

Expression of hedgehog signalling pathway in anaplastic thyroid cancer

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Abstract The purpose of this work is to study the activation of the hedgehog signalling pathway is associated with tumour progression in various types of cancer, hence the development of specific antagonists raises hope for new therapeutic strategies. Therefore, the expression of hedgehog pathway components in anaplastic thyroid cancer (ATC) and effects of the hedgehog inhibitor Cyclopamine on ATC cells were investigated in this study. Expression of the ligand Sonic Hedgehog (SHh), the transmembrane protein Smoothed (Smo), the receptor Patched (Ptc) and the target gene Gli-1 was evaluated in two ATC cell lines (Hth 74, C643) by RT-PCR and in tumour specimens by immunohistochemistry. The corresponding gene products were examined by western blotting analysis. After treatment with different concentrations of Cyclopamine the time-dependent course of cell viability in ATC cell lines was evaluated by MTT assay. SHh, Smo, Ptc and Gli were clearly expressed on mRNA and protein levels in both cell lines and in tumour samples (41 %SHh, 65 %Smo,

65 %Ptc and 65 %Gli). Treatment with Cyclopamine showed a time- and dose-dependent inhibition of cell numbers with IC50 values between 1 and 4 μ M in both cell lines, comparable to other types of cancer. In conclusion, we believe that the hedgehog pathway is expressed in anaplastic thyroid carcinoma specimens and proliferation of ATC cell lines can be influenced by the Hh inhibitor Cyclopamine. Aberrant activation of this pathway might be involved in the aggressive biology of anaplastic cancer and further evaluation regarding a possible clinical impact of pathway inhibition is warranted.

Keywords Hedgehog signalling pathway · Cyclopamine · Anaplastic thyroid cancer · MTA

Introduction

Carcinomas of the thyroid gland are the most common malignant diseases of the endocrine system, displaying a rising incidence in recent years. Although only 1–2 % of thyroid cancers (TC) are anaplastic [1] they contribute to up to 50 % of the TC associated mortality with a median survival of 3–5 months [2]. The reason for this is the failure of standard therapeutic procedures in anaplastic thyroid cancer (ATC), as well as its resistance to conventional chemotherapy, resulting in an urgent need for effective new strategies. Anaplastic thyroid cancer is distinguished from other types of thyroid cancer by extremely fast growth and aggressive behaviour. Recent advances in understanding the genetic and molecular pathogenesis of ATC hold promise for targeted therapy, based on identification of novel selective inhibitors in recent years. One promising new approach identified, especially for tumours with aggressive behaviour, is the hedgehog-signalling

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pathway which influences cell growth and cell differentiation during embryonic development. In adult tissue it is important for regeneration after tissue damage.

In the last few years, it became evident that the hedgehog-signalling pathway plays a role in the tumorigenesis of various cancers, including pancreatic, breast, hepatocellular, basal cell cancer and medulloblastomas [3–8]. This pathway is initiated by binding of one of three secreted ligands found in mammals, Sonic (SHh), Indian (IHh) and Desert (DHH) Hedgehog to Patched (Ptc), a transmembrane receptor with twelve domains and intracellular situated N- and C-terminus. Patched has two hydrophilic extracellular slings that act as a binding site for the ligand SHh [9, 10].

In the absence of a ligand, Patched represses the signalling of the seven-transmembrane G-protein coupled protein Smoothed (Smo) [11]. In humans, germline mutations of Patched resulted in the development of medulloblastoma and basal cell carcinoma, suggesting that its role in inhibiting Hh signalling is associated with a critical function as a tumour suppressor [12]. If the repression of Smo is stopped, the transcription factor Gli is modulated so that Gli-1 acts as a transcription activator, Gli-3 as a repressor and Gli-2 either activates or represses the transcription. The balance between the activating and repressing forms of Gli results in the expression of target genes, including Ptc and Gli-1 [13, 14]. Gli-1 is the most potent isoform inducing cellular transformation [15]. Many of the mutations in human cancers result in overexpression of the Hh ligand, inactivation of Patched, or increased activity of Smo. In some types of cancer, for example the basal cell carcinoma, the pathway is activated by increased activity of Gli-1 [16]. The transcription of Ptc is induced by Hh pathway signalling, thus generating a negative feedback loop and serving as a convenient indicator of pathway activation. The SHh pathway was suggested as a possible predictor of malignancy in follicular and papillary carcinoma of the thyroid gland [17]. The Hh pathway has recently been investigated in a panel of thyroid cancer specimens and cell lines, focussing on differentiated thyroid cancer (DTC). Expression of the main components of the Hh pathway as well as the ability to inhibit cell proliferation and the induction of cell cycle arrest by cyclopamine was demonstrated for DTC; however, only preliminary data was given regarding ATC [18]. To investigate the role of Hh pathway activity in cell proliferation of ATC we also used cyclopamine, a plant-derived pathway antagonist that acts specifically at the level of Smo [19]. In previous investigations, antiproliferative and antitumourigenic effects have been shown by cyclopamine for medulloblastoma [20], basal cell carcinoma, hepatocellular carcinoma and pancreatic carcinoma [21]. It has been demonstrated that the effect of cyclopamine treatment in

medulloblastomas is due to inhibition of cell proliferation and initiation of cell differentiation [20]. Cyclopamine and some other Smoothed antagonists are currently entering phase I trials [22]. An analogue of cyclopamine, GDC-0449 (Hh antagonist) is being tested in phase II clinical trials in metastatic colorectal cancer with concurrent chemotherapy and Bevacizumab as first-line therapy, however results have not been published yet (ClinicalTrials.gov).

This is a report about activation of hedgehog signalling in anaplastic thyroid carcinoma cells and tissue and of the functional aspects of Hh inhibition. The results presented here suggest that inhibition by specific agents might be a future option in the therapy of this fatal disease.

Materials and methods

Cell cultures

Two anaplastic thyroid cancer cell lines (C643 and Hth74), which were obtained from Dr. Nils-Erik Heldin (University of Uppsala, Sweden) [23], were used. For maintenance, cells were grown in standard medium (DMEM-h21/Ham's F12 1:1 (v/v) supplemented with 10 % FCS) with 1 % antibiotic (Penicillin/Streptomycin) at 37 °C, 5 % CO₂ in fully humidified air. During in vitro experiments standard medium was changed to serum-starved conditions (2 % FCS = DMEM-h21/Ham's F12 1:1 (v/v) supplemented with 2 % FCS). To assess cell viability, the trypan blue exclusion test was used. A pancreatic cancer cell line (Panc1), known to express Hh components [24] was used as a positive control.

Drugs

Cyclopamine (3 β , 23R)-17,23-Epoxyveratraman-3-ol) was purchased from LC Labs (LC Laboratories, Woburn, MA, USA) and stock solutions of 10 mM were prepared in 100 % Ethanol. For use in experiments, further dilutions were carried out using serum-starved medium (2 % FCS).

Western blot analysis

Cells grown in FGM/standard medium were lysed in RIPA containing protease inhibitor cocktail from Roche (Roche, Basel, Switzerland) and stored at –80 °C until use. Protein concentration was calculated with the BCATM Protein Assay (Pierce, Rockford, IL, USA). For the Western blot analysis 50 μ g protein per lane were used. After electrophoresis on 8 % SDS-PAGE proteins were transferred onto nitrocellulose membranes (HybondTM-ECL 0.45 μ m) (Amersham, Piscataway, NJ, USA). Membranes were blocked in 3 % skimmed milk powder in TBS (20 mM Tris, 150 mM NaCl, pH 7.6) for 1 h at room temperature.

Then anti-Gli-1 (H-300) (1:200), anti-Smo (H300) (1:200), anti-Ptc (G-19) (1:100) and anti-SHh (H-160) (1:2000) polyclonal antibodies (all: Santa Cruz, Heidelberg, Germany) were applied overnight at 4 °C. Afterwards membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark, 1:2,000) for 1 h at room temperature. The final detection of specific proteins was carried out using ECL™ Western Blotting Detection Reagent (Amersham, Piscataway, NJ, USA) and visualization on X-ray films. To assess equal loading anti-β-Actin staining was used (AC-74, Sigma, St. Louis, MA, 1:3,000).

RNA collection, cDNA synthesis and reverse transcription PCR analysis

RNA was extracted from cells grown in standard medium by Trizol. Further purification was made using the RNeasy® Mini kit (Qiagen, Hilden, Germany) and contaminating DNA was eliminated by treatment with DNaseI RNase-free DNase Set (Qiagen). Then RNA (5 µg each) was reverse transcribed into cDNA (Superscript III Reverse Transcriptase; Invitrogen, Grand Island, NY, USA) and stored at -80 °C until use.

For semiquantitative analysis cDNA (2 µl each) was subjected to RT PCR using ReadyMix™ Taq PCR Reaction Mix (Sigma, Munich, Germany) and specific primers for Smo, Gli1, Ptc, SHh and β-Microglobulin, as detailed in Table 1. Amplification was performed at annealing temperatures of 55 °C (Smo, Gli-1, β-Microglobulin) or 60 °C (Ptc and SHh) using an Eppendorf cycler. Cycles of amplification were 35–40. The amplification products were separated by electrophoresis on 1.5 % agarose gels and visualized by ethidium bromide staining. The human pancreatic carcinoma cell line Panc-1, which is known to express Hh proteins, served as a positive control.

Real-time PCR

RNA were prepared and reverse transcribed into cDNA as described above. Real Time PCR was performed on a Step One Plus Cyclers (Applied Biosystems, Foster City, CA,

USA) using TaqMan® Fast Universal PCR Master Mix and TaqMan Gene Expression Assay for Gli1-1, Patched and GAPDH (all: Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

The cycling parameters were: *Taq* activation 95 °C for 10 min, denaturation 95 °C for 15 s and annealing/extension 60 °C for 1 min (40 cycles). Data were analysed in Microsoft Excel using the comparative C_T ($\Delta\Delta C_T$) method. The amount of the targets Gli1 and Ptc1 ($2^{-\Delta\Delta C_T}$) was obtained by normalization to GAPDH as an endogenous reference. Estimations were made for each sample triplicate.

Proliferation array

Cellular proliferation was assessed by triplicate plating cells at a density of 1×10^4 cells/well in a 96-well plate. Cyclophamide at varying concentrations (5, 4, 3, 2, 1 and 0.5 µM) or vehicle alone (1 % EtOH) were added in media containing 2 % FCS to the cells 24 h after plating. Fresh medium containing drug or vehicle was added after 72 h. Cell viability was evaluated by MTT assay after 24, 72 and 144 h. Experiments were repeated three times.

Multi tissue array (MTA)

A Multi tissue array (MTA) was prepared from formalin-fixed, paraffin-embedded tumour tissue from 24 ATC-patients. The accuracy of ATC diagnosis was confirmed by additional histopathologic examination by an independent pathologist. For preparation punches of 1 mm in diameter were used. As a positive control a punch of a neuroendocrine tumour (NET) of the ileum was used and normal thyroid tissue as negative control. For immunohistochemical analysis 4 µM sections were made and processed for immunohistochemistry.

Immunohistochemical staining procedure

For staining standardized protocols were used. After melting, antigen retrieval was performed by incubation in citric acid buffer (10 mM, pH 6) for 20 min in a

Table 1 Primers for RT-PCR (Eurofins MWG Operon, Ebersberg)

Gli-1	Sense	5' CTC CCG AAG GAC AGG TAT GTAAC 3'
	Anti-sense	5' CCC TAC TCT TTA GGC ACT AGA GTTG 3'
Patched (Ptc)	Sense	5' GTT GGA AGA AAA CAA ACA GC 3'
	Anti-sense	5' AGC CGT CAG GTA GAT GTA GA 3'
Smoothered (Smo)	Sense	5' GTT CTC CAT CAA GAG CAA CCAC 3'
	Anti-sense	5' CGA TTC TTG ATC TCA CAG TCA GG 3'
Sonic hedgehog (SHh)	Sense	5' TGC AGG ACT CTA ATC CAA GTT TCC 3'
	Anti-sense	5' GTG GGA TGG CTG CCA GC 3'
β-Microglobulin	Sense	5' CTA TCC AGC GTA CTC CAA AG 3'
	Anti-sense	5' AAG TCA CAT GGT TCA CAC GG 3'

microwave oven at 600 Watt. Then slides were deparaffinized and rehydrated in graded alcohol. Endogenous peroxidase was blocked by H₂O₂ (3 %) and unspecific binding by treatment with 10 % BSA (Sigma Aldrich, Munich, Germany) in PBST for 1 h at room temperature. Antibody incubation was carried out at 4 °C overnight. The following antibodies were used: monoclonal rabbit anti-SHh (C9C5) (cell signalling, 1:50), polyclonal rabbit anti-Smo (H 300) (Santa Cruz, 1:50), polyclonal goat anti-Ptc (Santa Cruz, 1:300) and polyclonal rabbit anti-Gli (H-300) (Santa Cruz, 1:200). Visualization was performed using ABC-staining kits (Vector Labs, Burlingame, CA) and slides were counterstained with haematoxylin, according to Mayer. Positive staining was in brown.

Immunohistochemical analysis

Each slide was analysed by microscopy (Leica DMLB, ×10, ×40) by two independent investigators. For evaluation of the staining results, a comparing score system of 5 items (positive, negative, not evaluable, double positive and three times positive), according to Wiseman [25], was used.

Statistical analysis

To analyse the effect of increasing Cyclopamine concentrations on optical density (OD) (144 h) for each cell line, we first tested for proof of action using a non-parametric trend test [26]. Next, we used the method of Hasler and Hothorn [27] to find the minimum concentration among those used at which a significant effect on OD was detectable. In order to estimate parameters from the dose-response relationship such as the maximum effect and dose resulting in 50 % inhibition of growth, we fit a sigmoid Emax model [28] to the data.

Results

Expression of the four main components of Hh signalling pathway

Western blot analysis

Using standardized Western blot analysis SHh, Smo, PTC and Gli-1 protein were found clearly expressed in the two ATC cell lines C643 and Hth74 (Fig. 1a). Both SHh and Ptc showed a comparable expression in C643 and Hth74 cells that was almost as high for SHh as in Panc-1 cells, which were used as a positive control. Ptc was, compared to Panc-1, expressed to a lesser extent. Smo seemed to display a weaker signal in the Hth74 cell line when

compared to C643 and Panc-1. Gli-1 protein was difficult to demonstrate by Western blotting. Only weak signals were found, which were most intense in Hth74 cells. A more distinct Gli-1 signal was detected by PCR (see Fig. 1b).

Reverse transcriptase PCR (RT-PCR)

Gene expression of the hedgehog components was analysed by RT-PCR. Positive results were found for all components in anaplastic C643 and Hth74 cells as well as in Panc-1 cells (pancreatic cancer), which were used as a positive control (Fig. 1b). Gli-1 (217 bp) and Smo (238 bp) could easily be displayed with a signal that was as strong as the one from Panc-1 both in C643 and Hth74. Differences were seen for Ptc (277 bp) with a low signal in C643 cells. SHh (476 bp) showed weak signals in all cell lines with a signal for Hth74 comparable with Panc-1 and a signal of lesser intensity for C643.

Real time PCR (qPCR)

Quantitative analysis of gene expression was performed by qPCR using the $\Delta\Delta$ CT method. Compared to Panc-1 cells, which are known to express high levels of hedgehog proteins, Gli-1 was notably less expressed in the ATC cell lines Hth74 and C643. Ptc expression in Hth74 cells, however, was similar to Panc-1 cells, whereas in C643 cells Ptc expression was only half as much as in the Panc-1 cell line (Fig. 1c).

Expression of four main components of hedgehog signalling pathway in human ATC samples

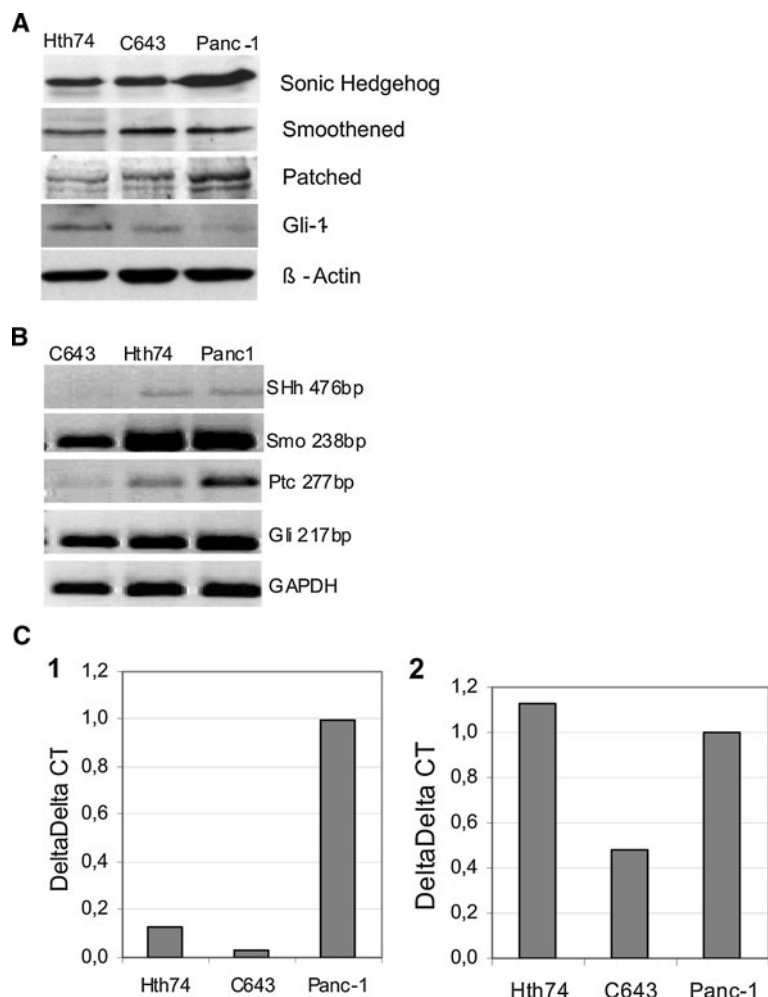
Immunohistochemistry

A Multi tissue array (MTA) containing 24 samples of anaplastic thyroid cancer tissue was analysed for the expression of hedgehog components by immunohistochemistry. Although the original tumours were carefully screened before conducting the punch biopsies, 7 samples had to be excluded because of necrosis and fibrosis. From the remaining 17 samples a total of 11 stained positive for hedgehog components.

In detail, 41 % of the samples stained positively for SHh (7/17), 65 % (11/17) for Smo, 65 % (11/17) for Ptc and 65 % (11/17) for Gli-1, as documented in Table 2, however with different staining intensities. For assessment of staining intensity an internal score system was used, as described in detail in [Materials and methods](#). According to this score, a total of four tissue punches displayed a weak staining (+), which was clearly distinguishable from negative staining. The other samples were assessed as double

Fig. 1 a Detection of hedgehog proteins Sonic Hedgehog (SHh), Smoothened (Smo), Patched (Ptc) and Gli-1 by Western blot analysis in ATC cell lines Hth74 and C643 in comparison to the pancreatic cancer cell line Panc-1 (positive control). β -Actin is shown as loading control.

b Gene expression of the Hedgehog components in anaplastic thyroid cancer cell lines C643 und Hth 74 and pancreatic cancer cell line Panc-1 as revealed by RT PCR. c Gli-1 (1) and Ptc (2) were quantitatively analysed by real time PCR using the DeltaDeltaCT method (which means relative gene expression). Shown: relative gene expression of Hth74 and C643 cells in comparison to the pancreatic carcinoma cell line Panc-1



or three times positive as documented in Table 2. Co-expression of all four components was found in 7 of the 11 positively stained samples. Immunohistochemical staining was found intracytoplasmatic in tumour cells. No nuclear localization was found. Tissue from the normal thyroid gland stained negatively for all hedgehog components. Examples of the original MTA specimens are given in Fig. 2.

Effect of pathway inhibiting drug (cyclopamine) on ATC cell lines

Treatment of proliferating Hth74 and C643 cells with increasing concentrations of cyclopamine (0.5–5 μ M) over 144 h resulted in a time- and dose-dependent inhibition of cell viability as assessed by MTT-assay (Fig. 3). For the Hth74 cells, a slightly delayed effect after exposure to the drug was seen, when compared to C643 cells. Altogether, the effect on cell viability was most pronounced in Hth74 cells. Here treatment with Cyclopamine at 5 μ M over 144 h resulted in a significant reduction of cell density of

Table 2 Summary of the immunohistochemical staining

Antigenes	+	++	+++	Positive	Negative
SHh	4 (24 %)	2 (12 %)	1 (6 %)	41 %	10 (59 %)
Smo	4 (24 %)	4 (24 %)	3 (18 %)	65 %	6 (35 %)
Ptc	4 (24 %)	4 (24 %)	3 (18 %)	65 %	6 (35 %)
Gli-1	4 (24 %)	6 (35 %)	1 (6 %)	65 %	6 (35 %)

The table shows the results of immunohistochemical staining of 17 samples of human anaplastic thyroid cancer tissues

+ Light positive reaction, ++ clear but weak reaction, +++ strong reaction

about 80 % (mean 0.56 ± 0.12 vs. 0.11 ± 0.04), whereas in the C643 cell line cell density decreased by about 68 % (mean 0.72 ± 0.16 vs. 0.23 ± 0.16) ($P < 0.05$). Treatment with Cyclopamine at a concentration as low as 1 μ M resulted in a reduction of 52 % (mean 0.56 ± 0.12 vs. 0.27 ± 0.06) ($P < 0.05$) in Hth74 and 43 % (mean 0.72 ± 0.16 vs. 0.41 ± 0.09) in C643 cells. IC_{50} values at 144 h were calculated as 0.8 ± 0.2 μ M for Hth74 cells and 2.2 ± 1.1 μ M for C643 cells.

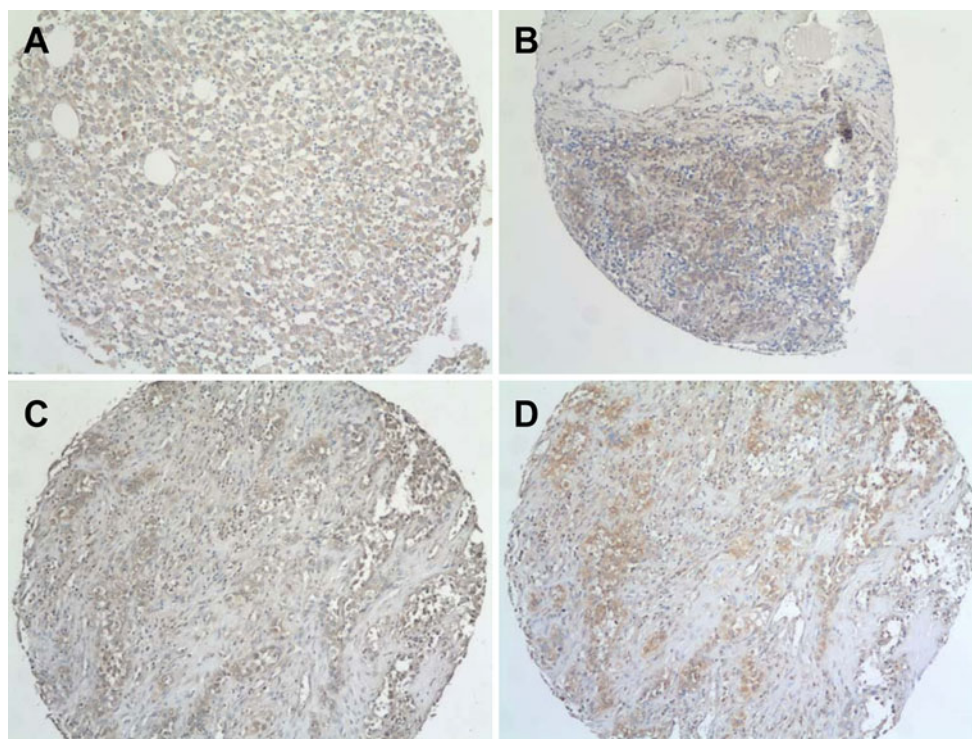


Fig. 2 Examples of Hedgehog protein detection **a** Gli-1, **b** Sonic Hedgehog (SHh), **c** Patched (Ptc) and **d** Smoothed (Smo) by immunohistochemical staining in punch biopsies of human ATC

tumour specimen. Representative examples from a multi tissue array (MTA) are depicted in 10× magnification

Discussion

The hedgehog (Hh) signalling pathway regulates cell proliferation, tissue polarity and cell differentiation during embryonic development. hedgehog signalling is regulated by the availability of SHh ligand and negative feedback mechanisms such as increased Patched (Ptc) expression in response to pathway activation.

To determine whether the proteins associated with the pathway are expressed in ATC, we examined ATC cell lines and human ATC specimens for transcripts related to Hh signalling such as Ptc, Smo, SHh and Gli-1. In the present study we could show the expression of the four main components in two anaplastic thyroid cancer cell lines (Hth74, C643) by western blotting analysis and confirmed the results by documenting gene expression of SHh, Ptc, Smo and Gli-1 using reverse transcriptase PCR, displaying more consistent results. High expression of the components is consistent with results from other tumour cell lines, such as ovarian and renal cell carcinoma cell lines described previously, showing an activated hedgehog pathway [29, 30] and confirm the results of recent investigations in single DTC and ATC cell lines [18].

Hh pathway activation is usually associated with increased expression of Ptc. Our data, revealed by quantitative real-time PCR, represent high levels of target gene

Ptc for Hth74 comparable to the pancreatic cancer cell line Panc-1, used for a positive control. This might be due to ligand-dependent pathway activation [31]. Ligand-dependent pathway activation is a prerequisite for the effectiveness of a Smo inhibitor as employed in our functional experiments. Since a strong Gli-1 expression was shown for both cell lines, a ligand independent activation might be a possible explanation. This type of activation was demonstrated for medulloblastomas [20] and ovarian cancer cell lines [29]. By definition, ligand responsiveness must be associated with some level of functional Ptc expression, as demonstrated especially for Hth74 (Fig. 1a, b). Bhattacharya et al. [29] suggested that the autonomous activation of the Hh pathway in ovarian cancer results from a combination of high levels of Hh ligand expression and low levels of Ptc that are unable to suppress the signalling response.

When evaluating human anaplastic thyroid cancer samples for Hh expression characteristics in a semiquantitative manner, we found a positive reaction for Smo, Ptc and Gli-1 in 65 % of the samples and for SHh in 41 % of the samples. In a series of 13 papillary carcinoma specimens a positive reaction for Smo was found in 23 %, for Ptc in 42 % and for SHh in 85 % [17].

A relationship between a high expression of SHh and carcinogenesis in papillary thyroid cancer has been

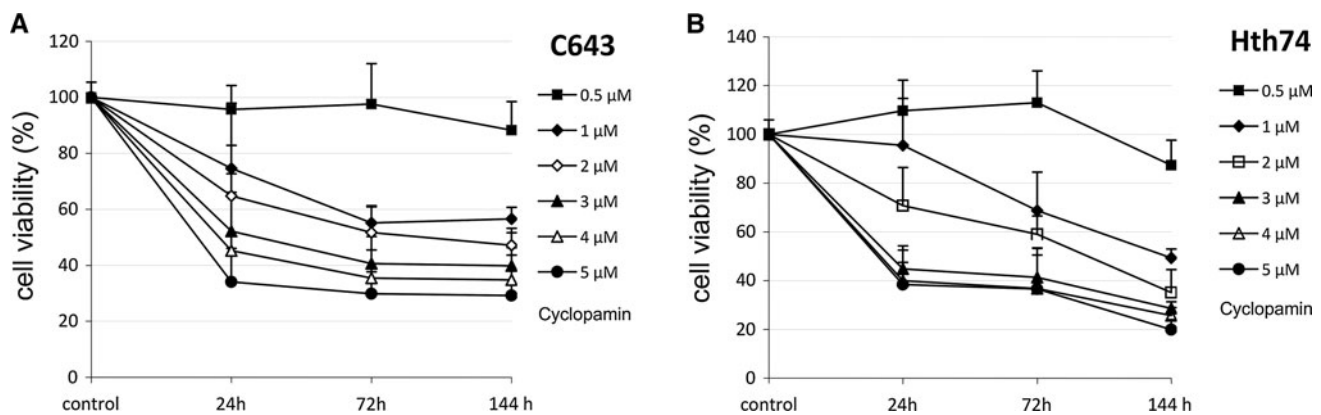


Fig. 3 Proliferation of anaplastic tumour cell lines under treatment with Cyclopamine in rising concentrations from 0.5 to 5 μM during 144 h, evaluated by MTT-Assay. A dose-dependent effect is shown for ATC cell lines C643 **a** on the left and Hth74 **b** on the right. IC_{50}

C643 = $2.2 \pm 1.1 \mu\text{M}$; IC_{50} Hth74 = $0.8 \pm 0.2 \mu\text{M}$. A significantly reduced cell number ($P < 0.05$) was detected at concentrations higher than 1 μM . Cell viability (%) means (%) of untreated cells at the given time point

suggested, based on the relationship to follicular adenoma that expressed SHh to a lesser extent (31 %) [17]. Another clinical series (Xu et al.) [18] mainly investigated activation of the SHh pathway in follicular adenoma, papillary and in a small group anaplastic carcinoma cells. They showed positive immunohistochemical staining for SHh (77–87.5 %), Smo (67–87.5 %), Ptc (65–87.5 %) and Gli (68–87.5 %). Since in our samples of human ATC we found a lower expression of SHh, we could not confirm the expression level to be a sign of aggressiveness [17]. Our lower rate of immunohistochemical staining of SHh might not necessarily be caused by reduced activation of the pathway, since high hedgehog activity and missing SHh in the immunohistochemical staining was shown for neuroendocrine tumours [32].

After verifying the expression of the pathway's components, we evaluated a possible impact on tumour cell proliferation by using Cyclopamine, a plant derivate that specifically inhibits Hh signalling at the level of Smo [16, 19, 33, 34]. Cyclopamine has been shown to slow the growth of various types of cancer including SCLC, neuroblastoma [35], lymphoma [36] and renal cell carcinoma [30] in a range between 10 and 95 %, however at concentrations of 15–20 μM .

In our study, inhibition of the Hh signalling pathway by Cyclopamine resulted in time and dose-dependent inhibition of proliferation of anaplastic thyroid carcinoma cell lines. Treatment with Cyclopamine at low doses (up to 5 $\mu\text{mol/l}$) resulted in a 70–80 % inhibition of proliferation of the two ATC cell lines tested. In comparison to Dormoy et al. [30] who suggested a high sensitivity of renal carcinoma cells because of a 80 % reduction of cell viability after 5-days treatment with 20 μM Cyclopamine, our cells seemed to be far more sensitive with a 70–80 % reduction after treatment with 5 μM Cyclopamine. Watkins and Berman [34] showed a growth-inhibiting effect of

Cyclopamine on SCLC cell lines only if both SHh and Gli-1 were expressed in the cells. Furthermore, our results fit well with the results of Bathacharya et al. [29] who showed a 70–90 % reduction of ovarian cancer cell lines after 48 h treatment with 5 $\mu\text{mol/l}$ Cyclopamine.

The cell line Hth74 was shown to be more sensitive to Cyclopamine-driven inhibition of tumour cell proliferation compared to C643 cells. Since we found a higher expression of Ptc and Gli-1 by quantitative PCR for Hth74 compared to C643 in the qPCR, we suggest a correlation between high levels of Hh components and sensitivity to Cyclopamine. A connection between levels of Gli target genes and possible inhibition by Cyclopamine has been postulated several times before [13, 24].

Xu et al. [18] used 9 μM Cyclopamine in ATC cell lines SW1736 and KAT-18 over 96 h. They could demonstrate a significant inhibition of proliferation by 24 % ($P < 0.05$). In comparison to that study we were able to show a significantly reduced cell number by treatment with lower doses between 1–2 μM Cyclopamine.

LoRusso et al. [37] recently published a phase I study with treatment of solid tumours with Cyclopamine and showed a response rate of 50 % with a toxicity between 8–30 % of the treated patients. Although so far no phase II studies were published, first results presented in an abstract at ESMO 2010 reported no clinical correlation between expression levels of Hh components and sensitivity to Hh targeting drugs. Regarding our quantitative results concerning Gli and Ptc content (Fig. 1c), a higher expression level accounted for a more time-dependent course of cyclopamine effects (Fig. 2). Other reports documented a Gli-3 level-dependent sensitivity to cyclopamine in the pancreatic cancer cell line Panc-1, where a knockdown of Gli-3 resulted in an increased sensitivity. In the same study they showed a reduced level of Ptc and Gli-1 after Cyclopamine treatment in Panc-1 [24].

Components of the hedgehog pathway are expressed in anaplastic thyroid carcinoma cells and samples of human ATC tissues and its activity can be influenced by Cyclopamine. We suggest a ligand-dependent activation of the pathway in the anaplastic thyroid carcinoma cell lines because of high Ptc levels that enables a possible inhibition of the signalling by Smoothed inhibitors. Therefore, in vivo and clinical investigation might be warranted, since—as outlined by Elisei [43]—innovative treatment strategies for ATC patients are desperately needed. However, additional mechanisms underlying Hh signalling mediated proliferation have been reported in cancers such as basal cell carcinoma, including a ligand-independent mechanism due to constitutive activation of the signalling. Ligand-independent tumour growth arises from loss of Ptc or SuFu repressor function due to mutation or activating mutations in the SHh effector Smo [38, 39], or to increased expression of other cross-talking signalling [40, 41], whereby the expression of Gli transcription factors can increase independently of Hh ligand [42]. Therefore, additional investigations are required to increase knowledge of Hh-dependent proliferation as a prerequisite for therapeutic approaches in ATC by Hh signalling inhibition.

Conflict of interest The authors declare that they have no conflict of interest.

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