MEDICAL UNIVERSITY OF VIENNA

Identification of prognostic biomarkers in patients undergoing pulmonary metastasectomy from primary colorectal cancer

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Submitted by

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"When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil."

Stephen Paget, English surgeon (1855 - 1926)

cited from Paget S, "The distribution of secondary growths in cancer of the breast" published in *The Lancet* (Volume 133, Issue 3421, 23 March 1889)

Declaration

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Abstract

Pulmonary metastases (PM) contribute to the high cancer-related morbidity and mortality in patients with solid malignancies including colorectal cancer (CRC). Despite advances in the diagnosis and treatment of patients with early-stage CRC, the optimal treatment of patients with distant metastases from CRC - especially PM - is currently unclear. To improve the clinical outcome of these patients, the biology of metastatic CRC needs to be further elucidated.

In this work we aimed to describe clinical as well as histopathological and genetic factors contributing to the diminished prognosis in patients with an unfavorable tumor biology. On DNA level, we assessed the mutation status of KRAS and BRAF, which are thought to play an important role in CRC progression. Moreover, the expression of EGFR in PM was described. In the second part of this work we focused on the tumor-surrounding stroma. To the best of our knowledge, we could describe for the first time that Hsp27 is highly expressed by activated fibroblasts adjacent to the disseminated tumor cells. Moreover, these activated fibroblasts are a prognostic marker after pulmonary metastasectomy.

In this work we demonstrated that patients with PM from CRC are a highly heterogenous group regarding the tumor biology and the outcome after pulmonary metastasectomy can be prognosticated by the underlying tumor biology. The accurate description of PM based on molecular markers might lead to an improved treatment of patients with metastatic CRC.

Zusammenfassung

Lungenmetastasen tragen zur hohen Morbidität und Mortalität von Patienten mit Krebserkrankung, insbesondere dem colorectalen Carcinom (CRC), bei. Obwohl deutliche Fortschritte in der Diagnose und Behandlung von Patienten CRC im Frühstadium der Krebserkrankung gemacht werden, ist die optimale Behandlung von Patienten mit Fernmetastasen – insbesondere Lungenmetastasen – derzeit unklar. Um die Behandlung dieser Patienten weiter zu verbessern, ist es notwendig die Tumorbiologie des metastasierten CRC besser zu verstehen.

Ziel dieser Arbeit war es histo-pathologische und genetische Faktoren zu identifizieren, die zur schlechten Prognose von Patienten mit besonders aggressiven Tumoren beitragen. Auf DNS-Ebene untersuchten wir Mutationen im KRAS- und BRAF-Gen, welche als wichtige Faktoren im Voranschreiten des CRC angesehen werden. Außerdem haben wir die Expression von EGFR in Lungenmetastasen untersucht.

Der zweite Teil unserer Arbeit fokussierte sich auf das tumor-assoziierte Stroma. Nach unserem derzeitigen Wissenstand konnten wir als erste Gruppe beschreiben, dass Hsp27 in aktivierten Fibroblasten in unmittelbarer Nachbarschaft zu Tumorzellen überexprimiert ist. Desweiteren sind diese aktivierten Fibroblasten prognostisch in Patienten mit CRC Lungenmetastasen.

In der vorliegenden Arbeit konnten wir zeigen, dass es große Unterschiede innerhalb der biologischen Eigenschaften von CRC Lungenmetastasen gibt. Unterschiede im Überleben dieser Patienten können durch die zugrunde liegende Tumorbiologie prognostiziert werden. Basierend auf molekularen Markern könnte die genaue Beschreibung von Lungenmetastasen zur Verbesserung der Behandlung von Patienten mit metastasiertem CRC beitragen.

Publications arising from this thesis

Schweiger, T., Lang, G., Klepetko, W. & Hoetzenecker, K. Prognostic factors in pulmonary metastasectomy: spotlight on molecular and radiological markers. *Eur J Cardiothorac Surg* **45**, 408-416 (2014).

Schweiger, T.*, et al.* EGFR, BRAF and KRAS status in patients undergoing pulmonary metastasectomy from primary colorectal carcinoma: a prospective follow-up study. *Ann Surg Oncol* **21**, 946-954 (2014).

Schweiger, T.*, et al.* Stromal expression of heat-shock protein 27 is associated with worse clinical outcome in patients with colorectal cancer lung metastases. *PLoS One* **10**, e0120724 (2015).

Abbreviations

95%CI... 95% confidence interval
alpha-SMA... alpha smooth muscle actin
CEA... carcinoembryonic antigen
COX-2… cyclooxygenase- 2
CRC... colorectal cancer
CT... computed tomography
CTC… circulating tumor cells
CXCL... C-X-C motif ligand
DFI... disease-free interval
EGFR... epidermal growth factor receptor (EGFR).
EMT… epithelial-mesenchymal transition
FAP... fibroblast-activation protein alpha
Hsp27... heat shock protein 27
KRAS... Kirsten rat sarcoma viral oncogene homolog
MVD... microvessel density
Nd:YAG... neodymium-doped yttrium aluminium garnet
NFkappa-B... nuclear factor kappa-light-chain-enhancer of activated B-cells
NRAS… Neuroblastoma RAS viral oncogene homolog
OR.... Odd's ratio
PI3K... phosphatidylinositol 3-kinase
TGF beta... transforming growth factor beta
TKR... tyrosine kinase receptors
TRAIL-R2… Tumor necrosis factor-related apoptosis-inducing ligand receptor 2
VEGF... vascular endothelial growth factor
Wnt... Wingless-related integration site

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CHAPTER ONE: INTRODUCTION

1. General introduction

In Europe, colorectal cancer (CRC) is the most commonly diagnosed type of cancer. In 2012, the age-standardized incidence in male was 55.7/100.000 and in female 34.7/100.000, resulting in an overall incidence of 43.5/100.000. Regarding the mortality, CRC is the second most common cause of cancer-related deaths, following lung cancer (Ferlay *et al*, 2013). Similar to other malignancies, distant metastasis is the cause of the high mortality of patients with CRC (Vatandoust *et al*, 2015).

1.1 Epidemiology of CRC pulmonary metastases

Second to the liver, the lungs are the most common site of distant spreading of CRC. During the course of disease, about 10-15% of all patients diagnosed with CRC will develop pulmonary metastasis (Mitry *et al*, 2010; Pihl *et al*, 1987). Metastasis can occur either synchronously, i.e. metastases are present at the time of diagnosis of the primary tumor, or metachronously, which means that the metastases are diagnosed during follow-up examinations. The median time between diagnosis of primary CRC and lung metastasis is 24.6 months, which is about 7 months longer than the average time to occurrence of liver metastases (Manfredi *et al*, 2006). This reflects the model of a stepwise metastatic spread of CRC, usually starting with the liver as first site of metastasis, afterwards involving the lung and finally further distant organs like the brain or the bones. Compared to colon cancer, patients with rectal cancer have a significantly higher risk of pulmonary metastases which is true for both, synchronous (OR (95%CI) 2.80 (1.65 to 4.76)) and metachronous (OR (95%CI) 2.63 (1.69 to 4.08) metastatic spreading. This can be explained by tumor cells bypassing the portal system through rectal veins and thus reaching the lungs as first filter organ. Patients with isolated lung metastasis have a significantly better overall survival than patients with at least one more site of metastatic spread, independently whether the lung metastases are synchronous or metachronous (Mitry *et al*, 2010).

1.2 Pathobiology of CRC lung metastases

The metastatic cascade comprises (1) local invasion at the primary tumor site, (2) intravasation into blood or lymphatic vessels, (3) circulating to the target organ, (4) arrest at the metastatic site, (5) extravasation and (6) outgrowth at the secondary organ (Valastyan & Weinberg, 2011).

Local invasion is characterized by the disintegration of the basement membrane, the degradation of the surrounding extracellular matrix and the detachment of the tumor cells. The process of detachment is called epithelial-mesenchymal-transition (EMT), which occurs physiologically during embryogenesis (Kalluri & Weinberg, 2009). Mainly by downregulation of E-cadherin by the transcription factor SNAIL, tumor cells can detach from the cell sheets and invade in the surrounding parenchyma and get in close contact to vessels (Oda *et al*, 1998; Thiery *et al*, 2009).

The intravasation into lymphatic vessels and blood vessels is a further keystep in the metastatic process. Evidence of lymphatic vessel invasion in the primary tumor is an important clinical prognosticator in various malignancies including CRC (Ishii *et al*, 2009). Our group could demonstrate that lymphatic invasion is of prognostic value in metastatic CRC (Schweiger *et al*, 2015). The intravasation of tumor cells in tumor-adjacent vessels is facilitated by the poor quality of intratumoral vessels, formed by excessive angiogenesis (Carmeliet & Jain, 2011).

As soon as the tumor cells reach the lumen of vessels, these tumor cells en route to metastatic organ sites are detectable in the peripheral blood and are termed circulating-tumor cells (CTC). The presence of these cells, which are a pre-requisite for metastatic disease, are also an negative prognostic marker in CRC (Romiti *et al*, 2014). CTC have to overcome several factors to form distant metastases. First of all, normal cells detached from their surrounding undergo anoikis. This programmed cell-death due to the loss of anchorage needs to be suppressed (Guo & Giancotti, 2004). Additionally, CTC have to be resistant to shear stress. Interestingly this seems to be mediated by coverage of the CTC with thrombocytes. By the same mechanism CTC can escape cytotoxic immune cells until they reach a distant capillary bed (Joyce & Pollard, 2009).

Whether the arrest of the tumor cell at the target site is a passive process (tumor microemboli) or merely a site-specific process is currently under discussion. Hematogenous dissemination is believed to be main route of metastasis to the lung, which is especially true for CRC (Woodard *et al*, 1998). As previously mentioned, in patients with rectal cancer metastases can be found more often in the lungs as initial metastatic site, compared to colon cancer in general and especially compared to right-sided colon cancer (Mitry *et al*, 2010). Thus, the first capillary bed encountered by a circulating tumor cell determined by vascular

anatomy is a key determinant of the metastatic process. However, there are limitations to this theory, which were firstly described by the English surgeon Stephen Paget (1855 - 1926) and the Viennese ophthalmologist Ernst Fuchs (1851 – 1930). Based on the observation that the frequency of metastases at different target organ sites can not be explained solely by anatomical considerations, the “seed and soil”- theory was proposed by Paget based on his findings in patients with breast cancer and a work published seven years before by Fuchs on patients with “uveal sarcoma” (Fuchs, 1882; Paget, 1889). According to this theory, the target organ site is also determined by characteristics of the tumor cell (“seed”) and the microenvironment of the secondary organ site (“soil”). Based on this hypothesis, the so-called *metastatic niche*, corresponding to the “soil”, has gained the attention of today’s oncological research (Psaila & Lyden, 2009). According to this model, the target organ forms a conductive environment by various mechanisms. For instance, by up-regulation of matrix-metalloproteinases, the extracellular matrix at the niche is degraded and pro-inflammatory cytokines are released, facilitating the extravasation and outgrowth of metastatic cells (Hiratsuka *et al*, 2002). Moreover, a site-specific colonization mediated by stromal-derived factor-1 (released by the target organ) and CXC-chemokine receptor 4 (expressed by the tumor cell) has been described (Feys *et al*, 2015).

The extravasation of intra-luminal CTC is the next step in the metastatic cascade. By the disintegration of endothelial cells and pericytes, tumor cells can advance into the parenchyma of the metastatic organ site and can therefore be seen as the opposite of intravasation. The underlying mechanisms are a matter of current research. Proteins increasing the permeability of capillaries like COX-2, VEGF and matrix-metalloproteinases were demonstrated to be secreted by tumors (Gupta *et al*, 2007). Interestingly, by releasing mediators capable to increase the permeability of the capillary bed, already the primary tumor can prime the secondary organ site (“pre-metastatic niche”) to facilitate the extravasation as soon as CTC arrive (Huang *et al*, 2009).

Finally, the metastatic tumor cells need to survive and grow at the distant organ site, despite the distinct environment. It was proposed that also the microenvironment is already modulated at an earlier stage of cancer progression (Psaila & Lyden, 2009). Cell proliferation needs to outnumber cell death at the distant site to grow out to a micro- and further on to a macro-metastasis. Increased neoangiogenesis is thought to be the prerequisite for the outgrowth as it confers the supply with oxygen and nutrients (Klauber-DeMore *et al*, 2001).

1.3 Clinical prognostic factors in pulmonary metastasectomy

The pre-existing literature describes mainly clinical risk factors for a decreased outcome after pulmonary metastasectomy. The most frequently discussed clinical prognostic factors are summarized in the following.

Carcinoembryonic antigen

The blood level of the tumor marker carcinoembryonic antigen (CEA) is thought to reflect the total tumor burden in CRC patients (Choi *et al*, 2005). Moreover, it was reported that an increased serum CEA level before pulmonary metastasectomy prognosticates a worse outcome after pulmonary metastasectomy. Suzuki *et al.* found a significantly decreased disease-specific survival for patients with elevated pre-metastasectomy CEA levels (5-year survival 57.0% vs. 30.9%) (Suzuki *et al*, 2015), without describing the used cut-off. In a cohort of 199 patients undergoing PM, CEA levels > 5ng/mL were associated with a significantly reduced overall survival (5-year survival 55% vs. 28%) (Zampino *et al*, 2014). In another study cohort Pfannschmidt *et al.* also applied a 5 ng/mL cut-off and found a 5-year survival of 48.3% for patients with low CEA levels and a 5-year survival rate of 22.7% for patient with elevated serum levels (Pfannschmidt *et al*, 2003). Additionally, a large multicenter study in Japan including 1030 patients confirmed these findings in univariate and multivariate survival analysis (Iida *et al*, 2013). 5-year survival was 60.4% in the group with normal CEA levels, compared to 43.4% in patients with increased serum levels.

Completeness of resection

A microscopic (R1) or even macroscopic (R2) incomplete resection is the most important and most widely accepted clinical prognosticator for decreased outcome after pulmonary metastasectomy. In an international survey of the European Society of Thoracic Surgeons, 98% of the participating surgeons consider an expected incomplete resection of pulmonary metastases as absolute (74%) or relative (24%) contraindication for pulmonary metastasectomy (Internullo *et al*, 2008). The reported 5-year survival drops to 8% -14% in patients with incomplete resection compared to 5-year survival rates in the respective studies of 47% - 55.8% for macroscopically and microscopically complete resection (Iida *et al*, 2013; Zampino *et al*, 2014). Moreover, an international registry (International Registry of Lung Metastases) including 5206 patients undergoing pulmonary metastasectomy confirmed the completeness of resection as a valuable prognostic marker (Pastorino *et al*, 1997). Thus, pulmonary metastasectomy should only be offered to patients if the pre-operative work-up

suggests that a complete resection of all pulmonary metastases is technically (and functionally) feasible.

Disease-free survival

The disease-free interval (DFI), which describes the time between the diagnosis of the primary tumor and distant metastases, is thought to reflect an aggressive tumor biology and thus is another proposed prognostic factor in various malignancies. The International Registry of Lung Metastases identified a DFI less than 36 months as prognosticator for decreased overall survival after metastasectomy. In other studies on pulmonary metastasectomy for metastatic CRC, the reported cut-off varies between 6 months and 60 months. Significant differences in survival dependent on the DFI could be demonstrated by some of the studies (Hwang *et al*, 2010; Onaitis *et al*, 2009; Yedibela *et al*, 2006). Although it appears to be legitimate to use the DFI as a measurable parameter for rapidly spreading tumors, most of the studies assessing clinical prognostic markers in pulmonary metastasectomy for CRC could not confirm the DFI as statistically significant (Iizasa *et al*, 2006; Kanemitsu *et al*, 2004; Melloni *et al*, 2006; Pfannschmidt *et al*, 2003; Saito *et al*, 2002; Suzuki *et al*, 2015; Welter *et al*, 2007a; Zampino *et al*, 2014). This might be due to various confounders like time of diagnosis of the primary tumor, different follow-up intervals, patients' compliance, etc.. Thus, patients with a short DFI should not be excluded from surgery. Excellent long-term outcome is achievable despite early evidence of pulmonary recurrence.

Lymph node involvement

In primary CRC, the evidence of lymph node metastases is an important prognostic factor and thus part of the staging system (TNM staging) of primary CRC (Gertler *et al*, 2009). Interestingly, in patients with pulmonary metastasis from CRC, the involvement of thoracic, i.e. hilar and mediastinal lymph nodes, has gained special attention in the scientific community as relatively novel prognostic factor. Patients with pulmonary CRC metastases and evidence for thoracic LN metastases have a dramatically decreased prognosis. For instance, in the Japanese multicenter study including 1030 patients, 5-year survival for patients with pathologically proven thoracic lymph node metastasis was 37.3%, compared to 59.4% in patients without evidence for lymph node involvement. In the vast majority of other studies, the 5-year survival drops to <20% as soon as thoracic lymph node metastases are detected (Iizasa *et al*, 2006; Inoue *et al*, 2004; Pfannschmidt *et al*, 2003; Renaud *et al*, 2014; Welter *et al*, 2007b; Zampino *et al*, 2014). Interestingly, the presence of lymph node metastases has only prognostic implications, the resection of these lymph node metastases does not add any survival benefit (Bolukbas *et al*, 2014; Renaud *et al*, 2014). These findings

suggest, that patients with CRC lung metastases and thoracic lymph node involvement, which corresponds to “metastasis-from-metastasis”, do not benefit from surgery. Our group could demonstrate in another recent study that evidence for infiltration to the lymphatic system in the pulmonary metastases is capable to predict the occurrence of lymph node metastases (Schweiger *et al*, 2015). Moreover, presence of lymphovascular invasion was associated with a diminished prognosis after pulmonary metastasectomy (see Figure 1).

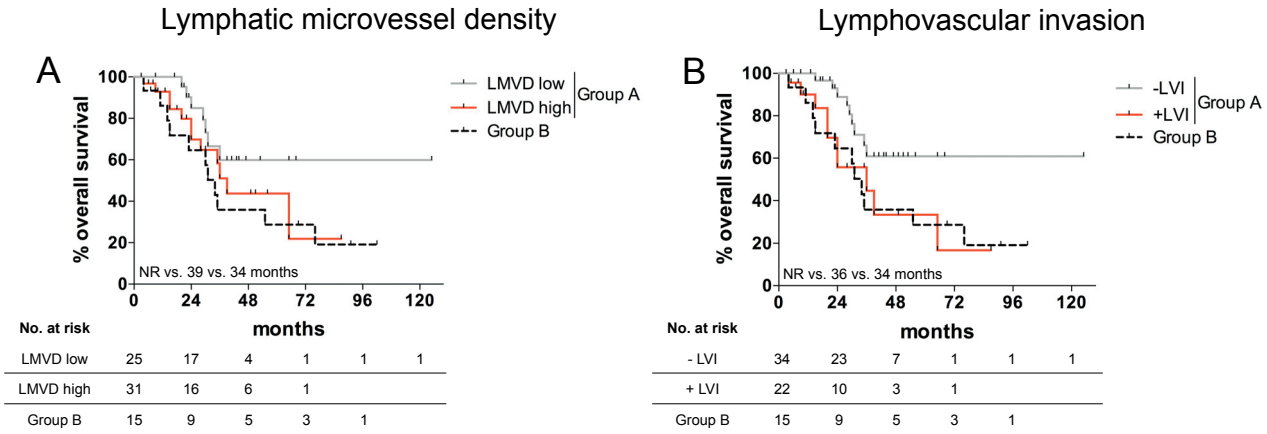

Figure 1: Overall survival of patients with CRC pulmonary metastases exhibiting sustained lymphangiogenesis (left) or with evidence of lymphatic invasion (right) have a diminished prognosis after pulmonary metastasectomy. The outcome is comparable to patients with pathologically verified thoracic lymph node metastasis (Group B; dashed line). Adopted from „Increased lymphangiogenesis in lung metastases from colorectal cancer is associated with early lymph node recurrence and decreased overall survival“ (Schweiger *et al*, 2015).

Number of nodules

Another proposed prognostic factor in patients undergoing pulmonary metastasectomy is the number of resected metastases (see Figure 2). Most studies differentiate between singular versus multiple metastases. Iida *et al.* described multiple metastases as significant poor prognosticator regarding the overall survival after pulmonary metastasectomy in univariate and multivariate analysis (5-year survival 42.0% versus 61.5%) (Iida *et al*, 2013). Also Zampino *et al.* found a significantly different survival between patients with single and multiple pulmonary metastases. 5-year survival was 51% for singular metastases compared to 44% for multiple metastases. After 10-year almost twice as many patients with single metastases were alive compared to the group of patients with multiple nodules (40% versus 23%) (Zampino *et al*, 2014). These recent works confirmed the prognostic impact of the number of pulmonary metastases, which was also described by several works on pulmonary metastasectomy for metastatic CRC in the past (Chen *et al*, 2009; Inoue *et al*, 2004; Kanemitsu *et al*, 2004; Onaitis *et al*, 2009; Pfannschmidt *et al*, 2003; Rama *et al*, 2009; Saito *et al*, 2002; Welter *et al*, 2007b; Yedibela *et al*, 2006). Moreover, the number of metastases

was also described as prognostic factor in the International Registry of Lung Metastases, which found a 5-year survival for patients with single metastasis of 43%, compared to 34% for patients with 2-3 metastases and 27% for patients with 4 or more metastases.

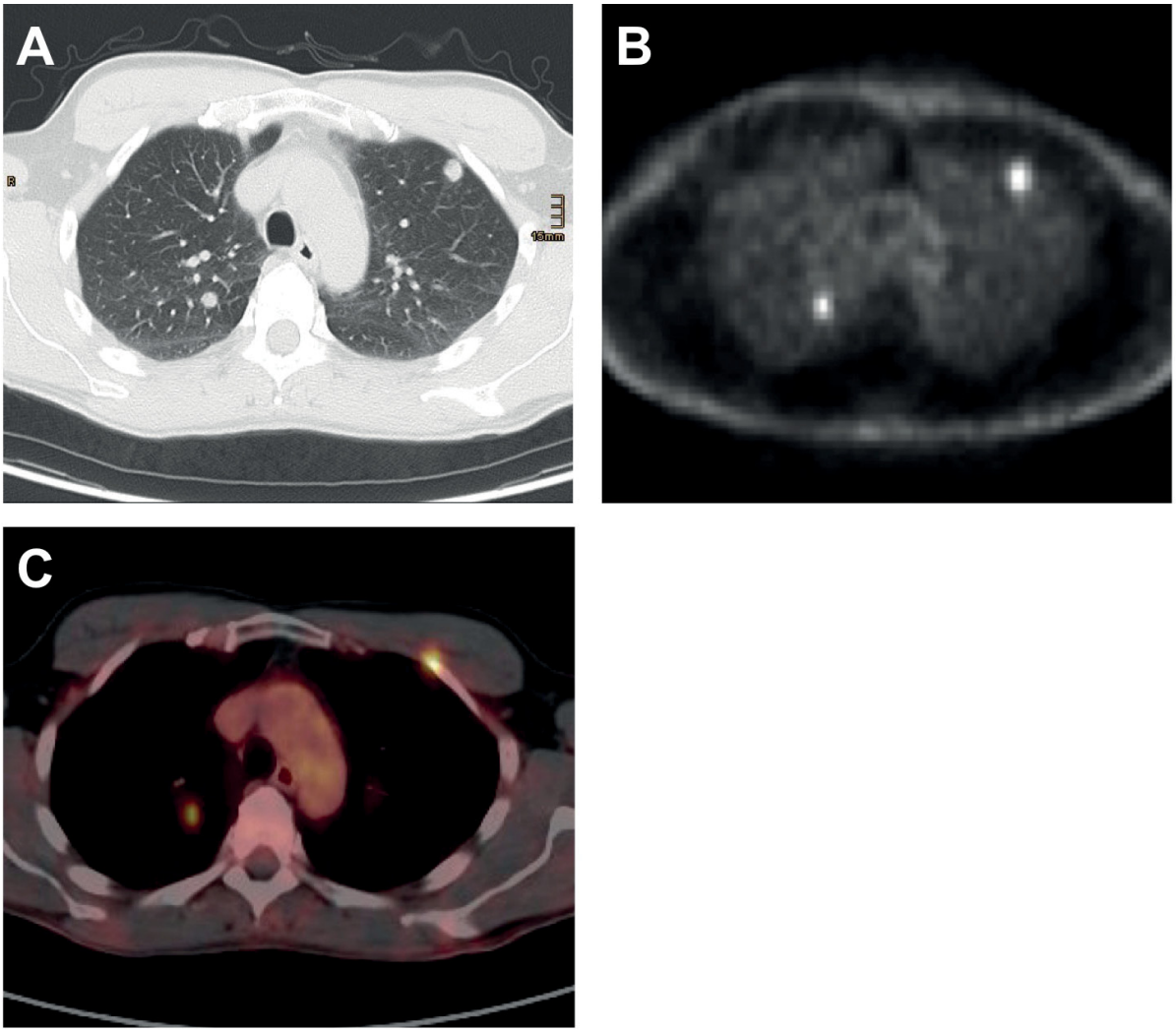

Figure 2: CT scan (A), PET scan (B) and merged PET-CT (C) showing strong 18F-FDG uptake of two lung metastases in a patient with a history of colorectal cancer.

1.4 Molecular prognostic factors in pulmonary metastasectomy

Prognostic factors in pulmonary metastasectomy: spotlight on molecular and radiological markers (published PDF)

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REVIEW

Prognostic factors in pulmonary metastasectomy: spotlight on molecular and radiological markers

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Summary

Pulmonary metastasectomy is nowadays a common practice in thoracic surgery. Clinical prognostic markers for poor outcome after pulmonary metastasectomy have been proposed in the late 1970's of the last century. Despite new insights in molecular mechanisms and advances in imaging techniques, neither molecular markers nor radiological features of metastases are used as prognosticators in routine clinical practice. Biomarkers associated with aggressive tumour behaviour came into the focus of clinical research and are the basis of personalized medicine. In addition, non-invasive imaging modalities like positron emission tomography and high-resolution computed tomography can provide further information on tumour aggressiveness. Regardless of a myriad of studies assessing prognostic markers in primary tumours, little is known about these markers in metastatic lesions. Furthermore, it has been emphasized that tumour biology of the primary might not reflect the behaviour of the corresponding metastasis. Therefore, information on the biology of metastases is necessary to treat patients adequately. This work reviews potential prognostic biomarkers in patients with pulmonary metastases, grouped in soluble tumour markers, tumour suppressor genes/proto-oncogenes and proteins involved in cell adhesion, tumour growth, cell metabolism and tumour angiogenesis.

Keywords: Prognostic marker • Pulmonary metastasectomy • Lung metastasis • Molecular biology • Radiology

INTRODUCTION

Since the first case report on a surgically removed pulmonary nodule by Weinlechner [1] in 1882, pulmonary metastasectomy has evolved into a standard procedure in patients with lung metastases. Widely accepted selection criteria are (i) a controlled or controllable primary tumour, (ii) the absence of extrathoracic metastases, (iii) the patient must be able to tolerate the degree of the planned lung resection and (iv) the non-availability of another superior effective therapy [2].

Although resection of pulmonary metastases is routinely performed in a variety of primary tumours, little is known on prognostic factors influencing the outcome of pulmonary metastasectomy. In times of individualized patient treatment, this knowledge is of great importance. Patients with a high risk of early tumour recurrence might benefit from pseudoadjuvant chemotherapy regimens. On the contrary, surveillance strategies may be sufficient for intermediate or low-risk patients. Moreover, a mapping of prognostic factors might help in the decision-making process when offering patients a remetastasectomy. A localized removal of pulmonary nodules is not recommendable if a recurrence within weeks is expected. Important clinical prognostic factors have been proposed by the International Registry of Lung Metastases (IRLM) including a short disease-free interval, multiple pulmonary nodules and an incomplete surgical resection [3]. However, contradicting studies can be found for other well-established clinical markers in regard to their prognostic impact after pulmonary metastasectomy [4, 5]. Thus, factors reflecting the tumour biology might be more accurate in defining the prognosis of patients with pulmonary tumour spread [6]. This review focuses on the prognostic markers predicting the clinical outcome in patients undergoing pulmonary metastasectomy that can be identified by molecular biological and radiological methods.

Using tissue or body fluid samples of patients to predict the individual risk of a certain disease or the success of a therapeutic approach has nowadays become routine in many diseases. In addition, biological markers are also of prognostic value in the course of some diseases [7]. The quest for reliable biomarkers is an important field of today's research in general and cancer research in particular. Compared with the bulk of literature regarding markers in primary tumours, little data are available on biological markers in pulmonary metastasectomy. Currently, evidence in literature is mostly based on small series of patients with primary colorectal carcinoma (CRC), melanoma and sarcoma.

In order to provide a clear overview, reviewed markers will be grouped into soluble tumour markers, cell adhesion, tumour growth, tumour suppressor genes/proto-oncogenes, cell metabolism and tumour angiogenesis (Fig. 1).

Soluble tumour markers

Soluble forms of tumour-associated proteins can be measured in blood samples to substantiate a diagnosis and for surveillance

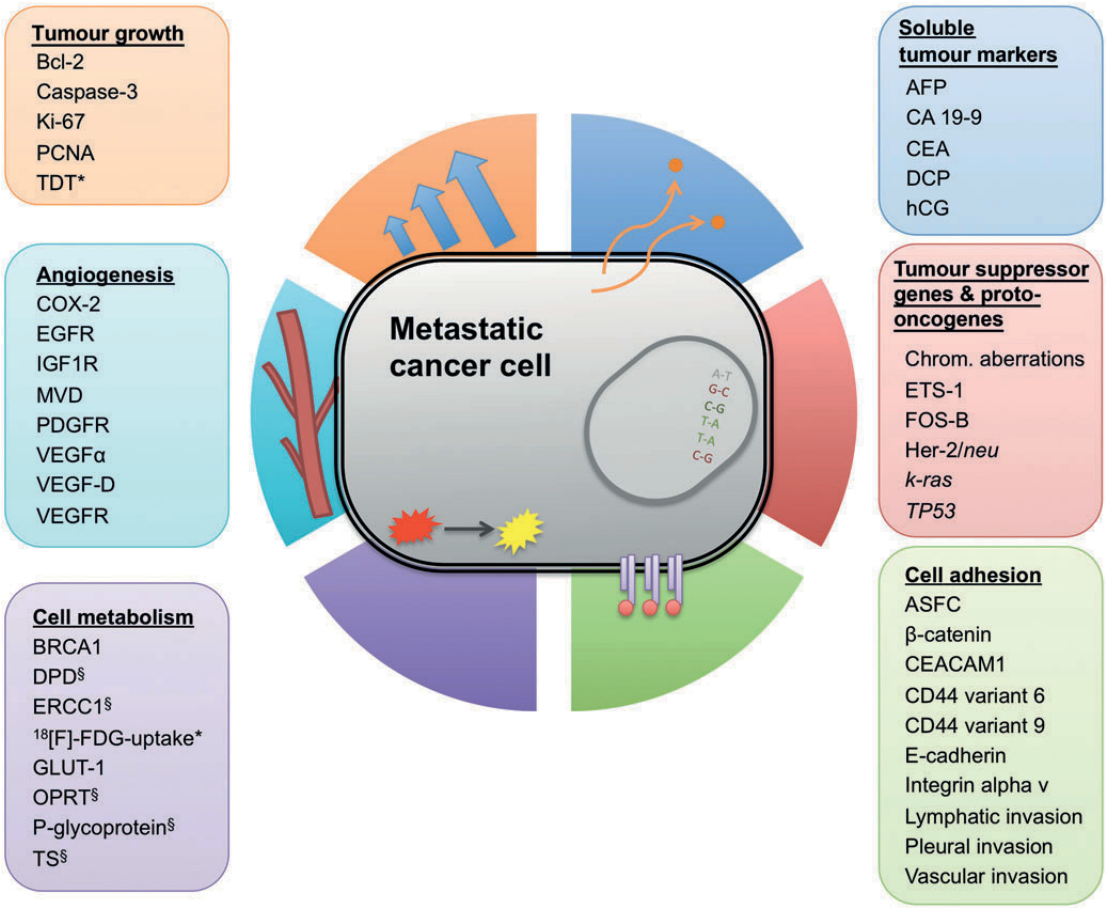

Figure 1: Potential prognostic biomarkers and radiological (*) markers in pulmonary metastases according to the classification chosen by the authors. Predictive markers for chemoresistance (§) have also been described in patients with pulmonary spreading of malignant disease.

purposes in the follow-up of patients with malignant disease. Some soluble factors have also been proposed to be capable of predicting the outcome of patients undergoing pulmonary metastasectomy.

Carcinoembryonic antigen. A heightened level of carcinoembryonic antigen (CEA) can be detected in patients with several types of cancer, *inter alia* CRC. Regarding its prognostic value in pulmonary metastasectomy, several contradicting studies with small sample sizes have been performed [4, 5]. Recently, two large studies including 1030 and 1112 patients confirmed the prognostic value of increased prethoracotomy CEA level (cut-off value >5 ng/ml) for 5-year survival in multivariate analysis [8, 9]. CEA expression persists during the metastatic process and has been identified in 93.3% of pulmonary metastases of CRC by immunohistochemistry [10]. Hence, it poses a helpful parameter in detecting the recurrence of disease.

CA19-9, alpha-fetoprotein and human chorionic gonadotropin. CA19-9 could be identified in 75% of pulmonary metastases and in 73.3% of paired primary CRC with high concordance in a work by Kayser *et al.* However, it did not influence the outcome [10]. Increased levels of alpha-fetoprotein (AFP; cut-off value ≥500 ng/ml), a marker associated with hepatocellular carcinoma (HCC), have been described as a negative prognostic factor for survival in patients undergoing resection for pulmonary nodules from HCC [11, 12]. These findings were confirmed by Kuo *et al.* [13], who found a significant association of high AFP and worse disease-free survival applying a cut-off value of 100 ng/ml. Although sample sizes of these three studies are small, they emphasize the potential value of serum markers to predict the postoperative outcome of patients following pulmonary metastasectomy. Besides AFP, des-gamma-carboxy prothrombin (DCP) is an established serum marker to monitor tumour burden and recurrence in patients with primary HCC. Kitano *et al.* [14] demonstrated a prognostic effect of DCP in their study population of 45 patients undergoing pulmonary metastasectomy from primary HCC (cut-off value: 40 mAU/ml) in univariate analyses regarding the disease-free survival and the overall survival. In germ cell tumours AFP and human chorionic gonadotropin (hCG) are accepted tumour markers. Elevated prethoracotomy serum levels of either AFP (cut-off value >15 μg/l) or hCG (cut-off value >5 Units/l) were significantly associated with reduced 5-year survival (50 vs 87.2 months) in patients with pulmonary spread of primary germ cell tumours [15].

Cell adhesion

The role of cell adhesion in cancer was first described in 1914 by Boveri. Since that time, the attention of metastasis research was drawn to molecules mediating cell–cell and cell–matrix adhesion [16]. Cell-adhesion molecules (CAMs) are capable of initiating

intracellular signalling and play a key role in epithelial-mesenchymal transition, which is a key mechanism in the metastasic cascade [17]. Loss of cell adhesion can be determined by demonstrating the presence of invasion of other structures with light microscopy. Shiono *et al.* demonstrated in pulmonary metastases from primary CRC that vascular invasion as well as intra-alveolar cancer cell clusters was associated with diminished prognosis after pulmonary metastasectomy in multivariate analysis (39.9 vs 85.8%; 46.9 vs 88.2%, respectively), and the presence of 10 or more of such infiltrative cell clusters predicted early local recurrence. Supporting this, lymphatic and pleural invasion were further variables with significant impact on 5-year survival in univariate analysis (47.0 vs 78.4 months; 49.5 vs 63.5 months) [18, 19]. Expression of membrane-bound adhesion molecules like cadherins, immunoglobulin-like CAMs (Ig-CAMs), CD44 and integrins can be determined by the utilization of immunohistochemistry.

Expression of cadherins. E(pithelial)-cadherin is one of the most extensively studied member of the cadherin-family. It mediates cell–cell adhesion, and thus its down-regulation plays a pivotal role in tumour dissemination [20]. In patients undergoing pulmonary metastasectomy subsequent to primary CRC, reduced expression of E-cadherin in resected nodules was an independent predictor for poor survival (5-year survival 74.5 vs 23.9 months). Loss of beta-catenin, which binds to E-cadherin at the intracellular side of the cell membrane, was also associated with worse 5-year survival in univariate analysis [21].

Expression of immunoglobulin-like cell-adhesion molecules. The expression of members of the Ig-CAM family was investigated in hepatoblastoma patients. Up-regulation of CEA-related CAM 1 (CEACAM1, also known as CD66a) predicted early metastasis to the lung and was associated with a decreased 5-year survival (92 vs 29%) [22]. CEACAM1 is also down-regulated in invasive HCC, however, data on expression levels in thoracic nodules and a possible prognostic value need to be elucidated [23].

Expression of CD44. The standard isoform of CD44 is expressed by many cell types and mediates cell–matrix adherence by binding hyaluronic acid. As it is also a molecular marker for cancer stem cells, extensive research has been undertaken to understand the role of this family of proteins in tumour progression and metastasis [24, 25]. Different CD44 variants, which are the result of alternative splicing of the *CD44* gene, are expressed in tumour tissue. The up-regulation of CD44 seems to play an important role in the homing of tumour cells to metastatic sites [25]. An increased expression of the CD44 gene in pulmonary metastases compared with corresponding primary CRC was found in a study by Kim *et al.* [26]. In a subsequent study, CD44 variant 9 expression was found in 88% of primary CRC with consecutive pulmonary dissemination, compared with 42% in patients without pulmonary dissemination. However, specimens from pulmonary nodules were not examined and follow-up after pulmonary dissemination was not described [27]. Additionally, an increased expression level of CD44 variant 6 in patients with primary CRC was related to a poor 2-year survival after pulmonary metastasectomy [28].

Expression of integrins. Another group of proteins relevant to cell–matrix interaction are integrins. They consist of an alpha- and a beta-subunit and mediate their adhesive effect by binding fibronectin and laminin [29]. Integrin alpha v was found to be expressed in 85.7% of lung metastases from primary CRC and in 52.4% of the paired primary tumours [30]. Yet, no data on the post-metastasectomy follow-up were provided by this study.

Tumour growth

Sustained cell proliferation is the key characteristic of malignant tumours. It is a consequence of attributes described as the hallmarks of cancer by Hanahan and Weinberg [31], such as resistance to cell death and up-regulation of proliferative signals. Proliferation can either be quantified by estimating the tumour growth radiologically or *ex post* by histological evaluation of the resected specimen.

Proliferation markers. Ki-67 (also known as MIB-1) is routinely used in histopathology to determine the proliferation rate of tumours. A high expression level at the primary tumour site was associated with pulmonary recurrence in osteosarcoma patients and mortality was increased in the strongly positive sub-group compared with patients with less proliferating osteosarcomas [32]. In contrast to that, a study on pulmonary metastases and matched primary osteosarcomas showed that Ki-67 expression did not correlate with the time to first pulmonary spreading. Follow-up until pulmonary recurrence after metastasectomy was not given and survival analysis dependent on Ki-67 was not described [33]. Besides, in patients with primary melanoma, the number of Ki-67 cells in the pulmonary nodules did not correlate with overall survival [34]. In patients with primary renal cell carcinoma (RCC) another marker for proliferation, the proliferating cell nuclear antigen (PCNA), was significantly higher in pulmonary metastases compared with primary tumours of patients with or without metastases. Moreover, mean PCNA expression was higher in patients with poor survival [35]. Though highly proliferating primary CRC was linked to tumour grade, lymphnode involvement, distant metastasis and an independent prognostic marker for 5-year survival in multivariate analysis, specific data after pulmonary metastasectomy are still lacking [36, 37].

Tumour doubling time. Rapid tumour proliferation can also be estimated *in vivo* by imaging techniques, e.g. computed tomography (CT). The tumour doubling time (TDT), indicating aggressive tumour biology, can vary between different types of tumours, within the same histological entities and even within the same patient (e.g. with multiple metastases) [38]. In patients with pulmonary metastases from CRC, a TDT <100 days was predictive for early pulmonary recurrence. Additionally, the overall survival was decreased in this sub-group with rapidly growing metastases; however, statistical significance was not reached [39, 40]. In patients with primary soft-tissue sarcoma, the TDT examined by chest X-ray was not associated with the patients' outcome [41]. Using CT for surveillance of patients with primary soft-tissue sarcoma, two studies identified the TDT of pulmonary nodules as a poor prognostic marker for overall survival [42, 43]. Moreover, in a series of 39 patients with primary osteosarcoma, the TDT of the pulmonary nodule was also predictive of poor overall survival, whereas this finding was not supported by Nakamura *et al.* in a small series of 11 patients [42, 43]. Ollila *et al.* [44] could identify a TDT <60 days as a significant prognostic marker for poor survival after pulmonary metastasectomy from primary melanoma using chest X-ray. Subsequently, the prognostic value and the cut-off value at 60 days were confirmed by Lee *et al*. It is noteworthy that the proliferative index determined by immunohistochemical staining for Ki-67 did not correlate with the radiologically determined TDT in their patient cohort [34].

Markers of apoptosis. As tumour growth is the result of cell division minus cell loss, antiapoptotic markers like bcl-2 or

proteins involved in apoptosis (e.g. caspases) might provide additional information on the cell turnover. The presence of bcl-2 in pulmonary metastases correlated with worse overall survival in patients with primary CRC and was divergent from the expression in the corresponding primary tumour [10]. This negative correlation between bcl-2 expression and patients' prognosis could not be confirmed in a series of osteosarcoma patients. Neither the expression level in the primary nor in the pulmonary metastases had influence on the recurrence-free survival and overall survival [45]. Caspase-3, a marker for cell apoptosis, was assessed in pulmonary metastases from primary melanoma, but was shown to have no effect on the clinical outcome after pulmonary metastasectomy [34] (Tables 1 and 2).

Tumour suppressor genes/proto-oncogenes

In general, tumour suppressor genes encode proteins, which have repressive functions on the cell-cycle. Proto-oncogenes are cellular genes, which are titled as oncogenes as soon they are mutated, amplificated or rearranged, leading to up-regulated cell growth [46]. Genes are indicated by *italic* letters, whereas proteins are denoted by normal letters. Alterations fo DNA or protein levels might have a diverging clinical impact.

Expression of p53. One of the most extensively studied tumour suppressors is the *TP53* gene, coding for the p53 protein, which plays an essential role in the cell-cycle arrest and apoptosis of damaged cells [47]. Kayser *et al.* found positive p53 protein staining in 26/60 pulmonary metastases and in 17/60 paired primary CRC, with a total of 45/60 concordant positive or negative stainings. Nevertheless, p53 expression was not associated with post-metastasectomy outcome [10]. Danner *et al.* compared chromosomal aberrations in the *TP53* gene region in paired primary and metastatic tissue specimens from CRC. Gain mutations at 20q, which correlates with altered *TP53*, were evident in 70 and 54% of primary tumours and metastases, respectively. Furthermore, they could confirm the finding by Kayser *et al.*, who found no significant correlation with recurrence-free survival after pulmonary metastasectomy or overall survival [48]. In patients with primary osteosarcoma, expression of p53 in the primary tissue failed to predict tumour progression [49]. However, in a more recent study including patients with pulmonary nodules from primary osteosarcoma, negative expression of p53 was significantly associated with poor survival after pulmonary metastasectomy in univariate analysis (3-year post-metastasectomy survival rate 17 vs 34%). It was concluded that the p53 might have a role as prognostic marker only in the subset of patients presenting with pulmonary dissemination [45].

Chromosomal aberrations. SMAD2, SMAD4/DPC4 and DCC, other well-investigated tumour suppressor genes, are clustered on the chromosome arm 18q, and a loss of 18q is associated with tumourigenesis [50]. In CRC patients, a loss of 18q was evident in 86% of pulmonary metastases, compared with 70% in corresponding primary tumours. However, a significant relationship between the loss of 18q and the clinical outcome could not be established [48].

K-ras mutation status. In CRC, mutations of the proto-oncogene *k-ras*, which encodes a small guanosine triphosphate-binding molecule involved in growth signalling pathways, are thought to be an early event in the progression from adenoma to carcinoma. However, only one-third of primary carcinomas have detectable alterations in this gene [46, 51]. *K-ras* mutation can more often be found in lung metastases (60%) than in liver metastases (33.3%). Additionally, in patients with *k-ras* mutation in the primary CRC, the lung is more often the initial site of metastasis compared with *k-ras* wild-type, thus *k-ras* might play an important role in metastatic dissemination, paticularly to the lung [52–54]. These findings were corroborated by data from the VICTOR study revealing that patients with *k-ras* mutation in the primary tumour have a significantly higher risk of lung relapse in multivariate analysis (hazard ratio: 1.9, 95% confidence interval: 1.1–3.1), whereas this effect could not be observed regarding liver metastases [53]. In support of this, Cejas *et al.* found a significantly decreased post-metastasectomy disease-free survival in patients with *k-ras* mutation (12.0 vs 18.0 months) without differentiating between lung and liver metastases.

Expression of Her2/neu. Another proto-oncogene, *c-erbB2*, encodes for the epidermal growth factor (EGF) homologon Her2/*neu* (alternatively named human EGF 2). Her2/*neu* is mostly known for its prognostic role and implications on treatment regimens in primary breast cancer. It could be shown that expression of Her2/*neu* in pulmonary metastases of primary breast cancer was associated with poor 5-year survival after metastasectomy (22 vs 74%). Additionally, loss of oestrogen receptor expression in the pulmonary metastases is associated with a worse post-metastasectomy 5-year survival in these patients (77 vs 12%) [55]. Her2/*neu* expression status is also strongly associated with pulmonary-specific metastasis in osteosarcoma patients [49, 56, 57]. Her2/*neu* could be detected immunohistochemically in 32% of primary osteosarcomas and in 53% of corresponding pulmonary metastases. Moreover, Her2/*neu* correlated with early pulmonary spreading (17.2 vs 31.8 months), but did not affect the post-metastasectomy survival [45]. Zhou *et al.* [58] confirmed these results and provided additional evidence of *c-erbB2* gene amplifications in some cases.

Expression of FOS-B and Ets-1. Addressing the prognostic value of proto-oncogenes in pulmonary metastasectomy, Finkel–Biskis–Jinkins murine osteosarcoma viral oncogene homologue B (FOS-B), has been investigated. Initially, FOS-B has been associated with tumour progression in primary CRC [59]. When detected in pulmonary metastases of CRC, FOS-B significantly affected the recurrence-free survival as well as the overall survival in univariate analysis, although this could not be confirmed in multivariate analysis [60]. Ets-1, an oncoprotein and transcription factor involved in matrix degradation is expressed in tumour cells as well as in the tumour-surrounding stroma, facilitating tumour progression. The expression level in pulmonary metastases correlated with corresponding primary CRC. Furthermore, stromal expression of Ets-1 significantly and independently predicted pulmonary recurrence of primary CRC [61]. Evaluating the prognostic value of Ets-1 regarding the post-metastasectomy outcome was not an aim of this study.

Cell metabolism

Glucose processing. An altered cell metabolism is common in various cancerous tissues. It is necessary for a tumour to be provided with sufficient amounts of carbohydrates, proteins, lipids and nucleic acids in order to meet the increased demand. One of the most investigated metabolic phenotype in cancer is the excessive glucose uptake of tumour tissue, the so-called Warburg

Table 1: Potential biomarkers in pulmonary metastases sorted by the entity of the corresponding primary tumour

Primary tumour	Marker	No. of patients	Outcome/result	First author	Year	Reference
Breast	ER	44	↑OS	Welter	2008	[55]
	HER-2/*neu*	31	↓OS			
Colorectal	ASFC	87	↓OS	Shiono	2005	[18]
	ASFC	61	↓Pulmonary recurrence-free survival	Shiono	2005	[19]
	bcl-2	60	↓OS	Kayser	2002	[10]
	β-catenin	86	↑OS (univariate)	Shiono	2006	[21]
	BRCA1	80	↑Outcome after PM and adjuvant chemotherapy	Kaira	2011	[70]
	CA 19–9 (tissue)	60	n.s.	Kayser	2002	[10]
	CD44 variant 6	20	n.s.	Indinnimeo	2003	[28]
	CD44 variant 9	42	No follow-up	Goi	2002	[27]
	CEA (no cut-off)	1030	↓OS	Iida	2012	[8]
	CEA (>5 ng/ml)	1112	↓OS	Salah	2012	[9]
	CEA (tissue)	60	n.s.	Kayser	2002	[10]
	Chromosomal aberrations	30	n.s.	Danner	2011	[49]
	DPD	80	n.s.	Kaira	2011	[70]
	E-cadherin	86	↑OS	Shiono	2006	[21]
	Ets-1	51	Prediction of lung metastases	Sato	2002	[61]
	ERCC1	80	Predictive marker for ↑outcome after PM and adjuvant chemotherapy	Kaira	2011	[70]
	FOS-B	39	n.s.	Pfannschmidt	2009	[60]
	IGF1R	86	n.s.	Shiono	2006	[21]
	Integrin alpha v	21	No follow-up	Sato	2003	[30]
	k-ras	17	↓Post-metastasectomy disease-free survival (liver and lung metastases combined)	Cejas	2009	[54]
	k-ras	45	No stratification depending on mutation in pulm met	Tie	2011	[53]
	k-ras	27	No follow-up	Kim	2012	[52]
	Lymphatic invasion	87	↓OS (univariate)	Shiono	2005	[18]
	MVD (CD34)	80	Predictive marker for ↓outcome after PM and adjuvant chemotherapy	Kaira	2011	[70]
	OPRT	80	Predictive marker for ↑outcome after PM and adjuvant chemotherapy			
	p53	86	n.s.	Shiono	2006	[21]
	p53	60	n.s.	Kayser	2002	[10]
	p53	80	Not predictive for better outcome after adjuvant chemotherapy	Kaira	2011	[70]
	Pleural invasion	87	↓OS (univariate)	Shiono	2005	[18]
	TS	80	Predictive marker for ↑outcome after PM and adjuvant chemotherapy	Kaira	2011	[70]
	Vascular invasion	87	↓OS	Shiono	2005	[18]
	VEGF-D	39	n.s.	Pfannschmidt	2009	[60]
	VEGFα	49	↓OS	Tamura	2004	[79]
Hepatocellular	AFP (≥500 ng/ml)	25	↓OS	Nakagawa	2006	[11]
	AFP (≥100 ng/ml)	34	↓OS (univariate)	Kuo	2007	[13]
	AFP (≥500 ng/ml)	20	↓OS ↓cancer-specific survival	Ohba	2012	[12]
	DCP (>40 mAU/ml)	45	↓OS ↓DFS	Kitano	2012	[14]
Hepatoblastoma	CEACAM1	19	↓LMFS ↓OS	Tsukada	2009	[22]
Melanoma	Caspase-3	20	n.s.	Lee	2009	[34]
	Glut-1	20	↓OS			
	Ki-67	20	n.s.			
	MVD (CD31)	20	↓OS			
Testis	Serum AFP (>15 μg/l)/hCG (>5 U/l)	41	↓OS	Pfannschmidt	2006	[15]
Osteosarcoma	Ki-67	30	No follow-up	Oda	2006	[33]
	bcl-2	19	n.s.	Ferrari	2004	[45]
	p53 (IHC)	19	↓3-year survival (univariate)			
	HER-2/*neu*	19	Lung-specific recurrence-free survival			
	HER-2/*neu*	25	Lung-specific recurrence-free survival	Zhou	2003	[58]
	P-glycoprotein	19	n.s.	Ferrari	2004	[45]
	COX-2	36	↓Disease-specific survival	Rodriguez	2008	[74]
Renal cell	PCNA	10	Increased in patients with survival <3 years	Gotoh	1998	[35]
Various	EGFR	35	No follow-up	Muehling	2010	[77]

Continued

Table 1: Continued

Primary tumour	Marker	No. of patients	Outcome/result	First author	Year	Reference
	PDGFR VEGFR GLUT1 Hexokinase 1 HIF-1 VEGF MVD (CD34)	146	Correlation with ^{18}[F]-FDG uptake	Kaira	2011	[65]

AFP: Alpha-fetoprotein; ASFC: aerogenous spread with floating cancer cell clusters; bcl-2: B-cell lymphoma 2; BRCA1: breast cancer susceptibility gene 1; CA: carbohydrate antigen; CD: cluster of differentiation; CEA: carcinoembryonic antigen; CEACAM: carcinomembryonic antigen-related cell-adhesion molecule 1 (CD66a); COX-2: cyclooxygenase 2; DCP: des-gamma-carboxy prothrombin; DFS: disease-free-survival; DPD: dihydropirimidine dehydrogenase; EGFR: epidermal growth factor receptor; ER: oestrogen receptor; ERCC1: excision repair cross-complementation group 1; FDG: 18F-fluorodeoxyglucose; FOS-B: Finkel–Biskis–Jinkins murine osteosarcoma viral oncogene homologue B; GLUT: glucose transporter; hCG: human chorionic gonadotropin; HER1/neu: human epidermal growth factor receptor 2/erb-B2; HIF-1: hypoxia-inducible factor 1; IGF1R: insulin-like growth factor 1 receptor; KRAS: Kirsten rat sarcoma viral oncogene homologue; LMFS: lung metastasis-free survival; MVD: microvessel density; n.s.: not significant; OPRT: orotate phosphoribosyltransferase; OS: 5-year overall survival; PCNA: proliferating cell nuclear antigen; PDGFR: platelet-derived growth factor receptor; PM: pulmonary metastasectomy; TS: thymidylate synthase; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor.

Table 2: Potential radiological markers in patients with pulmonary metastases sorted by the entity of the corresponding primary tumour

Primary tumour	Modality	Parameter	No. of patients	Outcome/result	First author	Year	Reference
Colorectal	CT	TDT	65	↓5-year intrapulmonary recurrence-free survival	Tomimaru	2008	[40]
Melanoma	CXR	TDT	20	↓OS	Lee	2009	[34]
	CXR	TDT	129	↓OS	Ollila	1998	[44]
Osteosarcoma	CT	TDT	39	↓OS	Roth	1985	[42]
	CT	TDT	11	n.s.	Nakamura	2011	[43]
Soft-tissue sarcoma	CXR	TDT	21	n.s.	Blomqvist	1993	[41]
	CT	TDT	67	↓OS	Roth	1985	[42]
	CT	TDT	29	↓OS	Nakamura	2011	[43]
Various	PET-CT	FDG uptake	146	↓OS; correlates with GLUT1, hexokinase I, HIF-1, VEGF and MVD	Kaira	2011	[65]

CT: computed tomography; CXR: chest X-ray; FDG: 18F-fluorodeoxyglucose; GLUT1: glucose transporter 1; HIF-1: hypoxia-inducible factor 1; MVD: microvessel density; n.s.: not significant; OS: 5-year overall survival; PET: positron emission tomography; TDT: tumour doubling time; VEGF: vascular endothelial growth factor.

effect [62]. Tumour cells generate adenosine triphosphate (ATP) to a high degree through aerobic glycolysis. Aerobic glycolysis is less efficient in generating ATP, therefore resulting in an increased glucose demand and consecutive uptake of glucose in tumour tissue. However, it has been speculated that aerobic glycolysis is superior to oxidative phosphorylation in hypoxic tumour tissue and leads to clonal selection of these cells [63]. The increased uptake of glucose via glucose transporters (GLUT) is also the basis for clinical tumour imaging by ^{18}[F]-fluorodeoxyglucose-positron emission tomography (FDG-PET). Hence, FDG uptake correlates with the expression of GLUT1 [64]. Kaira *et al.* published a work comparing ^{18}[F]-FDG uptake and histological markers associated with glucose metabolism in patients with either NSCLC or pulmonary metastases from extrapulmonary malignancy and a control group with benign nodules. A ^{18}[F]-FDG uptake beyond the calculated cut-off value was evident in 60, 29, 36, 44, 66, 66, 71 and 50% of pulmonary nodules of primary CRC ($n = 80$), sarcoma ($n = 17$), head and neck cancer ($n = 14$), breast cancer ($n = 9$), genital cancers ($n = 12$), oesophageal cancer ($n = 3$), gastrointestinal cancer ($n = 7$) and others ($n = 4$), respectively. Furthermore, ^{18}[F]-FDG uptake correlated with expression of GLUT1, hexokinase I, HIF-1alpha vascular endothelial growth factor (VEGF) and microvascular density in the pulmonary metastases and the size of the pulmonary nodules. The 5-year survival for all patients with confirmed metastases from an extrathoracic primary was 75.6 months. Assigning them to groups according the ^{18}[F]-FDG uptake, patients with highly positive pulmonary nodules were shown to have a significantly shorter median survival time. This finding was, however, limited by the fact that patients with different primary tumours were included in this analysis [65]. GLUT1 overexpression was also identified as a prognostic factor for poor overall survival after pulmonary metastasectomy in patients with primary melanoma. A potential correlation with ^{18}[F]-FDG uptake was not assessed in this work [34]. Further studies are needed to elucidate the glucose metabolism imaged by ^{18}[F]-FDG uptake as a prognostic tool.

Predictive markers indicating chemoresistance. Another important aspect with regard to cell metabolism lies in the changes leading to resistance to chemotherapeutics. In patients with pulmonary dissemination receiving an inter-disciplinary treatment, surgical removal of lung metastases is often accompanied by adjuvant chemotherapy. For that reason, markers predicting the response of patients to cytotoxic agents might affect the prognosis after metastasectomy. In the following, we discuss interesting findings regarding predictive markers in patients with pulmonary metastases. Thymidylate synthase (TS) is an enzyme that is involved in the metabolism of 5-fluorouracil (5-FU), pemetrexed and raltitrexed. An increased expression level of TS in primary CRC has been described as predictive for 5-FU response and patients' prognosis. Jensen *et al.* [66] underlined the importance of differentiating between tumour tissues from the primary tumour site or the metastasis, as they possess divergent characteristics regarding their pattern of chemoresistance. Supporting this, an increased expression of TS was detected in the pulmonary metastases of primary CRC, compared with the corresponding primary tumour and/or liver metastases [67–69]. Patients with CRC receiving 5-FU-based adjuvant chemotherapy after pulmonary metastasectomy and sufficient expression of TS in lung metastases benefited most from adjuvant chemotherapy. In the same study, this could also been shown for other proteins, which are involved in chemoresistance (orotate phosphoribosyltransferase (OPRT), excision repair cross-complementation group 1 (ERCC1) and breast cancer susceptibility gene 1 (BRCA1)) [70]. In patients with primary osteosarcoma, the expression of P-glycoprotein is significantly increased in pulmonary metastases compared with the primary tumours [45]. P-glycoprotein is a product of the multidrug resistance gene 1 and mediates the resistance to doxorubicin [71]. Expression of P-glycoprotein in primary tumours or pulmonary metastases is not associated with recurrence-free survival or survival after metastasectomy [45]. Cyclooxygenase-2 (COX-2) is involved in metastasis and resistance to chemoradiotherapy, particularly in patients with osteosarcoma [72, 73]. Increased expression of COX-2 in lung metastases predicts a worse outcome of patients with primary osteosarcoma and lung metastases, whereas the expression level of COX-2 seems not to be capable to prognosticate the outcome [74, 75]. Therefore, COX-2 seems to be a valuable prognostic marker in the sub-population of patients undergoing pulmonary metastasectomy.

In summary, existing data on the prognostic and predictive effect of cell metabolism markers is contradictory, and the contribution of these enzymes to the treatment outcome of patients with metastatic disease needs to be further elucidated.

Tumour angiogenesis

Sufficient blood supply is crucial for the growth of a tumour. Pivotal proangiogenetic mediators are VEGF, EGF, platelet-derived growth factor (PDGF) and their receptors (VEGFR, EGFR and EGFR, respectively) [76]. The increased activity of the proangiogenetic pathways leads to strongly vascularized tumours. This hypervascularization can be determined histologically and/or radiologically.

Receptor tyrosine kinases and ligands. The expression level of receptor tyrosine kinases has been described in various primary tumours and paired pulmonary metastases, irrespective of their potential role as biomarkers [77, 78]. An increased expression of VEGFα in pulmonary nodules compared with corresponding primary CRC could be determined by reverse transcription polymerase chain reaction [26]. Additionally, this increased expression of VEGF in CRC pulmonary metastases was found be an independent prognostic marker for poor 5-year survival in multivariate analysis (18 vs 46.1%) [79]. In metastatic osteosarcoma, the expression of VEGF in the metastatic lesion was comparable to that of the primary tumour. Nevertheless, the high expression level of VEGF in the primary tumour was associated with decreased survival in univariate analysis, whereas expression of VEGF in the metastatic lesion did not affect the outcome [33]. In a further work assessing pulmonary metastases from primary CRC, expression of VEGF-D, a VEGF sub-type that mediates lymphangiogenesis, was not associated with recurrence-free survival or overall survival [60]. Interestingly, VEGF expression was shown to correlate with ^{18}[F]-FDG uptake in pulmonary metastases from primary epithelial cancers [65]. This finding emphasizes the potential of PET-imaging to reflect tumour biology. Another growth factor involved in angiogenesis is the insulin-like growth factor. The increased expression of the corresponding receptor (IGF1R) seems not have prognostic value in metastatic CRC [21].

Microvessel density. Microvessel density can be determined immunohistochemically by staining of e.g. CD31 or CD34 and calculating the number of vessels per area. Pulmonary metastases from primary CRC with low microvessel density are associated with better outcomes after pulmonary metastasectomy and 5-FU-based adjuvant chemotherapy, whereas this is unaffected by the expression of VEGF [70]. As mentioned above, microvessel density correlates with ^{18}[F]-FDG uptake in various tumours [65]. Hyper-vascularized pulmonary metastases from primary melanoma are associated with worse survival after pulmonary metastasectomy [34].

CONCLUSION

Currently, the prognosis of cancer patients is based on the TNM classification. As this system is of limited value for patients undergoing pulmonary metastasectomy [4], further prognostic markers are warranted. The most prominent literature on prognostic factors following pulmonary metastasectomy was published 1997, reviewing over 5000 data sets from the IRLM [3]. The risk factors described in this publication are solely based on clinical observations. However, these factors correspond to tumour aggressiveness, characterized by a rapid, invasive and diffuse pattern of metastasis (short disease-free interval, incomplete resection and number of nodules). Each molecular and radiological marker of our review can be linked to the tumour phenotype described by the IRLM. Two examples illustrate this relationship: E-cadherin expression and markers of tumour angiogenesis. A loss of the epithelial marker E-cadherin is a key factor in the process of a diffuse tumour spreading. It enables the disaggregation of cancer cells from one another, which is the first step of the metastatic cascade. Thus, E-cadherin expression may be directly linked to the number of nodules and the disease-free interval. Supporting this, decreased levels of E-cadherin are still evident in metastatic tissue. On the other hand, angiogenesis is considered an essential component of tumour metastasis. Most of the disseminated tumour cells undergo apoptosis or remain in a silenced state on the secondary site, because sufficient vascularization limits the outgrowth of micrometastases to clinically detectable macrometastases. Therefore, the up-regulation of TRK-pathways as described in this

review may contribute to the number of metastasis—one of the IRLM risk factor for poor survival.

Methods in molecular biology and radiological imaging techniques have provided clinicians with a myriad of novel prognostic markers in cancer research. The majority of the findings reviewed here underline the divergent biology of primary tumour and corresponding pulmonary metastases. Compared with the existing literature on prognostic markers in primary tumours, only few studies have focused on the subset of patients with pulmonary metastases, which widely consists of a small series of highly selected patients. The best evidence for prognostic significance that is already clinically applicable are the findings on CEA (pre-metastasectomy serum level ≥5 ng/ml) in patients with primary CRC. Detection of CEA is easy and reproducible by commercially available assays and has pioneered the use of novel biomarkers in pulmonary metastasectomy. Besides this promising serum marker, non-invasive methods in radiology/nuclear medicine, e.g. PET-CT might be an important prognostic tool in the future. All other suggested markers of this review are still in a preclinical or even experimental phase and therefore a transition to a clinical application is currently not possible. It is important to notice that identifying poor prognostic markers in patients with pulmonary metastases remains a desirable aim, but should not lead to an increase in the long-term survival of our patients solely by stronger patient selection. Rather than withholding surgery from patients with a putative poor prognosis, aggressive treatment regimens with a multidisciplinary approach should be applied, whereas in low-risk patients, cost-intensive and burdening treatment and surveillance strategies might be unnecessary.

Conflict of interest: none declared.

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KRAS in metastatic colorectal cancer

A key-characteristic of cancer is the autonomous, dysregulated growth. This is achieved by enhanced signaling via tumor oncogenes or loss of tumor suppressors (Hanahan & Weinberg, 2011). A well-described tumor oncogene in CRC is V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS). KRAS is a GTPase located at the cell membrane and exhibits its function by transducing signals from the cell surface downstream to other intra-cellular mediators (Schmick *et al*, 2014). Most extensively described downstream signaling pathways are the PI3K/Akt pathway and the MAPK pathway (Normanno *et al*, 2009). Activated, GTP-bound KRAS activates these pathways and thus contributes to cancer progression by anti-apoptotic, proliferative and cell transforming effects (Bryant *et al*, 2014; Velho *et al*, 2008; Yang *et al*, 2015). KRAS itself is activated by tyrosine kinase receptors (TKR), e.g. the epidermal growth factor receptor (EGFR). These TKR are valuable therapeutic targets in a variety of cancer types, especially CRC. The monoclonal anti-EGFR antibodies cetuximab and panitumumab were shown to be effective anti-tumor drugs in CRC. As activating mutations in the *KRAS* gene, especially codon 12 and 13 of exon 2, lead to an EGFR- independent activation of the downstream signaling cascades, *KRAS* mutation is a predictive marker for the treatment response to anti-EGFR therapy (Bokemeyer *et al*, 2011; Van Cutsem *et al*, 2009).

Beyond the predictive value of *KRAS* mutations, *KRAS* mutant CRC might exhibit a different metastatic behavior compared to *KRAS* wild-type tumors. Tie *et al.* found *KRAS* mutations in 62.0% of CRC lung metastases, compared to 56.5% of CRC brain metastases and 32.3% CRC liver metastases. Furthermore, patients with *KRAS* mutant CRC had a higher risk for developing pulmonary metastases (HR 2.2; 95%CI 1.2-3.5; P = 0.007) (Tie *et al*, 2011). Cejas *et al.* found a significantly higher prevalence of *KRAS* mutations in CRC patients with lung metastases compared to patients with liver metastases (Cejas *et al*, 2009).

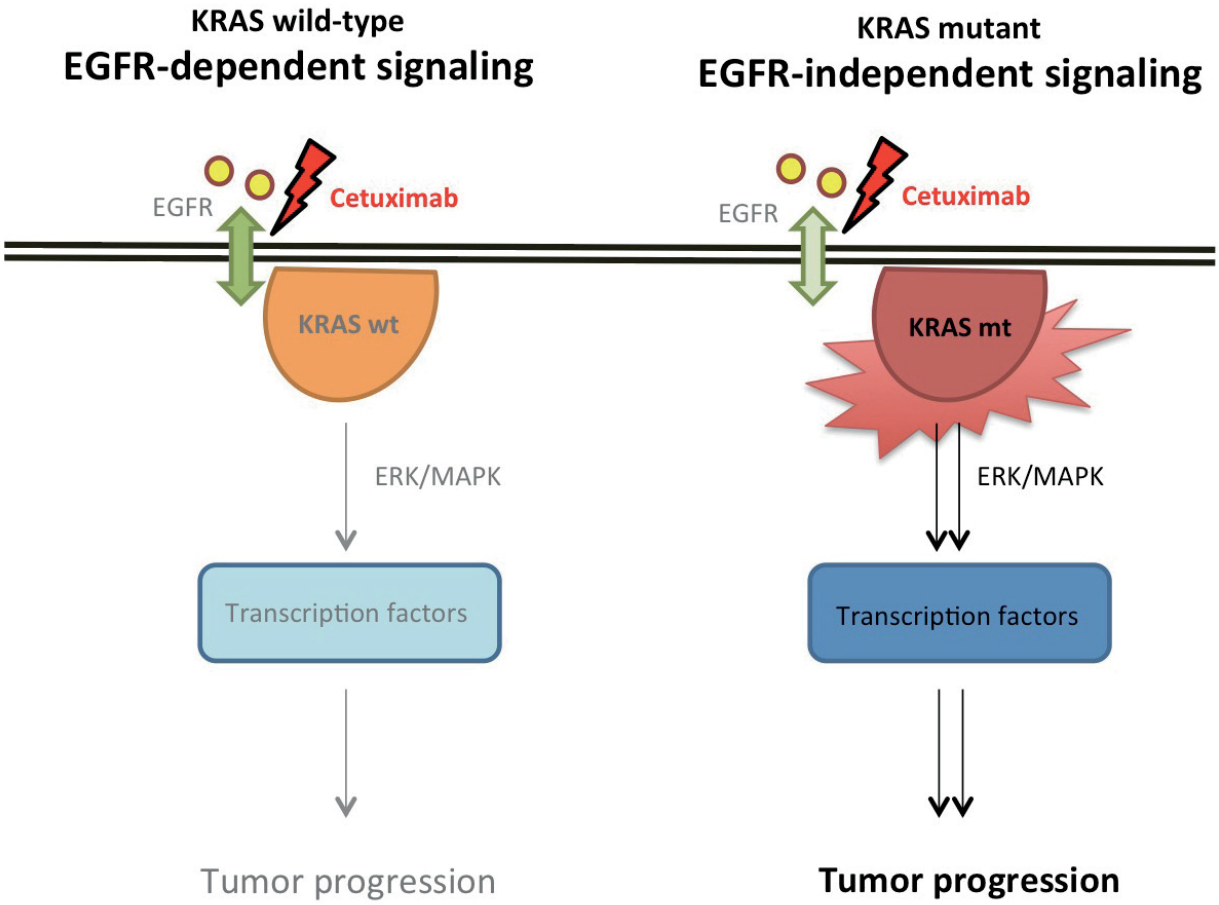

Figure 3: KRAS mutational status as predictive marker for anti-EGFR therapy. Anti-EGFR therapy, i.e. cetuximab or panitumumab, is capable to suppress the KRAS pathway downstream to EGFR (left side). Tumors with activating mutations of KRAS are characterized by constantly increased downstream signaling, e.g. ERK/MAPK pathway, which leads to tumor progression (right side). Anti-EGFR targeted therapy upstream to KRAS is considered being inefficient in a majority of these patients.

Heat shock protein 27 and fibroblasts

Extensive scarring, i.e. fibrosis is a common feature of various benign and malignant diseases. On a cellular level, fibrosis consists of activated fibroblasts, also called myofibroblasts as they express high levels of alpha-smooth muscle actin (alpha-SMA), extracellular matrix (mainly produced by myofibroblasts) and inflammatory cells (Wynn & Ramalingam, 2012). Physiologically, myofibroblasts play an essential role during wound healing. As they can also be found in the tumor stroma of solid cancer, the role of these cells in malignant disease is of increasing interest.

Beyond the over-expression of alpha-SMA, myofibroblasts are considered positive for fibroblast-activation protein alpha (FAP), palladin, osteopontin and vimentin (Brentnall *et al*, 2012; Henry *et al*, 2007; Nakagawa *et al*, 2004; Ngan *et al*, 2007; Pardo *et al*, 2005; Tsujino *et al*, 2007). Moreover, the small heat shock protein Hsp27 has been described to be over-

expressed and functional relevant in myofibroblasts in the context of benign disease (Hirano *et al*, 2004; Suarez *et al*, 2013). Moreover, our group – amongst others –recently demonstrated a systemic elevation of Hsp27 in benign and malignant lung disease (Hacker *et al*, 2009; Zimmermann *et al*, 2012). Using FAP as marker for myofibroblasts, Henry et al demonstrated that high density of FAP+ fibroblasts was associated with a decreased survival in patients with metastastic CRC (median 671 versus 428 days, $P = 0.042$). To the best of our knowledge, the prognostic value of Hsp27+ fibroblasts was never assessed in the context of CRC.

1.7 Treatment of pulmonary metastases

The first report on pulmonary metastasectomy was published by the Viennese surgeon Josef Weinlechner in 1882 (Weinlechner, 1882). The prerequisites for the surgical removal of pulmonary metastases were defined by Thomford in 1965 and basically remained valid until today (Thomford *et al*, 1965):

I) Functional resectability, which means that the patient needs to be in general condition to tolerate surgery and the expected remaining lung function after metastasectomy needs to be sufficient

II) The primary tumor site must be under control

III) No extra-thoracic metastatic tumor site or resectability of these extra-thoracic lesions

IV) A complete resection of all pulmonary metastases is technically feasible

Indication for pulmonary metastasectomy should only be set, if all of these criteria are met. The aim of pulmonary metastasectomy is a complete resection of all nodules together with the preservation of as much lung parenchyma as possible. As pulmonary metastases might recur after the first metastasectomy, the remaining functional lung parenchyma after the first procedure is crucial for further pulmonary metastasectomies.

There are several surgical devices and techniques available to resect lung metastases. Enucleation of the nodule can be performed using either electrocautery or a Nd:YAG laser device. Compared to other resection techniques, enucleation with either electrocaautery or laser allows the preservation of as much lung parenchyma as possible. This makes enucleation the resection technique of choice especially for multiple metastases (e.g. complete resection of >100 metastases during one procedure can be performed) (Rolle *et al*, 2006). The second application of enucleation techniques are centrally located nodules. These could theoretically also be resected extra-anatomically, but with a concomitant

substantial loss of lung parenchyma. Thus, enucleation is preferred for deeply intraparenchymal and/or multiple lesions.

The most commonly applied technique to resect pulmonary metastases is an extra-anatomical ("wedge") resection using stapler devices with an integrated blade. These devices allow an optimal sealing of the lung parenchyma and have widely replaced the traditional technique requiring multiple steps, including clamping of an extra-anatomical segment, resecting it with a surgical blade and oversewing the resection line afterwards with a running suture. These steps are all integrated in mechanical stapling devices.

Depending on the location of the pulmonary metastases and the functional capacity of the patient, anatomical resection of metastases are possible to achieve a complete resection of all nodules. Lung segments can be resected following the inter-segmental, anatomical border ("segmentectomy"). Moreover, lobectomy or even pneumonectomy can be indicated in selected cases (Migliore *et al*, 2010). Even repeated metastasectomy is possible in some patients. A low peri-operative morbidity and mortality and a reasonable long-term survival makes re-metastasectomy a valid option for recurrent lung metastases (Jarabo *et al*, 2011; Kandioler *et al*, 1998; Migliore *et al*, 2010; Park *et al*, 2010; Salah *et al*, 2013).

1.8 Aims of this thesis

a. Description of the EGFR expression, *KRAS* and *BRAF* mutations in CRC lung metastases

b. Correlation of EGFR, *KRAS* and *BRAF* alterations with clinical characteristics

c. Detection and quantification of myofibroblasts in CRC primary tumors, liver metastases and lung metastases

d. Description of the microvessel density dependent on the expression of Hsp27 in tumor-associated fibroblasts

e. Measurement of systemic Hsp27 before and after pulmonary metastasectomy

f. Evaluation of the assessed histological characteristics as prognostic markers in patients receiving pulmonary metastasectomy

CHAPTER TWO: RESULTS

2. Prologue

Pulmonary metastases are a significant contributor to cancer-associated morbidity and mortality in patients with CRC (La Vecchia *et al*, 2010; Malvezzi *et al*, 2011). Despite pulmonary metastasectomy is commonly performed, the selection of patients in which long-term survival can be achieved remains an obstacle in thoracic surgery. The underlying tumor biology, which determines the fate of the patient, has rarely been assessed in the context of pulmonary metastases.

After assessing lung, liver and brain metastases of CRC, Tie *et al.* found that *KRAS* mutations were significantly more frequent in patients who presented with lung recurrence (HR 2.1; 95% CI 1.2-3.5) (Tie *et al*, 2011). Similar to this work, Cejas *et al.* described the increased propensity of *KRAS* mutant CRC to spread to the lungs by assessing tissue samples from primary CRC (Cejas *et al*, 2009). In our work "EGFR, *BRAF* and *KRAS* status in patients undergoing pulmonary metastasectomy from primary colorectal carcinoma: a prospective follow-up study" we investigated the time and pattern of disease recurrence after pulmonary metastasectomy in patients with metastatic CRC dependent on the *KRAS* and BRAF mutation status and the expression level of EGFR. We could describe that *KRAS* mutant tumors were significantly associated with recurrent lung metastasis after the first pulmonary metastasectomy and had a significantly shorter time to pulmonary recurrence (Schweiger *et al*, 2014). Thus, we could show that *KRAS* is - specifically in patients with CRC pulmonary metastases undergoing surgery – a valuable prognostic marker.

2.1 EGFR, BRAF and KRAS status in patients undergoing pulmonary metastasectomy from primary colorectal carcinoma: a prospective follow-up study (published PDF)

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ORIGINAL ARTICLE – THORACIC ONCOLOGY

EGFR, *BRAF* and *KRAS* Status in Patients Undergoing Pulmonary Metastasectomy from Primary Colorectal Carcinoma: A Prospective Follow-Up Study

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ABSTRACT

Background. Pulmonary metastasectomy is an integral part of the interdisciplinary treatment of patients with pulmonary metastases (PMs) from colorectal carcinoma (CRC). Although alterations in the epidermal growth factor receptor (EGFR) pathway are common in CRC, there is still insufficient data regarding PM. We hypothesized that EGFR expression and Kirsten rat sarcoma viral oncogene homolog (*KRAS*)/*BRAF* mutations (Mts) might be associated with clinicopathological variables and the outcome in patients undergoing pulmonary metastasectomy.

Methods. In this single-center study, 44 patients undergoing pulmonary metastasectomy from primary CRC were included and prospectively followed up. Tissue specimens of resected PMs were assessed. Restriction fragment length analysis was used for *BRAF V600E* and *KRAS* codons 12 and 13 Mt analyses. EGFR expression was evaluated by immunohistochemistry. Patients were followed up in 3–6-month intervals.

Results. EGFR expression was evident in 49 % of the PMs, whereas Mts in *KRAS* and *BRAF* were detected in 48 and 0 %, respectively. Time to lung-specific recurrence after metastasectomy was significantly decreased in patients with *KRAS* mutated PMs in univariate ($p = 0.013$) and multivariate analysis ($p = 0.035$), whereas EGFR expression had no impact on recurrence free survival. Moreover, KRAS Mts were associated with the number of PMs ($p = 0.037$) and with the lung as first site of recurrence after metastasectomy ($p = 0.047$).

Discussion. This is the first evaluation of EGFR pathway alterations in the setting of pulmonary metastasectomy. Our data suggest that patients with *KRAS* Mts are at high risk for early pulmonary recurrence and have a more diffuse pattern of metastasis. These findings may have impact on the therapeutic management of CRC patients with pulmonary spreading.

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world. Despite advances in the treatment of the disease, CRC remains one of the leading causes of cancer-related death.[1] One of six patients develops pulmonary metastases (PMs) during the course of the disease, whereas the identification of patients with increased risk for PMs is a matter of current research.[2–4] Pulmonary metastasectomy is an integral part of the interdisciplinary treatment of these patients. Although randomized, controlled trials on pulmonary metastasectomy are still missing, the resection of metastases embedded in a multidisciplinary treatment plan is a widely accepted concept for selected patients.[5,6] In addition to that the concept of re-metastasectomy for recurrent disease is associated with good long-term survival in metastatic CRC.[7,8]

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Prediction of the outcome of patients undergoing pulmonary (re-)metastasectomy remains an obstacle in surgical oncology.[9] An a priori identification of patients with high risk for pulmonary recurrence may affect the treatment strategy. Thus, a quest for accurate biomarkers in pulmonary metastasectomy has evolved.[10]

The epidermal growth factor receptor (EGFR) and its downstream signalling cascade are key regulators of cellular growth, proliferation, and survival. Mutations (Mts), gene amplifications, or protein overexpression in this pathway are a frequent finding in many neoplasms.[11,12]

EGFR expression can be found in high frequency in primary CRC. Although 25–90 % of tumors present with a positive staining pattern, the prognostic value of this finding is still controversial.[13–15] However, the therapeutic application of anti-EGFR antibodies led to promising results.[16]

The BRAF protein, a serin–threonin protein kinase, is a downstream signal transductor in the EGFR–MAPK (mitogen-activated protein kinase) pathway. *BRAF* gene Mts are less frequent than Kirsten rat sarcoma viral oncogene homolog (*KRAS*) Mts. They can only be found in less than 10 % of all primary CRCs.[17] In contrast to *KRAS* Mts, alterations in the *BRAF* oncogene strongly correlate with shorter progression-free and overall survival.[18]

The V-Ki-ras2 *KRAS* is part of the EGFR–MAPK pathway. As most patients with *KRAS* Mt are resistant to anti-EGFR therapy, *KRAS* Mt has to be excluded in patients potentially receiving such a therapy.[19] Therefore, *KRAS* is especially useful as a predictive biomarker, whereas its prognostic value is still under discussion.[20] Interestingly, recent studies have shown that *KRAS* Mts interfere with other relevant pathways promoting metastasis and that *KRAS* Mts occur in a high percentage of primary tumors with subsequent pulmonary spreading.[21–24]

Therefore, we hypothesized that alterations in the EGFR-pathway might be relevant in the subset of patients undergoing pulmonary metastasectomy.

MATERIALS AND METHODS

Study Population

From April 2009 to October 2012, 44 patients who underwent pulmonary metastasectomy after primary CRC were included in this prospective, single center study (Fig. 1). Inclusion criteria were the first pulmonary metastasectomy, the absence of any extrapulmonary spreading, a controlled primary tumor site, and an expected complete resection based on preoperative CT images. 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. All patients were treated according to current guidelines for pulmonary metastasectomy with curative intent.[25] The majority of chemotherapy regimens before or after metastasectomy were based on 5-fluorouracil/oxaliplatin ± bevacizumab. For singular subpleural metastases, a VATS approach was applied; all other patients underwent a muscle-sparing anterior or posterior thoracotomy with a bimanual palpation of the lung. Complete resection was achieved in all patients, and a systematic lymph node sampling was performed if enlarged lymph nodes were found intraoperatively. Formalin-fixed, paraffin-embedded (FFPE) specimens and pathological data were provided by our pathology department. The mean diameters of the histologically confirmed metastatic nodules were measured, and the estimated tumor volume was calculated by assuming a spherical shape ($4/3 \times \pi \times r^3$) based on the radius ($r$) of the nodule given in the pathological report. Patients were

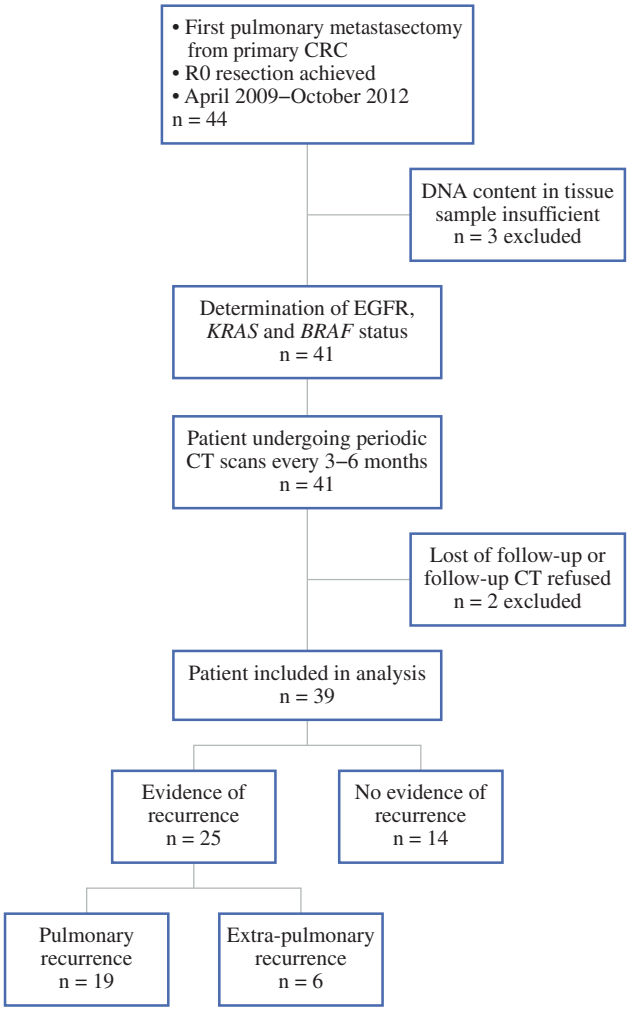

FIG. 1 Study scheme of the patients included in the analysis

followed-up in the outpatient ward with a CT scan every 3 months within the first year. If no recurrence was detected within the first 12 months, follow-up intervals were changed to biannual controls. Thirty-nine patients were included in the final analysis. Lung metastasis-free survival (LMFS) was defined as the time between the diagnoses of the primary and metastatic tumors. Time to recurrence was defined as the time from pulmonary metastasectomy to the evidence of tumor recurrence irrespective of the recurrence site. Time to lung-specific recurrence was calculated from the time of pulmonary metastasectomy to pulmonary recurrence of the disease identified by CT scan.

Immunohistochemistry and Scoring

Immunohistochemical staining was performed following a standard protocol. Briefly, FFPE specimens were assessed using a Benchmark Ultra Immunostainer (Ventana, Tucson, AZ). The binding of rabbit anti-EGFR antibody (ready to use; Ventana) was visualized with DAB substrate, and the tissue slides were counterstained with hematoxylin. In negative controls, the primary antibody was omitted. Staining intensity was scored as described previously.[26] In brief, sections showing membranous staining in $\geq$1 % of the tumor cells were scored as positive. Two independent, blinded observers rated the staining intensity. In case of a disagreement, a consensus score was determined.

KRAS and BRAF Mt Analysis

Restriction fragment length analysis was performed to detect KRAS codons 12 and 13 and BRAF codon 600 Mts as described previously.[27] Briefly, macrodissection of paraffin-embedded tumors was performed following the pathologists' assessment of tumor enriched area and tumor cell content. Next, genomic DNA was isolated using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). PCR was performed with AmpliTaqGold PCR Master Mix (Applied Biosystems, Foster City, CA) using primers designed to introduce restriction sites in the products generated from the wild-type (Wt) allele. The primer sequences were as follows: for KRAS codon 12 forward 5′-GAATAT AAACTTGTGGTAGTTGGACCT-3′ and reverse 5′-GGT CCTGCACCAGTAATATG-3′, for KRAS codon 13 forward 5′-GAATATAAACTTGTGGTAGTTGGACCT-3′ and reverse 5′-GGTCCTGCACCAGTAATATG-3′. The products obtained were then digested with *Bst*nI and *Bgl*I, respectively. BRAF codon 600 Mt was analysed in a two-step PCR and restriction digestion. The first primers sequences were as follows: forward (Mt–F) 5′-TAAAAA TAGGTGATTTTGGTCTAGCTGC-3′ and reverse (Wt-R) 5′-CCAAAAATTTAATCAGTGGAAAAATA-3′. The products obtained were then used in a second-stage PCR, which was performed under the same conditions as the first-stage PCR but with Mt–F and -R primer (5′-AAAAATTTAAG CAGTGGAAAAATAGC-3′). The PCR products were then digested with *Bts*I (New England Biolabs, Beverly, MA). All products were visualized on 3 % agarose gels stained with ethidium bromide.

Statistical Analysis

All data collected in this study were evaluated statistically using SPSS 19 (SPSS, Inc., Chicago, IL) and GraphPad Prism 6 (GraphPad Software, Inc., California) software. Nonparametric data were expressed as median and range. Mann–Whitney *U* test was used to compare medians between two groups. The Kaplan–Meier method, log-rank test, and Cox regression were used to compare survival functions. χ^2 test and Fisher's exact test were used to compare binominal variables. All tests were two-sided. $p \leq 0.05$ was considered statistically significant.

RESULTS

Clinicopathologic Characteristics

Twenty male and 19 female patients with a median age of 64 (range 37–79) years were included in the final analyses. Three patients were excluded because the low tumor DNA content of their samples impeded reliable mutational analyses; two additional patients were excluded because they refused to participate in the follow-up program. Tumor stage and grading of the primary tumor is depicted in detail in Table 1. The mean follow-up period was 27 months; the median follow-up was 23 months and did not differ in the assessed subgroups. Five patients had already stage M1 disease at the time of diagnosis. Mean LMFS was 27 (range 0–115) months. Twelve patients had already undergone liver metastasectomy when presenting with their first pulmonary spreading. An average number of 1.85 ± 0.28 (mean $\pm$ SEM) histologically confirmed pulmonary nodules were resected per patient, ranging from one to eight per patient. Complete resection could be achieved in all of the included patients. Forty-one enucleations (20 Nd:YAG laser enucleations, 21 electrocautery enucleations), 25 wedge resections, and 6 lobectomies were performed. Nine patients had bilateral metastases. Forty-two of the 48 accesses (30 patients had unilateral and 9 had patients bilateral nodules) were anterolateral or posterior muscle-sparing thoracotomies; in 6 patients a VATS approach was applied. In eight patients, a repeated pulmonary metastasectomy was performed due to tumor recurrence within the study period. Two patients died during follow-up (15 and 39 months after metastasectomy

TABLE 1 Correlation of clinicopathologic characteristics and KRAS mutational status in patients with CRC metastasizing to the lungs ($N = 39$)

	Total		*KRAS* Wt		*KRAS* Mt		χ^2 test	EGFR−		EGFR+		χ^2 test
	$N = 39$	%	$N = 21$	%	$N = 18$	%	p value	$N = 20$	%	$N = 19$	%	p value
Patients												
Sex												
Male	20	51.3	14	70	6	30	0.079	8	40	12	60	0.148
Female	19	48.7	17	36.8	12	63.2		12	63.2	7	36.8	
Age (years)												
Median	64		68		57		0.013*[a]	58.5		68		0.079[a]
Range	37–79		47–79		37–76			37–78		51–79		
Follow-up												
Median (range)	23 (4–42)		26 (5–37)		22 (4–42)		0.621[a]	21 (4–41)		27 (5–42)		0.634[a]
Mean ± SD	27 ± 12		21 ± 11		22 ± 14		0.87[c]	22 ± 13		24 ± 13		0.916[c]
Primary tumor												
Location												
Right-sided	4	11.1	0	0	4	100	0.022*	3	75	1	25	0.468
Left-sided	11	30.6	5	45.5	6	54.5		6	54.5	5	45.5	
Rectum	21	58.3	15	71.4	6	28.6		9	42.9	12	57.1	
Unknown	3											
T stage												
pT1	1	2.8	0	0	1	6.3		1	100	0	0	
pT2	7	19.4	5	71.4	2	28.6	0.482	2	28.6	5	71.4	0.248
pT3	23	63.9	13	56.5	10	43.5		11	47.8	12	52.2	
pT4	5	13.9	2	40	3	60		4	80	1	20	
Unknown	3							3				
N stage												
pN0	16	44.4	9	56.3	7	43.7	0.996	8	50	8	50	0.904
pN1	9	25	6	55.6	4	44.4		4	44.4	5	55.6	
pN2	11	30.6	6	54.5	5	45.5		6	54.5	5	45.5	
Unknown	3							3				
M stage												
pM0	31	86.1	19	61.3	12	38.7	0.149	15	48.4	16	51.6	0.630
pM1	5	13.9	1	20.0	4	80.0		3	60	2	40	
Unknown	3							3				
Grading												
G1	2	5.1	1	50	1	50		0	0	2	100	
G2	31	79.5	16	51.6	15	48.4	0.790	17	54.8	14	45.2	0.322
G3	6	15.4	4	66.7	2	33.3		3	50	3	50	
First pulmonary metastasectomy												
LMFS												
Median	27		31		23		0.279[b]	26		28		0.768[b]
Range	0–115		12–115		0–98			0–115		9–110		
Chemotherapy before metastasectomy												
Yes	31	79.5	16	51.6	15	48.4	0.582	14	45.2	17	54.8	0.132
No	8	20.5	5	62.5	3	37.5		6	75.0	2	25.0	
Chemotherapy after metastasectomy												
Yes	33	84.6	17	51.5	16	48.5	0.493	19	57.6	14	42.4	0.065
No	6	15.4	4	66.7	2	33.3		1	16.7	5	83.3	

TABLE 1 continued

	Total		*KRAS* Wt		*KRAS* Mt		χ^2 test	EGFR−		EGFR+		χ^2 test
	N = 39	%	*N* = 21	%	*N* = 18	%	*p* value	*N* = 20	%	*N* = 19	%	*p* value
Previous liver metastasis												
Yes	12	30.8	5	41.7	7	58.3	0.448	8	66.7	4	33.3	0.200
No	27	69.2	16	59.3	11	40.7		12	44.4	15	55.6	
Number of nodules												
1	28	71.8	18	64.3	10	35.7	0.037*	13	46.4	15	53.6	0.333
>1	11	28.2	3	27.3	8	72.7		7	63.6	4	36.4	
Site of first recurrence												
Pulmonary	19	76	7	36.8	12	63.2	0.047*	11	57.9	8	42.1	0.294
Extra-pulmonary	6	24	5	83.3	1	16.7		2	33.3	4	66.7	
No recurrence	14											

LMFS lung-metastasis-free survival after primary tumor

[a] Mann–Whitney test

[b] Log-rank test

[c] Student's *t* test

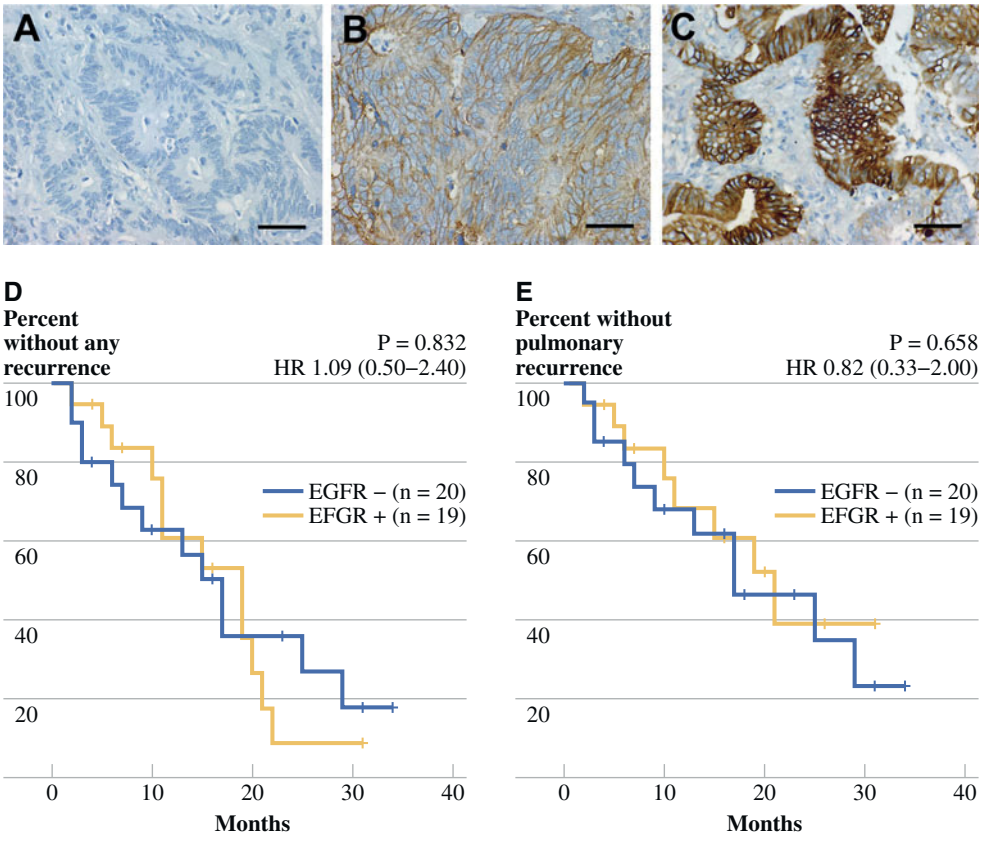

FIG. 2 Pulmonary metastases showed negative (**a**) or positive membranous staining for EGFR (*brown*; DAB substrate), which varied from moderate to highly positive intensity (**b** and **c**, respectively; ×40 magnification; *black bars* indicate 50 μm) EGFR expression was not associated with time to any recurrence (**d**) or time to lung-specific recurrence after pulmonary metastasectomy (**e**)

because of end-stage disease). Tissue samples of repeated metastasectomies also were evaluated according the EGFR, *KRAS*, and *BRAF* statuses, but these data were not included in the analysis.

EGFR Expression

EGFR overexpression was a frequent finding in resected PMs (Fig. 2). Nineteen of 39 patients had positive staining

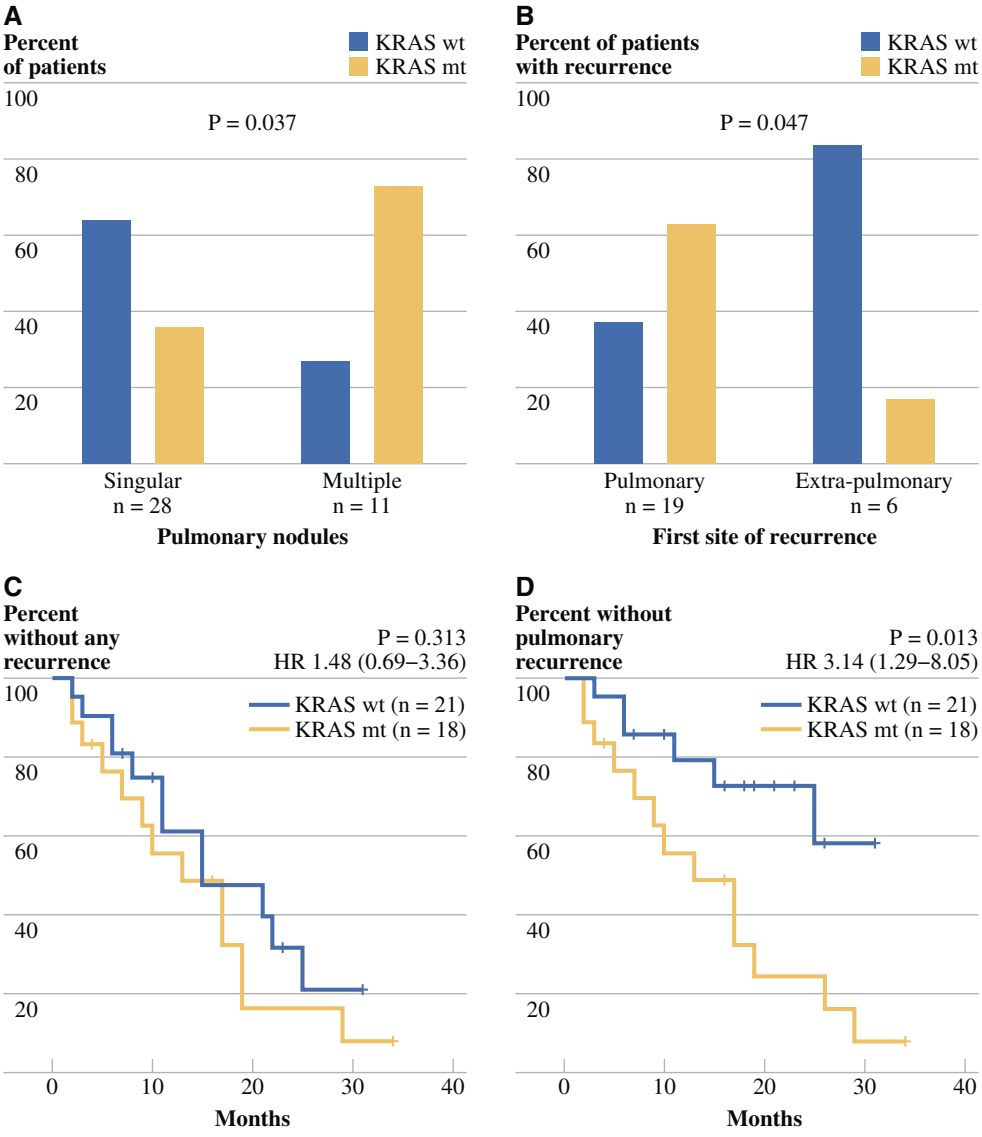

FIG. 3 **a** *KRAS* mutation was associated with multiple pulmonary metastases. **b** After the first metastasectomy, the lung was significantly more often the initial site of recurrence in patients with *KRAS* mutant metastases. **c** The time to any recurrence of patients harboring *KRAS* mutations did not differ from those with *KRAS* wild-type tumors. **d** However, the lung-specific time to recurrence was significantly decreased in patients with *KRAS* mutant tumors

for EGFR (49 %). EGFR expression was not associated with clinicopathologic characteristics (Table 1). Regarding the follow-up, Kaplan–Meier analysis revealed no impact of the EGFR expression levels on time to any recurrence (Fig. 2d) or time to pulmonary recurrence (Fig. 2e). Moreover, EGFR expression was not associated with Mts in the *KRAS* gene (Fisher's exact test, $p = 0.341$).

KRAS and BRAF Mt Status

Forty-six percent of resected metastases had a *KRAS* gene Mt. In 89 % of these cases, codon 12 was mutated, whereas codon 13 Mt was found in only two cases (11 %). Significant correlations between *KRAS* Mt status and clinicopathological parameters could be found regarding the age and the site of primary tumor (Table 1). This is in line with published data, showing that the worse prognosis of right sided CRC might be due to an unfavorable genetic profile.[28] Mucinous differentiation could not be observed in the right-sided tumors. In one patient, hereditary nonpolyposis CRC was suspected and ruled out by immunohistochemical staining for MSH2, MSH6, and MLH1. The time between the diagnoses of the primary tumor and first PM was shorter in patients harboring a *KRAS* Mt than in those with Wt tumors (23 vs. 31 months, respectively) without reaching the level of significance ($p = 0.279$). Moreover, in patients presenting with multiple metastases at the first metastasectomy *KRAS* was more likely to be mutated than in patients with a singular node at initial presentation (73 vs. 27 %; $p = 0.037$; Fig. 3a). In absolute numbers, patients with KRAS Mt in the tumor tissue had significantly more metastases than the *KRAS* Wt group ($p = 0.029$), whereas no significant

TABLE 2 Clinicopathological variables and time to recurrence and time to pulmonary recurrence of patients ($N = 39$) with CRC metastasizing to the lungs in the log-rank and Cox proportional hazards models

			Time to any recurrence		Time to pulmonary recurrence				
	Total		Univariate analysis (log-rank)		Univariate analysis (log-rank)		Multivariate analysis (Cox-regression)		
	$N = 39$	%	Months	p value	Months	p value	Exp(B)	95 % CI	p value
Sex									
Male	20	51.3	20	0.409	17	0.326	–	–	–
Female	19	48.7	17		21				
Age (years)									
<64	20	51.3	17	0.994	29	0.871	–	–	–
≥64	19	48.7	15		19				
Location									
Colon	15	41.7	15	0.991	15	0.4	–	–	–
Rectum	21	58.3	17		21				
Unknown	3	–	–	–	–	–	–	–	–
T stage									
pT1 + pT2	8	22.2	17	0.983	17	0.846	–	–	–
pT3 + pT4	28	77.8	17		25				
Unknown	3	–	–	–	–	–	–	–	–
N stage									
pN0	16	44.4	22	0.252	29	0.119	–	–	–
pN1 + pN2	20	55.6	13		13				
Unknown	3	–	–	–	–	–	–	–	–
Chemotherapy before metastasectomy									
Yes	31	79.5	15	0.162	17	0.222	–	–	–
No	8	20.5	25		25				
Chemotherapy after metastasectomy									
Yes	33	84.6	17	0.283	25	0.071	0.35	0.12–1.01	0.053
No	6	15.4	15		15				
Previous liver metastasis									
Yes	12	30.8	13	0.37	13	0.137			
No	27	69.2	19		21				
KRAS									
Wt	21	53.8	20	0.313	25	0.013*	2.77	1.08–7.11	0.035*
Mt	18	46.2	13		23				
EGFR									
−	20	51.3	17	0.832	17	0.658	–	–	–
+	19	47.7	19		21				

difference could be found regarding the estimated total tumor volume ($p = 0.272$; see Supplementary material). Twenty-five patients evidenced a disease recurrence within the study period. Within this subgroup, KRAS Mt was associated with the lung as initial site of recurrence ($p = 0.047$; Fig. 3b).

All *KRAS* Wt samples were further evaluated for their *BRAF V600E* status. No BRAF Mts were found in the assessed PMs. This finding is in line with published evidence on primary CRC.[21]

Univariate and Multivariate Analysis

We evaluated the impact of clinicopathological characteristics of the primary tumor and EGFR and *KRAS* status on time to tumor recurrence in univariate and multivariate analyses (Table 2). Neither clinicopathological characteristics nor EGFR protein expression or *KRAS* status were associated with the time to tumor recurrence irrespective of the recurrence site in univariate analysis. Likewise, we assessed the impact of clinical and molecular factors on

time to lung-specific recurrence. In univariate analysis, we found that patients receiving adjuvant chemotherapy after pulmonary metastasectomy had longer recurrence-free survival than those treated by surgery alone (25 vs. 17 months, respectively). However, this difference remained nonsignificant ($p = 0.071$). Mt in *KRAS* resulted in a significantly decreased time to pulmonary recurrence ($p = 0.013$; Fig. 3d). We further investigated all factors reaching $p < 0.1$ in log-rank tests in multivariate analysis (chemotherapy postmetastasectomy and *KRAS* Mt status). In multivariate analysis, the *KRAS* Mt status remained a poor prognostic factor associated with significantly decreased time to pulmonary recurrence after the first metastasectomy ($p = 0.035$).

DISCUSSION

Pulmonary metastasectomy has become an accepted approach in the treatment algorithm of CRC. However, there is a lack of well-designed prospective studies in this field. The two major obstacles are the low number of cases per year—even in high-volume centers—and the heterogeneity of the patient population in question. Thus, evidence is limited to retrospective analyses; most of the studies represent single-center experiences with an observation period of two to three decades.[5,10] To overcome this problem, we established a prospective follow-up study with the goal to evaluate various histological and clinical characteristics on patients' outcome after metastasectomy. Within an observation period of 4 years, 44 patients underwent metastasectomy.

This study represents the first systematic evaluation of EGFR expression and *KRAS*/*BRAF* Mt status in the setting of pulmonary metastasectomy with curative intent. We evaluated resected metastases for EGFR expression and *KRAS* and *BRAF* Mts.

Up to date, only two descriptive studies on EGFR expression in PMs from CRC are available.[26,29] In our patient cohort, we found no impact of EGFR expression on tumor recurrence. This confirms studies on the primary tumor site, where prognosis was independent from EGFR expression.[14]

We found a mutated *KRAS* gene in nearly half of our patients. This is in line with already published evidence from genetic analysis of the primary and metastatic tumor sites, with high occurrence of *KRAS* Mts in patients with PMs.[21,22,30] However, to date no evidence on the impact of *KRAS* status on metastasis-free survival after first pulmonary metastasectomy has been published. We were able to show that patients with *KRAS* mutated tumors had a higher likelihood to develop pulmonary recurrence compared with the *KRAS* Wt group. Moreover, *KRAS* Mt was associated with multiple pulmonary nodules, which supports the idea of an aggressive, lung-specific behavior of primary cancers harboring a *KRAS* Mt. Because only two patients died during follow-up, determining a potential impact of EGFR pathway alterations on overall survival was not possible. Noteworthy, in a cohort of patients undergoing liver or lung metastasectomy, *KRAS* did not affect the overall survival.[22] As *KRAS* and *BRAF* Mts are, in general, mutually exclusive, we further assessed KRAS Wt specimens regarding the *BRAF V600E* Mt.[31] In contrast to moderately frequent Mts in the *KRAS* gene, the *BRAF V600E* Mt could not be detected in any tissue specimen.

Both *KRAS* and *BRAF* Mts are thought to be highly concordant during the progression of the disease.[21,32] For this reason, we did not routinely examine tissue from the primary tumor. When more than one nodule was resected, genotyping revealed a consistent *KRAS* and *BRAF* status in all metastases. Furthermore, we analysed resected metastases of eight patients who underwent re-metastasectomy within the study period. Mt status also was highly conserved in all of these patients.

It is generally agreed that the identification of patients who will benefit at the utmost from pulmonary metastasectomy is difficult.[7] This is especially true when patients present with pulmonary recurrence for a second, third, or fourth metastasectomy. Thus, the identification of risk factors for early recurrence might help the surgeon to weight a local surgical approach against a systemic chemotherapeutic regime.

Our study is limited by the fact that there was a variation in the received chemotherapy schemes. Due to the typically complex and heterogeneous history of patients with metastatic disease, this limitation can only be avoided by conducting large collaborative studies. In our series, patients with adjuvant chemotherapy after metastasectomy showed a tendency toward increased recurrence-free survival. However, we believe that our findings on altered EGFR signaling in PMs might have an impact on the individualized therapeutic approach and, moreover, on the postoperative tumor surveillance of patients with metastatic spread to the lungs.

In summary, our work underlines the association of *KRAS* Mt with lung-specific metastasis in a closely followed up study cohort. Additional large international collaborative studies are required to define the precise and optimal role of EGFR expression measurements and KRAS mutational testing in the diagnostic and therapeutic algorithm of CRC metastasizing to the lungs.

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CONFLICT OF INTEREST None declared.

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2.1.1. Interlude

After assessing molecular markers in tumor cells themselves, we focused in the work "Stromal expression of heat-shock protein 27 is associated with worse clinical outcome in patients with colorectal cancer lung metastases" on the tumor surrounding stroma. The tumor-surrounding stroma consisting of immune cells, fibroblasts and vessels gained growing attention in oncology. Activated fibroblasts are believed to contribute to disease progression and resistance to chemotherapy in solid malignancies, including CRC (Tsujino *et al*, 2007) (Lotti *et al*, 2013). The prognostic value of myofibroblasts, characterized by strong expression of alpha-smooth muscle actin (alpha-SMA) and vimentin, in CRC lung metastases remained elusive.

Therefore we assessed occurrence of alpha-SMA$^+$, vimentin$^+$ fibroblasts in CRC lung metastases, liver metastases and paired primary tumors. We could demonstrate that the small heat shock protein Hsp27 is co-expressed in these activated fibroblasts, introducing a novel and targetable marker for myofibroblasts. Hsp27$^+$ fibroblasts were more common in lung metastases than in liver metastases, supporting the theory of a site-specific composition of the tumor microenvironment. Tumor vascularization, measured by CD31$^+$ vessels, was increased in patients with strong stromal reaction. Moreover, patients with lung metastases harboring a highly active, Hsp27$^+$ tumor stroma had a worse outcome after pulmonary metastasectomy.

2.2 Stromal expression of heat-shock protein 27 is associated with worse clinical outcome in patients with colorectal cancer lung metastases (published PDF)

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RESEARCH ARTICLE

Stromal Expression of Heat-Shock Protein 27 Is Associated with Worse Clinical Outcome in Patients with Colorectal Cancer Lung Metastases

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Abstract

Background

Pulmonary metastases are common in patients with primary colorectal cancer (CRC). Heat-shock protein 27 (Hsp27) is upregulated in activated fibroblasts during wound healing and systemically elevated in various diseases. Cancer-associated fibroblasts (CAFs) are also thought to play a role as prognostic and predictive markers in various malignancies including CRC. Surprisingly, the expression of Hsp27 has never been assessed in CAFs. Therefore we aimed to investigate the expression level of Hsp27 in CAFs and its clinical implications in patients with CRC lung metastases.

Methods

FFPE tissue samples from 51 pulmonary metastases (PMs) and 33 paired primary tumors were evaluated for alpha-SMA, CD31, Hsp27 and vimentin expression by immunohistochemistry and correlated with clinicopathological variables. 25 liver metastases served as control group. Moreover, serum samples (n=10) before and after pulmonary metastasectomy were assessed for circulating phospho-Hsp27 and total Hsp27 by ELISA.

Medical University of Vienna. Preliminary results with a limited number of patients have been presented at the annual meetings of the Austrian Society of Cardiothoracic Surgery and the Austrian Society of Surgery. HJA declares competing financial interests (patent number WO 2010/000820 (serum Hsp27 for COPD diagnosis) and WO 2012/080303 (serum Hsp27 for NSCLC diagnosis)). This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials. The other authors declare no conflicts of interest.

Results

Stromal expression of Hsp27 was observed in all PM and showed strong correlation with alpha-SMA ($P<0.001$) and vimentin ($P<0.001$). Strong stromal Hsp27 was associated with higher microvessel density in primary CRC and PM. Moreover, high stromal Hsp27 and αSMA expression were associated with decreased recurrence-free survival after pulmonary metastasectomy ($P=0.018$ and $P=0.008$, respectively) and overall survival ($P=0.031$ and $P=0.017$, respectively). Serum levels of phospho- and total Hsp27 dropped after metastasectomy to levels comparable to healthy controls.

Conclusions

Herein we describe for the first time that Hsp27 is highly expressed in tumor stroma of CRC. Stromal α-SMA and Hsp27 expressions correlate with the clinical outcome after pulmonary metastasectomy. Moreover, serum Hsp27 might pose a future marker for metastatic disease in CRC.

Introduction

Colorectal cancer (CRC) is, after lung cancer, the second most common cause of cancer-related death in Europe [1]. More than one fifth of the patients with CRC present with metastases already at time of diagnosis of the primary cancer and the same proportion will develop metastases during the course of disease [1, 2]. The lungs are the second most common site of distant metastasis, making pulmonary metastases (PM) an essential contributor to the high mortality of CRC.

Besides cancer cells themselves, a tumor comprises stromal cells. The interactions of stromal and cancer cells is thought to be a major determinant of the tumor behavior and response to therapy [3, 4]. Cellular components of the stroma are fibroblasts, endothelial cells, immune cells and pericytes [5]. Cancer-associated fibroblasts (CAF), and especially activated fibroblasts, play a major role in the tumor-stroma network, similar to dermal fibroblasts in wound healing. This contributed to the description of tumors as "wounds that do not heal" by Dvorak *et al.* in the late 80's [6]. CAF contribute to various tumor-promoting characteristics like extra-cellular matrix turnover, tumor growth, angiogenesis and metastasis [7, 8]. Due to the expression of α-smooth muscle actin (α-SMA), activated CAF are often described as myofibroblasts. They have also been shown to be positive for fibroblast-activation protein-α/seprase, palladin and vimentin [9–12]. Recently, efforts have been made to characterize the "signature" of these fibroblasts by proteome and gene expression profiling [13, 14].

In the context of benign diseases, wound healing and keloid formation it is well known that activated fibroblasts express high levels of heat-shock protein 27 (Hsp27), which is crucial for fibroblast adhesion, contractility and motility [15–17]. The TGF-beta induced p38-MAPK pathway is the key regulator in the induction of Hsp27 in smooth muscle cells and myofibroblasts [18, 19]. Once synthesized, two main functions of Hsp27 are critical in wound healing process: promoting myofibroblast motility and angiogenesis. Hsp27 is involved in the stabilization of actin filaments and of SNAIL, an inducer of epithelial-mesenchymal transition. Both mechanisms contribute to the induction of the myofibroblastic phenotype [20, 21]. Another function of Hsp27 executed in a paracrine manner is enhancing angiogenesis. It was demonstrated that extracellular Hsp27 leads to NfκB activation and subsequent expression of the

proangiogenic factors VEGF and interleukin-8 (IL-8) in endothelial cells [22]. Together with others, our group could show that an overexpression of Hsp27 is strongly linked to several benign and malign pathologies of the lung associated with fibroblast activation, including emphysema/chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis and non-small cell lung cancer [21, 23–26].

Given the fact that activated fibroblasts play an important role in the progression of malignant disease as well as in various non-malignant diseases of the lung, we aimed to investigate the prevalence of Hsp27 positive tumor stroma in CRC lung metastases and corresponding primary tumors. Furthermore, we sought to describe the implications on the clinical outcome after metastasectomy and the presence of cellular and secreted Hsp27 in these patients.

Materials and Methods

Study population

From April 2009 to November 2013, all consecutive cases of pulmonary metastasectomy from primary CRC and appropriate tissue samples were included in this study. Patients received diagnostic work-up including thoracic and abdominal computed tomography (CT). If patients had undergone pulmonary metastasectomy before, specimen of the first metastasectomy was also examined and the date of the first metastasectomy was used for outcome calculation. Lung metastasis free survival (LMFS) was defined as the time between diagnosis of the primary tumor and diagnosis of the metastatic spreading to the lung. R0 resection was achieved in all patients. Of 33 patients, specimens of the corresponding primary tumor could be obtained. Follow-up examinations were carried out in 3 to 6 months intervals. Recurrence-free survival (RFS) was defined as the period from the first pulmonary metastasectomy to evidence of recurrent disease at any site verified by CT scans. In 10 consecutive cases, serum samples obtained before metastasectomy and during follow-up were available. Follow-up serum samples were collected 3 to 6 months after surgery. Additionally, serum samples from age-, gender- and smoking status- matched healthy volunteers were collected. All patients gave their written informed consent prior to blood collection and participation. A study cohort of 25 consecutive patients with resected CRC liver metastases served as additional control group. The study was approved by the ethics committee of the Medical University of Vienna (EK 91/2006, EK1194/2011 and EK1044/2012) and was conducted according to the declaration of Helsinki. The current study cohort is based on previous published works by our group.[27, 28]

Immunohistochemistry and immunofluorescence

Immunohistochemical staining was performed according to a standard protocol. Shortly, formalin-fixed, paraffin-embedded tissue specimens were cut in 4-μm thick sections and deparaffinized. Heat-mediated antigen retrieval was performed. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide. Sections were incubated with the appropriate primary antibody for 1h at room temperature. For immunohistochemistry, as secondary step, the polymer- based ImmPRESS kit (Vector Laboratories, Burlingame, California) was used according to the manufacturer's protocol. 3,3'-Diaminobenzidine (DAB) was used as substrate (Vector Laboratories, Burlingame, California). Finally, the sections were counterstained with hematoxylin. Vimentin staining was performed in a Ventana ES Immunostainer System (Ventana Medical Systems Inc., Tucson, AZ, USA). For immunofluorescence, appropriate fluorescent secondary antibodies were used and cell nuclei were counterstained with DAPI (Sigma Aldrich, St. Louis, MO, USA). As negative controls, the primary antibody was omitted. Positive controls were tissue samples with known presence of the respective target protein. A detailed list of antibodies and dilutions is provided as supplementary information (S1 Table).

Scoring of stained tumor cells. Immunohistochemistry staining score (IHC score) was calculated as described previously [29]. The percentage of positive tumor cells could reach values between 0 and 100%, and were multiplied by the staining intensity (0 to 3). Thus, IHC scores could range from 0 to 300. Two blinded observers rated the staining. In case that the two ratings differed, the slide was discussed and re-evaluated. The continuous IHC score was dichotomized by applying the median score of metastases or primary tumors as cut-off value.

Scoring of stained stromal cells. The sections were scored semiquantitatively as described previously [10]. Briefly, the slides were screened at low magnification and evaluated for their staining intensity in the stromal cells in the tumor center. The stromal staining was assessed as grade 0 (negative, <1% positive stromal cells), grade 1+ (low,1–10% positive stromal cells), grade 2+ (intermediate, >10–50% positive stromal cells) and grade 3+ (strong, >50% positive stromal cells) by two blinded observers. In case that the two ratings differed, the slide was discussed, re-evaluated and a consensus was reached on all slides.

Determination of microvessel density. Microvessel density (MVD) was measured using the "hotspot" method, as published elsewhere [30]. In brief, the slides were screened at low magnification to identify the area with the greatest number of CD31-positive microvessels ("hotspot"). MVD was determined by counting all microvessels at 200x magnification (corresponding to 0.95 mm^2). Mean values were calculated from two independently counted densities. In case of strong inter-observer discrepancy, the slide was reevaluated.

Enzyme-linked immunosorbent assay (ELISA)

Serum samples were assessed by commercially available human Hsp27, phospho-Hsp27 and interleukin-8 ELISA kits (all R&D Systems, Minneapolis, USA). Measurement was conducted according to the manufacturer's instructions. The samples were assayed in duplicates. The absorbance was measured at 450nm using a plate reader (PerkinElmer, Waltham, USA), compared to a standard curve with known protein content and converted to pg/mL.

Statistical analysis

All obtained data was evaluated statistically using SPSS 19 (SPSS Inc., Chicago, USA) and GraphPad Prism 6 (GraphPad Software Inc., California, USA). Student's t-test was used to compare means of two independent groups, paired t-test for dependent groups and expressed as mean±standard deviation (SD). Kaplan-Meier curves and log-rank test were used to compare survival functions. Chi-square test and Fisher's exact test were used to compare binominal variables. All tests were two-sided. P-values equal or below 0.05 were considered as statistically significant.

Results

Tissue specimens of PM from a total of 51 consecutive patients were available. Median age at metastasectomy was 63 years (range 33–83). 29 (56.9.1%) male and 22 (43.1%) female patients were included. The clinicopathological characteristics of the included patients are summarized in Table 1.

Hsp27 is highly expressed in cancer-associated stroma of lung metastases

To assess the expression level of Hsp27 in cancer-associated stroma, tissue sections were stained for Hsp27 and vimentin (Fig. 1). The Hsp27 expression of tumor stroma in PM was scored in 0 (0%), 17 (33%), 17 (33%), 17 (33%) cases as 0, 1+, 2+ and 3+, respectively. The

Table 1. Descriptive data on patient, primary tumor and metastases characteristics stratified by stromal Hsp27 and α-SMA expression (N = 51).

				Stromal Hsp27 expression in PM								Stromal alpha-SMA expression in PM							
		Total		low		intermediate		high		X^2		low		intermediate		high		X^2	
		N = 51	%	N = 17	%	N = 17	%	N = 17	%	p-value		N = 14	%	N = 23	%	N = 14	%	p-value	
Patients																			
Sex	**Male**	29	56.9	10	19.6	8	15.7	11	21.6	0.571		8	15.7	12	23.5	9	17.6	0.771	
	Female	22	43.1	7	13.7	9	17.6	6	11.8			6	11.8	11	21.6	5	9.8		
Age (years)	**Median**	63		66		62		63		0.618[a]		67		62		61.5		0.383[a]	
	Range	33–83		47–83		37–78		33–74				50–83		37–78		33–74			
Primary tumor																			
Location	**Colon**	27	52.9	9	17.6	8	15.7	10	19.6	0.790		6	11.8	14	27.5	7	13.7	0.549	
	Rectum	24	47.1	8	15.7	9	17.6	7	13.7			8	15.7	9	17.6	7	13.7		
T stage	**pT1**	1	2.1	1	2.1	0	0.0	0	0.0			1	2.1	0	0.0	0	0.0		
	pT2	7	14.6	1	2.1	3	6.3	3	6.3	0.109[b]		1	2.1	3	6.3	3	6.3	0.032[b]	
	pT3	34	70.8	11	22.9	11	22.9	12	25.0			8	16.7	16	33.3	10	20.8		
	pT4	6	12.5	2	4.2	3	6.3	1	2.1			3	6.3	3	6.3	0	0.0		
	N/A	3	-																
N stage	**pN0**	21	43.8	9	18.8	6	12.5	6	12.5	0.169[b]		7	14.6	9	18.8	5	10.4	0.299[b]	
	pN1	11	22.9	2	4.2	6	12.5	3	6.3			2	4.2	7	14.6	2	4.2		
	pN2	16	33.3	4	8.3	5	10.4	7	14.6			4	8.3	6	12.5	6	12.5		
	N/A	3	-																
Grading	**G1**	2	3.9	1	2.0	1	2.0	0	0.0			1	2.0	1	2.0	0	0.0		
	G2	42	82.4	13	25.5	14	27.5	15	29.4	0.487[b]		11	21.6	19	37.3	12	23.5	0.713[b]	
	G3	7	13.7	3	5.9	2	3.9	2	3.9			2	3.9	3	5.9	2	3.9		
Stromal HSP27	**low**	6	18.2	0	0.0	2	6.1	4	12.1			0	0.0	3	9.1	3	9.1		
	intermediate	15	45.5	6	18.2	6	18.2	3	9.1	0.053[b]		5	15.2	8	24.2	2	6.1	0.081[b]	
	high	12	36.4	4	12.1	4	12.1	4	12.1			4	12.1	5	15.2	3	9.1		
	N/A	18	-																
Stromal alpha-SMA	**low**	8	24.2	2	6.1	3	9.1	3	9.1			3	9.1	3	9.1	2	6.1		
	intermediate	14	42.4	4	12.1	6	18.2	4	12.1	0.711[b]		3	9.1	7	21.1	4	12.1	0.779[b]	
	high	11	33.3	4	12.1	3	9.1	4	12.1			3	9.1	6	18.2	2	6.1		
	N/A	18																	
Pulmonary metastasis																			
Microvessel density	**Mean**	39.0		33.0		40.9		43.0		0.169[e]		32.4		39.5		44.6		0.136[e]	
	Range	12–96		12–56		16–76		19–96				12–76		18–65		19–96			
Previous liver metastasis	**Yes**	16	31.4	5	9.8	6	11.8	5	9.8	0.913		1	2.0	12	23.5	3	5.9	0.004[b]	
	No	35	68.6	12	23.5	11	21.6	12	23.5			13	25.5	11	21.6	11	21.6		
No. of nodules	**1**	33	64.7	12	23.5	9	17.6	12	23.5	0.462		8	15.7	14	27.5	11	21.6	0.236[b]	
	>1	18	35.3	5	9.8	8	15.7	5	9.8			6	27.5	9	45.1	3	27.5		

[a]Kruskal-Wallis test;

[b]Fisher's exact test;

[e]Oneway-ANOVA; LMFS: Lung-metastasis free survival after primary tumor

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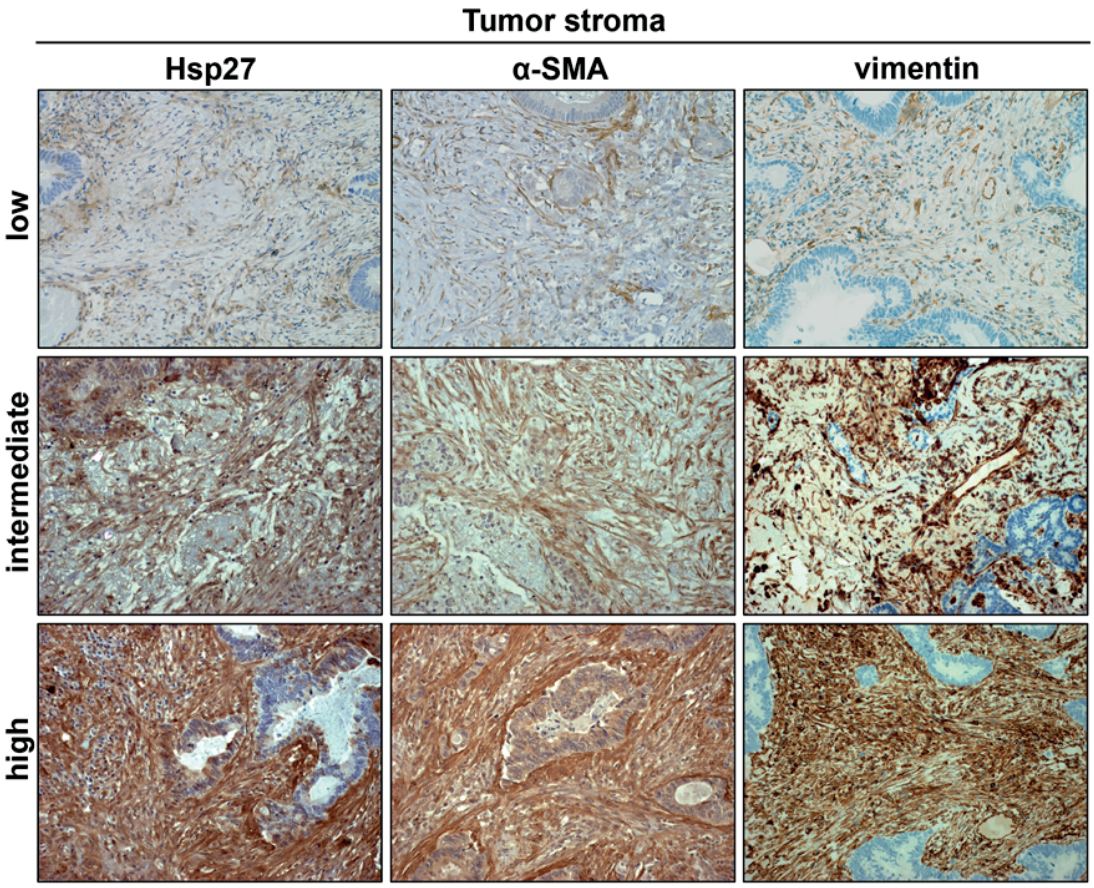

Fig 1. Representative images showing pulmonary metastases with low, intermediate and high intensity of positive tumor stroma stained for Hsp27 and α-SMA. Stromal fibroblasts were further identified by vimentin staining. (DAB substrate, same tumor specimen per row, 200x magnification).

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correlation of the stromal Hsp27 staining with patient and tumor characteristics is depicted in Table 1. Staining intensity of the tumor cells was determined separately (S1A Fig). 23 (45%) metastases were scored as Hsp27 highly positive and 28 (55%) metastases as low/negative (IHC score range 0–160; median/cut off 30). There was no correlation between tumor and stromal Hsp27 expression in pulmonary metastases (Chi square test; $P = 0.237$). 33 corresponding primary tumors were available. The stromal Hsp27 expression in the primary tumors 0 (0%), 6 (18%), 15 (46%) and 12 (36%) were scored as 0, 1+, 2+ and 3+, respectively. Determining the Hsp27 staining in the tumor cells in the primary tumor tissue, 14 (42%) of the cases were scored as Hsp27 positive and 19 (58%) as low/negative (IHC score range 5–100; median/cut off 70).

Hsp27 is co-expressed with α-SMA and vimentin in the stroma of PMs

Activated tumor stroma has been described as highly α-SMA positive in primary and metastatic CRC [12, 31]. Therefore, the specimens were stained for α-SMA. The expression of α-SMA in the stroma was rated in the same semiquantitative manner as the Hsp27 staining. 0 (0%), 13 (25.5%), 24 (51%) and 13 (25.5%) cases were scored as 0, 1+, 2+ and 3+, respectively. The staining score distribution according to the clinicopathological variables is depicted in Table 1.

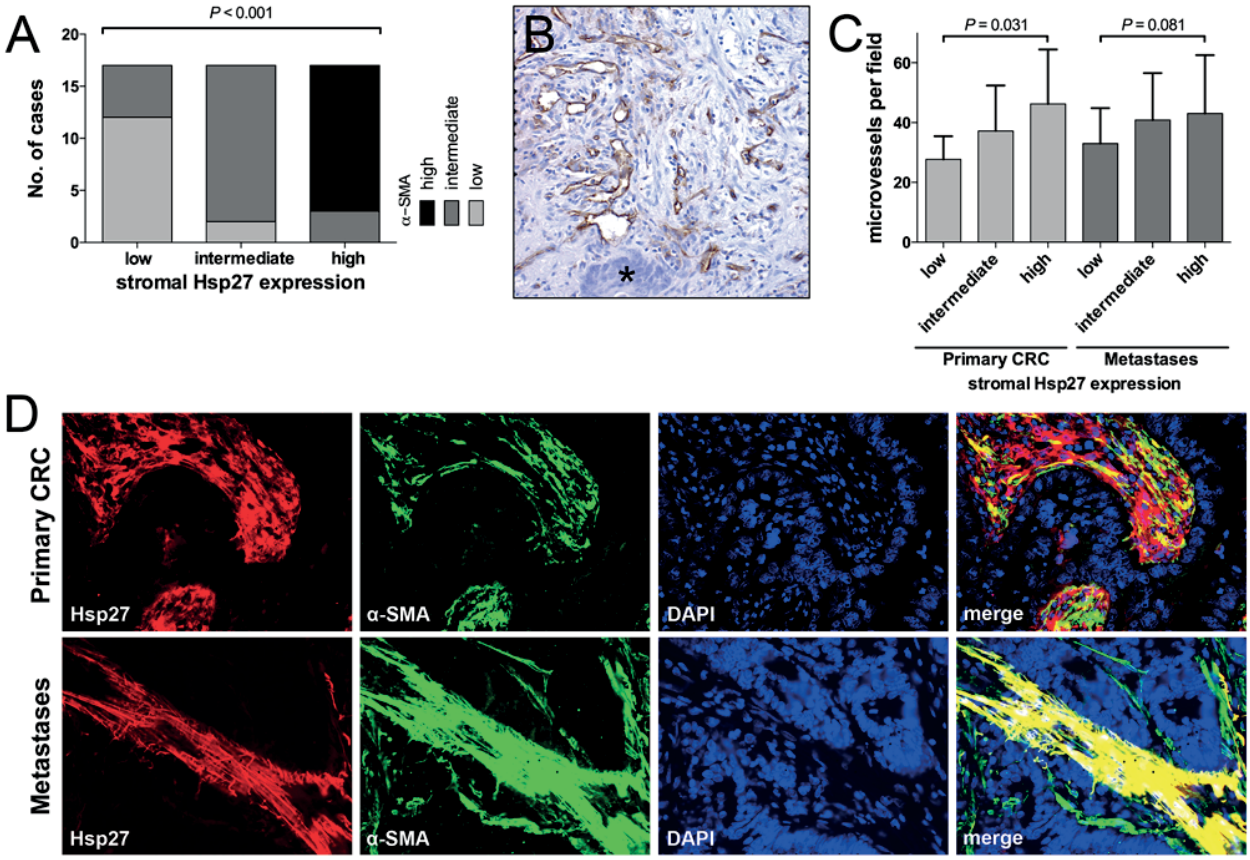

Fig 2. The degree of Hsp27 expression score in the tumor stroma correlated significantly with the expression of stromal α-SMA (A). CD31-positive microvessels surrounded by tumor stroma next to tumor cells (asterisk) (B). MVD was significantly increased in primary tumors and metastases with strong stromal Hsp27 expression (C). Immunofluorescence showed a co-expression of stromal Hsp27 and α-SMA especially in PM (400x magnification) (D).

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Expression levels of α-SMA correlated significantly with Hsp27 expression in PMs ($P < 0.001$, Fig. 2A). This was also observed in liver metastases (S2 Fig). To further assess the stromal co-expression of α-SMA and Hsp27, representative slides of primary and metastatic tumors were co-labeled for α-SMA and Hsp27 by immunofluorescence. A strong co-expression could be observed especially in PMs (Fig. 2D). Tumor stroma with strong Hsp27 and α-SMA staining was also highly positive for vimentin (S3 Table).

MVD is increased in tissue samples with Hsp27-positive tumor stroma

Recently it was shown that Hsp27 mediates angiogenesis [22]. We therefore determined the MVD by CD31 staining and correlated it with the Hsp27 expressions (Fig. 2B and 2C). In PM, we found a trend towards higher MVD in samples with high Hsp27 levels. MVD was 33.0±2.9, 40.1±3.8 and 43.0±4.7 for 1+, 2+ and 3+ Hsp27 intensity (mean±SD). However, this trend did not reach the level of significance ($P = 0.081$). Similarly, in primary CRC with high stroma levels of Hsp27, significantly more microvessels could be found (27.7±3.2, 37.2±4.0 and 46.4±5.2 for 1+, 2+ and 3+ Hsp27 intensity, respectively; $P = 0.031$). A detailed description of MVD stratified by clinicopathological characteristics is provided in S2 Table.

Table 2. Univariate analysis assessing clinicopathological variables and lung metastasis free survival, recurrence free survival after metastasectomy and overall survival of patients (N = 51) with CRC metastasizing to the lung.

				Lung metastasis free survival			Recurrence free survival			Overall survival		
		Total		Univariate analysis (log-rank)			Univariate analysis (log-rank)			Univariate analysis (log-rank)		
		N = 51	%	Months	HR (95% CI)	p-value	Months	HR (95% CI)	p-value	Months	HR (95% CI)	p-value
Sex	**Male**	29	43.1	24	1	0.091	17	1	0.125	39	1	0.464
	Female	22	56.9	29	0.62 (0.35–1.09)		11	1.63 (ß.86–3.09)		52	0.72 (0.29–1.77)	
Age (years)	**< 64 yrs**	26	51.0	24	1	0.759	15	1	0.820	39	1	0.377
	≥ 64 yrs	25	49.0	28	0.92 (0.52–1.61)		11	0.93 (0.49–1.77)		65	0.67 (0.28–1.64)	
Location	**Colon**	27	52.9	28	1	0.926	15	1	0.730	52	1	0.671
	Rectum	24	47.1	23	1.03 (0.58–1.81)		11	0.90 (0.47–1.70)		36	1.21 (0.50–2.93)	
T stage	**pT1+pT2**	8	16.7	24	1	0.932	15	1	0.977	31	1	0.650
	pT3+pT4	40	83.3	25	1.03 (0.48–2.24)		14	0.99 (0.41–2.37)		52	0.78 (0.26–2.35)	
	unknown	3	-									
N stage	**pN0**	21	43.7	29	1	0.643	17	1	0.465	39	1	0.827
	pN1+pN2	27	56.3	24	1.15 (0.64–2.07)		11	1.28 (0.65–2.50)		52	0.91 (0.37–2.23)	
	unknown	3	-									
Previous liver metastasis	**Yes**	16	31.4	25	1	0.691	9	1	0.118	30	1	0.401
	No	35	68.6	24	0.89 (0.48–1.62)		17	0.60 (0.31–1.16)		52	0.69 (0.28–1.69)	
stromal Hsp27	**low**	17	33.3	25	1		19	1	0.018[a]	52	1	0.031[a]
	intermediate	17	33.3	28	0.80 (0.40–1.59)	0.575[a]	17	1.50 (0.68–3.48)		NR	0.48 (0.13–1.70)	
	high	17	33.3	24	1.15 (0.58–2.26)		10	2.65 (1.31–6.77)		31	1.98 (0.75–5.37)	
stromal alpha-SMA	**low**	14	27.5	29	1		22	1	0.008[a]	52	1	
	intermediate	23	45.0	24	1.18 (0.60–2.30)	0.784[a]	15	1.83 (0.77–4.30)		NR	0.58 (0.17–1.90)	0.017[a]
	high	14	27.5	24	1.29 (0.61–2.72)		7	3.99 (1.53–10.41)		30	2.57 (0.84–7.84)	
No. of nodules	**1**	33	64.7	25	1	0.278	15	1	0.490	52	1	0.653
	>1	18	35.3	24	1.38 (0.76–2.50 =		14	1.26 (0.65–2.43)		36	1.23 (0.50–3.03)	
LMFS	**<36**	36	70.6	-	-	-	15	1	0.948	39	1	0.512
	>36	15	29.4		-		11	1.02 (0.51–2.07)		NR	0.72 (0.26–1.98)	

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Stromal Hsp27 is associated with poor outcome after pulmonary metastasectomy

We assessed the association of relevant clinicopathological variables with LMFS, RFS and OS after metastasectomy (Table 2). No variable had significant impact on the LMFS. Female patients had a strong trend towards a shorter time to PMs ($P = 0.091$). The stromal expression of neither α-SMA nor Hsp27 was associated with a significantly decreased LMFS. However, both histological markers had a significant prognostic impact on the RFS after pulmonary metastasectomy ($P = 0.018$ and $P = 0.008$ for stromal Hsp27 and α-SMA, respectively) (Fig. 3). Again, female patients showed a strong trend towards decreased RFS. Moreover, patients with a history of previous liver metastases had a decreased RFS compared to patients without liver metastases in the history. However, both variables, sex and the history of previous liver metastases, had no significant influence on the RFS. Additionally to that, extensive stromal Hsp27 stromal Hsp27 and α-SMA were associated with a decreased overall survival after metastasectomy ($P = 0.031$ and $P = 0.017$ for stromal Hsp27 and α-SMA, respectively). Due to the high rate of concordance, stromal Hsp27 and α-SMA were no independent factors in the outcome analysis when adding both variables into a multivariate analysis (data not shown).

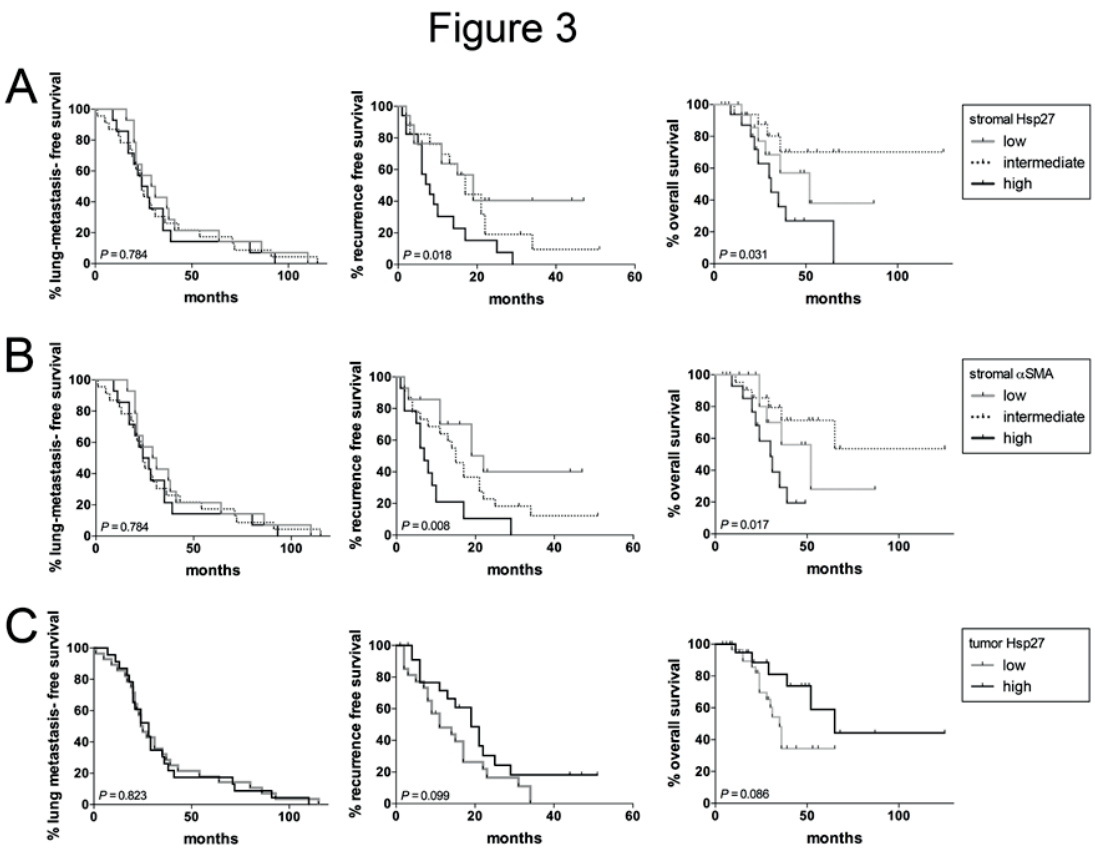

Fig 3. Kaplan-Meier plots showing the lung-metastasis free survival, recurrence free survival and overall survival after metastasectomy dependent on stromal Hsp27 (A), stromal α-SMA (B) and tumor Hsp27 (C) scoring.

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Soluble Hsp27 is systemically increased in patients before pulmonary metastasectomy

Elevated levels of soluble Hsp27 can be detected systemically in various malignant and non-malignant diseases. In a subset of 10 of our patients paired serum samples (pre- and post-metastasectomy) were available (Fig. 4). We compared the serum level of total Hsp27 and phospho-Hsp27 to matched healthy volunteers (S3 Table). Serum total Hsp27 levels were 2276 ±905, 3245±1684 and 2064±1226 pg/mL (mean±SD) for control, pre- and post-metastasectomy samples, respectively. Total Hsp27 decreased significantly after metastasectomy ($P = 0.016$). Serum phospho-Hsp27 levels were 84±63, 156±82 and 88±114 pg/mL (mean±SD) for control, pre- and post-metastasectomy samples, respectively. A significant difference was found between healthy controls and pre-metastasectomy samples ($P = 0.041$). Although the levels dropped after metastasectomy, the difference was not significant. IL-8, a proangiogenic cytokine involved in the Hsp27 signaling, was detected at very low concentrations of 15±8, 45 ±58 and 9±8 pg/mL (mean±SD) in the serum samples. The differences of systemic IL-8 levels between the three groups did not differ significantly from each other. No correlation was found between the expression level of stromal Hsp27, tumor Hsp27 and systemic levels (data not shown). Moreover, no significant correlation between total or phospho-Hsp27 and standard inflammatory markers (CRP and fibrinogen) was found (S1B and S1C Fig).

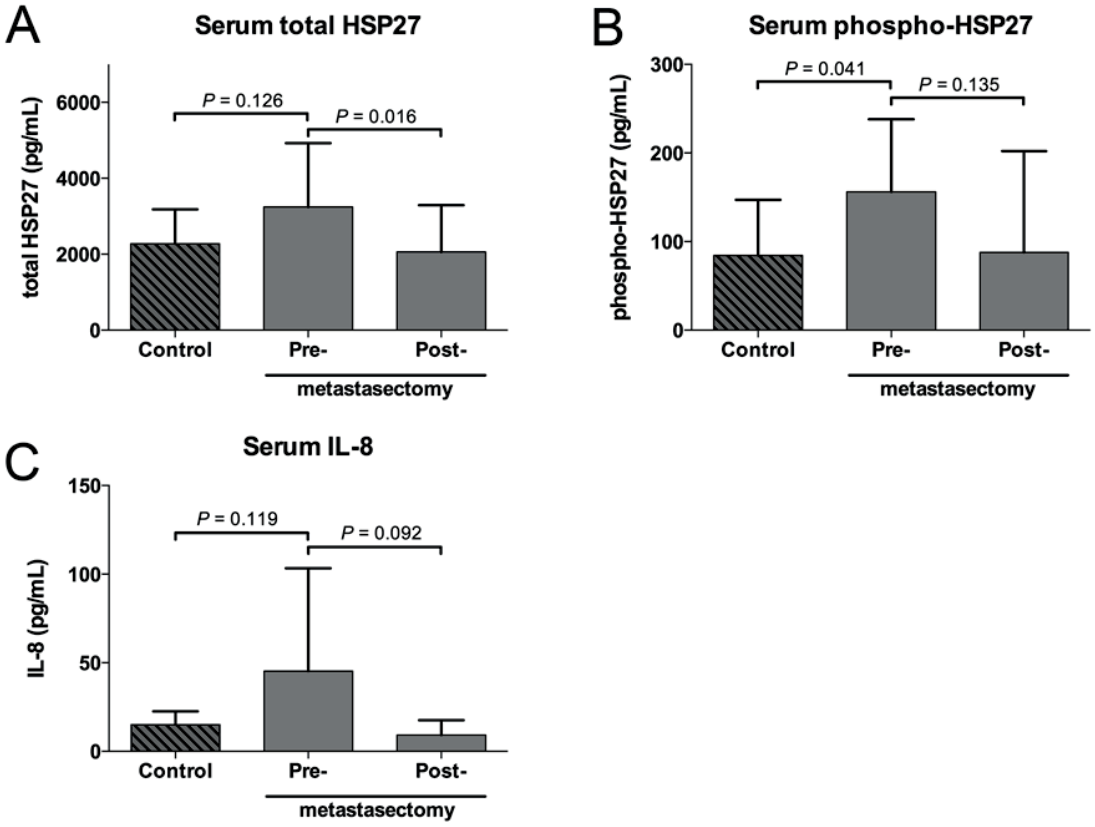

Fig 4. Total Hsp27 (A), phospho-Hsp27 (B) and IL-8 (C) were measured in serum samples of healthy volunteers, patients with CRC lung metastases before and 3 months after metastasectomy (each n = 10; whiskers indicate standard deviation).

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Stromal Hsp27 expression in pulmonary metastases is significantly increased compared to liver metastases

We further evaluated the stromal expression of stromal Hsp27 and α-SMA in liver metastases of 25 patients. Regarding the stromal Hsp27 expression 0 (0%), 12 (48.0%), 11 (44.0%) and 2 (8.0%) cases were scored as 0, 1+, 2+ and 3+, respectively. Stromal α-SMA expression was rated as expression 0 (0%), 8 (32.0%), 11 (44%) and 6 (24.0%) cases were scored as 0, 1+, 2 + and 3+, respectively. We found a strong correlation between stromal Hsp27 and α-SMA ($P < 0.001$). Furthermore, stromal Hsp27 positivity was significantly more often found in pulmonary metastases compared to liver metastases ($P = 0.014$), whereas this difference was not significant for α-SMA ($P = 0.625$). Moreover, expression of Hsp27 in tumor cells (median 120 (range 0–270)) was significantly increased in liver metastases compared to pulmonary metastases ($P = 0.037$). We further analyzed the overall survival of the patients with liver metastases depending on their stromal Hsp27, α-SMA and tumor Hsp27 expression, but significant differences were not found (S3 Fig).

Discussion

Distant metastases are the main cause of cancer-related mortality. Today, the tumor microenvironment and especially fibroblasts become of growing interest, providing additional information on tumor behavior and potential therapeutic targets [32]. Myofibroblasts can be found in

the microenvironment of various tumors and extensive fibroblast-activation could be linked to rapid disease progression in malignant disease [32].

Herein we could demonstrate in a well-defined study cohort that vimentin$^+$αSMA$^+$ stromal cells are highly present in pulmonary metastases of primary CRC. Wikberg *et al.* outlined that myofibroblasts in the tumor center are a better prognosticator for poor prognosis than fibroblasts at the tumor margin [33]. We, therefore, restricted the scoring of the putative fibroblast activation to the central portion of the tumor. Compared to Henry *et al.*, who identified myofibroblasts by FAP-staining in primary colon cancer, we found a similar distribution of the stromal scoring (0%, 20%, 38% and 35% for negative, low, intermediate and strong staining, respectively). Additionally, they described the association of high amounts of activated fibroblasts with a decreased overall survival in patients with colon cancer, especially in the metastasized situation [10]. This goes in line with our findings showing a decreased RFS after pulmonary metastasectomy.

Interestingly, α-SMA positive phenotype of tumor-associated stroma was accompanied by the expression of Hsp27. On the one hand, this small heat-shock protein has been examined in CRC tumor cells themselves [34–36]. An increased expression of Hsp27 in tumor cells of primary CRC was described as negative prognosticator for survival [37, 38]. On the other hand, Hsp27 expressing fibroblasts were examined in the context of wound healing and fibrosis. To the best of our knowledge, stromal Hsp27 has not been described as a potential prognostic marker in CRC. Interestingly, strong stromal Hsp27 expression was associated with high MVD in the primary tumors (P = 0.031) and in PM (P = 0.081). Due to the strong correlation of Hsp27 with α-SMA as an established marker for fibroblast activation, the over-expression of Hsp27 was associated with significantly worse clinical outcome after pulmonary metastasectomy (median RFS 19, 17 and 8 months for 1+, 2+ and 3+, respectively), comparable to the prognostic impact of stromal α-SMA expression. The decrease RFS was also translated in a significantly different OS between the low, intermediate and high stromal Hsp27 or α-SMA, respectively (Fig. 3). Similarly, Tsujino *et al.* described α-SMA positive fibroblasts as being capable to predict disease recurrence in a cohort of patients with stage II and III primary CRC [12]. Kahlert *et al.* demonstrated by microdissection of primary CRC, lung and liver metastases that a panel of pro-angiogenic factors are differentially expressed in tumor cells and the stromal compartment. The stromal expression of angiopoietin-2 in pulmonary metastases was an independent prognosticator for poor survival after surgery in multivariate analysis ($P = 0.044$), even in a rather small study cohort (n = 25) [39]. These findings support the results of our work, in which we could describe Hsp27 as a further proangiogenic, stromal marker with prognostic impact after metastasectomy. It is also important to mention, that Sato *et al.* described high stromal expression of the description factors ETS1 as a predictor of CRC lung metastases [40]. Interestingly, ETS1 is strongly interacting with the small heat-shock protein network [41].

The analysis of blood samples in a subgroup of patients revealed that metastatic CRC is a relevant inducer even of systemic Hsp27 expressions. The level of soluble total Hsp27 and phospho-Hsp27, a polymerized form of Hsp27, significantly decreased after complete removal of the PMs. Moreover, compared to a matched control group of healthy volunteers, preoperative total Hsp27 and phospho-Hsp27 levels were increased and dropped after surgery to comparable low levels. Similar to our findings, Zhao *et al.* could demonstrate increased serum levels of Hsp27 in ovarian cancer with peritoneal metastases. In the same work it was also shown that the levels dropped after chemotherapy [42]. IL-8, which is thought to mediate locally the proangiogenic effect of Hsp27, was also elevated before metastasectomy without reaching significance [22]. Nontheless, the circulating levels of Hsp27 did not correlate with the stromal or tumor expression. Furthermore it did not correlate with the systemic levels of CRP or

fibrinogen. Thus, the circulating Hsp27 might be influenced by other factors like secretion, degradation and elimination. Based on these preliminary results, further studies with adequate cohort sizes will be necessary to further clarify the possible prognostic and predictive role of circulating Hsp27 levels in patients with CRC.

In patients with CRC liver metastases the tumor stroma Hsp27$^+$ vimentin$^+$αSMA$^+$ fibroblasts were less evident than in pulmonary metastases. In contrast to this, the Hsp27 expression in tumor cells was significantly higher in resected liver metastases compared to pulmonary metastases. This goes in line with the observation that the p38 MAPK signaling, a pivotal kinase for Hsp27 phosphorylation and activation, is downregulated in CRC pulmonary metastases compared to liver metastases [43].

The findings of this work are of translational relevance because of two aspects: first, Hsp27, systemically, or confined to the tumor stroma, might possess potential prognostic and predictive value. Pulmonary metastasectomy is a widely offered treatment option in patients with CRC PM. However, the identification of patients who will benefit from surgery alone or an optional adjuvant chemotherapy is a matter of current research [44]. Biomarkers like Hsp27 might help to identify patients with high risk of early recurrence of disease after surgery. Adjuvant chemotherapy and a stringent follow-up could be offered to these patients. During the follow-up of patients with malignant disease, serum markers, e.g. CEA, CA19–9 and beta-HCG, are routinely used nowadays and additional markers might potentiate the accuracy of these established markers. A limitation to this study is, that CEA and CA19–9 levels were not routinely determined before metastasectomy. Correlation of these established tumor markers with serum Hsp27 will be addressed in a future study.

Secondly, Hsp27 is a promising drug target. Apatorsen (OGX-427, Oncogenex, Bothell, Washington, USA), a modified antisense oligonucleotide, binds to the Hsp27 mRNA transcript and therefore inhibits the Hsp27 expression [45]. Currently ongoing Phase II studies are recruiting patients with advanced prostate, bladder, pancreatic and non-small cell lung cancer [46]. The elevated expression levels of Hsp27 in the tumor-associated stroma provide a rationale for the use of Hsp27 also in patients with primary CRC. Given that not only CRC lung metastases, but also liver and lymph node metastases exhibit strong myofibroblast recruitment, especially patients with metastatic disease might benefit from Hsp27 inhibition.

In conclusion, we could demonstrate that Hsp27 is co-expressed with αSMA in the tumor stroma of CRC lung metastases and, moreover, that its over-expression is associated with worse clinical outcome after metastasectomy. Of note, soluble Hsp27 was also systemically measurable, making serum Hsp27 a potential future serum marker in CRC.

Supporting Information

S1 Table. Antibodies and dilutions used for immunohistochemistry and immunofluorescence.
(DOCX)

S2 Table. Descriptive data on patient, primary tumor and metastases characteristics stratified by MVD and vimentin (N = 51).
(DOCX)

S3 Table. Characteristics of matched patients and healthy controls.
(DOCX)

S1 Fig. Hsp27 expression was also found in tumor cells itself (anti-Hsp27; DAB substrate; 200x magnification) (A). Correlation of pre-operative Hsp27 and C-reactive protein (CRP) or

fibrinogen (n = 10) (B and C). Neither total Hsp27 (B), nor phospho-Hsp27 (C) correlated significantly with CRP or fibrinogen.
(TIF)

S2 Fig. In CRC liver metastases stromal Hsp27 and α-SMA scoring correlated significantly (n = 25) (A).
(TIF)

S3 Fig. The distribution of stromal Hsp27 (A) differed significantly in liver metastases (n = 25) compared to lung metasatses (n = 51). This difference did not reach significance for stromal α-SMA (B). Hsp27 expression in tumor cells was significantly higher in liver metastases compared to lung metastases (C). No significant differences were observed between the subgroups regarding overall survival (D-F).
(TIF)

Author Contributions

Conceived and designed the experiments: MM PB BD WK KH HJA. Performed the experiments: TS CN DT MZ BH. Analyzed the data: TS DT KH HJA. Contributed reagents/materials/analysis tools: PS MB MZ PB BH BD MM WK KH HJA. Wrote the paper: TS PS MB MZ PB BH BD MM WK KH HJA.

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CHAPTER THREE: DISCUSSION

3.1 General discussion

In this thesis we could describe multiple clinical and tissue- based prognostic markers in a well-defined cohort of patients with CRC lung metastases. We demonstrated that various alterations on DNA and protein level - which can be present in tumor cells or in the tumor-surrounding stroma – are capable of prognosticating the outcome after pulmonary metastasectomy. The herein described findings might contribute to a better understanding of pulmonary metastasis in CRC.

In the first work included in this thesis, patients with *KRAS* mutant metastases were demonstrated to have a worse clinical outcome after pulmonary metastasectomy. This finding was confirmed in various other study cohorts. Renaud *et al.* described in a retrospective cohort of 180 patients receiving CRC pulmonary metastasectomy a similar high incidence of *KRAS* mutations (51.7%). In multivariate analysis, *KRAS* wild-type tumors had a significantly better overall survival after metastasectomy than *KRAS* mutant tumors (HR 0.04; 95% CI 0.02–0.1, *P*<0.0001). In accordance to our study, time to lung-recurrence was significantly longer in patients with KRAS wild-type tumors (HR 0.16; 95%CI 0.07–0.33, *P*<0.0001). Interestingly, if the recurrent metastases were resected during a second metastasectomy in *KRAS* mutant patients, the overall survival was not inferior to patients with *KRAS* wild-type tumors (Renaud *et al*, 2015). In a study conducted at the MD Anderson Cancer Center (Houston, Texas, USA) including patients with metastatic CRC, patients with *KRAS* mutant tumors had a significantly shorter time to occurrence of lung metastases (22.4 versus 15.2 months; HR 1.40; 95% CI 1.12–1.75; *P*=0.002). The overall survival did not differ between the two groups (Pereira *et al*, 2015). In contrast to this, Ghidini *et al.* found a significantly decreased overall survival in patients with *KRAS* mutant after pulmonary metastasectomy (60.9 versus 36.6 months, HR 2.17, 95% CI 1.19-3.96, *P* = 0.012) (Ghidini *et al*, 2016). The heterogeneity in the management of recurrent disease (chemotherapy plus surgery versus chemotherapy or surgery alone) might contribute to a substantial difference in the outcome analysis in these studies.

Interestingly, also in CRC with isolated liver metastasis and liver metastasectomy, *KRAS* mutant tumors have an increased propensity to form lung metastases subsequently. Shindoh *et al.* showed in a patient cohort with 163 CRC liver metastases that patients with *KRAS* mutant CRC had a nearly 3-fold risk of lung recurrence after liver metastasectomy (HR 2.56; 95% CI 1.54-4.25; *P* < 0.001). The time to surgical failure was defined as the time between between initial liver surgery and diagnosis of metastatic recurrence deemed unresectable.

Time to surgical failure was significantly shorter in *KRAS* mutant tumors compared to the *KRAS* wilt-type tumors (18.8 versus 39.7 months, *P* = 0.001). Even more striking, the occurrence of unresectable (i.e. multiple, diffuse) lung metastases after liver metastasectomy was significantly more common in patients with *KRAS* mutant compared to *KRAS* wild-type CRC (18% versus 41%, *P* = 0.048). This conferred also a marked decrease in the 3-year disease-specific survival of the *KRAS* mutant group (59.8 versus 83.6 %; *P* = 0.016). (Shindoh *et al*, 2016). The authors draw the conclusion that especially lung recurrence after isolated liver metastatasectomy hinders long-term survival in patients with *KRAS* mutant tumors. Thus, these patients might benefit from adjuvant chemotherapy after liver resection and should be closely monitored by chest CT imaging. Stremitzer *et al.* described in a cohort of 60 patients receiving liver metastasectomy that the recurrence-free survival as well as the overall survival was decreased in patients with *KRAS* mutant CRC (Recurrence-free survival: HR 2.48, 95%CI 1.26 – 4.89; *P* = 0.009. Overall survival: HR 3.51, 95%CI 1.30 – 9.45; *P* = 0.013) (Stremitzer *et al*, 2012). Furthermore, Vauthey *et al.* showed in a study cohort from the U.S. that the time to lung-specific recurrence after liver resection was significantly decreased in patients with *RAS* (including *KRAS* and *NRAS*) mutant tumors (HR 2.0, 95%CI 1.1 – 3.4; *P*=0.01). The time to liver recurrence did not significantly differ between the two groups. Again, the increased likelihood to involve the lungs transduced into inferior 3-year survival of *KRAS* mutant CRC patients (52.2% versus 81%, *P*=0.002) (Vauthey *et al*, 2013).
The underlying mechanism for the increased capability of *KRAS* mutant CRC to metastasize to the lungs remains widely unknown. Pollock *et al.* demonstrated *in vitro* using the *KRAS* mutant HCT116 colon carcinoma cell line that cell adhesion is decreased and cell motility is increased in *KRAS* mutant cells. The disruption of the cytoskeleton was conferred by uncoupling the Rho-ROCK pathway from the capability to form fibers. Moreover, the increased cell motility was mediated by an increased PI3-kinase activity (Pollock *et al*, 2005). Schramm *et al.* demonstrated in SW480 colon cancer cells that *KRAS* mutant cells exhibit a strongly different phenotype regarding integrin expression at the cell surface (Schramm *et al*, 2000). This might have implications on the metastatic capability and especially on a potential organ-tropism. Another group demonstrated an increased expression of pro-angiogenic chemokines (CXCL-1 and -8) concomitant to a decreased expression of the anti-angiogeneic chemokine CXCL-10 in KRAS mutant cell lines (Khan *et al*, 2014). Watanabe *et al.* compared the expression of 30 cancer progression-related genes in 35 *KRAS* mutant and 78 *KRAS* wilt-type primary CRC. Differentially activated pathways included the Wnt-, NF-Kappa B and TGF beta- signaling pathway (Watanabe *et al*, 2011). In a recent work, it was demonstrated that the up-regulation of TRAIL-R2 is another possible mechanism of *KRAS* mutant tumors to rapidly proceed (von Karstedt *et al*, 2015). These findings shed light on the distinct biological characteristics of *KRAS* mutant CRC and provide possible explanations for

the different clinical presentation of patients with CRC harboring KRAS mutations. Beyond the routinely tested *KRAS* mutations in codons 12 and 13, there are several rare and atypical but - despite this- also activating mutations in the *KRAS* (codons 61 and 146) and *NRAS* (codons 12, 13 and 61) gene. Interestingly, tumors harboring these atypical mutations show a similar lung specific metastatic pattern like tumors with the traditional mutations in *KRAS* codons 12 and 13. Furthermore, the signatures of expressed genes were comparable between tissue samples of typical KRAS mutant tumors and atypical *KRAS* mutant tumors (Morris *et al*, 2014). Therefore, further studies assessing *KRAS* and other relevant genes using e.g. next generation sequencing might provide an even more precise prognostic information in patients with metastatic CRC.
In conclusion, we could demonstrate that *KRAS* mutation is a valuable prognostic marker after pulmonary metastasectomy. There is rising evidence in the literature that *KRAS* might be one of the most important prognosticators in metastatic CRC so far.

In the second work included in this thesis we assessed the tumor microenvironment of CRC lung metastases and corresponding primary tumors. We could demonstrate that large differences exist between pulmonary metastases regarding accumulation of $Hsp27^{+}$ fibroblasts in close neighborhood to the tumor cells. Moreover, the density of myofibroblasts in pulmonary metastases significantly exceeded the detected myofibroblasts in liver metastases. Interestingly, patients with CRC lung metastases consisting of highly Hsp27 and alpha-SMA positive stromal cells had a worse clinical outcome in terms of recurrence-free survival and overall survival after pulmonary metastasectomy. Moreover, angiogenesis was increased in tumor samples with highly Hsp27 positive stromal cells compared to samples with low stromal Hsp27 expression. This clinical observation was recently confirmed in an experimental metastasis model using C57BL/6 mice. Injecting activated fibroblasts – by TGF-beta pretreatment – resulted in highly angiogenic and proliferating metastases. Treating animals with a TGF-beta inhibitory peptide altered the angiogenic phenotype resulting in decreased CD31+ vessel counts (Gonzalez-Zubeldia *et al*, 2015). Thuringer *et al.* demonstrated a possible mechanism of Hsp27 mediated angiogenesis. Treating endothelial cells with recombinant Hsp27 resulted in autocrine and paracrine VEGFR2 activation and vessel sprouting *in vitro*. In a rat model of xenograft colon tumors, intraperitoneal treatment with OGX-427 – an antisense oligonucleotide to Hsp27 – significantly reduced microvessel density and tumor growth (Thuringer *et al*, 2013). This supports our findings of concomitant Hsp27 expression and increased angiogenesis in the tumor-surrounding stroma. Gene expression assays comparing fibroblasts from CRC tumor samples to normal fibroblasts of the colon mucosa revealed the highly distinct characteristics of cancer-associated fibroblasts. More than 2400 genes were significantly ($P < 0.05$) differentially expressed (Mrazek *et al*,

2014). Also in other solid tumors cancer-associated fibroblasts are thought to play a role in metastasis. Her2+ breast cancers are characterized by their capability of rapid progression and thus diminished prognosis. The expression of HSPB1, the gene coding for Hsp27, was demonstrated to be significantly upregulated in cancer-associated fibroblasts of Her2+ tumors compared to triple negative or estrogen receptor positive tumors (Tchou *et al*, 2012). Park *et al.* demonstrated in a recent work using a lung fibrosis model and human samples of idiopathic pulmonary fibrosis that pulmonary myofibroblasts are highly positive for alpha-SMA and Hsp27, which are co-localized at the cellular level. Delivery of HSP27 siRNA reversed the bleomycin-induced fibrosis in a mouse model (Park *et al*, 2016). This finding in benign lung disease supports our findings in lung metastases regarding the Hsp27-alpha-SMA co-expression in tumor- associated myofibroblasts.
To the best of our knowledge, we described for the first time that Hsp27 is strongly expressed by activated fibroblasts in the tumor-surrounding stroma of primary and metastatic CRC. The presence of these Hsp27^{+} alpha-SMA^{+} fibroblasts provides prognostic information after pulmonary metastasectomy.

Of course there are several limitations to this work. First of all, patients receiving pulmonary metastasectomy are a highly selected subgroup of patients with metastatic CRC. The findings in this selected cohort of patients can not necessarily be extrapolated to the whole cohort of patients with metastatic CRC. Second, the included patients are a heterogeneous group regarding the previously received treatment. As the patients were referred for metastasectomy from different institutions, various treatment plans were applied for the primary tumors, previous liver metastases etc.. This might have implications on the phenotype of the pulmonary metastases and might affect the outcome. Third, confirmation of our findings in an independent validation cohort is needed. However, despite these limitations, we could investigate and describe a variety of tissue-based prognostic markers in one of the most detailed and stringently followed-up cohorts of patients with CRC lung metastases.

3.9 Conclusion & future prospects

This thesis adds novel and promising prognostic markers in patients with pulmonary metastases of CRC. These markers might help to describe and stratify patients with CRC lung metastases according to the biological characteristics of the tumor rather than to the current clinical presentation of the patient. This might impact the treatment of these patients

as well as the interpretation of studies assessing the outcome of patients with CRC lung metastases.

CHAPTER FOUR: MATERIALS & METHODS

4.1 Materials

Study population

From April 2009 to October 2012 (study I)/from April 2009 to November 2013 (study II), all patients receiving R0 (histologically complete) pulmonary metastasectomy from primary CRC at the Department of Thoracic Surgery, Medical University of Vienna and appropriate tissue samples were included in this study. Of 33 patients corresponding primary CRC samples were available. Samples from a study cohort of 25 patients receiving liver metastasectomy were used as control group in study II. The diagnostic work-up before surgery included thoracic and abdominal computed tomography (CT). Time from surgery of the primary tumor to pulmonary metastasis was termed lung metastasis free survival (LMFS). Time to recurrence was defined as the period from the first pulmonary metastasectomy to evidence of recurrent disease irrespective of the organ site of recurrence. Time to lung specific recurrence was defined as the period of time between the first pulmonary metastasectomy and pulmonary recurrence verified by CT. Follow-up examinations were carried out in 3 to 6 months intervals. In patients receiving pulmonary metastasectomy before the inclusion period, tissue samples of the first lung metastasis were also assessed and the data of the first metastasectomy was used for outcome calculation.

In 10 patients receiving pulmonary metastasectomy, serum samples before and 3-6 months after metastasectomy were available. Moreover, serum samples from age-, gender- and smoking status- matched healthy volunteers served as control. Patients receiving blood draws gave written informed consent prior to participation. The studies were approved by the ethics committee of the Medical University of Vienna (EK 91/2006, EK1194/2011 and EK1044/2012) and was conducted according to the declaration of Helsinki.

4.2 Methods

KRAS and BRAF mutation analysis

KRAS codons 12 and 13, and *BRAF* codon 600 mutations were detected using restriction fragment length analysis as descibed previously.(Szabo *et al*, 2011) Briefly, macrodissection of FFPE tumor samples was performed. DNA was isolated using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). AmpliTaqGold PCR Master Mix (Applied Biosystems, Foster

City, CA) was used for polymerase chain reaction using specific primers to introduce restriction sites in the products generated from the wild-type allele. The primer sequences were as follows: for KRAS codon 12 forward 5′-GAATATAAACTTGTGGTAGTTGGACCT-3′ and reverse 5′-GGTCCTGCACCAGTAATATG-3′, for KRAS codon 13 forward 5'-GAATATAAACTTGTGGTAGTTGGACCT-3' and reverse 5'-GGTCCTGCACCAGTAATATG-3'. BstnI and BglI were used for digestion, respectively. BRAF codon 600 mutation was analysed in a two-step PCR and restriction digestion. The first primers sequences were as follows: forward (Mt-F) 5'-TAAAAATAGGTGATTTTGGTCTAGCTGC-3' and reverse (Wt-R) 5'-CCAAAAATTTAATCAGTGGAAAAATA-3'. The products obtained were then used in a second-stage PCR, which was performed under the same conditions as the first-stage PCR but with Mt-F and Mt-R primer (5'-AAAAATTTAAGCAGTGGAAAAATAGC-3'). BtsI (New England Biolabs, Beverly MA) was used for digestion. The digested products were visualized on 3% agarose gels stained with ethidium bromide.

Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunoflouroscence conducted following standard protocols. 4-µm thick, formalin-fixed, paraffin-embedded tissue samples were deparaffinized. Heat-mediated antigen retrieval was performed if necessary. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide. Afterwards an appropriate primary antibody was added for 1h at room temperature. For manual immunohistochemistry the polymer- based ImmPRESS kit (Vector Laboratories, Burlingame, California) was used as secondary step. For automated stainings Benchmark Ultra Immunostainer (Ventana, Tucson, AZ, USA) was used with appropriate secondary antibodies according to the manufacturer's protocol. 3,3'-Diaminobenzidine (DAB) was used as substrate (Vector Laboratories, Burlingame, California). Finally, the sections were counterstained with hematoxylin. Vimentin staining was performed in a Ventana ES Immunostainer System (Ventana Medical Systems Inc., Tucson, AZ, USA). Fluorescent secondary antibodies were used for immunofluorescence. DAPI (Sigma Aldrich, St. Louis, MO, USA) was used as cell nuclei dye. A detailed list of antibodies and dilutions is provided in Table 1.

Scoring of Hsp27, vimentin and alpha-SMA in stromal cells

The samples were rated in a semiquantitatively manner as described previously (Henry *et al*, 2007). The tumor center was screened at low magnification regarding the staining intensity in the stromal cells. The stromal staining was scored from grade 0 (negative, <1% positive stromal cells), grade 1+ (low,1-10% positive stromal cells), grade 2+ (intermediate, >10-50% positive stromal cells) to grade 3+ (strong, >50% positive stromal cells) by two blinded observers.

Scoring of target protein expression in tumor cells

The immunohistochemistry staining score (IHC score) was used to quantify protein expression in tumor cells according to the literature (Driessen *et al*, 2006). Staining intensity (0/1+/2+/3+) was multiplied by the percentage of positive tumor cells, resulting in IHC scores ranging from 0 to 300. The median was used as cutoff to dichotomize the IHC score. Staining intensity for EGFR was scored as described by Scartozzi *et al.* (Scartozzi *et al*, 2004). Samples showing membranous EGFR staining in $\geq$1% of the tumor cells were scored as positive. Stainings were assessed by two blinded observers and re-discussed if the ratings differed.

$CD3^+$ microvessel density

Microvessel density (MVD) was determined using the "hotspot" method (Weidner *et al*, 1992). At low magnification the area with the greatest number of CD31-positive microvessels was identified ("hotspot"). MVD in this hotspot was determined by counting all CD31+ vessels at 200x magnification (corresponding to 0.95 mm^2). Mean values from two independently counted densities were calculated.

Enzyme-linked immunosorbent assay (ELISA)

Commercially available ELISA kits (R&D Systems, Minneapolis, USA) were used to assess serum levels of human Hsp27, phospho-Hsp27 and interleukin-8 following the manufacturer's protocol. The samples were assayed in duplicates. The absorbance was determined at a wavelength of 450nm (PerkinElmer, Waltham, USA) and converted to pg/mL.

Antibody	Manufacturer	Application	Dilution
Rabbit anti-EGFR antibody (790-4347)	Ventana, Tuscon, AZ, USA	IHC	prediluted
Rabbit polyclonal alpha-SMA (ab5694)	Abcam; Cambridge, UK	IF/IHC	1:300/1:600
Alexa Fluor® 488 Goat Anti-Rabbit IgG (A-11008)	Molecular probes, Carlsbad, CA, USA	IF	1:500
ImmPRESS Reagent Kit Anti-	Vector Laboratories,	IHC	prediluted

Rabbit IgG (MP-7401)	Burlingame, CA, USA		
Mouse monoclonal CD31 (JC70A)	Dako, Carpenteria, CA, USA	IHC	1:20
ImmPRESS Reagent Kit Anti-Mouse IgG (MP-7402)	Vector Laboratories, Burlingame, CA, USA	IHC	prediluted
Mouse monoclonal Hsp27 (sc-13132)	Santa Cruz Biotechnology, Santa Cruz, CA, USA	IF/IHC	1:400
Alexa Fluor® 546 Goat Anti-Mouse IgG (H+L) (A-11003)	Molecular probes, Carlsbad, CA, USA	IF	1:500
ImmPRESS Reagent Kit Anti-Mouse IgG (MP-7402)	Vector Laboratories, Burlingame, CA, USA	IHC	prediluted
Mouse monoclonal Vimentin (#M0725)	Dako, Carpenteria, CA, USA	IHC	1:300
UltraVision LP detection system	Lab Vision Corporation, Fremont, CA, USA	IHC	prediluted

Table 1: Specification of antibodies and dilutions (IF= immunofluorescence; IHC= immunohistochemistry)

Statistical analysis

Data was statistically processed using GraphPad Prism 6 (GraphPad Software Inc., California, USA) and SPSS 19 (SPSS Inc., Chicago, USA). Fisher's exact test and chi-square test were used to compare binominal variables. Mann-Whitney U-test was used to compare medians between two groups. Student's t-test was used to compare means of two independent groups, paired t-test for dependent groups and expressed as mean±standard deviation (SD). Kaplan-Meier method, log-rank test and Cox regression were used to compare survival functions. All tests were two-sided. P-values < 0.05 were considered statistically significant.

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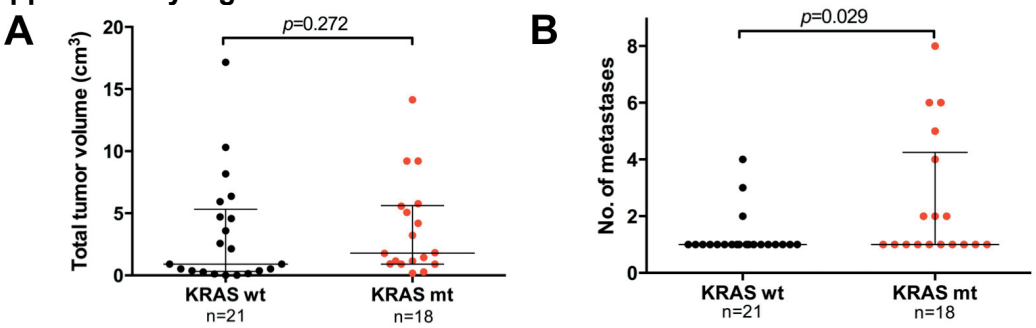
APPENDIX

Supplementary material "EGFR, *BRAF* and *KRAS* status in patients undergoing pulmonary metastasectomy from primary colorectal carcinoma: a prospective follow-up study"

Supplementary Figure 1



C

Patient No.	EGFR	KRAS mutation Codon12	KRAS mutation Codon13	BRAF mutation V600E
1	negative	positive	negative	-
2	++	positive	negative	-
3	negative	positive	negative	-
4	negative	positive	negative	-
5	negative	negative	negative	negative
6	+	negative	negative	negative
7	negative	positive	negative	-
8	negative	negative	negative	negative
9	+	negative	negative	negative
10	++	negative	positive	-
11	++	negative	negative	negative
12	+++	negative	negative	negative
13	+	negative	negative	negative
14	+++	negative	negative	negative
15	negative	negative	negative	negative
16	+	positive	negative	-
17	negative	negative	negative	negative
18	++	negative	negative	negative
19	negative	negative	negative	negative
20	negative	positive	negative	-
21	++	positive	negative	-
22	negative	negative	negative	negative
23	negative	positive	negative	-
24	negative	positive	negative	-
25	negative	negative	negative	negative
26	+	positive	negative	-
27	negative	positive	negative	-
28	negative	negative	negative	negative
29	++	positive	negative	-
30	negative	negative	negative	negative
31	+	negative	negative	negative
32	++	negative	negative	negative
33	negative	positive	negative	-
34	+	negative	negative	negative
35	++	negative	negative	negative
36	++	positive	negative	-
37	+	negative	negative	negative
38	negative	negative	positive	-
39	negative	positive	negative	-

Supplementary material “Stromal expression of heat-shock protein 27 is associated with worse clinical outcome in patients with colorectal cancer lung metastases.”

Supplementary Table 1

Antibody	Manufacturer	Application	Dilution	Antigen retrieval
Rabbit polyclonal alpha-SMA (ab5694)	Abcam; Cambridge, UK	IF/IHC	1:300/1:600	heat-mediated, buffer EDTA pH=9
Alexa Fluor® 488 Goat Anti-Rabbit IgG (A-11008)	Molecular probes, Carlsbad, CA, USA	IF	1:500	-
ImmPRESS Reagent Kit Anti-Rabbit IgG (MP-7401)	Vector Laboratories, Burlingame, CA, USA	IHC	prediluted	-
Mouse monoclonal CD31 (JC70A)	Dako, Carpenteria, CA, USA	IHC	1:20	heat-mediated, buffer EDTA pH=9
ImmPRESS Reagent Kit Anti-Mouse IgG (MP-7402)	Vector Laboratories, Burlingame, CA, USA	IHC	prediluted	-
Mouse monoclonal Hsp27 (sc-131132)	Santa Cruz Biotechnology, Santa Cruz, CA, USA	IF/IHC	1:400	heat-mediated, buffer EDTA pH=8
Alexa Fluor® 546 Goat Anti-Mouse IgG (H+L) (A-11003)	Molecular probes, Carlsbad, CA, USA	IF	1:500	-
ImmPRESS Reagent Kit Anti-Mouse IgG (MP-7402)	Vector Laboratories, Burlingame, CA, USA	IHC	prediluted	-
Mouse monoclonal Vimentin (#M0725) UltraVision LP detection system	Dako, Carpenteria, CA, USA Lab Vision Corporation, Fremont, CA, USA	IHC IHC	1:300 prediluted	heat-mediated, Target Retrieval Solution (Dako, Carpenteria, CA, USA)

Supplementary Table 2

	healthy controls n= 10	CRC patients n= 10	p-value
Sex **male/female**	7/3	7/3	*p*= 1.000[a]
Age **range (median)**	45-88 (52.5)	44-76 (62.5)	*p*= 0.430[b]
Smoking history **never smoker/ever smoker**	4/6	3/7	*p*= 1.000[a]
Months after primary tumor **resection (range)**	-	29 (7-91)	-

[a]Chi square test; [b]Mann-Whitney test

Supplementary Table 3

				MVD					Vimentin						
		Total		low		high		X^2	low		intermediate		high		X^2
		N=51	%	N=25	%	N=26	%	p-value	N=4	%	N=21	%	N=26	%	p-value
Patients															
Sex	**Male**	29	56.9	16	31.4	13	25.5	0.313	2	3.9	13	25.5	14	27.5	0.913
	Female	22	43.1	9	17.6	13	25.5		2	3.9	8	15.7	12	23.5	
Age (years)	**Median**	63		67		58.5		0.048	63.5		67		57.5		0.180[a]
	Range	33-83		33-83		45-77			50-68		44-83		33-78		
Primary tumor															
Location	**Colon**	27	52.9	14	27.5	13	25.5	0.668	1	2.0	12	23.5	14	27.5	0.585
	Rectum	24	47.1	11	21.5	13	25.5		3	5.9	9	17.6	12	23.5	
T stage	**pT1**	1	2.1	0	0.0	1	2.1	0.848	0	0.0	1	2.1	0	0.0	0.186
	pT2	7	14.6	3	6.2	4	8.3		0	0.0	3	6.2	4	8.3	
	pT3	34	70.8	19	39.6	15	31.2		4	8.3	10	20.8	20	41.7	
	pT4	6	12.5	3	6.2	3	6.2		0	0.0	5	10.4	1	2.1	
	N/A	3	-												
N stage	**pN0**	21	43.8	12	25.0	9	18.8	0.709	1	2.1	9	18.8	11	22.9	0.456
	pN1	11	22.9	6	12.5	5	10.4		0	0.0	5	10.4	6	12.5	
	pN2	16	33.3	7	14.6	9	18.8		3	8.3	5	10.4	8	16.7	
	N/A	3	-												
Grading	**G1**	2	3.9	2	3.0	0	0.0	0.319	1	2.0	0	0.0	1	2.0	0.218
	G2	42	82.4	19	37.3	23	45.1		2	2.9	18	35.3	22	43.1	
	G3	7	13.7	4	7.8	3	5.9		1	2.0	3	5.9	3	5.9	
Pulmonary metastasis															
Previous liver metastasis	**Yes**	16	31.4	8	15.7	8	15.7	0.925	0	0.0	7	13.7	9	17.6	0.442
	No	35	68.6	17	33.3	18	35.3		4	7.8	14	27.5	17	33.3	
No. of nodules	**1**	33	64.7	16	31.4	17	33.3	0.918	2	3.9	11	21.6	20	39.2	0.195
	>1	18	35.3	9	17.6	9	17.6		2	3.9	10	19.6	6	11.8	
Stromal HSP27	**low**	17	33.3	10	19.6	7	13.7	0.577	3	5.9	12	23.5	2	3.9	<0.001
	intermediate	17	33.3	8	15.7	9	17.6		1	2.0	8	15.7	8	15.7	
	high	17	33.3	7	13.7	10	19.6		0	0.0	1	2.0	16	31.4	
Stromal alpha-SMA	**low**	14	27.5	9	17.6	5	9.8	0.315	4	7.8	10	19.6	0	0.0	<0.001
	intermediate	23	45.1	11	21.6	12	23.5		0	0.0	11	21.6	12	23.5	
	high	14	27.5	5	9.8	9	17.6		0	0.0	0	0.0	14	27.5	

[a]Kruskal-Wallis test

CV

Curriculum vitae

Thomas Sebastian Schweiger

Personal background

Date of birth:	May 25th, 1987
Nationality:	German

Education

2015/05/–/present	Residency in Thoracic Surgery, Division of Thoracic Surgery (Head: Prof. Walter Klepetko), Medical University of Vienna, Austria
2014/09/–/2015/05/	Transplant coordination, Division of Thoracic Surgery (Head: Prof. Walter Klepetko), Medical University of Vienna, Austria
2012/10/–/present	MDPhD excellence program (Vascular biology),/Medical University of Vienna, Austria
2010/04 –/present	Research Fellow at the Christian Doppler Laboratory for Diagnosis and Regeneration of Cardiac and Thoracic Diseases, Medical University of Vienna, Austria
2008/10 –/2014/07	Human medicine (Degree: Dr.med.univ./MD),/Medical University of Vienna, Austria
2006/07/-/2007/04	Military Service
2006/06	High School Graduation
1997/-/2006	Katharinen-Gymnasium Ingolstadt, Germany
1993/-/1997	Primary School, Manching, Germany

Clinical Training

2014/04/-/2014/05	Clinical Clerkship at the/Division of Thoracic Surgery, Medical University Vienna, Austria

2012/08/-/2012/09	Clinical Clerkship at the Department of Internal Medicine, Evangelisches Krankenhaus, Vienna, Austria
2012/07 -/2012/08	Clinical Clerkship at the Department of Pathology, Medical University Vienna, Austria
2011/06/-/2011/07	Clinical Clerkship at the Department of Emergency Medicine, Klinikum Ingolstadt, Germany
2010/07/-/2010/08	Clinical Clerkship at the Department of General, Visceral, Vascular/and Thoracic Surgery, Charité Berlin, Germany

Scholarships and Honors

2014/12	Stipend of the Medical University of Vienna for outstanding study performance
2014/10	Poster Prize of the German Society for Thoracic Surgery
2014/06	ACO-ASSO Prize 2014
2014/05	Research Scholarship of the Medical University of Vienna/
2014/04	Stipend of the “Hans und Blanca Moser-/Stiftung”
2014/01	Research Scholarship of the Medical University of Vienna/
2013/12	Stipend of the Medical University of Vienna for outstanding study performance
2013/05	Research Scholarship of the Medical University of Vienna/
2012/12	Stipend of the Medical University of Vienna for outstanding study performance
2012/04	Research Scholarship of the Medical University of Vienna/
2011/10	Research Scholarship of the Medical University of Vienna/

Workshops

2014/03	Nahtkurs Gefäßchirurgie (OA Dr. C Senekowitsch), Wilhelminenspital, Vienna, Austria
2011/08	Theodor-Billroth-Academy/Surgical/Summer School 2011/(Prof. Dr. B Brücher, FACS), University of Tuebingen, Germany

Congresses and Meetings

2015/10	Annual Meeting oft he Austrian Society for Pulmonology, Graz, Austria
2015/09	7th ÖGMBT Anuual Meeting, Salzburg, Austria
2015/05	23rd European Conference on General Thoracic Surgery, Lisbon, Portugal
2014/10	ACO/ASSO Annual meeting 2014, St. Wolfgang, Austria
2014/06/	Annual Congress of the Austrian Society of Surgery, Vienna, Austria
2014/03	Management of Laryngotracheal Problems, Vienna, Austria
2014/01	EACTS Academic Thoracic Surgery Club, Bern, Switzerland

2013/10	Meeting of the Austrian, German and Swiss Society of Thoracic Surgery, Basel, Switzerland
2013/10	ACO/ASSO Annual meeting 2013, St. Wolfgang, Austria
2013/05	Annual Congress of the German/Society of Surgery, Munich, Germany
2012/11	36th/Seminar of the Austrian Society for Surgical Research, Vienna, Austria
2012/06/	Annual Congress of the Austrian Society of Surgery, Vienna, Austria
2012/04	Annual Congress of the German Society of Surgery, Berlin, Germany
2011/10	ACO/ASSO Annual meeting/2011, St. Wolfgang, Austria
2011/06	Annual Congress of the Austrian Society of Surgery, Vienna, Austria
2011/04	SkillsLab Symposium, Würzburg, Germany
2010/12	EACTS Meeting on Cardiac and Pulmonary Regeneration, Vienna, Austria
2010/10	Meeting of the Austrian, German and Swiss Society of Thoracic Surgery, Vienna, Austria
2010/04	SkillsLab Symposium, Münster, Germany

Original articles (peer-reviewed)

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Clinical impact of c-MET expression and mutational status in patients with colorectal cancer lung metastases.
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Schweiger T, Nikolowsky C, Graeter T, Seebacher G, Laufer J, Glueck O, Glogner C, Birner P, Lang G, Klepetko W, Ankersmit HJ, Hoetzenecker K.
Increased lymphangiogenesis in lung metastases from colorectal cancer is associated with early lymph node recurrence and decreased overall survival.
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Hypoxic phenotype in pulmonary metastases of different primary tumors *(Oral presentation)*
53. Österreichischer Chirurgenkongress 2012, Salzburg, Austria

K Hoetzenecker, T Schweiger, L Lehmann, F Gittler, HJ Ankersmit, G Lang, W Klepetko
Chirurgische Therapie pulmonaler Metastasen beim Mammakarzinom *(Poster presentation)*
Senologie-ACO 2011, St. Wolfgang, Austria

P Angleitner*, T Schweiger*, Michael Schmidts und Martin Schindl
Chirurgia Practica – Peer teaching chirurgischer Fertigkeiten *(Poster presentation)*
Skills Lab Symposium 2011, Würzburg, Germany

Review activity

2015-present	Transplant International

Clinical Trials

Schweiger T/(Study author), Klepetko W, Hoetzenecker K, Lang G –/EUDRACT: 2013-001725-10
Bronchsoscopic indocyanine green fluorescence imaging for the evaluation of tracheal perfusion after surgery – A monocenter, prospective open-label feasibility study

Teaching activity

2016/03	JC Current Topics in Applied Immunology/(summer term 2016; 861.111), Medical University of Vienna
2016/03	TS Applied Immunology and Tissue Regeneration/(summer term 2016; 861.119), Medical University of Vienna
2013/10	Student teaching assistant „Themenspezifische Untersuchungtechniken I/Chirurgische Grundfertigkeiten“ (winter term 2013/14; 805.008), Medical University of Vienna
2012/10	Student teaching assistant „Themenspezifische Untersuchungtechniken I/Chirurgische Grundfertigkeiten“ (winter term 2012/13; 805.008), Medical University of Vienna
2012/03	Hands-on Workshop „Introduction to surgical suturing techniques“, MedSuccess 2012, Medical University of Vienna
2011/10	Student teaching assistant „Themenspezifische Untersuchungtechniken I/Chirurgische Grundfertigkeiten“ (winter term 2011/12; 805.008), Medical University of Vienna
2011/10	Student teaching assistant „Themenspezifische Untersuchungtechniken I“ (/ winter term 2011/12; 805.008), Medical University of Vienna
2011/03	Hands-on Workshop/„Introduction to surgical suturing techniques“, MedSuccess 2011, Medical University of Vienna
2010/10/-/ongoing	Co-Founder of „Chirurgia Practica“ (peer-teaching of surgical techniques) together with Dr. Philipp Angleitner (Supervisor: Ao. Univ.-Prof. Dr. Martin Schindl), Medical University of Vienna

Memberships

2015/-/present	Membership at Austrian Society of Cardio-Thoracic Surgery (ÖGHTC)
2015/-/present	Membership at European Society of Thoracic Surgeons (ESTS)
2014/-/present	Membership at Austrian Society of Surgical Oncology (ACO-ASSO)
2011/-/present	Membership at Berufsverband Österreichischer Chirurgen (BÖC)