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Soluble cMet expression in the serum of patients with different stages of prostate cancer

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Summary. *Aim:* Hepatocyte growth factor (HGF) and its receptor, cMet, have been shown to be involved in prostate cancer. Many proteins are proteolytically released from the surface by a process known as ecdodomain shedding. Among the many receptors for which the ectodomain shedding has been shown is cMet. The aim of this study was to determine the expression of soluble cMet (s-cMet) in the serum of patients with different stages of prostate adenocarcinoma (PCa). *Materials and Methods:* Fifty seven serum samples from controls and 63 samples from patients with different stages of PCa were studied (n=20 stage I, n=16 stage II, n=14 stage III and n=13 stage IV). The total protein concentration (TPC) was measured using a Bio-Rad protein assay and the expression and concentration of s-cMet was determined by western blotting and enzyme linked immunosorbent assay (ELISA). *Results:* No significant change in the serum TPC of patients with different stages of PCa was seen as compared to controls. The relative s-cMet expression in the samples of patients with different stages of PCa is increased as compared to controls. It was shown that the serum concentration of s-cMet in the patients with various stages of PCa is significantly increased compared to normal controls (P<0.001). *Conclusions:* The result of this study suggests that s-cMet might be involved in the pathophysiology of PCa and the progression of PCa. We suggest that a possible role for the c-Met ectodomain as a regulator of HGF/c-Met activity should be examined in PCa.

Key words: serum, cMet, prostate cancer, hepatocyte growth factor

«Espressione del fattore di cMet solubile nel siero di pazienti affetti da cancro prostatico a diversi stadi di progressione»

Riassunto. *Scopo:* È stato dimostrato che il fattore di crescita degli epatociti (HGF) e il suo recettore cMet, sono coinvolti nel cancro della prostata. Diverse proteine vengono rilasciate proteoliticamente dalla superficie della membrana cellulare attraverso un processo chiamato rilascio dell'ectodominio. Fra i diversi recettori per i quali il rilascio dell'ectodominio è stato dimostrato, è presente il cMet. Lo scopo di questo studio è quello di determinare l'espressione di cMet solubile (s-cMet) nel siero di pazienti con adenocarcinoma prostatico (PCa). *Materiali e Metodi:* Sono stati studiati 57 campioni di siero derivati da pazienti del gruppo di controllo e 63 campioni da pazienti con PCa. La concentrazione di proteina totale (TPC) è stata misurata utilizzando Bio-Rad protein assay mentre l'espressione e la concentrazione di s-cMet è stata determinata da Western blotting ed enzyme linked immunosorbent assay (ELISA). *Risultati:* Non è stato notato nessun significativo cambiamento del TPC nel siero di pazienti con PCa quando paragonati al controllo. La relativa espressione s-cMet nei campioni di pazienti con PCa è aumentato se paragonato al controllo. E' stato inoltre dimostrato che la concentrazione di siero di s-cMet nei pazienti con PCa è significativamente aumentata paragonata al normale controllo (P<0.001). *Conclusioni:* Il risultato di questo studio suggerisce che s-cMet può essere coinvolto nella patofisiologia e nella progressione del PCa. Suggeriamo che venga preso in considerazione il possibile ruolo dell'ectodominio c-Met come regolatore dell'attività HGF/c-Met.

Parole chiave: Siero, c-Met, cancro prostatico, fattore di crescita degli epatociti

Introduction

Prostate cancer (PC) is extremely common affecting 15% of white men and 18% of African American men throughout their lifetime, and it will result in death in 3% of men in North America. In 2010 approximately 220,000 new cases of PC were diagnosed in the United States, resulting in about 32,000 PC related deaths (1). Early diagnosis is crucial to successful management of PC patients.

Prostate specific antigen (PSA) is a protein produced by the cells of the prostate glands. It is a tissue specific serine protease (2). As men age, both benign and malignant prostate lesions become more frequent. While PSA does not allow distinguishing between a benign prostatic condition and cancer, an elevated PSA level may indicate that other tests are necessary to determine whether cancer is present.

Polypeptide growth factors have been implicated in stromal-epithelial interactions in prostate adenocarcinoma (PCa) as well as in a multitude of biological behaviors of prostate cancer cells (3). Hepatocyte growth factor (HGF) and its receptor, the cMet protein tyrosine kinase which is a cell surface receptor, form a classic ligand-receptor system for epithelio-mesenchymal communications in the normal and cancerous prostate. HGF is a 90 kDa multidomain glycoprotein that is highly related to members of the plasminogen serine protease family. It is secreted as a single chain, active heterodimer extracellular form by a number of proteases (4). The cMet receptor is a disulfide-linked heterodimer consisting of extracellular α and β chains. The binding of HGF to the Met receptor induces activation of Met tyrosine kinase and the autophosphorylation of tyrosine residues in Met (5).

HGF and Met control several cellular processes essential for life. Knock-out of either HGF or Met causes embryonic lethality due to placental and liver failure and leads to an impaired development of several other organ systems (6, 7). HGF was subsequently shown to be identical to scatter factor (SF), a ligand inducing cell dissociation of epithelial cells. HGF supports a complex morphogenetic program called invasive growth, which is operational during embryogenesis and wound healing in adults. This HGF/Met-mediated program has also been implicated in cancer development and progression, in particular in tumor invasion and in progression to metastatic disease. It has been shown that stromal-derived HGF enhances the invasive growth of endometrial carcinoma cells (8). On the other hand, overexpression of Met has been observed in several types of malignant tumors including gastric and breast cancers (9, 10).

A variety of integral membrane receptors, including c-Met, can be released from the lipid bilayer by proteolysis to form soluble, truncated proteins. The proteases that generate soluble forms of membrane proteins are predominantly metalloproteinases or serine proteinases. The soluble receptors are smaller, consisting of the extracellular origin of the membrane-bound receptor and, in general, are able to bind to ligands with reduced affinity (11). The overexpression of HGF and c-Met in PCa tissues has been reported (12). In this study we measured the levels of soluble c-Met in the serum of patients with PCa. We hypothesized that the overexpression of c-Met characteristic of many malignancies might result in increased ectodomain shedding, and that measuring it could be a useful biomarker of tumor progression.

Materials and methods

Patient samples

After informed consent, a total of 57 samples of serum from normal subjects and 63 samples from patients with different stages of PCa were collected (20, 16, 14 and 13 samples for stages I, II, III and IV, respectively). Samples were aged matched between the two groups, analyzed and ranged in age between 54 and 73 years. None of the patients suffered from known diabetes mellitus, earlier diagnosed tumors or infection. The controls were not suffering from infections, diabetes or any diagnosed tumors. None of the patients were receiving hormonal therapy or other anti-cancer treatments at the time of blood sampling. The serum samples were frozen immediately and stored at -70°C until used.

Total protein concentration and western blot

The total concentration of proteins in serum was determined by the Bio-Rad protein assay based on the

Bradford dye procedure. For Western blot analysis, serum samples were mixed with a sample buffer containing 3.2% SDS, 15% glycerol, 2.8 M b-mercaptoeyhanol and 0.0015% bromophenol blue. Samples were applied to a 10% SDS-PAGE gel (Bio-Rad, Milan, Italy) according to Laemly and the proteins obtained were transferred to nitrocellulose sheets, pore size 0.45 lm (Bio-Rad). After incubation for 2 hours at room temperature in the blocking solution (PBS containing 5% skimmed milk), the nitrocellulose sheets were exposed overnight, at 4 °C, to anti-DL-21 monoclonal antibody (against s-cMet) (Upstate Biotechnology, USA) and identified with a peroxidase-labeled mouse IgM PK 4010 Vectastain Avidin Biotin complex kit (Vectorlab, Peterborough, UK). The peroxidase activity was revealed with diaminobenzidine (0.5 mg/ml in PBS with 0.02% hydrogen peroxide). ß-Tubulin expression was determined as a protein loading control. After Western blotting, the data were quantified by scanning densitometry.

Analysis of s-cMet concentration by ELISA

s-cMet concentration in CSF and serum was measured using sensitive two sited ELISA and antiserum against human s-cMet. Microtiter plates (Dynatech, Chantilly, VA) were first coated with 80 ng primary anti-s-cMet antibody per well in 0.1 M Tris buffer. After overnight incubation, the plates were blocked with EIA buffer (50 mM Tris, pH 7.5, 0.3 M NaCl, 0.1% Triton X-100, 1% BSA and 1% Gelatine). The samples and standards were placed in triplicate wells and incubated overnight at room temperature. After washing, a biotinylated secondary antibody (8 ng/ml) was added to each well and the incubation was carried out overnight at room temperature. b-Galactosidase coupled to avidin was then added and after two hours was followed by washing. Finally 200 μM 4-methylumbelliferyl-b-galactoside (Sigma, Poole, UK) in 50 mM sodium phosphate and 10 mM MgCl2 buffer were added and the amount of fluorescence was measured after 40 minutes incubation at 37 °C using a fluorimeter (Dynatech).

Statistical analysis

All data presented are expressed as means ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) and only values with $P \leq 0.05$ were considered as significant.

Results

Total protein concentration

The total protein contents of the serum samples from control group and patients with stages I to IV PCa was 0.37±0.006, 0.38±0.007, 0.38±0.004, 0.37±0.007 and 0.39±0.007 (g/l), respectively. No significant increase in the total protein concentration was seen in the serum of patients with stages of PCa as compared to the control group ($P>0.05$) (Figure 1).

Analysis of s-cMet expression by western blotting

A western blot analysis using anti-DL-21 antibody as a probe confirmed the presence of s-cMet in all samples from healthy subjects and patients with various different stages of PCa (Figure 2A). Since s-cMet was detected in all samples analyzed in this study, s-cMet appears to be a constant component of the serum. An image analyzer was used to determine the intensities of the bands in the respective lanes. The relative serum s-cMet level in patients with different stages of PCa and normal subjects was determined. Quantification of Western blot gels from repeated experiments showed that the amount of s-cMet was increased in the serum of patients with different stages of PCa as compared with normal controls (Figure 2B).

Analysis of s-cMet concentration by ELISA

Using ELISA, it was shown that the concentration of serum s-cMet in the patients with different stages of PCa was higher than in normal subjects. The mean±SEM s-cMet concentration in the serum of patients with stages I to IV PCa was 296.90±21.66, 326.09±40.32, 336±42.93 and 384.63±56.78 ng/ml, respectively, which was significantly higher than the 261.94±50.41 ng/ml of the normal subjects ($P<0.0001$) (Figure 3). We found that the levels of serum s-cMet were significantly higher in the group of PCa as com-

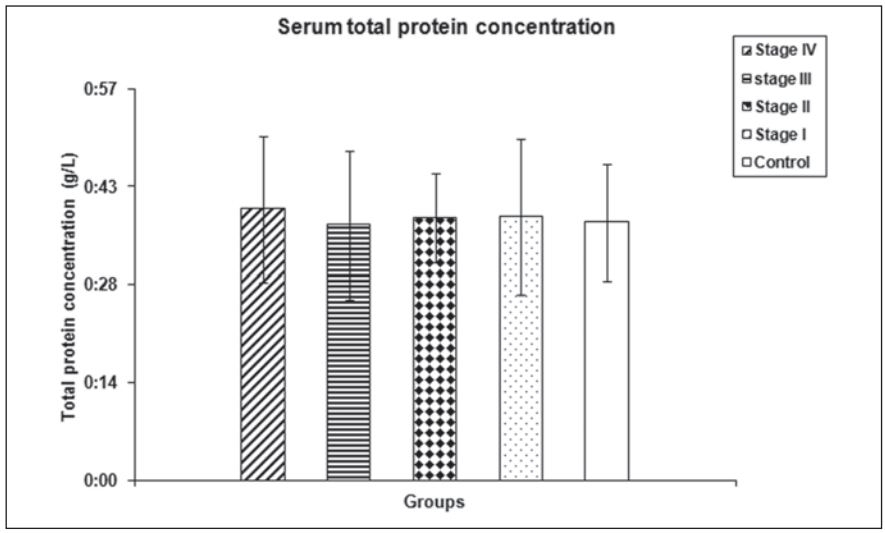

Figure 1. Total protein concentration in the serum of normal subjects and patients with various stages of PCa (g/L). No significant difference was seen in the total protein concentration between the groups (P=0.4).

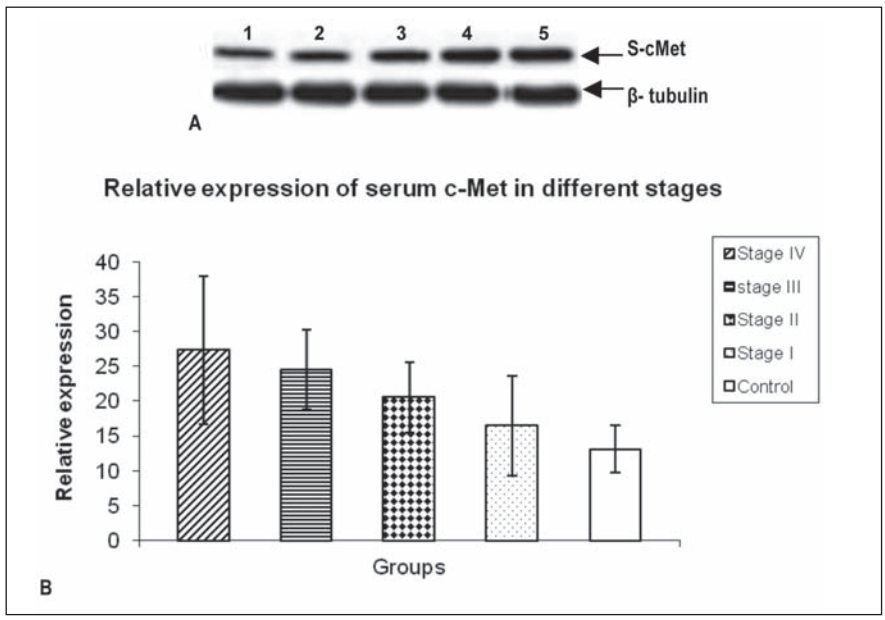

Figure 2. A) Expression of s-cMet in the serum of patients with various stages of PCa and control groups (lane 1; control group, lanes 2 to 5: stages 1 to 4, respectively). β-tubulin (50-kDa) expression was determined as a protein loading control. B) Signal intensities of s-cMet expression in the serum from the control and different stages of PCa immunoblotting experiments were determined by densitometric analysis. 3 stars: P<0.001.

pared to the healthy control group. Moreover, among the PCa patients, the concentration of s-cMet was significantly higher in more advanced stages of the disease.

Discussion

The present study indicates that concentration of s-cMet is elevated in the serum of patients with PCa compared to normal subjects. No significant change in the TPC was seen between the two groups. This result demonstrated the possible involvement of the HGF/Met pathway in the pathogenesis of PCa.

HGF is a heparin-binding growth factor with a pleiotrophic action. HGF is known to stimulate mitogenic, motogenic and morphogenic activities of a variety of cells including several carcinoma cell lines. HGF has the ability to stimulate the proliferation and migration of endothelial cells, indicating that the vascular wall is an important target for this growth factor (13, 14).

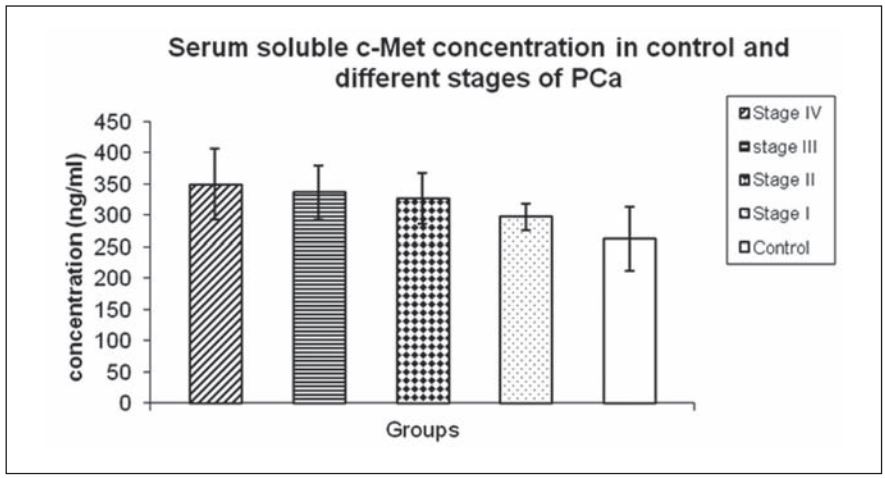

Figure 3. s-cMet concentration in serum samples from controls and patients with various different stages of PCa (ng/ml). A significant increase in serum s-cMet level was seen in various stages of PCa samples when compared with normal controls. Significance values are shown as stars: 3 stars $P < 0.0001$.

Ectodomain shedding is a process by which many transmembrane proteins are proteolytically released from the cell surface. Shedding is an important process during normal development and shedding defects are known to contribute to certain pathologies (15). Among the many receptors for which ectodomain shedding has been shown is c-Met, the HGF receptor tyrosine kinase (16). A wide variety of cell surface proteins are proteolytically cleaved to release their ectodomains into the extracellular milieu. Ectodomain shedding is an important post-translational modification. Ectodomain shedding rapidly regulates the expression of cell surface proteins and liberates biologically active soluble ectodomains that can function in an autocrine or paracrine manner. Once released, protein ectodomains exhibit functions similar to or distinct from their cell surface counterpart. Regulated shedding is typically a mechanism that modulates cellular processes such as adhesion, migration and proliferation (17). Thus dysregulation or lack of shedding results in diverse pathologies such as inflammation and cancer.

In this study we showed that the serum concentration of s-cMet was significantly increased in the group of PCa as compared to the control group. In addition, among the PCa patients, the concentration of s-cMet was significantly higher in more advanced stages of the disease. The HGF/Met system is involved in the pathogenesis of PCa by promoting cell proliferation and migration (8). HGF levels are elevated in a variety of disease states such as solid tumors (18-20). It has been shown that the soluble cMet concentration in the serum and peritoneal fluid of patients with endometriosis increases as compared to normal subjects (21). It was shown that HGF can induce the shedding of c-Met (22). Thus elevation of s-cMet concentration in the serum of patients with PCa may be the consequence of the elevation of HGF. It has also been shown that the tumor environment is a rich source of proteases causing cMet ectodomain shedding (23). This may be the other reason for the increased levels of s-cMet in the serum of patients with PCa.

Conclusion

The result of this study suggests that c-Met shedding may provide a reliable and practical indicator of malignant potential, tumor progression and overall tumor burden. It is also concluded that s-cMet might be involved in the pathophysiology of PCa. Clinical studies are necessary to see whether s-cMet is useful in therapeutic monitoring or as a diagnosis/prognosis marker either alone or in combination with other markers.

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