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Departamento de Química

MARIA JOÃO DA SILVA VIEIRA

CARACTERIZAÇÃO MOLECULAR E IMUNOHISTOQUÍMICA DE CARCINOMAS BASOCELULARES DE INDIVÍDUOS DA ZONA DE VILA REAL

MOLECULAR CHARACTERIZATION AND IMMUNOHISTOCHEMISTRY OF BASAL CELL CARCINOMAS OF INDIVIDUALS FROM VILA REAL

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, ramo Métodos Biomoleculares, realizada sob a orientação científica da Doutora Paula Boaventura, Investigadora no Instituto de Patologia e Imunologia Molecular da Universidade do Porto, e do Professor Pedro Domingues do Departamento de Química da Universidade de Aveiro

Dedico este trabalho aos meus pais, irmã e restante família pelo apoio incondicional ao longo de todos estes anos

o júri

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Palavras-chave Carcinoma Basocelular, radiação UV, subtipos histológicos, Via de Hedgehog, Síndrome de Gorlin, mutações no promotor TERT

Resumo

O carcinoma basocelular (BCC) constitui o tipo mais comum de cancro de pele, cuja origem continua em debate, com possível resposta nas céulas estaminais. Apesar da histologia mista ser comum, existem três subtipos principais de BCC (nodular, superficial e infiltrativo), que têm características histológicas distintas e diferentes níveis de agressividade. Este tipo de cancro de pele tem alguns fatores de risco associados, bem como mecanismos moleculares que estão implicados no seu desenvolvimento. O supressor tumoral *p53*, responsável pela resposta a danos no DNA, está presente em níveis elevados em vários tipos de cancro, incluindo o BCC. A transcriptase reversa da telomerase (TERT) é a subunidade da telomerase que é responsável pela manutenção do comprimento dos telómeros, e mutações no seu promotor têm sido consideradas como fator de prognóstico para vários tipos de cancro, inclusive para o BCC. O principal objetivo desta tese é descrever as mutações do promotor da *TERT* (*TERTp*) e a expressão da p53 no BCC, possibilitando a descoberta de possíveis biomarcadores desta patologia. A expressão da p53 foi avaliada através de imunohistoquímica de 165 lesões de 97 pacientes. Foi ainda realizada análise molecular das mutações do *TERTp* em 52 BCCs. Através da revisão histológica das lesões observámos a existência de uma associação estatisticamente significativa entre tumores maiores, diminuição de pigmentação e elevada frequência de infiltrado linfocítico, com os tumores mais agressivos. Os tumores com tecido necrótico apresentaram significativamente maior expressão da p53 e tumores com padrão micronodular apresentaram mais frequentemente mutações no *TERTp*. Tendo em conta os dados reportados em estudos anteriores, quer sobre a expressão da p53, quer sobre as mutações no *TERTp*, esperávamos encontrar mais associações positivas com outras características dos BCCs estudados, o que não se verificou. Dado que o estudo das mutações do *TERTp* não foi realizado em todas as lesões, seria interessante completá-lo para se poder estabelecer uma relação entre os dois biomarcadores - expressão de p53 e mutações do *TERTp* – incluindo todos os casos. Novos estudos, como a análise molecular da *p53* e o estudo da expressão da TERT no BCC, poderão ser realizados para uma compreensão mais abrangente do seu papel nesse tipo de cancro de pele.

Keywords Basal cell carcinoma, UV-radiation, BCC histological subtypes, Hedgehog Pathway, Gorlin Syndrome, *TERTp* mutations

Abstract Basal cell carcinoma (BCC) constitutes the most common malignancy among skin cancer, whose origin is still under debate, with a possible response in stem cells. Although mixed histology is common, there are three main subtypes of BCC (nodular, superficial and infiltrative), which have different histological appearances and different levels of aggressiveness. This type of skin cancer has some risk factors associated, as well as molecular mechanisms which are implicated in BCC development. Tumor suppressor *p53*, responsible for DNA-damage response, was found to be very frequent in several cancer cell lines, including BCC. Telomerase reverse transcriptase (TERT) is the subunit of telomerase which is responsible for maintaining telomeres length, and mutations in its promoter have been considered a prognostic factor for various types of cancers, including for BCC. The main goal of this thesis is to describe *TERT* promoter (*TERTp*) mutations and p53 expression, which could possibly lead to find biomarkers for this pathology. P53 expression was evaluated through immunohistochemistry of 165 lesions from 97 patients. *TERTp* mutations molecular analysis was also performed in 52 BCCs. Through the histological revision of the lesions we observed a significant association between larger tumors, decreased pigmentation and high frequency of lymphocytic infiltrate, with more aggressive tumors. Tumors with necrotic tissue showed significantly higher expression of p53 expression and tumors with a micronodular pattern presented a higher frequency of *TERTp* mutations. Taking into account data reported in previous studies, both for p53 expression and for *TERTp* mutations, we expected to find more positive associations with other characteristics of the studied BCCs, which did not occur. Since the *TERTp* mutations were not evaluated in the entire series, it would be interesting to complete this study, in order to establish a relationship between the two biomarkers - p53 expression and *TERTp* mutations - upon including all the cases. Further studies, such as the molecular analysis of *p53* and the study of TERT expression in BCC, may be undertaken for a more comprehensive understanding of the role of these biomarkers in this type of skin cancer.

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Abbreviations

ARF	Alternative reading frame
BAX	BCL-2 associated X
BCC	Basal cell carcinoma
BCNS	Basal cell nevus syndrome/ Gorlin Syndrome
BOC	Brother of CDO
cAMP	Cyclin adenosine monophosphate
CDO	CAM-related downregulated by oncogenes
CK1	Casein kinase 1
CKI	Cyclin-dependent kinase inhibitor
CLEC	C-type lectin-like receptor 2
CM	Cutaneous melanoma
CPDs	Cyclobutane pyrimidine dimers
CpG	Cytosine islands
DAB	Diaminobenzidine chromogen
DHH	Desert Hedgehog
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EVC	Ciliary proteins Ellis-van Creveld syndrome
FasR	Fas-receptor
FDA	Food and Drug Administration
GAS1	Growth arrest-specific 1
Gprk2	G-protein coupled receptor kinase 2
Gsk3β	Glycogen synthase kinase 3β
HH	Hedgehog
IFN-α	Interferon alpha
IHH	Indian Hedgehog
IPATIMUP	Instituto de Patologia e Imunologia Molecular da Universidade do Porto
KIF3A	Kinesin family member 3A
laBCC	Locally advanced BCC
LRP2	Low-density lipoprotein receptor-related protein-2
mBCC	Metastatic BCC
MDM2	Murine double minute 2
MITF	Melanocytic transcription factor
MSH	Melanocyte-stimulating hormone
mtDNA D-loop	Mitochondrial DNA displacement-loop
NFκB	Nuclear factor kappa B
NMSC	Nonmelanocytic skin cancer
NOXA	Phorbol-12-myristate-13-acetate induced protein 1
PBS	Phosphate-buffered saline
PC	Primary cilium
PKA	Protein kinase A
POMC	Pro-opiomelanocortin gene

PTCH1	Patched gene 1
PTCH2	Patched gene 2
PTM	Post-translational modifications
PUMA	P53 upregulated modulator of apoptosis
RT-PCR	Real-Time PCR
SCC	Squamous cell carcinoma
SPSS	Statistical package for social sciences
SHH	Sonic Hedgehog
SMO	Smoothened
SUFU	Suppressor of fused gene
TAD	Transactivation domain
TERC	RNA subunit of telomerase
TERT	Telomerase reverse transcriptase
TET	Tetramerization domain
TERTp	Telomerase reverse transcriptase promoter
TNF-α	Tumor necrosis factor alpha
UV	Ultraviolet light
WHO	World Health Organization

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1. Introduction

Among all types of cancer, skin cancer is the most common malignancy worldwide, with a particularly higher prevalence in the white population[1,2]. This type of malignancy can be the result of long-term ultraviolet-light (UV) exposure of human skin epidermis, and it is very recurrent in fair-skinned older people with scaly indurated lesions on sun-exposed areas, mostly on the head and neck[3,4]. In recent years, skin cancer prevalence has been increasing among the young population, probably due to higher exposure to solar radiation and frequent use of tanning beds[3]. A skin cancer diagnosis is usually made through a dermatoscopy, but it must be confirmed with a biopsy[5]. Skin cancer can be classified into three main types: nonmelanocytic skin cancer (NMSC), which includes BCC and squamous cell carcinoma (SCC), and melanoma [2]. BCC and SCC arise from malignancies occurring in epidermal keratinocytes, and its prevalence has been increasing over the past years. BCC constitutes the majority of NMSC cases worldwide[6] and usually develops in skin areas with a higher UV exposure, such as face, neck and arms[2,7]. BCC is characterized by mutations in several genes, including the ones involved in the Hedgehog pathway (HH), the *p53* tumor suppressor and the *TERTp* genes, with HH pathway being the most commonly studied pathway associated to BCC. Searches for an accurate biomarker for BCC and efficient therapies have been the focus of many studies. This thesis will focus on BCC and its epidemiological, histological and molecular characteristics. More particularly, we will study the p53 protein, which is frequently mutated and highly active in cancer cells, and *TERT*, a subunit of the enzyme responsible for preventing telomere shortening, which has been considered a prognostic factor for several types of cancer.

1.1. Basal cell carcinoma

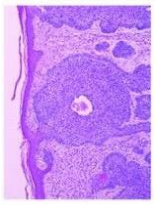
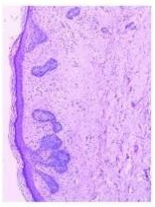
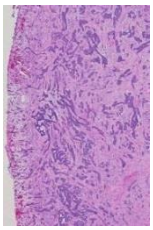
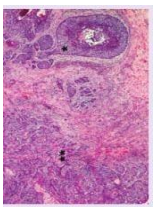
a. Epidemiology and histological characteristics of BCC

BCC is the most common form of skin cancer, and its origin is still controversial, but it has already been proposed a possible arise in epidermal stem cells[1]. Although this type of cancer has a relatively low mortality rate, as it rarely metastasizes, it causes significant morbidity through local invasion and destruction of visible tissue areas, mainly in the head and neck[9]. BCC can also be destructive in the surrounded areas if the patient does not receive treatment[10]. Its prevalence is higher in Caucasians, representing about two-thirds of skin cancer in this population[3]. However, establishing an incidence rate has been difficult due to the lack of registration practice in most countries, and because most cases of BCC are only registered once as an NMSC, without differentiation between BCC and SCC, leaving out multiple cases of BCC[9,11]. In Portugal, according to information obtained from the Northern Regional Cancer Registry (RORENO), BCC cases are registered one time per topographic region (head, face, etc.), i.e. if the second occurrence of BCC appears in the same region, it is not registered again. In European countries, BCC incidence has been increasing in the last few years, with Switzerland and Italy being the countries with higher incidence rates and Croatia with the lowest rates[11]. Worldwide, the country with the highest incidence rate is Australia; it has been estimated that 50% of Australians will suffer from BCC by the age of seventy[9,11]. Even though BCC incidence increases with age, especially in men, BCC cases have recently been increasing in young women[12]. This is probably due to greater attention to their appearance which leads them to use tanning beds (increasing BCC risk), and to visit the dermatologist more often (increasing BCC detection) to assure their skin health[11,13]. SCC can arise from preexisting lesions such as Bowen's disease or actinic keratosis, but BCC does not present a precursor lesion, delaying BCC diagnosis[14].

BCCs can be differentiated in three main histopathological subtypes: nodular, superficial and infiltrative[12], although BCCs with mixed histology are a common pattern[15]. Nodular BCC is the most common subtype and it appears mostly on the head and neck[1]. This subtype is clinically characterized by a white or pink papule with a pearly border with irregular growth pattern[1], presenting ulceration and bleeding of the central region as very common features[15]. Histologically, there are multiple circumscribed masses of basaloid cells, with peripheral palisading and retraction from surrounding stroma, and with different levels of keratinization[1,15]. Superficial BCC is mostly diagnosed in young women[16], occurring as

rounded red plaques with a thin pearly white border with no invasion in surrounding areas[1,17]. Typical basaloid cell buds arising from the basal layer of epidermis and regression areas with fibrosis are some of the histological characteristics of this subtype[1,15]. Infiltrative BCC is an aggressive BCC subtype, presenting perineural invasion; its histological characteristics rely on the presence of collagen and fibroblasts in the tumor stroma, and basaloid tumor cells in small spiky or angular nests[18]. Mixed BCC is a coexistence of two or more BCC subtypes, and it is a very common feature of BCC, comprising 10 to 38.5% of BCC cases[15,19,20]. Table 1 summarizes all the main BCC subtypes with its histological features and clinical appearance.

Table 1. BCC subtypes histological features and clinical appearance (adapted from Dormitive et al[1] and Correia de Sá et al[15]).

		Clinical	Histological	Location and Frequency
Nodular		White/pink papule with a pearly border Irregular growth pattern Ulceration and bleeding	Multiple circumscribed masses of basaloid cells Peripheral palisading Retraction from surrounding stroma Different levels of keratinization	Head and neck (50%)
Superficial		Rounded red plaques with a thin pearly white border No invasion in surrounding areas	Typical basaloid cell buds arising from the basal layer of the epidermis Regression areas with fibrosis	Trunk, limbs, face (15%)
Infiltrative		Perineural invasion Group of cells with an irregular outline with pointed spiky projections	No palisading Presence of collagen and fibroblasts in the tumor stroma Basaloid tumor cells in small spiky or angular nests	Head and neck (10–20%)
Mixed		Mixed clinical pattern	Mixed histological pattern	Various locations (10–38,5%)

Although these are the main BCC subtypes, there are other subtypes and other features we should consider, such as the micronodular BCC, that usually arises within other histological subtypes and which is characterized by the appearance of small nodules with peripheral palisading[19]. Its classification as a BCC histological subtype is controversial: some authors classify it as a histological feature of the nodular subtype[19], while others assume micronodular BCC as an individual and distinctive subtype[21]. Morpheaform BCC constitutes a variant of the infiltrative subtype, in which small groups of cells in cords with sclerotic neoplastic stroma are present, extending through the dermis[15,19]. This subgroup has an aggressive behaviour, similarly to another BCC subtype, which is the basosquamous carcinoma (or metatypical carcinoma)[15]. Basosquamous carcinoma is characterized by a prominent squamous carcinoma component which makes this subtype more likely to recur and metastasize[19]. It was described by the World Health Organization (WHO) as "basal cell carcinoma that is associated with squamous differentiation"; due to its mixed morphology it can lead to confusion between this subtype and SCC[22]. Histologically, basosquamous carcinoma cells exhibit a large amount of cytoplasm and increased keratinization[15,22]. A small percentage of BCC cases belong to another important BCC subtype called fibroepithelial BCC or fibroepithelioma of Pinkus. This is an unusual BCC subtype which typically appears in the trunk and extremities, in the form of arborizing cords of basaloid cells in a loose connective tissue stroma extending through the epidermis[1,19]. Efforts have been made in order to find accurate markers which could allow physicians to distinguish between aggressive and non-aggressive BCC subtypes. Bcl-2 was found to be a possible marker to distinguish the aggressive subtypes of BCC, as low bcl-2 levels were detected in aggressive subtypes of BCC, in opposition to non-aggressive BCCs[23]. Also, cyclin D1 has shown to be a possible diagnostic factor for aggressive BCC subtypes, as it has a high expression in these BCCs[24]. In 2009, stromal expression of α-smooth muscle actin was shown to be an accessible biomarker for aggressive subtypes as shown by Adegboyega and his colleges[25].

As mentioned above, BCC rates have been increasing throughout the world, varying between less and more aggressive subtypes. This aggressiveness can be measured through quantification of the levels of several molecules which have shown to be augmented in aggressive cases of BCC (infiltrative, mixed subtypes, etc.). BCC arises from several risk

factors which explain the high percentages registered worldwide, and they will be described in the following chapter.

b. BCC associated risk factors

The combination of genetic predisposition to skin cancer and environmental risk factors are at the origin of BCC development. UV and ionizing radiation exposure constitute the main environmental risk factors with a major impact in BCC development. Other important risk factors are high sun-sensitivity and family history of skin cancer, considered as phenotypic risk factors. Recently, the intake of hormone contraceptives has also been considered a risk factor for the development of BCC[26].

The major environmental risk factor is related with intense and intermittent UV exposure (sunlight or indoor tanning), which is responsible for causing devastating effects through different mechanisms, including gene mutations, formation of cyclobutane pyrimidine dimers (CPDs), immunosuppression, and oxidative stress[27,28]. This group ofrisk factors has a crucial role and a major impact in the development of BCC, with most of the mutations arising from DNA-damage due to UV exposure. In addition to UV exposure, ionizing radiation exposure is an important risk factor. Atomic bomb survivors and professionals working daily with ionizing radiation, such as radiologists, have an increased risk of developing BCC[29,30]. Moreover, individuals who have received treatment with ionizing radiation and immunosuppressive agents[31], such as cancer survivors and hematopoietic cell transplantation survivors, are also at risk for BCC development[29,32]. Environmental factors also include arsenic contamination through well water consumption[33,34].

Among phenotypic characteristics, advanced age constitutes an increased risk of being diagnosed with BCC, due to a decrease in DNA repair capacity as age advances, leading to an accumulation of DNA damage[10,35]. There have been controversial data related to BCC prevalence according to gender, but the majority of the studies have shown a higher incidence in men[13,36]. Because outside workers have continuous exposure to the sun, they have a higher risk of developing BCC, which could be the explanation for the higher incidence of BCC registered in men[37]. Highly sun-sensitive individuals include fair skin and clear eyes individuals, with low ability to tan and with family or personal history of skin cancer. This group is characterized by lower levels of melanin (a shield to UV radiation) and

constitutes a risk group for BCC development[38]. Patients with Xeroderma Pigmentosum have a genetic mutation in nucleotide excision repair genes, decreasing their ability to repair DNA damage and increasing the risk of developing multiple BCCs[12,39].

Melanin density in the skin, which is one of the main factors responsible for skin pigmentation, has proven to be an indirect marker of sun exposure and an indicator of risk of developing skin cancer[40]. High levels of melanin correspond to a higher ability for skin tanning and a lower risk of skin cancer[41,40]. Erythema, which indicates the redness of the skin, is related with hemoglobin levels and is characteristic of abundant sun exposure, and increased risk of skin cancer[41,42]. The Fitzpatrick phototype and melanin content have proven to be associated with a higher (I, II, III) or lower risk (IV, V) of developing cancer[41,43](Figure 10).

Von Luschan scale	Fitzpatrick Phototype	Characteristics	Sun tanning
1-5	I	Very fair skin Light eyes Light hair	Always burns No tanning
6-10	II	Fair skin Light or dark eyes Light or dark hair	Easily burns Light tanning
11-15	III	Light Intermediate skin Light or dark eyes Light or dark hair	Often burns Moderate tanning
16-21	IV	Brown skin Dark eyes and hair	Rarely burns Easy tanning
22-28	V	Brown skin Dark eyes and hair	Rarely burns Extensive tanning
29-36	VI	Very dark skin Dark eyes and hair	Never burns Abundant tanning

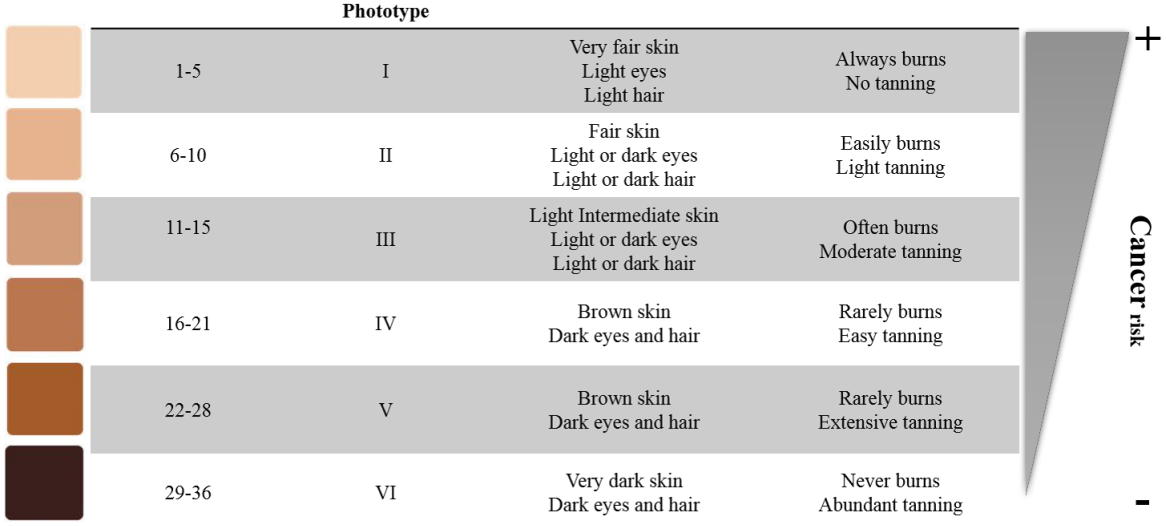


Figure 1. Fitzpatrick phototypes associated with Von Luschan scale and risk of cancer development (adapted from J. Orazio et al[41] and M. Bakare[44]).

Nowadays, most of the population, mainly women, use hormones which makes them a risk group for the development of BCC. A recent study by Lawrence Kuklinski *et al*.[26] showed that the use of hormones, such as oral contraceptives and hormone replacement therapy, is related to the development of aggressive BCC subtypes. This information could be an explanation for the increasing rate of BCC in women[26].

The awareness of the risk factors previously described is crucial to prevent BCC development and implement its early diagnosis. All these risk factors (Figure 1) play a very important role in BCC appearance constituting a key to the full understanding of this biological cancer model. Individuals presenting molecular deficiencies in genes with important roles in cell proliferation, cell differentiation and DNA-damage response, constitute a risk group for BCC development, as it will be further discussed.

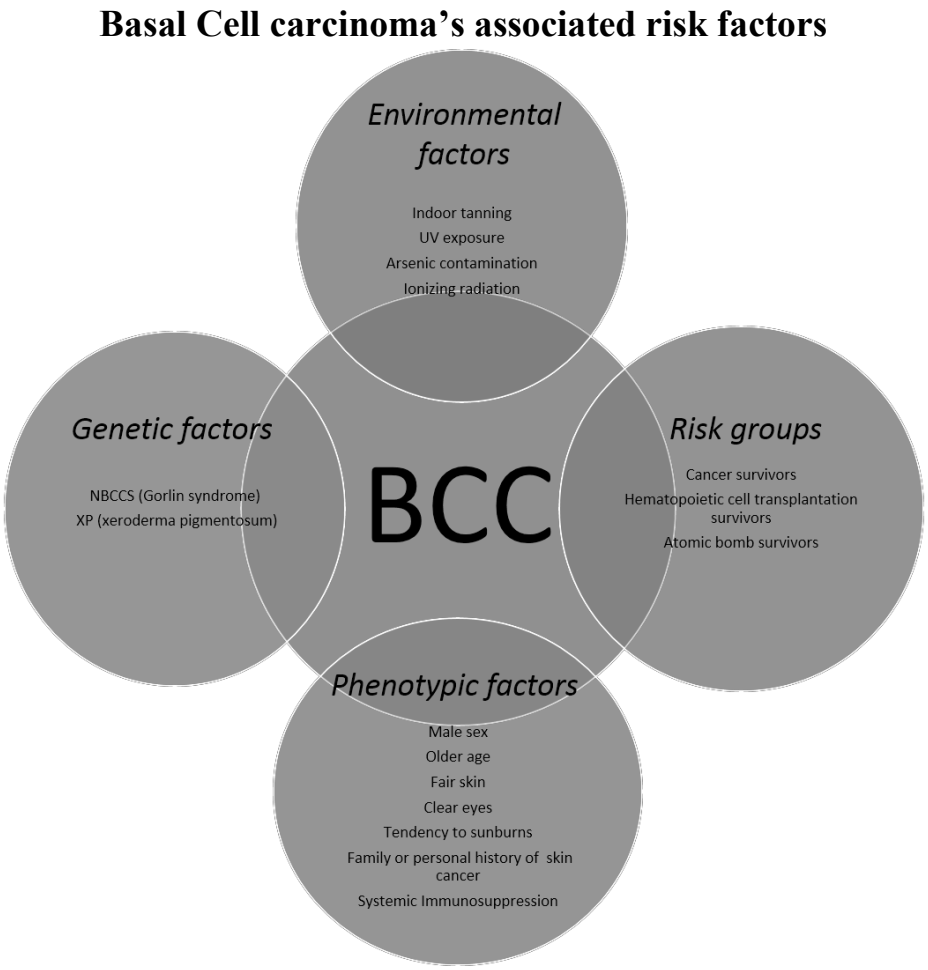


Figure 2. Main risk factors for BCC development (adapted from J. Verkouteren et al [12]).

c. Molecular Pathogenesis of BCC

Cancer cells are known for their higher ability to proliferate and continuous spreading through the organism when compared with normal cells[45]. Mutations in several genes are common events leading to tumor development and contribute to tumor spreading within the affected organism. To fully understand the mechanism behind cancer development, several studies have been focusing in the investigation of these mutations and their impact in pathways such as the HH pathway, and in genes with important roles in the maintenance of cell homeostasis, like *TERT* and tumor suppressor *p53*.

i. The Hedgehog pathway

The Hedgehog (HH) pathway plays a role in many important mechanisms such as cell differentiation and proliferation, tissue polarity and tumorigenesis[46,47]. This pathway includes four major classes of molecules: 1) three ligands, homologues of the Hedgehog gene [Sonic Hedgehog (SHH), Indian Hedgehog (IHH) and Desert Hedgehog (DHH)], 2) two receptors coded by *patched gene 1* (*PTCH1*) and *patched gene 2* (*PTCH2*), 3) one key signal transducer smoothened (SMO)[48], 4) Gli transcription factors (Gli1, Gli2, Gli3)[47,49]. In vertebrates, the HH pathway occurs in the primary cilium (PC), and it has two different actions depending on whether there is a ligand to PTCH or not (Figure 2). In the absence of an HH ligand, the HH pathway is inactivated, and PTCH inhibits SMO activity, which cannot migrate to the membrane, leading to its accumulation in the cytosol. Gli proteins are associated to suppressor of the fused gene (SUFU) and get phosphorylated by Protein kinase A (PKA), Casein kinase 1 (CK1), Glycogen synthase kinase 3β (Gsk3β) and G-protein coupled receptor kinase 2 (Gprk2). Instead of migrating to the membrane, Gli proteins are processed by the proteasome and segregated as their repressor form (Gli3). Gli3 is translocated to the nucleus and inhibits the transcription of HH target genes, but in the presence of an HH ligand, the HH pathway is activated. The binding between PTCH and the HH ligand is facilitated through the action of CAM- related/downregulated by oncogenes (CDO), brother of CDO (BOC), growth arrest- specific 1 (GAS1) and low density lipoprotein receptor-related protein 2 (LRP2)[50]. The action of these compounds inhibits the binding of PTCH to SMO, leading to PTCH degradation through the proteasome[47,49]. SMO is phosphorylated and interacts with β-arrestin and Kinesin Family Member 3A (KIF3A), entering the membrane associated with the ciliary proteins Ellis-van Creveld syndrome

(EVC proteins). Gli proteins (Gli1 and 2) bind to kinesin like-protein KIF7 and dissociate from SUFU, inhibiting its phosphorylation and allowing its translocation to the nucleus, enhancing the transcription of HH target genes[47,49]. In addition to this pathway, the activation of Gli proteins is also regulated by other molecules like *KRAS*, *TGF-β*, PI3K-AKT, PKC-α (positive regulation), p53, PKA, and PKC-δ (negative regulation) and constitutes an alternative non-canonical HH pathway[3].

Initially, the HH pathway was found to be related to cancer due to the discovery of a mutation in the *PTCH1* gene in patients with basal cell nevus syndrome (BCNS), also known as Gorlin Syndrome[51,52]. BCNS is an autosomal dominant disorder with a high degree of penetrance and a variable expressivity which is characterized by the appearance of multiple BCCs appearing at young age[51]. Dyskeratotic palmar pitting and keratocystic odontogenic tumors are also very common features of Gorlin Syndrome[3,53]. Patients with this disorder are a risk group for the development of other types of cancer such as medulloblastomas[51,54]. BCNS molecular pathogenesis relies on mutations in *PTCH1*, *PTCH2* or *SUFU* genes, which induce aberrant activation of the HH pathway with its upregulation[15]. In BCC, HH pathway promotes tumorigenesis through mutations in the *PTCH* gene, leading to a loss of function that activates several other mutations in *SMO* and promotes continuous activation of Gli proteins (Figure 2)[55,56]. However, different studies have shown that the HH pathway interacts with other pathways such as the *RAS/RAF/MEK/ERK* pathway which is involved in cell growth and survival[57]. The *mTOR*/*S6K1* pathway participates in the regulation of Gli activity through modulation of PKA-dependent phosphorylation[56,58].

Epidermal growth factor (EGF) plays an important role in cell proliferation and survival, tumor invasion, and metastasis[56,59]. This transmembrane protein has shown to be a negative regulator of HH genes[60]. Another pathway implicated in the development of BCC is the NOTCH pathway, in which a deficiency in *NOTCH1* causes a decrease of Gli2 expression [61,62]. The SUFU loss of function promotes deregulation of WNT/β-catenin and HH pathways, contributing to tumorigenesis[59,63].

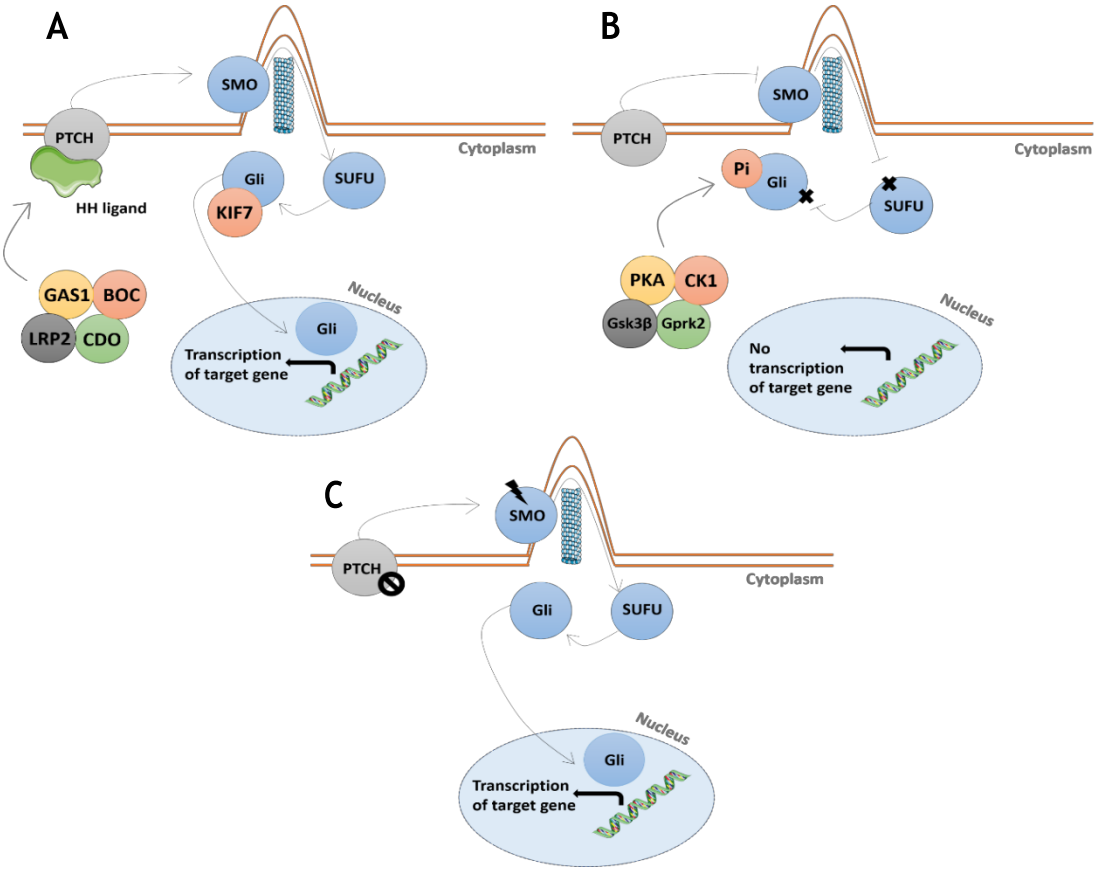


Figure 3. The Hedgehog pathway: (A) in the presence of a HH ligand, this pathway is active and promotes transcription of target genes; (B) absence of a ligand promotes inhibition of HH pathway; (C) mutations in *PTCH* gene and alterations in SMO protein, activate an aberrant function of this pathway, leading to formation of cancer cells (adapted from C. Pellegrini et al[3]).

In addition to all molecules and pathways mentioned above, there have been several studies showing the relation between other molecules and the HH pathway, which seem to have an impact in BCC development. For example, BCC is associated with the expression of D-type cyclin, which activates the HH signalling pathway, leading to cell cycle progression[64]. Deregulation of PKA was also found to be one of the triggers of BCC development, being a negative regulator of the HH pathway[65]. Reduced levels of PKA lead to aberrant activation of HH signalling, promoting tumor growth[66,67]. Among all the crosstalk with the HH pathway, tumor suppressor gene *p53* has been implicated in negative regulation of this pathway, as it represses the activation of Gli proteins in tumor cells[68].

ii. Tumor suppressor *p53*: association with BCC

Tumor suppressor p53 protein is encoded by the *TP53* gene, located, in humans, in chromosome 17p13.1. This transcriptional factor structure is composed of several functional domains: a transactivation domain (TAD), a proline-rich region, a DNA-binding core domain, a tetramerization domain (TET), and a C-terminal regulatory domain. TAD is located in its N-terminal part and is responsible for the recruitment of RNA polymerase and initiation of transcription mechanisms. The proline-rich region is crucial for the repression, apoptosis and response to radiation. Characterized by a higher rate of mutations, the DNA-binding core domain is rich in arginine and lysine residues, which bind to the local site of DNA response elements. TET is located in C-terminal which establishes the connection between p53 monomers. The C-terminal regulatory domain is rich in basic amino acids which controls p53 turnover and suffers post-translation mutations (Figure 4.A)[69,70]. TP53 family is composed of 12 p53 isoforms regulated by different promoters: p53(α, β, γ), Δ40p53 (α, β, γ), Δ133p53 (α, β, γ) and Δ160p53 (α, β, γ)[71]. These isoforms are characterized by a lack of several amino acids, having the same DNA-binding core domain but different transactivation and regulatory domains[71]. Proteins p63 and p73 encoded by genes *TP63* and *TP73*, respectively, are also members of the p53 family resulting of two different functional promoters, P1 and P2, and alternative splicing at multiple sites, similarly to *TP53*[72,73]. p63 and p73 proteins are composed by an extra domain (SAM) which confers higher stability and less plasticity to their structure when compared with p53 protein[74]. These proteins also have isoforms which can be divided into two groups: the ones that lack the TAD domain (ΔN) and those where the TAD domain is present (TAD variants) [74,75]. In this thesis, we will focus on p53 due to its major role in DNA-damage repair, its correlation to carcinogenesis, and its consistent mutations in several types of cancer.

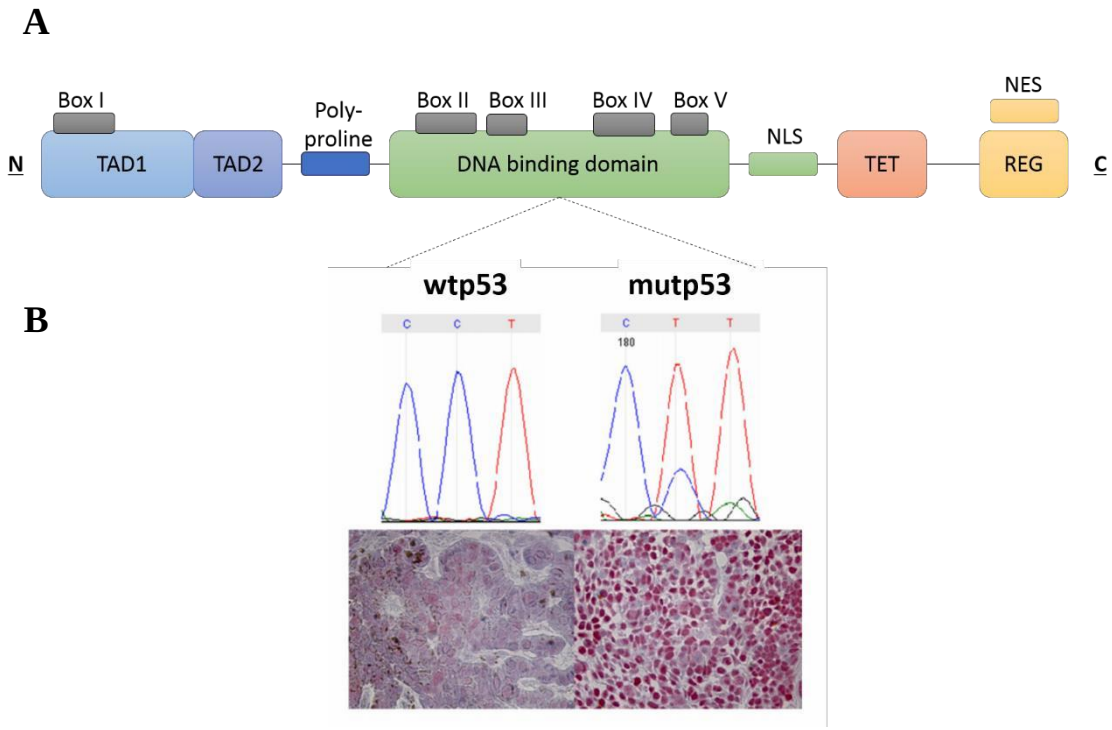


Figure 4. Tumor suppressor *p53*: (A)The *p53* gene structure; (B) Example of a mutation which can be detected by immunohistochemistry (adapted from C. Rutao et al[76]).

The p53 protein, also known as the "Guardian of the genome", is responsible for the cell damage response; mutations and overexpression of this protein are highly common in BCC cases[77]. The UV light is also one of the factors responsible for DNA damage, therefore constituting one of the main causes of BCC development, leading to p53 induced cell cycle arrest or cell apoptosis[64]. Under normal conditions, p53 levels are low, and p53 eventually becomes undetectable when it is not needed[73,78]. However, when a cell suffers several mutations and becomes malignant, p53 levels increase in the G1/S checkpoint, where p53 acts[78]. Through events of acetylation and phosphorylation, p53 production is activated, acting as a transcription factor which enhances secretion of cell arrest protein p21[79]. Protein p21 (also known as cyclin-dependent kinase inhibitor) (CKI), inhibits the activity of cyclins and CDK, inhibiting cell cycle progression during the G1 and S phases and leading to cell arrest[79,80].

Moreover, in stress conditions, p53 also induces the production of proteins for DNA repair or apoptosis, when the DNA damage is irreparable and inhibits angiogenesis[79]. This

tumor suppressor protein has been implicated in the pigmentation response, as it stimulates the pro-opiomelanocortin gene (*POMC*) in response to UV exposure[76,81]. Enhanced stimulation of this gene via p53, activates the production of melanocyte stimulating hormone (MSH) by keratinocytes, which binds to its receptor, melanocortin-1 receptor, in melanocytes[76]. This binding increases the cyclin adenosine monophosphate (cAMP) levels, promoting the transcription of the melanocytic transcription factor (MITF), and the production of melanin through tyrosine[76]. Melanin is transported through melanosomes to keratinocytes, where the latter accumulate to protect the cell from DNA exposure, forming the tanning of the skin[76,82]. When epidermal keratinocytes are damaged (for example in sunburn), p53 is phosphorylated and induces cell cycle arrest and apoptosis through activation of target genes like *BCL2 Associated X* (BAX), *Fas*-receptor (FasR), phorbol-12-myristate-13-acetate-induced protein 1 also known as NOXA, and p53 upregulated modulator of apoptosis (PUMA)[73,83].

P53 activity is regulated by several components such as murine double minute 2 (MDM2), which acts through a negative feedback mechanism of ubiquitylation, keeping p53 at low levels until DNA damage occurs[84]. Post-translational modifications (PTM), mainly phosphorylation and acetylation, which occur very frequently in p53 protein, also play an important role in p53 regulation and, consecutively, in DNA-damage response[70]. Alternative reading frame (ARF) also regulates p53 activity through its ubiquitination, and it is responsible for activating p53 expression when a high rate of uncontrolled proliferation is detected[64,73].

Missense mutations and consecutive inactivation of p53 deregulate this system of cell viability control and cause malignant cells to bypass the checkpoints of the cell cycle and continuously grow and proliferate (Figure 5)[4,73]. Usually, these mutations occur in the DNA-binding core domain and cause the impairment of DNA binding and formation of mutant variants of *p53*, leading to lower efficiency of its tumor suppressor activity[70]. Mutations in *p53* have been detected in 50% of BCC cases, and are commonly associated with BCC developed after sun exposure[3,10]. Usually, *p53* mutations occur in three main hotspots: codons 177, 196 and 245, with codon 177 being specific for BCC cases[85]. Expression of p53 in BCC has been detected through immunohistochemistry (Figure 4.B), with a higher percentage of expression in malignant tissue, making this a possible biomarker for mutations occurring in *p53* gene[85]. Most p53 mutations are nucleotide transitions

characterized by pyrimidine single and tandem changes, C-T and CC-TT, respectively, as described in Figure 4.B[3,86], and are associated with aggressive BCC subtypes[64,85]. As it was already mentioned above, in BCC, aberrant activation of HH pathway induces DNA-damage response, and, consecutively, a higher expression of p53[55].

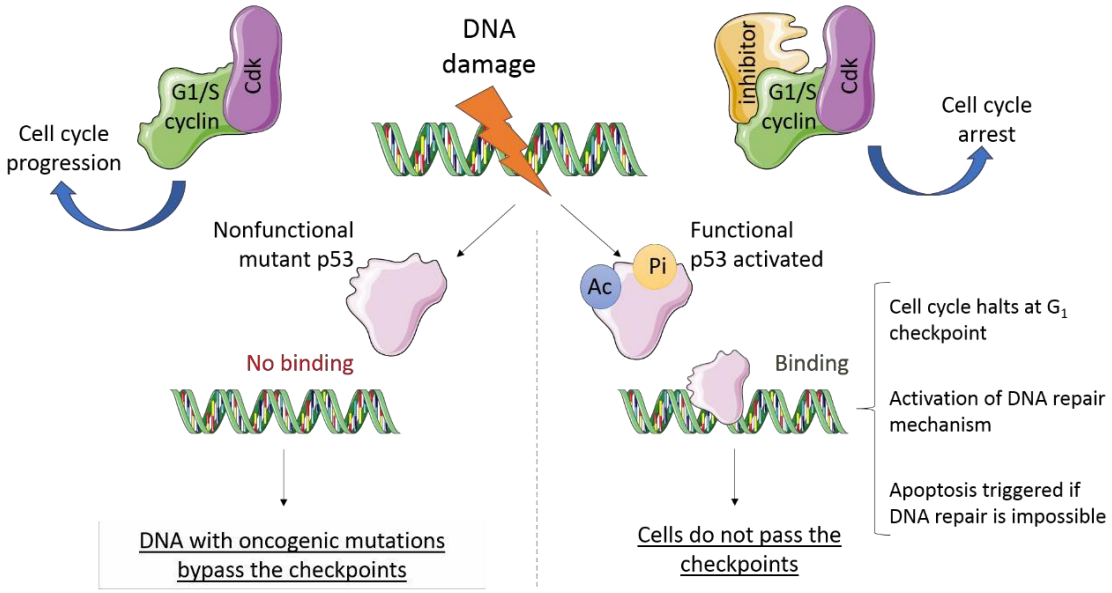


Figure 5. Action of p53 in response to DNA damage (adapted from Niazi, S. et al [73]).

p53 is the main coordinator of DNA-damage response as it mediates cell fate: whether it passes through the cell cycle checkpoints or goes under apoptosis. Mutations occurring in this protein encoded gene lead to an aberrant p53 which cannot direct a DNA-damage response, allowing cancer cells to proliferate. High levels of p53 and mutations in its encoded gene have been registered in BCC cases, making this protein a possible biomarker for BCC prognosis.

iii. TERT: a possible biomarker for BCC

Along with p53, TERT has also been implicated in cancer cells molecular mechanism. Being a subunit of telomerase with major importance for the enzyme's function, TERT has been the focus of many studies related to cancer. Several mutations in *TERTp* have been found in different types of cancer, including BCC, which makes it a possible biomarker for this type of malignancy.

Telomeres are nucleoprotein complexes consisting of 5'-TTAGGG-3' DNA sequence repeats at the end of the chromosomes[87]. These structures have the main function of chromosome integrity maintenance and genome stability, as telomeres act as shelters for end-to-end junction and degradation[88]. Shortening of telomeres is prevented by an enzyme with reverse transcriptase activity which adds small repeated DNA segments to the end of the chromosomes, after cell division. This enzyme is telomerase and it consists of a ribonucleoprotein enzyme complex composed by two major subunits: a RNA component (TERC) that provides the template to create the new DNA repeated sequence for telomere elongation; and a catalytic subunit also known as telomerase reverse transcriptase (TERT) responsible for adding this new segment of DNA to the end of the chromosomes[87,88]. Together, TERC and TERT assure the good functioning of telomerase and contribute to immortalization of several cells[8887]. In somatic cells, telomerase is either inactive or at very low levels[89]. However, in cells with rapid growth and division, such as bone marrow cells and cells from the gastrointestinal tract, this enzyme is active, with high levels, allowing cell continuous growth towards immortality[87,90]. Studies have shown that TERT levels are the main regulator of telomerase activity[87].TERT is encoded by *TERT* gene which is located at chromosome 5p15.33, has a length of 42-kb and it is composed of 16 exons. *TERTp* has 260 base pairs, and it is the most important component of the *TERT* gene for the regulation of telomerase activity[88]. *TERTp* includes binding sites (GC-boxes, E-boxes) for transcriptional factors such as p53, p21, SP1, ETS, E2F, AP1, HIF1, nuclear factor kappa B (NFκB) and c-myc, which are responsible for gene transcription regulation and several cellular events like apoptosis and cell growth[91]. Mutations in these binding sites are associated with a decrease in E2F transcription factors which are involved in the regulation of *TERTp* activity, acting simultaneously as an activator and a repressor of *TERT* transcription[92]. Tumor suppressor *p53* and *NFκB* overexpression lead to suppression of TERT expression[93,94]. Hypermethylation of cytosine islands (CpG) located in GC rich regions of *TERTp* results in lower telomerase activity and inhibition of *TERT* transcription[91,95]. Cooperation between telomerase activity and loss of function in p53 may induce tumorigenesis[87]. Apart from NFκB and p53, it has been proved that TERT expression can be regulated by several other compounds such as B-catenin[96] and tumor necrosis factor alpha (TNF alpha)[94], contributing to oncogenic events like metastization[87].

Mutations in *TERTp* are highly common among human cancers (80-90%), including BCC (56-80%)[87,91,97]. These mutations are associated with reactivation of *TERT* expression and enhanced telomerase activity (2-4-fold increased promoter activity), due to the creation of new consensus binding sites (CCGGAA) for Ets/TCF transcription factors. Continuous activation of TERT transcription leads to the continuous growth of telomeres length, allowing tumor growth and proliferation[87]. As it was already mentioned in **chapter b**, UV radiation is responsible for the formation of CPDs at dipyrimidine sites, so it would be expected to found alterations in two consecutive pyrimidines DNA bases[98]. In fact, two main hotspots have been reported at positions -124 and -146 bp with C>T or G>A changes and also tandem changes CC>TT or GG>AA, providing strong evidence that *TERTp* mutations have a UV-signature (Figure 6)[3,98].

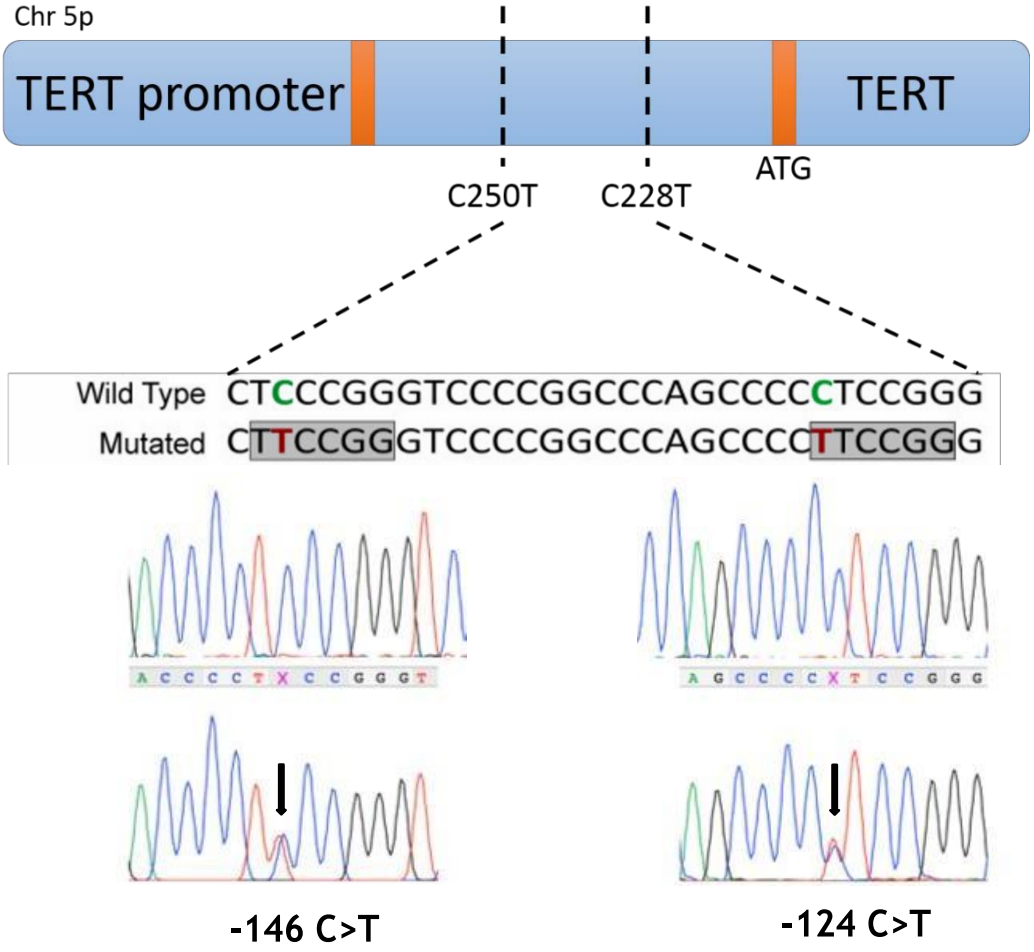


Figure 6. *TERTp* gene structure and common mutations occurring in BCC (adapted from C. Pellegrini et al[3]).

Boaventura *et al.*[97], have shown the prevalence of these two hotspots in BCC, and also, the tandem mutation at position -138/-139 was observed in two BCCs (3%) and at positions -124/-125 in BCCs (3%)[97]. Activation of MAPK and PI3-Akt pathways were proposed as an explanation for the correlation found between these mutations[90,99]. Mutations in *TERTp* influence the localization of TERT within the cell, which can promote injuries in mitochondrial and nuclear DNA and metabolic alterations in cancer cells[87]. Cleft lip and palate transmembrane 1-like (*CLPTM1L*) is a transmembrane gene located in *TERT* locus that plays a role in the genesis of multiple tumor types, including BCC. Polymorphisms on 1p36, 1q42, and 5p15 of the complex TERT-CLPTM1L were associated with BCC, more specifically, the rs2736098 in *TERT* gene and the rs401681 in *CLPTM1L* gene[100,101,99].

As it is shown in this chapter, TERT has a key role in the maintenance of telomeres length as it avoids their degradation. Mutations in *TERTp* are frequent in several cancers, including BCC. This gene will be the focus of this thesis, as it seems to represent an accurate biomarker for aggressive BCC subtypes.

iv. Mutations found in other genes in BCC cases

Very recently, it has been shown that high levels of epidermal growth factor receptors (EGFRs) are associated with the appearance of aggressive BCC subtypes[102]. In addition to all genes and pathways described above, there are others related to the development of BCC, but with lower importance, such as the *MYCN* gene and the *DPH3* promoter[3]. Many authors have tried to track mutations in *BRAF* and *RAS* genes, but until now, there is no evidence of mutations in these genes BCC cases[103,104]. Mitochondrial DNA displacement-loop (mtDNA D-loop) constitutes a vulnerable region with higher susceptibility to damage and mutations in this region have been found in several cases of BCC[14]. Table 2 summarizes the most important molecules that may contribute to BCC development[3].

Table 2. Common mutations detected in BCC cases (adapted from C. Pellegrini *et al*[3]).

Gene	Mutation frequency in BCC	Reference
DPH3 promoter	42%	3,105
mtDNA D-loop	25%	14
MYCN	30%	3,106
PTCH 1	90%	3,107
SMO	10-20%	3,106
SUFU	8%	3,106
TERTp	56-80%	87,91,97
Tp53	50-82%	10,85

Apart from *TERTp* and *p53*, there are several other important genes which were found to be mutated in some BCC cases. Additional investigations should be considered in order to confirm the data already obtained about the genes above mentioned, aiming at a better understanding of this type of skin cancer. The discovery of a relationship between mutations in different types of genes and BCC allows the investigators to pursue a more accurate and efficient therapy.

d. BCC treatment options

As mentioned before, BCC is a tumor which occurs in highly visible areas, causing a high rate of morbidity, so it is important to eradicate this tumor with minimum visual damage and with maximum preservation of the surrounding tissue[15]. Choosing a therapeutic approach for BCC (Figure 6), depends mainly on the BCC subtype which characterizes its aggressiveness, the location of the tumor (head, neck, arms, etc.), tumor size and patients preferences[107]. Low-risk primary BCC and localized BCC are usually treated through surgical approaches (Mohs surgery, curettage, etc.) and other conventional therapies such as chemotherapy. Molecular therapies such as Vismodegib, Itraconazole and Sonidegib, are being studied for locally advanced BCC (laBCC) and metastatic BCC (mBCC).

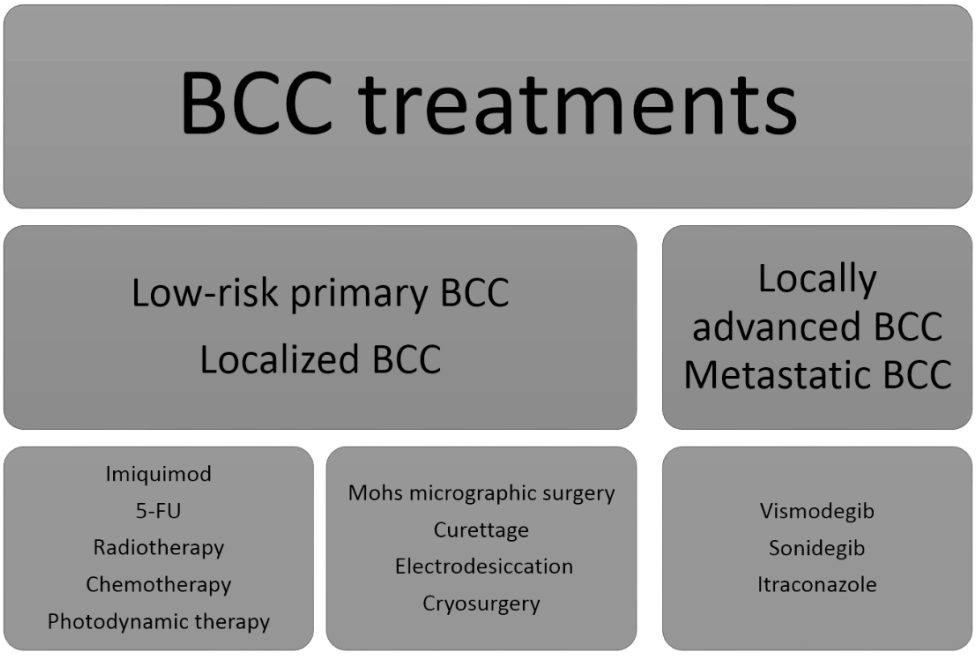


Figure 7. Therapeutic approach concerning BCC eradication[15].

The understanding of the affected genes and pathways in BCC, like in another disease, is crucial for developing effective molecular therapies. These molecular therapies, when compared with the above-mentioned approaches, have several advantages such as good cosmetic outcome, preservation of surrounding tissue, non-invasiveness and the possibility of home application[15]. These molecular therapies rely on the interference of genes and pathways, as previously described. Inhibitors of the HH pathway are currently being studied for the treatment of locally advanced BCC (laBCC) and metastatic BCC (mBCC)[108]. These two BCC variants cannot be treated with common surgery because it would lead to severe damage and large scar tissue[107].

Vismodegib is an inhibitor of the HH pathway, binding to the SMO protein and blocking the aberrant transcription of target genes mediated by Gli proteins (Figure 7)[109,110]. In 2012, it was approved by the Food and Drug Administration (FDA) as an effective treatment for laBCC and mBCC, and it is effective in BCC eradication when combined with other therapies, like photodynamic therapy[109]. Sonidegib is similar to Vismodegib, and it is also being studied as an alternative therapy for BCC treatment (Figure7). However, the development of resistance to these drugs is frequent, through *de novo* mutations in the target protein: positions 497 and 473 in Vismodegib and position 477 in Sonidegib[110]. Itraconazole

is another inhibitor of the HH pathway, with a different action from Vismodegib and Sonidegib: it inhibits SMO translocation to the primary cilium[110]. Other SMO inhibitors and Gli antagonists are being studied for BCC treatment and management, such as Ingenol mebutate, which was shown to reduce BCC lesions when applied in small portions. Taledigib, Saridegib and Glasdegib are also known to inhibit SMO's activityand have been tested in patients with advanced solid tumors, including BCC [3,111,112].

Topical application of Imiquimod constitutes one of the strategies used for BCC treatment, and it relies on the chemical destruction of the tumor[113]. It has an agonistic toll-like receptors 7 and 8 activity, which activates NFκB, inducing the activation of pro-inflammatory agents and an immune response[114]. Imiquimod acts like a tumor suppressor as it induces tumor apoptosis through interference with NOTCH and adenosine receptor signalling pathways[113,114]. It is used to treat primary nodular and superficial BCC, when patients cannot be submitted to surgery, but it may be associated with undesirable effects such as erosion, burnings and pain[15,114]. Its action relies on the chemical destruction of the tumor, and it constitutes one of the cheapest BCC therapies[113], with a success rate near to 90%[113,115].

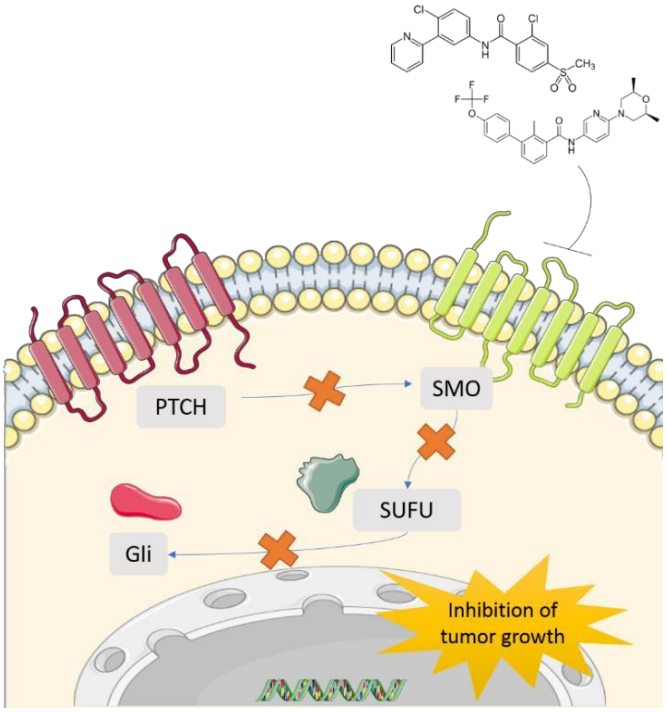


Figure 8. Molecular therapeutic approach concerning BCC eradication: Vismodegib and Sonidegib mechanism of action[15,110].

As previously mentioned, TERT was found to be a common biomarker among several types of cancer, so telomere and telomerase therapies have been largely studied as an attempt to induce apoptosis in cancer cells, towards tumor eradication[88]. These therapies include telomerase immunotherapy, telomerase interference, molecular inhibitors of TERT and telomerase activity, telomere-based strategies and *TERT/TERC* promoter driven therapy[88]. Although most of these therapies are still in phase trial, major efforts have been made in order to prove their efficacy for cancer treatment[89].

Therapies focused in telomerase immunotherapy consist in telomerase vaccines which cause direct immune cell death through two main strategies: direct inoculation, using TERT derived peptides GV-1001[116], Vx-001[117] and GX-301[118]; and ex vivo activation through the use of dendritic cells GRVNVAC1[119]. This therapy provides activation of immune responses by induction of cytotoxic T-lymphocyte, including $CD8^+$ and $CD4^+$ responses, which leads to the releasing of cytokine cell death[89]. Vaccines based on this mechanism are being studied and improved so that they could be used safely and efficiently in BCC patients[120,121].

Therapies relying on direct telomerase inhibition are based on the direct binding to TERT subunit, which leads to disruption of telomerase structure and its activity[122]. Direct inhibition of telomerase promotes the inhibition of cell proliferation in cancer cells, causing telomere shortening and, eventually, cell death[89]. Imetelstat, whose mechanism remains unclear, has been used in several clinical trials, showing positive results in cancer eradication, although it has not been accepted by the FDA yet. On the other hand, strategies based on telomerase interference have also been studied as efficient tools for rapid killing of cancer cells, as they interfere with telomerase action through techniques such as lentiviral infection, leading to cell senescence[123]. *TERT/TERC* promoter driven therapies (oncolytic virus and suicide gene therapy) are characterized by the expression of cytotoxic agents through the insertion of adenoviruses under control of *TERTp* in target cells, inducing cell lysis and tumor cell death[124]. These strategies based on telomerase disruption and interference have not been accepted as accurate treatments for BCC, but some of them have been used in several clinical trials[122,124]

Last but not least, telomere-based strategies rely on the interference with telomere structure and have already shown to be promising in cancer cells extinction[89]. These strategies include the G-quadruplex stabilizers which prevent telomere lengthening process, thus promoting its shortening and cell death[125]; t-oligo structures are also part of this group and are responsible for inducing DNA-damage response followed by apoptosis, by mimicking the presence of unprotected telomeres[126]. Finally, tankyrase inhibitors prevent dissociation of TRF1 which would be essential for telomere lengthening process[127]. Although none of these TERT therapies have been approved by the FDA, there have been large advances in proving their efficiency on the eradication of several types of cancer[88,89].

Finding a therapy for BCC is a very important step towards tumor eradication and better outcomes. Surgery, mainly Mohs surgery, and chemotherapy seem to be the most common therapies used in this type of malignancy, although molecular therapies including Vismodegib, Sonidegib, Imiquimod and Itraconazole, and TERT related therapies (Table 3) have been intensively studied and tested in recent years. A better understanding of the molecular mechanisms of BCC should contribute to these investigations to achieve accurate and improved novel therapies.

Table 3. TERT based therapies for cancer (based on Y.Xu *et al.*[89]).

TERT therapies				
Telomere-based strategies	*Direct Telomerase Inhibition*	*Telomerase Interference*	*TERT/TERC promoter driven therapy*	*Telomerase immunotherapy*
G-quadruplex T-oligo structures Tankyrase inhibitors	Imetelstat	Lentiviral infection	Oncolytic virus Suicide gene therapy	GV1001 Vx-001 GX-301 GRNVAC1

Major advances in BCC treatment have been achieved, but it is important to prevent the appearance of this malignancy in the first place, through awareness of BCC risk factors. The usage of sunscreen has not been proved to have an impact on the development of BCC, although its application was found to reduce *p53* mutations in this type of skin cancer[3,15]. However, sunscreen application has been proved to have beneficial effects in other types of skin cancer such as SCC and melanoma[128,129], emphasizing the importance of sunscreen application before and during sun exposure. Nevertheless, it seems more efficient to reduce the time of sun exposure, especially during the hottest hours to avoid sunburns[15].

e. Methodologies used to study mutations in the TERTp

Polymerase chain reaction (PCR) is a method widely used in several fields including cancer cell lines study[97,92]. It is based on generating multiple copies of a DNA specific region, using specific primers and the enzyme *Taq* DNA polymerase, which allows the PCR to proceed[130]. The reaction is achieved through consecutive cycles of heating and cooling of this specific region which will be, in the end, properly amplified[130]. Different PCR techniques have been used to study mutations in cancer cell lines, with Real-Time PCR (RT-PCR) and Sanger sequencing as the most commonly used to study *TERTp* mutations[131,132].

RT-PCR relies on the use of DNA probes with a fluorescence molecule and a quencher, which will allow the determination of the sequence through emitted fluorescent energy, further detected by the fluorometer present in the thermocycler[130]. This technique has proven to be verysensitive, allowing a rapid and accurate genome analysis and tracking

of mutations[130,133]. However, if the aim is to find non-specific mutations in the *TERTp*, to characterize BCC, this technique is not the most adequate, as it determines specific mutations using specific probes.

Sanger sequencing, also known as dideoxy sequencing, is characterized by the use of specially modified dideoxy nucleotides (ddNTPs) which will be responsible for the termination of the sequence being copied, due to its lack of an hydroxyl group at the 3' terminal carbon, inhibiting the formation of the phosphodiester ligation between this and the next nucleotide. The final sequence is determined through capillary electrophoresis and detected by fluorescence[134]. This technique is less specific and less sensitive than RT-PCR, allowing the tracking of different mutations across the whole genome[135]. It also requires less expertise, and it is cheaper than RT-PCR, which makes it a highly used technique in several fields, including the study of mutations in numerous genes[136]. Sanger sequencing has been the method chosen for *TERTp* analysis in BCCs as described in the literature[97,131,137]. It is important to use this method in the present work to allow the validation of our results.

1.2. Aims of the work

Contrary to SCC, which can arise from lesions such as Bowen's disease and actinic keratosis, BCC does not present a precursor lesion[14]. Finding a biomarker which could provide a quicker and more efficient diagnosis would be an important tool and also allow a better and more efficient therapy for BCC patients. Additionally, BCC behaves as a locally invasive tumor, despite rarely metastasizing, which makes it an interesting cancer model to study.

The *PTCH* gene has a wide content of exons, and many studies have already addressed its role in SCC (exons 2-23 have already been studied)[86,138]. The *TERTp* gene has been tested as a possible prognostic and diagnostic factor for several types of cancer, namely thyroid cancer and SCC[139,140], and for that reason, it was chosen as the main tool for the current investigation. Telomerase activity has been associated with tumorigenesis, as its activation constitutes a necessary phase to achieve the neoplastic step[141]. Continuous activation of telomerase promotes the resistance to senescence and apoptosis, leading to the continuous growth of the malignant cells[141]. Moreover, mutations in *TERTp*, constitute a major characteristic of BCC development, as it is shown in Table 2. Also, p53 has already been detected at high levels in several cancer cell lines, and it was considered as a biomarker of tumorigenesis. BCC cases have also shown alterations in this protein, including mutations in the encoded gene. As so, this study will focus on molecular characterization of this type of malignancy through the detection of mutations in *TERTp* and also immunohistochemistry of p53 to detect possible enhanced expression among BCC cases from Vila Real.

2. Materials and Methods

2.1. Samples

The patient samples used for this study were retrieved from a series of BCC patients from the Ear, Neck and Throat (ENT) Department of the Centro Hospitalar de Trás-os-Montes e Alto Douro (CHTMAD), collected from March 1990 to April 2018. From all the patients primarily diagnosed with BCC, 183 patients had a head and neck BCC histopathologically confirmed, managed and followed at the Department of ENT of CHTMAD. Afterwards, the 183 patients were contacted, but only 153 were reachable and committed to the study. However, of the 153 eligible patients, only 97 patients concomitantly filled an in-person interview questionnaire and had complete medical and pathological records. These were the inclusion criteria for the 97 patients of our series, which presented 165 BCCs, histologically reviewed at the Hospital de São João. Patients who did not fulfil the criteria mentioned above were excluded from this study. Melanin and hemoglobin contents of each patient were measured using a derma spectrometer in two body regions with different sun exposure: forehead, with higher sun exposure and the inner forearm, with lower sun exposure. Sun exposure was evaluated according to patients' professions. Von Luschan and Fitzpatrick scales were used for evaluating the patients' skin color and respective phototypes.

2.2. Molecular characterization: hTERT mutations

2.2.1. Hematoxylin-eosin staining

Deparaffination of the sample slides was achieved through two 10 minutes incubations in xylol (Enzymatic, Portugal), followed by hydration with decreasing concentrations of ethanol (Enzymatic, Portugal) of 5 minutes each: two incubations in 100% ethanol, one incubation in 96% ethanol and a final incubation in 70% ethanol. The slides were then washed with running water for 5 minutes, stained with hematoxylin (DIAPATH, Italy) for 4.5 minutes and washed again in running water for 5 minutes. After these procedures, the slides were stained with eosin-Y alcoholic (Thermo Scientific, USA) for 3 minutes and then dehydrated through consecutive incubations of 5 minutes each in increasing concentrations of ethanol (Enzymatic, Portugal): one incubation in 96% ethanol and two incubations in 100% ethanol. Stain fixation and material conservation were assured by two incubations in

xylol, for 10 minutes each. After mounting with mounting medium (Thermo Scientific, USA) and coverslips, the slides were histologically reviewed in the Hospital de São João Anatomical Pathology Department, to select the tumor area to dissection and evaluate its histological subtype.

2.2.2. DNA extraction and quantification

The DNA extraction was performed with the GRiSP kit (GRiSP, Portugal), which contains all the material and reagents needed, except xylol (Enzymatic, Portugal), 100% ethanol (Enzymatic, Portugal), and proteinase K (Enzymatic, Portugal).

Firstly, the slides were deparaffinized with xylol with two incubations of 10 minutes each, followed by two incubations of 5 minutes each in 100% ethanol. After a drying period of 15 minutes, the tumor area of each sample was dissected and collected to 2 ml vials. Disruption of cell membranes and protein digestion were accomplished by adding 200 µl of buffer 1 and 10 µl of proteinase K (20 mg/ml), respectively, to each vial. Then, the samples were incubated in a shaker, at 60°C with 750 rpm overnight, and in the next morning, an additional 5 µl of proteinase K (20 mg/ml) was added to the samples which showed a non-homogeneous appearance. DNA precipitation was assured by adding 200 µl of buffer 2 and 200 µl of absolute ethanol to the samples, which were then vortexed. The obtained products were transferred to gDNA plus spin columns and centrifuged at 16 000 g for 1 minute at room temperature. These columns were then transferred to new collecting tubes, washed with the first washing buffer and centrifuged at 16 000 g for 30 seconds (Legend Micro21R centrifuge, Thermo Scientific, USA). The content on the collecting tube was discarded and 600 µl of the second washing buffer was added. After centrifugation at 16 000 g for 30 seconds, the content was again discarded, and the centrifugation columns were dried through another centrifugation at 16 000 g for 3 minutes. To elute the DNA from the centrifugation columns, 50 µl of elution buffer were added to the samples. After the last centrifugation, DNA of the samples was quantified using the Nanodrop (ND-1000, Thermo Fisher Scientific, Lithuania), which provided information about the correct amount of sample to use in the next step (50 ng/µl). There was no available information concerning the composition of buffers 1 and 2, washing buffers 1 and 2, and elution buffer.

2.2.3. Polymerase chain reaction (PCR)

Screening of possible mutations present in the extracted DNA samples was achieved using the polymerase chain reaction (PCR) kit from Bioline (MyTaq HS Mix 2X, USA). The master mix composition is described in Figure 9A, and it was added to an adequate volume of DNA sample, according to the corresponding concentration obtained in the previous step. The PCR products were then amplified in a thermocycler system with the conditions described in Figure 9B.

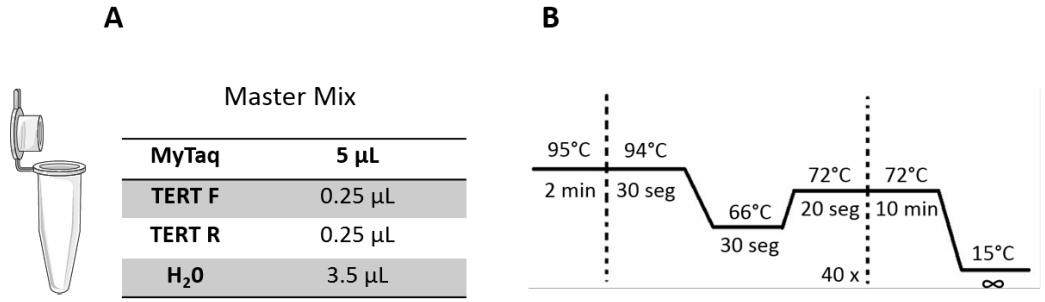


Figure 9. PCR (A) master mix composition and (B) conditions applied to *TERTp* amplification, using the Bioline kit.

2.2.4. PCR products evaluation and purification

Evaluation of DNA amplification by PCR was accomplished through electrophoresis, using a 1% agarose gel (GRS Agarose LE, GRiSP, Portugal). First, 2.5 µl of each PCR product was mixed with 1.5 µl of loading buffer dye containing gel red (VWR International-Material de Laboratório, Lda.). A ladder (Invitrogen, CA, USA) for molecular weight evaluation was used in each electrophoresis. The resulting gel was revealed through the ChemiDoc™ XRS+ System, from BIORAD (Universal Hood II, Hercules, CA, USA – 50/60 Hz).

If no aberrant bands were observed, the samples were purified by adding 1.5 µl of exoSAP to each PCR product. This procedure degrades the remaining DNA not processed in the previous PCR reaction and also removes possible excess of primers. ExoSAP consists of a mixture of Exonuclease I (Thermo Fisher Scientific, Lithuania) and Shrimp Alkaline Phosphatase (Thermo Fisher Scientific, Lithuania). To assure an efficient action of the enzyme, this procedure was performed on ice and the samples were then incubated at 37°C for 30 minutes, followed by another incubation at 85°C for 15 minutes.

2.2.5. Sanger Sequencing

To track the hotspot mutations, present in each sample, PCR products were sequenced by the Sanger sequencing method, using BigDye v3.1 Sequencing Kit (Applied Biosystems, Washington). The sequencing mix composition is described in Figure 10A, and it was added to an adequate volume of the purified PCR product. Finally, the sequencing products were amplified in a thermal cycler system according to the conditions described in Figure 10B.

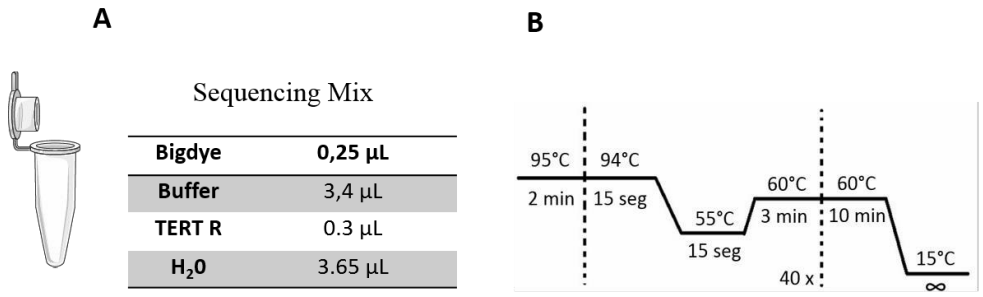


Figure 10. Sequencing (A) Mix composition and (B) Sequencing conditions applied to *TERTp*.

2.2.6. Sequencing products purification

The sequencing products were then purified to eliminate possible remaining DNA, which could interfere with the sample signal. This step was achieved by transferring the sequencing products to columns with Sephadex^{TM} G-50 resin (GE Healthcare Life Sciences, UK) and centrifuged at 3200 rpm for 4 minutes (Centrifuge 5417R, Eppendorf, Germany). Afterwards, 15 µl of Hi-Di^{TM} Formamide (Applied Biosystems, USA) were added to each sample to maintain the DNA in a denaturated state. Samples were then analyzed by capillary electrophoresis with fluorescence using the Applied Biosystems 3130/3130xl instrument. Validation of the detected mutations was assured by the repetition of the process starting from PCR amplification.

2.3. p53 immunohistochemistry

p53 expression in BCCs was evaluated through immunohistochemistry. Deparaffination of the sample slides was achieved through two 10-minute incubations in xylol, followed by hydration with decreasing concentrations of ethanol: two incubations in 100% ethanol (10 minutes each), 96% ethanol (5 minutes) and 70% ethanol (5 minutes). Then, the slides were

washed in pouring water for 10 minutes and incubated in concentrated citrate buffer 10% in the steamer at 90°C for 45 minutes, for antigene retrieval. After a cooling period of 20 minutes at room temperature, the slides were washed twice, for 10 minutes, in 10% phosphate-buffered saline (PBS) and 0.02% tween washing buffer. Using the hydrophobic pen, the tissue area was restricted so the reagents would stay in this area. Two incubations of 10 minutes each with ultravision hydrogen peroxide block (REF TA-060-H202Q, Thermo Scientific) and protein block (REF TA-125-PBQ, Thermo Scientific) were used to block endogenous peroxidase followed by a protein blocker (REF TA-125-PBQ, Thermo Scientific). After that, the slides were incubated with the primary antibody anti-p53 (NCL-L-p53, Leica), diluted in diluent 1:550 (REF TA-125-ADQ, Thermo Scientific), for 60 minutes. The slides were then washed three times in washing buffer (PBS) and incubated with primary antibody amplifier (REF TL-060-QPB, Thermo Scientific) for 10 minutes, and washed two times again in washing buffer. Then, incubation with HRP polymer quantum (REF TL-060-QPH, Thermo Scientific) for 10 minutes was done to further reaction with 3% diaminobenzidine chromogen (DAB, REF K3468, Dako) (3-minutes incubation). The slides were then washed in pouring water for 5 minutes, stained with hematoxylin for 1 minute and washed again in pouring water for 5 minutes. Dehydration of the slides was done through incubation in increasing concentrations of ethanol: 96% ethanol and 100% ethanol, for 5 minutes each. After two incubations in xylol for 5 minutes, the slides were mounted with the mounting medium, let to dry, and analyzed at the lab microscope.

The slides were digitalized, and the stained cells were counted recurring to digital media, allowing the quantification of the staining extension. For p53 staining evaluation, approximately 2000 cells were counted in each lesion. The scoring was done as follows: staining <10% was considered as negative staining, and staining ≥10% corresponded to positive cells[104,142]. Digitalization of the slides was performed with the scanner D. Sight flus (A-MENARINI diagnostics, UK) using its program D. Sight with automatized analysis and a p53 algorithm previously optimized by the Slides Digitalization Department of IPATIMUP.

2.4. Statistical analysis

Statistical analysis was performed using the statistical package for social sciences Software (IBM SPSS Statistics 23). Proportions were compared using X^2 test or Fisher's exact test when appropriate (Yates correction in case of multiple entries); the significance of differences between means was assessed by Student's unpaired t-test. A p-value <0.05 was considered statistically significant with a 95 % confidence interval.

3. Results

3.1. Demographic and Clinicopathological data

This study included 97 patients and their respective 165 BCC lesions. Within the 97 patients, there was an equivalent distribution between genders (Table 4), with 50 females (51.5%) and 47 males (48.5%).

Table 4. BCC patient's clinicopathological features.

Clinicopathological characteristics	
Number of patients	97
Age at diagnosis (mean ± SD) *	72.5 ± 11.5
Gender *	**N (%)**
Male	47 (48.5)
Female	50 (51.5)
BCC dimension (mm) (mean ± SD) *	13.2 ± 9.7
Von Luschen Scale (mean ± SD) *	11.9 ± 3.8
Melanin (mean ± SD) *	
Forearm	28.3 ± 3.2
Forehead	33.8 ± 4.0
Erythema (mean ± SD) *	
Forearm	7.5 ± 3.0
Forehead	18.0 ± 5.6
Solar exposure **	**N(%)**
Low	28 (32.9)
High	57 (67.1)
Phototype (mean ± SD) ***	2.4 ± 1.0
Multiple BCC *	**N(%)**
Single	60 (61.9)
Multiple	37 (38.1)

*N=97, **N=85, ***N=86

The mean age of the patients was 72.5 ± 11.5 years, ranging between 41 and 100 years old. Patients' skin color was evaluated with the Von Luschan scale, resulting in a mean value of 11.9 ± 3.8, which was then associated with the Fitzpatrick phototypes I, II, III and IV. The forehead presented higher values of melanin (33.8 ± 4.0) and erythema (18.0 ± 5.6) comparing with the inner forearm: 28.3 ± 3.2 and 7.5 ± 3.0 respectively. Most of the individuals had high sun exposure (67.1%). Regarding the 165 BCCs, the tumor dimension was 13.2 ± 9.7 mm, obtained through clinical evaluation. During the histological review, tumor thickness (2.40 ± 1.70 mm), lateral margin (1.99 ± 1.69 mm), and deep margins (2.59 ± 2.38 mm) were evaluated (Table 5).

Table 5. BCCs histopathological features.

Histopathological characteristics	
Number of lesions	165
Tumor thickness (mm) (mean ± SD) *	2.4 ± 1.7
Margins (mm) (mean ± SD) **	
Lateral	2.0 ± 1.7
Deep	2.6 ± 2.4
Topography of the lesion ***	**N (%)**
Nose	40 (24.2)
Face	36 (21.8)
Eyelid	32 (19.4)
Periauricular region	27 (16.4)
Scalp	13 (7.9)
Lip	6 (3.6)
Other	11 (6.7)
Histological Subtype ***	**N (%)**
Nodular	62 (37.0)
Infiltrative	53 (32.1)
Mixed	44 (26.7)
Superficial	7 (4.2)

*N=158, **N=161, ***N=165

Table 5. BCCs histopathological features (cont.)

Histopathological characteristics	
Invasion Level ****	**N (%)**
Papillary Dermis	10 (6.8)
Reticular Dermis	88 (55.0)
Subcutaneous	60 (37.5)
Intramuscular	2 (1.3)
Tumor characteristics ***	**N (%)**
Ulceration	98 (60.9)
Necrosis	34 (21.0)
Micronodular Pattern	24 (14.5)
Pigmentation	15 (9.3)
Elastosis	14 (10.0)
Actinic keratosis	13 (7.9)
Lymphocytic infiltrate ***	**N (%)**
Absent/Rare	89 (54.3)
Moderate/Intense	75 (45.7)
Lymphovascular invasion ***	
Not identified	153 (92.7)
Present	5 (3.0)
Undertemined	7 (4.2)
Perineural invasion ***	**N (%)**
Not identified	150 (90.9)
Present	9 (5.5)
Undertemined	6 (3.6)
Growth Pattern ***	**N (%)**
Expanded growth	75 (45.5)
Infiltrative growth	90 (54.5)

***N=165, N=162

BCC most common location was the nose (24.2%), followed by the face and the eyelid (21.8% and 19.4%, respectively). Some lesions were also found in the scalp, periauricular region, lip and other locations, with a lower frequency (7.9%, 16.4%, 3.6% and 6.7%, respectively). According to the histopathological revision (Figure 11), the nodular subtype was the most frequent (37.0%), followed by the infiltrative (32.1%), mixed (26.7%), and superficial (4.2%). The micronodular pattern was present in (14.5%) of the BCCs.

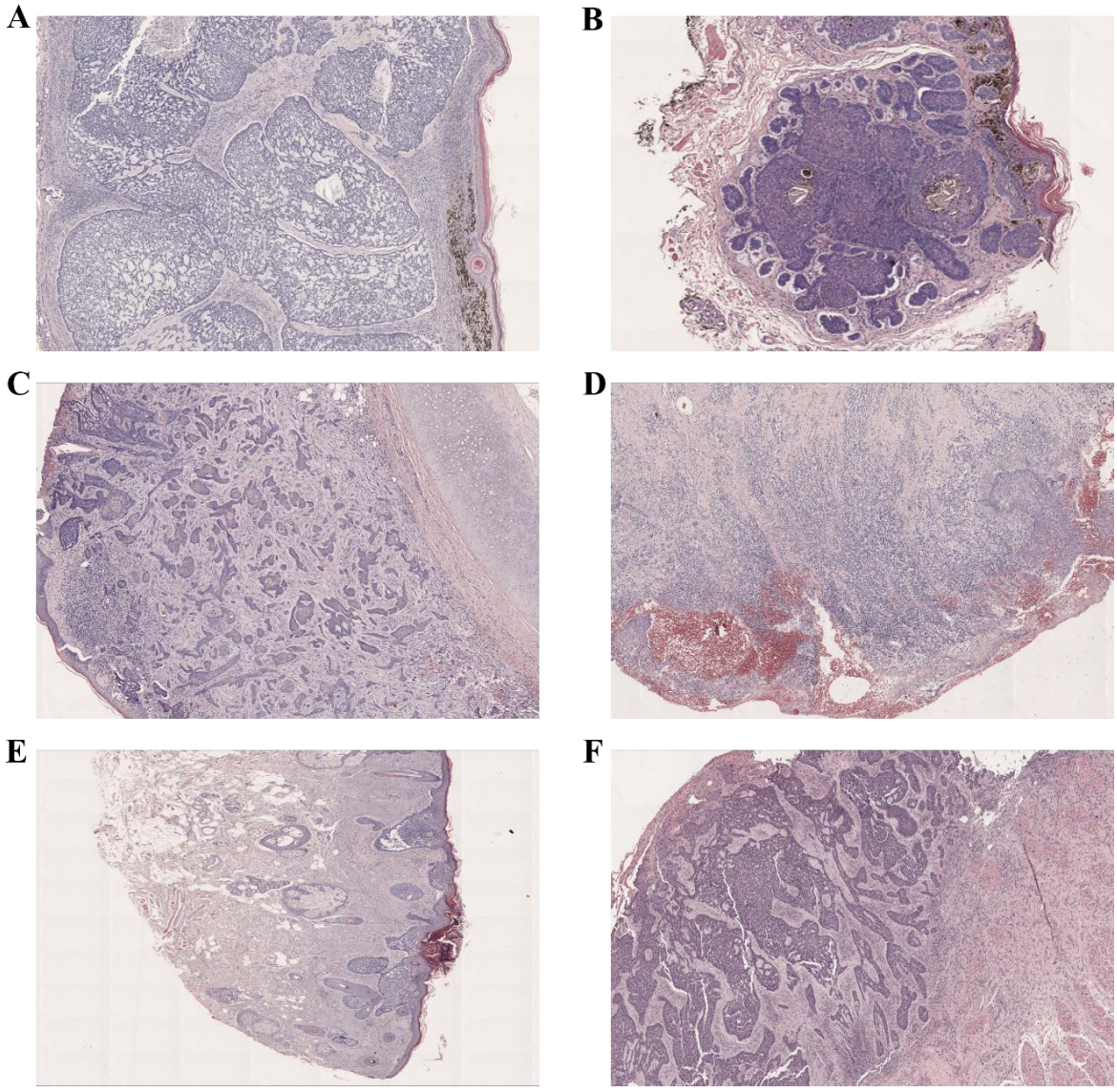


Figure 11. BCCs histopathological features - hematoxylin and eosin: (A) nodular subtype (40x); (B) nodular subtype with micronodular pattern (40x); (C) infiltrative subtype (40x); (D) lymphocytic infiltration (40x); (E) superficial subtype (20x); (F) nodular subtype with infiltrative features (mixed subtype, 40x).

Other histopathological characteristics evaluated were invasion level, ulceration, elastosis, necrosis, actinic keratosis and pigmentation. Actinic keratosis was present in 7.9% of the BCCs and 9.3% were pigmented. Clinical elastosis was observed in 63.6% (105/165) of the cases, but only 10.0% showed a pattern of elastosis in histopathological evaluation. 14.5% presented a micronodular pattern. Almost 61% of the BCCs showed a pattern of ulceration. 21.0% had necrosis, corresponding mostly (44.1%) to the nodular subtype, with no significant association with aggressive subtypes. The great majority of the BCCs presented reticular dermis and subcutaneous invasion (92.5%).

Presence of moderate/intense lymphocytic infiltrate was detected in 45.7% of the cases, being significantly higher (p=0.047) in the more aggressive cases. The majority of lesions did not show lymphovascular invasion (92.7% of the cases), nor perineural invasion (90.9% of the cases).

Evaluation of the growth pattern showed that only 45.5% of the lesions were characterized by an expanded growth, which comprises the less aggressive subtypes nodular and superficial, so most of the lesions had an infiltrative growth pattern. We looked for possible associations between the clinical and histopathological features above described and tumor aggressiveness, and only for tumor dimension and pigmentation, a significant association was observed. BCCs with infiltrative growth pattern were larger (15.0 ± 12.2) than expanded growth BCCs (10.8 ± 4.4) (p=0.003). Contrarily, pigmentation was significantly more frequent (16.0%) in the expanded growth BCCs comparing with the aggressive ones (3.4%) (p=0.007).

3.2. p53 immunohistochemistry

Within the analyzed BCCs, we observed distinctive patterns of expression: some presented a high percentage of p53 positive cells (nuclear brown staining) and others a higher percentage of negative cells (blue staining), as it is shown in Figure 12. From the 165 cases which were histologically reviewed, 3 cases showed non-specific staining, so it was only possible to analyze 162 BCCs. 10 cases (6.2%) had complete absence of p53 expression.

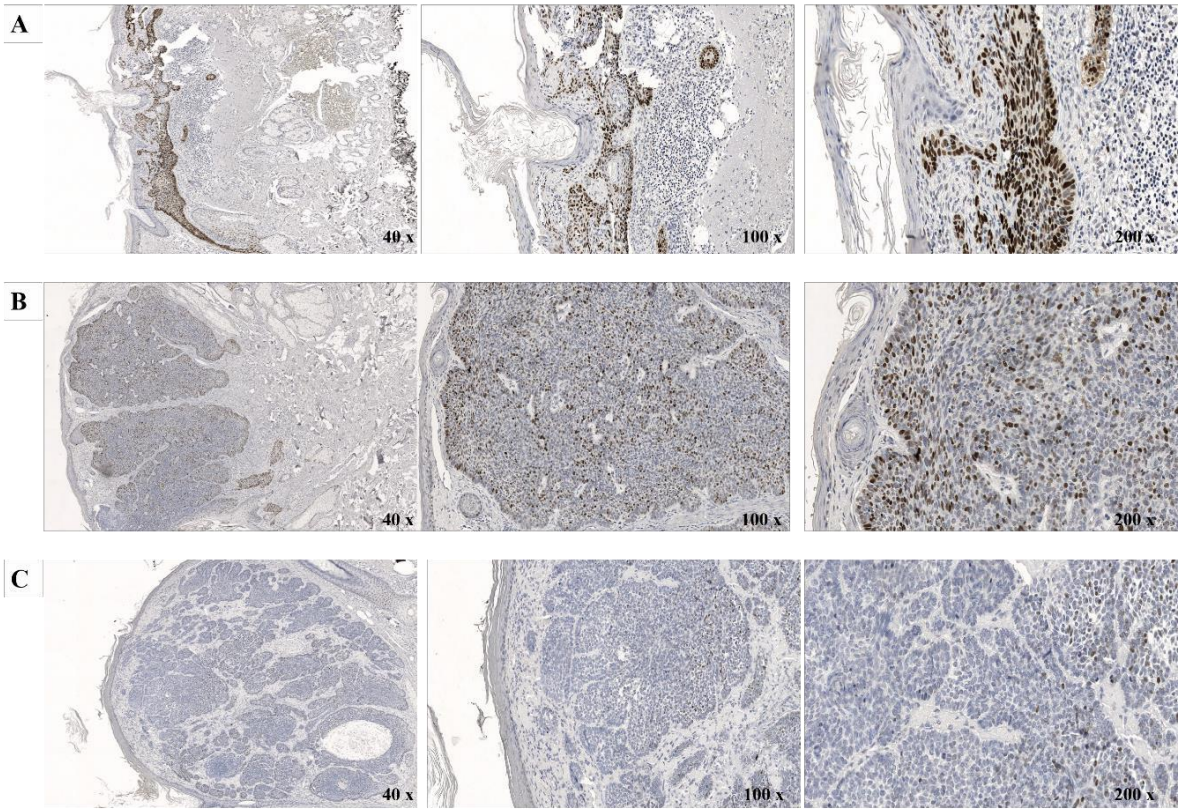


Figure 12. Extent of p53 expression: (A) high expression with 90.6% of positive cells; (B) moderate expression with 41.7% of positive cells; (C) low expression with 2.3% of positive cells.

We observed a similar patients' age in cases positive and negative for p53. Also, no differences were found between genders, as shown in Table 6. The same occurred with the other variables studied: time of diagnosis (time from appearance of the lesion until surgery was performed), tumor dimension, Von Luschen scale and phototype, melanin and erythema contents in the forearm and forehead, and sun exposure. There were no significant differences concerning p53 staining between single and multiple BCC. None of these differences were statistically significant ($p>0.05$).

Table 6. p53 staining pattern association with the BCCs clinicopathological features.

	p53 negative (<10%)	p53 positive (>10%)	p-value
Age at diagnosis (mean ± SD) *	71.9 ± 12.6	73.1 ± 10.6	0.527
Gender *	**N (%)**	**N (%)**	
Male	37 (56.9)	49 (50.5)	0.423
Female	28 (43.1)	48 (49.5)	
Time of diagnosis/ months (mean ± SD)**	22.9 ± 15.6	19.1 ± 14.3	0.112
Tumor dimension (mean ± SD) ***	13.2 ± 8.1	13.2 ± 10.8	0.987
Von Luschen Scale (mean ± SD) ******	11.6 ± 3.8	11.9 ± 3.7	0.646
Melanin (mean ± SD) *******			
Forearm	28.3 ± 2.9	28.3 ± 3.5	0.935
Forehead	34.1 ± 2.5	33.7 ± 4.8	0.596
Erythema (mean ± SD) *******			
Forearm	7.1 ± 2.1	7.7 ± 3.5	0.207
Forehead	18.1 ± 5.9	17.9 ± 5.3	0.870
Solar Exposure ********	**N (%)**	**N (%)**	
Low	14 (25.9)	22 (25.9)	0.995
High	40 (7.1)	63 (74.1)	
Phototype (mean ± SD) ********	2.4 ± 1.0	2.4 ± 1.1	0.699
Multiple BCC *	**N (%)**	**N (%)**	
Single	25 (38.5)	37 (38.1)	0.968
Multiple	40 (61.5)	60 (61.9)	

*N=162, **N=159, ***N=158, ****N=134, *****N=132, ******N=139, *******N=147

Higher percentage of positive cells were obtained for BCCs occurring on the nose (26.6%), face (22.3%), periauricular region (18.1%) and other locations (8.5%). BCCs located on the eyelid (16%), scalp (5.3%), and lip (3.2%) presented lower percentages of positive cells. Regarding the histological subtype, similar distributions of positive cells were observed for the nodular, infiltrative, superficial, and mixed subtypes. BCCs with reticular dermis or subcutaneous invasion had similar percentages of positive cells.

Table 7. p53 staining pattern according to the BCCs histopathological characteristics.

	p53 negative (<10%)	**p53 positive (>10%)**	**p-value**
Topography **	**N (%)**	**N (%)**	
Nose	15 (22.4)	25 (26.6)	
Face	14 (20.9)	21 (22.3)	
Eyelid	13 (19.4)	15 (16.0)	
Preiauricular region	12 (17.9)	17 (18.1)	0.835
Scalp	8 (11.9)	5 (5.3)	
Lip	3 (4.5)	3 (3.2)	
Other	2 (3.0)	8 (8.5)	
Histological Subtype ***	**N (%)**	**N (%)**	
Nodular	26 (40.0)	33 (34.0)	
Infiltrative	21 (32.3)	31 (32.0)	0.934
Mixed	16 (24.6)	28 (28.9)	
Superficial	2 (3.1)	5 (5.2)	
Invasion Level ****	**N (%)**	**N (%)**	
Papillary Dermis	3 (4.8)	7 (7.4)	
Reticular Dermis	31 (49.2)	55 (58.5)	0.547
Subcutaneous	27 (42.9)	32 (34.0)	
Intramuscular	2 (3.2)	0 (0.0)	
Tumor characteristics	**N (%)**	**N (%)**	
Ulceration **	34 (55.7)	62 (63.9)	0.305
Actinic keratosis ****	5 (8.1)	8 (8.4)	0.937
Necrosis ***	8 (12.7)	25 (26.0)	**0.042**
Pigmentation ***	9 (14.3)	6 (6.3)	0.090
Elastosis **	6 (10.9)	8 (9.4)	0.811
Micronodular pattern ***	10 (15.4)	13 (13.4)	0.723

*N=158, **N=161, ***N=162, ****N=160

Table 7. p53 staining pattern according to the BCCs histopathological characteristics (cont.).

	p53 negative (<10%)	p53 positive (>10%)	p-value
Lymphocytic infiltrate **	**N (%)**	**N (%)**	
Absent/Rare	37 (57.8)	50 (51.5)	0.435
Moderate/Intense	27 (42.2)	47 (48.5)	
Lymphovascular invasion ****	**N (%)**	**N (%)**	
Not identified	59 (92.2)	91 (94.8)	
Present	3 (4.7)	2 (2.1)	0.809
Undertemined	2 (3.1)	3 (3.1)	
Perineural invasion ****	**N (%)**	**N (%)**	
Not identified	59 (92.2)	88 (91.7)	
Present	4 (6.3)	5 (5.2)	0.991
Undertemined	1 (1.6)	3 (3.1)	
Growth Pattern ***	**N (%)**	**N (%)**	
Expanded growth	31 (47.7)	41 (43.3)	0.582
Infiltrative growth	34 (52.3)	55 (56.7)	

**N=161, ***N=162, ****N=160

BCCs with positive cells had similar frequencies of ulceration, actinic keratosis, elastosis and micronodular pattern comparing with the ones with negative cells. BCCs with positive cells had less pigmentation, but this difference was not significant (p=0.090). BCCs with positive cells had more necrosis (26.0%) than BCCs with negative cells (12.7%), and this difference was significant (p=0.042).

BCCs with peritumoral lymphocytic infiltrate had similar percentages of positive and negative cells. The same was observed for lymphovascular invasion and perineural invasion. BCCs with infiltrative growth pattern had identical percentages of positive (56.7%) and negative cells (52.3%).

Additionally, to try to find more possible associations between different parameters, we performed the same analyses separately for multiple and single BCCs. For single BCCs there were no associations. Contrarly for multiple BCCs, we found a significant association for elastosis: BCCs with elastosis had more positive cells (p=0.036).

3.3. Analysis of TERTp mutations

TERTp mutations analysis was performed in 52 of the 165 histological revised BCCs, belonging to 21 patients. TheDNA extraction was performed in 71 cases, but in 19 cases of very small BCCs, the low concentration and/or bad quality of the DNA obtained did not allow PCR amplification. DNA concentrations ranged from 1.44 to 197.84 ng/μL. Due to time constraints, it was not possible to analyze all the series, and we started with the cases for which we already had 10 μm cuts for DNA extraction.

TERTp mutations were observed in 18 of the 52 BCCs analyzed (34.6%). None of the clinicopathological features evaluated showed any association with the presence of *TERTp* mutations (Table 8).

Table 8. *TERTp* mutation association with BCCs clinicopathological features

	***TERTp* WT**	***TERTp* mutated**	**p-value**
Age (mean ± SD) *	72.6 ± 11.0	71.7 ± 12.2	0.790
Gender *	**N (%)**	**N (%)**	
Male	22 (64.7)	11 (61.1)	0.798
Female	12 (35.3)	7 (38.9)	
Time of diagnosis/ months (mean ± SD)*	20.9 ± 17.1	21.7 ± 15.0	0.875
Tumor dimension (mean ± std) *	17.2 ± 16.5	12.2 ± 5.8	0.224
Von Luschen Scale (mean ± SD) *	11.0 ± 3.4	10.8 ± 3.3	0.877
Melanin (mean ± SD) *			
Forearm	27.2 ± 2.4	26.6 ± 1.3	0.374
Forehead	33.5 ± 5.0	34.5 ± 2.1	0.465
Erythema (mean ± SD) *			
Forearm	7.6 ± 3.4	7.3 ± 2.4	0.780
Forehead	17.9 ± 4.8	18.4 ± 4.9	0.764
Solar Exposure**	**N (%)**	**N (%)**	
Low	4 (12.5)	1 (6.7)	1
High	28 (87.5)	14 (93.3)	
Phototype (mean ± SD) *	2.6 ± 1.1	2.7 ± 1.2	0.680
Multiple BCC*	**N (%)**	**N (%)**	
Single	8 (23.5)	2 (11.1)	0.462
Multiple	26 (76.5)	16 (88.9)	

*N=52, **N=47

There were no significant differences concerning the prevalence of *TERTp* mutation and age, gender, time until diagnosis, tumor dimension, Von Luschen scale, phototype, melanin and erythema contents in sun-exposed and sun-protected areas, sun exposure and the presence of multiple BCCs ($p>0.05$).

BCCs located on the face presented higher percentages of *TERTp* mutated cases (27.8%), compared with wild type BCCs (11.8%) (Table 9). The inverse was observed for BCCs located on the eyelid, which presented a higher frequency of wild type (32.4%) than *TERTp* mutated cases (16.7%). BCCs located on the nose, periauricular region and other regions had similar prevalence of *TERTp* mutations. Regarding the histological subtype, the mixed subtype had a similar frequency of mutated and wild type cases, the nodular subtype had higher frequency of mutated cases (55.6%) compared with the wild type (32.4%), the infiltrative subtype had a lower frequency of mutated cases (22.2%) compared with the wild type (38.2%), and the superficial subtype did not have mutated cases. Once again, these differences were not significant ($p>0.05$).

Table 9. *TERTp* mutation pattern according to the BCCs histopathological characteristics

	***TERTp* WT**	***TERTp* mutated**	**p-value**
Topography*	**N (%)**	**N (%)**	
Nose	5 (14.7)	4 (22.2)	
Face	4 (11.8)	5 (27.8)	
Eyelid	11 (32.4)	3 (16.7)	0.891
Periauricular region	8 (23.5)	3 (16.7)	
Scalp	3 (8.8)	1 (5.6)	
Other	3 (8.8)	2 (11.1)	
Histological Subtype*	**N (%)**	**N (%)**	
Nodular	11 (32.4)	10 (55.6)	
Infiltrative	13 (38.2)	4 (22.2)	0.643
Mixed	8 (23.5)	4 (22.2)	
Superficial	2 (5.9)	0 (0.0)	

*N=52

Regarding tumor invasion, BCCs with subcutaneous invasion presented higher frequencies of *TERTp* mutations (55.6%) comparing with wild type cases (33.3%) (Table 9). BCCs with *TERTp* mutations showed higher percentages of ulceration (82.4%) when compared with the wild type BCCs (64.7%). BCCs with *TERTp* mutations presented lower frequencies of actinic keratosis (11.1%) and elastosis (5.6%) comparing with the wild type BCCs. None of these differences were statistically significant. BCCs with *TERTp* mutations had more frequently a micronodular pattern (38.9%) than wild type BCCs (11.8%) (p=0.034)

Table 9. *TERTp* mutation pattern according to the BCCs histopathological characteristics (cont.).

	***TERTp* WT**	***TERTp* mutated**	**p-value**
Invasion Level**	**N (%)**	**N (%)**	
Papillary Dermis	3 (9.1)	0 (0.0)	
Reticular Dermis	18 (54.5)	7 (38.9)	0.434
Subcutaneous	11 (33.3)	10 (55.6)	
Intramuscular	1 (3.0)	1 (5.6)	
Tumor characteristics	**N (%)**	**N (%)**	
Ulceration**	22 (64.7)	14 (82.4)	0.192
Actinic keratosis**	4 (12.1)	2 (11.1)	1
Necrosis**	7 (21.2)	4 (22.2)	1
Pigmentation**	4 (12.1)	3 (16.7)	0.686
Elastosis***	3 (9.4)	1 (5.6)	1
Micronodular pattern*	4 (11.8)	7 (38.9)	**0.034**
Lymphocytic infiltrate*	**N (%)**	**N (%)**	
Absent/Rare	20 (58.8)	14 (77.8)	0.172
Moderate/Intense	14 (41.2)	4 (22.2)	
Lymphovascular invasion*	**N (%)**	**N (%)**	
Not identified	30 (88.2)	18 (100)	
Present	2 (5.9)	0 (0)	0.889
Undertemined	2 (5.9)	0 (0)	
Perineural invasion*	**N (%)**	**N (%)**	
Not identified	31 (91.2)	17 (94.4)	
Present	1 (2.9)	1 (5.6)	0.921
Undertemined	2 (5.9)	0 (0)	
Growth Pattern*	**N (%)**	**N (%)**	
Expanded growth	13 (38.2)	11 (61.1)	0.115
Infiltrative growth	21 (61.8)	7 (38.9)	

*N=52, **N=51, ***N=50

BCCs with moderate/intense lymphocytic infiltrate were less frequent in mutated (22.2%) than in wild type cases (41.2%), but this difference did not reach significance (p=0.172). None of the cases lymphovascular invasion presented *TERTp* mutations. Perineural invasion was rare, with a similar frequency for cases with or without mutation (5.6% *vs* 2.9%). BCCs with infiltrative growth pattern had a lower frequency of *TERTp* mutations (38.9%) when compared with wild type BCCs (61.8%), although this difference was not significant (p=0.115).

TERTp mutations found within the hotspots analyzed are described in Table 10. The most common mutation was the -146, comprising 89% of the cases. Less frequent were the mutations in the hotspot -124 and the tandem mutation -124/-125.

Table 10. *TERTp* mutations identified.

	***TERTp* mutations**	
	Frequency	*Percentage (%)*
-124	2	11.1
-146	14	77.8
-124 and -146	1	5.6
-124/-125 and -146	1	5.6
Total	18	100

We looked for an association between the presence of p53 positive cells and *TERTp* mutations, as shown in Table 11. The BCCs with positive presented a similar frequency of *TERTp* mutated (58.8%) and wild type cases (61.8%).

Table 11. *TERT*p mutation association with p53 expression.

	***TERTp* mutation**		**p-value**
	Wild type **N(%)**	*Mutation* **N(%)**	
p53 negative (<10%)	13 (38.2)	7 (41.2)	0.839
p53 positive (>10%)	21 (61.8)	10 (58.8)	

4. Discussion

4.1. Demographic, clinicopathological and histopathological data

In the present series, the patients had an advanced age (72.5 ± 11.5), one of the risk factors for BCC development. Previous studies have reported similar mean ages, ranging from 54 to 80 years old[97,143,144,145,146].

A high percentage of patients had professions related to intense solar/UV exposure (data not shown). Using Von Luschan and Fitzpatrick scales for skin type evaluation, patients were distributed between phototypes II and III which are characterized by easily burned skin and a moderate ability to tan. Individuals with these phototypes have a relatively high risk of developing skin cancer. Regarding the levels of melanin and erythema in sun-exposed areas (forehead), they were compatible with patients ethnicity[42]. Tadokoro *et al.* have shown a significant relation between erythema levels and DNA damage after sun exposure, with higher DNA damage in groups with higher values of erythema[147].

Melanin has shown to be a photoprotective agent, as it acts as a shield for UV exposure[38]. However, there are no available data correlating melanin content with sun exposure in Caucasians. The levels of melanin and erythema obtained in the present study are in accordance with the skin color evaluation based on phototypes, which pointed to phototypes with moderate ability to tan and to sunburn (II and III)[41]. Melanin content of sun-exposed areas (forehead) was higher than the melanin content of sun-protected areas, what was expected due to the high levels of sun exposure experienced by the patients of our series (67.1%).

Presence of multiple BCCs was observed in 38.1% of the 97 patients, which is in conformity with what has been reported in other series (16.4-52.8%)[97,143,144,145].

The majority of the BCCs was located on the nose and the face, both corresponding to areas with high sun exposure. Regarding the histological BCC subtypes, nodular BCC was the most frequent (37.0%). This frequency was slightly below the range previously described for this subtype (44.6-78.7%)[97,145,148]. Contrarily, the infiltrative subtype, considered as more aggressive, was more frequent in our series (32.1%) in comparison with what was reported in previous studies (7.5-16.6%)[97,131,145,143].

We observed a higher percentage of BCCs with an infiltrative growth pattern than with expanded growth, which has already been associated with tumor recurrence[149]. More than half of the BCCs showed invasion between the reticular dermis and subcutaneous layer, which could also be an indicator of aggressiveness[149,150].

In terms of tumor characteristics, the majority of the lesions showed ulceration, previously described as a predictor of tumor aggressiveness and shown to be associated with a high rate of proliferation[149,151]. This result, again, points to the BCC aggressiveness of our series.

Actinic keratosis, which is a precursor lesion of SCC, and is caused by UV exposure, was present in a low percentage of the cases (7.9%), similarly to elastosis (10.0%). There are no studies relating actinic keratosis and with BCC aggressiveness, being considered as controversial BCC risk factors[11]. However, since actinic keratosis and elastosis are the main indicators of sun exposure, and since we obtained high levels of sun exposure, it could be expected to obtained higher percentages for these two characteristics.

In our series, 21.0% of the BCCs presented necrosis, a lower frequency than the values obtained in a previous study of Welsch *et al*, where 39% of the lesions had necrosis, and were less deep than lesions without necrosis[149]. In fact, our cases showed an invasion level between the reticular dermis and the subcutaneous layer, but no significant association with necrosis was found ($p>0.05$).

Pigmentation was significantly more frequent in less aggressive cases, which leads to the assumption that tumor pigmentation might be inversely associated with tumor aggressiveness. Griewank *et al*. reported 3.1% of pigmentation among all cases, which represents a third of what we obtained in our study (9.3%). No significant association was observed between pigmentation and p53 expression, although pigmentation has already been associated with a higher expression of p53 in a previous study[76].

We observed more cases with absent/rare lymphocytic infiltrate (54.3%) than cases with moderate/intense lymphocytic infiltrate (45.7%), although the frequencies were rather similar. The lymphocytic infiltrate was significantly more common in more aggressive tumors, which could point once again to a higher aggressiveness of our BCC series. Contrarily to our results, the absence of lymphocytic infiltrate has shown to be an indicator of bad prognosis in some types of cancer, such as melanoma and glioblastoma. Patients with BCCs with lymphocytic infiltrate or brisk, according to Clark *et al.*, have shown high rates

of survival[152,153,154] and high rates of proliferation[151]. However, some authors studied the content of the lymphocytic infiltrate and came to the conclusion that, according to the lymphocyte infiltrate, patients would have a poor prognosis (when this infiltrate was composed of $CD8^+$, $CD4^+$ and $Foxp3^+$ lymphocytes, macrophages and mast cells,) or better prognosis (when it was composed of $CD3^+$ and $CD8^+$ lymphocytes, and C-type lectin-like receptor 2 (CLEC2))[155,156,157]. The majority of the BCCs did not present lymphovascular and perineural invasion, which is in accordance with previous findings in non-melanoma skin cancer[140]. These two parameters have shown to be indicators of a lower survival rate and tumor recurrence in gastric cancer, as they represent aggressiveness prognostic factors[158]. However, there are no described data suggesting this type of relation in BCC.

4.2. p53 immuno expression

We did not observe a significant association between p53 expression and sun exposure, similarly to what has been described in a previous study[159]. An increase of p53 positive cases with high sun exposure could be expected as UV-light can lead to DNA damage, enhancing p53 activity and expression[64]. It is also important to consider that, 10 cases showed complete absence of p53 expression, which might indicate the presence of a mutation.

In our series, cases with multiple BCC had more p53 expression than cases with only one BCC, but this difference was not significant. Previous studies have shown that patients with single BCCs are more prone to develop multiple BCCs and other types of skin malignancies when compared to healthy patients[12,144]. Being the presence of multiple BCC considered as a pattern of aggressiveness[53,144], we could expect to observe more p53 positive cases in multiple BCC, when compared with single BCC.

In this study, it was observed a similar distribution of positive and negative cells for each histological subtype. Auepemkiate *et al.* found a significant correlation between p53 immune expression and the presence of the infiltrative subtype, confirming it as an aggressive subtype with a higher likelihood to recur and invade[159]. However, the methodology used was no the same as the one used in the present study, as it is not possible to make valid comparisons.

In our series, most cases presented invasion between reticular dermis and subcutaneous layer, so it could be expected to detect a high number of p53 positive BCCs presenting invasion until the subcutaneous layer, once it has already been described that p53 overexpression and mutations precede tumor invasion[83,160]. Contrarily to the observations made by Enache *et al.*[148], we observed a higher percentage of positive cases in BCCs with invasion until the reticular dermis (58.5%). Due to the high number of aggressive and invasive cases, it was expected to have an increase in p53 expression in BCCs with a high level of invasion, i.e. less p53 expression in the papillary dermis (more superficial layer of

the skin) and more p53 expression in the intramuscular layer (deeper layer)[148]. However, our results did not show any significant differences, so it is not possible to establish an association between invasion level and p53 expression.

Significant associations between p53 expression and tumor characteristics were not found, except for necrosis. However, there is no evidence in the literature, linking necrosis with either tumor aggressiveness or p53 expression. On the other hand, pigmentation has already been related to higher p53 expression and aggressiveness in BCC, as it was previously mentioned. Considering that our cohort is composed of a high number of aggressive BCCs, it was expected to observe a high amount of p53 positive cells in pigmented BCC, what did not happen[76].

No significant association was found between the presence of lymphocytic infiltrate and p53 expression. Considering p53 expression as one of the factors involved in tumor progression, which has been associated both with BCC aggressiveness and poor prognosis, it could be expected to observe an increase of positivity with the absence of lymphocytic infiltrate and with an infiltrative growth pattern, as it was already seen in melanoma[153,161]. Additional studies, such as the evaluation of the lymphocytic infiltrate content, could help to find an eventual relationship between the lymphocyte subset and BCC prognosis and aggressiveness. Perineural and lymphovascular invasions did not show any significant associations with p53 expression. There are no data showing any relationship between these types of invasion and p53 expression.

We found a positive and significant association between aggressiveness and tumor dimension. However, we found no significant differences between small and large tumors according to the p53 expression. BCCs' dimension has already been considered as a marker of tumor aggressiveness, i.e., larger tumors tend to have a more aggressive pattern[25,137].

4.3. TERTp mutation pattern

A lower frequency of *TERTp* mutated cases was observed in our series comparing with the results of previous studies in which the prevalence of *TERTp* mutations ranged between 51 and 80%[97,87,91,131]. Within mutated cases, we observed a high percentage of mutations in the -146 hotspot (89%), higher than the one previously reported in similar studies (25-39%)[97,131,162].

No association between solar exposure and *TERTp* mutations was found. However, considering *TERTp* mutations may have a UV-signature[97,131,162], we expected to observe a significant increase of mutated cases in BCCs with high sun exposure.

Also, for multiple BCC and *TERTp* mutation patterns, no significant differences were observed. As previously mentioned, the presence of multiple BCC is a predictor of aggressiveness, as well as the presence of *TERTp* which has already been described as an aggressiveness biomarker for SCC[137]. However, our results are in accordance with those reported by Boaventura *et al*[97], as no significant differences were found in *TERTp* mutations frequency between multiple and single BCCs.

In nodular BCCs we observed a 55.6% frequency of *TERTp* mutation which was higher than the ones reported in previous studies (44.0- 44.5%)[97,131]. Also, the frequency of mutation for infiltrative BCC (22.2%), which is considered the most aggressive subtype[18], exceeded the percentages obtained in other studies (14.7-16.7%)[97,131]. However, the differences in the frequency of *TERTp* mutation according to the histological subtype were not significant.

We also did not observe differences in the frequency of *TERTp* mutations according to the invasion level. Considering *TERT* as a tumor invasion influencer[87], we could expect to see higher percentages of *TERTp* mutations in deeper layers of the skin. However, there are no published data relating these two parameters.

The presence of ulceration, elastosis, necrosis, pigmentation or actinic keratosis did not show a significant association with *TERTp* mutation. There is no evidence relating these tumor characteristics with *TERTp* mutation pattern in BCC. *TERTp* mutations were associated with the presence of a micronodular pattern (p=0.034). This pattern has shown to have an aggressive behaviour[21], and it has also been associated with a high p53 activity[163]. Lymphocytic infiltrate, perineural and lymphovascular invasion showed no significant association with *TERTp* mutation. In previous studies with SCC this association was also not reported[131,140].

We did not observe significant differences in the frequency of *TERTp* mutations according to BCC dimension. A previous study has shown increased *TERTp* mutations in larger tumors[164].

No significant relation between *TERTp* mutation pattern and p53 expression was found in our series. Two possible scenarios could be expected: either to find a higher amount of p53 positive cells in *TERTp* mutated lesions, as these two parameters are both biomarkers of aggressiveness and poor prognosis in several types of cancer[132,139,163,165], including SCC[140,166]; or to find a loss of function of p53 associated with *TERTp* mutations as it was already been described[87]. None of these was observed in our series, but there are no available data establishing a significant association between p53 expression and *TERTp* mutations in BCCs.

To sum up, it was observed that larger tumors were significantly associated with a more aggressive pattern, as well as decreased pigmentation and presence moderate/intense lymphocytic infiltrate. Previous data have shown correlations between p53 expression and some tumor characteristic such as pigmentation, but only the presence of necrosis was found to be significantly correlated with a high p53 expression. Also, regarding *TERTp* mutations it could be expected to observe an association with characteristics linked to BCCs aggressiveness, namely the presence of the infiltrative subtype. However, only the presence of a micronodular pattern was related with an increased frequency of *TERTp* mutations. The small amount of BCCs analyzed for *TERTp* mutations constitutes one of the main limitations of our study, once it may have led to low statistical significance due to low figures. To make a more powerful analysis it will be important to evaluate the remaining BCCs of our series.

5. Conclusion and Future Perspectives

Our results suggest that pigmentation, tumor dimension and lymphocytic infiltrate could be used as indirect markers for BCCs aggressiveness, as we have seen that aggressive BCCs corresponded to larger tumors and presented lower frequencies of pigmentation and higher frequencies of lymphocytic infiltrate.

Regarding p53 expression, we observed that BCCs with necrosis have a higher expression of this protein. Considering that there are no previous studies with these results, it could be interesting to perform immunohistochemistry in a larger series of BCCs, in an attempt to confirm our findings.

We expected to observe an association between *TERTp* mutations and BCC aggressiveness, considering this association has already been described in SCCs. However, from our results, we can only conclude that BCCs with a micronodular pattern have a higher frequency of *TERTp* mutations what could led us to suppose that these tumors could be more aggressive.

As it was previously mentioned, *TERTp* molecular analysis achieved in our cohort, was not done for every BCC included in our series. It would be relevant to perform this study in all the remaining BCCs, in order to try to increase the statistic value of our results.

To sum up, our data did not allow to establish any significant relation between the two biomarkers – p53 expression and *TERTp* mutations. Further studies such as molecular analysis of *p53* and study of TERT expression in BCC, should be achieved in the future so that we could get a complete understanding of their role in this type of skin cancer.

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