

# Expression profile of the amino acid transporters SLC7A5, SLC7A7, SLC7A8 and the enzyme TDO2 in basal cell carcinoma\*

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## Summary

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### Conflicts of interest

None to declare.

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**Background** The incidence of basal cell carcinoma (BCC) is increasing and the costs for care rising. Therefore, the need for simplified and cost-effective treatment choices is substantial. Aberrant signalling in several pathways, induced by ultraviolet radiation, is of importance in the development of BCC. Alterations in tumour metabolic activity are part of general carcinogenesis; however, these alterations are only partially recognized in skin cancer.

**Objectives** To study expression profiles in BCCs compared with individually matched nontumour skin, with a focus on finding differences associated with tumour metabolism.

**Materials and methods** Gene expression in biopsies from BCCs ( $n = 14$ ) compared with biopsies from nontumour gluteal skin was analysed with microarrays ( $n = 4 + 4$ ) and/or quantitative real-time polymerase chain reaction (qPCR,  $n = 14 + 14$ ). Protein expression and localization was assessed using immunohistochemistry (IHC) in formalin-fixed and paraffin-embedded BCC samples.

**Results** Microarray analysis revealed increased expression of the amino acid transporters SLC7A5, SLC7A7 and SLC7A8 as well as the cytosolic enzyme tryptophan 2,3-dioxygenase (TDO) 2 in BCC. Higher expression of SLC7A5 ( $P < 0.001$ ), SLC7A8 ( $P < 0.001$ ) and TDO2 ( $P = 0.002$ ), but not SLC7A7 ( $P = 0.50$ ), was confirmed by qPCR, and IHC demonstrated correlating tumour cell protein expression of SLC7A5 and SLC7A8. Protein expression of SLC7A7 was observed in the stratum granulosum, and TDO2 in immune cells.

**Conclusions** This study highlights the upregulation of SLC7A5, SLC7A8 and TDO2 in BCC compared with nontumour skin. Our findings imply that amino acid transporters may be further explored as potential targets for future medical treatment.

### What's already known about this topic?

- The incidence of basal cell carcinoma (BCC) is increasing and consequently also the costs of care.
- The transport and metabolism of amino acids are often altered in tumours although the knowledge of whether this applies to BCCs is limited.

### What does this study add?

- Alterations of amino acid transporters SLC7A5 and SLC7A8 and the cytosolic enzyme TDO2 is suggested in BCC and are possible potential targets for treatment.
- SLC7A7 (transporter of e.g. lysine) is expressed in the stratum granulosum of normal epidermis and may be involved in the cornification process.

### What is the translational message?

- We have found tumour-specific changes in proteins involved in nutrient transport and metabolism.
- These changes may be of importance for carcinogenesis and should be explored further for future drug development.

Basal cell carcinoma (BCC) is the most common type of skin cancer and the incidence is increasing worldwide.<sup>1</sup> The tumours generally grow slowly with limited soft tissue invasion.<sup>2</sup> Metastasizing BCCs are rare but the tumours occasionally grow in a local invasive manner with destruction of bone and cartilage.<sup>3</sup> Due to rising costs of care, BCC is becoming a global public health problem and the need for cost-effective noninvasive treatment choices is substantial.<sup>4</sup>

The mechanism for BCC carcinogenesis is only partially identified, although DNA damage following ultraviolet radiation (UVR) exposure (mainly UVB) is widely accepted as a fundamental cause of most skin cancers. UVR is associated with aberrations in multiple signalling pathways that regulate DNA repair, metabolism, checkpoint signalling and proliferation.<sup>5</sup> Alteration in the Hedgehog signalling pathway, with frequent mutations of genes in the patched gene family (e.g. PTCH1), sonic hedgehog (SHH) and/or smoothed (SMO), has been identified.<sup>6,7</sup> These findings have led to the development of new target drugs such as the smoothed receptor (SMO) inhibitor vismodegib, which has improved the treatment of metastasizing BCC.<sup>8</sup>

Altered metabolic activity in tumours mainly involves uptake and turnover of essential nutrients for the survival of tumour cells. Amino acids are fundamental building blocks in structural maintenance and are components of cell signalling.<sup>9</sup> Several enzymes and membrane transporters participate in metabolic changes favouring tumour growth, such as indoleamine 2,3-dioxygenase (IDO), which degrades the essential amino acid tryptophan. Expression of IDO promotes immune evasion and angiogenesis, which is a reason that IDO inhibitors are being evaluated in cancers such as metastatic melanoma.<sup>10,11</sup> The large neutral amino acid transporter small subunit 1/solute carrier family 7 member 5 (LAT1/SLC7A5) has been described in various malignancies and with higher expression in BCC compared with normal epidermis.<sup>12</sup> As upregulation promotes tumour cell proliferation and inhibition leads to decreased growth, LAT1/SLC7A5 is a promising target for cancer therapy.<sup>13,14</sup> In this study, by comparing expression profiles between BCC tumours and nontumour skin tissue, we aimed at exploring metabolism-related factors that may serve as future targets for inhibitor drugs. We conducted microarray analysis and observed tumour upregulation of tryptophan 2,3-dioxygenase (TDO2), a gene with a similar function as IDO1, as well as upregulation of the genes SLC7A5, SLC7A7 and SLC7A8, all involved in tryptophan transport. Thus, the study emphasis is on these four candidate genes.

## Materials and methods

### Patient recruitment and tissue handling

Fourteen patients, seven men and seven women, were included in the present cohort study. All patients had been diagnosed with BCC at the Department of Dermatology, Örebro University Hospital, Örebro, Sweden. Patients with recurrent tumours or tumours from where biopsies had already been taken for diagnostic purposes were excluded from the study. The patients' ages ranged from 64 to 92 years and the size of the tumours varied between 8 and 20 mm in diameter. The tumours were excised from the face ( $n = 7$ ) and the trunk ( $n = 7$ ). The histopathological evaluation of the included tumours showed nodular growth ( $n = 11$ ), mixed superficial/nodular ( $n = 1$ ) and more aggressive growth ( $n = 2$ ) patterns. At the time of excision, a 4-mm punch biopsy was taken from the centre of the tumour as well as from gluteal skin (nontumour tissue) from the same patient. All biopsies ( $n = 28$ ) were immediately put in Allprotect Tissue Reagent (Qiagen, Solna, Sweden) at room temperature (RT) and then transferred to  $-80^{\circ}\text{C}$  storage. Control skin from two healthy donors was taken in conjunction with surgical removal of excess skin after weight loss. Two biopsies were taken from each donor's abdominal skin, then formalin-fixed and paraffin-embedded (FFPE) according to clinical protocols. The donors' identities were decoded and no personal information was recorded.

The study was approved by the local Ethics Committee in Uppsala, Sweden (Uppsala/Örebro no. 2011/242) with the requirement of written consent from all the participants.

### RNA extraction

RNA was extracted using the RNeasy Microarray Tissue Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration and absorbance ratios (260/280 and 260/230) were measured using the NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Thermo Fisher Scientific, Wilmington, DE, U.S.A.). RNA was further controlled by determining the RNA integrity number (RIN) of each sample using the Agilent RNA 6000 Nano Kit for the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, U.S.A.). The RNA was stored at  $-80^{\circ}\text{C}$ .

### Microarray data acquisition

Eight samples (tumour  $n = 4$ , nontumour  $n = 4$ ) were considered to have acceptable RNA quality for microarray analysis (RIN >

6.5, 260/280 and 260/230 > 1.8). From each sample, 25 ng of total RNA was used for the generation of amplified, fluorescent complementary RNA (cRNA), labelled with cyanine dye (Cy3) with the one-color Low Input Quick Amp Labeling Kit (Agilent Technologies). The cRNA was purified using the RNeasy Mini Kit (Qiagen), and hybridized to SurePrint G3 Human Gene Expression 8x60K v2 Microarrays (Agilent Technologies) for 17 h at 65 °C before the fluorescent pattern was read with a G2565CA Microarray Scanner (Agilent Technologies). Analysis of the raw microarray images was performed using Agilent Feature Extraction software v10.7.3.1 (Agilent Technologies).

### Quantitative real-time polymerase chain reaction

For quantitative real-time polymerase chain reaction (qPCR) analysis, 100 ng of RNA from tumour and nontumour tissues ( $n = 28$ ) was converted to complementary DNA (cDNA) using the SuperScript IV First-Strand Synthesis System (Invitrogen, Thermo Fisher Scientific) run in triplicate reactions and pooled into a total volume of 60  $\mu\text{L}$ . Expression of the target genes SLC7A5, SLC7A7, SLC7A8 and TDO2 as well as the reference genes ABL1, TOP1 and ACTB were measured with qPCR using TaqMan<sup>®</sup> Gene Expression Assays (Table 1; Applied Biosystems, Foster City, CA, U.S.A.) and the TaqMan<sup>®</sup> Fast Advanced Master Mix (Applied Biosystems). The cDNA samples (5 ng  $\mu\text{L}^{-1}$ ) were set in triplicate in 96-well plates and in a final volume of 20  $\mu\text{L}$ . The plates were run, using the 7900HT Fast Real-Time PCR system (Applied Biosystems), at 50 °C for 2 min, 95 °C for 20 s and then 95 °C for 1 s followed by 60 °C for 20 s, repeated for 40 cycles. The quantification cycle ( $C_q$ ) was set automatically. Expression of the four target genes was normalized against the mean value of the three reference genes. The fold change (FC) gene expression (tumour vs. nontumour tissue) was calculated using the  $2^{-\Delta\Delta C_q}$  method.<sup>15</sup>

### Immunohistochemistry

Blocks of FFPE tissue were available from the patients' tumours only and not from nontumour tissue. Protein expression of SLC7A5, SLC7A7, SLC7A8 and TDO2 was assessed

using immunohistochemistry (IHC). For reference, protein expression was also assessed in FFPE abdominal skin from healthy donors. All antibodies were optimized for ideal dilution and incubation time (Table 1).

In brief, 4- $\mu\text{m}$  sections of FFPE tissue were mounted onto microscope glass slides, oven-dried at 60 °C for 1 h, deparaffinized and rehydrated in xylene, decreasing concentration series of ethanol and finally placed in deionized water. Subsequently, the slides were placed in DIVA decloaker buffer, pH 6 (Biocare Medical, Walnut Creek, CA, U.S.A.) and heat-induced epitope retrieval was performed in a decloaking chamber (Biocare Medical) for 10 min at 110 °C. Staining was carried out using the automated slide stainer IntelliPATH FLX<sup>™</sup> (Biocare Medical) and the MACH 1 Universal HRP-Polymer Detection system (Biocare Medical) with Betazoid DAB chromogen according to the manufacturer's instructions. After counterstaining with Mayer's haematoxylin (5 min, RT), the tissues were dehydrated in increasing concentrations of ethanol and finally xylene prior to mounting with Pertex<sup>®</sup> mounting medium. Along all runs tissues with known protein expression were included as positive and negative staining controls, as well as the healthy donor tissues.

### Evaluation of protein expression

The slides were scanned using the Panoramic 250 Flash II (3DHistech Ltd, Budapest, Hungary) and the digital images analysed using the software CaseViewer (3DHistech Ltd). In each tumour, 10 annotations with sizes corresponding to  $\times 40$  magnification vision fields were placed randomly in areas with BCC and the staining intensity in 20 consecutive tumour cells was assessed. The staining was graded as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong) and the H-score algorithm was used for estimation of protein expression ( $1 \times \% \text{ cells with staining grade 1} + 2 \times \% \text{ cells with staining grade 2} + 3 \times \% \text{ cells with staining grade 3}$ ) resulting in a number of 0–300. The final H-scores were based on consensus scores from two separate observers (S.P. and E.T.) who graded the staining individually. Protein expression in skin from healthy donors was recorded descriptively only.

**Table 1** Immunohistochemistry (IHC) staining of proteins in tumours, including the results from evaluation (H-scores) and information of the specific TaqMan gene expression assays used in quantitative real-time polymerase chain reaction (qPCR) analysis

	SLC7A5	SLC7A7	SLC7A8	TDO2
<b>IHC</b>				
Antibody	ab208776	HPA036227	UM500058	PA5-42759
Species	Rabbit monoclonal	Rabbit polyclonal	Mouse monoclonal	Rabbit polyclonal
Distributor	Abcam, Cambridge, U.K.	Sigma-Aldrich, St Louis, MO, U.S.A.	Origene, Rockville, MD, U.S.A.	Thermo Fisher, Waltham, MA, U.S.A.
Dilution	1 : 500	1 : 135	1 : 100	1 : 500
Incubation time	1 h (RT)	30 min (RT)	30 min (RT)	30 min (RT)
<b>qPCR</b>				
TaqMan assay ID <sup>a</sup>	Hs01001183_m1	Hs00374418_m1	Hs00794796_m1	Hs00194611_m1
RT, room temperature. <sup>a</sup> TaqMan assay ID for the reference genes: ABL1 (Hs01104728_m1), TOP1 (Hs00243257_m1) and ACTB (Hs99999903_m1) (Applied Biosystems, Foster City, CA, U.S.A.).				

## Statistical data analysis

The raw microarray data was normalized using the 75th percentile shift and baseline adjusted to median prior to statistical analysis. The detected entities were filtered on normality by the Shapiro–Wilks test ( $P < 0.05$ ). Entities that were expressed differentially between tumour and nontumour tissues were retrieved based on statistical significance (corrected  $P \leq 0.05$ ) using a moderated *t*-test with the Benjamini–Hochberg multiple testing correction method. Aiming to leave out entities with lower biological significance, the level for absolute FC was set to  $FC \geq 5.0$ . The differentially expressed genes (DEGs) were further analysed through Gene Ontology (GO) term enrichment ( $P \leq 0.1$ ). The microarray data was analysed using GeneSpring v.14.9 (Agilent Technologies).

In order to perform statistical calculations of qPCR data of SLC7A5, SLC7A7, SLC7A8 and TDO2 gene expression, all  $2^{-\Delta Cq}$  values were logarithmized. Gaussian distribution of the  $\log 2^{-\Delta Cq}$  values was tested using the Shapiro–Wilks test and normality was assumed for  $P > 0.05$ . Differences in gene expression ( $\log 2^{-\Delta Cq}$  values) between tumour and nontumour tissues were calculated using the paired *t*-test. For graphical visualization of gene expression, the  $\log 2^{-\Delta Cq}$  values were transformed into *z*-scores where a *z*-score of 0 represents the mean and *z*-scores of  $\pm 1$  equals 1 SD from the mean. Spearman's correlation coefficient ( $\rho$ ) for nonparametric data was used as a measure of correlation between gene expression ( $\log 2^{-\Delta Cq}$  value) and protein expression (H-score) for each patient's tumour tissue. A simple linear regression model was used to estimate  $R^2$  as well as the regression linear equation. *P*-values  $\leq 0.05$  were considered as significant. All data analysis was performed using IBM SPSS Statistics v. 22.0 (IBM, Armonk, NY, U.S.A.) and GraphPad Prism v. 7.03 (GraphPad Software, La Jolla, CA, U.S.A.).

## Results

### Gene expression profile in basal cell carcinoma

The analysis of microarray data resulted in 58 341 detected entities, of which 58 306 remained after filtering on normality. A moderated *t*-test (with Benjamini–Hochberg multiple testing correction) generated a list of 4864 entities that were differentially expressed in tumours compared with nontumour tissues (corrected  $P < 0.05$ ). The list of entities was further filtered by using a threshold FC of  $\geq 5.0$  (1883 remaining, Table S1; see Supporting Information). After eliminating entities represented by long noncoding RNA and pseudogenes 1225, differentially expressed genes remained (Table S2; see Supporting Information). Of those, 669 DEGs were upregulated (FC ranging from +5.0 to +341.7) and 556 DEGs were downregulated (FC ranging from –5.0 to –256.8).

Among all 669 upregulated DEGs ( $FC > 5.0$ ) were also members of Hedgehog and Wnt signalling (e.g. PTCH1, PTCH2, GLI1 and WNT5A). The 100 most upregulated DEGs ( $FC > 22$ ) comprised matrix metalloproteinases (MMP1, MMP12, MMP11,

MMP3 and MMP10), chemokines (e.g. CXCL9, CXCL13, CXCL10, CCL18, CXCL11), various cancer genes (e.g. SPP1, OLFM4) and genes involved in amino acid metabolism and membrane transport of solutes (e.g. TDO2, SLC01A2, SLC6A1). Among the 100 most downregulated DEGs ( $FC > -15$ ) were those

**Table 2** Genes with upregulation in BCC by microarray analysis. The curated list of DEGs includes genes selected through GO analysis, literature mining and on the basis of overlapping with results from other studies of BCC molecular signatures<sup>16–20,23</sup>

The 25 most upregulated genes			Curated DEGs		
Gene symbol	FC	Log 2 FC	Gene symbol	FC	Log 2 FC
IGLL5	341.7	8.4	EDN2	64.1	6.0
SOX11	177.0	7.5	MYCN	43.9	5.5
CXCL9	146.9	7.2	OLFM4	43.8	5.5
ABCC12	139.7	7.1	SLC01A2	37.1	5.2
PCDH8	135.7	7.1	CHGA	35.8	5.2
CHRD12	123.1	6.9	CXCL11	35.6	5.2
S100A7A	108.6	6.8	TDO2	35.3	5.1
MMP1	106.1	6.7	MMP11	31.2	5.0
TCL6	101.6	6.7	MMP3	25.8	4.7
KRTAP17-1	98.2	6.6	SLC6A1	25.6	4.7
HHIP	93.8	6.6	GLI1	24.2	4.6
RBFOX3	83.7	6.4	SH3GL3	23.4	4.5
CASC9	83.6	6.4	MMP10	22.4	4.5
FBN3	83.6	6.4	PTCH1	18.5	4.2
HMG2	81.3	6.3	TRO	18.3	4.2
ADAMTS3	74.9	6.2	MSR1	17.9	4.2
CXCL13	74.8	6.2	GPR161	17.6	4.1
ERC2	73.9	6.2	SLC39A14	16.1	4.0
ALK	72.0	6.2	MDK	13.8	3.8
CXCL10	71.8	6.2	VCAN	12.6	3.7
CCL18	70.9	6.1	SLC7A8	12.3	3.6
PNOC	70.2	6.1	CSPG4	12.0	3.6
SPP1	70.0	6.1	SLC16A3	11.9	3.6
MMP12	68.4	6.1	LRP8	11.2	3.5
KIF12	66.6	6.1	SHOX2	10.4	3.4
			DIO2	10.3	3.4
			PDGFA	9.7	3.3
			PTCH2	8.9	3.2
			NINL	8.8	3.1
			DUSP10	8.8	3.1
			SOX13	8.6	3.1
			SLC1A4	8.1	3.0
			HEPH	8.0	3.0
			KRT16	8.0	3.0
			SLC7A5	7.5	2.9
			STMN1	7.4	2.9
			COL5A2	7.0	2.8
			SLC7A7	6.8	2.8
			TSPAN4	6.7	2.7
			EPCAM	6.6	2.7
			CDH8	6.6	2.7
			CDH3	6.5	2.7
			TOP2A	6.4	2.7
			LOXL2	6.4	2.7
			TUSC3	5.9	2.6

BCC, basal cell carcinoma; DEGs, differentially expressed genes; FC, fold change; GO, Gene Ontology.

associated with adipose tissue (eg. PLIN4, LEP, PLIN1, ADIPOQ) and inhibition of peptidases and proteinases (SERPINA12, TIMP4). Tables 2 and 3 present the top 25 most upregulated

**Table 3** Genes with downregulation in BCC by microarray analysis. The curated list of DEGs include genes selected through GO analysis, literature mining and on the basis of overlapping with results from other studies of BCC molecular signatures<sup>16–20,23</sup>

The 25 most downregulated genes			Curated DEGs		
Gene symbol	FC	Log 2 FC	Gene symbol	FC	Log 2 FC
PLIN4	-256.8	-8.0	ADH1A	-29.7	-4.9
DCD	-250.3	-8.0	PRLR	-27.1	-4.8
LEP	-177.8	-7.5	SOX21	-26.5	-4.7
PLIN1	-163.9	-7.4	WNT16	-25.5	-4.7
PIP	-134.5	-7.1	RBP4	-24.9	-4.6
TMEM213	-75.9	-6.2	MUCL1	-24.8	-4.6
CA6	-73.3	-6.2	FABP7	-23.7	-4.6
SERPINA12	-72.9	-6.2	ADH1B	-23.4	-4.5
ADIPOQ	-63.3	-6.0	TUSC8	-22.2	-4.5
GDPD2	-62.2	-6.0	FOXA1	-21.9	-4.5
MYOC	-62.1	-6.0	CDHR4	-20.9	-4.4
PSAPL1	-52.3	-5.7	CDHR4	-20.9	-4.4
SCGB1D1	-51.5	-5.7	FOXP2	-20.7	-4.4
HOXA-AS3	-49.5	-5.6	NOX1	-18.4	-4.2
FA2H	-46.1	-5.5	TIMP4	-18.1	-4.2
ACADL	-42.9	-5.4	ADH1C	-17.1	-4.1
SCGB1D2	-42.9	-5.4	LAMB4	-17.0	-4.1
CDHR1	-41.5	-5.4	LIPK	-16.7	-4.1
SOX6	-41.1	-5.4	TNMD	-15.9	-4.0
FABP4	-38.3	-5.3	WNT3	-15.9	-4.0
CHP2	-37.9	-5.2	COBL	-14.5	-3.9
ISL1	-37.5	-5.2	SPINK1	-12.4	-3.6
MRAP	-36.9	-5.2	CLDN10	-12.0	-3.6
CLDN3	-36.8	-5.2	LIPE	-12.0	-3.6
HOXA9	-36.7	-5.2	SLC2A4	-11.9	-3.6
			LIPH	-11.4	-3.5
			ENDOU	-11.4	-3.5
			EXPH5	-11.1	-3.5
			ERBB4	-10.0	-3.3
			AZGP1	-9.5	-3.2
			FCGBP	-9.3	-3.2
			DMKN	-9.2	-3.2
			ZBTB16	-8.2	-3.0
			TGFBR3	-7.7	-2.9
			GPR126	-7.6	-2.9
			AKR1C1	-7.5	-2.9
			CA12	-7.4	-2.9
			ABCC3	-7.3	-2.9
			NRG2	-6.8	-2.8
			C2CD2	-6.6	-2.7
			SLC25A48	-6.1	-2.6
			NRG4	-6.1	-2.6
			WNT4	-5.9	-2.6
			CRYAB	-5.9	-2.6
			LPL	-5.2	-2.4

BCC, basal cell carcinoma; DEGs, differentially expressed genes; FC, fold change; GO, Gene Ontology.

and downregulated genes including genes overlapping with results from other studies of BCC gene expression.<sup>16–20,23</sup> GO analysis presented 211 enriched terms (Table S3; see Supporting Information) and one of the top candidate terms was the term Plasma membrane part (corrected  $P < 10^{-8}$ , GO:0044459), branching into Intrinsic component of plasma membrane (GO:0031226) and Plasma membrane region (GO:0098590). Among the genes enriched, within the latter GO term (Table S4; see Supporting Information), were the upregulated SLC7A5 (FC = 7.5), SLC7A7 (FC = 6.8) and SLC7A8 (FC = 12.3), solute carriers involved in amino acid transport across the cell membrane.

### Gene expression of SLC7A5, SLC7A7, SLC7A8 and TDO2

The gene expression of SLC7A5, SLC7A7, SLC7A8 and TDO2 was further examined in all samples (tumours  $n = 14$  with matched nontumour tissue  $n = 14$ ) using qPCR. The genes SLC7A5 ( $P < 0.001$ ), SLC7A8 ( $P < 0.001$ ) and TDO2 ( $P = 0.002$ ) were found to have higher expression in tumour tissues while SLC7A7 gene expression showed no difference in tumour compared with nontumour tissue ( $P = 0.50$ , Fig. 1). The median FC was as follows: for SLC7A5, 7.7 (min = 2.8, max = 24.1); for SLC7A7, 1.2 (min = 0.26, max = 8.0); for SLC7A8, 8.6 (min = 4.2, max = 21.5) and for TDO2, 7.6 (min = 0.37, max = 105.7).

### Protein expression of SLC7A5, SLC7A7, SLC7A8 and TDO2

Protein expression was assessed in FFPE BCCs from 13 of the 14 individuals included in the study. The overall tissue staining for each protein was evaluated descriptively but grading of staining intensity (i.e. level of protein expression) was restricted to tumour cells. The H-scores for each protein are shown in Figure 2.

SLC7A5 protein expression (Fig. 3) in the BCC tissues was primarily located in the cell membranes and to some extent in the cytoplasm of tumour cells, with H-scores varying between 8 and 263 (median H-score = 101, Fig. 2). The staining was heterogeneous and tumour cells in the centre part of the BCC were generally more intensely stained than the palisading cells in the tumour margins. The epithelia close to the tumour areas was more stained than the epithelia in the periphery. Staining was also present in the sebaceous glands as well as in hair follicles closer to the apical part with weakened intensity deeper down in the hair follicle. In the samples of skin from healthy donors staining was almost absent in the epidermis.

The staining intensity of SLC7A7 and the fraction of stained cells in tumour areas, epithelia and follicular epithelia were generally low. However, strong membrane staining was observable in the stratum granulosum of the epidermis (Fig. 4). All but one patient's BCC showed no SLC7A7 protein expression in tumour cells, with H-scores of 0 and 1 (median H-score = 0, Fig. 2). The skin tissues from healthy donors showed strong staining in the stratum granulosum and were similar to the epidermis of tumour tissues.



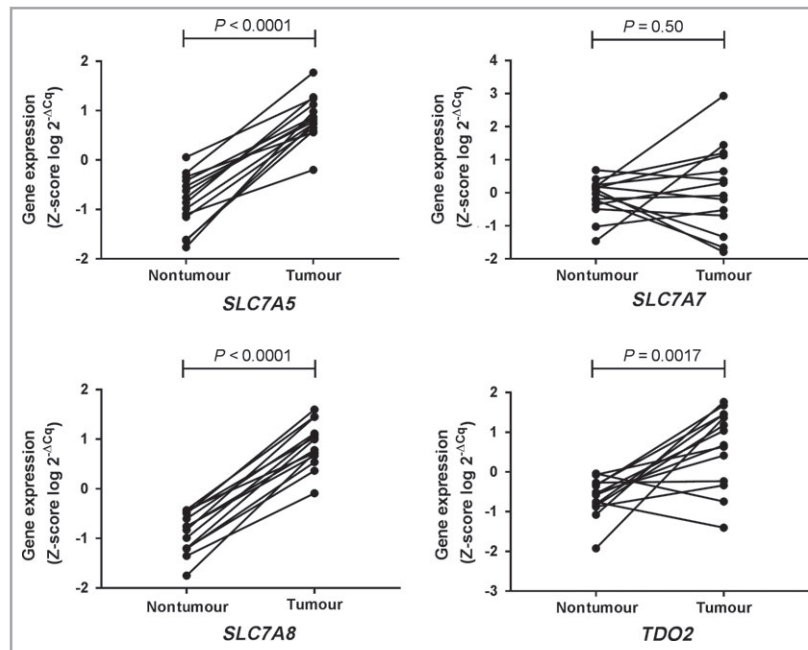


Fig 1. Expression of candidate genes selected from microarray data and validated using quantitative real-time polymerase chain reaction analysis. The statistical differences in gene expression of *SLC7A5*, *SLC7A7*, *SLC7A8* and *TDO2* between basal cell carcinoma ( $n = 14$ ) and individually matched nontumour tissues (gluteal skin,  $n = 14$ ) were calculated using a paired *t*-test.

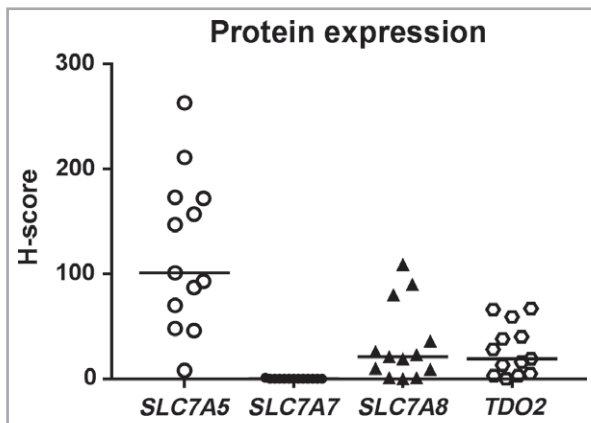


Fig 2. Distribution of H-scores after evaluation of protein staining of *SLC7A5*, *SLC7A7*, *SLC7A8* and *TDO2*. Horizontal bars indicate mean H-score.

*SLC7A8* protein expression was located in cell membranes and to some extent in cytoplasm, and presented with heterogeneity among tumour areas (Fig. 5). Although the overall *SLC7A8* staining intensity in BCC was low with H-scores between 0 and 109 (median H-score = 21, Fig. 2), a stronger staining intensity was observed in the tumour cells than cells in epithelia and hair follicular epithelia. Strong staining was observed in the hair bulbs. There was no visible *SLC7A8* staining in the epidermis taken from healthy donor skin.

The staining intensity of *TDO2* (Fig. 6) was generally low in the tumour cells with H-scores of 0–67 (median H-score = 19, Fig. 2) as well as in the follicular epithelia. There was a

clearly visible, and for several cases even strong, staining in lymphocyte infiltrates, in the stroma in dermis and in sebaceous glands. In the skin samples from healthy donors, the epithelial cells showed strong staining intensity.

Protein expression levels for *SLC7A5*, *SLC7A7*, *SLC7A8* and *TDO2* in abdominal skin from healthy donors are shown in Figure 7.

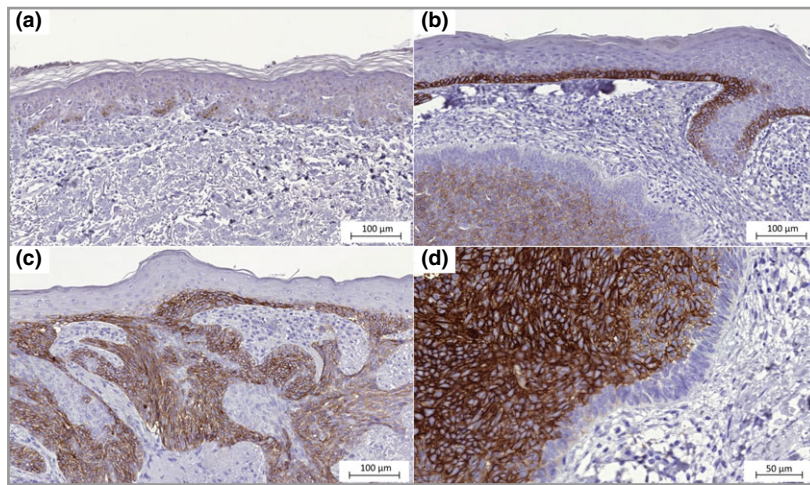
#### Correlation between gene and protein expression in tumours

In order to evaluate whether higher gene expression in tumour tissues reflects higher amounts of protein, correlations between the  $\log 2^{-\Delta Cq}$  values and H-scores were calculated. Higher gene expression of *SLC7A5* and *SLC7A8* correlated with higher protein expression ( $\rho = 0.64$ ,  $P = 0.019$  and  $\rho = 0.74$ ,  $P = 0.004$ , respectively, Fig. 8). There were no relationships found between gene and protein expression of *SLC7A7* ( $\rho = 0.08$ ,  $P = 0.80$ ) or *TDO2* ( $\rho = 0.19$ ,  $P = 0.54$ ).

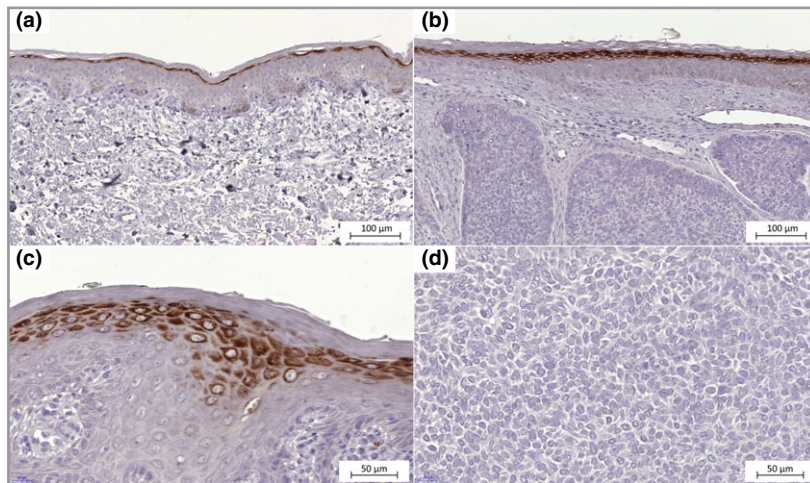
#### Discussion

Aiming to identify factors that may be important in BCC carcinogenesis, we conducted gene expression analysis with a focus on amino acid metabolism and transport. Our results show that the genes *SLC7A5*, *SLC7A8* and *TDO2* are upregulated in BCC compared with individually matched nontumour tissue (gluteal skin).

Firstly, we performed microarray analysis and compared our results with those of other studies to evaluate the



**Fig 3.** SLC7A5 expression in basal cell carcinoma. (a) Epidermis with normal appearance adjacent to the tumour, weak immunohistochemistry staining ( $\times 20$ ). (b) Epidermis directly above the tumour mass shows strong SLC7A5 staining ( $\times 20$ ). (c) The tumour is growing in a nodular and superficial pattern ( $\times 20$ ). (d) The inner mass of tumour cells with membrane staining and negative palisading cells ( $\times 40$ ).



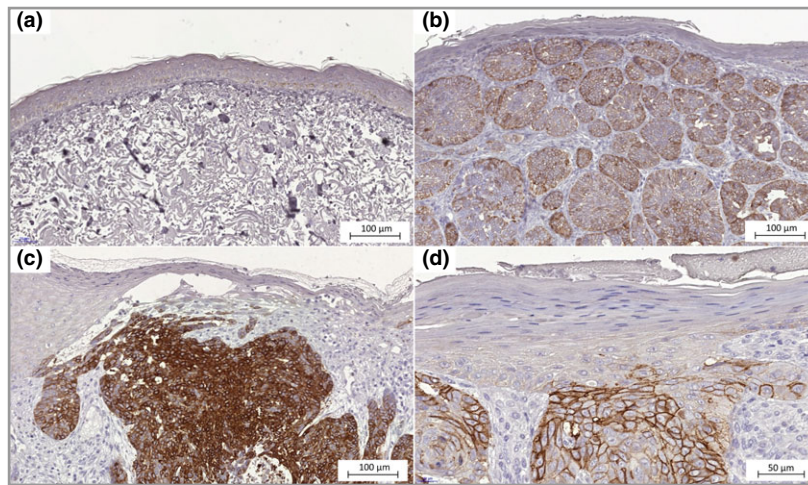
**Fig 4.** SLC7A7 expression in basal cell carcinoma. (a) Epidermis with normal appearance adjacent to the tumour, weak immunohistochemistry staining in basal cell layer and strong staining in the stratum granulosum cells ( $\times 20$ ). (b) Epidermis directly above the tumour mass shows strong SLC7A7 staining in stratum granulosum cells similar to normal cells ( $\times 20$ ). (c) Cells in the stratum granulosum with SLC7A7 protein expression in membrane and cytoplasm ( $\times 40$ ). (d) No SLC7A7 protein expression in tumour cells ( $\times 40$ ).

correspondence. Bonifas *et al.*<sup>20</sup> conducted qPCR to analyse gene expression in BCC in comparison with normal skin and found upregulation of *PTCH1*, *GLI1*, *HHIP*, *WNT5A* and downregulation of *WNT4*. Heller *et al.*<sup>16</sup> integrated microarray analysis with literature mining and reported genes that overlap those in other publications. According to their list of DEGs in BCC, several occur among our DEGs (e.g. upregulated *MDK*, *DUSP10*, *TSPAN4*, *SLC7A8*, *TOP2A*, *SHOX2* and downregulated *FABP4*, *ADH1B*, *ABCC3*, *LPL*, *COBL*, *TGFBR3*).<sup>16,17,19</sup> The gene coding for osteopontin (*SPP1*), a multifunctional protein involved in cancer development and with promising clinical utilization, was upregulated 70-fold.<sup>21–23</sup>

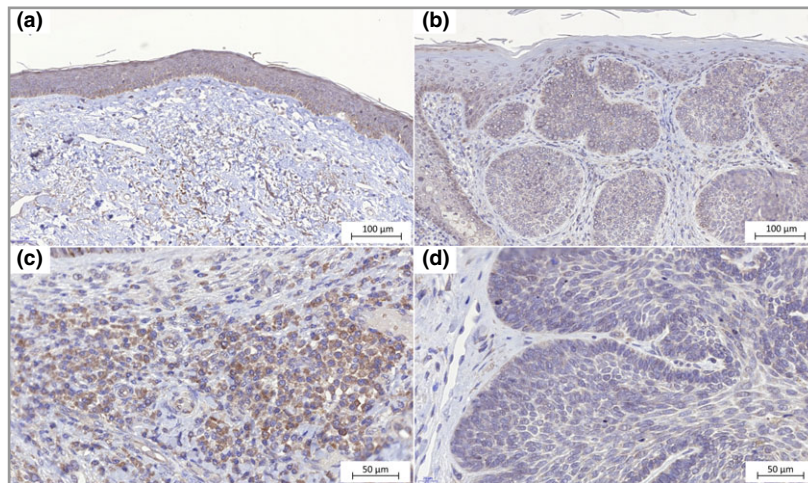
Microarray analysis requires high-quality RNA, and extraction from skin is generally difficult due to the high presence of

RNases. We aimed at seeking differences between tumours and matched nontumour tissues ( $n = 14$ ), and by matching thus minimizing the impact of variations between individuals. Only eight of 28 samples had RIN  $> 6.5$  and 260/280 and 260/230 absorbance ratios  $> 1.8$ , which is the reason why the microarray data analysis was based on group-wise differences between tumours ( $n = 4$ ) and nontumour tissues ( $n = 4$ ). Low study power was taken into consideration when processing data by using a stringent multiple correction method to adjust false-discovery rates. The more classical approach of selecting biologically relevant DEGs is using an FC cut-off point of 2, an arbitrary number not necessarily reflecting the most important changes as even small changes may be of relevance.<sup>24</sup> As our data are restricted with the small number of patients and an





**Fig 5.** SLC7A8 expression in basal cell carcinoma. (a) Epidermis with normal appearance adjacent to the tumour, none-to-weak immunohistochemistry staining ( $\times 20$ ). (b) Epidermis directly above the tumour mass shows none-to-weak staining. The tumour cells seem to express SLC7A8 heterogeneously as they display various degrees of staining intensity ( $\times 20$ ). (c) Some tissues had areas of tumour cells with strong membrane and cytoplasmic staining ( $\times 20$ ). (d) Staining of SLC7A8 in membranes of tumour cells ( $\times 40$ ).



**Fig 6.** TDO2 expression in basal cell carcinoma. (a) Epidermis with normal appearance adjacent to the tumour shows weak immunohistochemistry staining ( $\times 20$ ). (b) Epidermis directly above the tumour mass shows none-to-weak staining. Stroma surrounding the masses of tumour cells shows immunoreactivity ( $\times 20$ ). (c) Immune cells with cytoplasmic TDO2 staining ( $\times 40$ ). (d) Staining of TDO2 in tumour cells ( $\times 40$ ).

assumption of heterogeneity among samples, we selected DEGs with  $FC \geq 5.0$  and made modest interpretations of the results. Despite study limitations, the DEGs with the highest FCs were both biologically relevant (e.g. MMPs and members of Hedgehog and Wnt signalling pathways) as well as in concordance with other studies of BCC gene expression.

Subsequently, in order to retrieve genes that fall into the focus of our research field and for restricting the number of DEGs, we conducted GO analysis. Our primary goal was to find DEGs involved in amino acid metabolism and transport across cellular membranes. Sorting on statistical significance, the top GO term was 'Plasma membrane part', branching further into 'Plasma membrane region' where several candidate genes were identified and selected for further validation

(SLC7A5, SLC7A7, SLC7A8 and TDO2). Sequentially, the candidate genes were analysed using qPCR, a method allowing lower RNA quality compared with microarray analysis, given that each target gene expression is normalized against a set of (here, three) reference genes measured in that same individual sample.<sup>25</sup> To confirm whether alterations in gene expression followed altered protein expression, we also performed IHC in corresponding FFPE BCCs. The grading of protein expression, or staining intensity, was restricted to tumour cells and generated H-scores that were further correlated to gene expression. However, the expression of selected proteins was evaluated descriptively in abdominal skin from two healthy donors.

SLC7A5 and SLC7A8 code for LAT1 and LAT2, respectively. Both are  $Na^+$ -independent heteromeric amino acid transporters



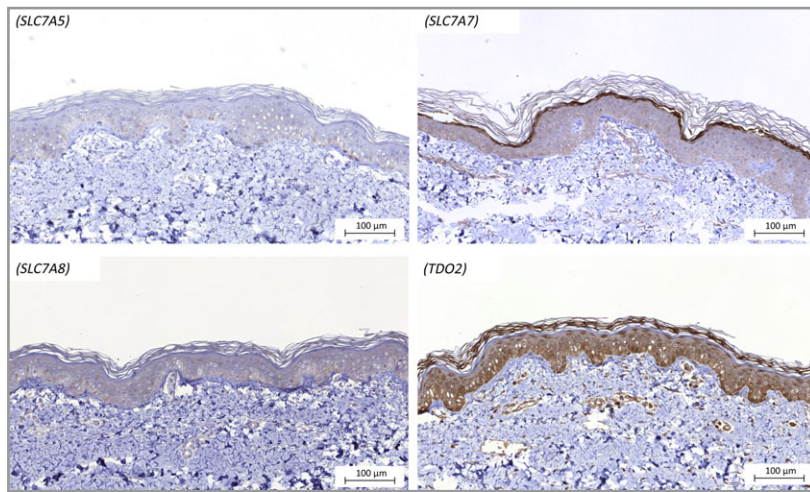


Fig 7. Representative micrographs of protein staining of SLC7A5, SLC7A7, SLC7A8 and TDO2 in abdominal skin from healthy donors.

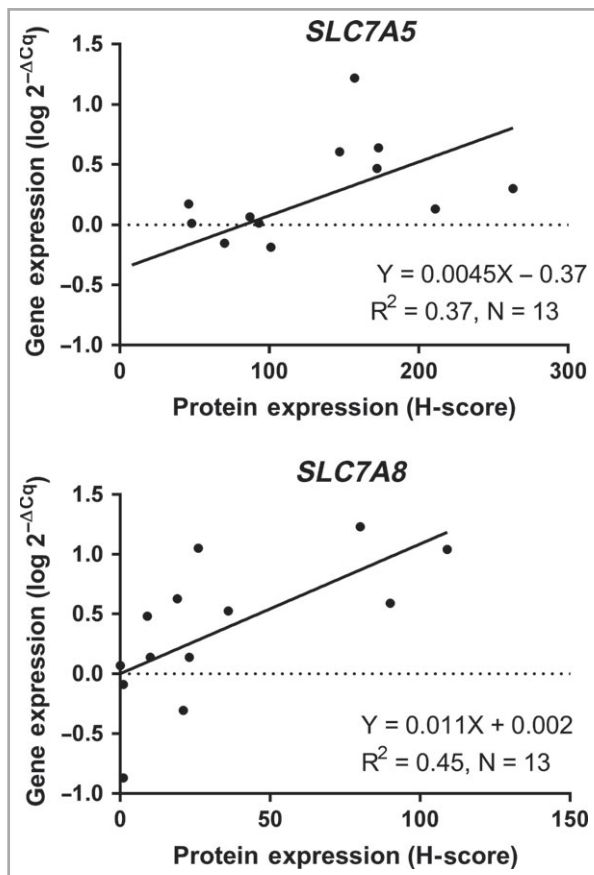


Fig 8. Estimation of  $R^2$  by linear regression and Spearman's correlation between basal cell carcinoma gene expression ( $\log 2^{-\Delta Cq}$  values) and the protein expression (H-score) of SLC7A5 and SLC7A8.

and associate with 4F2hc (SLC3A2) to be functional.<sup>26</sup> SLC7A5 and SLC7A8 transport large neutral amino acids (e.g. tyrosine, tryptophan and alanine) across the cell membrane. Increased expression of SLC7A5 in tumours is suggested to be of

importance for cell growth and survival<sup>26</sup> and the effects of inhibitory drugs are being evaluated for cancer treatment.<sup>13,14,27</sup> SLC7A8 is less well studied in cancer, but found to be overexpressed in neuroendocrine tumours.<sup>28</sup> We found both SLC7A5 and SLC7A8 overexpression in BCC (Figs 1 and 8), verified by gene and protein analysis, which supports previous reports of altered expression in tumour cells.

Upregulation of SLC7A7, which encodes a Y<sup>(+)</sup>L-type amino acid transporter (y<sup>+</sup>LAT1), could not be verified when analysing gene expression with qPCR as there was no statistically significant difference between the tumours and their matching nontumour tissues. The function of SLC7A7 is transporting amino acids (e.g. lysine) across the membrane in both a Na<sup>+</sup> independent and dependent manner in association with 4F2hc.<sup>26</sup> Interestingly, protein expression of SLC7A7 was detected in the stratum granulosum in the epithelia (Figs 4a–c and 7). During the cornification process, the cells in the stratum granulosum become more flat and a specific gene cluster named 'epidermal differentiation complex' generates expression of specific proteins such as profilaggrin, involucrin and loricrin. The nuclei and other cellular organelles degrade and an intracellular increase of Ca<sup>2+</sup> activates transglutaminases. The transglutaminases cross-link cellular proteins to form the cornified envelope close to the cell surface and eventually the only cellular proteins left are keratins (containing lysine), providing mechanical strength to the stratum corneum.<sup>29</sup> Our results show that SLC7A7 protein is strongly expressed in the stratum granulosum, indicating that SLC7A7 may have a role in the process of cornification, possibly by transporting lysine. Studies have shown upregulation of SLC7A7 in glioblastoma;<sup>30,31</sup> however, after qPCR validation, our study fails to demonstrate any alterations in BCC.

Among the most upregulated genes was TDO2, encoding the cytosolic enzyme TDO. TDO catalyses the first step of degradation of the essential amino acid tryptophan. A similar reaction is catalysed by IDO, a protein well studied for its immunosuppressive effects on T lymphocytes.<sup>32</sup> The effects of TDO are relatively unstudied but suggested to be analogous to those of

IDO. Previous studies have shown TDO2 upregulation in, for example, malignant melanoma, bladder carcinoma, hepatocarcinoma and triple-negative breast cancer.<sup>32,33</sup> According to our results, the majority of BCC cells did not express TDO2 protein. Similarly to IDO, IHC staining was confined to stromal cells (i.e. immune cells) and to the tumour microenvironment. There was no correlation between TDO2 protein and gene expression as scoring of protein was restricted to tumour cells and gene expression generated from whole tumour tissue, including stroma and stromal cells. TDO2 is suggested to promote carcinogenesis, as tryptophan catabolism seems to be an important factor in the immunosuppressive microenvironment of many types of cancers.<sup>34</sup> Because IDO inhibitors have shown promising results in melanoma treatment, it is plausible that TDO2 may also serve as a target in BCC,<sup>10</sup> although abdominal epidermis from the two healthy donors was, according to IHC, indicated to have higher TDO2 protein expression than epidermis in BCCs. In order to elucidate any biological importance of the findings in present study, *in vitro* functional studies with gene modulation will be a first start.

In conclusion, by using different methods we have identified upregulation of the amino acid transporters SLC7A5, SLC7A8 and the cytosolic enzyme TDO2 in BCC. This study thereby adds knowledge to forthcoming studies of therapeutic modalities. In addition, we found that the lysine transporter SLC7A7 was exclusively expressed in the stratum granulosum of normal epidermis, which, to the best of our knowledge, has not been shown previously.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Table S1** Genes and entities with differential expression in basal cell carcinoma tumours compared with nontumour tissue.

**Table S2** Differentially expressed genes (DEGs) in basal cell carcinoma after removal of entities corresponding to pseudogenes and lncRNA etc.

**Table S3** Gene Ontology analysis with 211 enriched terms (corrected  $P < 0.1$ ).

**Table S4** Genes included in GO:term Plasma membrane part; Intrinsic component of plasma membrane; Plasma membrane region.

**Powerpoint S1** Journal Club Slide Set.

**Video S1** Author video.