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Mutational Analyses of the Tumor Suppressor Gene PATCHED1

Role in Non-Melanoma Skin Cancer and Nevoid Basal Cell Carcinoma

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Stockholm 2008

Cover: “Creating PTCH1 mutations” © Erika Lindström
Published by Karolinska Institutet. Printed by Universitetsservice US-AB
Nanna Svartz väg 4
SE-171 77 Solna, Sweden

ISBN 978-91-7357-590-4

ABSTRACT

Skin cancer is the most common cancer in Western countries, with a rapidly increasing incidence. The majority of these tumors are non-melanoma skin cancer (NMSC). The most common NMSC is basal cell carcinoma (BCC) followed by squamous cell carcinoma (SCC). In the US more than 1.3 million NMSCs are diagnosed each year, while in Sweden approximately 43000 cases were diagnosed in 2006. BCCs are slow growing tumors that are locally invasive but rarely metastasize. In contrast, SCCs are rapidly growing, invasive and have a metastatic potential. Exposure to UV radiation is the most important risk factor for developing BCCs and SCCs, followed by fair skin. NMSC occurs mostly as sporadic cases, but is also associated with certain genetic diseases, including the Nevoid basal cell carcinoma syndrome (NBCCS). NBCCS is characterized by multiple BCCs, developmental defects and predisposition to other types of tumors. Additionally, Multiple-self healing squamous epithelioma (MSSE) is a genetic disease with tumors similar to SCCs, as its hallmark. Both the NBCCS and MSSE responsible gene(s) are mapped to 9q22.3. The following studies were performed in order to investigate the role of genetic components in the development of these diseases and in NMSCs.

In **Paper I**, the NBCCS gene was further mapped to the 9q22.3 region and confirmed to be involved in familial and sporadic BCCs. Moreover, we obtained evidence that (a) the NBCCS gene is indeed a tumor suppressor gene (TSG) and (b) another TSG in the same chromosomal region is likely to have a role in the development of the squamous type of skin cancer.

In **Paper II**, the NBCCS gene, *PTCH1*, was verified to be the gene underlying NBCCS in Swedish patients, as inactivating *PTCH1* mutations in the blood from these patients were identified. We also obtained evidence that the *PTCH1* gene is an important TSG involved in the development of both sporadic and familial BCCs.

In **Paper III**, we investigated whether genetic alterations in the *PTCH1* and *XPA* genes are critical for the development of the squamous type of skin cancer. However, no mutations but only a high degree of polymorphism of the *PTCH1* gene could be detected. Therefore, both *PTCH1* and *XPA* were excluded as of major importance for the development of this type of skin cancer. It is likely that other gene(s), distal to *PTCH1*, are involved in SCCs development.

The *PTCH1* gene is mutated in different cancer types, but mainly in BCCs and other tumors associated with NBCCS. To better understand the role of PTCH1 in human disease, a locus-specific database, the PTCH Mutation Database, was set up in order to collect all relevant mutations. In **Paper IV**, the distribution pattern of *PTCH1* mutations and single nucleotide polymorphisms that were compiled from the database were investigated. Unique distribution and mutation-type patterns that characterize the NBCCS disease, sporadic BCCs, BCCs from Xeroderma pigmentosum patients and sporadic medulloblastomas were identified. Additionally, domains and regions in the PTCH1 protein that are critical in the development of sporadic tumors and NBCCS were revealed.

Conclusion: *PTCH1* mutations result in deregulated Hedgehog signaling and are of major importance in the pathogenesis of NBCCS and BCCs but not SCCs.

LIST OF PUBLICATIONS

This thesis is based on following publications which are referred to in the text by their roman numerals:

I. **Holmberg E**, Rozell B L och Toftgård R. Differential allele loss on chromosome 9q22.3 in human non-melanoma skin cancer. British Journal of Cancer, 1996, 74, 246-250.

II. Undén AB*, **Holmberg E***, Lundh-Rozell B, Ståhle-Bäckdahl M, Zaphiropoulos PG, Toftgård R and Vorechovsky I. Mutations in the human homologue of drosophila patched (PTCH) in basal cell carcinomas and the Gorlin syndrome: different in vivo mechanisms of PTCH inactivation. Cancer Research, 1996, 56, 4562-4565.

III. Eklund LK, **Lindström E**, Undén AB, Lundh-Rozell B, Ståhle-Bäckdahl M, Zaphiropoulos PG, Toftgård R, Söderkvist P. Mutation analysis of the human homologue of Drosophila patched and the xeroderma pigmentosum complementation group A genes in squamous cell carcinomas of the skin. Molecular Carcinogenesis, 1998, 21(2), 87-92.

IV. **Lindström E**, Shimokawa T, Toftgård R and Zaphiropoulos PG. PTCH mutations: Distribution and analyses. Human Mutation, 2006, 27(3), 215-219.

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TABLE OF CONTENTS

1. INTRODUCTION 1
1.1 Cancer 1
1.1.1 Oncogenes 1
1.1.2 Tumor suppressor genes 1
1.1.3 Stability genes 2
1.1.4 Cancer is a complex disease 2
1.1.5 Cancer contributing factors 3
1.1.6 Human genome variation 3
1.1.7 Metastasis 3
1.1.8 Treatments 4
1.2 Skin 4
1.3 Skin cancer 5
1.3.1 Basal cell carcinoma 6
1.3.2 Squamous cell carcinoma 7
1.3.3 Actinic keratoses and Bowen's disease 7
1.3.4 Keratoacanthoma 7
1.3.5 Treatments of NMSC 8
1.4 Genetic disorders associated with NMSC 8
1.4.1 Nevoid Basal Cell Carcinoma Syndrome 8
1.4.2 Xeroderma pigmentosum 9
1.4.3 Multiple self-healing squamous epithelioma 10
1.4.4 Other syndromes 10
1.5 Molecular etiology of NMSC 11
1.5.1 BCC 11
1.5.2 SCC 11
1.6 Hedgehog signaling network 12
1.6.1 Hedgehog signaling 12
1.6.2 Patched1 14
1.6.3 Patched2 15
1.6.4 Other core components of Hedgehog signaling 15
1.7 Animal models of Hedgehog signaling 16
1.8 Deregulated Hedgehog signaling in human diseases 17
1.8.1 NBCCS 18
1.8.2 BCC 18
1.8.3 SCC 20
1.8.4 Medulloblastoma 20
1.8.5 Other tumors 20
1.8.6 Treatment of overactivated HH signalling 21
1.9 Final remark 21
2. AIMS OF THE THESIS 22
3. METHODS 23
4. RESULTS AND DISCUSSION 24
4.1 PAPER I: Fine Mapping of the NBCCS gene and Differential 9q Loss in Non-Melanoma Skin Cancer 24
4.1.1 NBCCS and BCCs 24
4.1.2 Squamous type of skin cancer 24

4.1.3 Conclusions 25
4.2 PAPER II: PTCH1 Mutations in Swedish NBCCS Patients and BCCs 25
4.2.1 Conclusions 26
4.3 PAPER III: The PTCH1 and XPA Genes are Not Mutated in the Squamous Type of Skin Cancer 26
4.3.1 *PTCH1* gene 26
4.3.2 *XPA* gene 26
4.3.3 Conclusions 27
4.4 PAPER IV: Distribution patterns of human PTCH1 mutations 27
4.4.1 General distribution pattern 27
4.4.2 Distribution in different groups 28
4.4.3 Distribution of different mutation types 29
4.4.4 Conclusions 29
4.5 GENERAL CONCLUSIONS 30
5. FUTURE PERSPECTIVES 31
6. ACKNOWLEDGEMENTS 32
7. REFERENCES 33

ABBREVIATIONS

AK	Actinic keratoses
BCC	Basal cell carcinoma
BD	Bowen's disease
C-terminal	Carboxyl terminal
Dhh	Desert hedgehog
Drosophila	Drosophila melanogaster
HH/Hh/hh	Hedgehog
HPE	Holoprosencephaly
Ihh	Indian hedeghog
LOH	Loss of heterozygosity
KA	Keratoacanthoma
MSSE	Multiple self-healing squamous epitheliomata
NBCCS	Nevoid basal cell carcinoma syndrome
NER	Nucleotide excision repair
NMSC	Non-melanoma skin cancer
NPC1	Niemann-Pick disease type C1
N-terminal	Amino terminal
OK	Odontogenic keratocyst
PCR	Polymerase chain reaction
PTCH/Ptch	Patched
RND	Resistance-nodulation-division
SCC	Squamous cell carcinoma
SCCA	Single strand conformation analysis
SCCP	Single strand conformation polymorphism
SHH/Shh	Sonic hedgehog
SMO/Smo	Smoothened
SNP	Single nucleotide polymorphism
SSD	Sterol-sensing domain
SUFU/Sufu	Suppressor of fused
TM	Transmembrane
TSG	Tumor suppressor gene
UV	Ultraviolet
XP	Xeroderma pigmentosum

Genes, italics (*Ptch*); human proteins, capital letters (PTCH); mouse and vertebrates proteins, initial capital letter (Ptch); Drosophila proteins, small letters (hh)

1. INTRODUCTION

1.1 Cancer

Cancer is a common disease and malignant tumors have been described in ancient cultures and diagnosed in humans as far as 3000 BC in Egyptian mummies (Tannock IF, 2005). The classical characteristic of cancer is the uncontrolled division of cells, which invade the nearby tissues through the lymphatic vessels or migrate to other parts of the body through the blood forming metastases. Cancer for a long time has been viewed as a cell-autonomous process and tumor development was thought to be a multi-step process, with a succession of genetic changes needed to drive the transformation of normal human cells into cancer cells. It has also been proposed that most but not all tumors share six properties; self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Several of the genes controlling these cellular processes are proto-oncogenes and tumor suppressor genes (TSG) (Hanahan & Weinberg, 2000). During the past few years the "reductionist" view of cancer cells and their genes has changed towards a more "holistic" approach. Thus, cancer is now viewed as a heterogeneous complex tissue with reciprocal interactions between epigenetically or genetically altered malignant cells and the surrounding, dynamic microenvironment (Radisky et al., 2001; Albini & Sporn, 2007; Kenny et al., 2007)

1.1.1 Oncogenes

Oncogenes are genes whose protein products are involved in the transformation of normal cells into a malignant state. Proto-oncogenes are genes that are crucial for normal cell cycling and division, differentiation and apoptosis. However, a proto-oncogene can function as an oncogene if illegitimately activated through mutations, amplifications, chromosomal rearrangements or overexpression. The oncogenic mutations are dominant, "gain-of-function", which means that only one allele needs to be altered for oncogenic function (Vogelstein & Kinzler, 2004; Tannock IF, 2005; Tilli et al., 2005; Croce et al, 2008). Most oncoproteins participate in signaling pathways and can be generally classified as; growth factors, growth factor receptors, signal transducers, transcription factors and regulators of cell-death (Croce, 2008). Examples of proto-oncogenes are *RAS*, *MYC*, and *GLI* (Kinzler et al., 1987; Croce, 2008).

1.1.2 Tumor suppressor genes

Tumor suppressor genes, also called anti-oncogenes, are genes that prevent tumor development. In general the TSG mutations are recessive at the cellular level, which means that both alleles have to be inactivated in order to observe "loss of function" of the TSG protein (Vogelstein & Kinzler, 2004; Tannock IF, 2005). This is also called the Knudson's two-hit hypothesis, which was based on incidence curves for familial and sporadic retinoblastoma, a childhood tumor of the eye. Knudson postulated that in familial retinoblastoma one mutated TSG is inherited via the germline cells, the first hit,

followed by a second hit in the somatic retinal cells. In sporadic retinoblastoma, both hits are thought to occur in the same somatic cell, which statistically is less likely to occur compared to the familial form of retinoblastoma (Knudson, 1971). This second allelic inactivation in tumors often occurs through deletion or chromosomal rearrangements, and is referred to as "loss of heterozygosity" (LOH) (Vogelstein & Kinzler, 2004; Tannock IF, 2005). Mutations in TSGs can be inherited, which predispose affected individuals and subsequent generations to early onset of disease, as the first hit is already present at birth. Recently, it has been shown that mutations in TSGs are not always recessive in the classical sense, and that only one mutated allele can contribute to cancer development, the phenomenon of haploinsufficiency. Several TSG have been shown to be haploinsufficient, including *p27* and *p53*. Dominant-negative mutations, when a mutant allele is interfering with the normal allele, have also been found in TSGs, suggesting that the two-hit dogma needs to be modified (Santarosa & Ashworth, 2004; Payne & Kemp, 2005). Typical examples of TSGs are *p53*, *APC*, *p16/INK4a* and *PTCH1* (Vogelstein & Kinzler, 2004).

1.1.3 Stability genes

Stability genes, also known as caretakers, are responsible for the integrity of the genome keeping genetic alterations to a minimum, and thus preventing tumor development. Many of the stability genes are involved in the DNA-repair systems. Mutations in these genes are recessive at the cellular level; both alleles have to be inactivated. Several chromosome instability disorders are known, where germline mutations result into genomic instability and predisposition to cancer. Examples of stability genes are *ATM*, *BRCA1-2*, *MSH2* and *XPA* (Levitt & Hickson, 2002; Vogelstein & Kinzler, 2004).

1.1.4 Cancer is a complex disease

Many different theories about the nature of cancer have been discussed during the last decades. For the past 50 years the somatic mutation theory, where cancer is thought to arise through a single mutation in a somatic cell followed by successive mutations, has dominated cancer research (Baker & Kramer, 2007). This theory resulted in the identification of many cancer-associated genes and their signaling pathways (Vogelstein & Kinzler, 2004). Recently, evidence for the importance of the tumor microenvironment, both inside the tumor itself and in the surrounding normal tissue, has challenged these prevailing views (Albini & Sporn, 2007; Kenny et al., 2007; Laconi, 2007). The classical perception of cancer as a disease of uncontrolled cell division has also been discussed, since this approach excludes the importance of cell differentiation. Many cancer-associated genes have been found to control cell division and /or the cell-cycle. Differentiation and cell division are reciprocally connected and important genes contributing to cancer progression are also involved in cell differentiation (Gottlieb et al., 2007).

1.1.5 Cancer contributing factors

Involuntary exposure to environmental factors such as microorganisms (viruses, bacteria and parasites), radiations (UV, radioactivity) and xenochemicals might play a more important role in cancer development than previously thought. Lifestyle related factors such as tobacco smoking, obesity, and alcohol consumption are also associated with carcinogenesis (Belpomme et al., 2007; Irigaray et al., 2007). In addition, hormones and immune system changes are important contributors (Frank & Nowak, 2004). Moreover, epigenetic factors regulate gene silencing at the chromatin level. Normally, this gene silencing is necessary for development and differentiation, but its deregulation can contribute to cancer development (Jones & Baylin, 2007; Stindl, 2008).

1.1.6 Human genome variation

Cancer can be sporadic, which is the most common form or familial. The inherited predisposition to cancer is the result of germline mutations in TSGs, stability genes or oncogenes. Cancer predisposition syndromes are rare, but have a high risk of malignancy. Examples of inherited cancer syndromes are the Li-Fraumeni syndrome, Xeroderma pigmentosum and the Gorlin syndrome (Vogelstein & Kinzler; Evans, 2007).
Additionally, the human genome contains genetic variations, which underlie phenotypic differences among individuals, such as eye color and blood group. These genetic variations determine the predisposition to complex diseases, such as cancer, and the responses to drugs and environmental factors. The International HapMap Project has created a public database of common variations in the human genome, single nucleotide polymorphism (SNP), to accelerate medical genetic research (HapMap, 2005; Frazer et al., 2007). This HapMap database is widely used and several susceptibility loci for different cancer types have been found (Easton et al., 2007; Gudmundsson et al., 2008).

1.1.7 Metastasis

Most cancer related deaths are caused by metastases, the distant settlements of tumor cells. The primary tumor cells are continuously dividing and invade the adjacent tissues, and in most tumors, cancer cells escape into the lymphatic and /or blood vessels. These tumor cells can either stay in the regional lymph nodes or spread to distant organs of the body. Metastases are then formed, if the tumor cells are supplied with enough nutrients through the formation of new blood vessels (neoangiogenesis) and appropriate interactions with the surrounding stroma (Hanahan & Weinberg, 2000; Fokas et al., 2007). It is known that different types of tumors spread to different organs, the “seed and soil” hypothesis, first dictated by Stephen Paget 1889, which postulates that tumor cells selectively form metastases at the distant organs that facilitate the survival of the tumor. Paget’s hypothesis has been updated and it is now known that also normal stromal cells and cells from the immune system contribute to cancer development and tumor angiogenesis (Albini & Sporn, 2007; Fokas et al., 2007; Kenny et al., 2007).

1.1.8 Treatments

The main treatments for cancer therapy are surgery, radiotherapy, chemotherapy or endocrine treatment. These are usually used in combinations, with or without complementing biological treatment such as immunotherapy and anti-angiogenesis. In the novel approach of target therapy, cancer drugs are designed to specifically target an aberrant signaling pathway in the tumor cells or the stromal cells that are believed to be critical for tumor growth or progression (Sawyers, 2004; Chabner & Roberts, 2005). Many cancer patients are cured from their tumors, but for the most, depending on the cancer type, the tumor progresses, metastasizes and eventually kills the patient mainly due to drug resistance, toxic side-effects and infections or by the growth of the tumor itself.

The paradigm shift that highlights the importance of the tumor microenvironment has resulted in the development of drugs targeting microenvironmental components. Examples already in clinical use are AvastinTM, which is an inhibitor of angiogenesis, the tyrosine kinase inhibitor GleevecTM/GlivecTM, one of the most successful drugs in target therapy, which has also been found to inhibit angiogenesis, tamoxifen, the first target therapy developed, which is an estrogen receptor antagonist, the aromatase inhibitors, which target estrogen biosynthesis in the microenvironment of estrogen-dependant tumors, and Zoledronic Acid, which targets the metastatic microenvironment of the bone (Sawyers, 2004; Albini & Sporn, 2007; Kenny et al., 2007). Another potential therapeutic approach is to induce the tumor cells to differentiate and thus switch off cell division and migration (Stindl, 2008). An example of a successful differentiation therapy is the treatment of acute promyelocytic leukemia with retinoic acid, which induces terminal differentiation of the malignant cells (Warrell et al., 1991).

1.2 Skin

The skin is the largest organ in mammals. It protects against injuries and serves as a barrier against different types of environmental hazards such as UV radiation, microbes and chemical carcinogens. It also has sensory and immunological functions.

In human, the skin is organized in three different layers; epidermis, dermis and subcutaneous layer (Figure 1). The epidermis is composed of different epithelial cell layers and the main cell type is the keratinocyte (Khavari, 2006). The keratinocytes migrate from the lowest layer, the basal layer, and differentiate up to the top, the horny layer, where they are constantly shed off as dead cells and replaced by cells from lower layers. The basal layer consists of basal keratinocytes and stem cells, which continually proliferate to produce new keratinocytes in an epidermal homeostasis (Braun & Prowse, 2006). An unbalance of the epidermal homeostasis may result into thin skin and loss of protection due to limited proliferation, whereas excessive proliferation results into skin diseases such as psoriasis and cancer (Fuchs, 2007). Several different appendages are present in the epidermis, including hair follicles, sebaceous glands and sweat glands. The pigment-producing melanocytes are also present in the epidermis, and the melanin

produced protects the deeper layers from UV radiation (Khavari, 2006). The epidermal homeostasis, as well as epidermal repair and hair regeneration is maintained by the stem cells found in epidermis, the hair follicle and the sebaceous gland (Fuchs, 2007).

Below epidermis is the dermis, which is much thicker. The main cell type in dermis is the fibroblast, which together with blood vessels and nerves is embedded into the extracellular matrix. The deepest layer is the subcutaneous layer, which consists of fat cells.
The structure of the skin differs between species, for example human and mice, and this should be considered when using mouse models for human skin cancer development (Khavari, 2006).

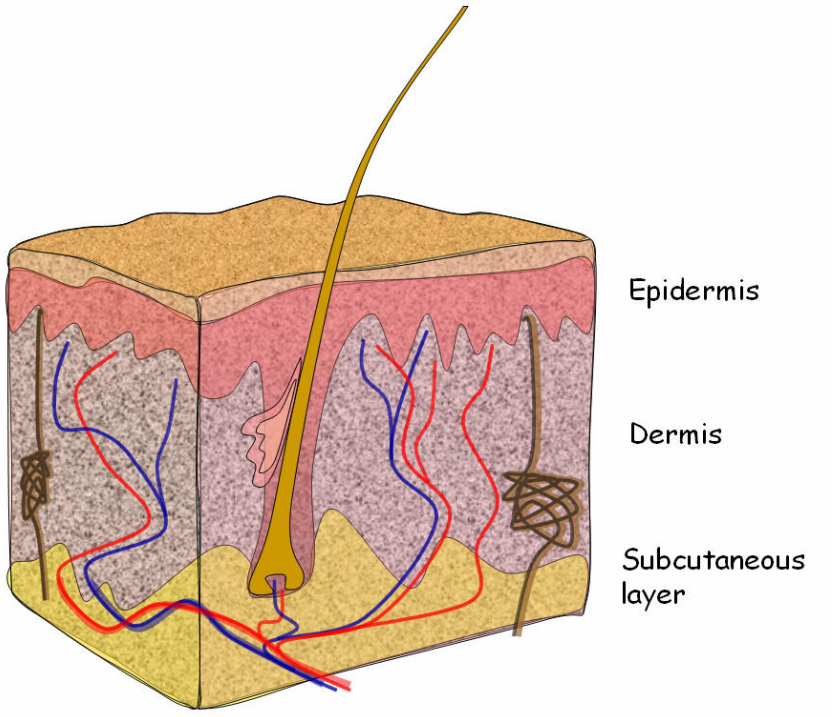

Figure 1. The organization of the human skin.
The human skin is organized into three different layers; epidermis, dermis and subcutaneous layer.
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1.3 Skin cancer

Skin cancer is the most common cancer in the Western countries. The majority of these tumors are non-melanoma skin cancer (NMSC). The most common NMSC is basal cell carcinoma (BCC), which accounts for 75% of the NMSC cases followed by squamous cell carcinoma (SCC) with 20% (Boyle et al., 2004; Neville et al., 2007). In the US more than 1.3 million NMSCs are diagnosed each year and in Sweden approximately 43000 cases were diagnosed in 2006 (Neville et al., 2007; Socialstyrelsen, 2007; Socialstyrelsen, 2008). The incidence in many countries is not exactly known as not all NSMCs are registered. In Australia, population-based studies and national surveys showed a rising incidence rate during the last 20 years for both BCCs and SCCs (Staples

et al., 1998; Staples et al., 2006; Lee et al., 2007). The incidence rates in the European countries have increased during the last decades, especially for BCCs (Ko et al., 1994; Boyle et al., 2004; Trakatelli et al., 2007). In Sweden, BCCs have not been registered until 2003, with the incidence increasing every year (Socialstyrelsen, 2006; Socialstyrelsen, 2008). For SCCs there is an annual increase of 3-4% in Sweden during the last 20 years and for the last decade it is even higher (Socialstyrelsen, 2007). The incidence rate of NMSC correlates with the latitude, with the highest being near the equator, illustrating therefore the importance of UV-radiation as one of the major risk factors (Staples et al., 2006). NMSC is basically a disease of ageing populations, with higher incidence in individuals over 55 years old. The increasing incidences together with the ageing populations will result in substantial economic costs, as NMSCs are among the most expensive cancers to treat due to their high frequencies. This is especially important for countries with high rates such as Australia (Housman et al., 2003; FitzGerald et al., 2006; Raasch et al., 2006; Trakatelli et al., 2007).

1.3.1 Basal cell carcinoma

BCCs (Figure 2) are derived from the epidermal keratinocytes, but the exact cellular origin of the tumors is still debated; interfollicular basal cells and/or skin stem cells in the hair follicle region (Lauth et al., 2004; Tilli et al., 2005). Characteristic features for BCCs are slow growth, local invasion and rare metastasis (Miller, 1991). The majority of the BCCs are according to the World Health Organization histologically classified into four major groups: nodular, superficial, micronodular and infiltrative, although mixed pattern also are found (LeBoit et al., 2005). Most common are the nodular and superficial BCCs, which both have a non-aggressive behavior. The micronodular and infiltrative BCCs are considered to be associated with a high risk of recurrence (Tilli et al., 2005; Crowson et al.., 2006; Raasch et al., 2006). BCCs occur both in sun-exposed and sun-protected skin, with the “head-and neck” region being the most common site, followed by the trunk (Crowson, 2006; Socialstyrelsen, 2008). UV exposure is one of the main risk factors, as is fair skin, ionizing radiation, chemical carcinogens such as arsenic and hydrocarbons, immunosuppression and certain genetic disorders such as Gorlin syndrome and Xeroderma pigmentosum (Tilli et al., 2005; Crowson et al., 2006; Trakatelli et al., 2007).

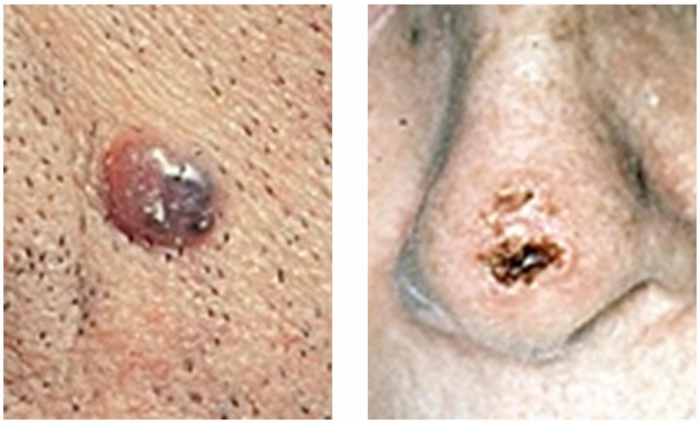

Figure 2. Examples of basal cell carcinoma (left) and squamous cell carcinoma of the skin (right).
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1.3.2 Squamous cell carcinoma

SCCs of the skin (Figure 2) are also derived from the epidermal keratinocytes, although the exact cellular origin is not yet known (Lauth et al., 2004). SCCs are rapidly growing, invasive and have a metastatic potential (Quinn et al., 1994a). Cutaneous SCCs are histologically divided into many diverse subtypes, with different clinical behavior from indolent to aggressive and metastatic. Tumors that arise in sun-damaged skin have the lowest risk of metastasis (LeBoit et al., 2005). SCCs mainly metastasize to the regional lymph nodes, but also to distant sites (Weinberg et al., 2007). SCCs may arise from progressed precursors/precancerous lesions such as actinic keratoses and Bowen's disease. Some SCCs arises "de novo" without any precursor lesion, with an aggressive behavior and a high incidence of metastasis (Cassarino et al., 2006a; Cassarino et al., 2006b). SCCs occur mostly on sun-exposed parts of the body such as the "head and neck" and the hands (Pearl & Scott, 1986; Staples et al., 2006; Trakatelli et al., 2007). Some of these "head and neck" tumors have a higher risk of developing metastasis (Weinberg et al., 2007; Veness, 2007). Risk factors for cutaneous SCCs are similar to BCCs and include chronic UV-exposure, fair skin, chemical carcinogens such as arsenic and hydrocarbons, ionizing radiation, human papilloma virus, immunosuppression and certain genetic disorders such as Xeroderma pigmentosum (Kwa et al., 1992; Trakatelli et al., 2007).

1.3.3 Actinic keratoses and Bowen's disease

Actinic keratoses (AK)s have for a long time been accepted as being precursors of SCCs, but there is a current debate whether AKs are simply precursors or early/ in situ SCCs from the start (LeBoit et al., 2005; Cassarino et al., 2006a; Chia et al., 2007). Irrespective of the debate, AKs can be classified into three different grades with regard to the degree of atypical keratinocytes of the epidermis (Roewert-Huber et al., 2007). AKs are mainly found in chronically sun-exposed skin of fair-skinned people, mainly middle-aged and elderly (Roewert-Huber et al., 2007). The prevalence of AKs is high, up to 50% in Australia and 26% in Western populations (Quatresooz et al., 2008). AKs can remain stable, but may also progress to invasive SCCs, and without treatment can metastasize and cause death (Cassarino et al., 2006a; Roewert-Huber et al., 2007; Trakatelli et al., 2007). Bowen's disease (BD) is an additional precancerous lesion, also known as SCC *in situ*, with atypical keratinocytes of full thickness of the epidermis (LeBoit et al., 2005; Cassarino et al., 2006b). BD is slow-growing, and is mainly found in sun-damaged skin of elderly people. Furthermore, BD can become invasive, and a large number of these patients develop metastases, therefore invasive BD is classified as a high-risk SCC (Cassarino et al., 2006b).

1.3.4 Keratoacanthoma

Keratoacanthoma (KA) is clinically, morphologically and biologically classified as benign, non-aggressive squamous neoplasm although some regard KA as a well-differentiated SCC (LeBoit et al., 2005; Cassarino et al., 2006b). These lesions grow rapidly for 1-2 months followed by spontaneous regression. KA occurs mainly in the face and extremities, and mostly in elderly males. Most cases are sporadic but multiple KAs

are associated with some genetic syndromes, including Ferguson-Smith and Muir-Torre syndromes. Some KAs are histologically indistinguishable from SCCs, but they hardly ever metastasize (Cassarino et al., 2006b).

1.3.5 Treatments of NMSC

Different parameters are considered when determining the treatment strategy for NMSCs, such as medical history, overall health status, age, histological tumor type, location, size, cosmetic aspects and primary versus recurrent tumor (Tilli et al., 2005; Ceilley & Del Rosso, 2006). The main modality used is surgery excision, electrodessication and curettage, cryosurgery or Imiquimod, an immunomodulating agent, for tumors located in low-risk areas. For tumors located in high-risk areas such as "head and neck" and in recurrent or large tumors, the Mohs micrographic surgery is the golden standard. Thin NMSCs are preferable treated with Imiquimod, 5-Fluorouracil or photodynamic therapy (Ceilley & Del Rosso, 2006; Neville et al., 2007). Cox-2 inhibitors such as Diclofenac are also used for treatment of AKs (Chia et al., 2007). Metastatic SCCs are mainly treated with surgery, radiation and chemotherapy, in combination. The survival of patients with metastatic SCCs depends on the extent of the spread to the lymph nodes (Weinberg et al., 2007).

1.4 Genetic disorders associated with NMSC

Most NMSCs are sporadic cases but in certain genetic disorders, there is an inherited predisposition to different types of NMSCs.

1.4.1 Nevoid Basal Cell Carcinoma Syndrome

The Nevoid Basal Cell Carcinoma Syndrome (NBCCS), OMIM 109400, also called Gorlin syndrome is an autosomal dominant disease, with a minimum prevalence of 1 in 55600 (Evans et al., 1993). However, 35-50% of the cases are sporadic and arise *de novo* without any family history (Gorlin, 1995). This disorder was first described by Gorlin and Goltz in 1960 (Gorlin & Goltz, 1960), and is traced back to mummies from 1000 BC (Satinoff & Wells, 1969). NBCCS has a high penetrance and variable expressivity (Evans et al., 1993; Kimonis et al., 1997). Characteristics for NBCCS are multiple, up to thousands BCCs, odontogenic keratocysts, skeletal abnormalities such as rib anomalies and calcification of the falx cerebri, palmar and plantar pits, ocular problems and predisposition to tumors such as medulloblastomas and ovarian and cardiac fibromas (Figure 3). The hallmark of NBCCS is BCCs, which appears mostly in sun-exposed sites such as face, back and chest, with onset being mainly in the second decade (Gorlin, 1987; Gorlin, 2004). The frequency of BCCs in African-American, Italian and Korean patients is lower and with a later onset, probably due to darker pigmentation (Kimonis et al., 1997; Lo Muzio et al., 1999a; Ahn et al., 2004; Gorlin, 2004). Odontogenic keratocysts is one of the first signs of NBCCS and can be detected in patients as early as in the seventh year of life (Lo Muzio et al., 1999b; Ahn et al., 2004; Gorlin, 2004). The incidence of

NBCCS associated medulloblastoma is ~5%, often appears during the first 2 years of life (Evans et al., 1993; Gorlin, 1995; Kimonis et al., 1997), and is associated with better prognosis than for sporadic medulloblastomas (Amlashi et al., 2003).

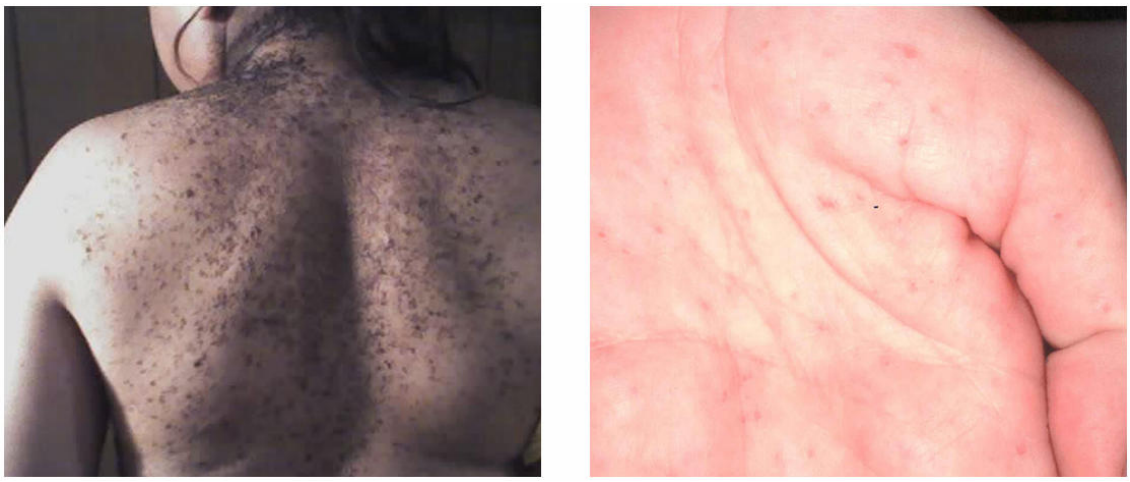

Figure 3. Clinical characteristics of NBCCS.

Multiple basal cell carcinomas on the back, after irradiation treatment for childhood medulloblastoma (left). Palmar pits (right).

The susceptibility gene for NBCCS was first mapped to 9q22.3-31 through linkage analysis, (Farndon et al., 1992; Gailani et al., 1992; Reis et al., 1992) then further fine-mapped (Farndon et al., 1994; Wicking et al., 1994; Unden et al., 1997a) and subsequently identified as the *PTCH1* gene (Hahn et al., 1996; Johnson et al., 1996). NBCCS is caused by a defective allele of *PTCH1* in the germline, with loss of the other allele occurring somatically in the tumors (Hahn et al., 1996; Unden et al., 1996; Tostar et al., 2006).

1.4.2 Xeroderma pigmentosum

Xeroderma pigmentosum (XP), OMIM 278700-278780 and 610651, is a rare autosomal recessive disease. The incidence is 1:250000 in Europe and North America, while it is higher in Japan (1:40000). XP patients are hypersensitive to UV-radiation due to defective DNA repair, and UV-light exposure leads to severe and chronic sun damage of the skin. XP patients develop precancerous lesions, AKs and malignant skin tumors at an early age and 2/3 of all patients die before adulthood due to metastases. The most common skin tumors are BCCs, SCCs and malignant melanomas. XP is also associated with neurological and ocular abnormalities. XP is a heterogeneous disease and is divided into 7 complementation groups (XP-A to XP-G) and the XP variant (XP-V), with XP-A being the most frequent form. These complementation groups are based on specific defective proteins involved in the nucleotide excision repair (NER), which is essential for repairing UV-induced DNA lesions. Treatments of XP patients mainly include restriction of sun-exposure, use of sunscreens and regular dermatological, neurological and ophthalmologic examinations. Application of topical DNA-repair enzymes on the skin

reduces the precancerous lesions and the skin cancer rate (Kraemer et al., 1987; Norgauer et al., 2003; Magnaldo & Sarasin, 2004).

1.4.3 Multiple self-healing squamous epithelioma

Multiple self-healing squamous epithelioma (MSSE), also known as Ferguson-Smith syndrome, OMIM 132800, is a rare autosomal dominant disease first described in 1934 by J Ferguson-Smith. Affected patients intermittently develop KAs or skin tumors that are morphologically similar to well differentiated SCCs. MSSE tumors are locally invasive but most spontaneously regress over 2-6 months, leaving pitted scars (Goudie et al., 1993; Cassarino et al., 2006b; D'Alessandro et al., 2007). Due to the disfigured scarring many tumors are removed by surgical excision or cryotherapy, especially if located in the face (D'Alessandro et al., 2007). The majority of MSSE patients are known to be of Scottish origin and possibly are all related to each other through a common affected ancestor, thus suggesting the occurrence of a founder mutation that is responsible for the disease (Ferguson-Smith et al., 1971; Goudie et al., 1993). Recently, MSSE on non-Scottish families have been reported, suggesting that MSSE is more common than originally thought and not caused by a founder mutation (D'Alessandro et al., 2007). The susceptibility gene for MSSE has been mapped to chromosome 9q22 through linkage analysis, and LOH of MSSE tumors suggested that the MSSE gene is a TSG (Goudie et al., 1993; Richards et al., 1997; Bose et al., 2006).

1.4.4 Other syndromes

Multiple BCCs are associated with **Bazex syndrome** also known as Bazex-Dupre-Christol syndrome, OMIM 301845, which is an X-linked dominant disease, with the BCCs having an early onset at the second decade (Lee et al., 2005; Torrelo et al., 2006). **Rombo syndrome**, OMIM 180730, is an autosomal dominant disease also associated with BCCs (Michaelsson et al., 1981). **Brooke-Spiegler syndrome** (BSS), OMIM 605041, **familial cylindromatosis** (FC), OMIM 132700, and **multiple familial trichoepithelioma** (MFT), OMIM 601606, are all autosomal dominant diseases with overlapping clinical features. BSS patients are predisposed to multiple trichoepitheliomas and cylindromas, both benign skin neoplasms with capability of malignant transformation. FC patients are characterized by the development of cylindromas, and MFT patients by trichoepitheliomas (Lee et al., 2005). The TSG CYLD was found to be mutated in patients either with BSS, FC or MFT, indicating phenotypic variations of a single gene defect (Lee et al., 2005; Massoumi & Paus, 2007; Saggar et al., 2008).

Muir-Torre syndrome, OMIM 158320, is an rare autosomal dominant disease characterized by tumors in the sebaceous gland or KA in association with one or more visceral malignancies such as colorectal cancers (Lee et al., 2005; Ponti & Ponz de Leon, 2005). The **recessive dystrophic epidermolysis bullosa** (RDEB), OMIM 226600, is a severe disease characterized by fragile skin and early development of highly aggressive metastatic SCCs (Yuspa & Epstein, 2005; Rodeck et al., 2007).

1.5 Molecular etiology of NMSC

The most important etiological factor for development of BCCs and SCCs is UV-radiation. UV exposure mainly induces DNA damage lesions such as pyrimidine dimers, which cause the UV- specific mutations, CC>TT and C>T. The pyrimidine dimers are repaired by the NER system, but if the repair system is deficient as in XP patients, then there is a dramatic increase in the risk of both NMSCs and melanoma skin cancer (Brash, 1997; de Gruijl et al., 2001). UV exposure of human skin upregulates the TSG *p53*, which then acts as a coordinator in the repair of the DNA-damage, by inducing and activating genes involved in DNA repair, cell cycle arrest and apoptosis (de Gruijl et al., 2001; Brash, 2006). UV-specific *p53* mutations have been found in 50% of the BCCs (Ziegler et al., 1993; van der Riet et al., 1994) and in over 90% of the SCCs (Brash, 1997). A high frequency of UV-related *p53* mutations has been found in AKs, as well as in Bowen's disease. *P53* mutations have also been found in normal sun-exposed skin, and UV-induced *p53* mutations in skin are proposed to act as *in vivo* dosimeters of UV-radiation (Brash, 2006; Benjamin & Ananthaswamy, 2007).

1.5.1 BCC

Historically, the molecular etiology of BCCs was significantly speeded up when a putative TSG was mapped to the NBCCS susceptibility region, 9q22.3-31 (Gailani et al., 1992; Quinn et al., 1994a; Quinn et al., 1994c; Holmberg et al., 1996) and proposed to be identical to the NBCCS gene. The final clue to the pathogenesis of BCCs was revealed when the NBCCS gene was identified as the *PTCH1* gene (Hahn et al., 1996; Johnson et al., 1996). This TSG, *PTCH1*, was also found to be somatically mutated in sporadic BCCs, suggesting that *PTCH1* is a classical TSG (Gailani et al., 1996; Hahn et al., 1996). Disruption of PTCH1, results into aberrant activation of the Hedgehog (HH) signaling pathway leading to overexpression of target genes (Pasca di Magliano & Hebrok, 2003). Both murine and *in vivo* human-tissue cancer models have shown that activated HH signaling is necessary and sufficient to induce BCCs (Fan et al., 1997; Oro et al., 1997; Grachtchouk et al., 2000; Nilsson et al., 2000; Khavari, 2006). The precise mechanism of how deregulation of the HH pathway contributes to BCC development or to the different BCC subtypes is, however, not yet revealed.
Additionally, *RAS* oncogenes were found to be mutated at a low frequency in sporadic BCCs, although their importance is unclear (Boukamp, 2005).

1.5.2 SCC

Sporadic cutaneous SCCs are associated with mutations in *p53*, *RAS* and in *CDKN2A*, but also with genomic instability, with a large number of chromosomal changes identified (Boukamp, 2005; Khavari, 2006). Loss of heterozygosity has been identified for certain chromosomes including 9p, 3p, 13p, 17p, 17q and 9q. In chromosome 9p a high frequency of LOH was found in the *CDKN2A* region, in addition to loss of the whole chromosomal arm (Boukamp, 2005). Also AKs are associated with genetic instability, with LOH in the same chromosomes as in SCCs (Rehman et al., 1994; Rehman et al., 1996). Aberration in 9q22.3-31 has also been reported in SCCs, in Bowen's disease and

in AKs (Quinn et al., 1994c; Holmberg et al., 1996; Ahmadian et al., 1998). This is especially interesting due to the similar localization of the MSSE susceptibility gene (Goudie et al., 1993). Recently, a high frequency of LOH in 9q22, including the *PTCH1* gene region, was found in SCCs, implying that the *PTCH1* itself or another gene in 9q22 may be involved in SCC development (Danaee et al., 2006). Other non-cutanous SCCs such as head and neck, bladder and esophagus tumors have also been reported to harbor LOH in 9q22-31 (Ah-See et al., 1994; Mori et al., 1994; Habuchi et al., 1995; Simoneau et al., 1999). These findings suggest that the 9q22-31 region may harbor genes involved in the squamous cell carcinoma phenotype. Some clue to the molecular etiology of the SCCs has been revealed through three dimensional human tissue cancer models, which include human tissue microenvironment (Khavari, 2006). Aggressive SCCs have been induced by expression of oncogenic HRAS combined with CDK4 or NF-κB blockade through IκBα, resulting in escape of growth arrest at the G1 phase of the cell cycle. These SCCs are indistinguishable from spontaneous SCCs (Lazarov et al., 2002; Dajee et al., 2003; Khavari, 2006).
Recently, using the same human tissue cancer model, overexpression of c-Myc in human primary keratinocytes, transformed by RAS and IκBα, activated the embryonic stem cell (ESC)-like program leading to tumor formation through the increase of tumor-initiating cells. These c-Myc-RAS-IκBα tumors were dedifferentiated and showed features of ESCs, including potential cancer stem cells (Wong et al., 2008).

1.6 Hedgehog signaling network

The Hedgehog (Hh) family of secreted signaling proteins is important in embryonic development and adult tissue maintenance (Bijlsma et al., 2004). These Hh proteins are crucial for the control of one of the most important networks of animal patterning, the Hh signaling network (Ruiz i Altaba et al., 2007). The Hh proteins were first discovered in Drosophila, where a mutation of the single *hh* gene resulted into a “hedgehog” like phenotype of the embryo (Nusslein-Volhard & Wieschaus, 1980).

The *hh* gene has been duplicated during evolution, and in mammals there are three *Hh* genes; Sonic hedgehog (*Shh*), Indian hedgehog (*Ihh*) and Desert hedgehog (*Dhh*) (Ingham & Placzek, 2006). Shh and Ihh are phylogenetically more closely related to each other and probably represent a more recent gene duplication. Shh is the most studied, as it is involved in neural tube development, left-right asymmetry, morphogenesis of different organs such as lung, skin, limbs, hair, teeth, and together with Ihh in skeletal development. Dhh is regulating spermatogenesis and is important in the formation of the peripheral nerve system (Umehara et al., 2002; Athar et al., 2006; Ehlen et al., 2006).

1.6.1 Hedgehog signaling

The Hh signaling network is very complex and not yet fully elucidated. Overall, most of the components in Hh signaling are conserved from insects to vertebrates, with a main difference in the number of genes in different species (Hooper & Scott, 2005). However,

it appears that there are also diverging signaling mechanisms (Varjosalo et al., 2006). Mammalian Hh signaling may be briefly summarized as follows. Hh proteins are synthesized, cleaved, modified and lipidated in the Hh-producing cells before being released by the 12-transmembrane protein Dispatched. Thereafter, the Hh proteins are trafficked to the Hh-receiving cells and bind to the Hh-receptor, the 12-transmembrane Patched 1 (Ptch1) protein. Additionally, the Hh-interacting protein (Hip) sequesters Hh and attenuates the signal, while the cell-surface proteins Cdo/Boc and Gas1 enhance Hh signaling (Chuang & McMahon, 1999; Tenzen et al., 2006; Allen et al., 2007; Jacob & Lum, 2007; Wang et al., 2007). The binding of Hh to Ptch1 abolishes the Ptch1 inhibitory function on the 7-transmembrane protein Smoothened (Smo). Activated Smo initiates a signaling cascade, which leads to activation of the glioma-associated (Gli1-3) family of zinc-finger transcription factors, which turn on target genes (Cohen, 2003; Rohatgi & Scott, 2007).

In the absence of Hh, the *Gli1* gene is transcriptionally silenced, and the Gli2/3 proteins are sequestered by Suppressor of fused (Sufu), and degraded or cleaved in the proteasome to act as repressors, inhibiting the transcription of target genes (Ruiz i Altaba et al., 2007) (Figure 4).

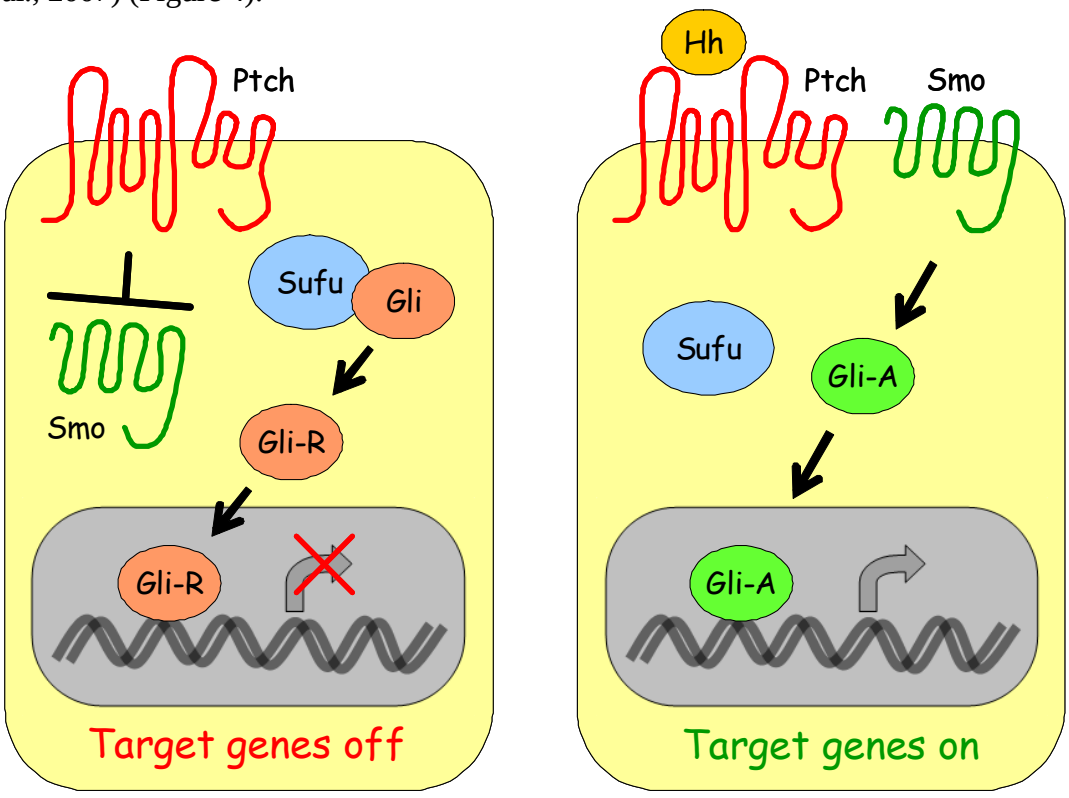

Figure 4. Model of mammalian Hedgehog signaling.
In the absence of Hh (left), Ptch inhibits Smo and this keeps the Gli transcription factors inactive, as these are sequestered by Sufu and cleaved to act as repressors (Gli-R) on target genes (left). Binding of Hh ligands to Ptch (right) abolishes the Ptch inhibitory function on Smo, which translocates to the cell membrane and activates the Gli transcription factors (Gli-A) that turn on target genes. © Erika Lindström

The direct downstream target genes that are upregulated by Hh signaling in vertebrates include *Ptch1*, *Ptch2*, *Bcl2*, *Cyclin D*, *FoxM1*, *FoxE1*, *Hip* and *Gli1*, with *Gli1* serving as positive and *Hip* and *Ptch1* as negative feedback regulators (Chuang & McMahon, 1999;

Teh et al., 2002; Yoon et al., 2002; Eichberger et al., 2004; Ikram et al., 2004; Rahnama et al., 2004; Regl et al., 2004; Ågren et al., 2004; Tang et al., 2007).

In mammals, the primary cilia are also suggested to have an important role in signaling, acting as sensory antennae for intercellular signals (Singla & Reiter, 2006). Ptch1 localizes to primary cilia and upon Shh binding, Shh triggers removal of Ptch1 and Smo accumulates in the cilium and activates the signaling cascade (Rohatgi et al., 2007).

1.6.2 Patched1

The *Patched1/PATCHED1* (*Ptch1/PTCH1*) gene is the mouse/human homolog of the Drosophila segment polarity gene, *patched*, and consists of 23 exons encoding a 1434/1447 amino acid transmembrane glycoprotein (Goodrich et al., 1996; Hahn et al., 1996; Johnson et al., 1996). In humans, *PTCH1* is responsible for the genetic disease NBCCS, acts as a TSG and maps to chromosome 9q22.3 (Gailani et al., 1996; Hahn et al., 1996; Johnson et al., 1996) with the *PTCH1* gene covering approximately 70kb of genomic DNA (Nagao et al., 2005). Ptch1 is the Hh receptor and has dual roles in Hh signaling, it transduces the signal and sequesters Hh (Chen & Struhl, 1996; Marigo et al., 1996; Stone et al., 1996). Several splice variants of Ptch1 are known (Hahn et al., 1996; Kogerman et al., 2002), with different tissue-specific expression patterns, protein stability and capacity to suppress Hh signaling (Shimokawa et al., 2004; Nagao et al., 2005; Shimokawa et al., 2007).

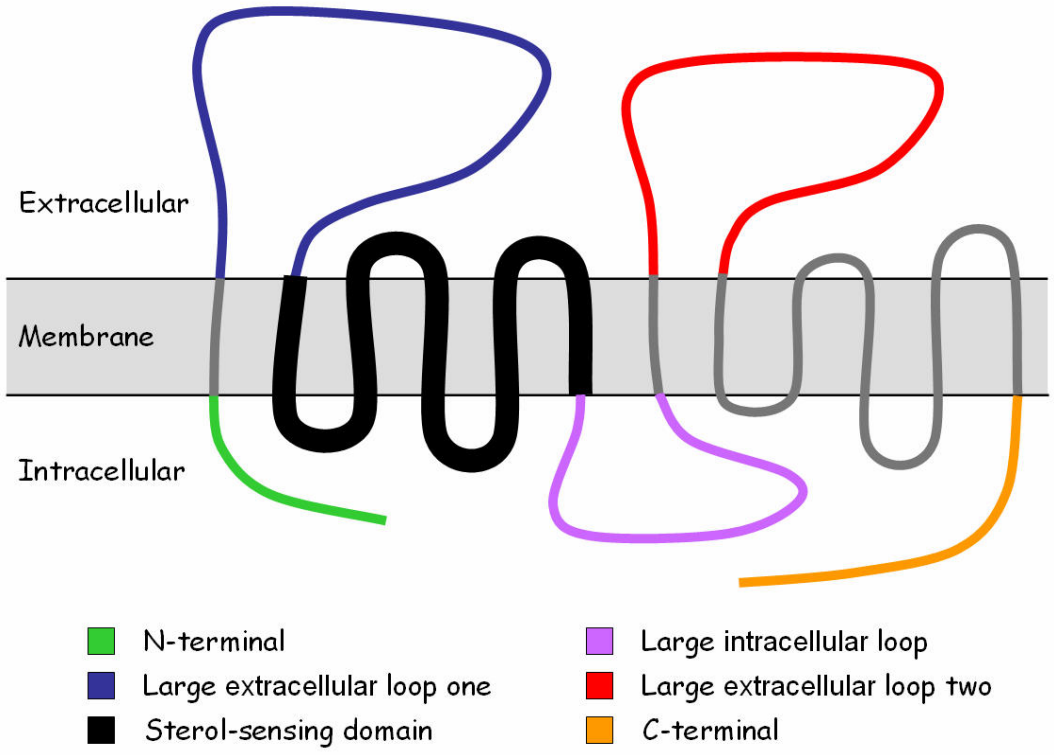

Figure 5. Topology of Ptch1.

The predicted secondary structure of Ptch1 is proposed to have 12 transmembrane domains, two large extracellular loops, one large intracellular loop and a sterol-sensing domain corresponding to transmembranes 2-6.

The predicted secondary structure of Ptch1 (Figure 5) is proposed to have 12 transmembrane (TM) domains, two large extracellular loops where Shh binds, and a smaller intracellular loop, where cyclin B1 binds (Goodrich et al., 1996; Marigo et al., 1996; Barnes et al., 2001). Ptch1 also contains a putative sterol-sensing domain (SSD) corresponding to TM 2-6 (Carstea et al., 1997; Loftus et al., 1997). There are several proteins with a SSD, including HMGCR, SCAP and the lipid storage disorder Niemann-Pick disease type C1 (NPC1), which are all known to be involved in cholesterol homeostasis (Kuwabara & Labouesse, 2002). Ptch1 shows structural homology to the resistance-nodulation-division (RND) family of prokaryotic permeases, which have the same 12 TMs topology. These RND permeases transport small molecules across the membranes, and based on the similar topology, Ptch1 has been suggested to be a putative transporter (Tseng et al., 1999). The RND permease AcrB, a multidrug transporter, is found to be a trimer of 12 TMs monomers, forming an active pore (Murakami et al., 2002). The crystal structure of Ptch1 has not yet been determined, but the Drosophila patched is found to be a possible trimer, and proposed to be a multisubunit transporter indirectly regulating the localization and function of smo (Lu et al., 2006).
Ptch1 is therefore proposed to function as a pump transporting a small molecule across the membrane that regulates the activity of Smo (Taipale et al., 2002). Several possible candidates of such small molecules have been reported, including pro-vitamin D3 and oxysterols (Bijlsma et al., 2006; Corcoran & Scott, 2006; Tang et al., 2007).

1.6.3 Patched2

There is a second patched gene in vertebrates, *Patched2/PATCHED2* (*Ptch2/PTCH2*) (Carpenter et al., 1998; Motoyama et al., 1998; Smyth et al., 1999; Zaphiropoulos et al., 1999), and human *PTCH2* consists of 22 exons encoding a 1203 amino acids protein, spans 15kb of genomic DNA, and is located on chromosome 1p33-34 (Smyth et al., 1999). PTCH2 shares 57% identity with PTCH1, with a high variability in the region between TM6 and TM7 (Zaphiropoulos et al., 1999), and moreover has lost part of the C-terminal tail compared to PTCH1 (Smyth et al., 1999; Zaphiropoulos et al., 1999). PTCH2 is also a HH receptor and binds to all HH ligands (Carpenter et al., 1998). There are several splice variants of PTCH2 with apparently distinct functions (Zaphiropoulos et al., 1999; Rahnama et al., 2004). The predicted secondary structure of PTCH2 is similar to that of PTCH1, with 12 TMs and two large extracellular loops (Carpenter et al., 1998). *PTCH2* has been proposed to be a TSG involved in a subset of sporadic BCCs and medulloblastomas (Smyth et al., 1999).

1.6.4 Other core components of Hedgehog signaling

Vertebrate Smoothened/SMOOTHENED (Smo/SMO) is a transmembrane protein (Stone et al, 1996), and human SMO encodes a 1024 amino acids protein and is mapped to 7q31-q32 (Xie et al., 1998). Smo has 7 TMs and a certain similarity to G protein-coupled receptors, although there is no evidence for interaction with a ligand. In humans SMO is a proto-oncogene being involved in development of sporadic BCCs and medulloblastomas (Rohatgi & Scott, 2007). How Smo initiates the signaling cascade, which inhibits Sufu repression of the Gli transcription factors still remains unclear in

mammals. Irrespective of this, Sufu appears to have an essential function for Hh signaling in mammals, whereas in Drosophila it seems to be dispensable (Svärd et al., 2006). Human SUFU, is a negative regulator of GLI11 (Kogerman et al., 1999), contains 12 exons and is mapped to chromosome 10q24-25 (Stone et al., 1999; Grimm et al., 2001). Additionally, *SUFU* is proposed to function as a TSG in certain medulloblastomas (Taylor et al., 2002). Human GLI1 was first identified in a malignant glioma as highly overexpressed (Kinzler et al., 1987). The GLI1-3 proteins consist of more than 1000 amino acids and belong to the Kruppel family of zinc finger proteins. In mammalian Hh signaling, Gli1 and Gli2 act mainly as activators, whereas Gli3 exists mostly as a cleaved repressor but also as a full-length activator (Rohatgi & Scott, 2007; Ruiz i Altaba et al., 2007).

1.7 Animal models of Hedgehog signaling

The use of animal models has provided insights into the importance of the Hh signaling network in embryonic development, adult tissue maintenance and tumor growth. These animal models, mainly using the mouse, have revealed phenotypes that are sometimes highly similar to certain human genetic diseases and tumors, increasing therefore our knowledge about development and cancer.

Homozygous inactivation of *Shh* in the mouse leads to holoprosencephaly, see 1.4.3, and limb deficiencies (Chiang et al., 1996). Treatment of different animal species with cyclopamine, a steroidal alkaloid from the desert plant *Veratrum californicum* (Cohen & Shiota, 2002), inactivates *Smo* and results into cyclopia, which is also associated with holoprosencephaly. Homozygous inactivation of *Smo* in the mouse also results into a phenotype consistent with holoprosencephaly (Cohen, 2003). Null mutations of *Dhh* result into male infertility in mice, implying an important role of Dhh in mammalian spermatogenesis (Bitgood et al., 1996).

The NBCCS disease in humans is caused by germline mutations in *PTCH1*, and heterozygous knockout *Ptch1* mice resemble to the NBCCS phenotype, with developmental abnormalities, spontaneous medulloblastomas and rhabdomyosarcomas (Goodrich et al., 1997; Hahn et al., 1998). However, BCCs develop only after irradiation (Aszterbaum et al., 1999). Homozygous inactivation of *Ptch1* is embryonically lethal in mice (Goodrich et al., 1997; Hahn et al., 1998). The medulloblastomas that develop in heterozygous *Ptch1* mice were found to be caused by haploinsufficiency of Ptch1, and this is in contrast to the Knudson's two-hit hypothesis for TSGs (Wetmore et al., 2000; Zurawel et al., 2000a; Wetmore, 2003). Mice with homozygous inactivation of *Ptch2* are born alive with a normal phenotype and are not cancer prone. But in combination with *Ptch1(+/-)* , both homozygous and heterozygous *Ptch2* mice result into a higher incidence and a larger spectrum of tumors compared to *Ptch1(+/-)* only mice. *Ptch2* elimination was therefore proposed to enhance tumor development via synergistic effects with *Ptch1* haploinsufficiency (Lee et al., 2006).

Interestingly, in a mouse model with *HRas* activation, mice were susceptible to develop SCCs if harboring a C-terminal *Ptch1* polymorphism, without deletion of the wild-type allele or increased Shh signaling. Therefore, it was suggested that both BCCs and SCCs could arise from the same target cell depending on the genetic background and the exposure to carcinogens (Wakabayashi et al., 2007).

Homozygous inactivated *Sufu* in mice is embryonically lethal, similar to homozygous inactivated *Ptch1*, while heterozygous *Sufu* mice develop frequent skin lesions and jaw keratocysts, which are characteristics of NBCCS (Svärd et al., 2006).

Mouse models of different mutations in *Gli1-3* result into many phenotypes, depending on the mutation and the genetic background. Homozygous inactivation of *Gli1* in mice produces normal phenotypes (Park et al., 2000), while overexpression of *Gli1* in the skin of transgenic mice results in BCCs and trichoepitheliomas (Nilsson et al., 2000). Homozygous inactivation of *Gli2* in mice results in multiple developmental anomalies (Mo et al., 1997; Matise et al., 1998), while heterozygous inactivation reveals a normal phenotype (Mo et al., 1997). Overexpression of *Gli2* in the skin results in BCCs with a similar histology as human BCCs (Grachtchouk et al., 2000). Heterozygous *Gli3* mutants in mice reveal limb defects and other deficiencies, similar to the human *GLI3*-related diseases (Cohen, 2003).

1.8 Deregulated Hedgehog signaling in human diseases

There are several human genetic disorders associated with deregulated HH signaling, with some being inherited through germline mutations, while others arise sporadically through genetic or environmental changes.

The Holoprosencephaly (HPE) disease is characterized by defects in separation of cerebral hemispheres and can be associated with craniofacial anomalies such as cyclopia. The incidence of HPE is high, reaching one of every 250 conceptuses, and 1:16000 live births (Ming et al., 1998). There are several mutated genes known to cause HPE, but mutations in *SHH* are the most common. Other HH signaling-related genes that are mutated in HPE are *GLI2* and in a few cases, *PTCH1* (Cohen, 2006; Dubourg et al., 2007). The molecular basis of 70% of the HPE cases remains unknown and other genes and/or environmental factors involved in this pathogenesis are likely to exist (Dubourg et al., 2007).

Mutations in *GLI3* are phenotype-genotype correlated in the two rare autosomal dominant syndromes, Grieg cephalopolysyndactyly (GCPS) and Pallister-Hall syndrome (PHS) (Biesecker, 2006). GCPS is characterized by extra fingers or toes and craniofacial anomalies. PHS is characterized by extra fingers or toes, hypothalamic hamartomas and bifid epiglottis (Ming et al., 1998; Biesecker, 2006). These two allelic syndromes with minimal overlapping phenotypes reveal the dual function of GLI3, acting both as a

repressor and an activator, with the distinct phenotypes caused by different classes of mutations and their different locations in the *GLI3* gene (Biesecker, 2006).

1.8.1 NBCCS

NBCCS or Gorlin syndrome is caused by germline mutations in one allele of the TSG *PTCH1* that activate HH signaling (Hahn et al., 1996; Johnson et al., 1996), as almost all mutations result in a truncated protein (Lindström et al., 2006). *PTCH1* mutations in NBCCS patients are detected in 60-85% of the cases, with this variation probably due to the methodology used for detection (Kimonis et al., 2004; Li et al., 2008). The NBCCS mutations are almost always spread throughout the entire gene (PTCH Mutation Database; www.cybergene.se/PTCH/), with a concentration of truncating mutations to the predicted two large extracellular loops, the N-terminal region and the large intracellular loop. The NBCCS missense mutations are mainly clustered into the predicted TMs regions, especially into the TMs located within the putative SSD (Lindström et al., 2006). TM 4 contains a GXXXD motif (where X is any amino acid), conserved from the ancient RND transporters (Taipale et al., 2002), with alterations of the aspartate reducing the transporter function in RND family (Goldberg et al., 1999; Guan & Nakae, 2001). Functional testing in mouse cells of NBCCS missense mutations within this motif reduces Ptch1 suppression of Hh signaling, suggesting that Ptch1 functions as a transmembrane molecular transporter (Taipale et al., 2002). In Drosophila, substitution of the glycine to valine results into a dominant-negative protein that activates Hh target genes (Hime et al., 2004). Furthermore, a functional SSD is important in NPC1, a protein closely related to PTCH1 that has a role in vesicular trafficking of cholesterol and other lipids (Ohgami et al., 2004).

There is no correlation between the type or position of the *PTCH1* mutations and the phenotype of NBCCS patients, indicating a possible role of modifier genes and environmental factors for the phenotypic diversity of NBCCS (Wicking et al., 1997; Boutet et al., 2003; Tanioka et al., 2005; Lindström et al., 2006). UV exposure could be a modifier factor, as heterozygous *Ptch* mice develop BCCs only after irradiation (Aszterbaum et al., 1999), and NBCCS patients with increased pigmentation such as Afro-Americans develop fewer BCCs and with a later onset (Kimonis et al., 1997).

Interestingly, one NBCCS family has recently been reported to have a *PTCH2* missense mutation, which cosegregates with the disease, affecting the second large extracellular loop (Fan et al., 2008). However, it is unclear if this mutation is disease-causing, although missense *PTCH1* mutations in the same domain have been found in the germline of NBCCS patients and in sporadic BCCs (PTCH Mutation Database; www.cybergene.se/PTCH/).

1.8.2 BCC

BCCs are characterized by aberrant activation of HH signaling leading to expression of target genes including *PTCH1* itself. Thus, overexpression of the PTCH1 mRNA is consistently found in tumor cells both in sporadic (Unden et al., 1997b; Nagano et al.,

1999) and familial BCCs (Unden et al., 1997b). This aberrant activation is mainly a result of inactivating mutations in the *PTCH1* gene or activating mutations in the *SMO* gene (Athar et al., 2006) (Figure 6). In sporadic BCCs, *PTCH1* is mutated in 40-60% and *SMO* in 10-20% of the tumors (Reifenberger et al., 1998; Xie et al., 1998; Lam et al., 1999; Reifenberger et al., 2005; Tilli et al., 2005). This high mutation rate implicates that PTCH1 and SMO are important in the pathogenesis of sporadic BCCs, which is further supported by the 60-70% frequency of LOH at the *PTCH1* locus in 9q22.3 (Holmberg et al., 1996; Reifenberger et al., 2005). A few sporadic BCCs have also been found to harbor *PTCH2* mutations (Smyth et al., 1999) or *SHH* mutation (Oro et al., 1997).

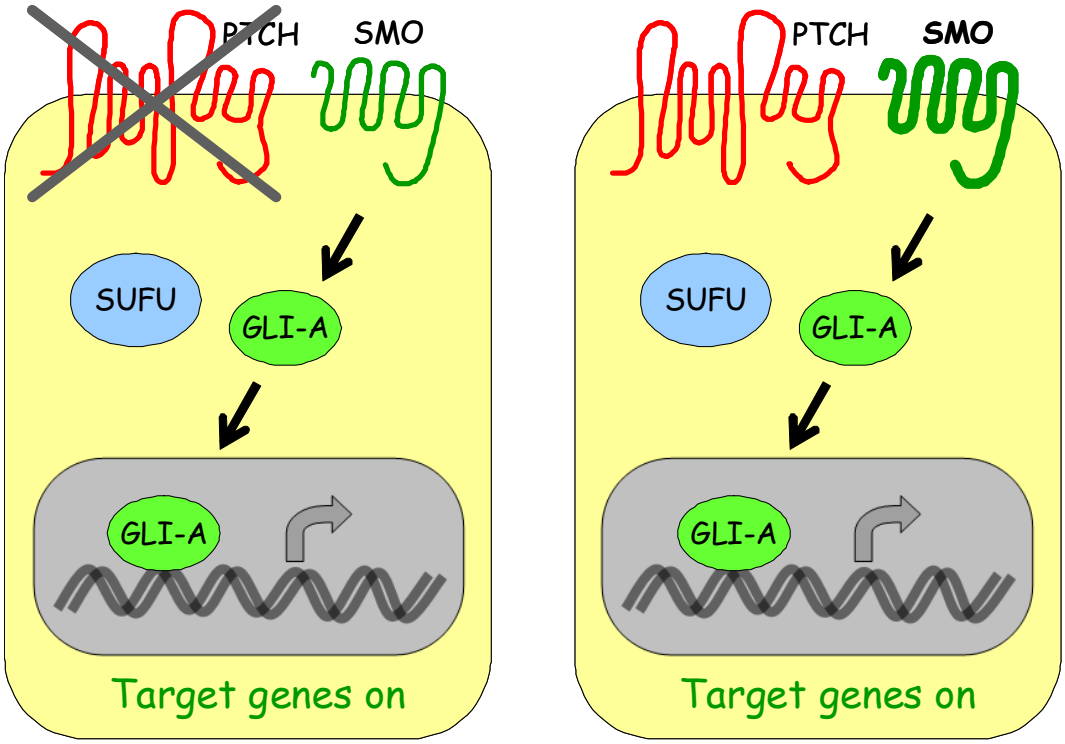

Figure 6. Aberrant activation of Hedgehog signaling in sporadic basal cell carcinomas.
Basal cell carcinomas are characterized by aberrant activation of hedgehog signaling. This is mainly due to inactivating mutations in the *PTCH1* gene (left) or activating mutations in the *SMO* gene (right).

The *PTCH1* mutations in sporadic BCCs are both truncating and missense (PTCH Mutation Database, www.cybergene.se/PTCH/), with the missense mutations showing a different distribution pattern compared to NBCCS, as these cluster into the predicted first large extracellular loop, the large intracellular loop and the C- terminus (Lindström et al., 2006). These domains are known to be functionally important, since Shh binds to both large extracellular loops (Marigo et al., 1996), cyclin B1 binds to the large intracellular loop (Barnes et al., 2001), and the C-terminal tail is important in suppressing Hh signaling and regulating turnover in Drosophila and the mouse (Johnson et al., 2000; Makino et al., 2001; Lu et al., 2006). Disrupting the interaction between Ptch1 and cyclin B1, by a truncating *PTCH1* mutation found in a sporadic BCC and in the germline of a NBCCS patient, results into constitutive activation of the Shh pathway with uncontrolled cell proliferation (Barnes et al., 2005). Several *PTCH1* mutations found in different types

of tumors and in the germline of NBCCS patients are located within or nearby the cyclin B1 binding site, suggesting that this unusual clustering of mutations represents a hot spot region (Barnes et al., 2005; Lindström et al., 2006).
A high frequency of UV-signature mutations in *PTCH1* is reported in sporadic BCCs (Lindström et al., 2006; Heitzer et al., 2007), especially in BCCs from XP patients that lack the capacity of DNA repair (Lindström et al., 2006), supporting therefore a role for UV-radiation in the pathogenesis of BCCs.

Some polymorphisms (SNP) in *PTCH1* are suggested to be a risk factor for sporadic BCCs, with the c.3944C/C genotype (corresponding to Pro/Pro) being more frequently found in BCCs compared to controls, especially in patients with multiple BCCs (Asplund et al., 2005; Liboutet et al., 2006). Other studies show that this genotype in combinations with additional *PTCH1* SNPs decreases, however, the risk of developing BCCs (Strange et al., 2004a; Strange et al., 2004b).

1.8.3 SCC

There are only a few *PTCH1* mutations, all UV-specific, found in SCCs of the skin, originating from individuals with a history of multiple BCCs due to sun-damaged skin (Ping et al., 2001). Analysis of cutaneous SCCs, premalignant AKs and BDs with LOH in the 9q22.3 region did not reveal any *PTCH1* mutations (Eklund et al., 1998). Two *PTCH1* mutations have been reported in esophageal squamous cell carcinoma, both with LOH in the *PTCH1* gene (Maesawa et al., 1998). Based on these studies, mutated *PTCH1* is apparently involved only in a subset of tumors of the squamous cell type. Importantly, some of these somatic missense mutations might not be “driver” mutations, but rather “passenger” mutations not contributing to oncogenesis (Futreal, 2007; Greenman et al., 2007).

1.8.4 Medulloblastoma

PTCH1 mutations are reported to affect the development of a subset of sporadic primitive neuroectodermal tumors, PNETs, often called medulloblastomas (Dong et al., 2000; Zurawel et al., 2000b). These mutations are mainly truncating, and the few missense mutations are clustered to the TMs, similarly to the germline mutations found in NBCCS patients (Lindström et al., 2006). Rare mutations are also found in the *SMO* , *SUFU* and *SHH* genes (Oro et al., 1997; Reifenberger et al., 1998; Taylor et al., 2002).

1.8.5 Other tumors

Odontogenic keratocysts (OK)s are associated with the NBCCS disease, and several somatic *PTCH1* mutations have been found in sporadic OKs (Barreto et al., 2000; Gu et al., 2006). Familial OKs are often associated with LOH of the normal *PTCH1* allele (Levanat et al., 1996). Recently, a germline missense *PTCH1* mutation was found in TM10 in a Chinese family with only familial OKs, without any symptoms of NBCCS. The mutation cosegregates with the disease, is highly conserved in vertebrates and is not

found in normal control chromosomes, suggesting that it is disease-causing (Song et al., 2006).

Somatic *PTCH1* mutations were found in a subset of sporadic trichoepitheliomas and associated with overexpression of the PTCH1 mRNA, implying that deregulated HH signaling is involved in the development of sporadic trichoepitheliomas (Vorechovsky et al., 1997). Two somatic *PTCH1* mutations have also been found in sporadic breast carcinomas (Xie et al., 1997). In breast cancer tumors, the *PTCH1* promoter was recently found to be epigenetically silenced, and correlated with low expression of PTCH1, implicating PTCH1 in the development of this tumor (Wolf et al., 2007).

Other cancer types with aberrant HH signaling are prostate, pancreas, malignant melanomas, stomach, intestine/colon, esophagus, small cell lung cancer, multiple myeloma and glioma (Jacob & Lum, 2007; Lauth & Toftgård, 2007).

"All roads go to ROME" is an old expression, and here it means that irrespective of the kind of mechanism that turns the signaling on, activation of the GLI transcription factors is the end result. This causes an upregulation of targets genes, leading to constant proliferation and reduced apoptosis, and consequently tumor development. Therefore, it is suggested that the ultimately target for inhibition of aberrant HH signaling is GLI.

1.8.6 Treatment of overactivated HH signalling

Targeting GLI has recently been reported, with two small-molecule antagonists of GLI, GANT61 and GANT58, found to inhibit cell proliferation *in vitro* and block cell growth *in vivo* using a xenograft model with human prostate cancer cells (Lauth et al., 2007). Another inhibitor of HH signaling is cyclopamine, which binds and inactivates SMO, but it does not inhibit HH signaling in tumors that are activated downstream of SMO (Lauth & Toftgård, 2007). Importantly, activated SHH signaling is also associated with resistance to chemoradiation, and inhibiting SHH signaling may improve cancer treatment responses (Chen et al., 2007).

1.9 Final remark

The rising incidence of NMSCs, with consequent increased economic costs for society, together with an ageing population highlights the need for prevention and inexpensive treatments.
New therapies are also needed due to recurrence, multiple tumors, complicated treatment methods, cosmetic outcomes and adverse side effects. The blocking of the HH signaling pathway with GANT compounds seems a promising strategy, although these have not yet been clinically evaluated. However, the most efficient way of decreasing the epidemics of NMSCs is prevention in different forms.

2. AIMS OF THE THESIS

The general aim of this thesis was to define the role of *PTCH1* mutations in the development of non-melanoma skin cancer and in the genetic disease Nevoid Basal Cell Carcinoma Syndrome (NBCCS).

The specific aims were to:

1. Further map the NBCCS gene in 9q22.3 and confirm that this gene is involved in familial and sporadic BCCs. Investigate if the NBCCS gene or another gene in the same chromosomal region has a role in the squamous type of skin cancer.

2. Verify that the *PTCH1* gene is the NBCCS gene in Swedish patients with NBCCS and acts as a tumor suppressor in familial and sporadic BCCs.

3. Investigate if the *PTCH1* gene is involved in the development of SCC. Examine if alterations in the *XPA* gene, located in the vicinity of the *PTCH1* gene in 9q22.3, is critical for SCC development.

4. Analyse and investigate the distribution pattern of *PTCH1* mutations compiled from the PTCH Mutation Database.

3. METHODS

Standard molecular biology techniques have been used in our analyses including the following:

DNA isolation and sequencing; Polymerase chain reaction (PCR); Loss of heterozygosity (LOH), which is a technique to identify genetic loss in tumor cells compared to normal cells, using highly polymorphic microsatellite markers; Single strand conformation polymorphism (SSCP)/Single strand conformation analysis (SSCA); *In situ* hybridization, a technique used to detect gene expression by hybridization of a probe to a specific mRNA in fixed tissue.

For statistical analysis, the two-sided Fisher's exact test (extended) was used.

4. RESULTS AND DISCUSSION

4.1 PAPER I: Fine Mapping of the NBCCS gene and Differential 9q Loss in Non-Melanoma Skin Cancer

4.1.1 NBCCS and BCCs

Previously, the NBCCS gene was mapped to chromosome 9q22.3 between microsatellite markers D9S196 and D9S180 through linkage analysis of affected families (Farndon et al., 1994; Wicking et al., 1994). A high frequency of LOH in 9q22-31 has been reported in sporadic and familial BCCs (Gailani et al., 1992; Quinn et al., 1994a), and this together with a case of a familial BCC, which showed loss of the non-disease carrying allele (Bonifas et al., 1994), the NBCCS gene was proposed to be a putative TSG that underlies both NBCCS and BCCs.

In an attempt to fine map the NBCCS gene region and to confirm the high frequency of loss of 9q22.3 seen in BCCs, we examined sporadic (N=16) and familial (N=2) BCCs for occurrence of LOH using the microsatellite markers D9S196, D9S280, D9S287 and D9S180. The analyses were done with PCR followed by GENESCAN™.

The LOH analyses of BCCs confirmed the high frequency (67%) of loss in 9q22.3, and we fine mapped the NBCCS gene in a region encompassing the microsatellite markers D9S280, D9S287 and D9S180. We also found supporting evidence for the NBCCS gene to be a TSG, as the non-disease carrying chromosome was lost in one familial BCC from a NBCCS patient.

4.1.2 Squamous type of skin cancer

The responsible gene for MSSE has been reported to map to the same region as the NBCCS gene through linkage analysis (Goudie et al., 1993; Povey et al., 1994). There were speculations that either the two genes reside in this region or that different mutations in the same gene could explain the different clinical features of the syndromes. The MSSE tumors are morphologically similar to well-differentiated SCCs (Goudie et al., 1993), and although no LOH studies of MSSE tumors were done, loss of 9q alleles in sporadic SCCs has been reported (Quinn et al., 1994b; Zaphiropoulos et al., 1994). Interestingly, LOH studies of squamous cell carcinomas from bladder, esophagus and head and neck showed high LOH frequency in 9q22-34, implicating putative TSG(s) residing in this region (Ah-See et al., 1994; Mori et al., 1994; Habuchi et al., 1995).

Our hypothesis was that a gene, NBCCS or another gene, which resides in the 9q22.3 region, may be involved in the development of the squamous type of skin tumors. We investigated this possibility using the microsatellite markers D9S196, D9S280, D9S287 and D9S180 for detecting LOH in this region in SCCs (N=11), premalignant skin lesions AKs (N=4) and BDs (N=13). The analyses were done with PCR followed by GENESCAN™.

The LOH analyses of sporadic SCCs, AKs and BDs showed a high frequency, 50%, of LOH at D9S180, but only 6% at the proximal nearby marker D9S287. We suggested therefore that the D9S287 microsatellite marker is the proximal border for a putative TSG involved in the development of the squamous type of skin cancer.

4.1.3 Conclusions

The high frequency of LOH in BCCs strongly implicates that the 9q22.3 region harbors a TSG, most likely the NBCCS gene, which is highly important in the development of BCCs. Interestingly, we found different LOH patterns in sporadic SCCs, AKs and BD compared to sporadic BCCs, implicating the possibility for another TSG being located within 9q22.3. We also suggested that loss of this putative TSG is important in the development of the squamous cell type of skin cancer, and is likely to be located distal to microsatellite marker D9S287.

4.2 PAPER II: PTCH1 Mutations in Swedish NBCCS Patients and BCCs

The NBCCS gene was identified in 1996 as a human homolog (*PTCH1*) of Drosophila *patched*, an important regulator in development. Initially, several *PTCH1* germline mutations were found in NBCCS patients and somatic mutations in sporadic BCCs (Hahn et al., 1996; Johnson et al., 1996).

We wanted to confirm that *PTCH1* is the gene underlying the NBCCS and has a role in the development of both sporadic and familial BCCs in Swedish patients. This was investigated by performing mutation analyses using PCR-SSCP and DNA sequencing.

The mutation analyses showed that the *PTCH1* gene is mutated in the germline of Swedish NBCCS patients. Two of these mutations were predicted to truncate the PTCH1 protein, whereas the third one was an in-frame deletion suggested to affect conserved amino acids in the putative transmembrane region. One of the truncating mutations was found in a three-generation family with different clinical outcomes of the affected family members, revealing an intra-familial phenotypic diversity. We suggested that this diversity might be explained by factors other than the mutation itself, such as environmental or genetic factors. The phenotype of the third family with the in-frame deletion was milder compared to the family with the intra-familial diversity, and a genotype-phenotype correlation was suggested. We also found distinct *PTCH1* inactivation mechanisms in two BCCs derived from one of the NBCCS patients. These two BCCs carried both a germline truncating mutation, together with either a somatic missense mutation or a loss of the normal copy of *PTCH1*.
The mutation analyses of the sporadic BCCs revealed three somatic *PTCH1* mutations, all of which resulted in truncation of the protein, together with LOH of the normal allele.

4.2.1 Conclusions

We verified that the *PTCH1* gene is the NBCCS gene in Swedish NBCCS patients. We also found evidence for the *PTCH1* gene being an important TSG involved in the development of both sporadic and familial BCCs in Swedish patients.

4.3 PAPER III: The PTCH1 and XPA Genes are Not Mutated in the Squamous Type of Skin Cancer

4.3.1 *PTCH1* gene

Chromosomal losses of 9q22-31 markers, including the *PTCH1* region, have been reported in AKs, BDs and SCCs (Quinn et al., 1994b; Rehman et al., 1994; Zaphiropoulos et al., 1994; Holmberg et al., 1996). However, the proximal border of these LOHs was only defined in one study (Holmberg et al., 1996). Furthermore, the MSSE gene was also mapped to the same region as the *PTCH1* gene (Goudie et al., 1993; Povey et al., 1994).

We wanted to examine if genetic alterations in *PTCH1* is critical for the development of the squamous type of skin cancer. Mutational analysis with PCR-SSCP and DNA sequencing was performed in 14 sporadic skin tumors with different squamous phenotypes. We also performed an *in situ* hybridization to examine if the PTCH1 mRNA was upregulated in BD and SCCs, as the mutant PTCH1 mRNA is known to be overexpressed in both sporadic and familial BCCs (Gailani et al., 1996; Unden et al., 1997b).

We did not find any *PTCH1* mutations in the tumors analyzed, but only a high degree of genetic variability of the *PTCH1* gene, since SNPs were found in 10/14 tumors and in the corresponding normal DNA. PTCH1 mRNA was not found to be upregulated in the tumors analyzed, which confirmed the lack of mutations in the *PTCH1* gene.

4.3.2 *XPA* gene

The *XPA* gene has been reported to be located in 9q22.3 (Hahn et al., 1996), and patients with a defective XPA protein frequently develop BCCs and SCCs due to deficient DNA repair of UV-induced DNA damage (Swift & Chase, 1979; Kraemer et al., 1987). Furthermore, XPA-deficient mice develop malignant SCCs when exposed to UVB-radiation (de Vries et al., 1995; Nakane et al., 1995).

Thus, we engaged into the investigation of whether the *XPA* gene was mutated in the same set of 14 sporadic skin tumors that were analyzed for *PTCH1* mutations. This was done using PCR-SSCP and DNA sequencing, but no alterations in the *XPA* gene could be identified in the tumors analyzed.

4.3.3 Conclusions

Our mutation analyses of *PTCH1* and *XPA* genes indicated that neither gene is of major importance for the development of the squamous type of skin cancer. Although frequent LOH of chromosome 9q22-34 have been found in other cancer types with a squamous cell phenotype, including head and neck, esophagus and transitional cell carcinoma of the bladder, no *PTCH1* mutations had been reported in these tumor types as of May 1997. We thus suggested that *PTCH1* mutations are restricted to tumor types associated with the NBCCS, i.e. BCCs, medulloblastomas and ovarian fibromas. Moreover, another gene or genes distal to the *PTCH1* locus could be involved in the development of SCCs.

4.4 PAPER IV: Distribution patterns of human PTCH1 mutations

Since the *PTCH1* gene was discovered 1996, hundreds of *PTCH1* mutations have been published in the scientific literature. One way of collecting mutations for genes important in human disease, is to set up a locus-specific database for the gene or genes of interest (Cotton et al., 2008). We therefore decided in 1997 to design and set up a mutation database, the PTCH Mutation Database, www.cybergene.se/PTCH, for collecting all known *PTCH1* mutations and SNPs, including the possibility of submitting new mutations/SNPs online. This database was publicly available in 1999, and is annotated by an editor, who controls all submitted mutations and SNPs before they are made publicly available. It is also possible to use a number of queries in the database. As of April 2008, the PTCH Mutation Database contained 424 mutations and 64 SNPs.

In order to better define the role of *PTCH1* mutations in NBCCS, BCCs and other tumor types, we decided to investigate the distribution pattern of the 284 *PTCH1* mutations and 48 SNPs that were included in the database as of 30 June 2005. Therefore, we compiled and categorized the mutations into five different groups; germline mutations found in NBCCS patients, sporadic BCCs, BCCs from patients with XP (XP-BCC), sporadic medulloblastomas and other sporadic tumors, which include odontogenic keratocysts, SCCs of the skin, trichoepitheliomas, esophagus squamous cell carcinomas and breast carcinomas. We used the two-sided Fisher's exact test (extended) for the statistical analyses.

4.4.1 General distribution pattern

In general, we found that the exonic *PTCH1* mutations were mainly clustered in the exons that correspond to the predicted two large extracellular loops and the large intracellular loop. On the contrary, the exonic SNPs were concentrated in the putative SSD and the second half of the protein. Especially the exonic missense SNPs were found in this second half of the protein, indicating a higher variability in that region compared to the first half. Moreover, some amino acids were highly overrepresented in the mutation pattern, and considered to be hot spots.

4.4.2 Distribution in different groups

We analyzed the distribution of the mutations of the different groups in relation to the domain structure, and identified significant differences (Figure 7). The NBCCS germline mutations (N=132) were mainly truncating and concentrated in the two large extracellular loops, the large intracellular loop and the N-terminus, which seems to harbor a slippage-sensitive region. The few NBCCS missense mutations, 17%, were instead predominant in the TM regions. The group of sporadic BCC mutations (N=86), were mainly truncating and concentrated in the two large extracellular loops, especially the first one. However, the missense mutations, 35%, were clustered into the first large extracellular loop, the large intracellular loop and the C-terminus.

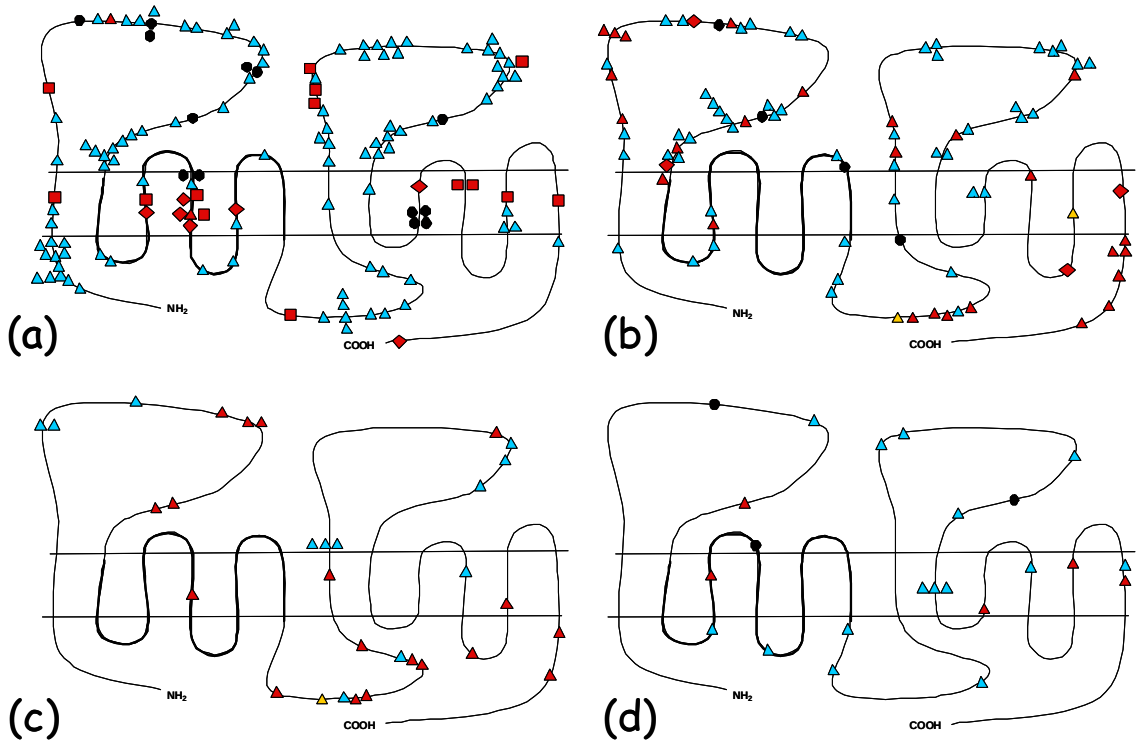

Figure 7. Distribution of PTCH1 mutations found in the germline of NBCCS patients and in different sporadic tumors in relation to the domain structure of the PTCH1 protein.

(a) Distribution of germline mutations in NBCCS patients. The black circles denote splice mutations in the adjacent exon junction, the blue triangles denote nonsense mutations, the red triangles denote de novo missense mutations, the red squares denote familial missense mutations, and the thick line denotes the SSD. (b)-(d) Distribution of mutations in different sporadic tumors; (b) sporadic BCCs, (c) XP-BCCs and (d) sporadic medulloblastomas. The black circles denote splice mutations in the adjacent exon junction, the blue triangles denote nonsense mutations, the red triangles denote somatic missense mutations, the red rombes denote conserved missense mutations, the yellow triangles denote silent mutations, and the thick line denotes the SSD.

In the group of XP-BCCs mutations (N=31), a different pattern compared to the sporadic BCCs and the germline NBCCS was seen, with a high frequency of missense mutations, 58%. The sporadic medulloblastoma mutations revealed, although low in numbers (N=23), a different pattern compared to the previous groups. Specifically, we found that these were predominant in the TMs, especially in the second half of the protein. The few

missense mutations, 22%, were distributed similarly to the NBCCS missense mutations, mainly in the TMs. There were too few mutations, (N=12), in the group of the different types of sporadic tumors to reveal any consistent distribution pattern.

4.4.3 Distribution of different mutation types

We found different mutation-type patterns among the groups analyzed. Mainly deletions and insertions were found in the NBCCS germline and the sporadic medulloblastomas. The sporadic BCCs, XP-BCCs and the group of other sporadic tumors, had substitutions as their main mutation type. We also found a high frequency of UV-related mutations in these groups compared to NBCCS germline and sporadic medulloblastomas.

4.4.4 Conclusions

In this meta-analysis of published *PTCH1* mutations, a unique distribution pattern and a unique mutation-type pattern for each group analyzed was consistently found. The truncating mutations found in all groups lead to an inactive PTCH1 protein. The missense mutations revealed domains and regions of likely importance for PTCH1 protein function and the development of sporadic tumors and NBCCS.

Specifically, the TMs appear to be important, and there are several NBCCS patients, whose disease is caused by a single amino acid change in one of the TMs. However, some TMs are more important than others, i.e. TM 4 with its clustering of NBCCS missense mutations. TM4 is within the SSD, which has been found to be functionally important in proteins that share homology with PTCH1, including NPC1 and RND permeases. Experimental studies by other groups have shown that some of the missense mutations in the TMs affect the PTCH1 ability to suppress HH signaling.

In sporadic BCCs, the missense mutations revealed other domains of importance, the first large extracellular loop, the large intracellular loop and the C-terminal region. We know that SHH binds to both large extracellular loops, and the identified missense mutations may highlight important residues for the interaction between HH and PTCH1. The C-terminal domain is known to be important in mouse and Drosophila, as it is critical for PTCH1 inhibition of Hh signaling. It is also known that cyclin B1 binds to the large intracellular loop, and disruption of this binding leads to uncontrolled cellular proliferation. In fact, this region appears to be a hot spot, as many mutations from all groups reside there.

The high frequency of missense mutations in XP-BCCs is not surprising, since these patients lack the capacity to repair UV-induced DNA damages, and this is indicated by the high frequency of UV-related mutations, 77%.

The distribution pattern of sporadic medulloblastoma mutations is also unique, as many are in the TMs in the second half of the protein, including the missense mutations. This further supports the important role of the TMs for a functional PTCH1 protein.

Finally, the strong impact of UV-radiation in the development of NMSC is exemplified by the high frequency of UV-related mutations in sporadic BCCs, XP-BCCs and in the group of other tumors, mostly SCCs from the skin.

4.5 GENERAL CONCLUSIONS

With Paper I, we further mapped the NBCCS gene and confirmed that this gene is most likely involved in the development of both familial and sporadic BCCs. We also provided evidence for another TSG in the same chromosomal region with a role in the development of the squamous type of skin cancer.

In Paper II, the transmembrane receptor of HH ligands, PTCH1, was verified to be the NBCCS gene in Swedish NBCCS patients. *PTCH1* was also confirmed to be an important TSG in the development of sporadic and familial BCCs.

In Paper III both the *PTCH1* and *XPA* genes were excluded as of major importance for the development of the squamous type of skin cancer. There is probably another gene(s) distal to *PTCH1* that is involved in SCCs development. Additionally, the *PTCH1* gene was also found to have a high degree of genetic variability.

Paper IV revealed that unique *PTCH1* mutation distribution and mutation-type patterns characterize the NBCCS disease, sporadic BCCs, BCCs from XP-patients and sporadic medulloblastomas. Moreover, domains and regions in the PTCH1 protein that are of importance in the development of sporadic tumors and NBCCS were identified.

5. FUTURE PERSPECTIVES

To further understand the importance of specific domains and regions of the PTCH1 protein in development of sporadic tumors and NBCCS, the following areas of future investigation are suggested:

Renewed genotype-phenotype analysis of NBCCS mutations compiled from the PTCH Mutation Database, as new entries have been submitted since 2005.
The missense mutations are of special interest, since the exact mechanism of how these are inactivating the PTCH1 protein is unclear. In analogy to the human diseases, Grieg cephalopolysyndactyly and Pallister-Hall syndrome, an analysis of different classes of mutations and their position on the *PTCH1* gene is tempting. Stratifications of ethnicity can also be done, as there is an increase in reports of *PTCH1* mutations found in Asian NBCCS patients. Some preliminary data show that missense TM mutations are more likely to be found in certain populations. The Ethnic perspective is interesting, since there are reports of fewer and later onset of BCCs in African-American, Korean and Italian NBCCS patients. Due to the large population in China, there is a potential group of undiscovered NBCCS patients. One could envision a collaborative project with Chinese medical doctors and scientists to find environmental modifier factors contributing to the diverse phenotypes seen in NBCCS patients.

Additionally, genotype-phenotype analysis of *PTCH1* mutations found in sporadic BCCs compiled from the PTCH Mutation Database, in relation to their clinical aggressive or non-aggressive behavior is of interest. This could be combined with analysis of reported p53 mutations/expression in the tumors analyzed, as there have been suggestions for a correlation between aggressive BCCs and p53 expression.

Moreover, the analysis could be complemented with stratifications of ethnicity, gender, and age or tumor location. The frequency of sporadic BCCs is known to be lower in populations with high skin pigmentation. Is therefore the *PTCH1* mutation spectrum different in BCCs from these populations?
Furthermore, there is a skewing of female gender in patients with early onset BCCs compared to older patients. The frequency of BCCs in the face is also lower in younger patients than in the older patients. These findings need therefore to be further investigated and analyzed in order to identify novel risk factors.

The specific hot spot residues and regions found in the *PTCH1* gene should also be further examined (Paper IV). The slippage-sensitive sequence in the N-terminal domain is of special interest, as there is an unusual clustering of truncating germline NBCCS mutations in this segment. Why are then these 35-40 base pairs mutated more frequently than the surrounding sequences?

Finally, silent mutations are mostly neutral, but some can activate cryptic splice sites. In Paper IV, we have reported several somatic silent *PTCH1* mutations, and it would be especially interesting to analyze how PTCH1 mRNA and protein may be affected.

6. ACKNOWLEDGEMENTS

My supervisor, Associate Professor Peter Zaphiropoulos. Thank you for all your patience, enthusiasm, support and correction of my misspellings. You have always time for me and know how to push me forward.

My co-supervisor and head of the group, Professor Rune Toftgård. Your enthusiasm and optimism have pushed me into the right direction. I am also very grateful for your support and the opportunity for me to manage the PTCH Mutation Database.

The Skin Cancer Foundation, http://www.skincancer.org/ for sharing pictures of skin cancer used in this Thesis. The "BCCNS Life Support Network", www.bccns.org, for sharing the Gorlin pictures used in this Thesis.

The former and present members of the RTO group. You are so many, but I am extra grateful to those of you who have contributed to this Thesis, thank you!
The former and present colleagues and friends at the Department, especially the former members in Bo Lambert's group. I would also thank Inger Moge, Marion Sandin, Kristina Bergholm and Ylva Svanberg, for all help, advices and for believing in me.
My co-authors; Barbro Lundh-Rozell, for the tumors and helpful discussions about skin cancer, Lena Thunell (former Eklund) for nice intercity collaboration and always being helpful when needed, Igor Vorechovsky, Takashi Shimokawa, for your help with the statistics and nice discussions, Anne-Birgitte Undén, for all our collaborations, Mona Ståhle-Bäckdahl, and Peter Söderkvist.
Britta Krynitz, for your valuable reading of this Thesis.
Jan and Helene Olsson at Cybergene AB.

All my private friends, you are all in my telephone list, I haven't forgotten you.
My grandmother, "mormor", you are one of my best supporters for this Thesis.
My mother and father, for life and been raised to be interested in all kinds of science.
My sister, for nice chats, and my brother for always being helpful and a good listener.

My parents in law, for first introducing me to long-distance skating. I am also happy for all the times spent with you.

Ronja, our sailing boat, for teaching me the real important things in life, and for exercising me to keep the focus in all kinds of weather.
My husband, for being my best friend. I am happy for all your support, and the illustrations to this Thesis. I will also thank you for introducing me into hiking in the mountains "fjällvandring", I am always looking forward to the next summer. I will never forget the Sarek tour with sun, mosquitos, heavy "ryggsäck" and stormy weather, what an adventure! You also introduced me into the "life of sailing", is there any better life?

At last I will thank the most important persons in my life, my daughters. You have almost been "living" at "fritids" during these last two years. You are my love, happiness and joy.

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