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Tumor hypoxia and expression of c-met in cervical cancer

Cornelia Leo [a,*,1], Lars-Christian Horn [b,1], Jens Einenkel [a], Bettina Hentschel [c], Michael Höckel [a]

[a] Department of Gynecology, Leipzig University, Philipp-Rosenthal-Strasse 55, 04103 Leipzig, Germany
[b] Division of Gynecologic Pathology, Department of Pathology, Leipzig University, Liebigstrasse 26, 04103 Leipzig, Germany
[c] Institute of Medical Informatics, Statistics and Epidemiology, Leipzig University, Härtelstrasse 12-14, 04103 Leipzig, Germany

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Abstract

Objectives. Hypoxia enhances malignant progression by promoting the development of metastases and increasing invasiveness. One key regulator that controls growth, invasion and metastasis in cancer cells is the growth factor receptor c-met. The aim of this study, therefore, was to investigate the expression of the c-met protooncogene in cervical cancers in relation to intratumoral hypoxia levels and to clinico-pathological parameters.

Methods. 43 Patients with cervical cancer were subjected to intratumoral pO_2 measurement with the Eppendorf electrode and biopsies were taken. The tissue was subsequently analyzed by immunohistochemistry with an anti-c-met antibody.

Results. c-met was expressed in 72% of cervical cancers. There was a significantly stronger expression in poorly differentiated tumors ($r=0.4$, $p=0.008$). Furthermore, c-met expression was significantly associated with a spray-like pattern of invasion ($p=0.008$). However, there was no significant relationship between c-met expression and intratumoral hypoxia, pT stage, FIGO stage, lymphovascular space involvement, tumor size or overall survival.

Conclusions. Although c-met has been shown to be hypoxia-induced in vitro, our results suggest that it is not the mediator of deleterious effects of hypoxia on clinical outcome in cervical cancer.

Keywords: Tumor hypoxia; Tumor microenvironment; Gene regulation; Cervix; Uterus; Metastasis; Cancer

Introduction

Tumor hypoxia is a feature of many solid tumors including cervical cancer [1], head and neck cancer [2] and soft tissue sarcoma [3]. Clinical studies performed by our group showed that hypoxia is an independent prognostic indicator of poor outcome in patients with cervical cancer. Patients with hypoxic cervical cancers had a significantly worse prognosis compared to patients with better oxygenated tumors regardless of treatment modality [1]. Mechanisms by which hypoxia enhances malignant progression, thereby promoting the development of metastases and increasing invasiveness, comprise changes in gene expression [4,5] and clonal selection of cells that have lost their apoptotic potential [6,7].

One key regulator that controls growth, invasion and metastasis in cancer cells is the protein product of the proto-oncogene c-met. c-met encodes for a transmembrane tyrosine kinase acting as a growth factor receptor [8–10]. The ligand for this receptor is the hepatocyte growth factor (HGF, scatter factor-1) [11]. Inappropriate activation of the HGF pathway has been described as one major pathway in the process of invasive growth and the development of metastatic disease in malignant tumors [12–14]. Moreover, the c-met receptor has been shown to be overexpressed in a variety of human malignancies, including breast cancer, nasopharyngeal cancer, colon cancer and cervical cancer [15–18]. Using cell lines from different tumors including the cervical cancer cell line SiHa, Pennacchietti et al. [19] demonstrated that hypoxia activates the transcription of the met protooncogene, resulting

* Corresponding author. Fax: +49 341 97 23409.
E-mail address: leo@medizin.uni-leipzig.de (C. Leo).
[1] Both authors contributed equally.

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in higher levels of the met protein. Furthermore, they showed that the met protein was highly overexpressed in hypoxic areas of the according experimental tumors. To the best of our knowledge, there are no studies to date addressing the relation between the expression of c-met in cervical cancer and intratumoral hypoxia levels. Therefore, we examined the expression of c-met in clinical samples of cervical cancer and investigated its relation to the hypoxia levels of the tumors determined by invasive measurements using the Eppendorf electrode. Furthermore, we analyzed the association between c-met expression and clinico-pathological parameters.

Material and methods

Patients, pO_2 measurement and tissue specimens

Archival tissue of cervical cancer samples with known intratumoral hypoxia levels was used for the analysis. The material was derived from a series of 43 consecutive patients for whom sufficient tumor material for analysis was available. These patients presented to the Department of Gynecology and Obstetrics at Leipzig University between 2001 and 2003 (FIGO stages IB to IV, Table 1) and prior to therapy underwent intratumoral oxygenation measurements with the Eppendorf histography system (Eppendorf, Hamburg, Germany) according to the standard procedure described earlier [20]. The patients were treated by Total Mesometrial Resection (TMMR [21]), Laterally Extended Endopelvic Resection (LEER [22]) or radiation therapy (Table 1). The procedure was performed after informed written consent was given by each patient. The study was approved by the medical ethics committee of Leipzig University. pO_2 measurement was performed in the conscious patient along at least two distinct tracks within the macroscopically vital tumor. Per track, approximately 30 data points were collected, starting at a tissue depth of 5 mm. To confirm that the measurement was performed within the tumor and not in necrotic or tumor-free areas, a needle core biopsy of approximately 2 mm in diameter and 20 mm in length was taken from the tissue of each measured track after the procedure. The biopsies were formalin-fixed and paraffin-embedded according to standard protocols, followed by an evaluation by a gynecologic pathologist. A correlation analysis between the median pO_2 of each track and the c-met protein expression in the corresponding biopsy was carried out (see below).

Immunohistochemistry

Immunohistochemical staining was performed according to standard procedures. 5-μm sections were stained for c-met with a rabbit polyclonal antibody (clone: c-12, Santa Cruz Biotechnology, Santa Cruz, USA, Cat-no: sc 10, dilution: 1:200). Briefly, slides were incubated overnight with the anti-c-met-antibody at 4 °C. This was followed by incubation with a biotinylated anti-rabbit secondary antibody (Dako CSA Rabbit Link) and the CSA system from DAKO (DAKO Cytomation, Glostrup, Denmark). Staining was visualized by using DAB chromogen (DAKO). Sections known to stain positively as well as placental tissue were included in each batch as positive controls and negative controls were performed by omitting the primary antibody.

Evaluation of immunostaining

For the assessment of the c-met staining results, the slides were evaluated semiquantitatively using a predefined scoring system based on the product of staining intensity and percentage of positive tumor cells [23]. Membranous and/or cytoplasmic staining was counted as positive as described by Cruz et al. [24]. The staining of the slides was evaluated based on the microanatomic distribution of staining results using established criteria [25,26]. Briefly, staining intensity was evaluated as negative (0=no staining of cytoplasm or cell membrane), weak (1=fine fibrillar staining of cytoplasm and/or punctuate and incomplete membrane staining), moderate (2=granular/dotted staining of cytoplasm and/or weak to moderate staining of entire membrane), or strong (3=diffuse dark staining of cytoplasm and/or strong staining of entire membrane). The percentage of positive tumor cells was categorized as follows: 0=0%; 1=1–10%; 2=11–50%; 3=51–80%; 4>80%. By multiplying both components, an expression score (0–12) was obtained. This score was used in correlation analyses. Tissue specimens with a score of 0–2 were considered negative for c-met expression, a score of 4–6 represented moderate staining and a score of 9–12 was counted as strong staining.

Table 1
Patient and tumor characteristics at the time of pretherapeutic *p*O2 measurements

	No. of patients
FIGO stage	
I	11
II	17
III	11
IV	4
Grade	
1	9
2	26
3	8
pT stage	
pT1b1	11
pT1b2	3
pT2b	5
pT4	1
NA	23
pN stage	
N0	16
N1	4
NA	23
LVSI	
L0	19
L1	24
Tumor diameter (mm)	
(*n*=40, ND 3)	
Median	45
Range	17–100
Patient age (years)	
Median (range)	46 (24–79)
Treatment modality	
TMMR with pelvic±paraaortic lymph node dissection	19
LEER	1
Radiation therapy	23
Histology	
Squamous cell carcinoma	40
Adenocarcinoma	3

Abbreviations: FIGO, International Federation of Gynecologists and Obstetricians; pT stage, pathologic tumor stage; pN stage, pathologic node stage; ND, not documented; LVSI, lymphovascular space involvement; TMMR, Total Mesometrial Resection; LEER, Laterally Extended Endopelvic Resection.

Statistical analysis

Correlations between two parameters were described by Spearman's rank correlation coefficient. The Mann–Whitney *U* test and Kruskal–Wallis *H* test were used for comparison of groups. Overall survival (OS), with deaths due to any cause as event, was calculated using the Kaplan–Meier method, and differences between groups were analyzed by log rank test.

A p value <0.05 was considered to indicate statistical significance. Statistical analysis was performed using the statistics package SPSS (version 11.5 for Windows; SPSS GmbH, Munich, Germany).

Results

c-met expression in cervical cancer

c-met protein expression was assessed by immunohistochemistry in 43 samples. Negative staining was found in 28%, moderate staining in 40% and strong c-met staining in 32% of the investigated cervical cancers (Fig. 1). Tumor cells with membranous and/or cytoplasmic staining were considered positive.

c-met expression and intratumoral hypoxia

The intratumoral oxygenation of the investigated 43 cervical cancers had been determined using the Eppendorf electrode. The median pO_2 was 6.8 mm Hg (mean: 10.2 mm Hg, range: 0.8 mm Hg–33.4 mm Hg). There was no correlation between c-met staining of the core biopsies and the median pO_2 levels of the corresponding measured tracks (Spearman rank correlation, $r=0.19$, $p=0.22$, Fig. 2).

Tumor oxygenation, clinico-pathological parameters and survival

There was no significant relationship between tumor hypoxia and FIGO stage, tumor size, lymphovascular space involvement, lymph node status, histological grade or histological type, respectively (data not shown).

The median follow-up period was 42 months (95% CI: 39–45). For two patients, no follow-up data were available for survival analysis. There were no significant differences in overall survival comparing patients having well-oxygenated cervical cancers ($pO_2 > 10$ mm Hg) with patients having hypoxic tumors ($pO_2 \leq 10$ mm Hg) (3-year survival rate: 81.3% vs. 68.0%, $p=0.59$).

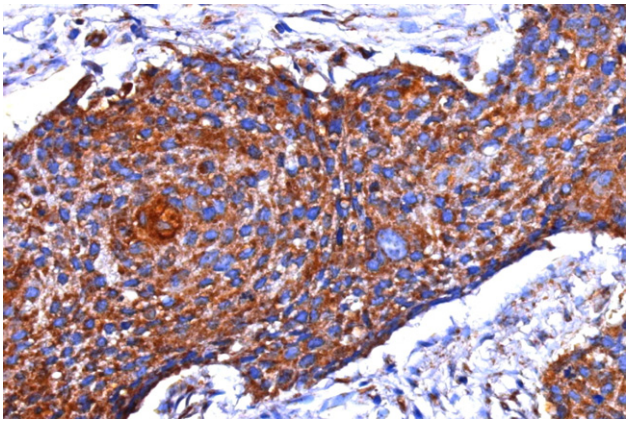

Fig. 1. Strong staining for c-met of tumor cells in a cervical cancer specimen (200× magnification).

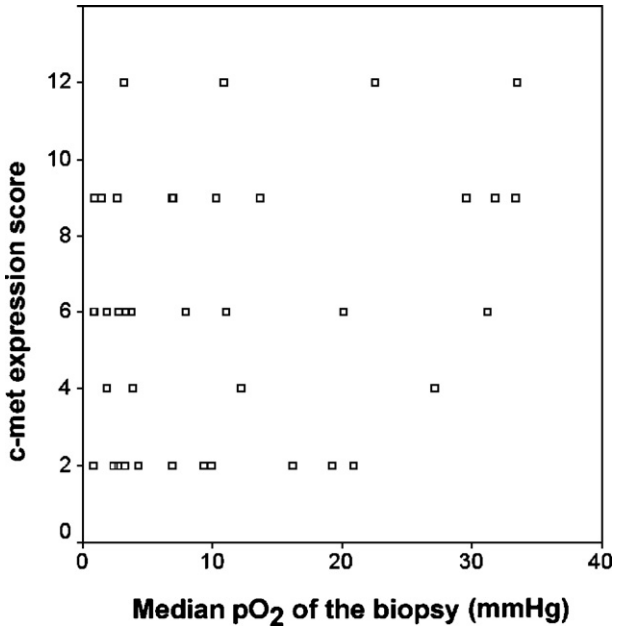

Fig. 2. Lack of correlation between the intratumoral median pO_2 levels and c-met expression in the corresponding biopsies.

c-met expression, clinico-pathological parameters and survival

c-met expression correlated significantly to histological tumor grade (Spearman rank correlation, $r=0.4$, $p=0.008$). This association was also evident in the box plot presentation (Kruskal–Wallis H test, $p=0.031$; Fig. 3). However, in the investigated cancers, there was no association between c-met expression and tumor size, lymph node status, lymphovascular space involvement, FIGO stage and histology (data not shown).

In the Kaplan–Meier analysis, there were no significant differences in overall survival comparing c-met-negative cervical

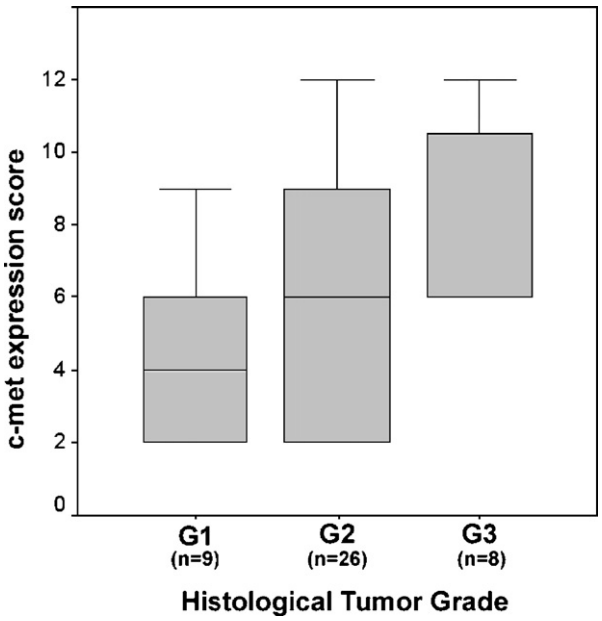

Fig. 3. Association between c-met expression in cervical cancers and histological tumor grade (G1, G2, G3).

cancers (score 0–2) with c-met-positive tumors (score>2) (3-year survival rate: 81.8% vs. 70.0%, $p=0.31$).

c-met expression and pattern of invasion

Of the 43 cervical cancers, 34 cases showed a finger-like pattern of invasion (Fig. 4A) and nine cancers exhibited a spray-like pattern (Fig. 4B). The degree of c-met expression was significantly associated with a spray-like pattern of invasion (Mann–Whitney U test, $p=0.008$, Fig. 4C).

Discussion

To our knowledge, this study explores for the first time the relation between c-met expression and intratumoral hypoxia levels in clinical samples of cervical cancer. We show that 72% of the investigated cancers displayed moderate or strong c-met staining. This is in line with a report by Baykal et al. who found c-met expression in 60% of the investigated cervical cancers [16]. A recent study by Tsai et al. [27] reported c-met expression in only 30% of their analyzed cancers, however, they exclusively studied adenocarcinomas of the uterine cervix. Likewise, we observed c-met expression in only one of the three adenocarcinomas present in our cohort.

In our study, c-met expression was significantly associated with a spray-like pattern of invasion that is related to a more aggressive phenotype resulting in poorer prognosis [28,29].

In keeping with previous results, the majority of investigated cervical cancers had a median pO_2 below 10 mm Hg, the commonly used threshold for hypoxic tumors [1]. In a previous study, we found that hypoxic cervical cancers were associated with a poorer survival when compared to well-oxygenated tumors [1]. Likewise, in the present study, patients with hypoxic tumors ($pO_2 \leq 10$ mm Hg) also had lower survival rates when compared to the other tumors, although this trend did not reach statistical significance.

In 2003, Pennacchietti et al. [19] demonstrated that hypoxia activates the transcription of the met protooncogene, resulting in higher levels of the met protein in vitro. They also showed that c-met was highly overexpressed in hypoxic areas of the investigated experimental tumors, as defined by expression of the hypoxia-inducible factor 1 (Hif-1). Although hypoxia is the strongest stimulus for Hif-1 expression, this transcription factor can also be induced by other factors, e.g., oncogenes [30]. In our study, we did not observe a correlation between intratumoral hypoxia levels and c-met expression. This is in line with a recent clinical study that did not show a correlation between Hif-1 expression and intratumoral hypoxia levels measured invasively in cervical cancer [31]. Although a variety of genes have been shown to be hypoxia-inducible in vitro, their association to intratumoral pO_2 levels in human cancer is less well defined [5]. Of the many hypoxia-induced markers identified in vitro, so far only the glucose transporter Glut-1 and carbonic anhydrase IX (CA IX) have been shown to correlate with the intratumoral oxygenation status in vivo [32,33]. These observations point towards a complex and finely tuned regulation of hypoxia-inducible genes in vivo. This regulation is only inadequately

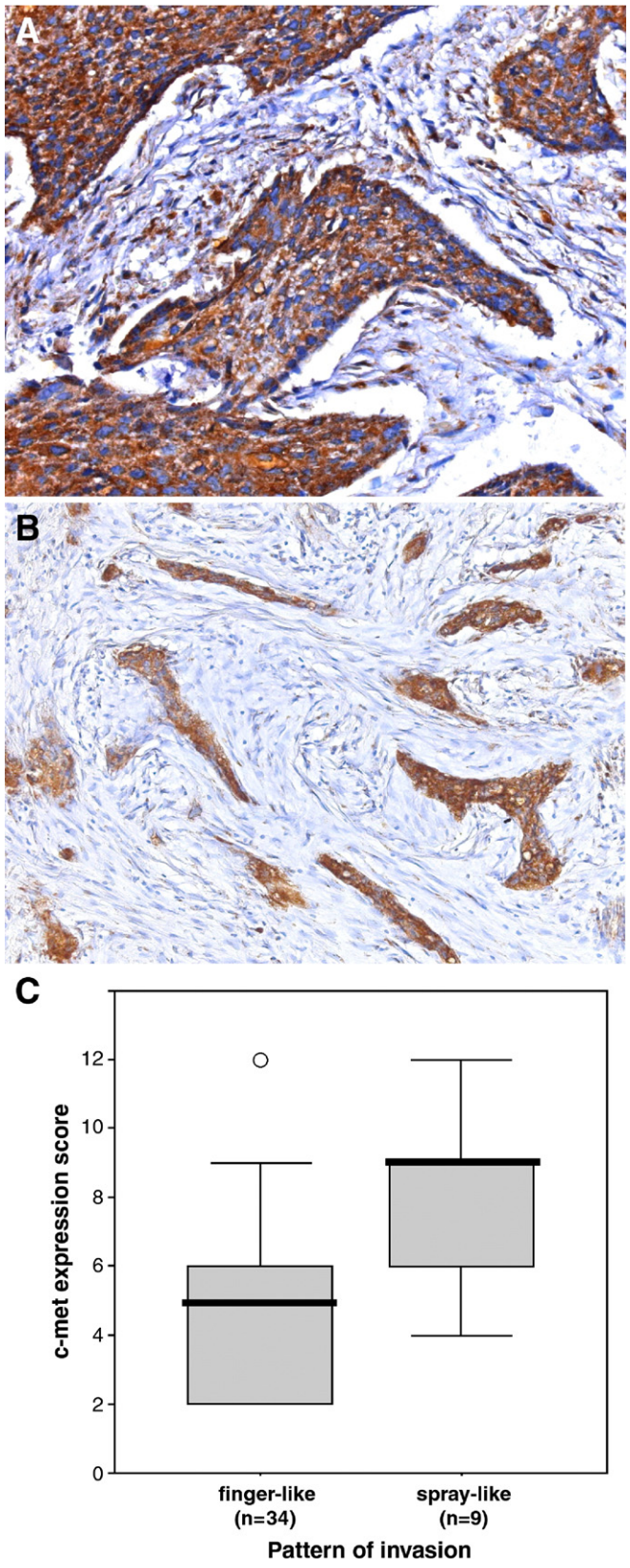

Fig. 4. (A) Cervical cancer exhibiting a finger-like pattern of invasion and strong c-met expression; (B) cervical cancer with a spray-like pattern of invasion showing strong c-met expression; (C) association between c-met expression and pattern of invasion.

represented in currently used in vitro models of hypoxia which typically consist in a one-time course of hypoxia lasting up to 48 h [34–36] neglecting the occurrence of chronic hypoxia and periods of reoxygenation.

Our results, therefore, suggest an additional or alternative regulation of c-met expression in vivo. Furthermore, it can be concluded that the deleterious effects of hypoxia on treatment outcome are likely independent of changes in c-met expression.

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