Carbonic anhydrase IX is associated with early pulmonary spreading of primary colorectal carcinoma and tobacco smoking

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Received 1 August 2013; received in revised form 4 October 2013; accepted 21 October 2013

Abstract

OBJECTIVES: Pulmonary metastasectomy is performed routinely in selected patients with metastatic spreading to the lungs. According to current guidelines, the tumour biology should be taken into account when selecting patients for a resection. Carbonic anhydrase IX (CA9) expression has been shown to be a common feature in primary tumour growth and metastasis and it negatively affects the clinical outcome in various malignancies. Data on CA9 in pulmonary metastases are lacking.

METHODS: Forty-four patients with primary colorectal cancer (CRC) who underwent curative pulmonary metastasectomy were included in this study. We determined the expression of CA9 in pulmonary metastases and corresponding primaries by immunohistochemistry. The expression level was correlated with clinical parameters and patients’ smoking habits. Furthermore, the impact of nicotine treatment on phosphorylation of STAT3, HIF-1α and CA9 expression was assessed in HT29 cells.

RESULTS: High expression of CA9 in resected pulmonary metastases and corresponding primary tumours correlated with early spreading to the lung (both \( P < 0.001 \)). Moreover, CA9 expression was affected by the smoking habits of the patients. Treating HT29 cells with nicotine resulted in an induction of CA9 in vitro. This induction was associated with STAT3 phosphorylation and was independent of HIF-1α.

CONCLUSIONS: This study provides first evidence of CA9 expression in pulmonary metastases of CRC and suggests a role of CA9 as a prognostic marker. Moreover, our in vitro and in vivo data indicate an association between tobacco smoking and CA9 expression. Immunohistochemical assessment of CA9 expression might serve as an additional tool in decision-making for selecting patients for pulmonary metastasectomy.

Keywords: Pulmonary metastasis • Colorectal cancer • Carbonic anhydrase • Cell biology

INTRODUCTION

Pulmonary metastases occur in 20–50% of all patients with malignancies [1]. Thus, the lungs are the most common organ of secondary lesions. Surgical resection of pulmonary metastases from various primary malignancies is daily routine in thoracic surgery [2]. Due to the fact that data from randomized controlled trials are missing so far, the first prospective study assessing the potential benefit of pulmonary metastasectomy from primary colorectal cancer has started to recruit patients in order to clarify the role of surgery in these patients [3, 4]. As the lung is frequently the only site of metastasis, a complete resection of all tumour masses poses a potentially curative treatment option. The criteria for the selection of patients include (i) the control of the primary tumour site, (ii) a complete resectability of all metastases, (iii) an adequate pulmonary function and (iv) the exclusion of a disseminated extrathoracic disease [5]. In addition, it is generally agreed that the biology of the tumour should be taken into consideration when selecting patients for pulmonary metastasectomy. However, to date, only few markers exist that reflect the biology of metastatic spreading and the patients’ prognosis.

Colorectal cancer (CRC) is one of the most common types of cancer and is the third leading cause of cancer-related death [6]. In patients with CRC, the cumulative incidence of pulmonary metastases is 14% [1]. Patients with untreated metastatic disease have a median survival of <10 months and a 5-year survival rate of <5% [7]. By the combination of repeated surgical interventions and novel systemic chemotherapeutic regimes, the 5-year survival after pulmonary metastasectomy could be increased to 39% according to a recent systematic review of the literature [8].
Carbonic anhydrase IX (CA9) catalyses the hydration of carbon dioxide to bicarbonate and protons, and in this way represents a linkage of metabolic processes and pH regulation [9]. CA9 overexpression is a frequent finding in various malignancies; however, the role of CA9 in tumour tissue is currently not completely understood. In vitro observations suggest that CA9 is involved in tumour progression and metastatic spreading [10]. Consequently, clinical observation revealed that CA9 overexpression is associated with a poor prognosis in various malignancies [11–13]. CA9 expression is regulated by a hypoxia-dependent, HIF-1α-driven mechanism as well as a hypoxia-independent mechanism, which has been shown to be regulated by the STAT3 pathway through the induction of Notch-3 [14].

Smoking is thought to be a major contributing factor to metastatic spreading to the lung [15, 16]. While tobacco-specific nitrosamines and other carcinogens present in the tobacco smoke are responsible for tumour genesis, signalling through the nicotinic acetylcholine receptors contributes to tumour progression and metastatic spreading [17].

The aim of this study was to determine CA9 expression and its impact on patients’ prognosis in pulmonary metastatic dissemination of CRC. Since tobacco smoking is a relevant risk factor for metastatic spreading to the lungs, we further investigated the association of nicotine exposure and CA9 expression in vitro and in vivo.

MATERIALS AND METHODS

Study population

From April 2009 to October 2012, 44 consecutive patients who underwent pulmonary metastasectomy after primary CRC were included in this retrospective study. All patients received thoracic and abdominal computed tomography for tumour staging prior to the metastasectomy. Complete resection was achieved in all patients. The mean diameters of the histologically confirmed metastatic nodules were measured and the estimated tumour volume was calculated by assuming a spherical shape. Lung metastasis-free survival (LMFS) was defined as the time between diagnosis of the primary tumour and diagnosis of the metastatic spreading to the lung. The study was approved by the ethics committee of the Medical University of Vienna (EK1044/2012) and was conducted according to the Helsinki declaration and the guidelines for good scientific practice of the Medical University of Vienna.

Immunohistochemistry and scoring

Immunohistochemical staining was performed following a standard protocol. Briefly, formalin-fixed, paraffin-embedded tissue specimens were assessed using the avidin–biotin–peroxidase complex technique. A mouse anti-carbonic anhydrase IX antibody (1:500; Abcam, Cambridge, UK) was combined with the Vectastain anti-IgG mouse kit (Vector Laboratories, Burlingame, CA, USA). The reaction was visualized with AEC substrate (Vector Laboratories) and counterstained with haematoxylin. As negative controls, the primary antibody was omitted. Two independent blinded observers rated the staining intensity. Immunohistochemistry staining score (IHC score) was calculated as the product of intensity (0–3) and percentage of positive cells (0–100%) as described previously [18]. If two ratings differed, the stained section was discussed and re-evaluated. The continuous IHC score was transformed into a dichotomous variable by calculating the median score for metastases or primary tumours. Tumour sections showing IHC scores above the median score were described as highly positive (CA9high) and sections below the median score were grouped as low expressing (CAlow). As no standardized, approved protocol for CA9 staining is available, the median IHC score in our cohort was chosen as cut-off point.

Cell culture

HT29 (ATCC# HTB-38) cells were cultured in McCoy's 5A medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum,
2 mM l-glutamine, 25 mM HEPES and 0.01% penicillin/streptomycin in a humidified incubator (37°C, 5% CO₂). 5 × 10⁵ cells/well were seeded on six-well plates. Thereafter, the adherent cells were treated for 16 h with 0.25, 0.50 or 1.00 μM (-)-nicotine (Sigma-Aldrich, St Louis, MO, USA) supplemented to the medium or medium alone. In some experiments, the cells were preincubated for 1 h with 200 nM α-bungarotoxin (Sigma-Aldrich). After the treatment, supernatants were removed and the cells were lysed using RIPA buffer with COMPLETE protease inhibitor (Sigma-Aldrich). All experiments were performed in triplicate. Cell viability was assessed by trypan blue exclusion test and was >95% in all experiments.

**Immunocytochemistry**

2 × 10⁵ cells/well were seeded on four-well culture slides (BD Falcon, Franklin Lakes, NJ, USA) and treated as described above. Cells were fixed in ice-cold methanol and immunocytochemical staining was carried out as described above, except that DAB was used as substrate.

**Enzyme-linked immunosorbent assay**

Cell lysates were assessed using a commercially available CA9 ELISA kit (DuoSet, R&D Systems, Minneapolis, MN, USA). All experiments were carried out at least three times and the samples were assayed in duplicate. The absorbance was measured at 450 nm and converted into pg/ml. The resulting amount of CA9 was adjusted by the total protein amount, determined by a bicinchoninic acid assay (BioRad, Richmond, VA, USA).

**Western blot analysis**

After protein concentration was determined, cell lysates were loaded on a 15% sodium dodecyl sulphate-gel, separated by electrophoresis under reducing conditions and blotted on a nitrocellulose membrane. The membrane was blocked and then incubated with primary antibodies (anti-HIF-1α antibody; Abcam) and anti-pSTAT3 antibody, detecting the phosphorylated tyrosine-705 (Cell Signaling, Boston, MA, USA). After a secondary antibody step,

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**Figure 1:** Negative (A), moderate (B) and intense (C) CA9 staining of pulmonary metastases. (D) Intense staining in perinecrotic areas. The star indicates the area of necrosis (×100/×200 magnification; bar = 200 μm). (E) Median total tumour volume of resected metastases (whiskers indicate the interquartile range). (F) Kaplan-Meier estimates of the lung metastasis-free survival depending on the expression of CA9 in the pulmonary.
antibody binding was detected using the enhanced chemiluminescence system and Hyperfilm (both Amersham Biosciences, Piscataway, NJ, USA). Band intensity was quantified by using the ImageJ software (National Institutes of Health, Hamilton, MT, USA).

Statistical analysis

All data collected in this study were evaluated statistically using SPSS 19 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, Inc., CA, USA). Non-parametric data were expressed as median, 25th and 75th percentile. The Mann–Whitney U-test was used to compare medians between two groups for non-parametric distributed data. Student’s t-test was used to compare means of two groups for parametric distributed data, expressed as mean ± standard error of the mean (SEM). The Kaplan–Meier method, log-rank test and Cox regression were used to compare survival functions. Chi-square test and Fisher’s exact test (if the expected frequency was <5) were used to compare binomial variables. All tests were two sided. P-values of ≤0.05 were considered statistically significant.

RESULTS

CA9 is expressed in pulmonary metastases of primary CRC and is associated with early pulmonary spreading

The demographic characteristics of the 44 patients are presented in Table 1. CA9 expression was evident in 90.1% of the resected metastases. The median IHC score was 167.5. CA9 was strongly expressed at the invasive front and around necrotic tumour areas (Fig. 1A–D). As the tumour volume is an indicator of tumour growth [19], the estimated metastatic tumour volume was compared between metastases with strong CA9 expression and low CA9 expression (Fig. 1E). The median in the low expression group was 1.49 vs 1.77 cm3 in the group with high expression of CA9. The LMFS of patients with CA9high and CA9low pulmonary metastases was compared using a Kaplan–Meier curve and log-rank test. We found a significantly decreased LMFS in patients with CA9high pulmonary metastases (median 21.0 vs 38.5 months; P < 0.001; Fig. 1F).

CA9 expression correlates with established negative prognostic markers

The International Registry of Lung Metastases (IRLM) has identified three negative prognostic markers for the overall survival of patients with pulmonary metastases [20]. We assigned the included patients into three groups according to their number of risk factors (Fig. 2A). Metastasectomy with histologically proven complete resection could be achieved in all patients, which enabled grouping of no, one or two risk factors. The CA9 expression increased with the number of risk factors (median 110, 160 and 185 for the groups with no, one and two risk factors, respectively (Fig. 2B)). The difference between the group with no risk factor and the group with two risk factors was highly significant (P < 0.001). CA9 staining intensity was further subdivided by grouping into numbers of metastases or DFI (Supplementary Fig. S1A).

Concordant high CA9 expression in pulmonary metastases and paired primary CRC is associated with lung metastasis-free survival

We further evaluated the expression of CA9 in corresponding tissue samples from the primary tumour site. Tissue specimens of 32 (72.7%) patients were available. Representative images are shown in Fig. 3A–D. Patients with CA9high expression in their primary tumour had a median LMFS of 23 months, compared with 37 months in the group with low IHC scores (P < 0.001, Fig. 3E). In the next step, we compared CA9 expression patterns of the primary tumour sites with the corresponding metastases. Twelve and 11 patients had concordant low/low or high/high CA9 expression.
expression in the primary and metastasis tissue specimens. The LMFS in concordant CA9low/low was 41 months vs 23.5 months in the concordant CA9high/high (P < 0.001; Fig. 3F).

Univariate and multivariate analyses

The impact of clinico-pathological characteristics on the time to the first pulmonary metastasis was examined by the log-rank test and Cox regression analysis. Sex, age, smoking history, location of the primary tumour, pT stage, pN stage, adjuvant chemotherapy and expression of CA9 of the metastases and the primary tumours were included in the analysis. The only parameter, which had a significant influence on the time to the first metastasis to the lung was the CA9 expression level in the primary tumour (P < 0.001 in univariate analysis). Interestingly, patients with a smoking history also had a decreased time to the first pulmonary metastasis, without reaching the level significance (Table 2 and Supplementary Fig. S1B). When adding the factors, which were most likely to affect the LMFS (sex, smoking history, location of the primary tumour and CA9 expression of the primary tumour) as covariates in the multivariate analysis, a high CA9 expression level in the primary tumour remained a significant prognostic factor (P = 0.004).

CA9 is increased in patients with a history of tobacco smoking

Tobacco smoking is thought to be a relevant factor for metastatic spreading to the lungs. Patients were grouped according to their smoking history into never smokers (n = 20) and former smokers, who stopped smoking more (n = 11) or less (n = 11) than 10 years before the diagnosis of CRC. Smoking status was unknown in two patients, who were excluded from further analysis. We found a strong association between the time of quitting tobacco smoking...
and median expression score of CA9 (157, 150 and 190 for never smokers, former smokers >10 and <10 years, respectively). The difference between never smokers and former smokers <10 years and between both groups of former smokers was significant ($P = 0.018$ and $P = 0.012$; Fig. 2C). Although LMFS was decreased by a positive smoking history (24 vs 34 months), this trend did not reach significance in our series of 44 patients (Supplementary Fig. S1B).

Nicotine induces CA9 expression in vitro

Given these clinico-pathological findings, we determined the influence of tobacco smoking on the expression of carbonic anhydrase IX in vitro. Therefore, HT29 cells were treated with nicotine (Fig. 4A). HT29 cells exposed to nicotine expressed significantly higher levels of CA9 than control HT29 cells treated with medium (14 634 ± 1386, 17 588 ± 2462, 20 381 ± 1222 pg/ml for medium, 0.25 nicotine, 0.5 μM nicotine, respectively; $P = 0.03$). This effect could be inhibited by α-bungarotoxin, a α7 nicotinic acetylcholine receptor antagonist (10 589 ± 1586 pg/ml; $P = 0.032$). Moreover, the impact of the nicotine on carbonic anhydrase IX expression was confirmed by IHC (Fig. 4B). In a further set of experiments, we evaluated the impact of nicotine exposure on pSTAT3 and HIF1α expression. We found a dose-dependent increase in pSTAT3 (1.1-fold, 1.5-fold, 2.0-fold increase compared with medium-treated cells for cells stimulated with 0.25 0.5 and 1 μM nicotine) expression. This effect could be attenuated by preincubation with α-bungarotoxin (Fig. 4C).

**DISCUSSION**

The identification of patients who will benefit at the utmost from a surgical procedure still remains a major problem in pulmonary metastasectomy. To date, selection criteria are limited to clinical data including disease-free interval, number of nodules and alternative treatment regimes [2, 8]. However, the impact of some of these clinical factors (e.g. number of nodules) on the prognosis is not a consistent finding in all conducted studies. Several studies have been undertaken to find prognostic biomarkers for pulmonary metastasectomy in CRC patients [21]. However, none of these molecular markers has reached general acceptance and is routinely used. Nevertheless, studies describing such markers are of further interest in translational cancer research, as they provide information on highly metastatic tumour clones capable of forming macrometastases.

CA9 is a transmembrane glycoprotein that catalyzes hydration of CO2 and thus is involved in a variety of physiological processes [9]. Overexpression of CA9 could be observed in several tumour entities, such as breast cancer, non-small-cell lung cancer and oesophageal cancer. Recent data suggest that CA9 expression is

<table>
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<th>Table 2: Univariate and multivariate analyses of the lung metastasis-free survival</th>
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<tr>
<td><strong>Total</strong></td>
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<td><strong>N</strong>= 44 (%)</td>
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<tr>
<td><strong>Sex</strong></td>
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<tr>
<td>Male</td>
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<tr>
<td>Female</td>
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<tr>
<td><strong>Age (years)</strong></td>
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<tr>
<td>&lt;64</td>
</tr>
<tr>
<td>≥64</td>
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<tr>
<td><strong>Smoking history</strong></td>
</tr>
<tr>
<td>Never smoker</td>
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<tr>
<td>Former smoker</td>
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<tr>
<td>Unknown</td>
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<td><strong>Location</strong></td>
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<td>Rectum</td>
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<tr>
<td>Unknown</td>
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<td>pT3 + pT4</td>
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<td><strong>N stage</strong></td>
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<td><strong>Chemotherapy</strong></td>
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<td>1</td>
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<td><strong>CA9 expression primary tumour</strong></td>
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directly involved in tumour progression and metastatic spreading and CA9 was linked to tumour cell migration and invasion. In addition, a facilitated epithelial–mesenchymal transition mediated by Rho-GTPases could be observed after CA9 transfection [10].

Our study gives first evidence that CA9 is highly expressed in CRC pulmonary metastases. Moreover, we were able to show that high expression of CA9 in metastases and corresponding primary tumours is associated with an early metastatic spreading to the lung. The IRLM has defined clinical prognostic markers after pulmonary metastasectomy in a huge cohort of over 5000 patients [20]. By grouping our patients according to the number of IRLM risk factors, we found a significant increase in CA9 expression in pulmonary metastases of high-risk patients. Thus, CA9 might be a potential biomarker to help identify those patients with CRC pulmonary metastases who will mostly benefit from a surgical procedure.

One very important finding of our study is the association of CA9 expression and smoking habits. Smoking is thought to be a major contributing factor not only for the development of primary lung cancer but also for the occurrence of pulmonary metastatic spreading of primary tumours outside the lung [22]. Therefore, we sought to investigate the association of nicotine exposure and CA9 expression in a set of in vitro experiments. By incubating the colorectal carcinoma HT29 cell line with different concentrations of nicotine, we observed an increased pSTAT3 and CA9, but an unaffected HIF1α expression. This finding indicates that in CRC, smoking leads to STAT3-dependent CA9 up-regulation, independent of the hypoxia-related pathway. Interestingly, a STAT3-dependent mechanism enhancing the metastatic spreading was recently shown in a murine model of pancreatic cancer by Momi et al. [23]. The expression of CA9, in turn, is thought to be a STAT3-dependent process as shown in an in vitro model with breast cancer cell lines [14]. This relationship was confirmed by histopathological evaluations of tissue specimens in patients with oesophageal cancer [24].

All patients in our cohort were never-smokers or ex-smokers who had unexceptionally quit smoking before or at the time of the diagnosis of CRC. An explanation for the constant CA9 expression in metastatic lesions even years after smoking cessation might be an initial induction of CA9 by nicotine and a subsequent selection of CA9 overexpressing clones. This theory is supported by the notion that some of our patients already received a second pulmonary metastasectomy and the CA9 expression of all resected metastases was constant throughout time (data not shown). Future studies are planned to validate this concept.

Considering the abnormal high expression of CA9 in different tumours, CA9 may represent an interesting candidate for future targeted anticancer therapy. First results in cell lines and animal models are promising. Most relevant to our study was work by Lou et al. showing that the formation of metastases could be inhibited by CA9 blocking. Mice injected with a CA9-depleted breast cancer cell line evidenced a reduced capacity to form lung

Figure 4: (A) Levels of CA9 in cell lysates of HT29. α-Bungarotoxin significantly reduced the level of CA9, which can be increased by treatment with nicotine. The decrease in CA9 below the medium control implicates an autocrine/endogenous ACh signalling (n = 3; mean ± SEM). (B) Immunocytochemical staining of HT29 cells for CA9 (DAB substrate). (C) Western blot analysis revealed an induction of pSTAT3 but not HIF1α by nicotine treatment (n = 3, pooled analysis. Lanes 1–4 and 5 were spliced from the same gel).
metastases as determined by bioluminescent imaging [25]. In the same study, several small molecule inhibitors, which could be introduced in a clinical setting in near future, were tested for their pharmacological activity.

Interestingly, CA9 expression showed a trend towards lower expression in patients with previous liver metastases. This supports the idea that tumours of patients with the liver as the first step of distant colonization might be biologically different compared with patients with the lungs as the first site of tumour spreading.

The study is limited by the fact that only formalin-fixed, paraffin-embedded specimens of the resected tumours were available. Confirming the results with additional methods like western blotting would have been desirable. Despite the interesting association of smoking history and pulmonary metastasis, the validity of the conclusions drawn by retrospective data is limited. A prospective study design including more patients is warranted to confirm this work's results.

Concluding, this study describes CA9 expression in pulmonary metastases and corresponding CRC primary tumours for the first time. We could show an association of CA9 expression with smoking habits and this correlation was reproduced by in vitro experiments. Further studies are warranted to expand the understanding of CA9 involvement in the process of metastasis and the impact of CA9 overexpression on selecting patients for curative metastasectomy.

**SUPPLEMENTARY MATERIAL**

Supplementary material is available at EJCTS online.

**Funding**

This study was supported by the Christian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration, the Medical University of Vienna and the German Research Foundation (GU 1271/2-1).

**Conflict of interest:** none declared.

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