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The host defence peptide LL-37/hCAP-18 is a growth factor for lung cancer cells

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Summary Cancer development can be viewed as dysregulated repair. Antimicrobial peptides (AMPs) are effector molecules of the innate immune system with direct antimicrobial activity. Beside this host defence function several AMPs play a role in the regulation of inflammation and tissue repair. The aim of the present study was to investigate whether the human cathelicidin AMP LL-37/hCAP-18 is involved in the biology of lung cancer.

Human cancer cell lines were found to express the human cathelicidin LL-37/hCAP-18 mRNA and peptide at different levels. Immunohistochemistry of human lung cancers showed that the peptide is expressed mostly in adenocarcinoma and squamous cell carcinoma. Application of exogenous LL-37 at low concentrations of 5 ng/ml to cancer cell lines increased proliferation and growth of anchorage-independent colonies. At the molecular level, LL-37 induced phosphorylation of the epidermal growth factor receptor (EGFR) and activation of downstream MAP kinase signalling pathways. Lung cancer cell lines that stably overexpress the peptide by means of a doxycycline-regulated promoter system also showed a faster growth. When these cell lines were injected subcutaneously into nude mice, cathelicidin overexpression resulted in increased tumourigenicity and the formation of significantly larger tumours.

In conclusion, cathelicidin is expressed in human lung cancers. The peptide activates tumour cells resulting in increased cell growth in vitro and in an animal model. The host defence peptide cathelicidin LL-37/hCAP-18 acts as growth factor for human lung cancer.

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1. Introduction

Lung cancer remains the most frequent cause of cancer death. An improved understanding of the mechanisms that are involved in cancer progression may identify new etiologic, prognostic or therapeutic targets that can improve patient management. An essential feature of tumour development is uncontrolled proliferation of a clone of transformed cells within the growth-supporting microenvironment. This microenvironment is closely linked to the processes which develop in tumour-surrounding tissues. One of these processes is the formation of new vessels that supply oxygen and nutrients [1]. Novel blood vessels are essential for tumours to grow beyond a critical size or metastasize to other organs. The progression of cancer appears also related to inflammatory processes [2]. Chronic inflammation can be viewed as a part of a host defense reaction including the activation of the innate immune system and associated repair mechanisms [3]. Cancer progression, angiogenesis, host defense and tissue repair are related processes and regulated by a variety of soluble factors [4].

Antimicrobial peptides (AMPs) are effector molecules of the innate immune system with direct antimicrobial activity [5]. The defensins and the cathelicidins are the two prototypical families of AMPs expressed in mammals. AMPs are expressed in epithelial cells and professional host defense cells such as macrophages or neutrophils. In addition to their role as antibiotics, AMPs have diverse activities on various cell types [6]. Some of these activities are related to the biology of cancer. Alpha-defensins stimulate epithelial cells to release proinflammatory cytokines that also have growth factor properties [7]. Furthermore, alpha-defensins stimulate the proliferation of epithelial cells [8,9]. LL-37/hCAP-18 is the only cathelicidin present in humans and has several properties that might be relevant for the progression of tumours [10]. LL-37 is the C-terminal cleavage product that carries the biological activity. LL-37 activates the formation of vessels by a direct effect on endothelial cells [11]. The peptide releases inflammatory mediators from epithelial cells by transactivation of the epidermal growth factor receptor (EGFR) pathway [12]. It also acts as epithelial repair factor involved in repair of lesions of the airway epithelium [13] and the skin [14].

Some AMPs are expressed in cancer cells or in myeloid cells that invade tumours. Human neutrophil peptides (HNP)-1, -2 and -3 are expressed in renal cell carcinoma and oral squamous cell carcinoma [15,16]. The peptides stimulated the growth of renal cancer cells at low concentrations [15]. Beta-defensins are present in oral squamous cell carcinoma [16] and are also involved in tumour angiogenesis by chemoattracting dendritic cell precursors into cancer areas, where they differentiate into endothelial-like cells [17]. The levels of serum beta-defensins are increased in patients with lung cancer [18]. LL-37/hCAP-18 is overexpressed in human breast cancer and highest levels were detected among the patients with advanced disease [19].

It was the aim of this study to determine whether lung cancer cells express LL-37/hCAP-18 and whether this AMP affects the behaviour of cancer cells. We characterized the effect of exogenous cathelicidin on various aspects of cancer cell biology. Cell lines with inducible overexpression of the peptide were generated and studied in vitro and in an animal model. Immunohistochemical analysis of lung cancer samples was performed to study the expression of LL-37/hCAP-18 in human disease.

2. Materials and methods

2.1. Patients and tissue samples

For the immunohistochemical analysis of cathelicidin expression we included patients admitted into the Pulmonary Division of the Gutenberg University Medical Center (Mainz, Germany). One hundred and six patients with newly diagnosed lung cancer and surgical resection of the primary lung lesion were included. The study was performed compliant to the rules and regulations of the Ethics Committee, all subjects gave written informed consent. Medical records were reviewed for clinical data including smoking status, Eastern Cooperative Oncology Group (ECOG) performance status, surgical and pathological classification. Clinical TNM staging (including clinical examination, CT scans, sonography, endoscopy, MRI, bone scan) was performed according to the IUCC/AJCC recommendations [20]. To determine a definite tumour stage [21], post-surgical pathological examination was included. The study population included patients of all stages and various treatment plans supplementing lung surgery. In more detail, 55% of patients were defined as stage I, 25% as stage II, 15% as stage III and 5% as stage IV. According to the WHO classification of lung tumours [22], the primary pulmonary lesion was classified as squamous cell carcinoma in 40 patients (37.8%), 33 (31.1%) as adenocarcinoma, 9 (8.5%) as bronchioloalveolar carcinoma and 5 (4.7%) as adenosquamous carcinoma. Seven patients had large cell carcinoma (6.6%) and seven patients had carcinoid tumours (classic carcinoid: five cases, 4.7%; atypical carcinoid: two cases, 1.9%). Five tumours (4.7%) were small cell carcinoma, all presented with limited disease. Since no serum samples were available from the cohort in Mainz, a second cohort of patients at University Hospital Giessen and Marburg (Marburg, Germany) was established for measurements of serum cathelicidin concentration. We included 46 subjects with histologically confirmed lung cancer. Thirty-three subjects (58%) suffered from non-small cell lung cancer (17 adenocarcinoma, 16 squamous cell carcinoma) and 13 patients (42%) were diagnosed with small cell cancer. Control samples were obtained from 12 individuals (5 female, 7 male) matched in age with the patients groups. The protocol was approved by the Ethical Committee of the University of Marburg and informed consent was obtained. Blood was drawn for further analysis.

2.2. Cell culture

The following lung cancer cell lines were used for the experiments: NCI-H24, NCI-H60, NCI-H69, NCI-H82 (SCLC) and U1810, NCI-H322, LCLC-97TM1, NCI-H23, EPLC-H272 (NSCLC). All cell lines were cultured in RPMI 1640 (GIBCO, Karlsruhe, Germany) supplemented with 2 mM L-glutamine, containing 10% (v/v) heat-inactivated fetal bovine serum

(FBS, GIBCO, Karlsruhe, Germany), 100 U/ml penicillin and 100 U/ml streptomycin (PAA Laboratories, Pasching, Austria) in a humidified 5% CO_2 atmosphere at 37 °C. The culture medium was changed every second day. Cells were passaged every 5 days using 0.05% (v/v) trypsin—EDTA (GIBCO, Karlsruhe, Germany). Cell lines were tested for the absence of mycoplasma infection (VenorGeM Minerva Biolabs, Berlin, Germany). Cell lysates were produced by collection of the cells in ice-cold PBS using a cell scraper. The cell suspension was centrifuged for 10 min at 1000 rpm at 4 °C. RIPA (radioimmunoprecipitation buffer) containing 10 mM Tris—HCl (pH 7.4), 0.5% (v/v) NP-40, 0.5% (v/v) deoxycholate, 150 mM NaCl, 10 mM NaF containing 1 μg/ml Aprotinin (Sigma, Munich, Germany) and 1 mM PMSF (Sigma, Munich, Germany) equivalent to the volume of the cell pellet was added and gently mixed. After incubation on ice for 30 min the lysed cell pellet was centrifuged for 20 min at 14,000 rpm at 4 °C. The supernatant was collected and stored at −80 °C. The lysates were diluted 1:100 with diluent (PBS with 1% BSA).

2.3. Construction of expression plasmids and generation of cell lines

A LL-37/hCAP-18-expression vector was generated by cloning the full-length cDNA of cathelicidin (NP_004336) into the Tet-off vector SSV9tetoffd2gfp [23]. To achieve stable expressing clones, SSV9tetoffhCAP-18 (only ampicillin resistance) and pcDNA3.1 containing the cassettes for neomycin resistance (Invitrogen, Carlsbad, CA, USA) were transfected into U1810, a large cell lung cancer cell line, and NCI-H322, an adenocarcinoma cell line, using FuGene 6 (Roche, Indianapolis, USA). Cells were selected using G418 (PAA Laboratories) for about 4 weeks by limiting dilution and singularized in 96-well plates. Monoclonal clones were expanded and tested for the expression of cathelicidin by RT-PCR. The resulting cell lines were called U1810_hCAP-18 and NCI-H322_hCAP-18. In the absence of doxycycline the cell lines express LL-37/hCAP-18. Doxycycline (1 μg/ml; Sigma, Taufkirchen, Germany) was added to the culture medium and substituted every second day to deactivate the expression of LL-37/hCAP-18.

2.4. Measurement of LL-37/hCAP-18 expression

Total RNA was isolated from cells using an RNeasy Mini Kit (Quiagen, Hilden, Germany). Isolated RNA was reverse transcribed by using the First Strand cDNA Synthesis Kit (Quiagen). Real-time quantitative PCR was performed with an iCycler (Bio-Rad, Munich, Deutschland) using the QPCR SYBR Green Fluorescein Mix (ABGene, Epsom, UK) through 40 cycles. Primers for hCAP-18 (5′: 5′-CCACCATGGGCCTGGTGATGCCTCTGGCCATC-3′ and 3′: 5′-TGTACACTAGGACTCTGTCCTGGGTACAAG-3′; annealing temperature: 65 °C) were obtained from TIB Molbiol (Berlin, Germany). To obtain quantitative PCR results the ΔΔCt (cycle threshold) method was used with β-actin (5′: 5′-AGCCTCGCCTTTGCCGA-3′ and 3′: 5′-CTGGTGCCTGGGGCG-3′; annealing temperature: 60 °C, TIB Molbiol) as a reference.

2.5. Cathelicidin ELISA

The total concentration of cathelicidin in cell culture lysates or serum from patients was measured by the cathelicidin ELISA. Before measuring cathelicidin the total protein concentration was measured with the bicinchoninic acid assay (BCA Protein Assay Kit, Pierce, Rockford) and adjusted to the same total protein concentration. Cell lysates were directly applied to the assay, serum was used with 0.1% Tween. Samples were incubated overnight at 4 °C in 96-well Nunc-Immuno Plates (F96 Cert Maxisorp, Nunc, Wiesbaden, Germany), washed, blocked with 0.1% (w/v) gelatine and incubated with a mouse anti-LL-37 antibody (Clone 1-1C12, Sanbio GmbH, Beutelsbach, Germany). A horseradish peroxidase-linked sheep-anti-mouse antibody (Amersham, Uppsala, Sweden) was used as secondary antibody. TMB (tetramethylbenzidine) was used as substrate and the absorbance was measured at 450 nm by using a Tecan Ultra 384 reader (Tecan, Crailsheim, Germany). Synthetic LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-COOH; Charite, Berlin, Germany) was used as control. Spiking experiments with addition of known amounts of peptide to serum or cell lysates showed adequate recovery of the peptide (data not shown).

2.6. Signal transduction analysis

Cancer cell lines were starved for 24 h and stimulated for the indicated time periods. The cell pellet was lysed with RIPA (radioimmunoprecipitation buffer) containing 10 mM Tris—HCl (pH 7.4), 0.5% (v/v) NP-40, 0.5% (v/v) deoxycholate, 150 mM NaCl, 10 mM NaF. Equal amounts of total protein were separated by SDS-PAGE (10% (w/v) Tris—HCl precast gels; Bio-Rad, München, Germany), transferred onto nitrocellulose (Blotting Nitrocellulose Membrane 0.45 μM; Bio-Rad) and blots were incubated with anti-phospho-EGFR (Upstate, Charlottesville, Virginia), anti-phospho-MEK 1/2 (Upstate), anti-phospho-JNK (Upstate), anti-total-EGFR (Upstate), anti-total-MEK (Sigma, Munich, Germany), anti-total-JNK (Upstate) or anti-total-p38 (Sigma, Munich, Germany) antibodies all at 1:1000 dilution in 5% (w/v) milk in PBS overnight. The membranes were immersed in ECL solution (Amersham Biosciences) for 60 s and then exposed to X-ray film (Eastman Kodak, Rochester, NY, USA). In separate experiments cell lines were incubated with a combination of neutralizing antibodies against the EGFR ligands HB-EGF, TGF alpha, amphiregulin (R&D Systems, Minneapolis, MN, USA) and EGF (Upstate) (concentration: all at 5 μg/ml) while stimulation with LL-37 or medium. In all experiments the membrane was reprobed with an antibody against β-actin (Abcam, Cambridge, UK) as control for equivalent protein loading.

2.7. Cell proliferation

Cell proliferation was measured by determining the number of viable cells by incubation with WST-1 (Roche) and measuring the absorbance at 450 nm using a Tecan Ultra 384 reader. Cells were seeded into 96-well plates, starved for 24 h and stimulated for 48 h. LL-37, the chemically synthesized all-D-form of LL-37, called D-LL-37,

a scrambled version of LL-37, called sLL-37 (RSLEGTDRFPFVRLKNSRKLEFKDIKGIKREQFVKIL-COOH) or epidermal growth factor (EGF 100 ng/ml, Sigma—Aldrich, Munich, Germany) were used at the indicated concentrations. Culture medium contained 1% (v/v) FBS.

For inhibition studies, the cell lines were incubated with the following inhibitors: 100 ng/ml pertussis toxin (PTx, inhibitor of G-protein coupled receptors; Sigma, Taufkirchen, Germany), 1 μM AG1478 (EGFR kinase inhibitor; Calbiochem, La Jolla, CA, USA), 25 μM PD98059 (MEK inhibitor; Alexis, Nottingham, UK), 10 μM LY294002 (phosphatidyl-inositol-3-kinase inhibitor; Biosource), 25 μM U0126 (MEK inhibitor; Sigma, Taufkirchen, Germany), 1 μM GM6001 (metalloproteinase inhibitor; Calbiochem), 1 μM U73122 (phospholipase C inhibitor; Calbiochem) or 1 μM GF1092032X (proteinkinase C inhibitor; Sigma) 1 h before stimulation with LL-37. A second experiment was performed with medium instead of LL-37 after incubation of inhibitors to exclude cytotoxic effects.

2.8. Anchorage-independent colony formation

Thirty-five millimeter dishes were precoated with 1 ml of 0.6% (w/v) agar containing 1% (v/v) or 10% (v/v) FBS as a bottom layer. Cell lines or stable LL-37-expressing clones were suspended in 0.8 ml 0.36% (w/v) agar containing 1% (v/v) FBS and plated onto the bottom layer. Test substances were used at the indicated concentrations in the media. Three to four weeks after plating and cultivating in a humidified 5% CO_2 atmosphere at 37 °C, the colony forming efficiency was determined. Micrographs were obtained using an inverted Axiovert 25 microscope (Carl Zeiss, Oberkochen, Germany) and analyzed using the Evolution LC Megapixel FireWire Camera Kit and Image-Pro Discovery software (Media Cybernetics, Silver Spring, MD, USA). The sizes of the largest colonies (more than 50 μm in diameter) on the image were determined by the Image J software and the sizes between the groups were compared. The size of the colonies is displayed as relative colony forming activity. Additionally the numbers of colonies more than 50 μm in diameter were counted by two independent blinded investigators.

2.9. LL-37-dependent tumour progression in nude mice

To examine the impact of LL-37/hCAP-18 overexpression on tumour progression in vivo, BALB/c-nu/nu mice (female, 6 weeks old, $n = 10$) were injected subcutaneously into both flanks with 4×10^6 U1810_hCAP-18 or 1.5×10^6 H322_hCAP-18 cells in 150 μl PBS. One group received doxycycline in the drinking water (1 mg/ml). The growth of the subcutaneous tumours was monitored every 1—3 days and the size was estimated from the product of the perpendicular diameters of the tumours. At the end of the experiments, the mice were euthanized and tumours were resected and fixed in 3.7% buffered formalin. The animal experiments were approved by the responsible federal institution, the Regierungspräsidium Giessen.

2.10. Immunohistochemistry of human samples

Freshly resected tumour specimens were immediately formalin-fixed and paraffin-embedded. Samples were cut and mounted on capillary gap slides and baked overnight. The paraffin was removed in xylene and rehydrated through series of graded alcohol. The slides were cooked in pH 9 buffer (Dako Target Retrieval Solution®, Dako, Germay) for 20 min at 96 °C. All slides were stained simultaneously using a computer controlled autostainer (Dako TechMate 500, Dako) and the Dako EnVison-System [24]. Unspecific tissue peroxidases were blocked by H_2O_2 (POD-Block Dako EnVison, 15 min, Dako) followed by incubation with the polyclonal primary rabbit anti-LL-37 antibody at a 1:100 dilution (60 min). After washes a secondary, peroxidase-labeled anti-rabbit-antibody (Dako EnVison) was added for 30 min and washed out. The staining reaction was initialized by adding DAB-substrate (Dako EnVision). After washes, the sections were counterstained in hematoxylin and covered with Entellan (Merck, Darmstadt, Germany). During the staining procedure 10 slides (9.4%) were damaged and could not be evaluated. The slides were classified by three investigators (S.B., R.W., H.S.) according to Remmele's immunoreactive score (IRS) [25]. For the evaluation of the IRS, only tumour cells were taken into account. Percentage of positive cells (0—10% = 1, 11—50% = 2, 51—80% = 3, 81—100% = 4 points) and staining intensity (weak = 1, moderate = 2, strong = 3) were evaluated and multiplied. An IRS ≥ 3 was considered as positive. The interobserver variability was low. Regarding LL-37/hCAP-18 positive staining (defined as an IRS ≥ 3), analysis differed in 2/96 cases (2%). For final assessment, all three observers commonly agreed on staining results.

2.11. Immunohistochemistry of tissue section from mouse experiments

Samples were deparaffinized as described above and incubated in 0.3% (v/v) H_2O_2 in methanol for 10 min to block endogenous peroxidase activity. The sections were pressure-cooked for 12 min in citrate buffer (pH 6.1) for antigen retrieval. As the primary antibody, lyophilised polyclonal rabbit anti-human-Ki-67 was used at 1:500 dilution (Novo Castra, Newcastle upon Tyne, UK), rabbit anti-LL-37 was applied at a 1:1000 dilution on adjacent serial sections. A goat anti-rabbit antibody (Histostain Plus Kits Zymed 2nd Generation, Zymed Laboratories Inc., Invitrogen, San Francisco, CA, USA) was used as secondary antibody and AEC Chromogen (Histostain Plus Kits Zymed 2nd Generation, Zymed Laboratories Inc.) was applied as substrate.

2.12. Data analysis

All experiments were performed as triplicates for each condition and repeated at least twice. All data are expressed as means ± S.D. Significance of the results was evaluated using the Sigma Stat 3.1 software package. Comparisons between groups were analyzed by the *t*-test (two-sided) or ANOVA for experiments with more than two subgroups. Results were considered statistically significant for *p*-values

less than 0.05. Statistical analysis of immunohistological results was performed using SPSS 13 software.

3. Results

3.1. Lung cancer cells express LL-37/hCAP-18

Cathelicidin is expressed in pulmonary epithelial cells [26]. It is also known that the peptide modulates processes that are relevant for tumour biology such as epithelial cell proliferation, migration [13,14] and angiogenesis [11]. We therefore hypothesized that the peptide might be produced in lung cancer cells and that this expression influences the properties of the tumour cells. We selected a variety of lung cancer cells and analyzed the expression of cathelicidin by quantitative RT-PCR. LL-37/hCAP-18 transcripts were detected in all cancer cell lines at different levels (Fig. 1a). The peptide was detected in cell lysates as measured by ELISA (Fig. 1b). These data show that the cathelicidin LL-37/hCAP-18 is expressed in human lung cancer cells on the transcript and the protein level at biologically relevant levels.

3.2. Expression of LL-37/hCAP-18 in lung cancer in vivo

We next studied whether the peptide is expressed in human lung cancer. One hundred and six lung cancer samples from patients who underwent surgery were immunostained for LL-37/hCAP-18 using a polyclonal antibody. The IRS scoring system [25] was used to analyze staining results. Overexpression of LL-37/hCAP-18 was observed in malignancy in 25% of all cases. Cathelicidin-positive slides showed clear membrane staining and/or cytoplasmatic staining with an IRS value of at least 3, with absence of nuclear staining (Fig. 2a and b). In SCLC and carcinoid tumours no staining for LL-37/hCAP-18 could be detected. NSCLCs stained positively at different frequencies depending on the histology, ranging from no positive staining (carcinoid) up to 28% positive staining (adenocarcinoma). Among the two main entities, adenocarcinoma (13 cases, including bronchioloalveolar carcinoma) and squamous cell carcinoma (10 cases, accounting for 23%) had comparable staining frequencies (Fig. 2c). Of note, the five adenosquamous carcinoma cases counted for both the squamous cell carcinoma group ($n = 37 + 5$) and adenocarcinoma group ($n = 40 + 5$), however all were negative for LL-37/hCAP-18. Crosstab tables showed no association of LL-37/hCAP-18 with any clinical or pathological parameters, including sex (two-sided Pearson Chi-square test, $p = 0.16$), age (dichotomised <70 years versus $\geq$70 years, $p = 0.08$), smoking status (never smokers versus ever smokers, $p = 0.87$), ECOG performance status ($p = 0.89$), lymph node status ($N = 0$ versus $N \geq 1$, $p = 0.59$), metastasis ($p = 0.181$), clinical and pathological tumour stage ($p = 0.70$ and $p = 0.85$, respectively), histology ($p = 0.52$) and grading ($p = 0.64$). We then measured cathelicidin levels in serum of patients diagnosed with cancer. We found that patients with squamous cell carcinoma have significantly increased cathelicidin blood levels (Fig. 2d). The apical line of epithelial cell, cilia and the stroma of mucin-rich bronchial glands were often LL-37/hCAP-18 positive, usually with light to moderate intensity (data not shown). Lymphocytes, cartilage and stroma never stained for LL-37/hCAP-18. These data show that cathelicidin is expressed in a significant number of human lung cancers.

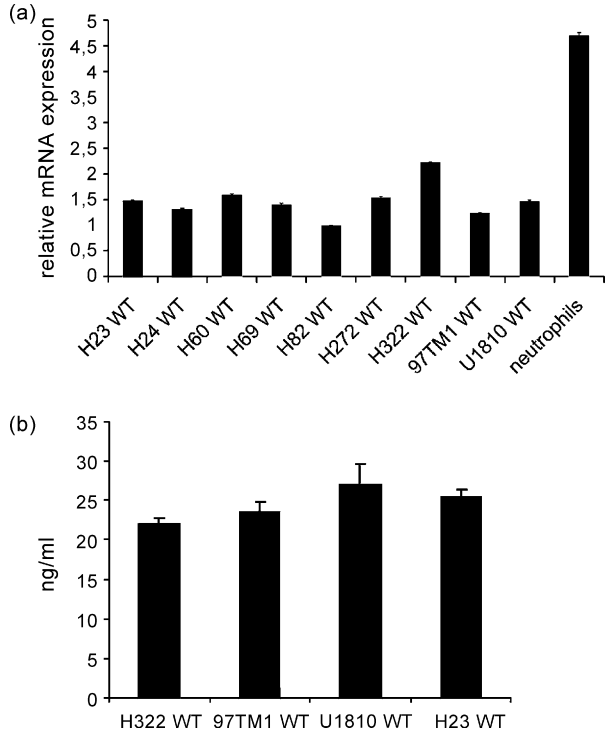


Fig. 1 Cathelicidin is expressed in human lung cancer cell lines. (a) Quantitative RT-CR detected variable amounts of LL-37/hCAP-18 transcripts (relative to β-actin) in the indicated cancer cell lines. Neutrophils were used as control ($n = 4$). (b) Cathelicidin is detectable in cell lysates of the indicated cancer cell lines by ELISA ($n = 4$).

3.3. LL-37 activates lung cancer cells in vitro

We next investigated whether LL-37 interacts with lung cancer cells and how this interaction modulates the biology of the tumour. To analyze whether LL-37 stimulates proliferation, the cancer cell lines H322 and U1810 were incubated with different concentrations of the peptide. We found a dose-dependent increase of the viable cell numbers after 48 h exposure to the peptide (Fig. 3a). The effect on proliferation was already detectable at concentrations as low as 5 ng/ml. A scrambled control peptide had no effect, whereas D-LL-37, a peptide consisting of only D-amino acids, had an equivalent effect on proliferation as the naturally occurring L-form (Fig. 3a). Next, the effect of LL-37 on cancer cells in an anchorage-independent assay was investigated. The application of LL-37 resulted in a significant increase of the size of colonies with a diameter greater than 50 μm (Fig. 3b and c). These data show that exogenous LL-37 activates cancer cells to proliferate and accelerates anchorage-independent growth.

To test whether expression of cathelicidin has an impact on tumour growth, stably overexpressing cell lines were generated by transfection of cancer cells with a Tet-Off

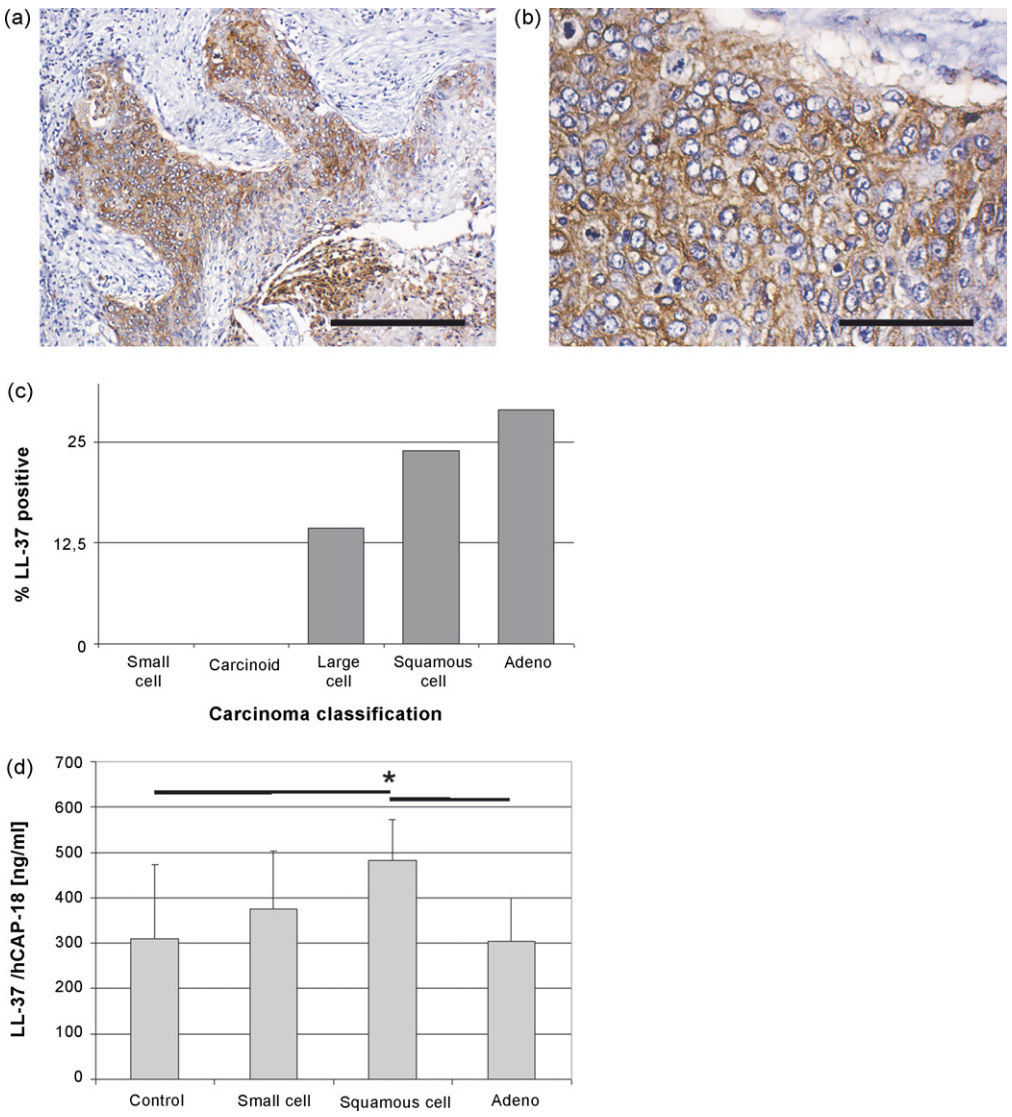


Fig. 2 Human lung cancers express cathelicidin. (a and b) Immunohistochemistry using an antibody against LL-37 shows a strong positive signal in a squamous cell cancer (IRS = 6). Bar in (a), 1200 μm and in (b), 100 μm. (c) Small cell carcinoma and carcinoid tumours do not express detectable levels of cathelicidin. Large cell, squamous cell and adenocarcinoma express the peptide. (d) Patients with squamous cell carcinoma have significantly increased levels of cathelicidin in their serum, $^{*}p < 0.05$.

expression system expressing the full-length cDNA coding for LL-37/hCAP-18. Omission of doxycycline from the culture medium resulted in the induction of LL-37/hCAP expression as determined by quantitative RT-PCR (Fig. 4a). The presence of doxycycline had no influence on the tumour cell proliferation of native cell lines (data not shown). The expression of the peptide varied between individual clones. Cells overexpressing the peptide released measurable amounts of LL-37/hCAP-18 into the culture medium as measured by ELISA (Fig. 4b). Overexpression of LL-37/hCAP-18 resulted in increased proliferation as compared to wildtype cells or cells with downregulated expression. The levels of mRNA expression as determined by RT-PCR correlated closely with the number of cells in the proliferation assay (Fig. 4c). Also the anchorage-independent growth of colonies was increased by the endogenous expression of the peptide (Fig. 4d). To test whether the effects of the overexpressed cathelicidin depend on secretion of soluble factors into the culture medium, conditioned medium from overexpressing cells was transferred to wildtype cells and were found to induce significant proliferation (Fig. 4e). This suggests that the LL-37/hCAP-18 is secreted from cancer cells and acts as soluble autocrine factor.

These results show that externally applied LL-37 and endogenously expressed peptide induce cell proliferation and anchorage-independent growth of lung cancer cells. The effect of endogenous overexpression of cathelicidin on cellularity is mediated by secreted factors.

3.4. Cathelicidin-dependent tumour promotion involves EGFR signaling

Several mechanisms have been implicated in the interaction of LL-37 and various host cells. EGFR-activation is involved in LL-37-dependent activation of primary airway epithelial

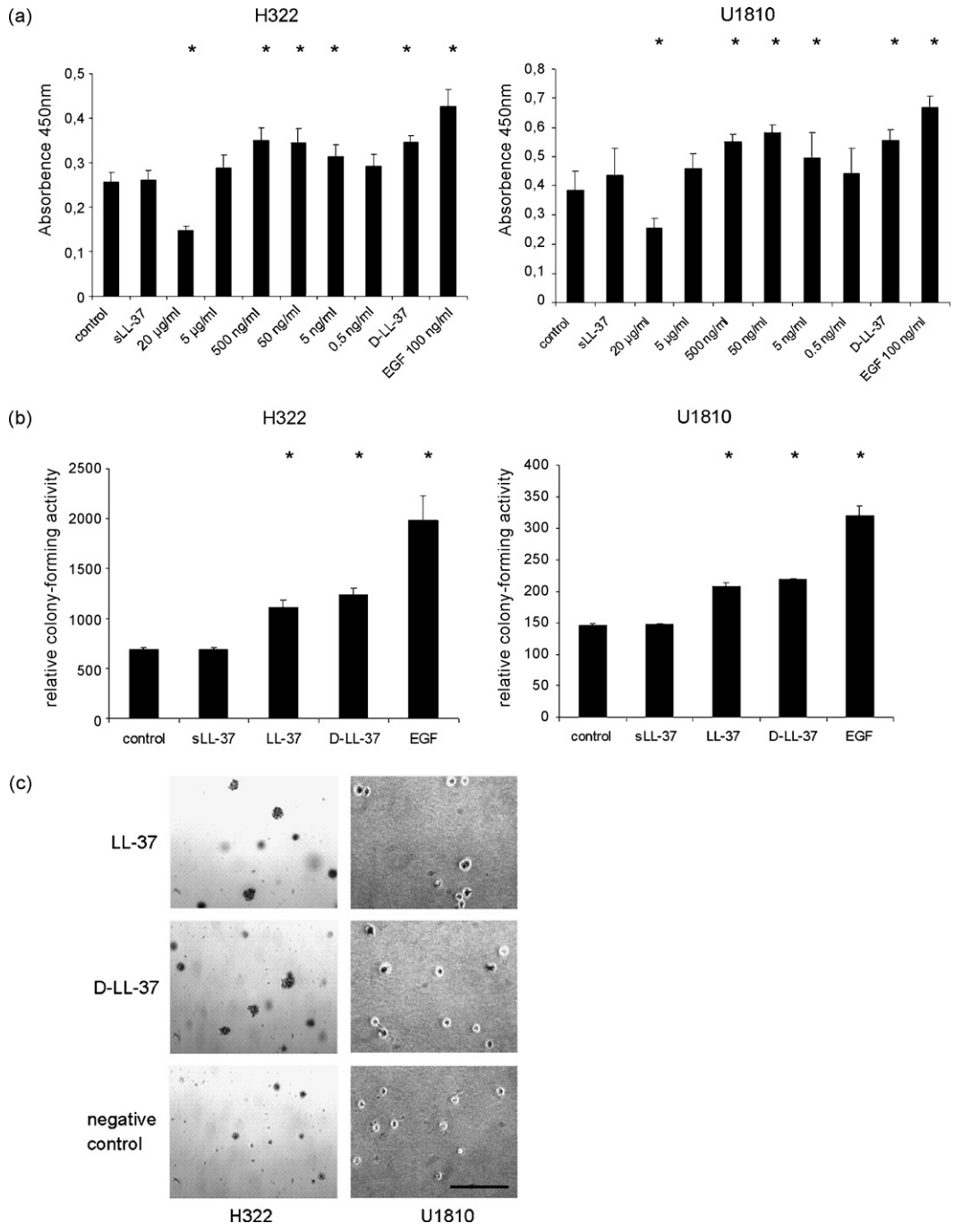
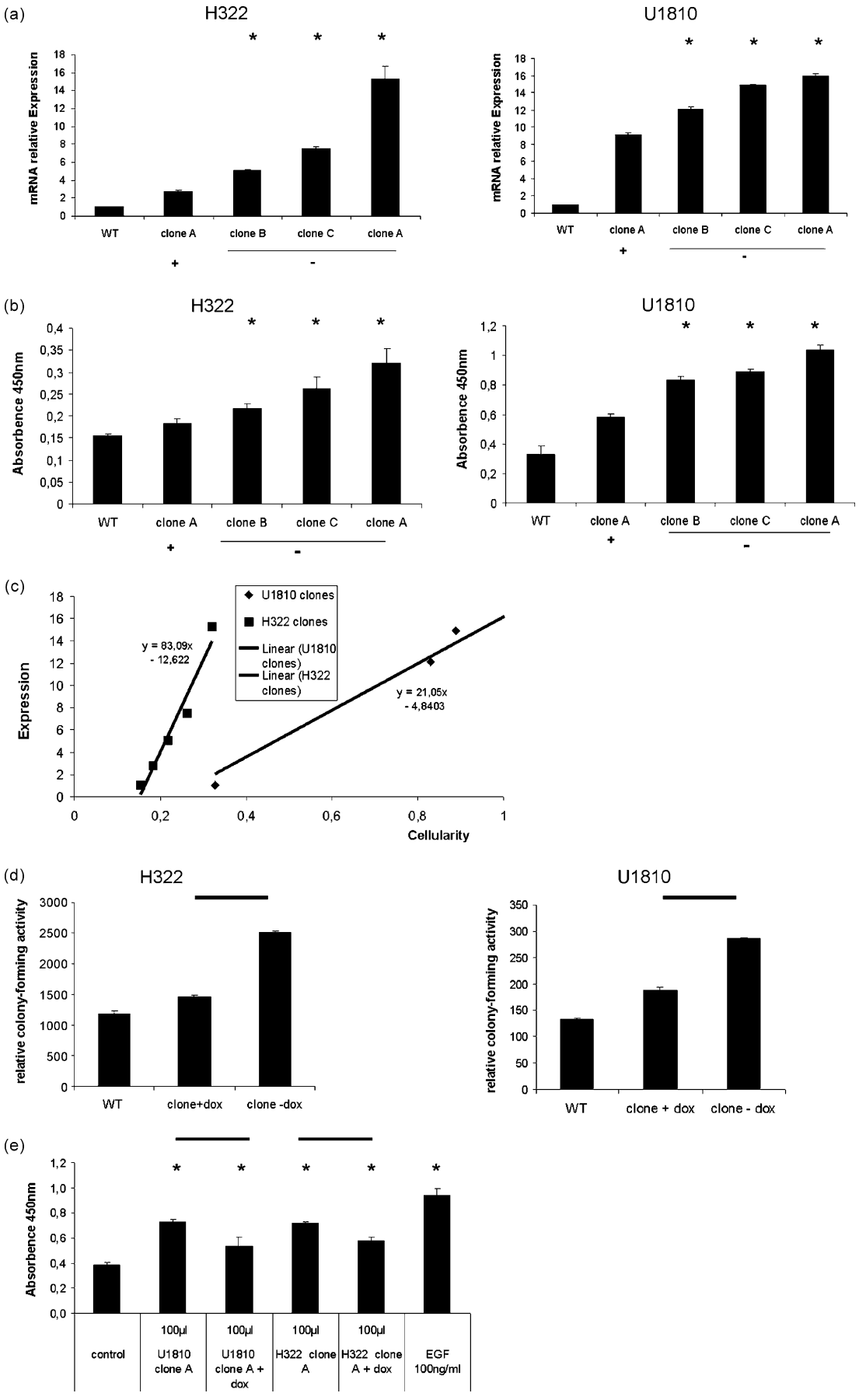


Fig. 3 Cathelicidin increases cellularity and anchorage-independent growth of the lung cancer cells H322 and U1810. (a) Application of LL-37 at the indicated concentrations resulted in increased cell numbers in a dose-dependent manner as determined by the WST 1 assay. The scrambled control peptide sLL-37 (H322 500 ng/ml, U1810 50 ng/ml) did not result in altered cell numbers. EGF was used as control, $^{*}p < 0.05$, $n = 8$. (b and c) LL-37 induced anchorage-independent growth in a soft-agar assay. The application of LL-37 and the all-D-form (D-LL-37) resulted in increased colony size, $^{*}p < 0.05$, $n = 8$. Bar = 100 µm.

Fig. 4 Overexpression of cathelicidin results in increased cell growth of lung cancer cells. The cell lines H322 and U1810 were stably transduced to express cathelicidin under the control of a Tet-Off system. (a and b) Clones a—c cultivated without doxycycline (—) showed higher levels of cathelicidin transcripts (a) and released measurable amounts of LL-37/hCAP-18 into the culture medium as measured by ELISA (b) as compared to the wildtype cells or clones cultivated in the presence of doxycycline (+), $^{*}p < 0.05$, $n = 8$. (c) The numbers of transcripts correlated closely with the cell numbers as determined by the WST 1 assay. (d) Transformed clones cultivated without doxycycline (—dox) showed larger colonies in an anchorage-independent growth assay as compared to the wildtype clones or clones cultivated in the presence of doxycycline (+dox). Bar indicates $p < 0.05$, $n = 8$. (e) Transfer of conditioned medium from overexpressing cell clones to native cells increased cellularity. Supression of hCAP-18 expression diminishes this effect. '*' indicates $p < 0.05$ as compared to the control group and bars indicate $p < 0.05$ between the indicated groups.


(a)
H322
U1810
mRNA relative Expression
WT
clone A
clone B
clone C
clone A
+
-
(b)
H322
U1810
Absorbence 450nm
(c)
U1810 clones
H322 clones
Linear (U1810 clones)
Linear (H322 clones)
y = 83,09x - 12,622
y = 21,05x - 4,8403
Expression
Cellularity
(d)
H322
U1810
relative colony-forming activity
WT
clone+dox
clone -dox
clone + dox
clone - dox
(e)
Absorbence 450nm
control
100µl U1810 clone A
100µl U1810 clone A + dox
100µl H322 clone A
100µl H322 clone A + dox
EGF 100ng/ml

cells at high concentrations [12]. The activation of epidermal growth factor receptor (EGFR) is linked to cancer progression and has been identified as a target of novel therapeutic approaches [27]. LL-37 was shown to signal via formyl peptide receptor like 1 (FPRL1) [28] and the P2X7 receptor [29]. We tested involvement of these pathways by the application of various inhibitors of key molecules. The application of the inhibitor of EGFR signaling AG1478 as well as MEK inhibitors PD98059, and U0126 resulted in significant inhibition of the LL-37 induced effect on proliferation (Fig. 5a). These results indicate the involvement of EGFR and MEK. We next tested the effect of LL-37 on the phosphorylation of EGFR. After exposure of different lung cancer cell lines to LL-37, a 175 kDa band was detected using a phosphorylation-dependent antibody to EGFR (Fig. 5b). To study the effect on the EGFR pathway, cell lines were incubated with a combination of neutralizing antibodies against the EGFR ligands HB-EGF, TGF alpha, amphiregulin and EGF while stimulation with LL-37. Neutralizing antibodies used at the recommended concentrations did not inhibit the phosphorylation of EGFR (Fig. 5b). Furthermore, we determined the activation of MAP kinases by the detection of phosphorylated proteins (Fig. 5c).These data show that the effects of LL-37 depend on the EGFR pathway with downstream activation of MEK and MAP kinases.

3.5. LL-37/hCAP-18 promotes cancer growth in a murine model

The marked effects of LL-37 on cell proliferation, anchorage-independent growth and signaling suggested that expression of cathelicidin might regulate tumour growth in vivo. Nude mice were injected with cell lines carrying a regulated LL-37/hCAP-18 expression cassette. Eighty percent of the animals subcutaneously injected with 4×10^6 U1810_hCAP-18 cells overexpressing cathelicidin developed detectable cancer growth as compared to only 20% of the animals injected with cells not expressing the peptide. The tumour take for the NCI-H322_hCAP-18 cell line was not significantly different between the two groups. The animals injected with 1.5×10^6 NCI-H322_hCAP-18 cells overexpressing the peptide developed significantly larger tumours than mice without overexpression of cathelicidin (Fig. 6a). We determined the percentage of Ki-67 positive cells as surrogate marker of local tumour cell proliferation. Animals with cathelicidin-overexpressing tumours showed significantly increased numbers of Ki-67 positive cells (Fig. 6b and c). As control, we determined the expression of cathelicidin in the excised tissues. As expected, tumours from animals that were fed without doxycycline showed a marked positive signal (Fig. 6c).

4. Discussion

The main finding of the present study is the ability of the human antimicrobial peptide cathelicidin to promote tumour growth by direct interaction with cancer cells. The peptide is strongly expressed in a significant percentage of human lung cancers. The application of LL-37 or the overexpression of the peptide stimulates in vitro surrogate markers of cancer growth. Overexpression of the peptide in experimental cancers is associated with increased growth in an animal model.

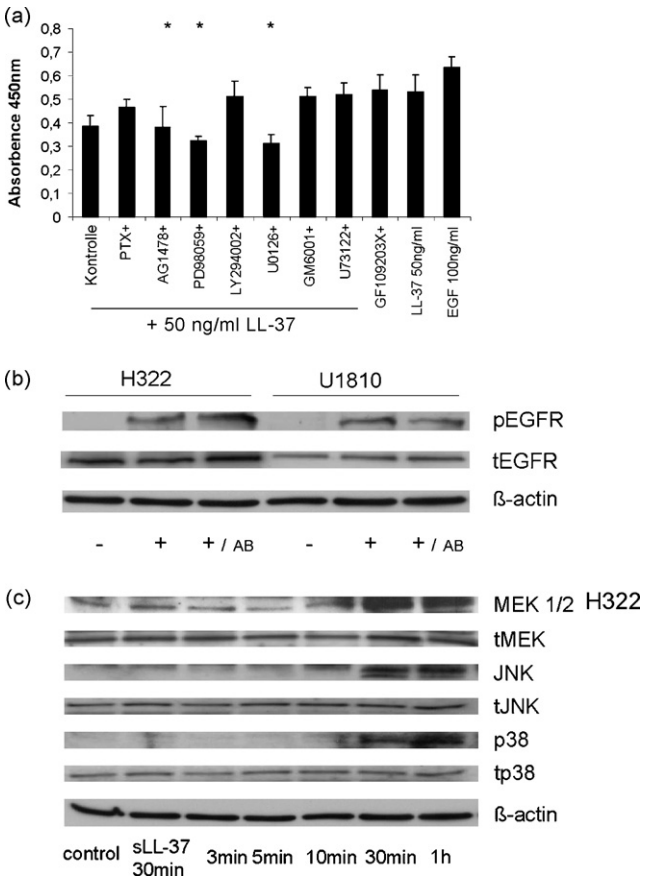


Fig. 5 Cathelicidin induced signalling through EGFR, MEK and MAP kinases. (a) LL-37-induced cell proliferation is suppressed by inhibitors of EGFR (AG1478) or MEK (PD98059, U0126), $^*p < 0.05$ as compared to the LL-37 group, $n = 8$. (b) Application of LL-37 (+500 ng/ml, 10 min) without serum results in phosphorylation of EGFR in the H322 and U1810 cell lines as compared to the control (−). Application of neutralizing antibodies to ligands of EGFR (+/AB) did not inhibit phosphorylation. (c) Application of LL-37 (500 ng/ml) to the cancer cell line H322 resulted in phosphorylation of MAP kinases. Three experiments with equivalent outcomes.

Cathelicidin is a multifunctional host defence peptide that links inflammation, host defence, tissue repair and cancer growth. Cancer is increasingly being viewed as a disease of dysregulated repair [30]. Repair is commonly the response to infectious or inflammatory tissue damage and is associated with a host defense reaction. Inflammation has been identified as an independent factor that contributes to the initiation and the progression of cancer [2]. In this context, antimicrobial peptides qualify as interesting candidate molecules that link innate host defence and inflammation with cancer development. Taken cathelicidin as example, this molecule is involved in host defence and cancer biology. It has direct antimicrobial activities and is expressed in classical host defence cells such as neutrophils [31—33], macrophages and epithelial cells [26]. The murine homologue, called CRAMP, shields animals from skin [34] and urogenital infections [35]. Expression of cathelicidin has a

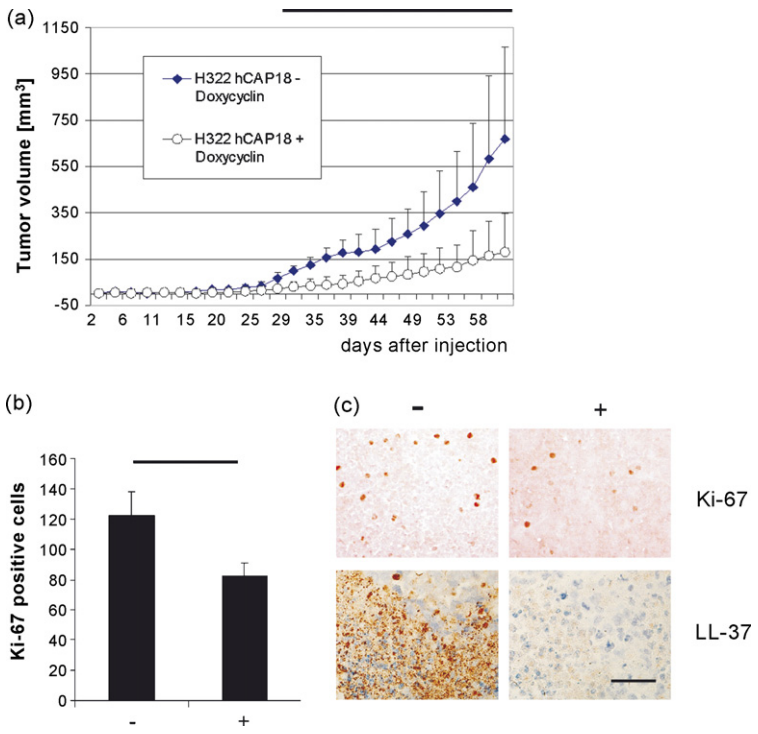


Fig. 6 Overexpression of cathelicidin results in increased tumour growth in vivo. Nude mice were injected subcutaneously with 1.5×10^6 with transformed H322 cells and the tumour size was measured. (a) Tumours overexpressing cathelicidin showed significantly faster growth ($n = 10$ mice). Bar indicates $p < 0.05$. (b and c) Immunohistological analysis showed increased numbers of Ki-67-positive cells (b and c) and a strong positive signal for LL-37 (c) in the tumours that overexpressed cathelicidin (−, omission of doxycyclin) as compared to the control (+, plus doxycycline). Bar in (b) indicates $p < 0.05$ and bar in (c), 100 μm.

role in the protection against tuberculosis [36]. In addition to these antibacterial activities, the peptide is involved in the regulation of host cells. It interacts with neutrophils [28] and macrophages [37] and stimulates epithelial cells via the EGFR pathway to release the inflammatory mediator IL-8 [12]. Own studies showed that the peptide stimulates angiogenesis by a direct interaction with endothelial cells [11,38]. Furthermore, the peptide regulates the repair of epithelial wounds of the skin [14] and the airways [13]. The peptide's role in host defense and tissue repair becomes detrimental in the context of cancer that is characterized by dysregulated growth. As in other organ systems, lung cancer is associated with inflammation. Cathelicidin expression in the lungs is increased during inflammatory and infectious lung diseases [39—42]. Increased levels of cathelicidin in inflamed lung tissues could promote local cancer growth.

The tumour promoting effect of cathelicidin could be mediated by different pathways. The data of the present study suggest that endogenous LL-37/hCAP-18 is secreted from cancer or possibly inflammatory cells. Secreted cathelicidin regulates the biology of tumour cells. Since we could not detect a signal in Western blotting, the molecular form is not known. Critical to the effect of cathelicidin is the presence of a protease that activates the hCAP-18 precursor. While it is known hCAP-18 is cleaved by neutrophil protease 3 [43] and by gastricsin in the vaginal milieu [44], the protease that cleaves the peptide's precursor in cancer cells has not been identified. Addition of LL-37 to incubated cells had the same effect as overexpression of the cDNA indicating that cancer cells contain the necessary proteases to cleave the peptide's precursor. One further mechanism of cathelicidin-dependent tumour growth could involve the angiogenic effect of the peptide. The growth of vessels is a critical factor in the expansion of tumours and pathological angiogenesis is a hallmark of cancer [1]. LL-37 is known to stimulate angiogenesis by a direct effect on endothelial cells [11,38]. The effects of LL-37 shown in this study and its known effect on angiogenesis may be two important independent effects of the peptide on cancer development.

The exact molecular mechanism how LL-37 stimulates tumour cell growth is not clear. The present data indicate the involvement of the EGFR pathway. Cathelicidin is known to interact with this pathway in epithelial cells by transactivation of EGFR [12]. Cathelicidin activated lung cancer cells as demonstrated by phosphorylation of EGFR and of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK). Cellular activation was suppressed by the inhibitors of EGFR and MEK. The activation of the EGFR-cascade is a principal signaling pathway involved in tumour pathogenesis [27]. Beside EGFR, LL-37 has been described to signal through other receptors such as the P2X7 [29] or the FPRL1 [28]. In the present study inhibition of these receptors did not modulate the effect of the peptide on cancer cells. Interestingly, the all-D-form D-LL-37 causes the same effects as the naturally occurring form. A possible explanation could be electrostatic interaction of the cationic peptide with negatively charged components of cell membranes potentially resulting in receptor-independent activation of membrane-associated signaling cascades. This kind of interaction will be independent on the chirality

of the peptide. Indeed, EGFR, which is activated in lung cancer cells and was affected by LL-37 in this study, is recently shown to localize in membrane-associated lipid raft microdomains [45]. A precise mode of interaction of the peptide with cancer cell membranes represents an intriguing issue for future studies.

The effect of cathelicidin on tumour biology appears to be relevant in vivo. Relatively low concentrations of the peptide are necessary to activate cancer cells in vitro. These concentrations are well within the rage that have been measured in samples from humans bronchoalveolar lavage [40]. An animal model also showed tumour-promoting effects of cathelicidin. Tumours that overexpress the peptide showed faster growth and increased numbers of proliferating cells. The immunohistochemical data from human cancer sections show that lung cancer cells are a source of cathelicidin within the tumour microenvironment. Interestingly, the different tumour entities differed in their expression of cathelicidin. SCLC and carcinoids, both are of neuroendocrine origin, do not express cathelicidin. Of note, the growth of NSCLC cells, which expressed LL-37, is known to be dependent on EGFR pathway that was activated by LL-37 in this study. Thus, it is possible that under certain circumstances the peptide serves as an autocrine stimulator of NSCLC progression. An important factor regulating LL-37 activity in cancer cells might be the presence of specific protease within tumour microenvironment, as already discussed above. Neutrophils and macrophages/monocytes are another possible source of peptide within the tumour environment. Whether the expression of the peptide by cancer cells or secretion by inflammatory cells results in an altered progression free survival or overall survival in NSCLC patients will be the scope of further investigations.

In conclusion, the human cathelicidin LL-37/hCAP-18 is expressed in a significant number of NSCLC. The peptide is known to have different roles in a host defense reaction such as direct antimicrobial, angiogenic and wound repair functions. Here we show that cathelicidin modulates the biology of cancer cells. In vitro and animal models demonstrated that LL-37/hCAP-18 results in faster tumour growth. The cathelicidin peptide links host defence, repair and inflammation with cancer progression.

Conflict of interest

None declared.

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