

Örebro Studies in Medicine 312



Sara Prosén

**Studies on expression profiles in keratinocyte
cancers with focus on basal cell carcinoma**

Author: Sara Prosén

Title: Studies on expression profiles in keratinocyte cancers with focus on basal cell carcinoma

Publisher: Örebro University, 2025

www.oru.se/publikationer

Print: Örebro University, Repo 02/2025

ISSN: 1652-4063

ISBN: 978-91-7529-624-1 (print)

ISBN: 978-91-7529-625-8 (pdf)

Abstract

Aims: This thesis aimed to investigate metabolic changes in keratinocyte carcinoma with a focus on basal cell carcinoma (BCC), to find potential treatment targets.

Material and Methods: Patients diagnosed with BCC (n=55) or cutaneous squamous cell carcinoma (cSCC, n=4) were included. Snap-frozen tumour tissue from BCC tumours, formalin-fixed paraffin-embedded tissue from BCC and cSCC tumours, and donor skin were investigated with quantitative real-time polymerase chain reaction (qPCR), microarray analysis, immunohistochemistry, and immunofluorescence. Cell lines from BCC, cSCC, and non-neoplastic keratinocytes were used to examine LAT1 inhibition with JPH203 in terms of decreased viability and changed gene expression in genes important for cell metabolism and carcinogenesis.

Results: SLC25A43 gene- and protein expression were significantly decreased in the BCC tumour samples (n=14) compared to the surrounding epidermis. Microarray examination of the tumour material (n=4+4) revealed increased expression of the amino acid transporters SLC7A5/LAT1 and SLC7A8/LAT2, which was confirmed with qPCR (n=14) and immunohistochemistry (n=14). The LAT1 expression was mainly in the centre of the tumours, and the fraction of LAT1-positive cells were significantly ($p<0.01$) inversely correlated to the proliferative active cells. Cleaved caspase 3 was significantly ($p=0.02$) increased in tumour areas with high LAT1 expression. In the patient cohort (n=57), the H-score for LAT1 was significantly higher ($p<0.001$) than for GLUT1 or GLI1. A sub-analysis of the BCC tumours also revealed a statistically significant correlation ($p<0.01$) between LAT1 and GLUT1 protein expression. The keratinocyte cell line (HEK001) showed significantly decreased viability when exposed to the LAT1 inhibitor JPH203 at concentration of 100 μM , and a low but significant upregulation of SLC7A5, SLC3A2, CCND1, ATF4 and GLI1 when exposed to a concentration of 10 μM JPH203.

Conclusions: Both SLC25A43 and LAT1 are altered in BCC tumours compared to normal skin suggesting metabolic changes in the tumours. The changed LAT1 expression might be explained by the harsh tumour environment. LAT1 could be a drug target for keratinocyte cancer, but needs further investigations in more advanced models.

Keywords: keratinocyte cancer, non-melanoma skin cancer, basal cell carcinoma, cutaneous squamous cell carcinoma, SLC25A43, LAT1

To my family

Table of Contents

List of papers	8
Abbreviations	9
Keratinocyte cancer	11
Epidemiology and aetiology of BCC	11
Clinical features of BCC	12
Histopathological growth patterns of BCC	12
Epidemiology and aetiology of cSCC	13
Clinical features of cSCC	14
Histopathology of cSCC	15
Socioeconomic perspective on skin cancer	15
Hallmarks of cancer	16
Cell proliferation in cancer cells	17
Deregulated pathways in keratinocyte cancer	17
Evading growth suppressors in keratinocyte cancer	19
PI3K/Akt/mTOR signalling in keratinocyte cancer	20
Genome instability and mutations: ultraviolet radiation and its cancerogenic effect on the skin	21
Inducing angiogenesis and avoiding immune destruction: the importance of the tumour microenvironment	22
Deregulating cellular energetics: the changed tumour metabolism	24
The mitochondria and cancer	24
Reactive oxygen species and cancer	25
SLC25A43 expression in cancer	26
Cell membrane transporters	27
Glucose and amino acid transporters in cancer	27
LAT expression in cancer	28
Keratinocyte cancer treatment	31
Standard keratinocyte cancer treatment in Sweden	31
A possible new drug target: inhibitors of LAT1	31

Aim of the thesis	33
Material	34
Patients	34
Cell lines.....	36
Ethical considerations.....	37
Research methods.....	38
Methods for analysing gene expressions.....	38
Tissue preparation and isolation of nucleic acids	38
Quantitative real-time polymerase chain reaction (qPCR).....	38
Microarray analysis	39
Methods for analysing protein expressions	40
Immunohistochemistry.....	40
Immunofluorescence staining	40
Antibodies.....	41
Drug exposure.....	42
Statistical data analysis	43
Results	45
The mitochondrial transporter SLC25A43 in BCC (Study I).....	45
LAT1 and LAT2 upregulated in BCC tumours (Study II).....	46
LAT1 expression and harsh tumour environment (Study III).....	50
Examination of LAT1 in the whole cohort (Study IV).....	53
Protein expression pattern of LAT1 and GLUT1 in BCC (Study IV).....	55
LAT1 expression in the cell lines TE354.T, A431, and HEK001 (Study IV)...	55
Effects of the LAT1 inhibitor JPH203 in keratinocyte cancer in vitro (Study IV).....	56
Discussion	57
Mitochondrial alteration in BCC.....	57
Searching for potential drug targets, upregulation of LAT1 and LAT2.....	57
LAT1 expression depending on tumour microenvironment.....	58
Limited effect on LAT1 inhibition in vitro.....	59
Limitations	60

Conclusions.....	62
Future perspectives	63
Acknowledgements.....	64
Sammanfattning på svenska.....	66
References	68

List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I. **Prosén S**, Eremo AG, Tsegai AD, Lindberg M, Tina E. Decreased expression of the mitochondrial solute carrier SLC25A43 in basal cell carcinoma compared with healthy skin. *Oncol Lett.* 2017 Aug;14(2):2218-2222
- II. Tina E, **Prosén S**, Lennholm S, Gasparyan G, Lindberg M, Göthlin Eremo A. Expression profile of the amino acid transporters SLC7A5, SLC7A7, SLC7A8 and the enzyme TDO2 in basal cell carcinoma. *Br J Dermatol.* 2019 Jan;180(1):130-140
- III. **Prosén S**, Tina E, Sneckenborg AH, Loinder C, Seifert O, Lindberg M, Eremo AG. Increased expression of LAT1 in basal cell carcinoma-implications for tumour cell survival. *Clin Exp Dermatol.* 2022 May;47(5):910-917
- IV. **Prosén S**, Tina E, Seifert O, Lindberg M, Eremo AG. Implications of LAT1 expression and inhibition in basal cell carcinoma and cutaneous squamous cell carcinoma. In manuscript

Papers I–III are reprinted with the permission of the original publishers.

Abbreviations

ABL1	ABL proto-oncogene 1, non-receptor tyrosine kinase
ACTB	actin beta
ADP	adenosine diphosphate
Akt	oncogene isolated from the thymoma cell line AKT-8, of Ak mouse strain (serine-threonine kinase)
ATF4	activating transcription factor 4
ATP	adenosine triphosphate
BCC	basal cell carcinoma
BRAF	v-Raf murine sarcoma viral oncogene homolog B1
CCND1	cyclin D1
c-MYC	cellular oncogene of avian myelocytomatosis virus
CoA	coenzyme A
CDKN2A	cyclin dependent kinase inhibitor 2A
CPD	cyclobutane pyrimidine dimer
Cq	quantification cycle
cSCC	cutaneous squamous cell carcinoma
CYC1	cytochrome c1
FC	fold change
FFPE	formalin-fixed paraffin-embedded
GLI	glioma associated oncogene
GLUT1	glucose transporter 1
GST	glutathione S transferase
HER	human EGF receptor
Hh	Hedgehog
HIF1	hypoxia induced factor 1
HRAS	Harvey-oncogene of rat sarcoma virus
IDO1	indoleamine 2,3-dioxygenase 1
IF	immunofluorescence
IHC	immunohistochemistry
Ki-67	Kiel-67
LAT1	large neutral amino acid transporter small subunit 1
L-DOPA	l-3,4-dihydroxyphenylalanine

MAPK	mitogen activated protein kinase
MEK	MAPK/ERK kinase
MYC	v-myc avian myelocytomatosis viral oncogene homolog
mTOR	mammalian (or mechanistic) target of rapamycin
mTORC1	mammalian (or mechanistic) target of rapamycin complex 1
n-MYC	v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog
P53	tumour protein P53
PET	positron emission tomography
PI3K	phosphatidylinositol-3 kinase
qPCR	quantitative real-time polymerase chain reaction
RAS	oncogene of rat sarcoma virus
ROS	reactive oxygen species
SLC	solute carrier
TDO2	tryptophan 2,3-dioxygenase 2
Topo	topoisomerase
UVR	ultraviolet radiation
VEGF	vascular endothelial growth factor

Keratinocyte cancer

The integumentary system is the body's largest organ, and its intricate structure provides a protective cover for the rest of the body (1). It is also an active component of the immune system, and protects the body from ultraviolet radiation (UVR), cold, heat, and mechanical and chemical stresses. The skin has three levels with different structures: the epidermis, the dermis, and the subcutis. Skin cancer can arise from different types of cells within the skin, with the three most common forms being basal cell carcinoma (BCC), cutaneous squamous cell carcinoma (cSCC) and malignant melanoma. Both BCC and cSCC arise from keratinocytes in the epidermis, and are therefore known as keratinocyte cancer.

Epidemiology and aetiology of BCC

Basal cell carcinoma (BCC) is the most common type of cancer in fair-skinned individuals worldwide (2). In Sweden, the incidence is rapidly increasing, and every year approximately 70 000 BCCs are biopsied or excised (3). There may also be a high number of unreported cases that are treated with locally destructive methods before histopathological evaluation (4). The incidence is similar in men and women, and increases with age (2); the median age at diagnosis is 74 years (3). The main risk factor for acquiring BCC is exposure to UVR, and although the risk increases with cumulative exposure, people with indoor work and occasional UVR exposure, such as sunny holidays, are also at higher risk (5). Immunosuppression and fair skin type are other risk factors (6). The cellular origin of BCC has historically been under debate (7-11). At the beginning of the 20th century, BCC was suspected to originate from the basal cell layer in the epidermis, and hence was given its present name. Later, during the 1940s, it was hypothesised that the cancer stem cell came from the epithelia of the hair follicle. The evidence is still inconclusive, as there are data supporting an origin from the hair follicle epithelia as well as from the interfollicular epidermis.

Clinical features of BCC

BCC may appear as a red eczema patch or a nodular tumour with central ulceration (12), but in some cases it grows more aggressively with borders that are hard to define resembling a scar with telangiectasia (Figure 1). Although BCCs are usually slow growing tumours, there may be high variety and heterogeneity in growth rate (13). BCC rarely metastasises but can become locally invasive, mutilating soft tissue and bone (12).

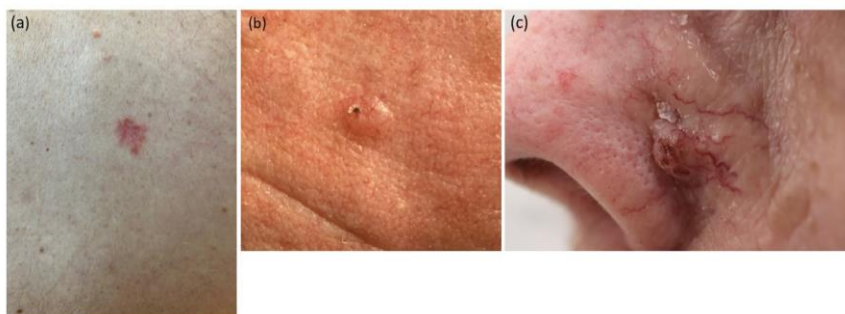


Figure 1 (a) A superficial basal cell carcinoma (BCC) mimicking an eczema patch. (b) Nodular BCC with a small ulceration. (c) Morphea BCC on the nose with an indurated scar-like appearance and telangiectasias. All patients gave their informed consent before these photographs were taken. Photographs taken by: (a) Martin Törnqvist, (b) Sara Prosén, (c) Petter Bengtsson, all at the Department of Dermatology, Örebro University Hospital, Sweden.

Histopathological growth patterns of BCC

Histopathologically, there are several different BCC growth patterns (14). The most common forms are the low-aggressive patterns as nodular and superficial growth, while the infiltrative or morphea patterns are rarer. The superficial type of BCC has small dense basophilic nests of basal carcinoma cells budding the basal membrane of the epidermis. The nodular type has palisading cells surrounding dense nodules protruding down into the dermis, and sometimes offshoot cells like the arms of an octopus. The infiltrative type and particularly the morphea type, contain cords with a thickness of one or two cells, consisting of BCC cells surrounded by eosinophilic hyalinised stroma. Other

subtypes include micro nodular, metatypical and basosquamous carcinoma. A BCC can have different subtypes within the same tumour but there is usually no progression from one subtype to another (15).

Epidemiology and aetiology of cSCC

After BCC, cSCC is the second most common type of skin cancer in Sweden. Its incidence is also increasing, and approximately 10 000 cases are reported in Sweden every year (3). Men are slightly overrepresented among cSCC patients compared to women (56% vs 44%) and the difference increases with age. A majority of patients acquiring a cSCC are elderly, and the median age at diagnosis is 79 years (3). The major risk factors for cSCC is chronic UVR exposure and a fair skin type. Other risk factors are previous radiotherapy and chronic inflammation in burns or wounds (16). Human papilloma virus exposure is a risk factor for squamous cell carcinoma in the genital and subungual area but there is contradictory evidence for its importance in cSCC in other parts of the body (17). The most common location for cSCC are the head and neck, which are often exposed to the sun (18).

The cell of origin in cSCC is the epidermal keratinocyte, and in a majority of cases different stages of squamous cell dysplasia of the keratinocytes will arise before the invasive cancer (19). The non-invasive precursor of cSCC is sometimes named intraepidermal carcinoma, and could also be divided into two types: Bowen's diseases (with sharper edges) and squamous cell carcinoma in situ (often a more poorly defined lesion sometimes with field cancerisation). However, today this division is considered to be less clear, and all the names are often used interchangeably. The first stage of dysplasia in sun-exposed skin is called actinic keratosis; in this stage, only parts of the epidermis are exchanged for atypical keratinocytes. It is estimated that approximately 0.1-10% of actinic keratosis cases will progress to a cSCC (20).

As the level of highly atypical and polymorphic keratinocytes increases, displacing the normal keratinocytes, eventually the whole layer of epidermis may be occupied by severely dysplastic cells; this condition is called squamous cell carcinoma in situ. This, in turn, can

progress further to invasive cSCC, with the risk of progression estimated at 3–5% (21). The risk of metastasis is approximately 3.7–5.2%, and the risk factors are invasion beyond the subcutaneous fat, perineural invasion, diameter >20 mm, poor differentiation, Breslow thickness >2 mm, location on the lip, ear, or temple and immunosuppression (22). Immunocompromised patients, for example those who have undergone organ transplants, have an approximately 100-fold increased risk of acquiring a cSCC, and are also more predisposed to develop metastasis (22, 23). Immunosuppression due to medication or chronic lymphocytic leukaemia has also been shown to increase the risk of cSCC (24, 25).

Several genetic disorders with defects in a wide range of genes involved in pigmentation, telomere maintenance, skin integrity, and nucleotide excision repair are associated with higher cSCC incidence (26-29). Some medications can increase the risk for cSCC; for example, thiazide diuretics and the antifungal agent voriconazole, both of which have a photosensitivity effect (30, 31). Moreover, vismodegib and v-Raf murine sarcoma viral oncogene homolog B1 (BRAF) inhibitors can enhance cSCC development due to paradoxical reactions with enhanced activation of the mitogen activated protein kinase (MAPK) pathway (17).

Clinical features of cSCC

A common symptom of cSCC is an ulcer or nodule (Figure 2), sometimes with a hyperkeratotic plug in the middle, on sun-exposed skin (32, 33). It is often infiltrated, and could have a crust or different levels of squamation or hyperkeratinisation. There may also be a tingling sensation or a slight aching pain in the nodule. An erythematous and/or scaly plaque might be a sign of early squamous cell dysplasia.

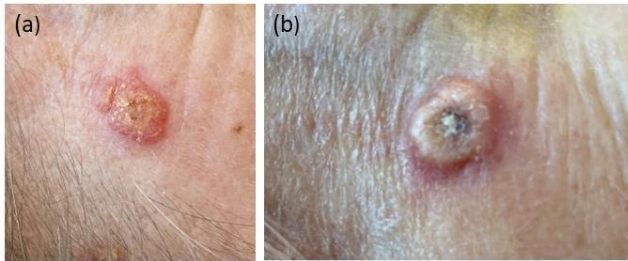


Figure 2 (a) A squamous cell carcinoma in situ. (b) A highly differentiated cutaneous squamous cell carcinoma on the temple. Both patients gave their informed consent before these photographs were taken. Photographs taken by Sara Prosén at the Department of Dermatology, Örebro University Hospital, Sweden.

Histopathology of cSCC

The histopathology of cSCC is characterised by atypical keratinocytes invading the basal cell membrane into the dermis. The tumour is often categorised according to the level of differentiation and keratinisation. In highly differentiated cSCC, the keratinocytes have some preserved ability to mature, and they build keratin bundles seen as keratin pearls. They often have an easily detectable squamous epithelium, minimal pleomorphism, and basally located mitotic figures. In poorly differentiated cSCC, the ability to keratinise has been lost, there are marked nuclear atypia, and it is sometimes difficult to determine a keratinocyte lineage (17). The cSCC could also have a differentiation towards other skin structures such as pseudovascular, acantholytic, metaplastic, and adenosquamous (34).

Socioeconomic perspective on skin cancer

The rising incidence of keratinocyte cancer is having an increasing impact on the healthcare system. Often the BCC and cSCC are diagnosed and treated at a dermatology department, and skin cancer treatment will need more resources in the future (35, 36). This huge forthcoming challenge motivates continued work for cost-effective treatments and an effective workflow through the healthcare system.

Hallmarks of cancer

The progression towards a developing tumour is a multi-step pathway in which the cells and their surroundings develop new or previously hidden abilities. In 2000, Hanahan and Weinberg published an article summarising different principles in carcinogenesis: sustaining proliferative signalling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death (37). In 2011, they expanded the concept with four additional emerging hallmarks and enabling characteristics: genome instability and mutation, deregulating cellular energetics, avoiding immune destruction, and tumour promoting inflammation (38) (Figure 3). Further hallmarks and enabling characteristics have been suggested since this, including the tumour microenvironment, unlocking phenotypic plasticity, non-mutational epigenetic reprogramming, polymorphic microbiomes, and senescent cells (39). Most of these aspects are known to be relevant in keratinocyte cancer even if the knowledge in many areas is very limited.

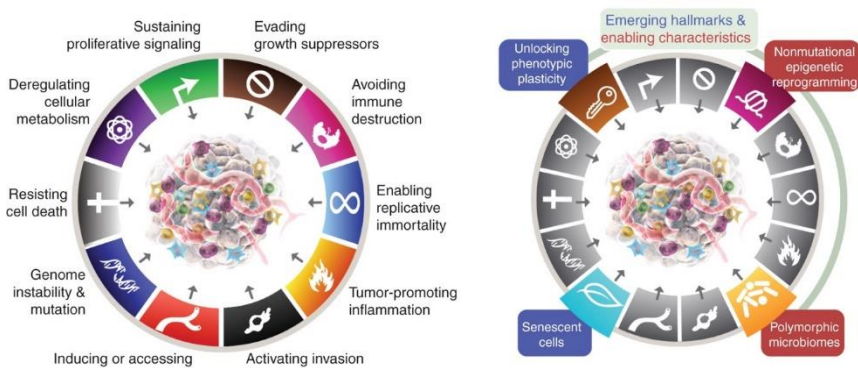


Figure 3 Left: The eight hallmark capabilities and two enabling characteristics of cancer, Right: Additional proposed emerging hallmarks and enabling characteristics: tumour microenvironment, unlocking phenotypic plasticity, nonmutational epigenetic reprogramming, polymorphic microbiomes, and senescent cells. Adapted from an article by Hanahan (39) with permission from American Association for Cancer Research.

Cell proliferation in cancer cells

Cell proliferation is often increased in cancer cells. In histopathology, cell proliferation is often measured through counting mitotic figures and immunostaining for the Ki-67 antigen. Ki-67 is expressed in cycling cells, but not in cells in the resting G0 phase (40) and is a clinically useful score that has been used in staging tools for many types of cancer (41-43). Studies have indicated that in BCC a higher Ki-67 staining is correlated with higher risk of recurrence, but also that the growth pattern of the tumour has a bigger impact on the risk of incomplete excision (13, 44-46).

Topoisomerase (Topo) II α is also expressed during cell proliferation. It disentangles the newly replicated sister chromatids, and is essential for cell viability (47). Topo II α is expressed in the mid S-phase, and is degraded upon mitotic completion. Its production and degradation are cell-cycle-regulated, and therefore reflect the cell's proliferation (48). As cancer cells often have a higher rate of division, chemotherapy such as etoposide directed against Topo II α is sometimes used. However, drug resistance due to changes in Topo II α activity can be a therapeutic problem (49).

Deregulated pathways in keratinocyte cancer

Sustained cell proliferation in cancer is often driven by changes in key regulatory genes known as oncogenes. One important oncogene in BCC, and to a lesser extent also in cSCC, is glioma associated oncogene (GLI) 1, a product of the Hedgehog (Hh) cell signalling pathway (50). The Hh pathway was first explored in the 1990s during studies on *drosophilae*, sporadic BCC, and the hereditary form of BCC called Gorlin's syndrome or naevoid BCC syndrome (51, 52). Gorlin's syndrome is autosomal dominant; affected individuals develop BCC at a young age and are also prone to medulloblastoma (53).

A majority of sporadic BCCs carry a mutation affecting the Hh pathway. These mutations are situated in human chromosome 9q22, in alleles coding for the proteins Patched or Smoothed. Patched is a receptor that confines a transmembrane protein called Smoothed to a cytoplasmatic vesicle. Under these inactivated circumstances, GLI is

cleaved to a protein that moves to the nucleus and acts as a repressor of transcription. When Patched is connected to its ligand Hedgehog, the inhibition of Smoothened breaks, and Smoothened starts a reaction that ends in an un-cleaved GLI entering the nucleus and activating transcription of target genes (54). There are three proteins in the GLI family; were GLI1 works as an activator, while GLI2 and GLI3 have both activation and repression functions.

The mutations of the Hh pathway result in a constantly active signalling pathway that favours oncogenesis. Overexpressed GLI has been found in several different neoplasms. The transcribed target genes of the Hh pathway also contain negative feedback loops (55). The Hh pathway is important during embryonal development, and regulates organogenesis, stem cell maintenance, and tissue repair and regeneration. In the skin, it also controls the development of hair follicles (56).

As described in the section below on keratinocyte cancer treatment, the first BCC-specific treatment, vismodegib, was a large step forward. Vismodegib, like the later-introduced sonidegib, targets the Hh pathway and is a Smoothened inhibitor. As clinical experience grew after its approval, it became obvious that some patients (approximately 20% per year) experience treatment failure and local relapse when treatment is discontinued. Some of the treatment resistance is due to mutations in the receptor of the drug target, Smoothened, and also to copy number changes in Suppressor of Fused and GLI2 (57). Another study (58) gives a theory of the mechanism behind the local relapse after treatment discontinuation. The authors used a mouse model to show that BCC cells, which initially show similarity to the hair follicle bulge stem-cells, transform their identity when under Hh-inhibition to other stem-cell-like properties, more closely resembling the stem cells in the isthmus and interfollicular epidermis. This transformation renders them quiescent, and they no longer require the Hh signalling. After Hh inhibition has ceased, they return to their original status and regrowth.

In addition to changes in the Hh pathway, v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (n-MYC)

mutations are commonly found in BCC. N-MYC is an oncogene, a transcriptional activator, and a potential downstream effector of the Hh pathway (59). It is a member of the MYC family that influences cell growth, proliferation, differentiation, and apoptosis (60). In a microarray study published in 2016, a missense mutation in the n-MYC gene was found in 30% of the studied BCCs (61).

The rat sarcoma virus (RAS) oncogenes are an important oncogene family in cSCC (17, 62). In melanoma patients treated with the BRAF inhibitor vemurafenib, a common side effect is the development of cSCC and keratoacanthomas. Mutations in Harvey-rat sarcoma virus (HRAS) is commonly seen in these vemurafenib-induced tumours. This phenomenon is due to paradoxical MAPK pathway activation and an enhanced cell growth in cells with a pre-existing HRAS mutation. This accelerated growth could be inhibited with a MAPK/ERK kinase (MEK) inhibitor (63).

Evading growth suppressors in keratinocyte cancer

One frequently mutated growth suppressor in keratinocyte cancer is p53, often described as “the guardian of the genome”. Approximately 60% of BCCs have a p53 mutation (64). Mutation in p53 is the second most frequent mutation found in BCC, often causing an inactivation of the gene (65), and is suspected to be an early event in BCC carcinogenesis. Moreover, p53 plays a vital role for the carcinogenesis in cSCC (66).

The majority of the p53 mutations in BCC and cSCC seem to be a result of UVR-induced damage such as C to T transitions and especially CC to TT double base changes (65, 66). UVR induces a stress response in the cell in order to handle the DNA damage. The protein p53 orchestrates the cell response including cell cycle arrest, DNA repair, and apoptosis. The p53 gene has a dominant-negative allele, which means that a mutation in just one of the two alleles coding for p53 is enough to produce a severe response in the cell.

P53 is localised in the nucleus, and the wild type is both synthesised rapidly and, under normal conditions rapidly degraded. Its usual steady-state level in the cell is low, but it can accumulate fast if

degradation is blocked in a stressed cell (55). In the 1990s, it was found that increased p53 could be induced by a variety of different agents such as radiation, UVR exposure, chemotherapeutic drugs that damage DNA, inhibitors of DNA synthesis, and agents that disrupt the microtubule components of the cytoskeleton. Later, hypoxia and the presence of the oncogene MYC were also found to stimulate a p53 increase. Today, the list of stimuli known to activate p53 has become long (55, 67).

Another important growth suppressor in cSCC is the family of Notch- receptors, which form part of a highly conserved signal transduction pathway important for the development, growth, and survival of a cell (68). In cSCC, Notch mutation is a gatekeeping event in the carcinogenesis (69).

The cyclin dependent kinase inhibitor 2A (CDKN2A) gene is mutated in several cancers. In malignant melanoma, for example, hereditary mutations in CDKN2A are a high-risk marker both for new melanomas and for pancreatic cancer. CDKN2A codes for two proteins that control the cell cycle, p14 and p16. P16 may be altered in BCC (70), while in cSCC, there is evidence for altered function of both p14 and p16 (71).

PI3K/Akt/mTOR signalling in keratinocyte cancer

The phosphatidylinositol-3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signal transduction pathway is the hub for several physiological functions in the cell, linking growth factors, nutrients and energy availability to lipid and protein synthesis, metabolism, cell growth, proliferation, survival, apoptosis, angiogenesis, and tissue development (72). There is also evidence that it plays an important role in normal keratinocytes and in keratinocyte cancer.

Normal keratinocytes that are exposed to UVA and UVB radiation have an activation of PI3K, with UVB giving the strongest activation. UVR exposure also leads to an increase in mTOR. Experiments with mTOR-deficient mice showed that UVB exposure led to increased apoptosis, supporting an important role for mTOR in UVR-stressed normal keratinocytes (73). There is emerging evidence for a changed

mTOR signalling in BCC tumours. Interactions have been identified between the Hh and the PI3k/Akt/mTOR pathways, and it has been hypothesised that mTOR is downstream of Hh signalling or that both signalling pathways co-regulate common downstream targets. One example is that Hh signalling enhances n-MYC expression while PI3K/Akt/mTOR reduces n-MYC phosphorylation and its proteolytic destruction (74, 75).

Different medications that affect the PI3K/Akt/mTOR pathway are used to try to inhibit the carcinogenesis in keratinocytes. An example of this is sirolimus, an mTOR inhibitor. In transplant patients with cSCC, and to some extent even in transplant patients with BCC, sirolimus is more protective against keratinocyte cancer than other immunosuppressing medications (76-79). The difference between cSCC and BCC could be due to the amount of phosphorylated mTOR in the cytoplasm (76).

Another group of drugs used for preventing keratinocyte cancers is retinoids. There are studies indicating that retinoids act on cell proliferation through the PI3K/Akt/mTOR pathway. Both oral intake and topical use of retinoids have been shown in different studies to inhibit carcinogenesis in BCC and even more in SCC; however, despite some evidence for their effectiveness, side effects often limit their use (80, 81).

Genome instability and mutations: ultraviolet radiation and its cancerogenic effect on the skin

One important aspect of the skin's physical cover is the protection it provides against UVR. There are three types of UVR, divided according to the wavelength of the radiation: UVA (320–400 nm), UVB (290–320 nm), and UVC (200–290 nm). UVC is mainly absorbed by the atmosphere, and unlike UVA and UVB does not reach the surface of the earth. UVR, especially UVB, is highly mutagenic, and is considered to be the major environmental factor contributing to skin cancer development (82). Both cSCC and BCC have a high mutagenic burden because of the high UVR exposure of the skin (69, 83).

The most common DNA damage found in skin cancers is due to two photoproducts of UVR: the cyclobutane pyrimidine dimer (CPD) and the pyrimidine (6-4) pyrimidone (64). DNA damage can also arise via indirect effects, particularly from oxidation and production of reactive oxygen species (ROS). ROS can damage not only the DNA but also lipids and proteins in the cell. UVA produces relatively more oxidation damage than photoproducts when compared to UVB. Still, UVA produce 3–6 times more CPD than 8-oxo-guanine (oxidation of guanine) (84). The CPD gives the DNA an altered structure with helix distortions, which is repaired by the nucleotide excision repair complex. If all the reparation systems fail to repair the DNA and too much damage remains, the cell activates apoptosis. Often the mutations make no difference to the cell, but sometimes the mutation occurs in certain key genes such as the p53 gene, contributing to cancer formation (64).

Inducing angiogenesis and avoiding immune destruction: the importance of the tumour microenvironment

A growing body of evidence has shown the important of the tumour microenvironment for the tumour's sustained survival (38). The tumours often live under harsh circumstances involving energy and oxygen deprivation, due to a rapid growth and a vasculature that is not sufficiently developed. Thus, in many tumours there is a “angiogenic switch” where normally quiescent vasculature starts to sprout new vessels. A well-known mediator of angiogenesis is vascular endothelial growth factor (VEGF). This gene is important during the embryologic and postnatal development, as well as during physiological and pathological situations in adults. The VEGF gene can be upregulated both by hypoxia and oncogenic signalling. In cSCC there is a higher expression of VEGF than in BCC, and cSCC tumours show a higher microvascular density than BCC tumours (85). Higher microvascular density has been associated with more aggressive growth pattern (85, 86). The patterns of the changed vasculature seen in dermoscopy can help in diagnosing both BCC and cSCC (87).

The microenvironment around the tumour is important for many reasons. Both the ability for invasive growth and metastasis and the

paracrine signalling of growth factors and evasion of immune destruction involve the tumour stroma. Inflammatory cells are often surround the neoplasm, and are sometimes found inside it. They can act both in a tumour-antagonising and a tumour-promoting way (38). An example of a tumour-promoting action is the effect of the tryptophan catabolic enzymes indoleamine 2,3-dioxygenase 1 (IDO1) and tryptophan 2,3-dioxygenase 2 (TDO2). These enzymes catalyse the rate-limiting step of the kynurenine pathway, where the essential amino acid tryptophan converts to biologically active secondary metabolites contributing to an immunosuppressive tumour microenvironment (88). Thus, inhibitors against IDO1 have been under development against malignant melanoma with the aim of enhancing the immunoreaction against the tumours (89).

Modulation of the immune system does not only take place under influence of the tumour; in addition, UVR induces an immunosuppression in the irradiated skin that is both local and also potentially systemic (64). The immunosuppressive effect involves many molecular and cellular events, and can involve suppressing both primary activation of immunity and reactivation of memory immunity. UVR immunosuppression in the skin could also have a negative impact on the immune rejection of skin cancer cells (90).

Deregulating cellular energetics: the changed tumour metabolism

Warburg was the first to observe a change in the energy metabolism of cancer cells, noting that even in the presence of oxygen, the cancer cells reprogrammed their metabolism to favour glycolysis before oxidative phosphorylation in the mitochondria (91, 92). To compensate for the less-efficient glycolysis, the cancer cells upregulate their glucose transporters. Glycolytic fuelling has been associated with activated oncogenes such as RAS, MYC, and the mutant tumour suppressor p53 (93, 94). Increased glycolysis also facilitates a diversion of glycolytic intermediates into different biosynthetic pathways; for example, biosynthesis of new macromolecules and organelles required for active cell proliferation (38). In BCC, there is growing evidence that links the Hh cell signalling pathway to the regulation of the cell metabolism (95). It has, for example, been shown that cells treated with a Smoothed agonist undergo a Warburg-like metabolic reprogramming.

The mitochondria and cancer

The main function of the mitochondria in the cell is energy production through the tricarboxylic acid cycle. In addition, many of the metabolites from this cycle are building blocks for nucleotides, amino acids, lipids, and haem. The mitochondria are involved in regulating cell proliferation, and are suggested to have a key regulatory role for the cell cycle in the G1 to S transition, where energy depletion activates a cell cycle checkpoint (96, 97). In addition, the mitochondria are important for mediating programmed cell death, and participate in several cell signalling pathways (98).

When the Warburg effect was first discovered, it was assumed that the mitochondria in the cancer cells were dysfunctional. It later became evident that these mitochondria have important parts to play, for example in providing building blocks for the cancer cell (99). Studies have shown that cancer cells lacking mitochondrial DNA have significantly slower growth and lower tumorigenic potential. Many tumour suppression genes and oncogenes, for example MYC oncogene, alter

the mitochondrial metabolism. The mitochondrion is now being evaluated as a new target for different anti-tumour drugs. Knowledge of mitochondrial dysfunction in keratinocyte cancer is limited, but one study showed that mutations in the mitochondrial DNA are more common in BCC than in cSCC (100).

Reactive oxygen species and cancer

ROS is a normal by-product of oxygen metabolism in the mitochondria (101), but the mitochondrion is also sensitive to reactive oxygen produced by UVA exposure (102, 103). Cancer cells often exhibit high oxidative stress through both intrinsic and extrinsic pathways (104, 105). The presence of ROS has sometimes been described as a two-edged sword. At low to moderate levels, ROS can act as signal transducers to activate cell proliferation, angiogenesis, migration, and invasion. However, high levels of ROS can damage the nucleic acid, membranes and lipids, and lead to cell death. Antioxidants have been shown both to increase the development of cancer metastasis in a mouse model of malignant melanoma (106), and to suppress the metastatic potential in highly metastatic cancer in mice (107). In addition, in one study on mouse skin, antioxidants had a potential to reduce UVR induced cSCC (108).

As skin cells are especially exposed to ROS due to their exposure to UVR, it is important for the cells in the skin to have all the major antioxidant enzymes present to protect them. Skin cells also have a mitochondrial-sited antioxidant with adaptive features, manganese-superoxide dismutase, that is upregulated after repeated UVA exposure (109). Another part of the antioxidant defence system, glutathione, has been shown to be depleted in cultured human skin cells by UVA and UVB radiation, rendering them highly sensitive to mutations and cell death (110). Hereditary differences in enzyme activity have been found for the antioxidant glutathione-S-transferase (GST), which might explain some of the heredity of individual skin sensitivity to UVR (110, 111). This association between antioxidant levels and skin cancer has been detected in BCC patients, where different genetic variants of GST have been associated with higher incidence of both sporadic BCC and BCC in Gorlin's syndrome patients (112, 113).

SLC25A43 expression in cancer

Solute carrier (SLC) family 25 member 43 (SLC25A43) is a transport protein in the inner membrane of the mitochondrion (Figure 4). The main functions of SLC family 25 are to transport molecules such as adenosine diphosphate (ADP), adenosine triphosphate (ATP), citrate, coenzyme A (CoA), and amino acids between the cytosol and the mitochondrial matrix. Dysfunction in SLC25 transport proteins has been related to several diseases involving mitochondrial and cellular metabolism, affecting mainly myopathy, encephalopathy, and neuropathy (114). The SLC25 transporters which are phylogenetically closely related to SLC25A43, transport ADP or CoA, but the substrate for SLC25A43 is still unknown (115). The gene is located at Xq24.

Studies on human EGF receptor (HER) 2-positive breast cancer have shown deletion and altered methylation pattern of SLC25A43 in breast cancer tumours, but also loss of heterozygosity in cervix tumours and adenocarcinoma in the lung (116, 117). SLC25A43 protein expression is related to the S-phase fraction of the cell, which could indicate a role for SLC25A43 in cell cycle progression. This hypothesis is strengthened by an *in vitro* study with gene silencing of SLC25A43 that affected the cell cycle progression and cell proliferation rate (118). In normal breast epithelial cells, with normal mitochondrial oxidative phosphorylation, the proliferation rate was low. In contrast, in HER2-positive breast cancer cells with dysregulated mitochondrial function, the SLC25A43 knockdown caused higher proliferation. Knockdown of SLC25A43 has also been shown to decrease the efficacy of paclitaxel in HER-positive and HER-negative breast cancer cell lines, indicating that SLC25A43 could have a role in drug resistance (119). A study published in 2022 with knockdown experiments of SLC25A43 in a mouse haploid embryonic stem cell line, showed enhanced viability and preserved mitochondrial function in a high-ROS cell environment in the cells with knockdown SLC25A43 (120). The knockdown of SLC25A43 protected the cells from ROS-mediated cell death, which suggests that SLC25A43 participates in the redox regulation in the cell.

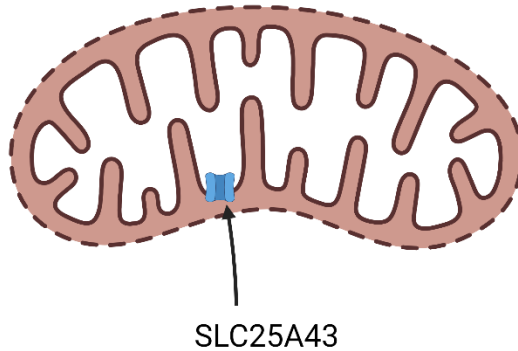


Figure 4 The mitochondrial transporter SCL25A43 is located in the inner mitochondrial membrane but the substrate of transport is still unknown. Image created in BioRender by the present author. <https://BioRender.com/h78z758>

Cell membrane transporters

Glucose and amino acid transporters in cancer

The enhanced proliferation in cancer cells demands a high intake of glucose and amino acids to serve as a source of energy and as building blocks (38, 94). Cancer cells also need to resist fluctuations in oxygen and nutrients, as the vasculature is often insufficient in parts of the growing tumour mass. Many of the currently-known tumour suppressors, play critical roles in suppressing growth under hypoxic conditions and when essential metabolites are reduced (94). An often-over-expressed glucose transporter in cancer is glucose transporter 1 (GLUT1) (121), which can be used in tumour detection during positron emission tomography (PET) examinations. The main method is ^{18}F -deoxyglucose PET, which takes advantage of the enhanced glucose uptake in tumours (122). A new and promising method instead uses L-3- ^{18}F -fluoro- α -methyl tyrosine PET to look for the increased uptake of amino acids using the elevated large neutral amino acid transporter small subunit 1 (LAT1) expression in the tumour cells (123, 124).

Amino acids play an important part in many cell activities, such as the redox balance, energetic regulation, biosynthetic support, and homeostatic maintenance. It is therefore important for the cancer cell to regulate the amount of amino acids and especially essential amino acids through the amino acid transporters in the cell membrane (125). Amino acid uptake is regulated by membrane-bound transport proteins that are important in transporting proteins both through the cell membrane and between different cell compartments. The human genome encodes for 66 different amino acid transporters belonging to 11 individual SLC families. Many amino acid transporters are uncharacterised, but the SLC7 family is one of the most well-studied (126).

LAT expression in cancer

Three important members of the SLC7 family of transporters are SLC7A5, which encodes the protein LAT1, SLC7A8, which encodes the protein LAT2, and SLC7A7, which encodes γ -LAT1 (127). LAT1, LAT2 and γ -LAT1 all separately form a complex with 4F2 cell-surface antigen heavy chain (4F2hc; the gene also known as SLC3A2), making them more stable and mediating the translocation to the cell membrane (128, 129). In the context of cancer initiation and progression, LAT1 is more well-studied than LAT2 or γ -LAT1 (130).

An overview of LAT1 is given in Figure 5. It is a sodium independent transporter that mediates uptake of large neutral amino acids such as phenylalanine, tyrosine, leucine, histidine, tryptophan, valine, methionine, and isoleucine. The amino acid uptake is coupled to the export of another amino acid, such as histidine, glutamine, or tyrosine. LAT1 can also transport amino acid derivatives including L-3,4-dihydroxyphenylalanine (L-DOPA), thyroid hormones, and drugs such as gabapentin (126). It is important not only in the cell membrane but also in the lysosome, where it is involved in the activation of mammalian (or mechanistic) target of rapamycin complex 1 (mTORC1) (131). In the cell, mTORC1 plays a vital role in regulating cell growth and metabolism. Studies have shown that inhibition or genetic deletion of LAT1 decreased the mTORC1 activity, while conversely mTORC1 upregulates LAT1 (126).

Amino acid depletion in the cell is one way to activate the integrated stress response, which is a conserved way for the cell to react to different threatening stresses. On amino acid depletion, a chain of reactions occur leading to translation of the transcript factor ATF4. ATF4 binds to its target genes, for example LAT1, mediating the cell to overcome the amino acid starvation.

LAT1 is upregulated in many different cancer forms, and has been associated with shorter patient survival (132). Upregulation of LAT1 could be the result of oncogenic alterations secondary to deregulated signalling pathways such as Hippo and MYC, or might stem from adaptive pathways in oxygen- and nutrient-deprived microenvironment (133). An example of oncogenic alterations is when c-MYC, which binds to the promoter of LAT1, leads to increased expression of LAT1 in cancer cells (134). LAT1-mediated uptake of essential amino acids then stimulates MYC mRNA translation, forming a feed-forward loop. Another regulation of LAT1 is when HIF2 α , a protein important in hypoxia, binds the LAT1 proximal promoter inducing its expression (135). This mediates mTORC1 activity even in hypoxia and could be an important requirement for sustained proliferation under harsh conditions.

Different avenues for cancer treatments have been proposed with the aim of taking advantage of the LAT1 upregulation in cancer cells. The main track has been to inhibit LAT1 in order to starve the cancer cells. However, there have also been attempts to deliver anti-tumour agents through LAT1 (136), using the specificity of high LAT1 upregulation in the cancer tissue, but not in the surrounding cells, for more targeted drug delivery. LAT1 protein expression in the skin was examined in a small study in 2014 (137). Both BCC (n=11) and cSCC (n=9) tumours had a significantly increased LAT1 expression compared to the normal epidermis, but it is still not known what role or function this transporter has in keratinocyte cancers.

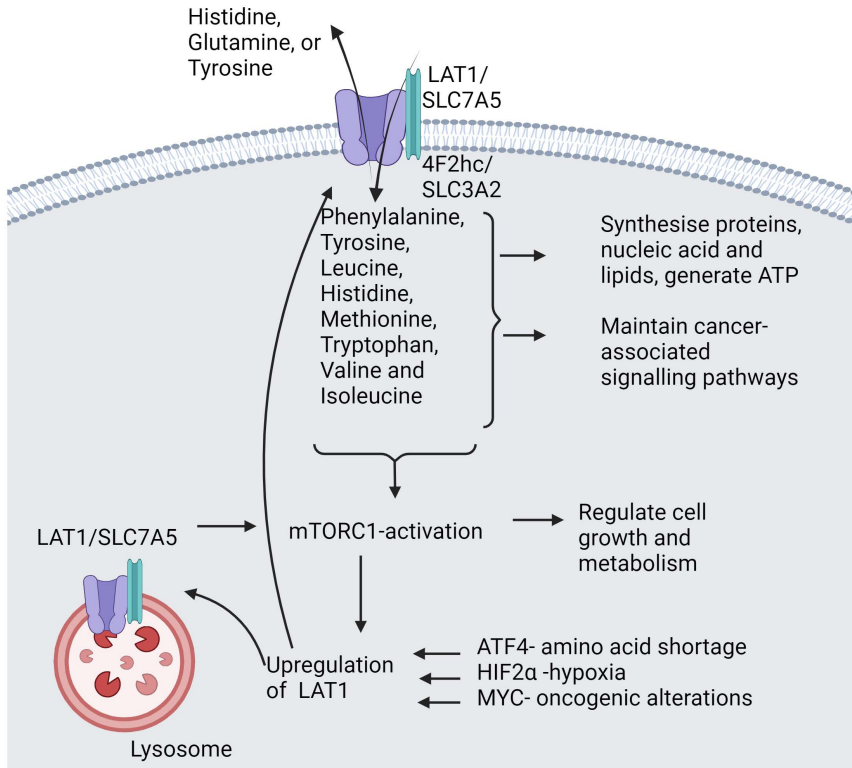


Figure 5 Effects of LAT1 expression in cancer cells. A schematic figure of LAT1 expressed in the cell membrane together with the 4F2hc/SLC3A2 protein, mediating uptake of large neutral amino acids such as leucine and tryptophan. The amino acid intake is coupled to export of the amino acids histidine, glutamine, or tyrosine. The amino acids are important for the synthesis of proteins, nucleic acid and lipids. LAT1 is also expressed in the lysosome, mediating the mTORC1 activation that is important for cell growth and metabolism. Image created in BioRender by the present author. <https://BioRender.com/x36i368>

Keratinocyte cancer treatment

Standard keratinocyte cancer treatment in Sweden

BCC and cSCC are often treated with excision. Other treatment options for BCCs include curettage and cryotherapy, curettage and electrodesiccation, photodynamic therapy, radiotherapy, CO₂ laser, and, for the superficial subtype, imiquimod or 5-fluorouracil ointment (45). Since BCC and cSCC are often located in exposed skin areas such as the face, scars from the treatment can be cosmetically disturbing, and for some people may cause substantial morbidity. BCC and cSCC that are locally advanced with limited possibilities for surgery, or that have metastasised, are indicated for systemic treatments.

The first drug approved in the European Union for BCC treatment targeting the Hh-signalling pathway was vismodegib, in the year 2013, and this was later joined by sonidegib (138, 139). The first immune-checkpoint inhibitor for locally advanced or metastasised cSCC or BCC that has relapsed after Hh inhibitors is the PD1 inhibitor cemiplimab (140, 141). There is still a need for new treatment modalities that are both simple to manage, in order to meet the needs of a growing group of patients, and effective, so no follow-ups are needed. There is also a need for more effective treatment for the smaller group of locally advanced and metastatic tumours.

A possible new drug target: inhibitors of LAT1

There are several inhibitors against LAT1. The most common is 2-Aminobicyclo-(2,2,1)-heptane-2-carboxylic acid, which is a classical system L inhibitor that inhibits both LAT1 and LAT2, and JPH203 (KYT-0353, nanvuranlat) a selective LAT1 inhibitor that was structurally developed from the thyroid hormone triiodothyronine (T3) (142).

JPH203 has been tested in a small clinical phase I trial on patients with gastrointestinal cancers or breast cancer as well as a phase II study that showed promising results, especially for biliary tract cancer (143, 144). The underlying mechanism is not totally clear, but it is hypothesised that the inhibition of LAT1 activates the amino acid stress

response and suppresses the mTORC1 signalling. Another hypothesis is that JPH203 treatment inactivates cell-cycle-related kinases and proteins, thereby inducing cell cycle arrest (126).

LAT1 is important in normal tissue, such as in the blood-brain barrier and for the immune cells. It is therefore important to pay special attention to the brain and immune system when evaluating potential side-effects of LAT1 inhibition treatment. LAT1 inhibitors have not previously been evaluated in BCC or cSCC. Keratinocyte cancers are easy to reach with a percutaneous drug formula, and their high and increasing incidence mean that new and innovative treatments are needed. It is therefore of value to investigate the possibilities of new drug targets as LAT1 inhibition in keratinocyte cancer.

Aim of the thesis

This thesis aimed to investigate metabolic biomarkers in keratinocyte carcinoma, with a focus on BCC, to find potential treatment targets.

The specific aims of each study were:

Study I: To determine the gene and protein expression of the mitochondrial transport protein SLC25A43 in BCC compared with that in non-tumoural skin in the same individuals, in both men and women.

Study II: To identify metabolism-related factors that may serve as potential future targets for inhibitor drugs in BCC.

Study III: To investigate LAT1 and its colocalisation with LAT2 in BCC, using fluorescence microscopy, and to describe the tumour expression pattern of LAT1 in relation to cell proliferation, apoptosis, and hypoxia.

Study IV: To examine LAT1, GLUT1, and GLI1 protein expression in BCC and cSCC. Additionally, to explore the effects of LAT1 inhibition with JPH203 on a BCC cell line (TE354.T), a cSCC cell line (A431), and a keratinocyte cell line (HEK001), focusing on cell viability and changes in gene expression related to metabolism and carcinogenesis.

Material

Patients

The patient cohort comprised 59 individuals, treated at the Department of Dermatology, Örebro University Hospital, Sweden. Their characteristics are described in Table 1. The inclusion criteria were: 1) the patient had a skin tumour suspected to be BCC or cSCC, 2) the tumour was planned for excision, 3) the tumour diameter was >6 mm, and 4) the patient was able to understand the information leaflet and give their written informed consent. Exclusion criteria were: 1) recurrent tumour, 2) a punch biopsy had already been performed, resulting in too much scarring for another punch biopsy to be taken and 3) the patient had a contagious disease or serious substance abuse.

After removal of the tumour, punch biopsies were taken from the middle of the tumour and from the patient's gluteal skin. If the tumour had a diameter >8 mm, a 4 mm punch biopsy was taken, and in other case a 3 mm punch biopsy was chosen. The excised tumours were formalin fixed for routine pathological-anatomical diagnose.

Table 1 Clinical characteristics of the patients.

Characteristics	
Sex, n (%)	
Male	39 (66)
Female	20 (34)
Age in years, median (range)	74 (54–98)
Tumour, n (%)	59 (100)
Basal cell carcinoma	
Low aggressive growth	54 (91)
High aggressive growth	1 (2)
Squamous cell carcinoma	
Squamous cell carcinoma in situ	1 (2)
Invasive squamous cell carcinoma	3 (5)
Tumour size in mm, median (range)	10 (6–29)
Tumour localisation, n (%)	
Head/neck	31 (53)
Trunk/back	23 (39)
Extremities	5 (8)

Study cohort in Studies I, II, and III

Studies I–III used formalin-fixed and paraffin-embedded (FFPE) BCC tumour samples from the first 7 female and first 7 male patients. Studies I and II also analysed fresh frozen tissue samples from the same patients and their normal gluteal skin. For Studies II and III, skin from two anonymised donors was collected and used as controls in method optimisation.

Study cohort in Study IV

Study IV investigated tumour FFPE samples from 57 patients. Two patients from the total cohort were excluded due to lack of sufficient tumour material.

Cell lines

In Study IV, *in vitro* experiments were performed on the three cell lines specified below. All the cell lines were obtained from American Type Culture Collection (ATCC), Manassas, VA, USA and were cultured according to the manufacturer's instructions at 37 °C in an environment with 5% CO₂ in a humidified incubator.

TE354.T

The TE354.T cell line (CRL-7762™) comprises fibroblast-like adherent growing cells from a female human BCC. This cell line has slow growing properties.

HEK001

The HEK001 cell line (CRL-2404™) comprises non-neoplastic epidermal basal cells. The cells were obtained from male scalp epidermal cells, and transfected with plasmid p1321 containing human papilloma virus 16 E6 and E7 genes. The cells express keratin 14 but not keratin 10, and they show a phenotype resembling proliferative basal epidermal cells.

A431

The A431 cell line (CTL-1555™), a cSCC-like cell line, was originally derived from a female patient's epidermoid cancer.

MCF7

The MCF7 cell line (HTB-22™) derives from a breast adenocarcinoma. This cell line has a known LAT1 expression (145, 146), and was used as a positive control for immunofluorescence (IF) staining in Study IV.

Basic culturing conditions

The cells were cultured in 37 °C, mainly in 75cm² cell culture flasks, in an incubator holding 5% CO₂. The medium for the TE354.T and A431 cells comprised Dulbecco's Modified Eagle Medium (DMEM) with 10% foetal bovine serum (FBS), 1% penicillin streptomycin, and 2.5% HEPES. The HEK001 cells were grown in Keratinocyte Serum-

Free Medium with 1% penicillin streptomycin. All handling of the cells, such as subculturing, thawing and cryopreservation, was performed according to the recommendations of the manufacture (ATCC).

Ethical considerations

According to the Helsinki Declaration approved by the World Medical Association in 1964, all research should follow the ethical rules stated in the declaration. One of the fundamental principles is the informed consent of patients. All patients included in Study I-IV gave both written and oral informed consent, and the research was approved by an ethics committee in line with the Act Concerning the Ethical Review of Research Involving Humans (2003:460).

The studies included in this thesis used tissue from tumours and matched gluteal skin from patients treated at Örebro University Hospital. These studies were approved by the local ethical Committee in Uppsala, Sweden (Uppsala/Örebro approval no.2011/242, with amendment 2011/242/1 from 2015 and 2011/242/2 from 2017). The extra procedure affecting the patients in these studies was an extra punch biopsy from the normal skin of the gluteal area.

A punch biopsy is always taken after the area has been cleaned and treated with injection of local anaesthetics, and is painless aside from the sensation of the needle in the syringe and the injection of local anaesthesia. It leaves a small wound that will heal in 2–4 weeks, sometimes with an almost invisible scar. Complications are rare but could include, a small amount of bleeding, or a wound infection that results in delayed healing. The punch biopsy taken from the tumour could not affect the patient, because it was taken after the removal of the tumour, and the tumour would have still been excised even if the patient had not been included in the study. The only risk of this procedure was that the pathologist might not have been able to make a complete examination if a punch biopsy had been taken in the middle of the tissue. To minimise this risk, we only included tumours that were at least 6 mm in diameter.

All patient information and tissues were handled in anonymised format.

Research methods

Methods for analysing gene expressions

Tissue preparation and isolation of nucleic acids

The punch biopsies were handled with one of three different techniques, due to evolving possibilities for quick freezing: snap frozen using dry ice with isopropanol (n=2), placed in Allprotect Tissue Reagent (Qiagen GmbH, Hilden, Germany) at room temperature (n=12), or snap frozen in liquid nitrogen (n=45). All the tissue material was then transferred to -80 °C for long term storage. At the time of RNA extraction, the punch biopsies were homogenised using TissueLyser II (Qiagen GmbH). In Study I, RNA extraction was performed using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen GmbH) according to the manufacturer's protocol. In Study II, the microarray analysis required high quality RNA, and therefore RNA was extracted using RNeasy Microarray Tissue Mini Kit (Qiagen GmbH) according to the manufacturer's instructions. In Study IV, RNA was extracted from the cell lines using RNeasy Plus Mini Kit (Qiagen GmbH) according to the manufacturer's instruction. RNA concentrations and purity of the samples were measured through absorbance ratios (260/280 nm and 260/230 nm) using the NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Thermo Fisher Scientific, Wilmington, DE, USA).

Quantitative real-time polymerase chain reaction (qPCR)

In Studies I, II, and IV, qPCR was used to examine target gene expression levels in comparison to the following reference genes; actin beta (ACTB) and ABL proto-oncogene 1, non-receptor tyrosine kinase (ABL1) in Study I; ACTB, ABL1 and Topo 1 in Study II, and cytochrome c1 (CYC1), ABL1, and Topo 1 in Study IV. RNA was transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Thermo Fisher Scientific Inc, Wilmington, DE, USA) in Studies I and IV, while in Study II the Superscript IV First Strand Synthesis System (Invitrogen, Thermo Fisher Scientific) was used according to the manufacturer's protocol. Expression of the

target and reference genes was measured using the TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA, USA) and Fast Advanced Master Mix (Applied Biosystems).

The plates were run using the 7900HT Fast Real-time PCR System (Applied Biosystems) in Studies I and II, and QuantStudio 7 (Applied Biosystems) in Study IV. The qPCR was first performed at 50 °C for 2 min and then at 95 °C for 20 sec, followed by 40–50 cycles of 95 °C for 1 sec and 60 °C for 20 sec. All the reactions were set in duplicates, and the quantification cycle (Cq) was set automatically. The target genes were normalised against the mean values of the reference genes, and the fold change gene expression (treated vs non-treated cells or tumour cells vs. non tumour cells) was calculated using the $2^{-\Delta\Delta Cq}$ method (147).

Microarray analysis

Study II used a microarray technique to simultaneously analyse differences in gene expressions between tumour tissue and gluteal skin samples. This technology is based on a microarray chip containing probes on its surface that can bind to the target RNA. The chip used in Study II was obtained from Agilent Technologies, contained 8 arrays each with 60 000 spotted probes, and was used according to the manufacturer's instructions.

In brief, RNA from tissue samples was labelled with a cyanine dye (Cy 3) using the one-color Low Input Quick Amp Labeling Kit (Agilent Technologies) and amplified to receive cRNA. Next, the cRNA was purified using the RNeasy Mini Kit (Qiagen GmbH). The cRNA samples were then hybridised to the microarray slides (SurePrint G3 Human Gene Expression 8x60K v2 Microarrays, Agilent Technologies) for 17 h at 65 °C. For fluorescence intensity measurement, a G2565CA Microarray scanner (Agilent Technologies) was used, followed by data analysis with version 10.7.3.1 of Agilent Feature Extraction Software (Agilent Technologies).

Methods for analysing protein expressions

Immunohistochemistry

In Study I, II, and IV, immunohistochemistry (IHC) was used to detect the amount and localisation of protein expressions. The FFPE tissue was sliced into 4 µm sections and placed on glass slides. Deparaffinisation and rehydration were performed, and the epitopes were recovered by heat induced antigen retrieval. The tissue was incubated with a primary and secondary antibody (Table 2). To allow visualisation, the secondary antibody was conjugated with horseradish peroxidase, which together with hydrogen peroxide oxidises 3,3'-diaminobenzidine into a brown pigment. The glass slides were finally counterstained with haematoxylin. To evaluate the antibodies and staining procedures, tissue from the liver, gallbladder, lung, gastrointestinal tract, placenta, thyroid gland, kidney, and normal skin was used. The IHC results were evaluated using a light microscope in Study I and with a digital scanner and the associated software in Studies II–IV. The individual slides were scored by two independent observers. The staining was manually evaluated according to the strength and amount of staining, and was summed up in a H-score (148, 149).

Immunofluorescence staining

Studies III and IV used IF staining to assess colocalisation of protein expression in tumour samples. Sections (4µm) of FFPE tissue from selected cases (n=14 in Study III, n=5 in Study IV) was stained using almost the same procedure as for the IHC, except for the last steps where the secondary antibody was added. In the IF technique, the primary antibody is chosen to target the protein of interest, and the secondary antibody is coupled to a fluorescent that emits light upon excitation via light of a shorter wavelength (150). The secondary antibodies used in Studies III and IV were AffiniPure anti-mouse for the mouse antibodies and AffiniPure anti-rabbit for the rabbit antibodies (Table 3).

The cell lines in Study IV were stained as follows. First, the cells were seeded into Permanox Chamber Slides (Thermo Fisher Scientific)

incubated for 48 h. They were then fixed and permeabilised using the Image-iT® Fixation/Permeabilization kit (Thermo Fisher) according to the manufacturer's instructions. The primary antibody was added, followed by a fluorescent secondary antibody; nuclei were stained with DAPI and F-actin with ActinGreen™ 488 Ready-Probes™ Reagent (Alexa Fluor™488 phalloidin, Invitrogen, Carlsbad, CA, USA).

Antibodies

Table 2 Primary antibodies used in immunofluorescence staining of the selected proteins.

Protein	Cat. no.	Host species and clonality	Distributor	Dilution	Incubation time
SLC25A43	HPA 035188	Rabbit polyclonal	Sigma-Aldrich, Merck KGaA, Darmstadt, Germany	1:175	½h
LAT1 (SLC7A5)	Ab208776	Rabbit monoclonal	Abcam, Cambridge, UK	1:500	1h
LAT2 (SLC7A8)	UM500058	Mouse monoclonal	OriGene, Rockville, MD, USA	1:100	½h
SLC7A7	HPA036227	Rabbit polyclonal	Sigma-Aldrich, St Louis, MO, USA	1:135	½h
TD02	PA5-42759	Rabbit polyclonal	Thermo Fisher, Waltham, MA, USA	1:500	½h
Ki-67	Clone MIB-1 M7240	Mouse monoclonal	Dako Denmark A/S, Glostrup, Denmark	1:100	½h
Topo II α	Ki-S1, MA5-12433	Mouse monoclonal	Thermo Fisher Scientific, Rockford, USA	1:50	1h
Cleaved caspase-3	Ab2302	Rabbit polyclonal	Abcam, Cambridge, UK	1:200	1h
HIF1 α	Ab8366	Mouse monoclonal	Abcam, Cambridge, UK	1:750	1h
GLUT1	IHC404	Mouse IgG2a monoclonal	Cell Signaling Technology, Danvers, MA, USA	1:1000	1 h
GLI1	JF09-08	Rabbit monoclonal	Invitrogen, Rockford, IL, USA	1:100	½h

Table 3 AffiniPure secondary antibodies used for detection of primary antibodies. Dye-swap experiments were used to check double-labelling of primary antibodies from the same species.

	Conjugate	Cat. no.	Host species and clonality	Dilution
<i>(AffiniPure) Anti-mouse</i>	Cy TM 5	715-175-151	Donkey IgG (H+L)	1:400
	Alexa Fluor [®] 488	715-547-003	Fab fragment donkey IgG (H+L)	1:100
<i>(AffiniPure) Anti-rabbit</i>	Cy TM 3	711-165-152	Donkey IgG (H+L)	1:400
	Alexa Fluor [®] 488 1:400	711-547-003	Fab fragment donkey IgG (H+L)	1:100

Note: All antibodies were obtained from Jackson ImmunoResearch Europe Ltd, Cambridge, UK.

Drug exposure

In order to test the viability of the TE354.T, A431 and HEK001 cells after 48 h and 96 h incubation with JPH203, the cells were seeded onto 96-well plates at 1×10^4 cells/well for the A431 and HEK001 cells and a lower concentration of 1×10^3 cells/well for the TE354.T-cells, due to slow growing cells and larger cell configuration. After 48 h, the normal growth medium was changed to a medium containing rising concentrations of JPH203 from 0.01 μM up to 100 μM and in one experiment up to 1000 μM JPH203. In the cell lines HEK001 and A431, inhibition by 5-fluorouracil at concentrations of 1 mg/ml, 100 $\mu\text{g}/\text{ml}$, and 10 $\mu\text{g}/\text{ml}$, both alone and in combination with JPH203 was also assessed.

After 48h, and for 2 plates after 96 h, the CellTiter-Blue Cell Viability Assay (Promega, Madison, WI, USA) was used. This assay detects the amount of resazurin reduced to fluorescent resorufin, indicating metabolically active viable cells. Fluorescence was measured at 579/584 nm using a Cytation 3 Cell Imaging Multi-Mode Reader (BioTek Instruments, Winooski, VT, USA) and analysed with version 3.05 of the Gen 5 software package (BioTek). The viability results were normalised to the growth medium and to the control cells with no JPH203, but with the addition of DMSO/ethanol (0.1%/0.9%) that was necessary to obtain a concentration of 100 μM JPH203.

Statistical data analysis

The statistical calculations were performed in versions 23.0 (Study I), 22.0 (Study II), and 29.0 (Study IV) of SPSS Statistics (IBM, Armond, N.Y, USA) and versions 6.0 (Study I), 7.03 (Studies II-III), and 8.2.1 (Study IV) of GraphPad Prism (GraphPad Software, La Jolla, C.A., USA). Statistical significance was set to <0.05 .

Study I: Differences in gene expression between tumour and gluteal skin were calculated with a paired sample t-test using normalised Cq values ($2^{-\Delta Cq}$). The Mann-Whitney U-test was used to evaluate difference in fold change and H-score between sexes. To calculate the difference in protein expression between adjacent epidermis and tumour, the Wilcoxon signed-rank test was used for associated samples.

Study II: The raw microarray data were normalised using the 75th percentile shift and baseline adjusted to the median prior to statistical analysis. The detected entities were filtered for normality with the Shapiro-Wilks test. Entities that were expressed differentially between tumour samples and gluteal skin samples were retrieved based on statistical significance using a moderated t-test with the Benjamini-Hochberg multiple testing correction method. The differentially expressed genes were further analysed through Gene Ontology term enrichment. The qPCR data were logarithmised and normality tested using the Shapiro-Wilks test. Differences in gene expression ($\log 2^{-\Delta Cq}$ values) between tumour and gluteal skin were calculated using the paired t-test. Spearman's correlation coefficient for nonparametric data was used as a measure of correlation between gene expression and the protein expression (H-score) for each patient's tumour tissue. The protein expression was also presented descriptively. A simple linear regression model was used to estimate R^2 as well as the regression linear equation.

Study III: The difference in staining was calculated using the Wilcoxon matched signed-rank test, Mann-Whitney U test and Kruskal-Wallis test with Dunn's correction. The correlation between the protein expressions was calculated using Spearman's correlation coefficient for nonparametric data and linear regression equation was

calculated. The expression pattern within the tumours was also presented in a descriptive way.

Study IV: The Mann-Whitney U test was used to compare the H-score of LAT1 with the H-scores of GLUT1 and GLI1. Spearman's correlation coefficient for nonparametric data was used to measure the correlation between the H-scores of different protein expressions. A linear regression equation was also calculated. The Kruska-Wallis test with Dunn's correction was used to evaluate the difference in viability between different concentrations of JPH203 and 5-FU, respectively. IC_{50} values were estimated by finding a straight line using the logarithm of the concentration values and then halving the maximal inhibitory concentration. To perform statistical analyses on the qPCR data, the logarithm of $2^{-\Delta Cq}$ was used. A small group of outliers (4.6%) was identified using the IQR-method of 3rd quantile + $1.5 \times$ interquartile range and 1st quantile - $1.5 \times$ interquartile range. After removal of the outliers, the Gaussian distribution was tested with the Shapiro-Wilks test and normality was assumed for $p > 0.05$. Differences in gene expression ($2^{-\Delta Cq}$) between treated and control cells were calculated using one-way ANOVA. The Sidak multiple comparison test was performed to correct for multiple testing. Fold change ($2^{-\Delta\Delta Cq}$) was calculated for all the examined genes.

Results

The mitochondrial transporter SLC25A43 in BCC (Study I)

When studying the mitochondrial transporter SLC25A43 in the BCC tumours, we found a significantly lower ($\geq 50\%$) gene expression of SLC25A43 in the tumour samples than in the patient's gluteal skin. There was no difference in gene expression between the sexes (Figure 6). In addition, the protein expression of SLC25A43 was absent in over 90% of the vision fields in the BCC and the H-score was significantly lower ($p < 0.05$) in the BCC than in the adjacent epidermis (Figure 7).

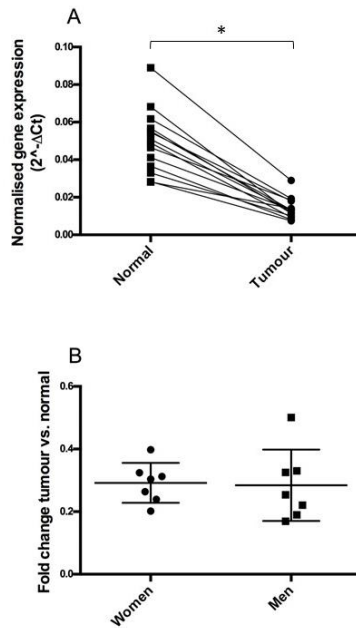


Figure 6 (A) The normalised gene expression of SLC25A43 was significantly lower in the basal cell carcinoma tissue than in the paired healthy gluteal skin $*p < 0.05$. (B) There was no difference in the SLC25A43 gene expression between women and men.

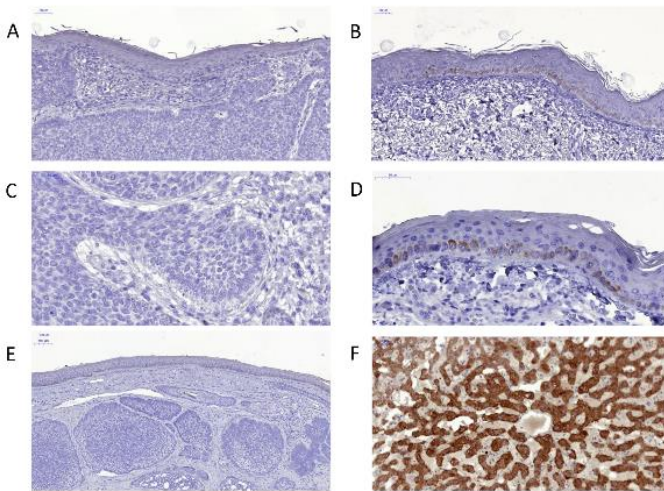


Figure 7 Immunohistochemical staining of solute carrier family 25 member 43. (A) Epidermis located directly above a basal cell carcinoma and (B) epidermis adjacent to the tumour exhibit different staining intensity (magnification $\times 20$). (C) Cells in a basal cell carcinoma and (D) cells in the adjacent epidermis (magnification $\times 40$). (E) Basal cell carcinoma (magnification $\times 10$) and (F) liver tissue, which served as a positive-staining control (magnification $\times 20$).

LAT1 and LAT2 upregulated in BCC tumours (Study II)

To explore new metabolism-related factors that may serve as future targets for inhibitor drugs we conducted a microarray analysis of the BCC tissue. This revealed increased expression of the amino acid transporters SLC7A5/LAT1, SLC7A8/LAT2, and SLC7A7/Y+LAT1 as well as the cytosolic enzyme TDO2, which is involved in tryptophan metabolism. The qPCR confirmed statistically significant higher amounts of SLC7A5 ($p < 0.001$), SLC7A8 ($p < 0.001$), and TDO2 ($p = 0.002$) in the tumour tissue but no difference was seen regarding SLC7A7 (Figure 8).

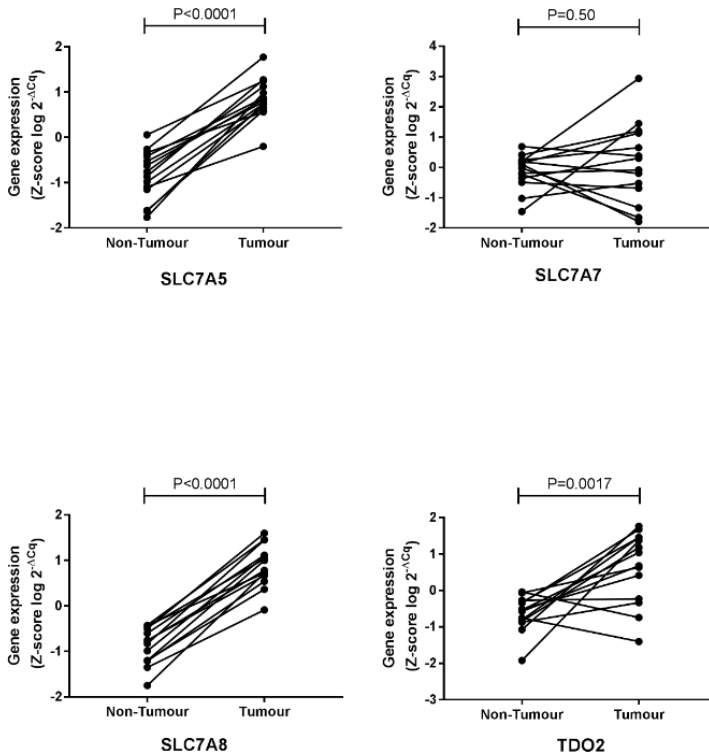


Figure 8 Gene expression of the amino acid transporters SLC7A5/LAT1, SLC7A7/Y+LAT1, and SLC7A8/LAT2 and the enzyme TDO2 in non-tumoural skin and basal cell carcinoma tumours, measured by quantitative real-time polymerase chain reaction.

Immunohistochemistry demonstrated correlating tumour cell protein expression of SLC7A5/LAT1 (Spearman $r=0.64$ $p=0.019$, linear regression $Y=0.0045 \times X - 0.37$) and SLC7A8/LAT2 (Spearman $r=0.74$, $p=0.004$ linear regression $Y=0.011 \times X + 0.002$) (Figure 9). The staining of SLC7A5/LAT1 was both membrane bound and cytoplasmic, with low staining in the peripheral palisading cells (Figure 10). The staining of SLC7A8/LAT2 was heterogeneous, with different degrees of staining (Figure 11). No correlation was found between gene and protein

expression for SLC7A7 and TDO2. The protein expression of SLC7A7 was low/negative in the BCC, but observed in the stratum granulosum of normal epidermis. The staining intensity of TDO2 was low in the tumour cells, while strong staining was observed in the stroma, sebaceous glands, normal epithelia, and the immune cells surrounding the tumour.

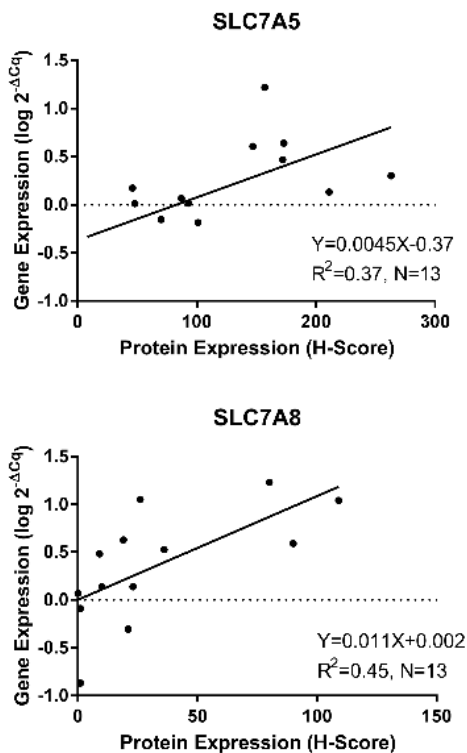


Figure 9 A statistically significant correlation was seen between the gene expression and the protein expression of SLC7A5/LAT1 ($p=0.019$) and SLC7A8/LAT2 ($p=0.004$).

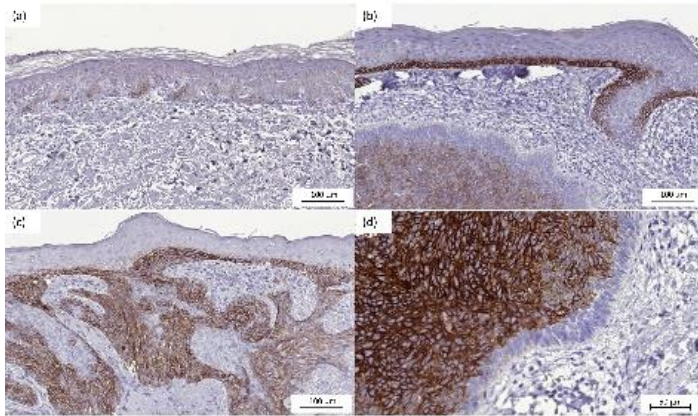


Figure 10 Immunohistochemistry staining of SLC7A5/LAT1 in basal cell carcinoma. (a) Adjacent to the tumour, the staining in the basal cell layer of the epidermis was weak or absent. (b) Directly above the tumour, the basal cell layer of the epidermis showed stronger staining of SLC7A5/LAT1. (c) The tumour showed both membrane bound and cytoplasmic staining. (d) The palisading tumour cells in the periphery of the tumour masses showed weak or no staining.

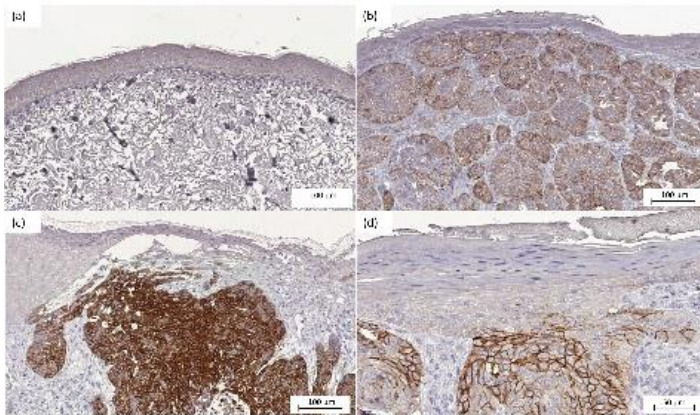


Figure 11 Immunohistochemistry staining of SLC7A8/LAT2 in basal cell carcinoma (BCC). (a) The epidermis adjacent to the tumour area showed weak or no staining (×20) (b) The BCC cells expressed SLC7A8/LAT1 heterogeneously, with different degrees of staining (×20). (c) Some tumours had areas with strong membrane and cytoplasmic staining of SLC7A8/LAT1 (×20), (d) Other tumour areas had mainly membrane staining (×40).

LAT1 expression and harsh tumour environment (Study III)

In Study III we further investigated the protein LAT1 with IHC and IF. First, we explored the colocalisation and correlation between the LAT1 and LAT2 expression. Both LAT1 and LAT2 were overexpressed, but we found no correlation between their protein expression and their colocalisation was low (Figure 12). The LAT1 cells were mainly expressed in the middle of the tumour masses, whereas the LAT2 cells showed a more scattered expression pattern.

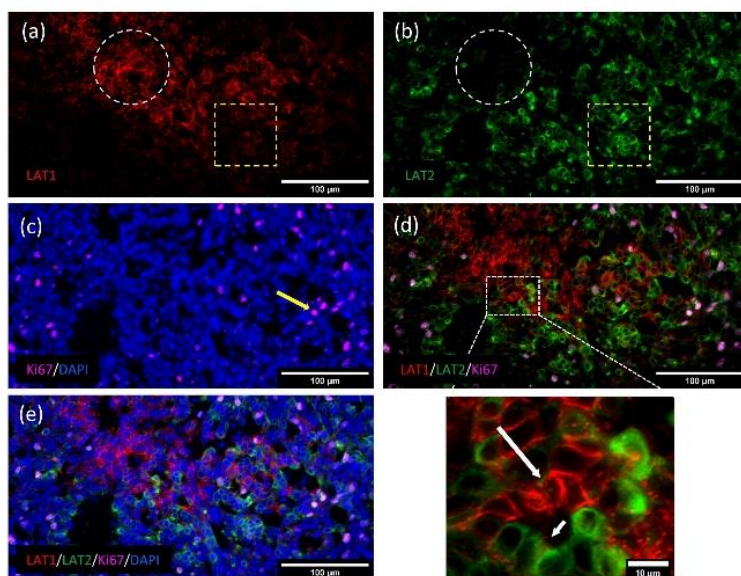


Figure 12 (a-c) Triple immunofluorescence staining of LAT1, LAT2, and Ki-67 in basal cell carcinoma. (a) The area in the white circle shows LAT1 expression whereas the area in the yellow square lacks LAT1 expression. (b) The corresponding areas are LAT2 negative in the white circle and LAT2 positive in the yellow square. (c) Cells with Ki-67 positive nuclei (yellow arrow) were more often LAT1 negative. (d) LAT1 and LAT2 were mainly not colocalised in the same cell. (e) Merged image with LAT1, LAT2, and Ki-67 expression when the cell nuclei were visualised with DAPI. Images were captured in CaseViewer at $\times 60$ magnification. Scale bars are 100 μm in the main images (a-e), and 10 μm in the magnified section of (d).

We compared LAT1 to the proliferation marker Ki-67 and also Topo II α , a proliferation marker which was upregulated in the microarray study. The expressions of both Ki-67 and Topo II α in the tumours were higher than in the surrounding epidermis ($p=0.01$ and $p<0.01$ respectively) (Figure 13a). The Topo II α positive cells in the BCCs were mostly expressed in the periphery, and the expression pattern was similar to Ki-67 (Figure 13b). As Ki-67 is a more commonly used proliferation marker in the clinic, we continued to compare the expression pattern of Ki-67 with that of LAT1. We found a significant negative correlation between the actively proliferating cells at the periphery of the tumours compared to the cells expressing LAT1 in the middle of the tumour masses (Spearman $r=-0.83$, $p<0.01$, linear regression: $Y=-0.35\times X+30.56$) (Figures 13c and 14).

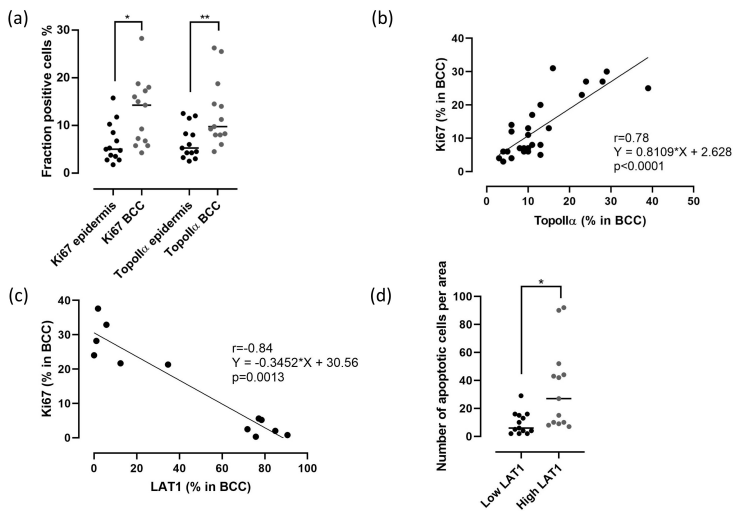


Figure 13 (a) Significant difference in Ki-67 and Topo II α expression between normal epidermis and basal cell carcinoma tissue (BCC). * $p<0.05$, ** $p<0.01$. (b) Correlation between Ki-67 and Topo II α expression in BCC tissue. (c) Negative correlation between the actively proliferating cells with Ki-67 expression and LAT1 expressing cells. (d) Significantly higher numbers of apoptotic cells in the areas of high LAT1 expression compared to the areas with low LAT1 expression. Horizontal bars indicate median number of cells. * $p<0.05$

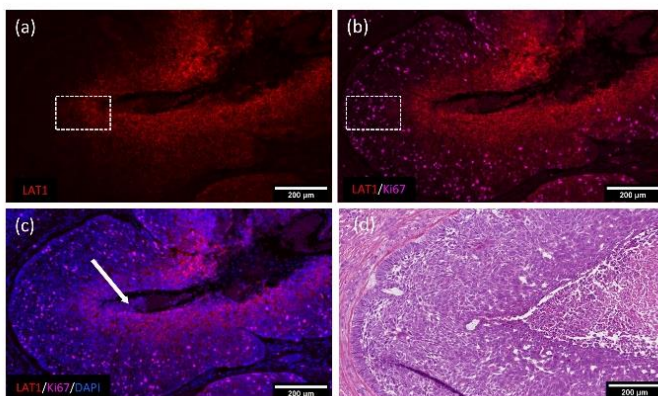


Figure 14 Double immunofluorescence staining of LAT1 and Ki-67 in BCC. (a) The white box shows increased LAT1 staining in the central parts of the tumour tissue. (b) Proliferating cells with Ki-67 staining were located in the peripheral area where LAT1 expression was low. (c) Merged image showing increased LAT1 protein expression in non-proliferating cells in the inner parts of the BCC. The white arrow points at the central part of the tumour with clear signs of necrosis. (d) BCC tumour with necrotic debris in the inner parts of the tumour mass (haematoxylin and eosin). Images captured with Case-Viewer software at $\times 60$ magnification and assembled with scalebars using Image J software. Scale bars in all images: 200 μm .

Since the LAT1 expression rose in the middle of the tumour masses, where there was often a cavity with necrotic cells and cell debris, we also stained the tumours for cleaved caspase-3, an important marker for apoptosis, and HIF1 α , a marker for lack of oxygen. We found that areas with high LAT1 expression also had a significantly higher fraction of cleaved caspase-3 expression ($p=0.02$) (Figure 13d), suggesting an association between LAT1 expression and harsh tumour environment. The HIF1 α staining was mainly cytoplasmic, and no tumour cells showed nuclear staining, suggesting normoxia.

Examination of LAT1 in the whole cohort (Study IV)

In Study IV we continued to examine the LAT1 expression in BCC, but we also included 4 cases of cSCC/cSCC in situ and examined the whole BCC cohort (n=53, excluding 2 cases for which insufficient material was available). We found a statistically significantly ($p<0.001$) higher protein expression of LAT1 in comparison to the expression of the glucose transporter GLUT1 and the oncogene GLI1. A sub-analysis of only the BCC tumours showed a statistically significant correlation ($p<0.01$) between LAT1 and GLUT1 expression (Figure 15). We found no correlation between either LAT1 and GLI1, or GLUT1 and GLI1.

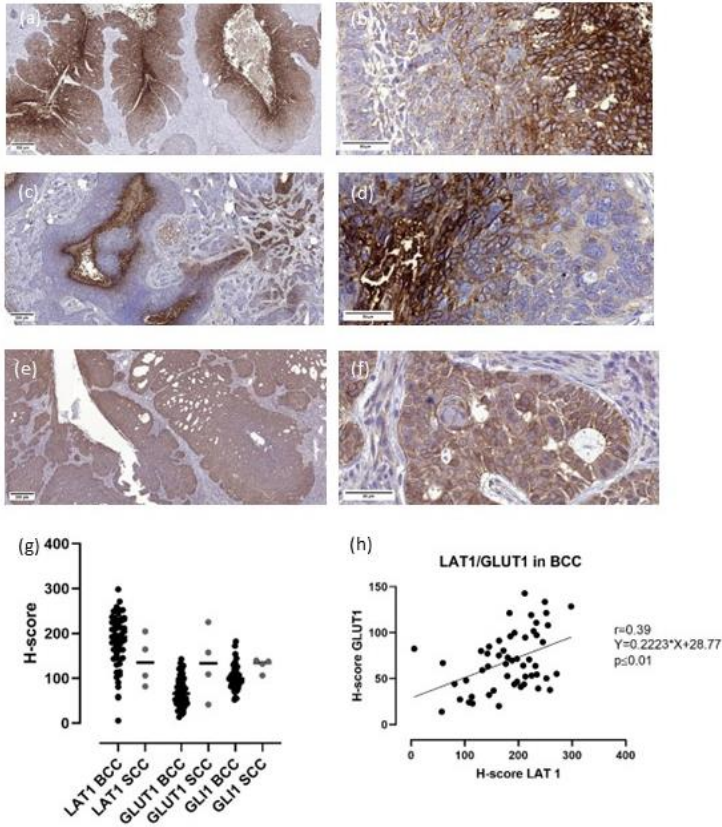


Figure 15 Protein expression of LAT1, GLUT1 and GLI1 in basal cell carcinoma (BCC) and cutaneous squamous cell carcinoma (cSCC). (a–b) Immunohistochemistry staining of LAT1 in BCC tissue showed increased staining in the centre parts of the tumour islands. (c–d) GLUT1 staining had a similar pattern to LAT1, with increased staining towards the inner centre of the tumour tissue in BCC. (e–f) GLI1 staining of the BCC tissue showed a homogeneous cytoplasmic staining pattern. (g) LAT1, GLUT1, and GLI1 H-score in the whole cohort including both BCC and cSCC tumours. (h) Statistically significant positive correlation was found between the H-score of LAT1 and GLUT1 in BCC tumours. Images were captured with version 2.7 of SlideViewer software package (3DHitech) and assembled with scale bars using ImageJ software. (a), (c), (e): $\times 5$ magnification and 200 μm scalebars. (b), (d), (f): $\times 40$ magnification and 50 μm scale bars.

Protein expression pattern of LAT1 and GLUT1 in BCC (Study IV)

The correlation of LAT1 and GLUT1 in BCC motivated a further exploration of whether the proteins were colocalised. Double staining of LAT1 and GLUT1 with IF in BCC tumours (n=5) revealed that even though the highest expression of both LAT1 and GLUT1 was in the centre of the tumours, often close to necrotic tissue, their colocalisation was low. The tumour cells with the highest expression of GLUT1 visually showed a lower expression of LAT1, and were closer to the necrotic centre of the tumours than the LAT1 positive cells.

LAT1 expression in the cell lines TE354.T, A431, and HEK001 (Study IV)

LAT1 expression in the BCC cell line TE354.T, the cSCC cell line A431, and the keratinocyte cell line HEK001 was investigated with IF, and found to be present in all three (Figure 16). In TE354.T, the LAT1 expression was only cytoplasmic, while the expression in A431 and HEK001, was both cell membrane bound and cytoplasmic.

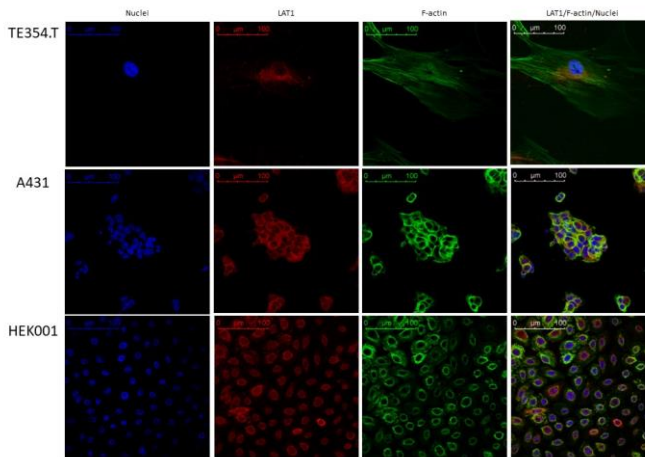


Figure 16 LAT1 in the basal cell carcinoma cell line TE354.T showed a cytoplasmic expression, while the cutaneous squamous cell carcinoma cell line A431 and the keratinocyte cell line HEK001 showed both cell membrane bound and cytoplasmic expression. Confocal images were captured using a TCS SP8 confocal microscope (Leica Microsystem GmbH). Scale bars indicate 100 µm.

Effects of the LAT1 inhibitor JPH203 in keratinocyte cancer in vitro (Study IV)

After exposure to the LAT1 inhibitor JPH203 for 48 h, cell viability in the TE354.T and A431 cells declined slowly at concentrations higher than 10 μM , but no significant decrease was seen, even with concentration of 100 μM (Figure 17a–b). The HEK001 cells showed more sensitivity to JPH203, with a statistically significant decrease in viability at 100 μM JPH203 compared to control cells ($p < 0.01$) (Figure 17c). The IC_{50} values, which correspond to concentrations at which 50% of the cells are no longer viable, were above the max concentration of 100 μM in the tests. However, after a new exposure, this time with 1000 μM JPH203, the median cell viability was 10% (range 7–15%) in A431 and 18% (range 6–19%) in HEK001 cells. It was not possible to show the decrease statistically, as the experiment was only performed once. Exposure to a combination of 10 μM JPH203 and 1 mg/ml 5-FU for 48 h did not show an additional effect on cell viability in the A431 and HEK001 cells compared to 1 mg/ml 5-FU alone (data not shown). We also investigated the gene expression of carcinogenic or metabolically important genes after inhibition with 10 μM JPH203 in the HEK001 and A431 cell lines. In the HEK001 cell line we found an upregulation of SLC7A5/LAT1 and SLC3A2/4F2 cell-surface antigen heavy chain, which codes for the other component of the transporter complex. We also found a small but significant upregulation of CCND1, ATF4, and GLI1 after JPH203 exposure.

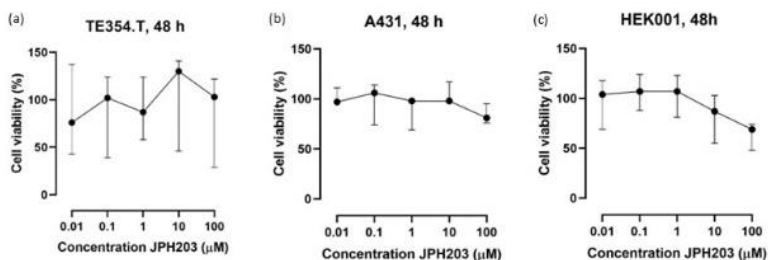


Figure 17 Cell viability after 48 hours of incubation with LAT1-inhibitor JPH203 in concentration range from 0.01 μM to 100 μM in (a) the basal cell carcinoma cell line TE354.T, (b) the cutaneous squamous cell carcinoma cell line A431 and (c) the normal keratinocyte cell line HEK001. Data presented as median and range.

Discussion

Mitochondrial alteration in BCC

Study I revealed a statistically significant downregulation of SLC25A43 in the investigated BCC tumours, which could indicate a changed mitochondrial function. The SLC25A43 gene is located on the X chromosome, and has previously mainly been studied in women (116). We examined tumours from both men and women, but found no differences in gene expression between the sexes. In a more recent publication, the knockdown of SLC25A43 has been found to be important for protection of the cell from ROS-mediated cell death (120). Downregulation of SLC25A43 therefore seems to be a protective strategy for the tumour cell to resist an environment with high ROS. The skin is under a high burden of UVR exposure that mediates ROS, and lower expression of SLC25A43 in the BCC tumours might be an adaptation in the cell that is beneficial for tumour cell survival.

Searching for potential drug targets, upregulation of LAT1 and LAT2

The general aim of this thesis was to find potential drug targets and we therefore continued to search for upregulated proteins that it might be possible to inhibit. We found that LAT1 and LAT2 seemed to be upregulated in the tumour tissue, and that TDO2 was expressed to a higher extent in immune cells around the tumours. The immune system reaction against the BCC tumours is heterogeneous in its intensity, and detailed knowledge is relatively sparse (90). It might be that the TDO2 expression around the tumour is a part of the tumour's ability to avoid immune destruction. This would parallel the actions of IDO1, which is involved in the kynurenine pathway and mediates an immunosuppressive tumour microenvironment (88). The upregulation of LAT1 and LAT2 in BCC tumours is also found in many other types of cancer, and in some of them is associated with poor prognosis (127, 151, 152). LAT1 has been studied in particular, and the selective LAT1 inhibitor JPH203 has been tested in phase I and II studies against biliary tract cancer (143, 144). LAT1 expression

is therefore an interesting drug target to study for keratinocyte cancer, as well.

LAT1 expression depending on tumour microenvironment

In Study III we continued to explore the expression of LAT1 and LAT2 in BCC. The two transporters have different protein expression patterns, and LAT1 seems to be more often induced in cancer than LAT2 (130, 152). In contrast to several other forms of cancer (127), LAT1 in BCC seems to be expressed not in the actively proliferating cells but instead in the cells inside tumour masses, where apoptosis and cell debris are more common. Low colocalisation of LAT1 and Ki-67 has also been seen in pancreatic ductal carcinoma (151). Studies on breast cancer samples and renal carcinoma cell line have shown a positive correlation between LAT1 expression and hypoxic markers (135, 153). However, no increased HIF1 α expression was detected in the BCC tissue in Study III. Despite this, the expression pattern of LAT1 could still indicate that the main role of LAT1 in BCC is not to provide the actively proliferating cells with nutrients, but to be a rescue for the cells inside a harsh environment of nutrient and oxygen deprivation. Our finding of a higher fraction of cleaved caspase-3 expression, a marker of apoptosis, in tumour areas with high LAT1 expression might strengthen this hypothesis.

In Study IV we examined LAT1 expression in the total cohort including both BCC and cSCC tumours, and looked for correlation with GLUT1, which is known to be upregulated in cancer (38), and the oncogene GLI1, the outcome of aberrant Hh signalling pathway activation (154). We found a statistically significant correlation between LAT1 and GLUT1 protein expression in the BCC tumours. This positive correlation could be attributed to the fact that both transporters are upregulated in response to hypoxia (135, 146, 155). Both proteins were expressed in the centre of the tumour masses, as previously described (156). When we stained these proteins with IF staining, we unexpectedly found that they were mainly not colocalised, as the GLUT1 positive cells were located one step closer to the centre of the tumours. This could be a sign of different mechanisms of regulation between the two transporters. There was no correlation between

LAT1 or GLI1, either in the total cohort or in the BCC subgroup. This suggests that the LAT1 expression in BCC is likely to be driven by the tumour microenvironment rather than by increased Hh pathway activation.

Limited effect on LAT1 inhibition in vitro

We investigated the effect of the selective LAT1 inhibitor JPH203 on cell viability in three cell lines. The decrease in viability was moderate, but this might have been influenced by the fact that JPH203 is a competitor inhibitor to the amino acids, and the artificial environment at the lab uses nutrient-rich cell medium to allow the cells to survive. These environmental conditions are very different from the ordinary tumour microenvironment. The effect of JPH203 has been shown to be more cytostatic than cytotoxic, with a limited ability to induce apoptosis (157). It might therefore be favourable to combine JPH203 with another drug for enhanced effect. We studied the effect of combining JPH203 with 5-FU, but found no additive effects. It would be of interest to test JPH203 in combination with other drugs such as rapamycin (an mTOR-inhibitor also known as sirolimus) in future studies.

Alterations in expression of different metabolically or carcinogenically important genes were elucidated after LAT1 inhibition with 10 μ M JPH203. The qPCR results indicated a difference in gene expression between exposed and control cells for SLC7A5, SLC3A2, CCND1, ATF4, and GLI1. The results need to be interpreted with caution due to one performed experiment, but a compensatory upregulation of LAT1 has been described previously, and is a common feedback mechanism (158, 159). Our results could also indicate a small change in CCND1, which is important in cell cycle regulation. Inhibition of LAT transporters has previously been found to lead to cell cycle inhibition in both prostate and pancreas cancer models (160, 161). ATF4 is important for the amino acid depletion in the cell, and is known to be upregulated in ductal pancreatic carcinoma cells after LAT1 inhibition (162). GLI1, the known oncogene in the Hh cell signalling pathway, regulates expression of c-MYC, a transcriptional regulator of LAT1 (163). Decreased expression of c-MYC after

exposition to JPH203 has been detected in a T-cell lymphoma mouse model (164), but knowledge of GLI1 expression after LAT1 inhibition with JPH203 is still scarce.

Limitations

One limitation in all four studies is that the results of IHC and IF are dependent on the accuracy of the antibodies. All the antibody stainings were optimised, and the results were compared to other publications, to the manufacturer's data, and to positive and negative control tissue with known staining patterns. When evaluating IF we also examined the background fluorescence, and autofluorescence, and performed dye-swap experiments to confirm the binding of the secondary antibody. HIF1 α , is known to be a difficult target to stain and despite careful optimisation we could not detect it to the same extent as in some other publications (165, 166). The hypoxic status in BCC tumours therefore needs further investigation. GLI1 has to some extent also shown a diverse expression pattern in previous studies, with different staining of the epidermis and cytoplasmic versus nuclear staining in the tumours (167-170). For both proteins, it is difficult to interpret our findings in comparison to previously published studies, as there are few studies and their quality varies. Future studies would be needed to further evaluate the importance of LAT1 in relation to HIF1 α and GLI1 in keratinocyte cancer.

Another limitation is the lack of available human BCC cell lines for in vitro studies. In Study IV we used TE354.T, which is the only available commercial cell line originating from BCC. This cell line has a fibroblast like growth pattern and is very slow growing. Substantial effort was put into the experiments at the cell culturing laboratory to allow us to use this cell line, but in the end we were only able to use the normal keratinocyte cell line HEK001 and the cSCC cell line A431 in the analysis of gene expression after LAT1 inhibition. LAT1 inhibition with 10 μ M JPH203 only changed the gene expression slightly, and during data analysis of the qPCR results we identified and removed outliers (4.6%) to obtain a Gaussian distribution for the remaining data. The exclusion of outliers took place after a careful examination the dataset which revealed that most of the faulty data

were control cells from the same well, indicating experimental error. The results should therefore be interpreted with caution. Due to the difficulty of finding a representative in vitro model for BCC tumours, alternatives for the future might be to start a primary cell culture from a patient's tumour or to test the LAT1 inhibition in animal models.

Cell signalling within the tumours is complex, and the result of both tumour-specific mutations in the genome and adaptation to the changing tumour microenvironment. Our hypothesis, which has grown stronger during the work of this thesis, is that the increased LAT1 expression in BCC tumours is a way for the tumour to survive in a harsh environment, and that the LAT1 inhibitor JPH203, when used in combination with other drugs, may have an inhibitory effect in keratinocyte cancers.

Conclusions

- The mitochondrial transport protein SLC25A43 is downregulated in BCC, indicating mitochondrial changes in the BCC tumour tissue.
- The amino acid transporters SLC7A5/LAT1 and SLC7A8/LAT2 are upregulated in BCC compared to non-tumoural skin.
- LAT1 is expressed to a higher degree in the centre of the BCC tumour masses, and is inversely correlated with the actively proliferating tumour cells.
- LAT1 expression is higher in areas that also have a higher expression of cleaved caspase-3, indicating a role in the tumour's adaptation to a harsh microenvironment.
- LAT1 expression is significantly correlated with GLUT1 expression in BCC tumours.
- The LAT1 inhibitor JPH203 suppresses the viability of the keratinocyte cell line HEK001 at concentrations of 100 μ M and above. However, it produces no significant change in viability in either the BCC cell line TE354.T or the squamous cell carcinoma cell line A431. The low responsiveness to LAT1 inhibition indicates that JPH203 needs to be further evaluated in more advanced models, and combined with other therapeutic agents for effective treatment of keratinocyte cancer.

Future perspectives

The incidence of skin cancer is an ongoing and growing challenge to the healthcare system. There is a need not only for new, easy, safe, and cheap treatments, but also for new biomarkers to predict cases with a risk of poor outcome. The LAT1 inhibitor JPH203 might not be sufficient alone for systemic treatment in BCC or cSCC, but it is still an interesting target for combined therapy or transcutaneous administration. It would be of value to investigate how cutaneous application of higher concentrations of JPH203 would affect the tumours and the surrounding skin, and which drug would best combine with JPH203 to produce synergistic effects. A next step could therefore be to develop a primary cell line from BCC, or to build an organoid cell culture. The conditions in the cell culture need to be modified, with lower oxygen pressure and lower amounts of amino acid components in the medium. Another possibility is to develop an animal model for BCC or cSCC, to allow testing the LAT1 inhibitors in an environment closer to the human tumour microenvironment.

A small proportion of keratinocyte cancers exhibit mutilating and sometimes even life-threatening growth. As important as it is to have an easy treatment modality for the large number of easy-to-treat tumours, it is of the same importance to have a precise and effective treatment at the right time for the tumours with life-threatening growth. There is a need both in BCC and especially in cSCC for precise and easy-to-use predictive biomarkers, for early detection of tumours with suspected adverse treatment results. Better knowledge of the function and molecular biology of keratinocyte cancer is therefore urgently needed.

Acknowledgements

There are many people whom I want to thank for all their encouragement and practical help. Without you, I would not have been able to write this thesis. Special thanks go to:

My first supervisor, Magnus Lindberg, whose great encouragement and curious attitude to life and science helped me to start this journey.

My supervisor, Elisabet Tina, who with steadfast and great help and encouragement has patiently stood by my side through the whole journey.

My co-supervisor, Anna Göthlin Eremo, whose enthusiasm, pedagogic and helpful attitude, and profound knowledge of cancer biology educated me in planning, performing, and evaluating the laboratory work, and who has also been a great help with the calculations and statistical assessments.

My co-supervisor, Oliver Seifert, for the inspiration, good ideas, and faithful support that have guided me and helped me to carry on over the years.

All my wonderful colleagues at the Department of Dermatology, Örebro University Hospital, particularly Karolina Evenhamre and Sofia Fall, who have supported me on both good and bad days. Great thanks also go to Helena Pellrud, current head of the department, and Anna Josefson, former head of the department, for supporting me and creating a workplace where clinical work can be combined with research.

The staff at the Clinical Research Laboratory, Örebro University Hospital, especially Hanna Arnesson, Lena Jansson, and Aron Sundell, for your laboratory help, general support, and fellowship during the lunch breaks.

Former medical students Alexander Duarte Tsegai, Sophie Lennholm, Camilla Loinder, Amalie Holst Sneckenborg, and Josefine Jordahl, for good collaboration and contribution to the data collection.

All the patients who were included in the patient cohort and generously donate a part of their gluteal skin to this research. Without you, this thesis would not be the same.

My mother Lena and father Jan, who have always encouraged me and supported my studies. Being able to reflect on my life and thoughts while surrounded by your love has helped me to grow, to believe in myself, and to be curious about life. My sister Hanna and brother Mikael, for being full of humour and generosity, and for giving me such positive energy.

My final thanks go to Jesus Christ, who gives my life meaning and to my family, for support and love. Pierre, Lukas, Moa and Evelina: you are my love and happiness.

This thesis was supported by ALF grants, Nyckelfonden at Örebro University Hospital, Lions Cancer Fund and Hudfonden (Edvard We-landers stiftelse, Finsenstiftelsen, Insamlingsstiftelsen Hudfonden).

Sammanfattning på svenska

Bakgrund: Hudcancer ökar kraftigt i den ljushyade befolkningen både i Sverige och internationellt. De vanligaste formerna av hudcancer är basalcellscancer (BCC) och skivepitelcancer i huden (cSCC) även kallade gemensamt för keratinocytcancer. Med anledning av den höga samt även stigande incidensen finns ett behov av ökad kunskap om cancerutvecklingen samt nya behandlingsstrategier.

Syfte: Syftet med denna avhandling har varit att belysa förändringar i cancermetabolismen vid keratinocytcancer med fokus på basalcellscancer.

Metod: Totalt 59 patienter med basalcellscancer eller skivepitelcancer har inkluderats i studien. Från patienterna har färskfrusen tumörvävnad och vävnad från samma patients gluteala hud samlats in. I delstudierna har även formalinfixerad och paraffinbäddad tumörvävnad samt normal hud studerats. För in vitro-försök av LAT1-inhibitorn JPH203 användes cellinjer med ursprung från BCC, cSCC och normal hud. Inom delstudierna har ett flertal olika analysmetoder använts så som qPCR, microarray, immunhistokemi/immunfluorescens samt viabilitetstest.

Resultat: I första delstudien (Study I) påvisades att gen- och proteinuttrycket av det mitokondriellt transportproteinet solute carrier (SLC) family 25 member 43 (SLC25A43) var signifikant lägre i BCC vävnad jämfört med patientens icke-tumorösa hud. Med microarray identifierades, i andra delstudien (Study II) ett förhöjt genuttryck av aminosyretransportörerna SLC7A5/ large neutral amino acid transporter (LAT) small subunit 1, SLC7A7/y+LAT1, SLC7A8/LAT2 samt enzymet Tryptophan 2,3 dioxygenase 2 (TDO2). Resultatet bekräftades med qPCR där ett signifikant högre genuttryck påvisades i tumörvävnad för SLC7A5/LAT1 ($p < 0,001$), SLC7A8/LAT2 ($p < 0,001$) och TDO2 ($p < 0,01$). Detta konfirmerades genom undersökning av proteinuttrycket med immunhistokemi och vi fann ökad nivå av SLC7A5/LAT1 och SLC7A8/LAT2 i tumörerna. I tredje delstudien (Study III) studerades samuttryck av proteinerna LAT1 och LAT2 i tumörcellerna, vilken visade sig var lågt och vi fann att LAT1 uttrycktes

framför allt i centrum av tumörmassorna. Andelen tumörceller med LAT1-uttryck var signifikant negativt korrelerat mot de aktivt prolifererande cellerna i tumörerna (Spearman $r=-0,83$, $Y=-0,35 \times X+30,56$, $p<0,01$). Resultaten visade även att klyvt caspase 3, talande för apoptos, programmerad celldöd, dessutom var signifikant ökat i tumörornåden med högt LAT1 uttryck ($p<0,05$). I den sista delstudien (Study IV) inkluderades hela kohorten för analys avseende proteinuttryck, vilket visade att LAT1-uttrycket i tumörvävnaden var signifikant högre än GLUT1 ($p<0,001$) respektive GLI1-uttrycket ($p<0,001$). Vi fann en statistisk signifikant positiv korrelation mellan proteinuttrycket av LAT1 och GLUT1 i BCC tumörerna (Spearman $r=0,39$, Linear regression $Y=0,22 \times X+28,77$, $p<0,01$) Under in vitro-försöken där cellinjerna exponerades för JPH203 visade resultaten en påverkan på cellviabiliteten och genuttryck för cellinjen HEK001.

Slutsatser: Resultaten visar att uttrycket av metabola markörer så som det mitokondriella transportproteinet SLC25A43 och aminosyretransportören LAT1 är förändrade i BCC tumörer jämfört med icke-tumörös hud. Det kan tala för anpassningar i tumörcellerna på grund av förändrad mikromiljö. LAT1 uttrycks även i cSCC men är inte lika välstuderat. LAT1 skulle kunna vara ett möjligt mål för läkemedelsbehandling mot keratinocyt cancer, framför allt i kombination med andra läkemedel men detta behöver studeras ytterligare och i mer avancerade modeller.

References

1. McGrath JA, Uitto J. Structure and function of the skin. In: Griffiths C, Barker J, Bleiker T, Hussain W, Simpson R (Eds.) *Rook's Textbook of Dermatology*. p. 1–50.
2. Lomas A, Leonardi-Bee J, Bath-Hextall F. A systematic review of worldwide incidence of nonmelanoma skin cancer. *Br J Dermatol*. 2012;166(5):1069–80.
3. Socialstyrelsen. Cancer i siffror 2023: Socialstyrelsen; 2023 Available from: <https://www.socialstyrelsen.se/globalassets/sha-repoint-dokument/dokument-webb/statistik/cancer-i-siffror-2023.pdf>.
4. Holm AS, Nissen CV, Wulf HC. Basal cell carcinoma is as common as the sum of all other cancers: implications for treatment capacity. *Acta Derm Venereol*. 2016;96(4):505–9.
5. Moan J, Grigalavicius M, Baturaite Z, Dahlback A, Juzeniene A. The relationship between UV exposure and incidence of skin cancer. *Photodermatol Photoimmunol Photomed*. 2015;31(1):26–35.
6. Verkouteren JAC, Ramdas KHR, Wakkee M, Nijsten T. Epidemiology of basal cell carcinoma: scholarly review. *Br J Dermatol*. 2017;177(2):359–72.
7. Asada M, Schaart FM, de Almeida HL, Jr., Korge B, Kurokawa I, Asada Y, et al. Solid basal cell epithelioma (BCE) possibly originates from the outer root sheath of the hair follicle. *Acta Derm Venereol*. 1993;73(4):286–92.
8. Howell BG, Solish N, Lu C, Watanabe H, Mamelak AJ, Freed I, et al. Microarray profiles of human basal cell carcinoma: insights into tumor growth and behavior. *J Dermatol Sci*. 2005;39(1):39–51.
9. Marzuka AG, Book SE. Basal cell carcinoma: pathogenesis, epidemiology, clinical features, diagnosis, histopathology, and management. *Yale J Biol Med*. 2015;88(2):167–79.

10. Trakatelli M, Morton C, Nagore E, Ulrich C, Del Marmol V, Peris K, et al. Update of the European guidelines for basal cell carcinoma management. *Eur J Dermatol.* 2014;24(3):312–29.
11. Youssef KK, Van Keymeulen A, Lapouge G, Beck B, Michaux C, Achouri Y, et al. Identification of the cell lineage at the origin of basal cell carcinoma. *Nat Cell Biol.* 2010;12(3):299–305.
12. Wong CS, Strange RC, Lear JT. Basal cell carcinoma. *BMJ.* 2003;327(7418):794–8.
13. Kramer E, Herman O, Frand J, Leibou L, Schreiber L, Vaknine H. Ki67 as a biologic marker of basal cell carcinoma: a retrospective study. *Isr Med Assoc J.* 2014;16(4):229–32.
14. Masterpol KS, Primiani A, Duncan LM. *Atlas of Essential Dermatopathology.* London: Springer; 2013.
15. Betti R, Radaelli G, Crosti C, Ghiozzi S, Moneghini L, Menni S. Margin involvement and clinical pattern of basal cell carcinoma with mixed histology. *J Eur Acad Dermatol Venereol.* 2012;26(4):483–7.
16. Alam M, Ratner D. Cutaneous squamous-cell carcinoma. *N Engl J Med.* 2001;344(13):975–83.
17. Que SKT, Zwald FO, Schmults CD. Cutaneous squamous cell carcinoma: incidence, risk factors, diagnosis, and staging. *J Am Acad Dermatol.* 2018;78(2):237–47.
18. Dal H, Boldemann C, Lindelöf B. Trends during a half century in relative squamous cell carcinoma distribution by body site in the Swedish population: support for accumulated sun exposure as the main risk factor. *J Dermatol.* 2008;35(2):55–62.
19. Boukamp P. Non-melanoma skin cancer: what drives tumor development and progression? *Carcinogenesis.* 2005;26(10):1657–67.
20. Salasche SJ. Epidemiology of actinic keratoses and squamous cell carcinoma. *J Am Acad Dermatol.* 2000;42(1 Pt 2):4–7.

21. Morton CA, Birnie AJ, Eedy DJ. British Association of Dermatologists' guidelines for the management of squamous cell carcinoma in situ (Bowen's disease) 2014. *Br J Dermatol*. 2014;170(2):245–60.
22. Thompson AK, Kelley BF, Prokop LJ, Murad MH, Baum CL. Risk factors for cutaneous squamous cell carcinoma recurrence, Metastasis, and Disease-Specific Death: A Systematic Review and Meta-analysis. *JAMA Dermatol*. 2016;152(4):419–28.
23. Krynitz B, Edgren G, Lindelöf B, Baecklund E, Brattström C, Wilczek H, et al. Risk of skin cancer and other malignancies in kidney, liver, heart and lung transplant recipients 1970 to 2008—a Swedish population-based study. *Int J Cancer*. 2013;132(6):1429–38.
24. Tseng HW, Huang WC, Lu LY. The influence of immunosuppressants on the non-melanoma skin cancer among patients with systemic lupus erythematosus and primary Sjögren's syndrome: a nationwide retrospective case-control study in Taiwan. *Clin Exp Rheumatol*. 2019;37(6):946–52.
25. Fried LJ, Criscito MC, Stevenson ML, Pomeranz MK. Chronic lymphocytic leukemia and the skin: implications for the dermatologist. *Int J Dermatol*. 2022;61(5):519–31.
26. Fogel AL, Sarin KY, Teng JMC. Genetic diseases associated with an increased risk of skin cancer development in childhood. *Curr Opin Pediatr*. 2017;29(4):426–33.
27. Patel T, Morrison LK, Rady P, Tyring S. Epidermodysplasia verruciformis and susceptibility to HPV. *Dis Markers*. 2010;29(3-4):199–206.
28. Schierbeck J, Vestergaard T, Bygum A. Skin cancer associated genodermatoses: a literature review. *Acta Derm Venereol*. 2019;99(4):360–9.
29. Ruggiero JL, Freese R, Hook KP, Polcari IC, Maguiness SM, Boull C. Skin cancer and sun protection practices in Fanconi anemia patients: A cross-sectional study. *J Am Acad Dermatol*. 2022;86(1):179–81.

30. Parza K, Singh P, Cvinar J, Zimmerman T, Watson B, Faris M. Voriconazole induced cutaneous squamous cell carcinoma in an immunocompetent patient. *Cureus*. 2022;14(5):e25508.
31. Nochaiwong S, Chuamanochan M, Ruengorn C, Noppakun K, Awiphan R, Phosuya C, et al. Use of thiazide diuretics and risk of all types of skin cancers: an updated systematic review and meta-analysis. *Cancers (Basel)*. 2022;14(10):228
32. Wysong A. Squamous-Cell Carcinoma of the Skin. *N Engl J Med*. 2023;388(24):2262–73.
33. Eisen DB, Asgari MM, Bennett DD, Connolly SM, Dellavalle RP, Freeman EE, et al. Guidelines of care for the management of actinic keratosis. *J Am Acad Dermatol*. 2021;85(4):e209–33.
34. Calonje E, Brenn T, Lazar A, McKee PH. Tumours of the surface epithelium. In: Calonje E, Brenn T, Lazar A, McKee PH (Eds.) *McKee's Pathology of the Skin with Clinical Correlations*. (4th ed): Elsevier; 2012.
35. Housman TS, Feldman SR, Williford PM, Fleischer AB, Jr., Goldman ND, Acostamadiedo JM, et al. Skin cancer is among the most costly of all cancers to treat for the Medicare population. *J Am Acad Dermatol*. 2003;48(3):425–9.
36. Gordon LG, Rowell D. Health system costs of skin cancer and cost-effectiveness of skin cancer prevention and screening: a systematic review. *Eur J Cancer Prev*. 2015;24(2):141–9.
37. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100(1):57–70.
38. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646–74.
39. Hanahan D. Hallmarks of Cancer: New Dimensions. *Cancer Discov*. 2022;12(1):31–46.
40. Sun X, Kaufman PD. Ki-67: more than a proliferation marker. *Chromosoma*. 2018;127(2):175–86.

41. Dowsett M, Nielsen TO, A'Hern R, Bartlett J, Coombes RC, Cuzick J, et al. Assessment of Ki67 in breast cancer: recommendations from the International Ki67 in Breast Cancer working group. *J Natl Cancer Inst.* 2011;103(22):1656–64.
42. Richards-Taylor S, Ewings SM, Jaynes E, Tilley C, Ellis SG, Armstrong T, et al. The assessment of Ki-67 as a prognostic marker in neuroendocrine tumours: a systematic review and meta-analysis. *J Clin Pathol.* 2016;69(7):612–8.
43. Luo Y, Ren F, Liu Y, Shi Z, Tan Z, Xiong H, et al. Clinicopathological and prognostic significance of high Ki-67 labeling index in hepatocellular carcinoma patients: a meta-analysis. *Int J Clin Exp Med.* 2015;8(7):10235–47.
44. Horlock NM, Wilson GD, Daley FM, Richman PI, Sanders R. Cellular proliferation characteristics of basal cell carcinoma: relationship to clinical subtype and histopathology. *Eur J Surg Oncol.* 1997;23(3):247–52.
45. Peris K, Fargnoli MC, Garbe C, Kaufmann R, Bastholt L, Seguin NB, et al. Diagnosis and treatment of basal cell carcinoma: European consensus-based interdisciplinary guidelines. *Eur J Cancer.* 2019;118:10–34.
46. Yerebakan O, Ciftçioğlu MA, Akkaya BK, Yilmaz E. Prognostic value of Ki-67, CD31 and epidermal growth factor receptor expression in basal cell carcinoma. *J Dermatol.* 2003;30(1):33–41.
47. Nitiss JL. Targeting DNA topoisomerase II in cancer chemotherapy. *Nat Rev Cancer.* 2009;9(5):338–50.
48. Lee JH, Berger JM. Cell cycle-dependent control and roles of DNA topoisomerase II. *Genes (Basel).* 2019;10(11):859
49. Ganapathi RN, Ganapathi MK. Mechanisms regulating resistance to inhibitors of topoisomerase II. *Front Pharmacol.* 2013;4:89.
50. Tanese K, Emoto K, Kubota N, Fukuma M, Sakamoto M. Immunohistochemical visualization of the signature of activated Hedgehog signaling pathway in cutaneous epithelial tumors. *J Dermatol.* 2018;45(10):1181–6.

51. Stone DM, Hynes M, Armanini M, Swanson TA, Gu Q, Johnson RL, et al. The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. *Nature*. 1996;384(6605):129–34.
52. Gailani MR, Ståhle-Bäckdahl M, Leffell DJ, Glynn M, Zaphiropoulos PG, Pressman C, et al. The role of the human homologue of *Drosophila* patched in sporadic basal cell carcinomas. *Nat Genet*. 1996;14(1):78–81.
53. Lo Muzio L. Nevoid basal cell carcinoma syndrome (Gorlin syndrome). *Orphanet J Rare Dis*. 2008;3:32.
54. Evangelista M, Tian H, de Sauvage FJ. The hedgehog signaling pathway in cancer. *Clin Cancer Res*. 2006;12(20 Pt 1):5924–8.
55. Weinberg RA. *The Biology of Cancer*. (2nd ed.) New York, London: Garland Science, Taylor & Francis Group; 2014.
56. Daya-Grosjean L, Couvé-Privat S. Sonic hedgehog signaling in basal cell carcinomas. *Cancer Lett*. 2005;225(2):181–92.
57. Sharpe HJ, Pau G, Dijkgraaf GJ, Basset-Seguín N, Modrusan Z, Januario T, et al. Genomic analysis of smoothened inhibitor resistance in basal cell carcinoma. *Cancer Cell*. 2015;27(3):327–41.
58. Biehs B, Dijkgraaf GJP, Piskol R, Alicke B, Boumahdi S, Peale F, et al. A cell identity switch allows residual BCC to survive Hedgehog pathway inhibition. *Nature*. 2018;562(7727):429–33.
59. Hatton BA, Knoepfler PS, Kenney AM, Rowitch DH, de Alborán IM, Olson JM, et al. N-myc is an essential downstream effector of Shh signaling during both normal and neoplastic cerebellar growth. *Cancer Res*. 2006;66(17):8655–61.
60. Welcker M, Orian A, Jin J, Grim JE, Harper JW, Eisenman RN, et al. The Fbw7 tumor suppressor regulates glycogen synthase kinase 3 phosphorylation-dependent c-Myc protein degradation. *Proc Natl Acad Sci U S A*. 2004;101(24):9085–90.
61. Bonilla X, Parmentier L, King B, Bezrukov F, Kaya G, Zoete V, et al. Genomic analysis identifies new drivers and progression

- pathways in skin basal cell carcinoma. *Nat Genet.* 2016;48(4):398–406.
62. Durinck S, Ho C, Wang NJ, Liao W, Jakkula LR, Collisson EA, et al. Temporal dissection of tumorigenesis in primary cancers. *Cancer Discov.* 2011;1(2):137–43.
 63. Su F, Viros A, Milagre C, Trunzer K, Bollag G, Spleiss O, et al. RAS mutations in cutaneous squamous-cell carcinomas in patients treated with BRAF inhibitors. *N Engl J Med.* 2012;366(3):207–15.
 64. Lim HW, Hönigsmann H, Hawk JL. *Photodermatology. US: CRC Press, Taylor & Francis Group; 2007, reprinted 2019.*
 65. Pellegrini C, Maturo MG, Di Nardo L, Ciciarelli V, Gutiérrez García-Rodrigo C, Fargnoli MC. Understanding the molecular genetics of basal cell carcinoma. *Int J Mol Sci.* 2017;18(11);2485
 66. Brash DE, Rudolph JA, Simon JA, Lin A, McKenna GJ, Baden HP, et al. A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc Natl Acad Sci U S A.* 1991;88(22):10124–8.
 67. Blattner C. Regulation of p53: the next generation. *Cell Cycle.* 2008;7(20):3149–53.
 68. Wang NJ, Sanborn Z, Arnett KL, Bayston LJ, Liao W, Proby CM, et al. Loss-of-function mutations in Notch receptors in cutaneous and lung squamous cell carcinoma. *Proc Natl Acad Sci U S A.* 2011;108(43):17761–6.
 69. South AP, Purdie KJ, Watt SA, Haldenby S, den Breems N, Dimon M, et al. NOTCH1 mutations occur early during cutaneous squamous cell carcinogenesis. *J Invest Dermatol.* 2014;134(10):2630–8.
 70. de Zwaan SE, Haass NK. Genetics of basal cell carcinoma. *Australas J Dermatol.* 2010;51(2):81–92.
 71. Brown VL, Harwood CA, Crook T, Cronin JG, Kelsell DP, Proby CM. p16INK4a and p14ARF tumor suppressor genes are

- commonly inactivated in cutaneous squamous cell carcinoma. *J Invest Dermatol.* 2004;122(5):1284–92.
72. Chamcheu JC, Roy T, Uddin MB, Banang-Mbeumi S, Chamcheu RN, Walker AL, et al. Role and therapeutic targeting of the PI3K/Akt/mTOR signaling pathway in skin cancer: a review of current status and future trends on natural and synthetic agents therapy. *Cells.* 2019;8(8)803.
 73. Leo MS, Sivamani RK. Phytochemical modulation of the Akt/mTOR pathway and its potential use in cutaneous disease. *Arch Dermatol Res.* 2014;306(10):861–71.
 74. So PL, Wang GY, Wang K, Chuang M, Chiueh VC, Kenny PA, et al. PI3K-AKT signaling is a downstream effector of retinoid prevention of murine basal cell carcinogenesis. *Cancer Prev Res (Phila).* 2014;7(4):407–17.
 75. Chow RY, Levee TM, Kaur G, Cedeno DP, Doan LT, Atwood SX. MTOR promotes basal cell carcinoma growth through atypical PKC. *Exp Dermatol.* 2021;30(3):358–66.
 76. Karayannopoulou G, Euvrard S, Kanitakis J. Differential expression of p-mTOR in cutaneous basal and squamous cell carcinomas likely explains their different response to mTOR inhibitors in organ-transplant recipients. *Anticancer Res.* 2013;33(9):3711–4.
 77. Dantal J, Morelon E, Rostaing L, Goffin E, Brocard A, Tromme I, et al. Sirolimus for secondary prevention of skin cancer in kidney transplant recipients: 5-year results. *J Clin Oncol.* 2018;36(25):2612–20.
 78. Smith A, Niu W, Desai A. The effect of conversion from a calcineurin inhibitor to sirolimus on skin cancer reduction in post-renal transplantation patients. *Cureus.* 2017;9(8):e1564.
 79. Gu YH, Du JX, Ma ML. Sirolimus and non-melanoma skin cancer prevention after kidney transplantation: a meta-analysis. *Asian Pac J Cancer Prev.* 2012;13(9):4335–9.
 80. Wright TI, Spencer JM, Flowers FP. Chemoprevention of non-melanoma skin cancer. *J Am Acad Dermatol.* 2006;54(6):933–46.

81. So PL, Fujimoto MA, Epstein EH, Jr. Pharmacologic retinoid signaling and physiologic retinoic acid receptor signaling inhibit basal cell carcinoma tumorigenesis. *Mol Cancer Ther.* 2008;7(5):1275–84.
82. Yoon JH, Lee CS, O'Connor TR, Yasui A, Pfeifer GP. The DNA damage spectrum produced by simulated sunlight. *J Mol Biol.* 2000;299(3):681–93.
83. Jayaraman SS, Rayhan DJ, Hazany S, Kolodney MS. Mutational landscape of basal cell carcinomas by whole-exome sequencing. *J Invest Dermatol.* 2014;134(1):213–20.
84. Courdavault S, Baudouin C, Charveron M, Favier A, Cadet J, Douki T. Larger yield of cyclobutane dimers than 8-oxo-7,8-dihydroguanine in the DNA of UVA-irradiated human skin cells. *Mutat Res.* 2004;556(1-2):135–42.
85. Loggini B, Boldrini L, Gisfredi S, Ursino S, Camacci T, De Jeso K, et al. CD34 microvessel density and VEGF expression in basal and squamous cell carcinoma. *Pathol Res Pract.* 2003;199(11):705–12.
86. Cernea CR, Ferraz AR, de Castro IV, Sotto MN, Logullo AF, Bacchi CE, et al. Angiogenesis and skin carcinomas with skull base invasion: a case-control study. *Head Neck.* 2004;26(5):396–400.
87. Lupu M, Caruntu C, Popa MI, Voiculescu VM, Zurac S, Boda D. Vascular patterns in basal cell carcinoma: dermoscopic, confocal and histopathological perspectives. *Oncol Lett.* 2019;17(5):4112–25.
88. Zhai L, Ladomersky E, Lenzen A, Nguyen B, Patel R, Lauing KL, et al. IDO1 in cancer: a Gemini of immune checkpoints. *Cell Mol Immunol.* 2018;15(5):447–57.
89. Cheong JE, Sun L. Targeting the IDO1/TDO2-KYN-AhR pathway for cancer immunotherapy - challenges and opportunities. *Trends Pharmacol Sci.* 2018;39(3):307–25.

90. Zilberg C, Lyons JG, Gupta R, Damian DL. The immune microenvironment in basal cell carcinoma. *Ann Dermatol.* 2023;35(4):243–55.
91. Warburg O. On the origin of cancer cells. *Science.* 1956;123(3191):309–14.
92. Warburg O. On respiratory impairment in cancer cells. *Science.* 1956;124(3215):269–70.
93. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metab.* 2008;7(1):11–20.
94. Jones RG, Thompson CB. Tumor suppressors and cell metabolism: a recipe for cancer growth. *Genes Dev.* 2009;23(5):537–48.
95. Hosseini M, Kasraian Z, Rezvani HR. Energy metabolism in skin cancers: a therapeutic perspective. *Biochim Biophys Acta Bioenerg.* 2017;1858(8):712–22.
96. Schieke SM, McCoy JP, Jr., Finkel T. Coordination of mitochondrial bioenergetics with G1 phase cell cycle progression. *Cell Cycle.* 2008;7(12):1782–7.
97. Mandal S, Guptan P, Owusu-Ansah E, Banerjee U. Mitochondrial regulation of cell cycle progression during development as revealed by the tenured mutation in *Drosophila*. *Dev Cell.* 2005;9(6):843–54.
98. McBride HM, Neuspiel M, Wasiak S. Mitochondria: more than just a powerhouse. *Curr Biol.* 2006;16(14):R551–60.
99. Huang M, Myers CR, Wang Y, You M. Mitochondria as a novel target for cancer chemoprevention: emergence of mitochondrial-targeting agents. *Cancer Prev Res (Phila).* 2021;14(3):285–306.
100. Durham SE, Krishnan KJ, Betts J, Birch-Machin MA. Mitochondrial DNA damage in non-melanoma skin cancer. *Br J Cancer.* 2003;88(1):90–5.
101. Starkov AA. The role of mitochondria in reactive oxygen species metabolism and signaling. *Ann N Y Acad Sci.* 2008;1147:37–52.

102. Birch-Machin MA, Swalwell H. How mitochondria record the effects of UV exposure and oxidative stress using human skin as a model tissue. *Mutagenesis*. 2010;25(2):101–7.
103. Lu J, Sharma LK, Bai Y. Implications of mitochondrial DNA mutations and mitochondrial dysfunction in tumorigenesis. *Cell Res*. 2009;19(7):802–15.
104. Szatrowski TP, Nathan CF. Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res*. 1991;51(3):794–8.
105. Nakamura H, Takada K. Reactive oxygen species in cancer: current findings and future directions. *Cancer Sci*. 2021;112(10):3945–52.
106. Le Gal K, Ibrahim MX, Wiel C, Sayin VI, Akula MK, Karlsson C, et al. Antioxidants can increase melanoma metastasis in mice. *Sci Transl Med*. 2015;7(308):308re8.
107. Ishikawa K, Takenaga K, Akimoto M, Koshikawa N, Yamaguchi A, Imanishi H, et al. ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. *Science*. 2008;320(5876):661–4.
108. Burns EM, Tober KL, Rigganbach JA, Kusewitt DF, Young GS, Oberyszyn TM. Differential effects of topical vitamin E and C E Ferulic® treatments on ultraviolet light B-induced cutaneous tumor development in Skh-1 mice. *PLoS One*. 2013;8(5):e63809.
109. Poswig A, Wenk J, Brenneisen P, Wlaschek M, Hommel C, Quel G, et al. Adaptive antioxidant response of manganese-superoxide dismutase following repetitive UVA irradiation. *J Invest Dermatol*. 1999;112(1):13–8.
110. Afaq F, Mukhtar H. Effects of solar radiation on cutaneous detoxification pathways. *J Photochem Photobiol B*. 2001;63(1–3):61–9.
111. Ibbotson SH, Dawe RS, Dinkova-Kostova AT, Weidlich S, Farr PM, Ferguson J, et al. Glutathione S-transferase genotype is

- associated with sensitivity to psoralen-ultraviolet A photochemotherapy. *Br J Dermatol.* 2012;166(2):380–8.
112. Lear JT, Smith AG, Bowers B, Heagearty AH, Jones PW, Gilford J, et al. Truncal tumor site is associated with high risk of multiple basal cell carcinoma and is influenced by glutathione S-transferase, GSTT1, and cytochrome P450, CYP1A1 genotypes, and their interaction. *J Invest Dermatol.* 1997;108(4):519–22.
 113. Yang XR, Pfeiffer RM, Goldstein AM. Influence of glutathione-S-transferase (GSTM1, GSTP1, GSTT1) and cytochrome p450 (CYP1A1, CYP2D6) polymorphisms on numbers of basal cell carcinomas (BCCs) in families with the naevoid basal cell carcinoma syndrome. *J Med Genet.* 2006;43(4):e16.
 114. Palmieri F, Scarcia P, Monné M. Diseases caused by mutations in mitochondrial carrier genes SLC25: a review. *Biomolecules.* 2020;10(4):655.
 115. Haitina T, Lindblom J, Renström T, Fredriksson R. Fourteen novel human members of mitochondrial solute carrier family 25 (SLC25) widely expressed in the central nervous system. *Genomics.* 2006;88(6):779–90.
 116. Tina E, Lindqvist BM, Gabrielson M, Lubovac Z, Wegman P, Wingren S. The mitochondrial transporter SLC25A43 is frequently deleted and may influence cell proliferation in HER2-positive breast tumors. *BMC Cancer.* 2012;12:350.
 117. Lindqvist BM, Farkas SA, Wingren S, Nilsson TK. DNA methylation pattern of the SLC25A43 gene in breast cancer. *Epigenetics.* 2012;7(3):300–6.
 118. Gabrielson M, Reizer E, Stål O, Tina E. Mitochondrial regulation of cell cycle progression through SLC25A43. *Biochem Biophys Res Commun.* 2016;469(4):1090–6.
 119. Gabrielson M, Tina E. The mitochondrial transport protein SLC25A43 affects drug efficacy and drug-induced cell cycle arrest in breast cancer cell lines. *Oncol Rep.* 2013;29(4):1268–74.

120. Zhang J, Zhao Y, Tian Y, Geng M, Liu Y, Zhang W, et al. Genome-wide screening in the haploid system reveals Slc25a43 as a target gene of oxidative toxicity. *Cell Death Dis.* 2022;13(3):284.
121. Adekola K, Rosen ST, Shanmugam M. Glucose transporters in cancer metabolism. *Curr Opin Oncol.* 2012;24(6):650–4.
122. Sengupta D, Pratz G. Imaging metabolic heterogeneity in cancer. *Mol Cancer.* 2016;15:4.
123. Kim M, Higuchi T, Nakajima T, Andriana P, Hirasawa H, Tokue A, et al. (18)F-FDG and (18)F-FAMT PET-derived metabolic parameters predict outcome of oral squamous cell carcinoma. *Oral Radiol.* 2019;35(3):308–14.
124. Achmad A, Bhattarai A, Yudistiro R, Heryanto YD, Higuchi T, Tsushima Y. The diagnostic performance of (18)F-FAMT PET and (18)F-FDG PET for malignancy detection: a meta-analysis. *BMC Med Imaging.* 2017;17(1):66.
125. Lieu EL, Nguyen T, Rhyne S, Kim J. Amino acids in cancer. *Exp Mol Med.* 2020;52(1):15–30.
126. Kahlhofer J, Teis D. The human LAT1-4F2hc (SLC7A5-SLC3A2) transporter complex: physiological and pathophysiological implications. *Basic Clin Pharmacol Toxicol.* 2023;133(5):459–72.
127. Wang Q, Holst J. L-type amino acid transport and cancer: targeting the mTORC1 pathway to inhibit neoplasia. *Am J Cancer Res.* 2015;5(4):1281–94.
128. Salisbury TB, Arthur S. The regulation and function of the L-type amino acid transporter 1 (LAT1) in cancer. *Int J Mol Sci.* 2018;19(8):2373.
129. Segawa H, Fukasawa Y, Miyamoto K, Takeda E, Endou H, Kanai Y. Identification and functional characterization of a Na⁺-independent neutral amino acid transporter with broad substrate selectivity. *J Biol Chem.* 1999;274(28):19745–51.
130. Yanagida O, Kanai Y, Chairoungdua A, Kim DK, Segawa H, Nii T, et al. Human L-type amino acid transporter 1 (LAT1):

- characterization of function and expression in tumor cell lines. *Biochim Biophys Acta*. 2001;1514(2):291–302.
131. Milkereit R, Persaud A, Vanoaica L, Guetg A, Verrey F, Rotin D. LAPTM4b recruits the LAT1-4F2hc Leu transporter to lysosomes and promotes mTORC1 activation. *Nat Commun*. 2015;6:7250.
 132. Hafliger P, Charles RP. The L-type amino acid transporter LAT1—an emerging target in cancer. *Int J Mol Sci*. 2019;20(10):2428.
 133. Parks SK, Cormerais Y, Pouyssegur J. Hypoxia and cellular metabolism in tumour pathophysiology. *J Physiol*. 2017;595(8):2439–50.
 134. Zhang J, Xu Y, Li D, Fu L, Zhang X, Bao Y, et al. Review of the Correlation of LAT1 with diseases: mechanism and treatment. *Frontiers in Chemistry*. 2020;8:564809.
 135. Elorza A, Soro-Arnáiz I, Meléndez-Rodríguez F, Rodríguez-Vaello V, Marsboom G, de Cárcer G, et al. HIF2 α acts as an mTORC1 activator through the amino acid carrier SLC7A5. *Mol Cell*. 2012;48(5):681–91.
 136. Kanai Y. Amino acid transporter LAT1 (SLC7A5) as a molecular target for cancer diagnosis and therapeutics. *Pharmacol Ther*. 2022;230:107964.
 137. Hirano K, Uno K, Kuwabara H, Kojima K, Ohno S, Sakurai H, et al. Expression of L-type amino acid transporter 1 in various skin lesions. *Pathol Res Pract*. 2014;210(10):634–9.
 138. Frampton JE, Basset-Seguín N. Vismodegib: A review in advanced basal cell carcinoma. *Drugs*. 2018;78(11):1145–56.
 139. Brancaccio G, Pea F, Moscarella E, Argenziano G. Sonidegib for the Treatment of Advanced Basal Cell Carcinoma. *Front Oncol*. 2020;10:582866.
 140. Stratigos AJ, Sekulic A, Peris K, Bechter O, Prey S, Kaatz M, et al. Cemiplimab in locally advanced basal cell carcinoma after hedgehog inhibitor therapy: an open-label, multi-centre, single-arm, phase 2 trial. *Lancet Oncol*. 2021;22(6):848–57.

141. Dumann K, Artz N, Ziemer M. Complete remission of basal cell carcinoma following treatment with cemiplimab after 2 years. *JAMA Dermatol.* 2021;157(8):1004–6.
142. Oda K, Hosoda N, Endo H, Saito K, Tsujihara K, Yamamura M, et al. L-type amino acid transporter 1 inhibitors inhibit tumor cell growth. *Cancer Sci.* 2010;101(1):173–9.
143. Okano N, Naruge D, Kawai K, Kobayashi T, Nagashima F, Endou H, et al. First-in-human phase I study of JPH203, an L-type amino acid transporter 1 inhibitor, in patients with advanced solid tumors. *Invest New Drugs.* 2020;38(5):1495–506.
144. Furuse J, Ikeda M, Ueno M, Furukawa M, Morizane C, Takehara T, et al. . Nanvuranlat, an L-type amino acid transporter (LAT1) inhibitor for patients with pretreated advanced refractory biliary tract cancer (BTC): primary endpoint results of a randomized, double-blind, placebo-controlled phase 2 study. *J Clin Oncol* 2023;41(4):494.
145. Krys D, Mattingly S, Glubrecht D, Wuest M, Wuest F. PET Imaging of L-type amino acid transporter (LAT1) and cystine-glutamate antiporter (xc⁻) with [¹⁸F]FDOPA and [¹⁸F]FSPG in breast cancer models. *Mol Imaging Biol.* 2020;22(6):1562–71.
146. Canhasi L, Tina E, Eremo AG. Hypoxia-mimetic by CoCl₂ increases SLC7A5 expression in breast cancer cells in vitro. *BMC Res Notes.* 2023;16(1):366.
147. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and 2^{-ΔΔC_T} method. *Methods.* 2001;25(4):402–8.
148. Wen Z, Luo D, Wang S, Rong R, Evers BM, Jia L, et al. Deep learning-based H-score quantification of immunohistochemistry-stained images. *Mod Pathol.* 2024;37(2):100398.
149. Detre S, Saclani Jotti G, Dowsett M. A “quickscore” method for immunohistochemical semiquantitation: validation for oestrogen receptor in breast carcinomas. *J Clin Pathol.* 1995;48(9):876–8.

150. Im K, Mareninov S, Diaz MFP, Yong WH. An Introduction to performing immunofluorescence staining. *Methods Mol Biol.* 2019;1897:299–311.
151. Yanagisawa N, Ichinoe M, Mikami T, Nakada N, Hana K, Koizumi W, et al. High expression of L-type amino acid transporter 1 (LAT1) predicts poor prognosis in pancreatic ductal adenocarcinomas. *J Clin Pathol.* 2012;65(11):1019–23.
152. Barollo S, Bertazza L, Watutantrige-Fernando S, Censi S, Cave-don E, Galuppini F, et al. Overexpression of L-type amino acid transporter 1 (LAT1) and 2 (LAT2): novel markers of neuroendocrine tumors. *PLoS One.* 2016;11(5):e0156044.
153. Törnroos R, Tina E, Göthlin Eremo A. SLC7A5 is linked to increased expression of genes related to proliferation and hypoxia in estrogen-receptor-positive breast cancer. *Oncol Rep.* 2022;47(1):17.
154. Skoda AM, Simovic D, Karin V, Kardum V, Vranic S, Serman L. The role of the Hedgehog signaling pathway in cancer: a comprehensive review. *Bosn J Basic Med Sci.* 2018;18(1):8–20.
155. Al Tameemi W, Dale TP, Al-Jumaily RMK, Forsyth NR. Hypoxia-modified cancer cell metabolism. *Front Cell Dev Biol.* 2019;7:4.
156. Abdou AG, Eldien MM, Elsakka D. GLUT-1 Expression in cutaneous basal and squamous cell carcinomas. *Int J Surg Pathol.* 2015;23(6):447–53.
157. Nishikubo K, Ohgaki R, Liu X, Okanishi H, Xu M, Endou H, et al. Combination effects of amino acid transporter LAT1 inhibitor nanvuranlat and cytotoxic anticancer drug gemcitabine on pancreatic and biliary tract cancer cells. *Cancer Cell Int.* 2023;23(1):116.
158. Yothaisong S, Dokduang H, Anzai N, Hayashi K, Namwat N, Yongvanit P, et al. Inhibition of L-type amino acid transporter 1 activity as a new therapeutic target for cholangiocarcinoma treatment. *Tumour Biol.* 2017;39(3):1010428317694545.

159. Cormerais Y, Pagnuzzi-Boncompagni M, Schrötter S, Giuliano S, Tambutté E, Endou H, et al. Inhibition of the amino-acid transporter LAT1 demonstrates anti-neoplastic activity in medulloblastoma. *J Cell Mol Med.* 2019;23(4):2711–8.
160. Zhou X, Ohgaki R, Jin C, Xu M, Okanishi H, Endou H, et al. Inhibition of amino acid transporter LAT1 in cancer cells suppresses G0/G1-S transition by downregulating cyclin D1 via p38 MAPK activation. *J Pharmacol Sci.* 2024;154(3):182–91.
161. Wang Q, Tiffen J, Bailey CG, Lehman ML, Ritchie W, Fazli L, et al. Targeting amino acid transport in metastatic castration-resistant prostate cancer: effects on cell cycle, cell growth, and tumor development. *J Natl Cancer Inst.* 2013;105(19):1463–73.
162. Ma Y, Okuda S, Okanishi H, Xu M, Jin C, Endou H, et al. Upregulation of ATF4 mediates the cellular adaptation to pharmacologic inhibition of amino acid transporter LAT1 in pancreatic ductal adenocarcinoma cells. *J Pharmacol Sci.* 2024;155(1):14–20.
163. Vallini G, Calabrese L, Canino C, Trovato E, Gentileschi S, Rubegni P, et al. Signaling pathways and therapeutic strategies in advanced basal cell carcinoma. *Cells.* 2023;12(21):2534.
164. Rosilio C, Nebout M, Imbert V, Griessinger E, Neffati Z, Benadiba J, et al. L-type amino-acid transporter 1 (LAT1): a therapeutic target supporting growth and survival of T-cell lymphoblastic lymphoma/T-cell acute lymphoblastic leukemia. *Leukemia.* 2015;29(6):1253–66.
165. Vaughan MM, Toth K, Chintala S, Rustum YM. Double immunohistochemical staining method for HIF-1 α and its regulators PHD2 and PHD3 in formalin-fixed paraffin-embedded tissues. *Appl Immunohistochem Mol Morphol.* 2010;18(4):375–81.
166. Seleit I, Bakry OA, Al-Sharaky DR, Ragab RAA, Al-Shiemy SA. Evaluation of hypoxia inducible factor-1 α and glucose transporter-1 expression in non melanoma skin cancer: an immunohistochemical study. *J Clin Diagn Res.* 2017;11(6):EC09–16.
167. Ghali L, Wong ST, Green J, Tidman N, Quinn AG. Gli1 protein is expressed in basal cell carcinomas, outer root sheath

- keratinocytes and a subpopulation of mesenchymal cells in normal human skin. *J Invest Dermatol.* 1999;113(4):595–9.
168. Kim HS, Kim YS, Lee C, Shin MS, Kim JW, Jang BG. Expression profile of sonic hedgehog signaling-related molecules in basal cell carcinoma. *PLoS One.* 2019;14(11):e0225511.
169. Pyczek J, Khizanishvili N, Kuzyakova M, Zabel S, Bauer J, Nitzki F, et al. Regulation and role of GLI1 in cutaneous squamous cell carcinoma pathogenesis. *Front Genet.* 2019;10:1185.
170. Dahmane N, Lee J, Robins P, Heller P, Ruiz i Altaba A. Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours. *Nature.* 1997;389(6653):876–81.

