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# *The Diagnostic Significance of the Sonic Hedgehog-Signalling Pathway in Lung Carcinoids*

***Andreas Kirschbaum[1]*; Joana Grups[1]; Pietro Di Fazio[1]; Inga Boeck[2]; Nikolas Mirow[3]; Detlef Bartsch[1]; Christian Meyer[4]***

*[1]Department of Visceral, Thoracic and Vascular Surgery, University Hospital Gießen and Mar-burg (UKGM), Baldingerstrasse, D - 35043 Marburg, Germany.*

*[2]ÜGP – CHC for Pathology, Cytology and Molecular Pathology, Forsthausstrasse 1-3, D - 35578 Wetzlar Germany.*

*[3]Department of Cardiac Surgery, University Hospital Gießen and Marburg (UKGM), Baldingerstrasse, D - 35043 Marburg, Germany.*

*[4]Surgical Department, Bad Wildungen City Hospital, Brunnenallee 39, D - 34537 Bad Wildungen, Germany.*

**Abstract**

**Background:** Typical and atypical carcinoid tumours are relatively rare lesions of the lung. Atypical carcinoid tumours show a more aggressive growth behaviour than the typical ones do. Unambiguous diagnostic classification using the established histological markers can be difficult. The purpose of the present work is to ascertain whether the proteins of the sonic hedgehog-signalling pathway could improve the diagnostic.

**Material and methods:** In 20 patients with a histologically confirmed carcinoid tumour of the lung (9 typical and 11 atypical carcinoid tumours), the expression of SMO, PTCH1, Twist, Vimentin, Snail1 and E-cadherin was examined by immunohistochemical staining. RT-qPCR for the detection of *Snail1* and *Vimentin* transcript was performed. In vitro, real-time growth of the neuro-endocrine lung tumour cell line NCI-H727 was monitored after treatment with the inhibitors of the sonic hedgehog pathway (cyclopamine, GN25 and PEG (polyethylene glycol).

**Results:** PTCH1 staining was detected in all examined samples. SMO was detected in 16 of the cases. Snail1 was stained in 13 and E-cadherin in 19 of the cases. Twist and Vimentin staining was negative in all analysed probes. *SNAIL1* and *Vimentin* transcripts were overall down-regulated. The viability of NCI-H727 cells was inhibited only by very high concentrations of the inhibitors.

**Conclusion:** The detection of the signal proteins of the sonic hedgehog pathway does not allow better diagnostic differentiation between typical and atypical carcinoid tumours. A better understanding of the mechanism of action of the sonic hedgehog pathway in the lung carcinoids could potentially open up new anti-tumour therapy options.

**Keywords:** Hedgehog Signalling Pathway; EMT; Typical Carcinoid Tumours; Atypical Carcinoid Tumours; SMO: PTCH 1; Snail 1; Vimentin; Twist; E-Cadherin.

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**Correspondance:** *Andreas Kirschbaum, Department of Visceral, Thoracic and Vascular Surgery, University Hospital of Gießen and Marburg (UKGM), Baldingerstraße, D-35043 Marburg, Germany.*

*Tel: +49-6421-58-61738, Fax:+49-6421-58- 62543; Email: akirschb@med.uni-marburg.de*

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## Background

Neuro-endocrine lung tumours are generally rare, with only about 1% recurrence of all lung tumours belonging to this group [1]. Bronchoscopy is usually performed for histology sampling. The type of tumour and degree of differentiation of the neuro-endocrine tumour can be determined by HE and PAS staining of the tissue. The mitosis rate in a 2 mm² field of view under the microscope, the occurrence of cell necrosis, and the shape of the nuclei are rated. The typical carcinoids have mitosis rates less than 2/2 mm² and no necrosis. The atypical carcinoids have 2 to 10 mitoses / 2 mm²; isolated cell necrosis can be found. The immunohistochemical marker Ki-67 was introduced to improve diagnostics. The more pronounced this marker is, the faster and more aggressively a neuro-endocrine tumour will grow. For improvement of morphological diagnosis, the Ki-67 marker was combined with the mitosis rate to form an index. However, this so-called Ki-67 index did not improve diagnostics [2]. A clear distinction between a typical and an atypical carcinoid tumour would be important, since atypical neuro-endocrine tumours have a much more aggressive growth behaviour. This parameter has not been achieved in every case yet.

The epithelial–mesenchymal transition (EMT) plays an important role not only in the normal development of cells but also in the tumorigenesis. It is characterised by loss of adhesion properties of epithelial cells, which convert them into cells with mesenchymal characteristics. This process is regulated by numerous glycoproteins. The important adhesion molecule E-cadherin is down-regulated during EMT [3], while the mesenchymal marker Vimentin is up-regulated [4]. Loss of cellular polarity also occurs, which triggers cell migration. Thus, the cytoskeleton becomes increasingly unstable, resulting in tumour invasion and distant metastasis [5]. This process is controlled and influenced by glycoproteins such as Snail1, Twist, E-cadherin, SMO and Vimentin [6].

Here, the sonic hedgehog pathway is an important element for the cellular development. The sonic hedgehog signalling pathway (SHH) is of great importance during the embryogenesis; in adults, its proteins can usually no longer be detected. If the sonic hedgehog signalling pathway is activated for any reason, this indicates development of malignancies in various organs, e.g. the lung, breast or pancreas [7]. Jiang et al. [8] showed that the sonic hedgehog pathway is activated in non-small-cell lung cancer. Hwang et al. [9] demonstrated that the activity of the sonic hedgehog pathway was not only increased but also correlated with the prognosis of the patients affected by advanced lung cancer. Furthermore, Jiang et al. [8] showed in an experimental study that inhibition of the sonic hedgehog pathway in lung cancer reduced tumour cell migration. In a phase-I study in small-cell neuro-endocrine tumours, Rimkus et al. [10] showed that the administration of a SMO inhibitor might be a potentially interesting therapeutic option for the future. In the present work, we address the question as to whether typical and atypical carcinoid tumours express glycoproteins of the sonic hedgehog pathway at all and whether there are any differences between the two lung carcinoids. This aspect could serve for improved diagnostic differentiation between the two tumours. In addition, it will be investigated whether inhibitors of the EMT/sonic hedgehog pathway affect the viability of an established human lung carcinoid cell line.

## Materials and methods

**Patient sample collection:** For the present study, tissue samples of 20 patients with histologically proven typical (n = 9) or atypical (n = 11) carcinoid tumours, who had been operated on at the University Hospital Marburg between 1996 and 2014, were included. The study was approved by the local ethic committee of the medical faculty of Philipps University of Marburg (AZ 68/14) and all patients signed out the inform consent. Four samples of tumour-free lung tissues were collected from formalin-fixed paraffin-embedded (FFPE) blocks obtained from patients who underwent surgical resection of lung adenocarcinoma and metastasis of colon carcinoma. The tissue was found tumour free after pathological examination and was used as control for the PCR experiments.

9 patients (4X ♂ and 5X ♀) were affected by a typical carcinoid tumour had a mean age of 59 years (38 – 79 years). 8 patients were diagnosed with a typical stage-1 carcinoid tumour, and one patient had a stage 2a with a peribronchial lymph node metastasis.

The mean age of the 11 patients (4X ♂ and 7X ♀) with an atypical carcinoid tumour was 53 years (31 – 72 years). 6 patients had stage 1, and 5 patients had stage 3B.

The diagnosis of a typical or atypical carcinoid tumour was unambiguously established for each tissue sample by haematoxylin-eosin (HE) standard staining and immunohistochemical staining for chromogranin and synaptophysin.

### Immunohistochemistry (ICH)

Immunohistochemical staining was performed on the FFPE slices with the respective antibodies against the following sonic hedgehog signal proteins: Vimentin (anti-Vimentin antibody (RV 202) by Abcam), E-cadherin (anti-E-cadherin antibody ab 15148 by Abcam), PTCH1 (anti-Patched/PTCH1 antibody ab 53715 by Abcam), Twist (anti-Twist antibody Twist2C1a-ChIP Grade9), Smoothened (Smoothened antibody NBP 2-24543 by Novusbio), Snail (Snail (CD15D3) rabbit mAb #3879 by Cell Signalling).

The immunohistochemical sections were independently appraised by two experienced pathologists. The colour intensities were classified as – = no staining, + = weakly positive, ++ = moderately positive and +++ = strongly positive. Furthermore, the criteria of Remmele and Stegner [11] were also applied. The colour intensity was estimated from 0 = no staining reaction to 3 = strong staining reaction, and the number of positive tumour cells 0 = no positive cells to 4 = > 80% positive tumour cells. A maximum score (% positive tumour cells × score staining intensity) of 12 can be achieved.

### RT-qPCR

Total RNA was isolated from FFPE slices and converted into cDNA (Bio-Rad Laboratories, Hercules, California, USA). A quantitative polymerase chain reaction (PCR) with specific oligos for *Snail1* (QT00010010 Qiagen, Hilden Germany) and *Vimentin* (QT00095795, Qiagen) was performed by using a RT-qPCR thermocycler CFX96 TM Real-Time System (Bio-Rad Laboratories, Hercules, California USA). Four samples of tumour-free lung tissue were included as control probes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, QT01192646, Qiagen) was used as reference transcript to verify the correct performance of the test procedure. It is therefore suitable as a reference and control gene [12]. Results

were analysed with the Bio-Rad CFX-Manager (Bio-Rad Laboratories) Raw data were further analysed with Rest2009 (relative Expression Software Tool V.2.0.13. Qiagen).

**Cell culture**

A cell line from a 65-year-old woman suffering from a lung carcinoid tumour was used. This cell line, called NCI-H727, was provided by ATCC, Manassas, Virginia, USA. The cells were kept in culture by following the company instructions (ATCC). The culture media were regularly tested for mycoplasma.

**Used substances for culture inhibition**

Cyclopamine (LC Laboratories, Woburn MA, USA, Cat.No. C – 8700, LotBAC – 116), GN25 (Calbiochem GmBH, Bad Soden, Germany; article number: 506179); Polyethylenglycol (PEG) (Sigma-Adrich House, Homefield Buisness Park, Haverhill, United Kingdom; article number: 83276-250G-F)

**Real time cell viability**

NCi-H727 cells were seeded on E-plates (05232368001, OLS, Bremen, Germany) and real-time cell viability was monitored with the xCelligence device for 120h, every 15 minutes, after the addition of the sonic hedgehog inhibitors cyclopamine (1 nM - 100 µM), GN25 (1 nM - 100 µM) and polyethylene glycol (0.5 – 10 %).

**Statistical analysis**

Statistical analysis was performed by EXCEL 2007 (Microsoft, Albuquerque, New Mexico USA) for the analysis of the PCR results. Significance was calculated using one-way-TTEST. $P < 0.05$ were regarded as significant.

## Results

**Detection of the protein level of the sonic hedgehog pathway markers in typical and atypical carcinoid tumours**

In relation to the totality of immunohistochemical staining (n= 20), the investigated signalling proteins showed different degrees of positivity. PTCH1 was detectable in 20 of the examined sections. In 16 of the cases, positive staining of the sections for SMO was found. Snail1 was detected in 13 of the sections examined. E-cadherin was detected in 95% of the sections. However, Twist was not detectable in any of the analysed samples. Staining for Vimentin was detected in 2 of the sections examined (Table 1 and Figure 1).

In addition, the two pathologists independently described the colour intensities as follows for the individual signalling proteins. Among the 9 typical carcinoid tumours examined, there were 5X „++" (55.5%) and 4X „+++" (44.5%) tumour probes positive for PTCH1 staining. For the atypical carcinoid tumours, the positive staining was 9X „++" (81%) and 2X „+++" (19%). The SMO staining intensity of typical carcinoid tumours was 2X „–" (22.2%), 4X „++" (44.4%) and 3X „+++" (33.3%). In the atypical carcinoid tumours, 2X „–" (18.2%), 3X „+" (27.3%), 3X „++" (27.3%) and 3X „+++" (27.3%) were found. For typical carcinoid tumours, Snail1 was found 2X „–" (22.2%), 1X „+" (11.1%), 1X „++" (11.1%) and 5X "+++" (55.5%) in terms of staining intensity. The atypical carcinoid tumours showed 5X „–" (45.45%), 2X „++" (18.18%) and 4X „+++" (36.36%). E-cadherin was strongly positive („+++") in all typical carcinoid tumours. In the atypical carcinoid tumours, 1X no staining was observed, 2X „++" (18.18%) and 8X „+++" (72.72%). Twist staining was found detectable neither in typical nor in atypical carcinoid tumours. In the typical carcinoid tumours, no Vimentin staining could be detected. In the atypical carcinoid tumours, Vimentin was found 1X „+" (9%), 1X „+++" (9%) and in 81.81% „–" (Figure 2). According to Remmele and Steger, the average score for the typical carcinoid tumours is: PTCH1 9.8; SMO 6.9; Snail1 6.2; E-cadherin 10.9; Twist 0, and Vimentin 0. For the atypical carcinoid tumours, the average score is: PTCH1 6.9; SMO 6.2; Snail1 3.1; E-cadherin 9.3; Twist 0 and for Vimentin 0.7 (Table 2).

**Expression of *Snail1* and *Vimentin* transcripts in typical and atypical carcinoid tumours**

The transcript of *Snail1* showed an increased expression compared to normal lung tissue in two analysed samples out of 15. No differences were found between typical and atypical carcinoid tumours. The analysis of *Vimentin* transcript level yielded a similar result. In 4 out of 20 samples examined, an increased gene expression was found in comparison to normal lung tissue. No relevant differences between typical and atypical carcinoid tumours were found (Figure 3).

**Effect of the EMT pathway inhibitors on the viability of the CRL-5815 cells**

The treatment with 100 µM of cyclopamine or GN25 caused a significant reduction of the cell viability (Figure 4A, B). Upon addition of PEG, a reduction of the cell viability was observed only at concentration of 10% (Figure 4C).

The experiments highlight that low concentrations of the three EMT signalling pathway inhibitors are not able to affect the viability of neuro-endocrine tumour cells.

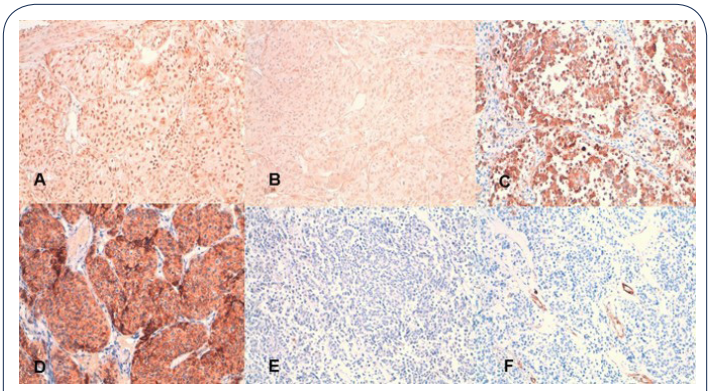


**Figure 1:** Examples of immunohistochemical staining of the sonic hedgehog signal proteins. A) PTCH1; B) SMO; C) Snail1; D) E-cadherin; E) Twist; F) Vimentin. Magnification is 200X.

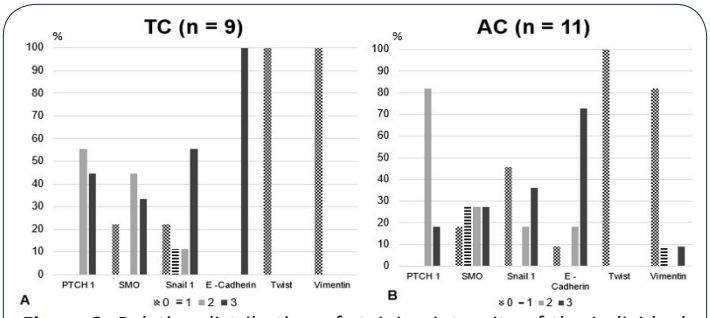


**Figure 2:** Relative distribution of staining intensity of the individual signal proteins in typical [TC] (n = 9) and atypical [AC] (n = 11) carcinoid tumours.

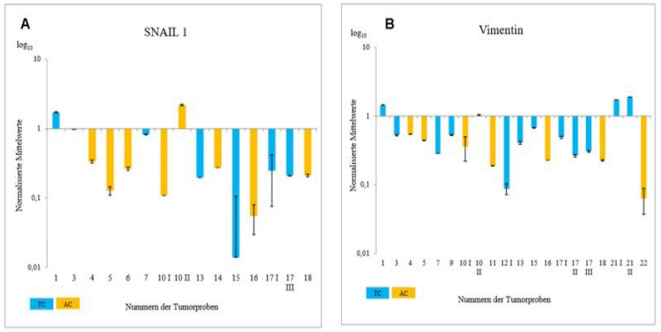


**Figure 3:** Overview of the PCR for A: Snail 1 and B: Vimentin, TC and AC each shown in a separate colour. Shown is the mean log relative expression normalised to GAPDH ± SEM (Standard Error Mean) of experiments performed in triplicates.

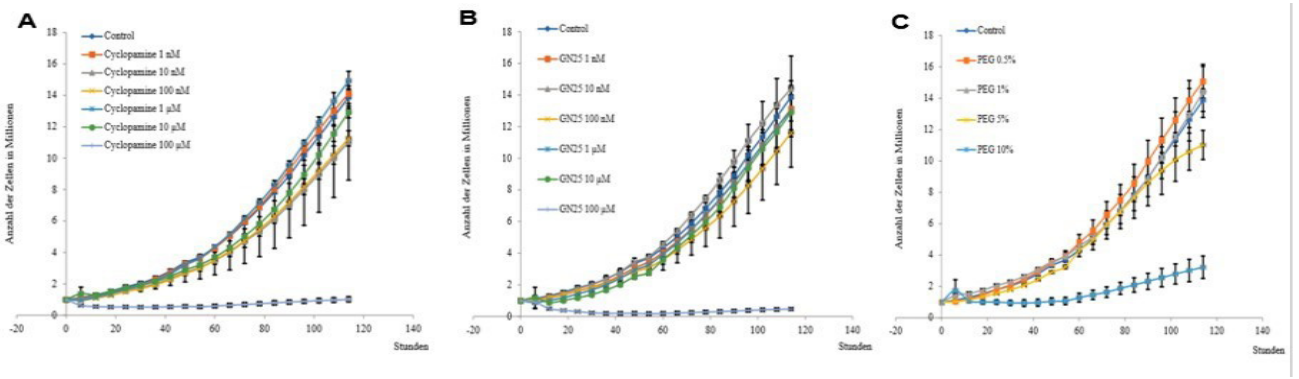


**Figure 4:** Growth curves of the neuro-endocrine lung tumour cell line NCI-H727 after administration of different concentrations of the sonic hedgehog inhibitors Cyclopamine (A), GN25 (B) and PEG (C). Control=untreated cells. The experiments were performed in triplicates.

**Table 1:** Overview of immunohistochemical staining for PTCH1, SMO, Snail1, E-cadherin, Twist and Vimentin (n = 20).

| Signalling protein | Positive detection | Negative detection |
|---|---|---|
| **PTCH 1** | 20 | 0 |
| **SMO** | 16 | 4 |
| **Snail 1** | 13 | 7 |
| **E-cadherin** | 19 | 1 |
| **Twist** | 0 | 20 |
| **Vimentin** | 2 | 18 |

**Table 2:** Results of the Remmele & Steger average score for typical (n=9) and atypical (n=11) carcinoid tumours. Significance based on p<0.05 (Mann–Whitney Test).

| Signalling proteins | TC | AC | P value |
|---|---|---|---|
| **PTCH 1** | 9.8 | 6.9 | 0.23 |
| **SMO** | 6.9 | 6.2 | 0.79 |
| **Snail** | 6.2 | 3.1 | 0.42 |
| **E-cadherin** | 10.9 | 9.3 | 0.31 |
| **Twist** | 0 | 0 | > 0.99 |
| **Vimentin** | 0 | 0.7 | 0.48 |

## Discussion

Neuro-endocrine lung tumours are relatively rare overall. Apart from the rapidly growing tumour types such as the small-cell neuro-endocrine tumour, there are the typical and atypical carcinoid tumours. The atypical carcinoid tumours show a more aggressive growth behaviour compared to the typical carcinoid tumours. Unambiguous diagnostic differentiation between the two types of tumours is of essential therapeutic importance, but it is often difficult yet. In the present study, we addressed the question as to whether the detection of signal proteins of the sonic hedgehog pathway would allow better differentiation.

Despite a previously described down-regulation of PTCH1 and SMO [15], PTCH1 protein was overall detectable and showed no significant differences between the typical and atypical neuro-endocrine carcinoids. Based on the study of Sari et al. [15] highlighting a non-canonical hedgehog pathway, SMO was found expressed in 80% of the sections. Nevertheless, no significant differences were found between typical and atypical carcinoid tumours. The expression of Snail1, a marker of EMT activation, was detected in 65% of the sections, which would support the previous discovery in colon cancer [16]. Once again, no significant difference was found between typical and atypical carcinoid tumours. As consequence of the expression of Snail1, down-regulation of E-cadherin would have to be expected. However, E-cadherin could be detected in 95% of tumour sections. Fendrich V. et al. [17] showed that Snail1

expression differed in strength between tumour segments of liver metastasis of the ileum. In the current study, Snail1 expression was highest at the tumour margins and weakest in the centre of the tumour. E-cadherin was very pronounced in both typical and atypical carcinoid tumours, and there was no significant difference between tumour segments.

The absence of Twist expression in typical and atypical carcinoids highlighted that neuro-endocrine lung tumours differ in terms of biological behaviour from other solid cancer such as breast cancer, endometrial carcinoma, hepatocellular carcinoma and malignant melanoma, which are characterised by a correlation between Twist expression and poor long-term survival [18]. Despite Twist is considered an important regulator of epithelial–mesenchymal transmission [19], it does not seem to exert a role in neuro-endocrine lung tumours.

Vimentin is considered an important factor in tumorigenesis and metastasis capacity in various tumours [20]. Thus, it seems to play a rather minor role in typical and atypical carcinoid tumours. The low expression of Vimentin could reflect an indication of the moderate growth behaviour of carcinoid tumours. Typical and atypical carcinoid tumours showed no significant differences.

Pharmacological influence on the sonic hedgehog pathway represents a potential ant-cancer therapy. This study evidenced that the cell viability could be reduced in neuroendocrine lung neoplastic cells only by the administration of high concentrations of cyclopamine, GN25 and PEG. As well, the SMO inhibitors „saridegib" and "sonidegib" were used for the treatment of pancreatic cancer without achieving a significant breakthrough in the therapy [22]. The antidiabetic drug metformin is known to decrease the expression of Twist and Vimentin [23]. In vitro, a growth-inhibiting effect could be proven for breast and ovarian cancer [24]. Since the sonic hedgehog pathway has not been fully clarified yet, it will certainly take years before the development of an effective anti-tumour therapy based on its inhibition.

A limiting factor for the results of our study is the small number of cases in a historical patient collective. In addition, cell changes may occur during fixation with formalin and embedding into paraffin. Gustaffson et al. [25] described differences in the detection of signal proteins in fresh tissue compared to tissue fixed in formalin. This view was shared already by Bellet et al. [26], who found significant differences in the protein patterns between freshly frozen tissue and tissue fixed in formalin. However, the storage period of samples does not seem to have any influence. Craven et al. [27] could not detect any measurement difference in renal cell carcinoma samples despite a storage period of 10 years.

Detection of the signalling proteins of the sonic hedgehog pathway could not allow characterizing the typical from the atypical carcinoid tumours. However, a larger patient cohort and fresh tumour material could improve the results obtained in the neuroendocrine lung carcinoids. Due to the rarity of the tumours, this would be possible only by considering a multicentre prospective study. A better understanding of the signalling pathways also opens new drug-based therapeutic approaches. Individual inhibitors of the sonic hedgehog pathway could be used in a more targeted manner. In addition to the in vitro cell line models, the effects of such inhibitors could be studied in a specific mouse model [28].

## Conclusion

Determination of the signalling proteins of the sonic hedgehog pathway for the purpose of a significant characterization of typical and atypical neuro-endocrine tumours is not expedient. However, it was found that the expression pattern of the signalling proteins differs from other solid cancer.

## Declarations

**Conflict of interest:** No

**Funding:** None

**Ethical concerns:** This study was approved by the responsible Ethics Committee of the Department of Human Medicine at the Philipps University of Marburg (Ref. №: 68/14).

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