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Reduced membranous MET expression is linked to bladder cancer progression

Martina Kluth [a,1], Kristina Reynolds [a,1], Michael Rink [b], Felix Chun [b], Roland Dahlem [b], Margit Fisch [b], Wolfgang Höppner [c], Walter Wagner [d], Ousman Doh [e], Luigi Terracciano [f], Ronald Simon [a], Guido Sauter [a], Sarah Minner [a,*]

[a] Institute of Pathology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [b] Department of Urology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [c] Department of Urology, Clinical Center Itzehoe, Itzehoe, Germany; [d] Department of Urology, German Armed Forces Hospital, Hamburg, Germany; [e] Department of Urology, Regio Clinic Wedel, Wedel, Germany; [f] Institute of Pathology, University of Basel, Basel, Switzerland

The MET protein is involved in the malignant progression of different tumors. This study aimed to analyze the relationship of MET expression with tumor phenotype and clinical outcome in bladder cancer and the role of gene amplification for MET overexpression. A bladder cancer tissue microarray containing 686 bladder cancers was analyzed by immunohistochemistry and by fluorescence in situ hybridization. MET immunostaining was seen in normal urothelium and was recorded in 459 of 560 analyzable urothelial carcinomas (82.0%). Low MET staining was associated with a more unfavorable tumor phenotype. MET staining was seen in 89.8% of 266 pTa, 81.1% of 132 pT1, and 69.4% of 160 pT2–4 cancers ($P < 0.0001$). MET staining was detectable in 92.4% of 66 grade 1, 85.6% of 257 grade 2, and 75.1% of 237 grade 3 cancers ($P = 0.001$). MET expression status was not associated with overall or tumor-specific survival in muscle-invasive cancers (pT2–4), tumor progression in pT1 cancers, or recurrences in pTa tumors. Only four of the analyzed tumors (0.8%) showed amplification of the *MET* gene. We conclude that MET is not overexpressed in urothelial cancer but rather downregulated in a fraction of cancers. Accordingly, rare amplification of the genomic area including the *MET* gene was not associated with MET protein overexpression.

Keywords Bladder cancer, MET, tissue microarray, prognosis, immunohistochemistry
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Bladder cancer is the seventh most-common cancer in males, with an estimated incidence of about 380,000 cases worldwide (1). Invasively growing bladder cancers (pT1–4) have a significant cancer-related mortality, and surgical treatment options vary from transurethral resection to cystectomy with the risk of substantial treatment-associated morbidity. Therefore, it is important to identify biomarkers that are useful for early diagnosis and exact assessment of individual patient prognosis and thus enable better decision-making in the individual treatment of bladder cancer patients. In recent years, numerous genetic alterations were found and linked to tumor growth, metastasis, and survival in bladder cancer (2–4). These include DNA copy number alterations that often pinpoint the localization of important tumor suppressor genes or proto-oncogenes in cancers. A major class of proto-oncogenes are protein tyrosine kinases (*PTK*) that are involved in various cell regulatory processes, such as proliferation and migration (5). Some of these proto-oncogenes, such as *HER2/neuII*, are well-known (2,3). But the role of most *PTK*s in bladder cancer is insufficiently clarified.

The *MET* gene, located on chromosome 7q31, encodes a transmembrane tyrosine kinase receptor for the hepatocyte growth factor/scatter factor (HGF/SF). Under physiological conditions, the HGF/MET signaling plays a role in various cellular processes, such as cell differentiation, cell motility, promotion of invasion, cell survival, and epithelial

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* Corresponding author.
E-mail address: s.minner@uke.de

[1] Both authors contributed equally to this work and both are considered first author.

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morphogenesis (6,7). The *MET* gene product has been found to play an oncogenic role in a variety of different human cancers such as breast (8), gastric (9), colorectal (10), and bladder cancer (11,12). In these cancers, MET overexpression is suspected to play a role in tumorigenesis and cancer progression and is often associated with shortened survival, advanced tumor stage, and high tumor grade (13). Aberrant activation of MET in cancer has been found to be due to transcriptional upregulation resulting in overexpression (14), gene amplification (15), germline mutations (16,17), or somatic mutations (18,19) leading to constitutive MET activations and ligand-dependent autocrine loops (20). Furthermore, MET inhibition is also investigated as a potential target for cancer treatment (21–23).

In bladder cancer, several studies using immunohistochemistry (IHC) have described MET overexpression, and some studies described a strong relationship between high MET protein expression and tumor grade, tumor stage, invasion, metastasis, and decreased survival (11,12,24–27). This study was undertaken to assess the role of *MET* gene amplification as a mechanism for MET overexpression in bladder cancer. In addition, we intended to further investigate the clinicopathological associations of MET protein expression levels in bladder cancer. For this purpose, an urothelial bladder cancer tissue microarray (TMA) containing samples from 686 patients was analyzed by fluorescence in situ hybridization (FISH) and IHC.

Materials and methods

Bladder cancer tissue microarray

A preexisting bladder cancer TMA containing 686 urothelial bladder cancer samples was used (28). One 0.6-mm tissue core had been punched out from each case and transferred into a tissue microarray (TMA) format as described previously (29). Tumor stage and grade were defined according to Union for International Cancer Control (UICC) and earlier World Health Organization (WHO) classification (30,31). (The tumors were not classified according to the current 2004 WHO classification, since the tumors for the TMA construction were reviewed before 2004.) Cancers with stromal invasion but absence of muscular bladder wall in the biopsy were classified as at least pT1 (pT1). A papillary tumor growth was assumed if at least one unequivocal papilla with similar atypia to the invasive tumor area was present. Time to recurrence was selected as the study endpoint for pTa tumors, and time to progression (to stage pT2 or higher) was selected as the study endpoint for pT1 cancers. Recurrences were defined as cystoscopically visible tumors. Tumor progression was defined as the presence of muscle invasion (stage pT2 or higher) in a subsequent biopsy.

Immunohistochemistry

Freshly cut TMA sections were analyzed on the same day in a single experiment. High temperature pretreatment of slides was done in an autoclave in Tris-EDTA-citrate buffer, pH 7.8, for 5 minutes. MET immunostaining was performed using a monoclonal antibody (clone EP1454Y, dilution 1:150; Abcam, Cambridge, UK). The TMAs were evaluated by one pathologist (S.M.). Only membranous staining was considered. The staining intensity (0, 1+, 2+, 3+) and the fraction of positive tumor cells were recorded for each tissue spot. A final score was built from these two parameters according to the following scores: Negative scores had a staining intensity of 0; weak scores had a staining intensity of 1+ in 70% or less of the tumor cells or a staining intensity of 2+ in 30% or less of the tumor cells; moderate scores had a staining intensity of 1+ in more than 70% of the tumor cells, a staining intensity of 2+ in more than 30% and 70% or less of the tumor cells, or a staining intensity of 3+ in 30% or less of the tumor cells; and strong scores had a staining intensity of 2+ in more than 70% of the tumor cells or a staining intensity of 3+ in more than 30% of the tumor cells. This is a standardized scoring system used by our group with the purpose of defining a group with high expression level, a group with low or absent expression, and two intermediate groups. In our experience, this system works well to define whether molecular features are associated with tumor phenotype and clinical outcome (32).

Fluorescence in situ hybridization

A 4-μm TMA section was used for dual-color FISH. For proteolytic slide pretreatment, a commercial kit was used (Vysis paraffin pretreatment reagent kit; Abbott Laboratories, Abbott Park, IL). TMA sections were deparaffinized, airdried, and dehydrated in 70%, 85%, and 100% ethanol, followed by denaturation for 5 minutes at 74°C in 70% formamide 2 × SSC solution. The dual-color FISH probe set consisting of an orange fluorochrome direct-labeled centromere 7 probe and a green fluorochrome direct-labeled *MET*-specific probe was used (ZytoVision GmbH, Bremerhaven, Germany). Hybridization was done overnight at 37°C in a humidified chamber, and slides were washed and counterstained with 0.2 μmol/L 4′-6-diamidino-2-phenylindole in an antifade solution. Each spot was evaluated, and the predominant gene and centromere copy numbers in tumor cells were estimated by one experienced technician. Data from our laboratory have previously shown that diagnosis of amplification based on signal number estimation is highly reliable (33). A tumor was considered amplified if the ratio of gene-specific signals/centromere signals was ≥2.0. In cases in which the ratio was not clearly below or above 2.0, a total of 20 cells were scored.

Statistics

For statistical analysis, the JMP 8.0 software (SAS Institute Inc, Cary, NC) was used. Contingency tables were calculated to study the association between MET overexpression or amplification and clinicopathological variables, and the χ^2 (likelihood) test was used to find significant relationships. Survival curves were calculated by the Kaplan–Meier method and compared with the log-rank test. Depending on the selected endpoint, patients were censored at the time of last clinical control at which they showed no evidence of disease or at the date when cystectomy was performed.

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