–Wallis one-way analysis test and then Mann-Whitney *U* test ($p \leq 0.05$ accepted as significant), were applied. The "Bonferroni correction" was also used for reducing the false-positive results (type I errors). The correlation between the parameters was investigated by Spearman's correlation test ($0.25 \leq r$; $p \leq 0.05$ accepted as significant).

For qRT-PCR data, statistical significance between the groups was evaluated by *Pair Wise Fixed Reallocation Randomization Test*© by the REST software.

2.7. Ethical Issues and Support.

This study was financially supported by the Scientific and Technical Research Council of Turkey (TUBITAK) (grant number SBAG-108S184). The project was approved by the Local Ethical Committee - Kırıkkale (07.04.2008/ 2008-039).

CHAPTER 3- RESULTS

3.1. Immunohistochemical Staining, HSCOREs and qRT-PCR Results.

The significant changes and comparative results between the HSCOREs of the groups are demonstrated in **Fig. 3.1.**

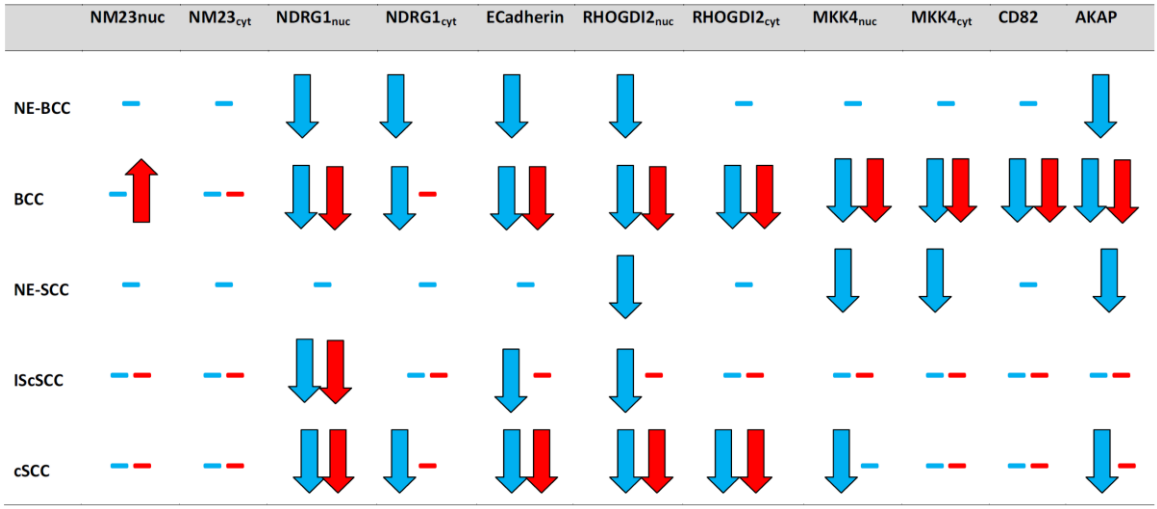


Fig. 3.1. Summary of HSCORE Data. A group of protein showed lower scores in adjacent epidermis of BCCs (NE-BCC) and SCCs (NE-SCC) when compared to normal skin. NM23-H1 HSCOREs were clearly protected in NMSC. $NDRG1_{cyt}$ was also protected in NMSC when compared to NE-BCC or NE-SCC. The other proteins were downregulated in BCCs. However, CD82 and $MKK4_{cyt}$ levels showed no difference in in-situ and invasive carcinomas. RHOGDI2, AKAP12 and $MKK4_{nuc}$ levels were also protected in in-situ SCC. AKAP12 levels in SCCs were downregulated when compared to normal epidermis but showed no difference when compared to NE-SCC. *Only statistically significant changes ($0.05 \leq p$) were used when creating this figure. Blue shows comparison to normal epidermis red points out comparison to adjacent epidermis of neoplasia.

Similar to immunohistochemistry study, BCCs were also compared to both the normal non-lesional skin tissue and the skin adjacent to neoplasia in qRT-PCR study. The fold changes of the studied genes are summarized in **table 3.1.**

Table 3.1: qRT-PCR results of BCC group.

Gene	BCC/N Fold Change	*p*	BCC/NS-BCC Fold Change	*p*
NM23-H1	**1,4 ↑**	**0,032**	-1,1	NS
NDRG1	1,1	NS	**2,2 ↑**	**0,001**
E-cadherin	-1,1	NS	-1,2	NS
RHOGDI2	-1,5	NS	1,1	NS
MKK4	1,3	NS	1	NS
CD82	1,1	NS	-1,4	NS
AKAP	**-1,2 ↓**	**0,006**	-1,3	NS

N=Normal Skin ,NS-BCC=Normal skin adjacent to Basal Cell Carcinoma. NS=Not significant.

3.1.1. NM23-H1

3.1.1.1. Immunohistochemical Analysis

In the normal epidermis (N), cytoplasmic NM23-H1 positivity was strong and easily detectable **(Fig.3.2A)**. However, nuclear NM23-H1 was only seen in the basal layers of the epidermis. Normal epidermis adjacent to BCCs (NE-BCC) showed more heterogeneous positivity with NM23-H1 when compared to the normal epidermis. In normal epidermis neighboring cSCCs (NE-SCC), NM23-H1 staining showed similar intensity and distribution with normal epidermis.

NM23-H1 cytoplasmic expression ($NM23\text{-}H1_{cyt}$) was also strong and homogeneous in all BCCs **(Fig. 3.2B, C)**, except in two BCCs (97.9%). Nuclear expression of NM23-H1 was weaker and expressed in 73 of 96 (76%) BCCs.

$NM23\text{-}H1_{cyt}$ positivity was detected as strong and homogeneous in all of the in-situ and invasive cSCCs **(Fig. 3.2D)**. However, nuclear NM23-H1 ($NM23\text{-}H1_{nuc}$) expression was significantly weak and detected in two of IS-SCC (33.3%) and only in 15 cSCCs (46.8%).

NM23-H1 showed strong cytoplasmic positivity in the two cell lines **(Fig. 3.2E, F).** However, focal nuclear positivity was stronger in the HaCaT cell line.

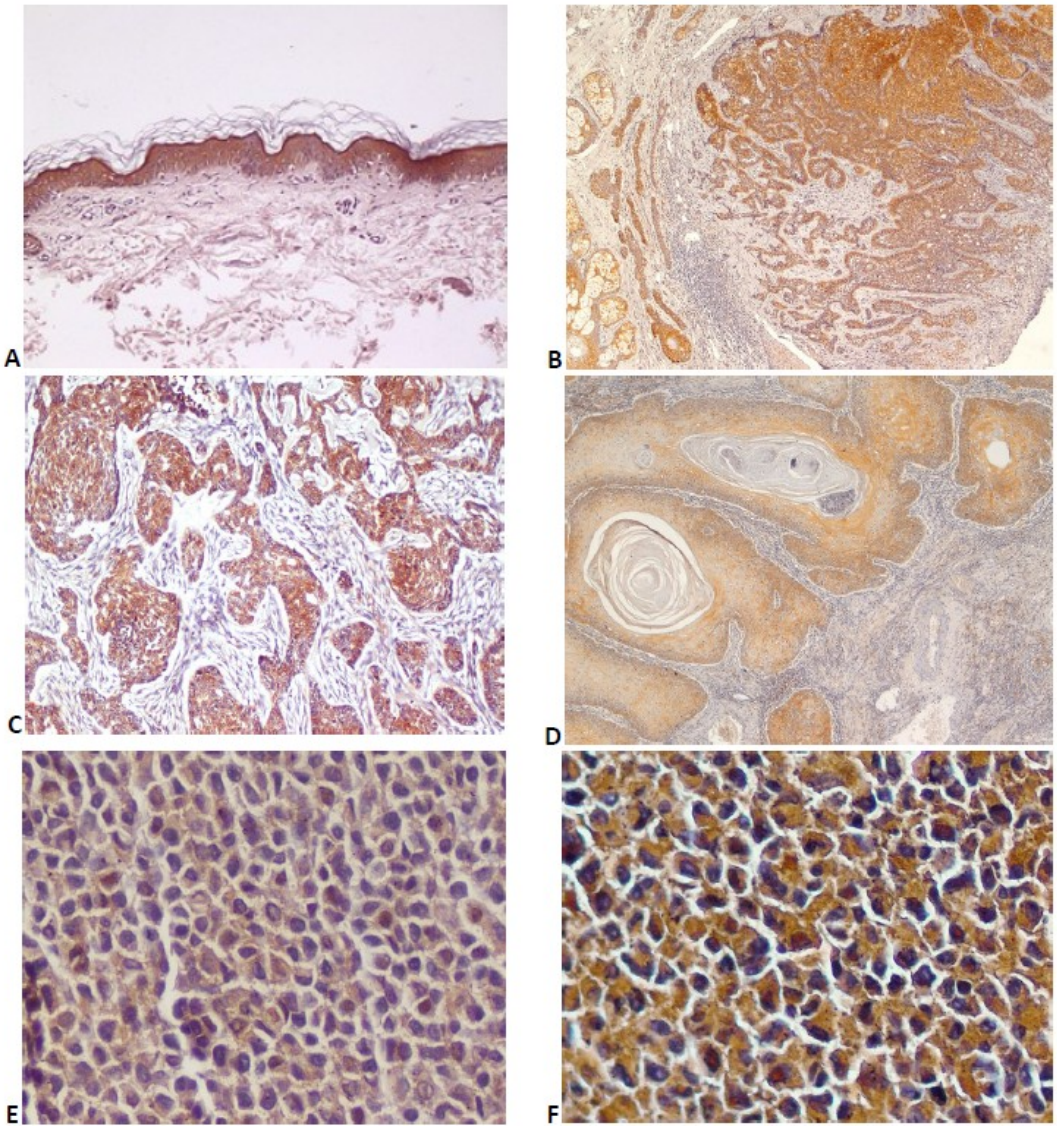


Fig. 3.2. NM23-H1 immunohistochemistry. Nm23-H1 positivity in normal skin(A). Strong cytoplasmic NM23-H1 expression in BCCs (B; C) and SCC(D). Easily detectable NM23-H1 positivity in HaCaT (E) and A-431 (F) cell lines. (A, B, x40; C, D, x100, E, F, x200) (Fig. 3.2C-Copyright; Int J Dermatol 2014)

3.1.1.2. HSCOREs

Nm23-H1$_{nuc}$ showed significantly lower scores in stage I cSCCs than stageII/III SCCs (p=0.049). But there was no statistical difference for NM23-H1$_{cyt/nuc}$, between the normal and tumor groups except NM23-H1$_{nuc}$ which showed higher scores in BCCs when compared to NE-BCC (p=0,013). These data clearly showed NM23-H1 protein was protected in NMSC **(Fig. 3.3A, B)**.

3.1.1.3. qPCR Results.

We found significant upregulation of *NM23-H1* (1.4 fold p=0.032) when BCC was compared to normal skin **(Fig. 3.6A).** There was no fold difference between A-431 and HaCaT cell lines.

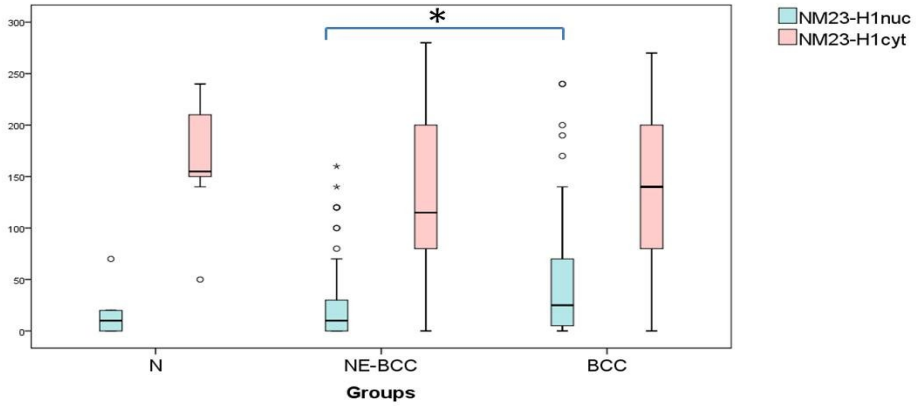
3.1.2. NDRG1

3.1.2.1. Immunohistochemical Analysis

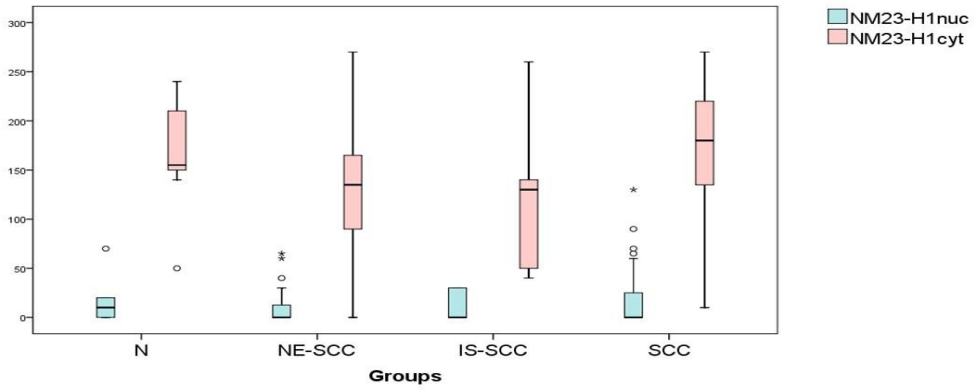
Normal epidermis showed strong cytoplasmic and nuclear NDRG1 positivity **(Fig. 3. 4A)**. Normal epidermis adjacent to BCCs (NE-BCC) expressed less intense positivity when compared to the normal epidermis. However, in NE-SCC, NDRG1 staining revealed similar intensity and distribution as in normal epidermis.

In BCCs, both cytoplasmic (NDRG1$_{cyt}$) and nuclear NDRG1 (NDRG1$_{nuc}$) positivity were seen homogeneous **(Fig. 3.4 B, C)**. NDRG1$_{cyt}$ was detected in all BCCs but only 74 of 96 (77%) BCCs showed nuclear positivity. Similarly, NDRG1 (NDRG1$_{cyt}$)

cytoplasmic positivity were detected strong and homogeneous in all of the in-situ and invasive cSCCs **(Fig. 3.4. D)**. Nuclear positivity of NDRG1 ($NDRG1_{nuc}$) was seen in 4 of 6 IS-SCCs (66.6%) and 29 of 32 cSCCs (90.6%). Significant positivity was seen by NDRG1 antibody in the two cell lines **(Fig.3.4E, F)**.



A



B

Fig. 3.3. Boxplot graphs of NM23-H1. (A) BCC (B) SCC groups. There was no statistical difference for $NM23\text{-}H1_{cyt/nuc}$, between the normal and tumor groups except $NM23\text{-}H1_{nuc}$ which showed higher scores in BCCs (A) when compared to NE-BCC. These data clearly showed NM23-H1 protein was protected in NMSC. *=$p \leq 0.05$; ** $p \leq 0.01$

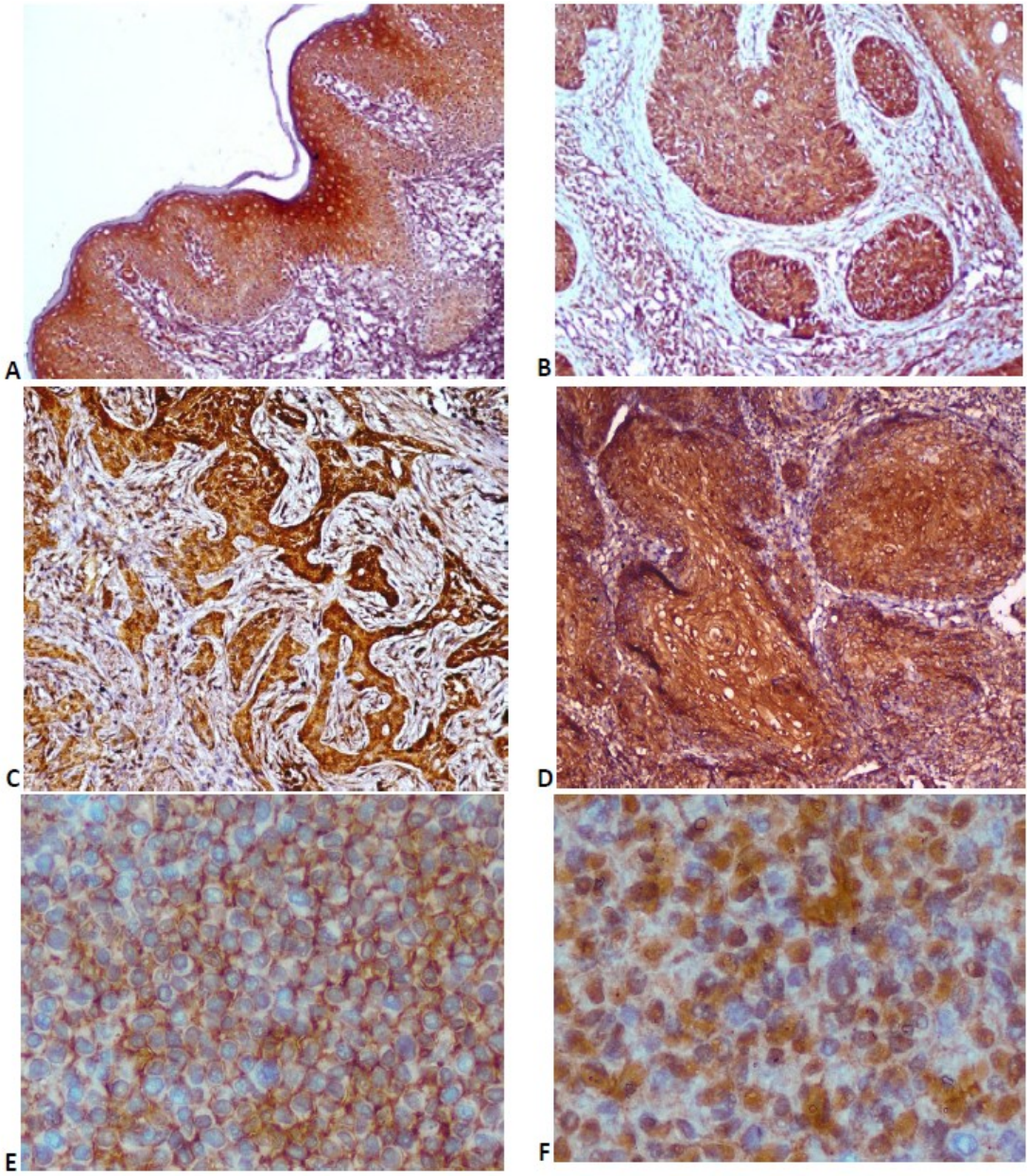


Fig. 3.4. NDRG1 immunohistochemistry. NDRG1 positivity in normal skin (A). Strong and diffuse NDRG1 positivity in BCCs (B, C) and SCCs (D). Similarly, significant positivity is seen by NDRG1 antibody in the two cell lines. HaCaT (E); A-431(F). (A, B, C, x100; D, E, F, x200)

3.1.2.2. HSCOREs

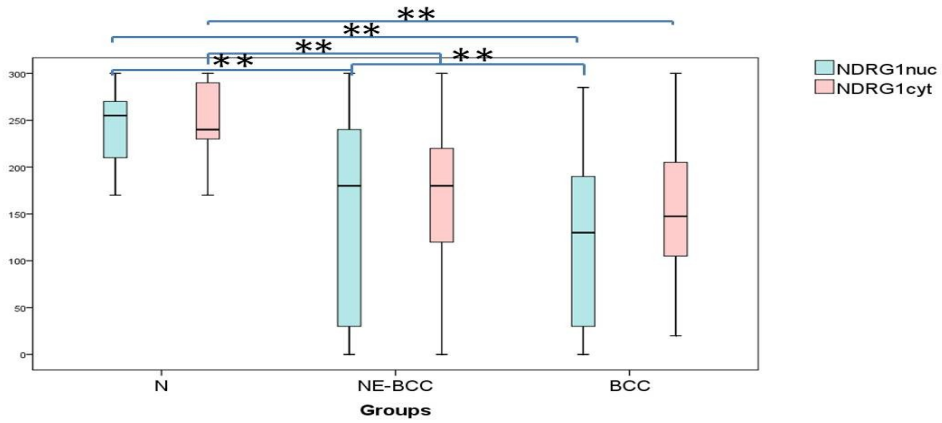
The HCORES of $NDRG1_{cyt}$ (p=0,001) and $NDRG1_{nuc}$ (p=0.007) showed a significant reduction in NE-BCC **(Fig. 3.5A)**.

When N was compared to BCCs, $NDRG1_{cyt}$ (p=0.001) and $NDRG1_{nuc}$ (p=0.001) staining showed significantly lower scores in the BCCs **(Fig. 3.5A)**. When NE-BCC was compared to BCCs, $NDRG1_{nuc}$ levels (p=0.003), but not $NDRG1_{cyt}$ reduced in the BCC group. Furthermore A-BCCs showed significantly higher $NDRG1_{cyt}$ scores (p=0.001) than NA-BCCs.

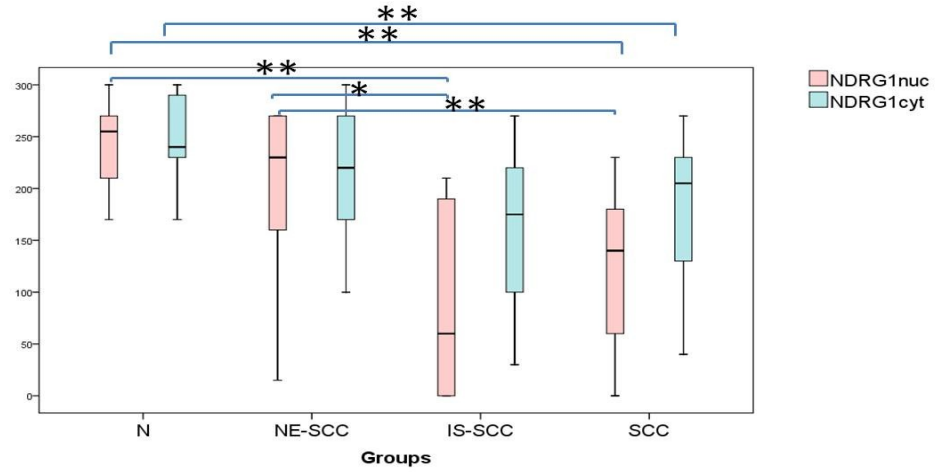
In SCCs, different from BCCs, there was no difference between N and NE-SCC **(Fig. 3.5B)**. But there was statistically significant difference for $NDRG1_{nuc}$ (p=0.003) between the normal skin and in situ carcinomas. This was also valid when compared to NE-SCC (p=0.011). In invasive cSCC, $NDRG1_{nuc/cyt}$ showed significantly lower HSCOREs than normal epidermis (p=0.001; p=0,007). However, only $NDRG1_{nuc}$ scores were decreased in cSCC (p=0.001) when compared to NE-SCC **(Fig. 3.5B)**.

3.1.2.3. qPCR Results.

NDRG1 showed statistically significantly higher levels (2.2 fold, p=0.001) in BCC when compared to the skin adjacent to the neoplasia, similar to the immunohistochemical results **(Fig. 3.6)**. *NDRG1* (34.4-fold, p=0.001) was also upregulated in A-431 cell line when compared to HaCaT.



A



B

Fig. 3.5. Boxplot graphs of NDRG1. (A) BCC (B) SCCgroups. The HCORES of $NDRG1_{cyt}$ and $NDRG1_{nuc}$ showed a significant reduction in NE-BCC(A). When N was compared to BCCs, $NDRG1_{cyt}$ and $NDRG1_{nuc}$ staining showed significantly lower scores in the BCCs. When NE-BCC was compared to BCCs, $NDRG1_{nuc}$ levels, but not $NDRG1_{cyt}$ was reduced in the BCC group. In SCCs, there was no difference between N and NE-SCC (B). But there was statistically significant difference for $NDRG1_{nuc}$ between the N and IS-SCC. This was also valid when compared to NE-SCC In invasive cSCC, $NDRG1_{nuc/cyt}$ showed significantly lower HSCOREs than normal epidermis. However, only $NDRG1_{nuc}$ scores were decreased in cSCC when compared to NE-SCC. *=$p \leq 0.05$; ** $p \leq 0.01$

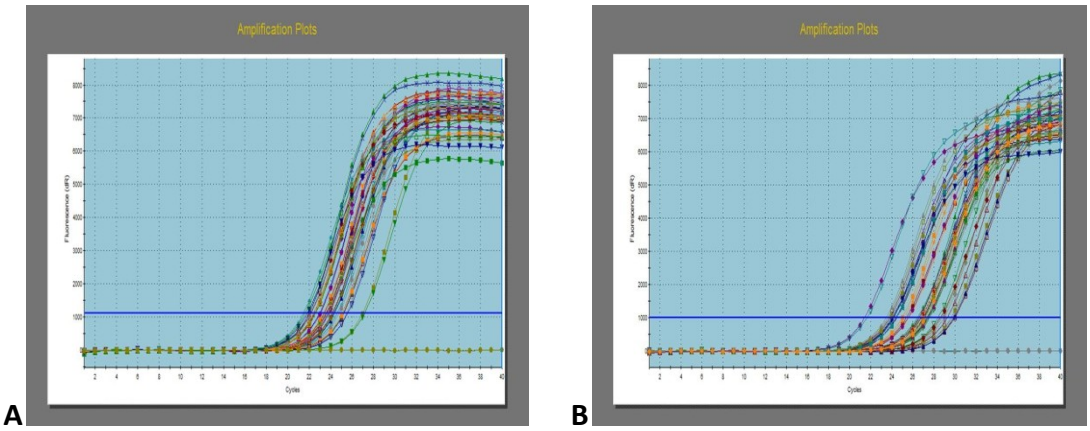


Fig. 3.6. Amplification plots of *NM23-H1* (A) and *NDRG1* (B) genes.

3.1.3. E-Cadherin

3.1.3.1. Immunohistochemical Analysis

In the normal epidermis (N), E-Cadherin immunostaining showed strong and easily detectable positivity **(Fig. 3.7A)**. However, its expression was heterogeneous and downregulated in NE-BCC. E-Cadherin staining was showed similar intensity and distribution with normal epidermis in NE-SCC.

E-Cadherin positivity was detected in all cases except in 7 BCCs (92.7%) **(Fig. 3.7B, C)**. Nuclear staining was seen very rarely and usually in strongly stained areas. Heterogeneous and less intense E cadherin staining was detected in all of the SCCs **(Fig. 3.7D)** except five cases of invasive tumors (84.3%)**.**

E-cadherin expression was stronger in the HaCaT cell line **(Fig. 3.7E)** compared to A-431**(Fig. 3.7F).**

3.1.3.2. HSCOREs

HSCOREs of E-Cadherin were downregulated in NE-BCC (p=0.01) but not in NE-SCC when compared to N **(Fig .3.8A, B)**.

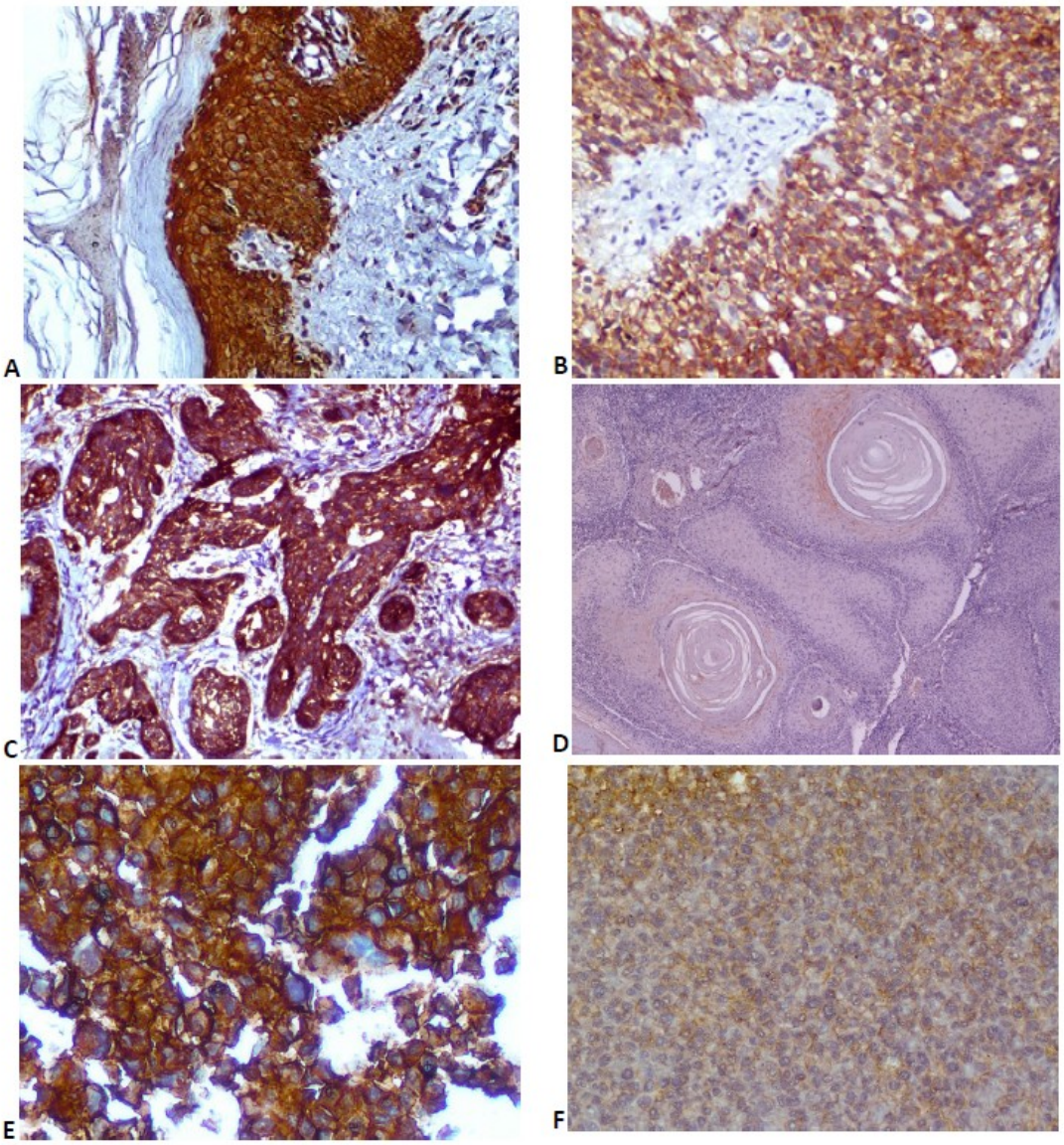
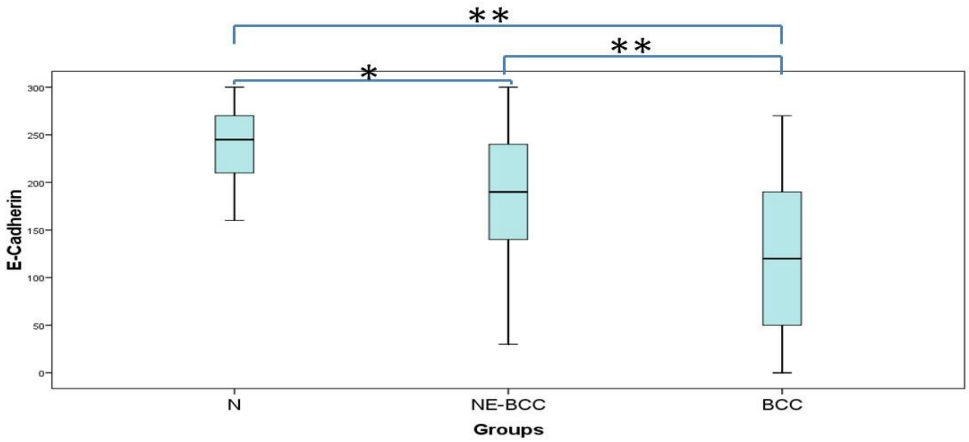


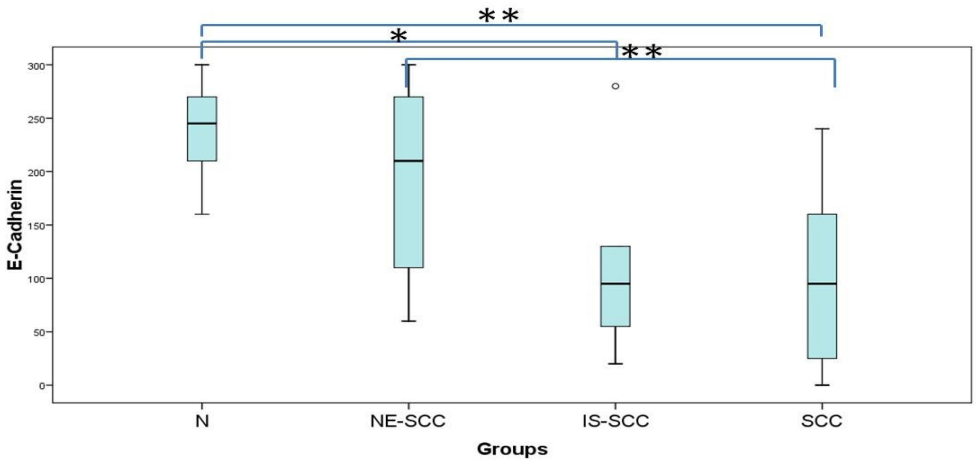
Fig. 3.7. E-Cadherin immunohistochemistry.Membranous E-Cadherin expression in normal epidermis (A). Significant E-Cadherin expression in nodular (B) and infiltrative (C) BCCs and also HaCaT cell lines (E). However downregulation is easily detected both squamous cell carcinoma and A-431 squamous carcinoma cell line. (D, X40; A, x100; B, C, E, F, x200)(Fig 3.C. Copyright; Int J Dermatol 2014)

There was statistically significant difference for E-cadherin (p=0.016) between the normal skin and in situ carcinomas. In in-situ carcinomas, the HSCOREs of E-

Cadherin were lower than the normal epidermis. However there was no statistical difference when compared to NE-SCC.



A



B

Fig. 3.8. Boxplot graphs of E-Cadherin. (A) BCC (B) SCC groups. HSCOREs of E-Cadherin were downregulated in NE-BCC (A) but not in NE-SCC (B) when compared to N. There were statistically significant differences for E-cadherin between the normal skin and in situ carcinomas. In in-situ carcinomas, the HSCOREs of E-Cadherin were lower than the normal epidermis (B). However there was no statistical difference when compared to NE-SCC. Invasive SCC (B) and BCCs (A) showed lower levels of E-Cadherin expression than normal epidermis and normal epidermis adjacent to neoplasia. *=$p \leq 0.05$; ** $p \leq 0.01$

BCCs (p=0.001; p=0.001) **(Fig. 3.8A)** and invasive SCCs (p=0.001; p=0.001) **(Fig. 3.8B)** showed lower levels of E-Cadherin expression than normal epidermis and normal epidermis adjacent to neoplasia.

3.1.3.3. qPCR Results.

We did not find any significant fold change of E-Cadherin expression between BCCs and normal skin groups **(Fig.3. 11A)**.When Squamous cell carcinoma cell line (A-431) was compared to normal immortalized cell line (HaCaT), There was no fold difference between two cell lines.

3.1.4. RHOGDI2

3.1.4.1. Immunohistochemical Analysis

Cytoplasmic RHOGDI2 positivity ($RHOGDI2_{cyt}$) was strong and easily detectable in N, NE-BCC, and NE-SCC **(Fig .3.9A)**. However, nuclear staining ($RHOGDI2_{nuc}$) intensities were reduced in NE-BCC and NE-SCC.

In BCCs, although RHOGDI2 staining showed cytoplasmic positivity ($RHOGDI2_{cyt}$) in 89 of 96 (92.7%) cases, the intensity was significantly reduced and heterogeneous staining was detected **(Fig. 3.9B)**. Nuclear expression ($RHOGDI2_{nuc}$) was hardly detectable and heterogeneous in 59 (61.4%) of 96 BCCs.

RHOGDI2 staining showed cytoplasmic positivity in 5 (83.3%) and 30 (93.7%), in

situ, and invasive cases, respectively, with weak-medium strength **(Fig. 3.9.C, D)**. $RHOGDI2_{nuc}$ was more weak and heterogeneous in 4 of 6 IS-SCCs (66.6%) and 14 of 32 cSCCs (43.7%). Tumor infiltrating lymphocytes were significantly positive in all tissue sections and used as an internal control **(Fig. 3.9B, D).**

RHOGDI2 showed strong but heterogeneous positivity in the HaCaT and A-431 cell lines **(Fig. 3.9E, F)**.

3.1.4.2. HSCORES

The HSCORES of $RHOGDI2_{cyt}$ in NE-BCC and NE-SCC show similar values as in N but $RHOGD1_{nuc}$ levels (p=0.001; p=0.037) were downregulated **(Fig. 3.10A, B).**

Both of the nuclear (p=0.001) and cytoplasmic (p=0.001) RHOGDI2 staining were downregulated in BCCs when compared to normal skin. Similarly $RHOGDI_{nuc/cyt}$ (p=0.001; p=0.001) showed reduced level in BCCs than NE-BCC **(Fig. 3.10A)**.

In the BCC group, the recurrent BCCs expressed higher $RHOGDI2_{nuc}$ levels (p= 0.01).

In in-situ SCCs, only $RHOGDI2_{nuc}$ (p=0.002) showed reduced levels than N. Invasive SCCs expressed reduced levels of $RHOGDI2_{nuc/cyt}$ than N (p=0.001; p=0.001) and also then NE-SCC (p=0.001; p=0.001).

3.1.4.3. qPCR Results.

We did not find any significant fold change of *ARHGDIB gene* between BCCs and normal skin **(Fig. 3.11B)**. However, when A-431 cell line was compared to HaCaT, ARHGDIB *(RHOGDI2)* (-4.7-fold, P=0.001) was downregulated in A-431 cell line.

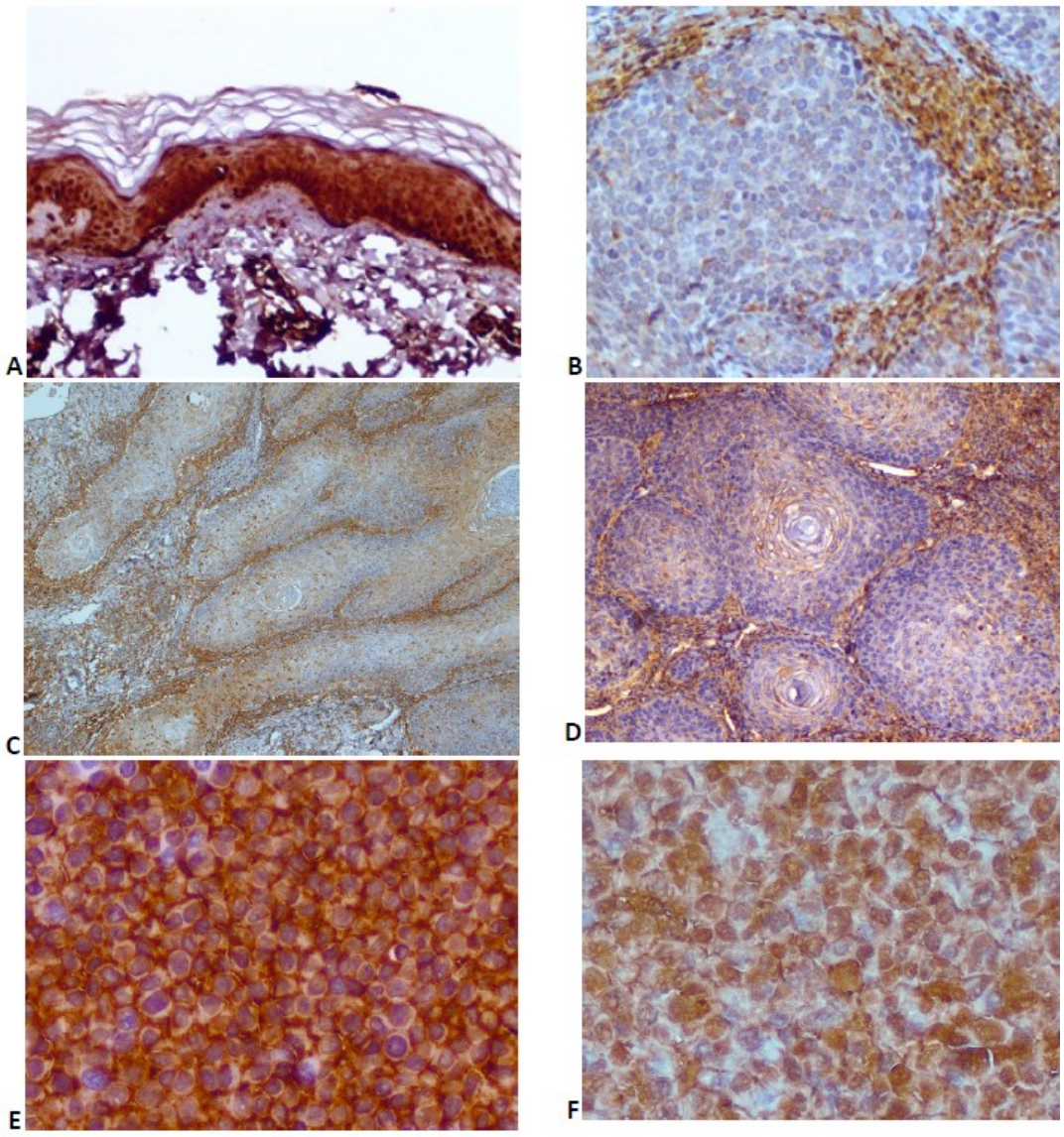
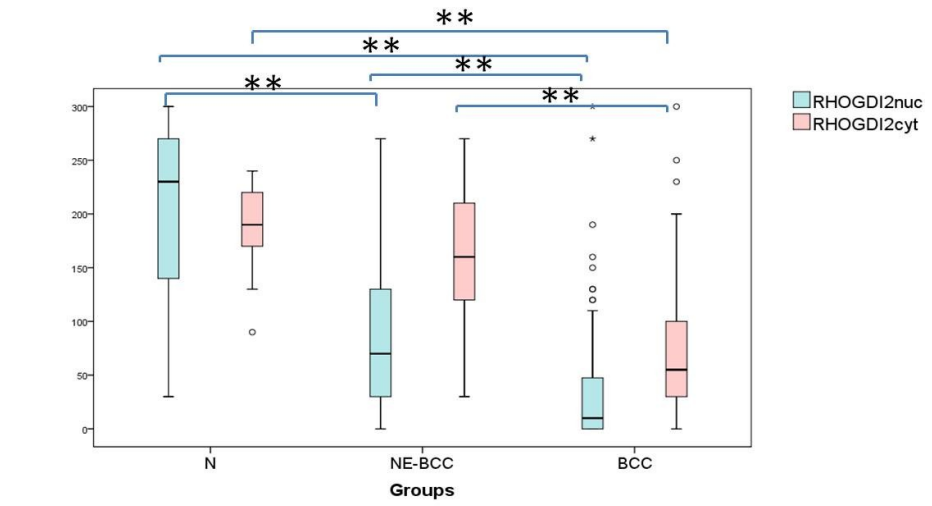
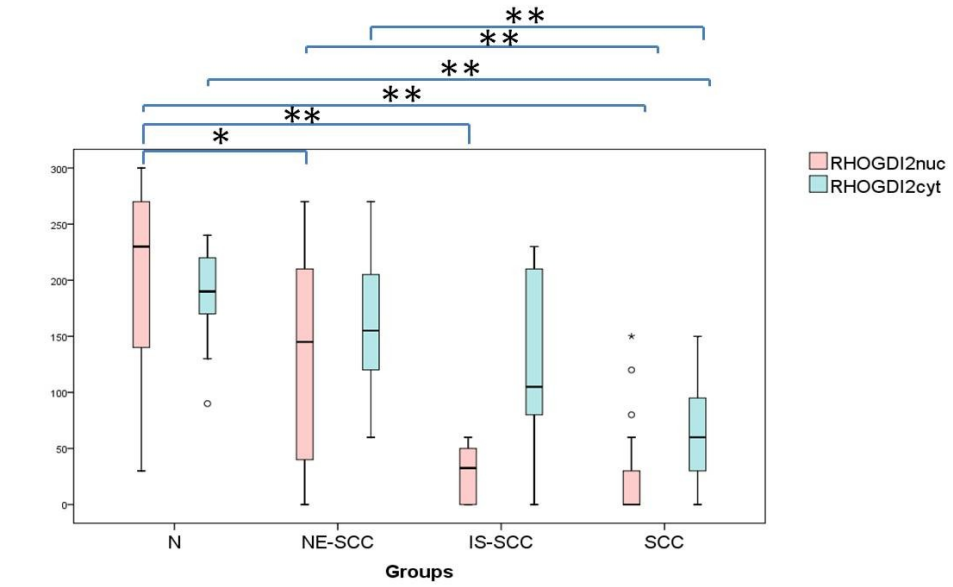


Fig. 3.9. RHOGDI2 immunohistochemistry. RHOGDI2 positivity in normal epidermis. (A) RHOGDI2 positivity is significantly reduced, positive staining cell are inflammatory cells in BCC (B).Reduced levels of RHOGDI2 in two different SCCs (C; D). Inflammatory cells around squamous islands can be used as an internal control (D). Strong mainly cytoplasmic positivity in HaCaT (E). Downregulated but basically nuclear positivity in A-431 (F). (A, x40; C, x100, B, D, E, F x200)

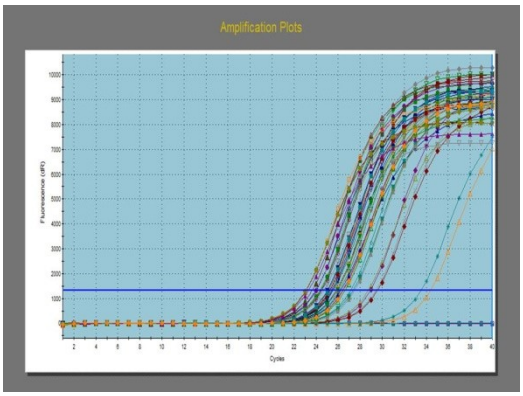


A



B

Fig. 3.10. Boxplot graphs of RHOGDI2. (A) BCC (B) SCC groups. The HSCORES of RHOGDI2cyt in NE-BCC (A) and NE-SCC (B) show similar values as in N but $RHOGD1_{nuc}$ levels were downregulated. Both of the nuclear and cytoplasmic RHOGDI2 staining were downregulated in BCCs when compared to normal skin (A). Similarly $RHOGDI_{nuc/cyt}$ expressed reduced level in BCCs than NE-BCC. In in-situ SCCs, only $RHOGDI2_{nuc}$ showed reduced levels than N. Invasive SCCs showed reduced levels of $RHOGDI2_{nuc/cyt}$ than N and also then NE-SCC (B). *=$p \leq 0.05$; ** $p \leq 0.01$

A 

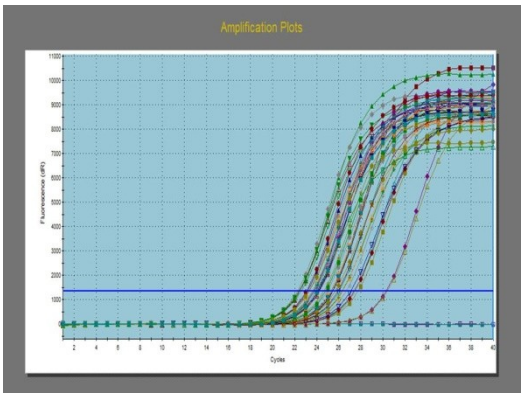
B 

Fig 3.11. Amplification plots of *CDH1 (E-Cadherin)* (A) and ARHGDIB *(RHOGDI2)* (B) genes in the skin tissue group.

3.1.5. MKK4

3.1.5.1. Immunohistochemical Analysis

In normal epidermis, cytoplasmic MKK4 ($MKK4_{cyt}$) was stained at medium intensities **(Fig. 3.12A)**. Nuclear staining of MKK4 ($MKK4_{nuc}$) was very weak and not easily detectable. Normal epidermis adjacent to BCCs (NE-BCC) showed more heterogeneous but not reduced positivity with $MKK4_{nuc/cyt}$ when compared to the normal epidermis. In normal epidermis neighboring cSCCs, $MKK4_{nuc/cyt}$ intensities were reduced.

MKK4 immunostaining of BCCs was weak/medium cytoplasmic positive in 73 (76%) and weak nuclear positive in only 35 (36.4%) of BCC **(Fig. 3.12B, C)**. $MKK4_{cyt}$ were detected in all of IS-SCCs and $MKK4_{nuc}$ was detected 5 of 6 IS-SCC(83,3%). Similar to BCCs, MKK4 immunostaining of cSCCs was showed weak/medium cytoplasmic positivity in 25 cases (78.1%), and weak nuclear positivity ($MKK4_{nuc}$) in 7 cSCCs (21.8%) **(Fig. 3. 12D)**.

MKK4 staining was very weak in the HaCaT cell line and completely negative in A-

431 **(Fig. 3.12E, F).**

3.1.5.2. HSCOREs

There was no statistical difference for MKK4 $_{nuc/cyt}$ scores between N and NE-BCC **(Fig. 3.13A).** However the scores were reduced in NE-SCC (p=0.014; p=0.031). When BCCs were compared to N (p=0.001; p=0.003) and NE (p=0.001; p=0.001), $MKK4_{nuc/cyt}$ levels were significantly showed lower scores in the BCCs.

In invasive cSCC, $MKK4_{nuc}$ showed significantly lower HSCOREs than normal epidermis (p=0.001) **(Fig. 3.13B)**. However, $MKK4_{cyt}$ levels were protected in cSCCs. In in-situ SCCs, $MKK4_{nuc/cyt}$ levels were also protected. Between in-situ carcinomas and invasive SCCs, $MKK4_{nuc}$ (p=0.011) showed higher scores in in-situ carcinomas.

3.1.5.3. qPCR Results.

We did not find any significant fold change of *MKK4* gene between BCCs and normal skin groups **(Fig. 3.16A)**. However, there was a reduction of *MKK4* gene expression in A-431 cell line than HaCaT (-2.1-fold, P=0.001).

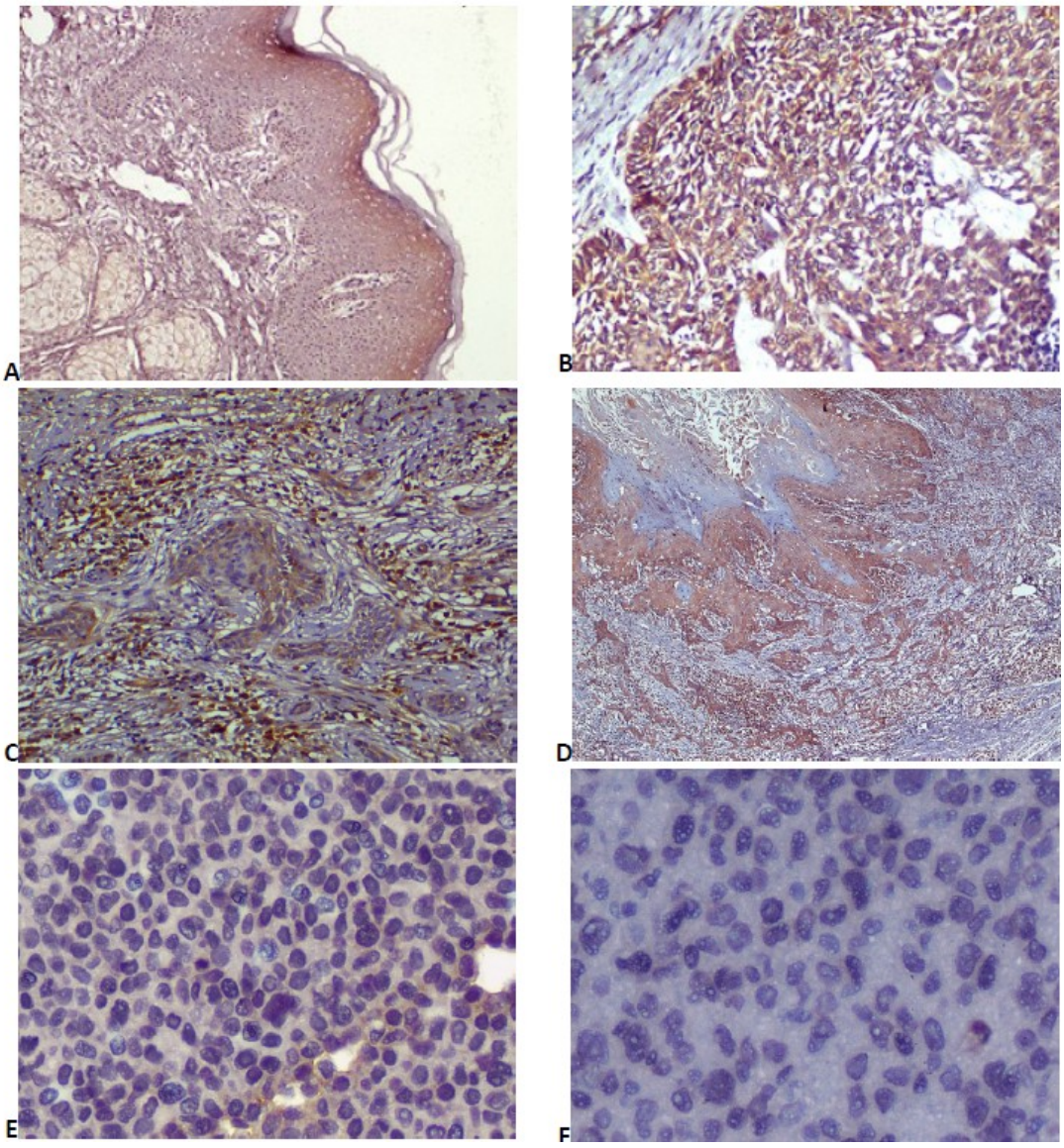
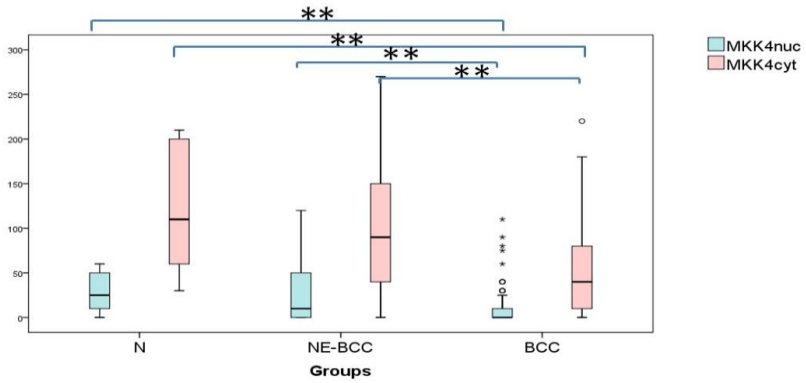
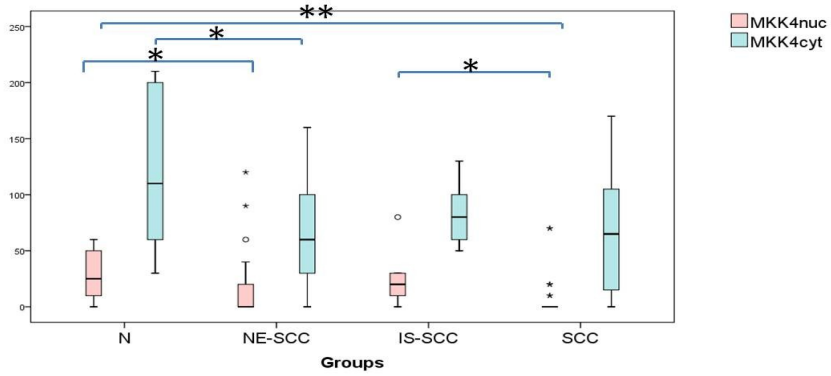


Fig. 3. 12. MKK4 immunohistochemistry. Medium intensity staining in normal epidermis (A).MKK4 positivity in NA- BCCs (B). In A-BCCs positivity is decreased (C). However invasive SCCs positivity is easily detectable (D) . Very weak positivity in HaCaT (E), but no positivity is detected in A-431 cell line (F). (D, x40; A, B, x100; C, E, F, x200) (Fig. 3.12C. Copyright; Int J Dermatol 2014)



A



B

Fig. 3.13. Boxplot graphs of MKK4. (A) BCC (B) SCC groups. There was no statistical difference for MKK4 $_{nuc/cyt}$ scores between N and NE-BCC (A). However the scores were reduced in NE-SCC. When BCCs were compared to N and NE, MKK4$_{nuc/cyt}$ levels were significantly showed lower scores in the BCCs (A). In invasive cSCC (B), MKK4$_{nuc}$ showed significantly lower HSCOREs than normal epidermis. However, MKK4$_{cyt}$ levels were protected in cSCCs. In in-situ SCCs, MKK4$_{nuc/cyt}$ levels were also protected. Between in-situ carcinomas and invasive SCCs, MKK4$_{nuc}$ showed higher scores in in-situ carcinomas. *=p≤0.05; ** p≤0.01

3.1.6. CD82/KAI1

3.1.6.1. Immunohistochemical Analysis

In normal epidermis, CD82/KAI was stained at medium intensities **(Fig. 3.14A)**. In normal epidermis neighboring BCCs and cSCCs, CD82/KAI staining showed similar intensity and distribution with normal epidermis **(Fig. 3.14B)**.

CD82/KAI positivity was reduced and only seen in focal areas of the BCCs in 14 (15.1%) of 96 cases **(Fig. 3.14B, C).** Different from BCCs group, CD82 positivity was protected all of the IS-SCCs and 22 of cSCCs (68.7%) **(Fig. 3.14D)**.

CD82/KAI1 showed medium intensity positive in both cell lines **(Fig. 3.14E, F)** , though positivity was more heterogeneous in A-431 cell line **(Fig. 3.14F)**.

3.1.6.2. HSCOREs

The only significant finding was significant downregulation of CD82/KAI1 in BCCs than N (p=0.001) and NE (p=0.001) **(Fig. 3.15A)**. No difference was detected between the others groups **(Fig. 3.15A, B)**.

3.1.6.3. qPCR Results

We did not found any significant fold change of *CD82* expression between BCCs and normal skin groups **(Fig. 3.16B)**. However, A-431 cell line showed less levels of *CD82/KAI* expression than HaCaT (-2.4-fold, P=0.001).

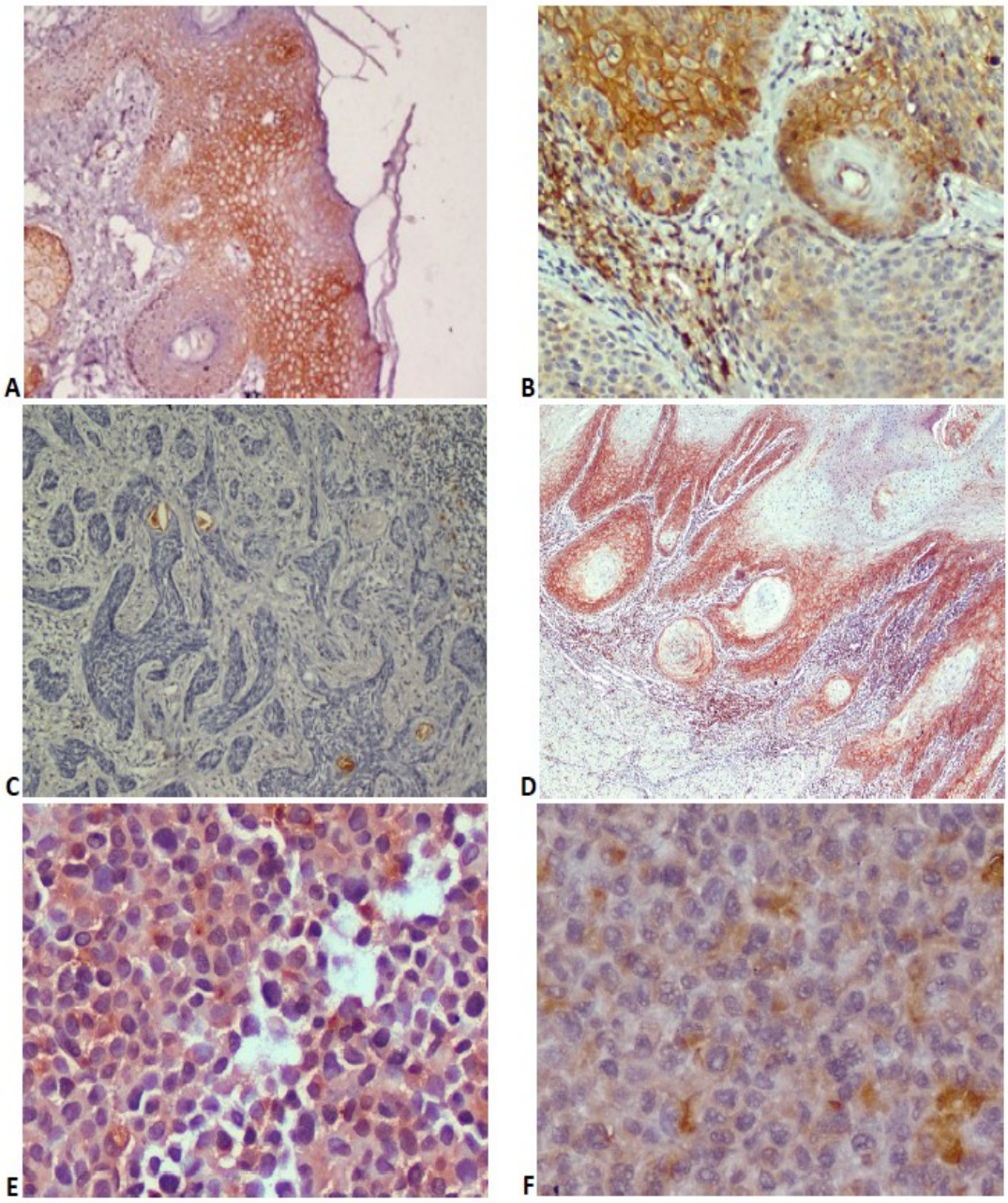
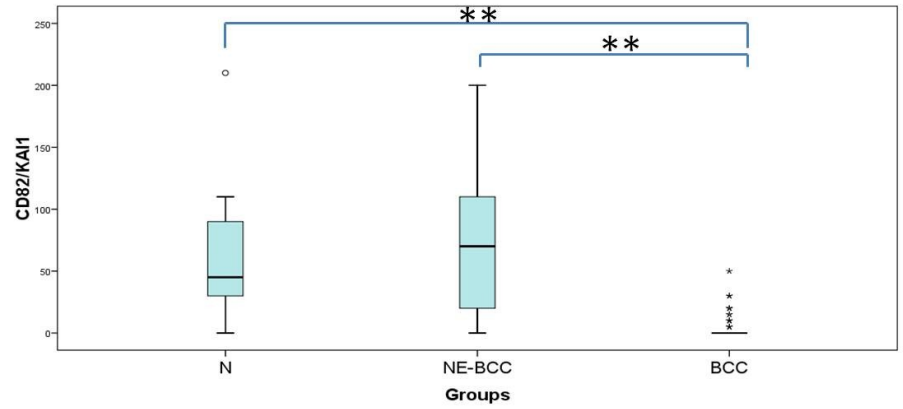
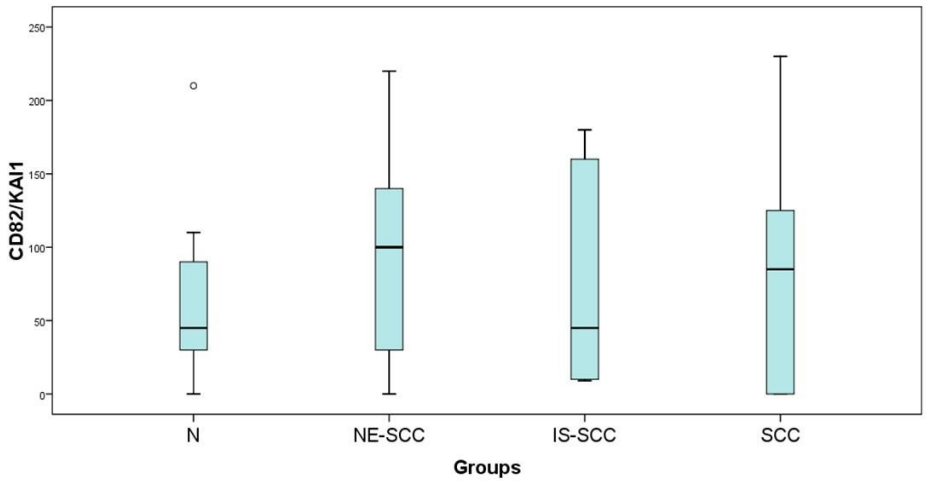


Fig. 3.14. CD82/KAI1 immunohistochemistry. Membraneous staining in normal skin (A). Normal skin show strong positivity adjacent to BCC, but BCC shows only focal weak positivity (B). In A-BCC CD82/KAI1 shows no positivity except focal keratinous differantiation areas (C). Contrast to BCCs, CD82/KAI1 is significantly positive in SCC (D). HaCaT cell line (E) shows stronger positivity than A-431 cell line (F). (A, C, D x100; B, E, F x200) (Fig. 3.14C. Copyright; Int J Dermatol 2014)

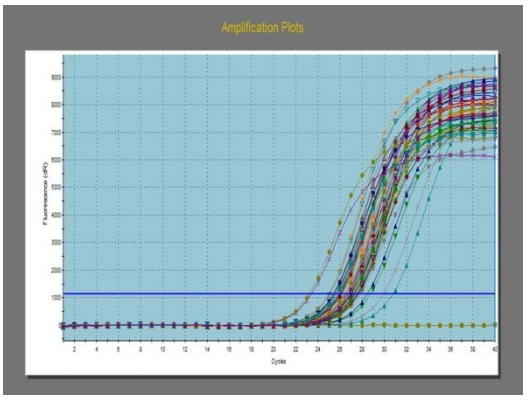


A



B

Fig. 3.15. Boxplot graphs of CD82/KAI1. (A) BCC (B) SCC groups. The only significant finding was downregulation of CD82/KAI1 in BCCs than N and NE. No difference was detected between the others groups.

A 

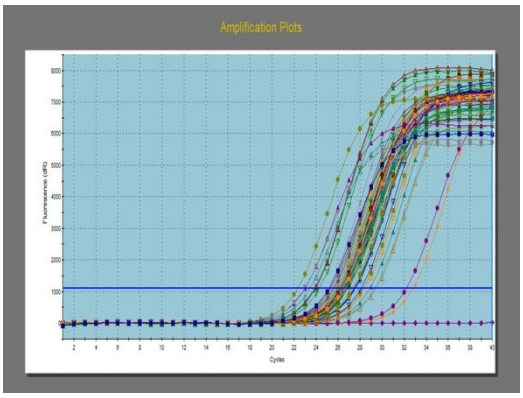
B 

Fig. 3.16. Amplification plots of *MKK4* (A) and *CD82 (*B) genes in the skin tissue group.

3.1.7. AKAP12

3.1.71. Immunohistochemical Analysis

In normal epidermis, AKAP12 positivity was seen at medium intensities **(Fig. 3.17A)**. NE-BCC and NE-SCC showed more heterogeneous and reduced positivity with AKAP12 antibodies when compared to the normal epidermis **(Fig. 3.17B)**.

AKAP12 positivity were significantly diminished and only seen in focal areas of the BCCs in 21 (21.8%) of 96 BCCs **(Fig. 3.17A, B)**. AKAP12 positivity was also significantly reduced and only seen in focal areas of 13 cSCCs (40.6%), and in 4 situ SCC (66.6%) **(Fig. 3.17D)**. Tumor stroma and nerves were significantly positive with AKAP12 antibody in tissue sections and used as an internal control **(Fig. 3.17C)**. AKAP12 staining was very weak or lost in both cell lines **(Fig. 3.17E, F)**.

3.1.7.2. HSCOREs

The HSCOREs of AKAP12 were downregulated both NE-BCC (p=0.015) and NE-SCC (p=0.026) when compared to N **(Fig. 3.18A, B)**. BCCs showed significantly reduced positivity than normal tissue groups (N, p=0.001; NE, p=0.001). Invasive SCC showed downregulation when compared to N (p=0.001). There were no differences between IS-SCC and normal groups, and also between cSCCs and NE-SCC.

3.1.7.3. qPCR Results.

We found significant downregulation of *AKAP12* expression (-1.2 fold; p=0.006) in BCCs when compared to normal skin **(Fig. 3.19A)**. Similarly, *AKAP12* (-9.7-fold, P=0.001) were also downregulated in A-431 cell line than HaCaT.

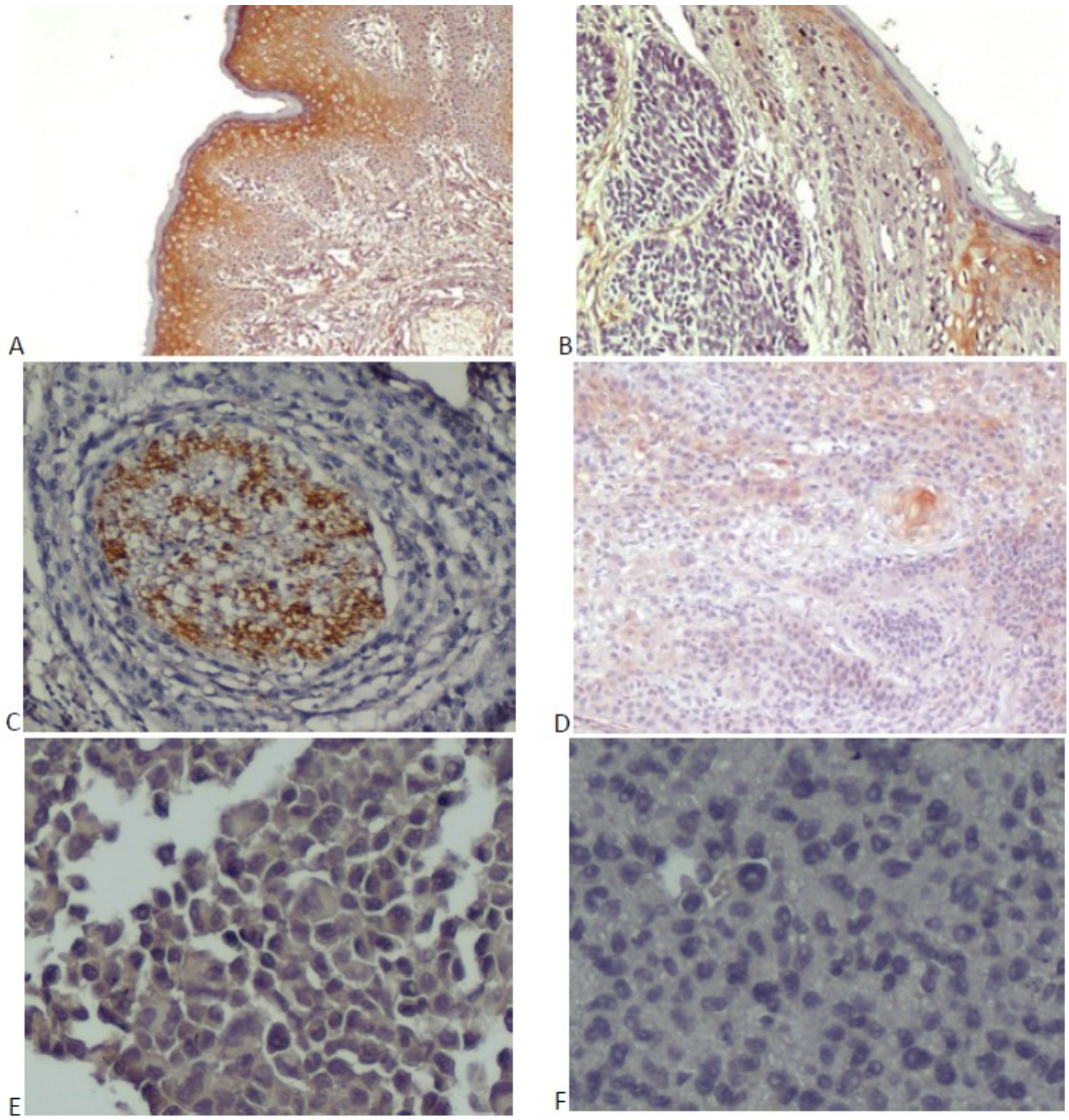
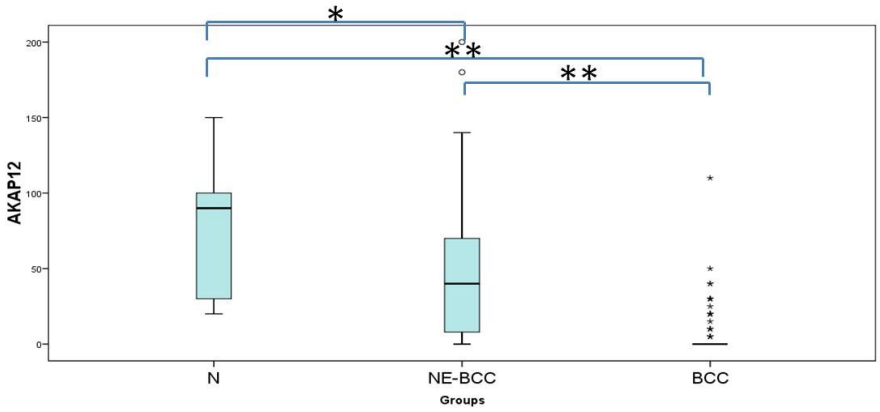
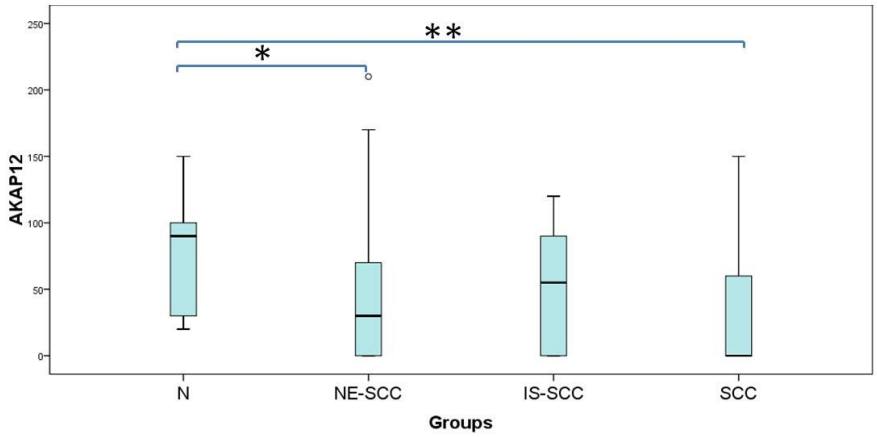


Fig 3.17. AKAP12 Immunohistochemistry. Medium intensity cytoplasmic positivity in normal epidermis (A). No positivity detected in BCC, however adjacent epidermis shows positivity (B). Perineural invasion. Strong AKAP12 expression of nerve and negative staining of tumor cells around (C). Focal medium intensity staining of AKAP12 in SCC (D). Weak cytoplasmic positivity in HaCaT cell line (E) but lost in A-431 (A). (A,B x100; D, x100, C, E, F, x200)



A



B

Fig 3.18. : Boxplot graphs of AKAP12. (A) BCC (B) SCC groups. The HSCOREs of AKAP12 were downregulated both NE-BCC (A) and NE-SCC (B) when compared to N. BCCs showed significantly reduced positivity than normal tissue groups (B). Invasive SCC showed downregulation when compared to N . There were no differences between IS-SCC and normal groups, and also between cSCCs and NE-SCC. *=p≤0.05; ** p≤0.01

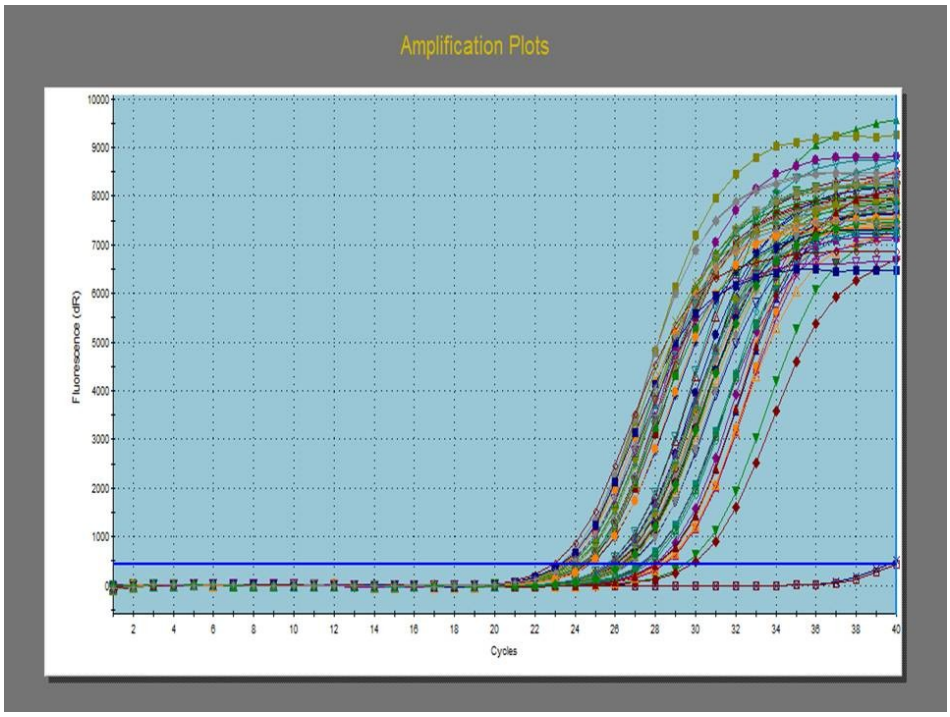


Fig. 3.19. Amplification plots of AKAP12 gene.

3.2. Correlation Analysis

In the BCC group, there were significant correlations (p=0.01 level) between several markers as follows **(Fig. 3.20)**; NM23-H1nuc-NM23-H1cyt (r=0.442); AKAP12-RHOGDI2cyt (r=0.333); AKAP12- NDRG1cyt (r=0.280); E-Cadherin-RHOGDI2cyt (r=0.303); E-Cadherin-NDRG1cyt (r=0.413), RHOGDI2nuc-RHOGDI2cyt (r=0.405); RHOGDI2nuc-MKK4cyt (r= -0.294); NDRG1nuc-NDRG1cyt (r= 0.356); MKK4nuc-MKK4cyt (r= 0.365). There were also significant negative correlations between AKAP12 and inflammation (r=-0.275; p=0.007).

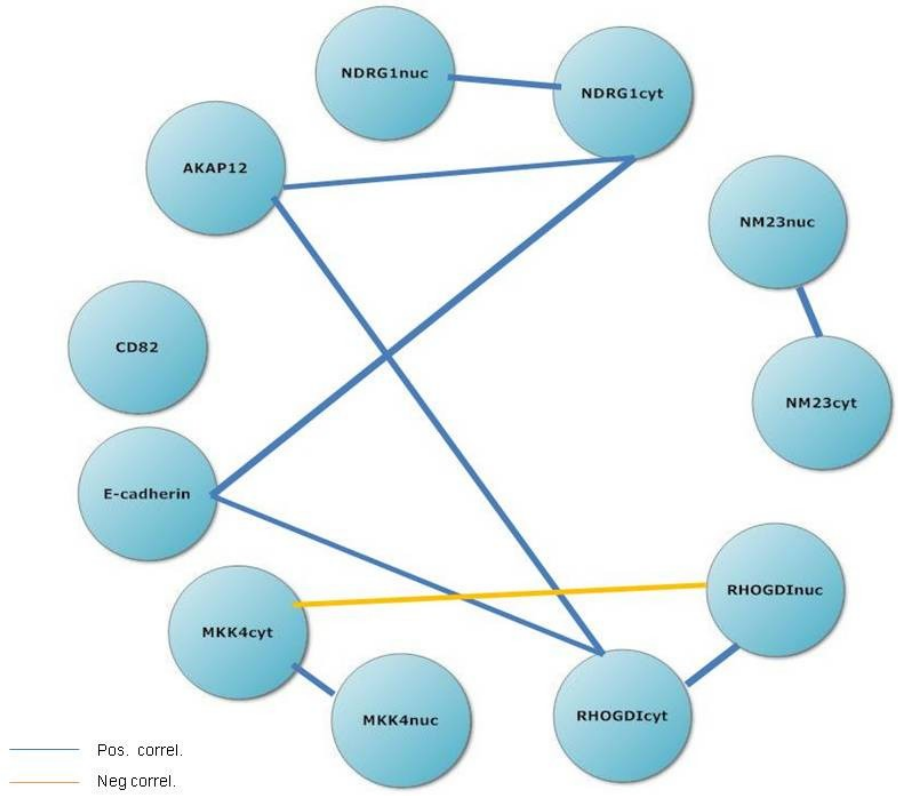


Fig 3.20. Schematic presentation of correlations in BCC study group.
NM23-H1nuc-NM23-H1cyt (r=0.442); AKAP12-RHOGDI2cyt (r=0.333); AKAP12- NDRG1cyt (r=0.280); E-Cadherin-RHOGDI2cyt (r=0.303); E-Cadherin-NDRG1cyt (r=0.413), RHOGDI2nuc-RHOGDI2cyt (r=0.405); RHOGDI2nuc-MKK4cyt (r= -0.294); NDRG1nuc-NDRG1cyt (r= 0.356); MKK4nuc- MKK4cyt (r= 0.365).

A randomly selected subgroup of 44 BCCs from the main group was stained with p53 primary antibody to show the correlations between MSPs and p53. However, we detected only a negative correlation with RHOGDI2cyt (r= -0.316; p= 0. 037).

In the SCC groups, there were significant correlations (p=0.01 level) between several markers as follows **(Fig. 3.21)**; E-cadherin-RHOGDI2cyt (r=0.452), RHOGDI2cyt-MKK4cyt (r=0.486), and MKK4nuc-MKK4cyt (r=0.481).

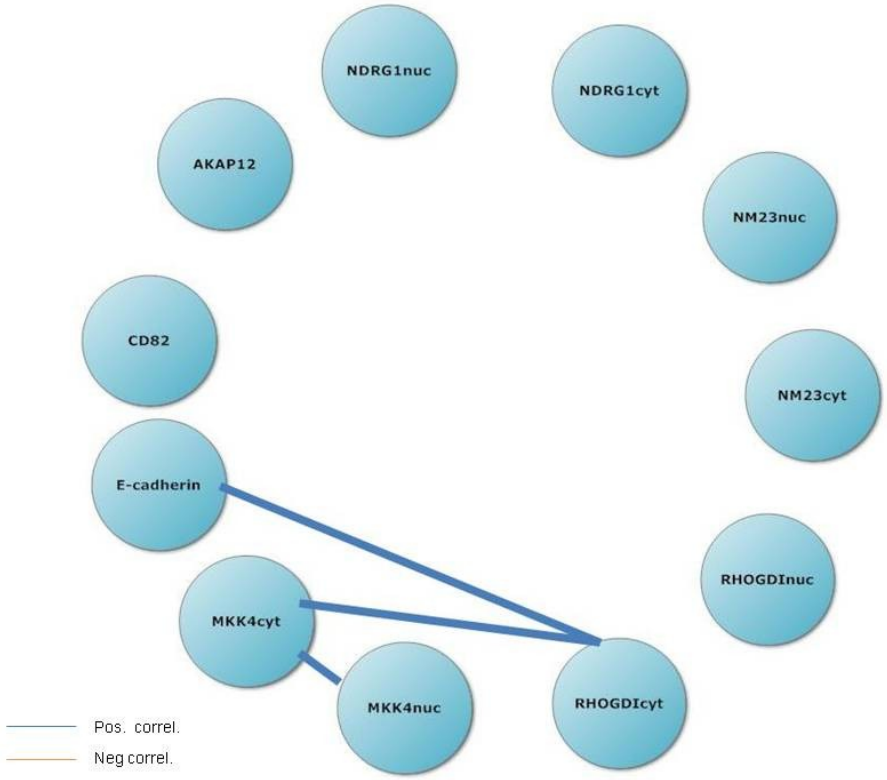


Fig. 3.21. Schematic presentation of correlations in SCC study group.
E-cadherin-RHOGDI2cyt (r=0.452), RHOGDI2cyt-MKK4cyt (r=0.486), and MKK4nuc-MKK4cyt (r=0.481).

3.3. Bisulfite Sequencing Results

3.3.1. MKK4

Promoter DNA methylation analysis of MKK4 gene in BCC and normal skin tissue samples was performed by bisulfite sequencing using primers amplifying -121 to +191 bp region of the gene. 58 colonies (34 BCCs, 24 normal skins) were sequenced from normal and tumor samples. Bisulfite sequencing of the 41 CpGs in the analyzed region revealed that none of the CpG dinucleotides were methylated in either non-tumor or tumor tissues **(Fig. 3.22).** Thus, the expression changes observed via immunohistochemical experiments of these tissues can not be related to the promoter methylation of MKK4 gene. Detailed methylation status of colonies

is demonstrated in **appendix B**

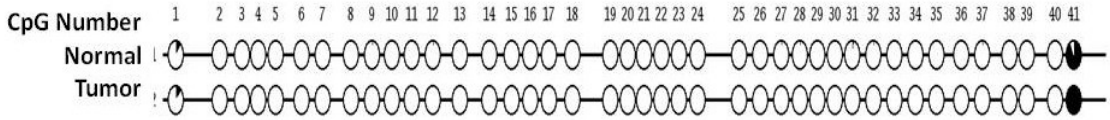


Fig. 3.22. Bisulfite sequencing of MKK4 gene promoter. Region covering -121bp to +191 bp of the MKK4 gene was analyzed using the internet based QUMA software. Each pie chart represents the percentage of methylated CpGs in either non tumor (upper) or tumor(lower) samples. 58 colonies (34 BCCs, 24 normal skins) were sequenced.

3.3.2. CD82/KAI1

A region spanning from -179 to +152 (331bp) of the gene was analyzed in tissue samples by bisulfite sequencing method. A total of 32 CpGs were analyzed. Total 68 colonies (40 BCC, 28 normal skin) were sequenced from samples but no differential methylation was found between BCC and normal groups. Actually both groups were heavily unmethylated **(Fig. 3.23)**. Detailed methylation status of colonies is demonstrated in **appendix B.**

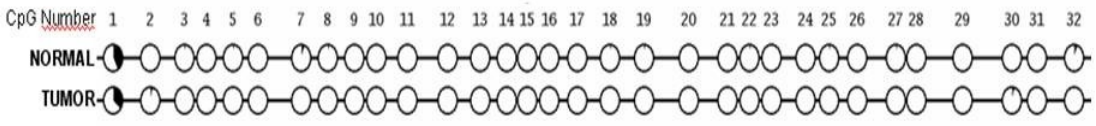


Fig 3.23. Bisulfite sequencing of CD82 gene promoter. A region spanning from -179 to +152 (331bp) was analyzed with QUMA. Each pie chart represents the percentage of methylated CpGs in either normal (upper) or tumor (lower) samples. Total 68 colonies (40 BCC, 28 normal skins) were sequenced.

CHAPTER 4- DISCUSSION AND CONCLUSION

Metastasis is the most important feature of cancer and significantly related to poor prognosis in clinical practice [109, 224]. It is a complex mechanism for the tumor cells because of various factors. First of all, tumor cells should gain same functional properties for metastasis [107]. These functional advantages are probably formed by mutations and epigenetic mechanisms [95]. Metastasis associated genes, classified as metastasis initiation, metastasis progression and metastasis virulence genes (Nguyen *et al.*) usually have functions in cell motility, invasivity, angiogenesis, immune evasion and colonization [113, 114]. There is a strong effort to include metastasis-associated genes in studies [225, 226]. Metastasis-associated genes supporting the metastasis cascade in different organ cancers are well defined [227]. On the contrary, a group of gene and coded proteins inhibit or suppress metastasis [121]. This “Yin and Yang” game probably determines the fate of the tumor. Metastasis suppressor proteins (MSPs) are crucial role control of metastasis and they often downregulated in different steps of metastasis cascade. In this study, we selected seven well-known MSPs that covers the different steps of metastatic cascade including E-cadherin in EMT and invasion, CD82/KAI1 in intravasation and transport, MKK4 in migration and colonization, NDRG1 in angiogenesis, invasion and colonization, AKAP12 in angiogenesis and migration, and Nm23-H1 and RhoGDI2 in migration and colonization [212, 215].

Non-melanoma skin carcinomas are common malignant neoplasms in the human population and are associated with significant morbidity [8]. Although more valid for BCCs, they show limited metastatic potential and usually stable as local invasive

neoplasms [1]. BCCs display all the features of the malignant tumors including invasion, with the exception of metastasis. To date, the question of why BCCs metastasize only rarely has not been adequately answered but It has been postulated that the strictly stromal dependence of BCC is decrease the rate of metastasis [228]. cSCCs have significant metastatic capacity when compared to BCCs. However, cSCCs are usually more quiet neoplasms when compared to similar internal organ malignancies [1, 8].

In this study, we analyzed seven imported MSGs and MSPs, which covers nearly all areas of metastasis steps, in basal cell and squamous cell carcinoma of the skin. We found a differential expression signature of MSPs in BCCs somewhat different from other malignant tumors. NM23-H1 was protected in all types of BCC. Similarly, preserved cytoplasmic expression of NDRG1 and E-cadherin were detected but AKAP12, CD82/KAI1 expressions were significantly decreased in BCCs. Despite downregulation compared to normal epidermis, the expressions of other proteins were somewhere between the two extremes. As we expected, a significant number of MSPs are downregulated in SCCs. However, we cannot find any difference of NM23-H1 as in BCCs, and no significant difference was detected for CD82 and cytoplasmic expression of MKK4 when compared to normal epidermis.

We detected that NM23-H1 HSCOREs were protected in BCCs and, along with immunohistochemistry, mRNA level of *NM23-H1 (1.4-fold)* in BCCs was higher than normal skin. Besides BCC, NM23-H1 are also significantly protected and showed higher scores in SCCs in this study. The expression of NM23-H1 in BCC and cSCC is controversial. Similar to our findings, Ro Y et al. showed that the expression of

NM23-H1 was well protected and stained intense in cSCCs of the skin and keratoacanthomas (KA). In this study, all of the BCCs were also positively stained but showed reduced levels when compared to cSCCs and KA.[229] Similar results also demonstrated by Stephenson *et al*. in cSCCs and KA.[230] However, Kanitakis et al. showed contradictory results and showed reduced NM23-H1 levels in cSCCs and higher levels in BCCs [231].

Despite the significant expression of NM23-H1 in BCCs and cSCCs, its importance is not well known. it has been shown that NM23-H1 expression is inversely related to the metastasis status in other human carcinomas [232]. The reduction of NM23-H1 levels significantly associated with aggressive behavior in several carcinomas including breast, gastric, ovarian cancer and melanoma [196, 198, 233]. However, NM23 is not a MSP for all type cancers. It has been well documented that NM23 act as a metastasis promoter in human neuroblastomas [234-236]. The significant NM23-H1 expression in BCCs ad cSCCs probably contributes to their non-metastasizing feature.

One of the important results of this study was the demonstration of significant cytoplasmic NDRG1 expression in BCC and the immunohistochemical data was also supported with the qRT-PCR study. We detected similar results in in-situ and invasive SCCs. Cytoplasmic NDRG1 expression are protected in cSCCs and mRNA expression of squamous cell carcinoma cell line A-431 were 34-fold increased when compared to normal immortalized cell line HaCaT. To our knowledge, NDRG1 expression has not been demonstrated in BCC in the English literature previously and only limited expression studies are reported to establish the importance of

NDRG1 in cSCCs. Dang, C et al. demonstrated that NDRG1 mRNA expression increased 5.87-fold in AKs, and 7.07-fold in cSCCs when compared to normal skin [132]. In the English literature, importance of NDRG1 has been examined in squamous cell carcinomas of the internal organs. Dos Santos M *et al* showed the significant upregulations of NDRG1 in oral and oropharyngeal SCCs and authors concluded that NDRG1 overexpression was significantly correlated to long-term survival [237]. Similar results were also reported by Chang JT. et al [134]. In esophageal SCCs, the prognostic importance of NDRG1 has also been demonstrated [238]. Besides oral and esophageal SCCs, the prognostic importance of NDRG1 expression has been pointed out in other human tumors including prostate, breast and colon [123, 124, 239, 240]. However, the MSP function is probably carcinoma-dependent. NDRG1 overexpression in hepatocellular carcinoma has been shown to be an indicator of poor prognosis [241]. In non small cell lung carcinoma, its expression is significantly associated with advanced stages and weak vascularization [242].

Despite slight downregulation, E-cadherin expression was generally preserved in BCCs and qRT-PCR studies also showed no significant difference from normal skin. The data from the literature and our study show that E-cadherin positivity is expected in BCC, even if reduced compared to the normal epidermis. Although we cannot find any difference between A-BCC and NA-BCCs, the reduction of E-cadherin expression in infiltrative BCCs has been shown [243, 244]. A recent article demonstrated E-cadherin expression to be significantly higher in metastatic BCCs than nodular BCCs [245]. Unexpected results of metastatic cases from the literature

and our study need to be investigated further regarding the importance of E-cadherin in BCCs.

In contrast to that found in BCCs, E-cadherin levels were significantly downregulated in cSCCs. Downregulation of E-cadherin levels has been demonstrated in cSCC before [246, 247]. It has also been reported that the reduced levels of E-cadherin expression is more evident in acantholytic subtypes of cSCCs [248]. Although downregulation of E-cadherin was seen in both BCCs and cSCCs, more significant downregulation in cSCCs may show that epithelial mesenchymal transition (EMT) is somewhat different in cSCC from BCCs.

CD82/KAI expressions were significantly reduced or lost in all types of basal cell carcinoma but not in cSCCs. Similar to our results, the downregulation of CD82/KAI1 expression in BCCs was demonstrated in a limited previous study [249]. It is clear that all types of BCCs show reduced expression of CD82/KAI1 protein levels similar to other carcinomas and CD82/KAI1 probably has no or minimal contribution to the non-metastatic features of BCCs [165]. Even though downregulation of CD82/KAI1 is an expected phenomenon in human carcinomas, the mechanisms of this down-regulation are not well known. As a preliminary study, we investigated the promoter DNA methylation of *CD82/KAI1* gene in a small group of BCC and normal skin tissues. However, no significant promoter DNA methylation was found in normal and neoplastic groups. Melanoma cell lines, oral squamous cell carcinoma, prostate carcinoma, and bladder carcinoma have also given similar results in the literature [222, 250-252]. However, Drucker *et al.* detected CD82/KAI1 promoter methylation in multiple myeloma cells [253]. Probably, promoter methylation of

CD82 is tissue specific and differential expression of CD82 in BCC and normal skin samples may not depend on promoter methylation in our samples. In the literature, other mechanisms have been proposed to explain the downregulation of CD82 in human carcinomas. Mashimo *et al*. demonstrated that the expression of CD82/KAI1 is regulated by p53 [254]. There are some clues of complex mechanisms between p53, CD82/KAI1 and with other important proteins Jun B and AP2 [255, 256]. This suggested correlation was examined in our study group at immunohistochemistry level. However, no significant correlation was found between p53 and CD82/KAI in BCCs. Other studies questioning this correlation also found negative results, similar to our results [250, 257-259].

In contrast from BCCs, CD82 is one of the protected proteins in cSCC. Similar to our findings Okachi et al. showed significant downregulation in basal cell carcinoma of the skin but protected in Bowen disease [249]. The importance of CD82 in other human squamous cell carcinoma is controversial. The downregulation was demonstrated in oral, cervical, penile, laryngeal, head and neck and lung SCCs [260] [172, 261]. Interestingly, when compared to cSCCs, all of these carcinomas showed significant metastasis potential. Relatively protected levels of CD82 may be helpful for the non-metastasing features of cutaneous SCCs.

In this study, we detected a significant downregulation or AKAP12 immunohistochemical staining and mRNA in BCC. Although we can demonstrate downregulation in cSCC, this decline is not sharp as seen in BCCs. The downregulation of AKAP12 in human carcinomas has been reported in various types of human cancer [205]. The expression pattern of AKAP12 is not known in skin

carcinomas. A recent study focused on epigenetic regulation of AKAP in skin cancer and the authors pointed out that the promoter methylation frequencies were significantly higher in carcinomas than normal skin tissue. Authors demonstrated that AKAP12 methylation frequiencies were in cSCC, BCC and AK, 89.6%, 87.1% and 51.2%, respectively [209]. We believe CD82/KAI and AKAP12 are interesting negative markers for BCCs but not for cSCCs, and further studies might show their importance in the differential diagnosis.

MKK4 were also downregulated in BCC when compared to the normal epidermis, but were not completely diminished, and we could not find any difference from normal skin in the qRT-PCR study. Slightly different from BCCs, cytoplasmic MKK4 expression was somewhat protected in SCCs in our study. In prostate and ovary carcinomas, it has been shown that MKK4 expression is reduced during cancer progression [262, 263]. Similar results were shown in other epithelial cancers including endometrial carcinoma, gastric carcinoma, and pancreatic cancer. [264-266] MKK4 is generally accepted as a metastasis suppressor but there are conflicting reports in the literature. Huang C *et al.* found higher expression of MKK4 in laryngeal squamous cell carcinomas in comparison with their normal counterpart, and furthermore, they showed a positive correlation between higher expression of MKK4 and metastasis [267] In a recent experimental article, Finegan *et al.* proposed that MKK4 has pro-oncogenic roles in skin carcinoma [268]. Although our data do not parallel their findings in basal cell carcinoma, it may support their hypothesis in cSCCs. This shows that the MKK4 profile is closely related to tumor type.

The mechanism of MKK4 down-regulation is not clear in human carcinomas. In this study, we could not demonstrate any correlation with p53 expression and showed that the expression changes observed via immunohistochemical experiments of these tissues is probably not related to the promoter methylation of MKK4 gene in basal cell carcinoma. Similarly, Spillman MA *et al.* detected no promoter methylation in 21 ovarian carcinomas [269]. Other possible down-regulation mechanisms have been also investigated in the literature. Genetic alteration of MKK4 was limited to 3% in the examined human carcinoma cell lines [270]. Mutation rate with loss of function was generally very low, approximately around 5% in human carcinoma [190, 266, 271].

We demonstrated significant downregulation of RHOGDI2 in non-melanoma skin cancer. To our knowledge, RHOGDI2 expression has not been studied in skin carcinomas previously. The MSP function of *RHOGDI2* was first shown as an MSG in bladder carcinoma [145]. Because of conflicting reports, the metastasis suppressor function of this protein is probably tissue or organ-dependent. RHOGDI2 has been shown to be upregulated in gastric and ovarian carcinoma and probably has a dual role in carcinogenesis [272].

RHOGDI2 is generally accepted as a cytoplasmic protein with occasional nuclear positivity [144]. In this study, nuclear expression was strictly found in the normal epidermis, and the sharp decline found in tumor groups was roughly correlated with cytoplasmic positivity in BCC and cSCCs. Several reports in the English literature have shown nuclear staining in the breast and gastric carcinoma but the importance of nuclear staining is not well known and further molecular functional

studies are needed [148, 150, 273]. However, significant downregulation of nuclear expression in both types of skin carcinoma may show different meanings of nuclear and cytoplasmic expression of RHOGDI2.

We have demonstrated significant but medium strength correlations between the markers in both BCCs and SCCs groups. Although not easy to interpret, the presence of such correlations between the proteins we studied might show these proteins work closely together in related MSP pathways. One of the relatively strong correlations which we found was between E-cadherin and $NDRG1_{cyt}$ (p=0.001) in BCCs and has also reported in the literature previously. Guan *et al.* showed that NDRG1 induces E-cadherin levels in colon carcinoma cell lines, and the authors suggested that NDRG1 induces colon cancer differentiation [274]. Furthermore, an *in-vitro* study showed that NDRG1 was involved in the recycling of E-cadherin and recently, the relationship between E-cadherin and NDRG1 has also been emphasized in prostate carcinomas [275, 276]. Our data clearly supports an E-cadherin/NDRG1 pathway in human carcinomas.

One of the goals of the study was to show the correlation between the MSPs and important clinicopathological parameters in non-melanoma skin cancer. In BCCs, we found AKAP12 to be inversely correlated with inflammation. This may be expected because of the close relationship of MSPs in the tumor microenvironment and the supportive role of inflammation in cancer progression [109, 272]. Another inverse relationship was between NM23-$H1_{nuc}$ and perineural invasion. NM23-$H1_{nuc}$ was also downregulated in high stage SCCs. Besides these expected correlations, we found that recurrences were correlated only with $RHOGDI2_{nuc}$. These results may be

explained by the dual and unpredicted role of RHOGDI2 in carcinomas as proposed by Griner and Theodorescu [272]. Although these correlations might indicate complex relationships between MSPs and clinicopathological parameters, it is not easy to interpret them correctly without large clinical and experimental studies.

The tumor suppressor p53 has critical functions in cell response to stress and shows significant interactions with various proteins. We found only an inverse correlation between $RHOGDI2_{cyt}$ and p53. Although the relationship between p53 and RHOGDI2 has not been demonstrated previously, interaction between p53 and CD82/KAI1, another MSP, has also been reported. Mashimo *et al*. demonstrated that the expression of CD82/KAI1 is regulated by p53 [254]. There are some clues for the presence of complex mechanisms between p53, CD82/KAI1 and other important proteins such as Jun B and AP2 [255, 256]. However, other studies questioning this correlation also found negative results, similar to our results [250, 257-259].

One of major questions in this study is the contribution of MSPs to the aggressive phenotype of BCCs. We detected upregulation of NDRG1 levels in the aggressive phenotype (p=0.001). Similarly, CD82/KAI levels (p=0.048) were downregulated. These results may show a slightly different profile of MSPs in aggressive carcinomas than NA-BCCs.

In this study, we detected that normal skin near the NMSC showed different MSP expression than normal non lesional skin. This fact may be related to complex relationship of tumor and tumor environment. However, because of age difference between our normal and tumour groups, we can not exclude the possibility that this

difference may be related to aging skin.

In conclusion, we have demonstrated differential expression patterns for the seven MSPs in BCCs and in situ and invasive cSCCs. The AKAP12, CD82/KAI1 levels were significantly reduced in BCCs. However, NM23-H1 and NDRG1 levels and also E-cadherin levels were minimally reduced and they were generally expressed in this neoplasm group. The other markers were also reduced but not lost in BCCs. In SCCs the metastasis suppressor gene expression is similar but not identical to BCCs. Although NM23-H1, and NDRG1 were protected and RGHOGDI2 and AKAP12 were downregulated in cSCCs as in BCCs, significant E-Cadherin downregulation also attracted our attention. Furthermore MKK4 and CD82 are also protected in cSCCs. Although this is a very simplified approach, preserved levels of NM23-H1 and NDRG1 may contribute to the non-metastatic features of non-melanotic skin carcinomas.Data from this study might also reveal possible pathways between MSPs, using the current knowledge on pathways. This relationship between these MSPs warrants further biological and experimental pathway research.

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APPENDIX-A

qRT PCR Study Group

No:	Age	M/F	Type
Q-1	61	M	Non-Aggressive,Focal infiltrative areas[#]
Q-2	83	M	Non-Aggressive,Focal infiltrative areas[#]
Q-3	36	F	Non-Aggressive,Focal infiltrative areas[#]
Q-4	73	M	Non-Aggrresive
Q-5*	67	M	Non-Aggrresive
Q-6*	67	M	Non-Aggrresive
Q-7*	67	M	Non-Aggrresive
Q-8	90	M	Non-Aggrresive
Q-9	86	F	Non-Aggrresive
Q-10	50	M	Non-Aggrresive
Q-11	76	F	Non-Aggrresive

* Different biopsies from same patient [#]=Infiltrative areas less than 50%

Basal Cell Carcinoma Study Group

No:	Age	M/F	Localisation	Type	Max.Dia.	Inv.Dep.	Anat.Inv.	Lymph.	Perineur.	Inf.	Rec.
B-1	77	M	Head and Neck	Non-aggressive	0,6	0,14	3	-	-	0	-
B-2	50	F	Head and Neck	Non-aggressive	1,5	0,9	4	-	-	1	-
B-3	57	F	Head and Neck	Non-aggressive	0,3	0,1	4	-	-	2	-
B-4	54	F	Head and Neck	Non-aggressive	0,7	0,2	5	-	-	3	-
B-5	59	F	Head and Neck	Non-aggressive	0,6	0,4	4	-	-	1	-
B-6	52	F	Head and Neck	Non-aggressive	1,5	0,3	3	-	-	2	-
B-7	80	M	Head and Neck	Non-aggressive	1,2	0,2	4	-	-	0	-
B-8	48	M	Head and Neck	Non-aggressive	0,8	0,34	5	-	-	1	-
B-9	52	M	Head and Neck	Non-aggressive	0,3	0,18	3	-	-	1	-
B-10	58	F	Head and Neck	Non-aggressive	0,6	0,4	4	-	-	2	-
B-11*	54	F	Head and Neck	Non-aggressive	0,3	0,25	4	-	-	0	-
B-12*	54	F	Head and Neck	Non-aggressive, adenoid	0,4	0,4	5	-	-	1	-
B-13	60	M	Head and Neck	Non-aggressive	0,7	0,1	3	-	-	2	-
B-14	49	M	Head and Neck	Non-aggressive	0,4	0,2	3	-	-	3	-
B-15	59	F	Head and Neck	Non-aggressive	0,7	0,3	5	-	-	2	-
B-16	81	F	Head and Neck	Non-aggressive	0,7	0,23	4	-	-	3	-
B-17	46	F	Head and Neck	Non-aggressive	0,4	0,14	4	-	-	1	-
B-18	74	F	Head and Neck	Non-aggressive	0,7	0,13	5	-	-	3	-
B-19	48	F	Head and Neck	Non-aggressive	0,6	0,23	4	-	-	1	-
B-20	85	M	Head and Neck	Non-aggressive	0,8	0,14	4	-	-	3	-
B-21	55	M	Head and Neck	Non-aggressive	0,2	0,5	2	-	-	3	-
B-22**	76	M	Head and Neck	Non-aggressive	2,2	0,2	4	-	-	3	-
B-23**	76	M	Head and Neck	Non-aggressive,superficial	5	0,1	3	-	-	3	-

No:	Age	M/F	Localisation	Type	Max.Dia.	Inv.Dep.	Anat.Inv.	Lymph.	Perineur.	Inf.	Rec.
B-24	53	F	Head and Neck	Non-aggressive	0,5	0,25	4	-	-	2	-
B-25	80	M	Head and Neck	Non-aggressive	1	0,27	4	-	-	3	-
B-26	34	M	Head and Neck	Non-aggressive	0,4	0,1	3	-	-	3	-
B-27	71	M	Head and Neck	Non-aggressive	0,6	0,14	3	-	-	3	-
B-28	80	M	Head and Neck	Non-aggressive	1,6	0,62	4	-	-	3	-
B-29	61	M	Head and Neck	Non-aggressive, adenoid	0,8	0,3	4	-	-	2	-
B-30	64	M	Head and Neck	Non-aggressive	0,4	0,25	3	-	-	1	-
B-31	70	F	Head and Neck	Non-aggressive	0,8	0,2	3	-	-	2	-
B-32	66	F	Trunk	Non-aggressive	2,5	1,5	5	-	+	1	-
B-33	74	M	Head and Neck	Non-aggressive	1	0,5	4	-	-	1	-
B-34	76	F	Head and Neck	Non-aggressive	0,7	0,6	4	-	-	2	-
B-35	56	M	Head and Neck	Non-aggressive	0,8	0,12	3	-	-	1	-
B-36	70	M	Trunk	Non-aggressive, superficial	12	0,04	2	-	-	2	-
B-37	67	F	Head and Neck	Non-aggressive	0,6	0,2	4	-	-	2	-
B-38	69	F	Trunk	Non-aggressive, superficial	1,5	0,6	2	-	-	2	-
B-39	51	F	Head and Neck	Non-aggressive	0,7	0,17	3	-	-	1	-
B-40* **	61	M	Head and Neck	Non-aggressive	1,3	0,25	4	-	-	0	+
B-41	69	F	Head and Neck	Non-aggressive	0,3	0,1	3	-	-	3	-
B-42	79	M	Head and Neck	Non-aggressive, superficial	0,6	0,15	4	-	-	3	-
B-43	75	M	Head and Neck	Non-aggressive, adenoid	1,5	0,5	4	-	-	1	-
B-44	36	F	Head and Neck	Non-aggressive	1	0,36	4	-	-	2	-
B-45* **	62	M	Head and Neck	Non-aggressive	0,6	0,15	3	-	-	2	-
B-46	75	F	Head and Neck	Non-aggressive, adenoid	1,1	0,25	4	-	-	1	-
B-47	60	M	Head and Neck	Non-aggressive, adenoid	1	0,6	5	-	-	1	-
B-48	82	F	Head and Neck	Non-aggressive	0,3	0,07	3	-	-	1	-
B-49	85	M	Head and Neck	Non-aggressive	1,5	0,36	5	-	-	2	-

No:	Age	M/F	Localisation	Type	Max.Dia.	Inv.Dep.	Anat.Inv.	Lymph.	Perineur.	Inf.	Rec.
B-50	72	F	Head and Neck	Non-aggressive	0,4	0,4	5	-	-	0	-
B-51	92	F	Head and Neck	Non-aggressive	0,7	0,28	3	-	-	2	-
B-52	59	M	Head and Neck	Non-aggressive	2	0,18	4	-	-	2	+
B-53	45	M	Head and Neck	Non-aggressive	1,5	0,4	4	-	-	1	-
B-54	57	F	Head and Neck	Non-aggressive	0,3	0,16	3	-	-	1	-
B-55****	74	M	Trunk	Non-aggressive, superficial	0,4	0,06	2	-	-	2	-
B-56****	74	M	Trunk	Non-aggressive, superficial	2,5	0,02	2	-	-	2	-
B-57	55	F	Head and Neck	Non-aggressive	0,9	0,1	4	-	-	3	-
B-58	60	M	Head and Neck	Non-aggressive	2	0,2	4	-	-	3	-
B-59	57	F	Head and Neck	Non-aggressive	0,6	0,34	4	-	-	3	-
B-60	75	M	Head and Neck	Non-aggressive	0,3	0,15	3	-	-	0	-
B-61	71	F	Head and Neck	Non-aggressive	0,4	0,2	4	-	-	1	+
B-62	37	M	Head and Neck	Non-aggressive	0,4	0,22	4	-	-	2	-
B-63	61	M	Head and Neck	Non-aggressive	0,4	0,17	4	-	-	3	-
B-64	83	M	Head and Neck	Non-aggressive	1	0,5	5	-	-	1	-
B-65	69	F	Head and Neck	Non-aggressive	0,5	0,23	4	-	-	1	-
B-66	72	M	Head and Neck	Non-aggressive	0,8	0,13	4	-	-	2	-
B-67	43	M	Head and Neck	Non-aggressive	3	0,2	4	-	+	1	-
B-68	84	M	Head and Neck	Non-aggressive	2	0,27	4	-	-	1	-
B-69	75	M	Head and Neck	Aggressive	3,5	0,15	4	-	-	2	-
B-70	79	M	Head and Neck	Aggressive	0,5	0,24	4	-	-	3	-
B-71	61	F	Head and Neck	Aggressive	1	0,4	5	-	+	2	+
B-72	75	M	Head and Neck	Aggressive	0,4	0,3	4	-	-	1	-
B-73	53	F	Head and Neck	Aggressive	0,3	0,26	5	-	+	1	-
B-74	80	F	Head and Neck	Aggressive, morpheiform	1,2	0,16	4	-	-	1	-
B-75	81	F	Head and Neck	Aggressive	3	0,65	5	+	+	1	+

No:	Age	M/F	Localisation	Type	Max.Dia.	Inv.Dep.	Anat.Inv.	Lymph.	Perineur.	Inf.	Rec.
B-76	57	M	Head and Neck	Aggressive	0,6	0,3	4	-	-	1	+
B-77	71	M	Head and Neck	Aggressive	0,3	0,1	3	-	-	1	-
B-78	76	F	Head and Neck	Aggressive	1,4	0,12	5	-	+	3	-
B-79	78	F	Head and Neck	Aggressive	2	0,5	5	-	-	2	-
B-80	84	F	Head and Neck	Aggressive	1,6	0,17	5	-	-	2	-
B-81	49	M	Head and Neck	Aggressive	0,5	0,09	3	-	-	2	-
B-82	75	M	Head and Neck	Aggressive	0,3	0,2	4	-	-	3	+
B-83	87	M	Head and Neck	Aggressive	1,5	0,12	3	-	-	2	-
B-84	71	F	Head and Neck	Aggressive	0,6	0,18	4	-	-	3	-
B-85	53	F	Head and Neck	Aggressive	0,4	0,13	4	-	-	3	-
B-86	62	M	Head and Neck	Aggressive	1,6	0,45	5	-	-	2	-
B-87	91	F	Head and Neck	Aggressive	Inc.Biop.	Inc.Biop.	Inc.Biop.	-	-	2	-
B-88	95	M	Head and Neck	Aggressive	1	0,8	4	-	-	3	-
B-89	74	M	Head and Neck	Aggressive	2,5	0,4	4	-	+	3	-
B-90	83	F	Head and Neck	Aggressive	2,3	0,5	4	-	-	3	-
B-91	67	F	Head and Neck	Aggressive	0,3	0,25	4	-	-	1	-
B-92	81	F	Head and Neck	Aggressive	0,3	0,32	4	-	-	1	-
B-93	60	M	Head and Neck	Aggressive	0,8	0,2	3	-	-	1	-
B-94	49	F	Head and Neck	Aggressive, focal micronodular component.	0,5	0,17	5	-	-	2	-
B-95	58	M	Head and Neck	Aggressive	1,2	0,16	3	-	-	2	-
B-96	74	F	Head and Neck	Aggressive	1,3	0,25	5	-	-	1	+

*Different biopsies from same patient. **Max.Dia**.= Maximum diameter (cm), **Inv.Dep**=Invasion depth(cm), **Lymph.Inv**=Lymphovascular space invasion, **Perineur.Inv**=Perineural invasion, **Anat.Inv=**Anatomical invasion level (Clark), **Inf=** Inflamation (Grade 0-3), **Rec=** Recurrence. **Inc. Biop:** Inc

Squamous Cell Carcinoma Study Group

No	Age	M/F	Loc.	Type	Gr.Dim	Tum Dept.	Anat.Inv	Lym.	Perineur.	Grade	Inf	TNM	Stage
IS-1	89	F	Trunk	SCC-Insitu			1					TisN0M0	0
IS-2	79	M	Trunk	SCC-Insitu			1					TisN0M0	0
IS-3	67	M	Extremities	SCC-Insitu			1					TisN0M0	0
IS-4	81	F	Extremities	SCC-Insitu			1					TisN0M0	0
IS-5	66	F	Head and Neck	SCC-Insitu			1					TisN0M0	0
IS-6*	86	M	Head and Neck	SCC-Insitu			1					TisN0M0	0
S-1	53	F	Head and Neck	SCC-NOS	0.4	0,08	2	-	-	1	1	T1N0M0	1
S-2	69	M	Head and Neck	SCC-NOS	0.5	0,08	3	-	-	1	2	T1N0M0	1
S-3	72	M	Head and Neck	SCC-NOS	1.5	0,09	3	-	-	1	3	T1N0M0	1
S-4	75	M	Lips	SCC-NOS	2.4	0,5	4	-	-	1	2	T2N0M0	2
S-5	40	M	Extremities	SCC-NOS	8	1	4	-	-	1	3	T2N0M0	2
S-6	73	M	Lips	SCC, verrucous carcinoma with invazive areas	1.7	0,2	3	-	-	1	2	T1N0M0	1
S-7	43	M	Head and Neck	SCC-NOS	N/A	0,5	5	-	-	1	3	T2N0M0	2
S-8	64	M	Lips	SCC-NOS	3	0,5	5	+	-	1	2	T2N0M0	2
S-9	59	M	Lips	SCC-NOS	1.8	0,3	5	-	-	1	2	T2N0M0	2
S-10	44	M	Trunk	SCC-NOS	4.2	0,8	5	-	-	1	1	T2N0M0	2
S-11	79	M	Head and Neck	SCC-NOS	1.1	0,5	5	-	-	1	2	T2N0M0	2
S-12	75	F	Head and Neck	SCC-NOS	0.8	0,09	3	-	-	1	3	T1N0M0	1
S-13	71	M	Head and Neck	SCC-NOS	3	1,5	5	+	+	1	2	T2N1M0	3
S-14	68	F	Head and Neck	SCC,keratoacanthoma like.	1.8	0,4	4	+	+	1	2	T2N0M0	2
S-15	57	F	Head and Neck	SCC-NOS	0.7	0,3	4	-	-	1	2	T2N0M0	2

No	Age	M/F	Loc.	Type	Gr.Dim	Tum Dept.	Anat.Inv	Lym.	Perineur.	Grade	Inf	TNM	Stage
S-16	83	F	Lips	SCC-NOS	2	0,4	5	-	-	1	1	T2N0M0	2
S-17	66	M	Head and Neck	SCC-NOS	0.8	0,3	5	-	-	1	2	T2N0M0	2
S-18	57	M	Head and Neck	SCC-NOS	6	0,9	3	-	-	1	2	T2N0M0	2
S-19	69	F	Head and Neck	SCC-NOS	2.3	0,8	5	+	+	1	2	T2N0M0	2
S-20	85	M	Head and Neck,Ear	SCC-NOS	0.6	0,2	5	-	-	2	1	T2N0M0	2
S-21	71	M	Head and Neck,Ear	SCC-NOS	0.6	0,1	4	-	-	2	1	T2N0M0	2
S-22	71	M	Head and Neck	SCC-NOS	0.6	0,6	5	-	-	2	3	T2N0M0	2
S-23	66	M	Lips	SCC-NOS	2.2	0,4	5	-	-	2	1	T2N0M0	2
S-24*	86	M	Head and Neck	SCC-NOS	2.5	0,6	5	-	+	2	2	T2N1M0	3
S-25	81	F	Head and Neck	SCC-NOS	0.6	0,3	2	-	-	2	2	T1N0M0	1
S-26	65	M	Lips	SCC-NOS	1.5	0,6	4	-	-	2	3	T2N0M0	2
S-27	74	M	Head and Neck, Ear	SCC-NOS	2	0,3	4	+	-	2	2	T2N0M0	2
S-28	79	M	Head and Neck, Ear	SCC-NOS	0.5	0,4	5	-	-	3	3	T2N0M0	2
S-29	74	M	Head and Neck, Ear	SCC-NOS	4.5	1	5	-	+	3	1	T2N0M0	2
S-30	73	M	Lips	SCC-NOS	0.7	0,4	5	-	-	3	3	T2N0M0	2
S-31	85	F	Head and Neck	SCC-NOS	1.8	N/A	3	-	-	3	1	T1N1M0	3
S-32	67	M	Head and Neck, Ear	SCC-NOS	2	0,4	5	-	-	3	2	T2N0M0	2

SCC-NOS: Squamous cell carcinoma-not otherwise specified. NA:Not applicable due to incisional biopy or other reason. *Different biopsies from same patient. **Gr.Dia**.= Greater diameter (cm), **Tum.Dep**=Tumor depth(cm), **Lymph**=Lymphovascular space invasion, **Perineur**=Perineural invasion, **Anat.Inv=**Anatomical invasion level (Clark), **Inf=** Inflamation (Grade 0-3),

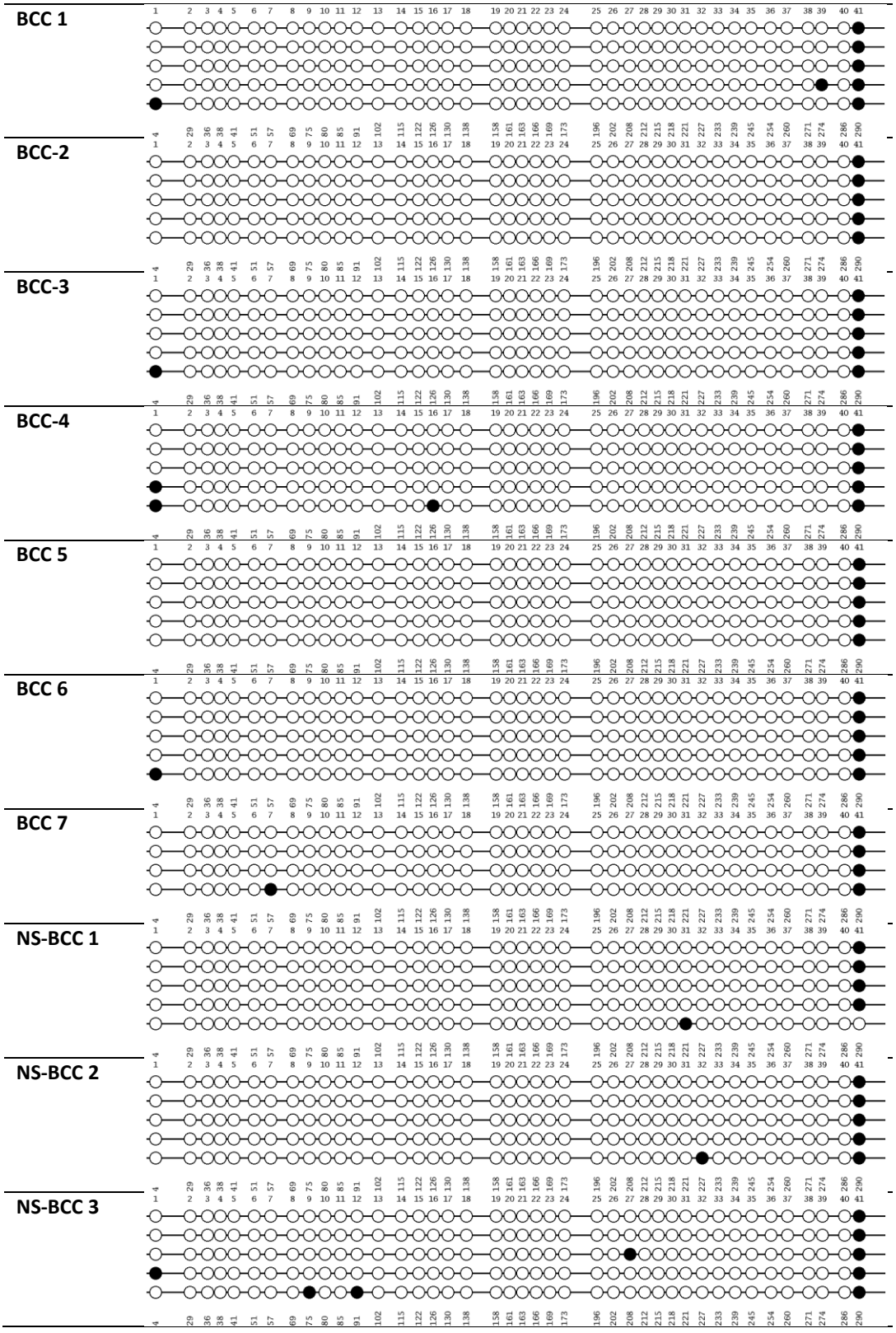
Anatomic Level *

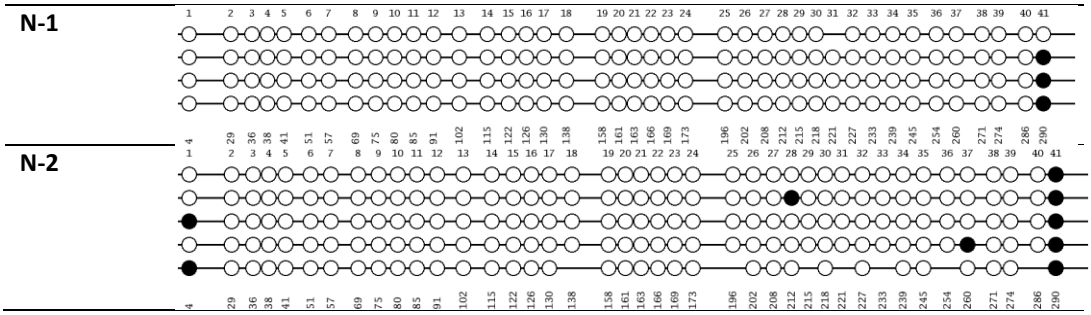
I (carcinoma in situ)
II (carcinoma present in but does not fill and expand papillary dermis)
III (carcinoma fills and expands papillary dermis)
IV (carcinoma invades reticular dermis)
V (carcinoma invades subcutaneum)

* www.cap.org

APPENDIX B

Bisulfite Sequencing of MKK4 Gene Promoter

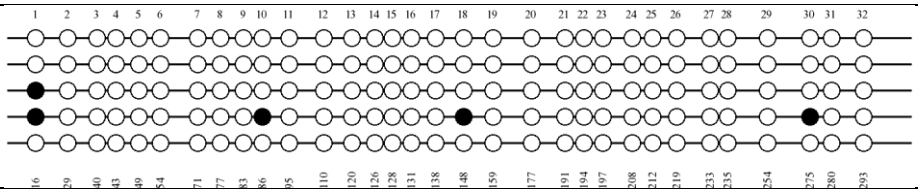




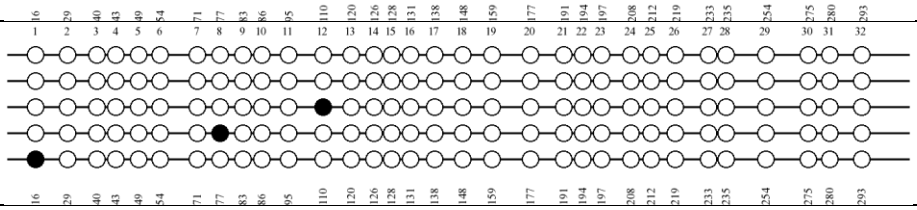
BCC=Basal cell carcinoma, NS=Normal skin adjacent to BCC, N=Normal non-lesional skin.

. Bisulfite Sequencing of CD82 Gene Promoter

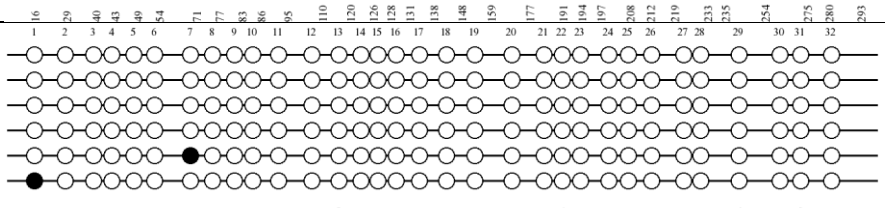
BCC 1



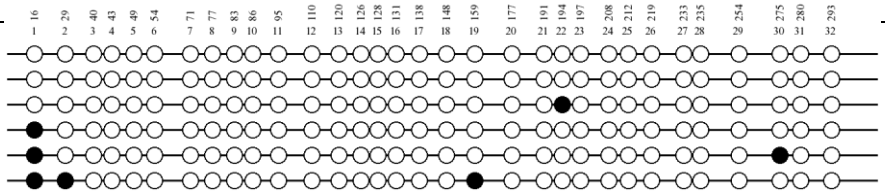
BCC-2



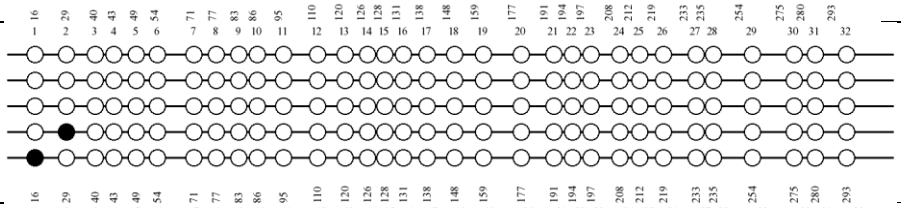
BCC-3



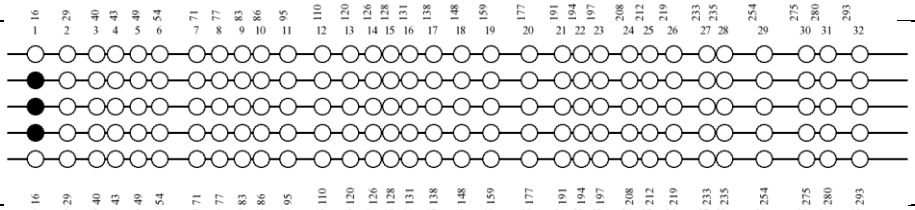
BCC-4



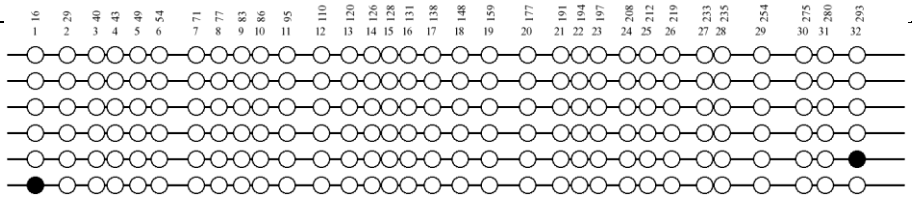
BCC 5



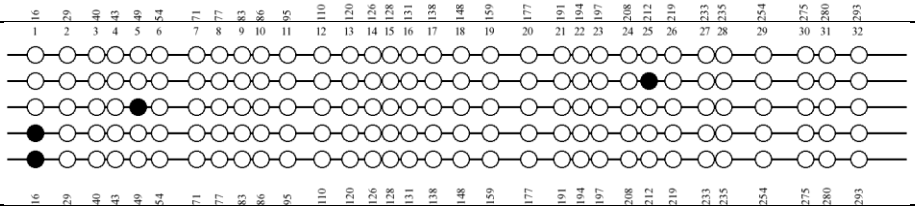
BCC 6

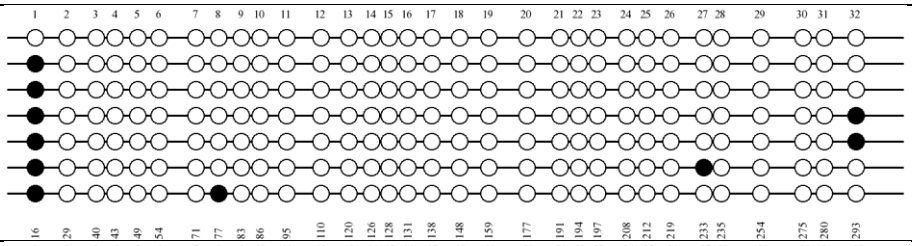
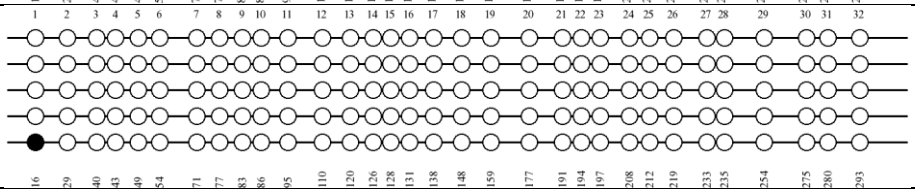
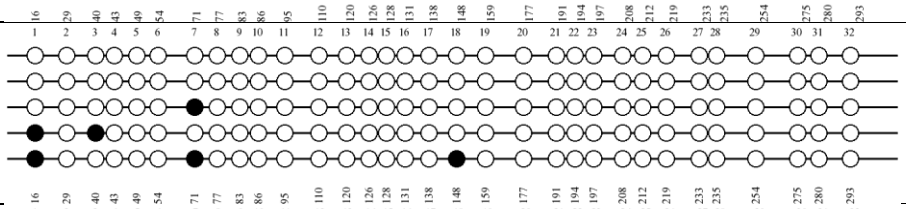
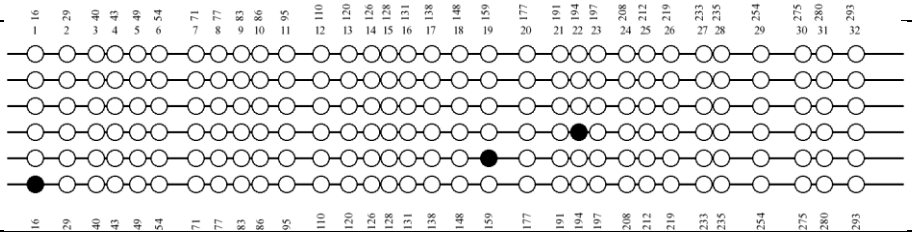


BCC 7



NS-BCC 1



NS-BCC 2	
NS-BCC 3	
N-1	
N-2	

BCC=Basal cell carcinoma, NS=Normal skin adjacent to BCC, N= Normal non lesional skin.