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Evaluation of Serum Biomarkers (FGF-2, HGF, MIF and PTN) in Patients With Testicular Germ Cell Cancer

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Abstract. *Background/Aim: FGF-2, HGF, MIF and PTN have been suggested as biomarkers for testicular germ cell cancer patients in earlier studies. Our study was designed to validate these potential novel tumor markers. Materials and Methods: Serum FGF-2, HGF, MIF and PTN levels were analysed using an ELISA technique in a screening cohort of 20 testicular germ cell cancer patients and 10 healthy men. MIF levels were measured in a validation cohort of 84 patients with testicular cancer, 24 with non-malignant testicular tumors and 64 healthy men. Results: Serum FGF-2, HGF and PTN levels did not differ in cancer patients and healthy males within the screening cohort, whereas MIF was significantly increased among cancer patients. Within the validation cohort, a modest but insignificant increase of serum MIF was observed in TGCT patients compared to healthy men. MIF levels were not correlated with adverse clinical-pathological parameters. Conclusion: FGF-2, HGF, MIF and PTN are not suitable as non-invasive biomarkers for testicular germ cell cancer patients.*

Testicular germ cell cancer (TGCT) is the most common malignancy in young men. The incidence of TGCT is still rising in many western countries, while mortality rates remain low. The successful treatment of patients with even advanced metastatic TGCT is mainly due to the effective multimodal therapy (polychemotherapy, radiotherapy and surgery) (1). The serum tumor markers alpha-fetoprotein (AFP) and human chorionic gonadotropin (hCG) are used for diagnosis, risk stratification, treatment monitoring, and surveillance (2). However, both biomarkers lack high sensitivity, especially in early tumor stages and seminoma TGCT patients. It is estimated that about 50% of TGCT patients have normally ranged tumor markers (3). Thus, novel non-invasive tumor markers with improved sensitivity could help improve the clinical management of TGCT patients.

A variety of potential TGCT biomarkers including circulating nucleic acids [*e.g.* microRNA (4, 5), DNA methylation (6), DNA concentration (7)] and serum proteins [*e.g.* Fas ligand (8), TNFRSF8 (9)] have been evaluated, but none of them have yet been applied in daily routine due to the lack of validation studies and the small case numbers (10). Aigner *et al.* reported in 2003 an increase in serum FGF-2 (fibroblast growth factor-2) and PTN (pleiotrophin) levels in TGCT patients (n=22) compared to healthy individuals (n=21) (11); both proteins are involved in tumor angiogenesis. Serum PTN [*e.g.* colorectal (12), lung (13), pancreatic (12)] and FGF-2 [*e.g.* colorectal (14), ovarian (15), lung (16)] levels were also increased in other cancer patients, indicating the possible diagnostic role of these proteins. In a recent study, we investigated the circulating levels of various cytokines in patients with human malignancies including TGCT patients. Among these, MIF (macrophage migration inhibitory factor) and HGF (hepatocyte growth factor) were significantly increased in TGCT patients compared to control subjects. In order to validate the findings of these earlier studies, we investigated serum biomarker levels in an enlarged cohort of patients with malignant and benign testicular disease, as well as in healthy men.

Materials and Methods

Sample collection. First, we investigated the serum concentration of FGF-2, HGF, MIF and PTN in a screening cohort of 20 testicular germ cell cancer patients (10 seminoma and 10 non-seminoma patients) and 10 healthy subjects; see Table I for clinical-pathological parameters. The validation of MIF expression levels

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was then performed in 108 patients undergoing inguinal exploration suspicious for testicular cancer; among these, 84 patients had malignant testicular germ cell cancer (seminoma n=48, non-seminoma n=35) and 24 had non-malignant diseases (histological diagnosis: intratubular germ cell neoplasia n=1; epidermoid cyst n=6; leydig cell tumor n=6; sertoli cell tumor n=1; mature teratoma n=2; adenomatoid tumor n=2; testicular infarction n=1; infection n=1; stromal spindle cell tumor n=1; scar tissue n=1; fibroma n=1; fibrosis n=1). We also studied an age-matched control cohort that consisted of 64 healthy male individuals. The detailed clinicopathological parameters are provided in Table II.

The collection of serum samples was performed according to local standard operating procedures within the framework of the Biobank initiative at the CIO Köln/Bonn. In brief, venous blood was collected in Serum S-Monovette Gel tubes with clotting activator (Sarstedt, Nümbrecht, Germany) prior to surgery. Serum was centrifuged after clotting for 10 min at 2500 g; the separated serum samples were stored in cryotubes at –80˚C until use. Sample collection was performed between 1997 and 2014. Written informed consent was obtained from each individual and the study was approved by the local ethic committee (approval number: 162/14). The study conformed with The Code of Ethics of The World Medical Association ("Declaration of Helsinki").

ELISA. The serum levels of FGF-2 (Quantikine FGF Immunoassay; R&D Systems, Minneapolis, MN, USA), HGF (Quantikine HGF Immunoassay; R&D Systems), MIF (Quantikine MIF Immunoassay; R&D Systems) and PTN (ELISA Kit for Pleiotrophin; Cloud-Clone, Houston, TX, USA) were quantified using ELISA kits according to the manufacturer's recommendations. We used 50 μl (MIF) or 100 μl (FGF-2, HGF, PTN) of serum for the analysis; each sample was measured in duplicate. A standard curve was constructed using the supplied standard to determine serum protein levels. The optical density of each well was detected at 450 nm on an SLT-reader (Tecan, Crailsheim, Germany).

Statistical analysis. The Mann-Whitney *U*-test and the Kruskal-Wallis-test were used to determine differences between cancer patients and controls, and to associate MIF expression with clinical-pathological parameters. The Pearson test was used to correlate AFP/HCG with MIF levels. The diagnostic sensitivity and specificity of the biomarkers was determined using receiver operator curve (ROC) analyses. Statistical significance was concluded at $p<0.05$; statistical analyses were performed using SPSS Statistics v22 (IBM, Chicago, IL, USA).

Results

Screening cohort. The serum levels of FGF-2, HGF, MIF and PTN were determined in a screening cohort of 10 seminoma and 10 non-seminoma patients, as well as 10 healthy male individuals (Figure 1). FGF-2, HGF and MIF were detected in all study patients. PTN was not detected in non-seminoma patients and only in one individual of the control group, whereas it was detectable in all seminoma patients. The serum levels of MIF were significantly increased in TGCT compared to healthy individuals (mean 7.47 ng/ml *vs*. 3.17 ng/ml; $p=0.005$), whereas FGF-2 ($p=0.965$) and HGF ($p=0.965$) were similar in both cohorts. Mean serum levels of all markers were similar in non-seminoma and seminoma TGCT patients (FGF-2 $p=0.307$; HGF $p=0.880$; MIF $p=0.762$). MIF levels were higher in seminoma ($p=0.013$) and non-seminoma ($p=0.019$) patients compared to controls (Figure 1).

Validation cohort. In order to validate the potential of MIF as a novel TGCT biomarker, we investigated its serum concentration in a larger cohort which included 84 patients with testicular cancer (seminoma n=48, non-seminoma n=35), 64 healthy male individuals and 24 patients with non-malignant testicular disease. The mean level of MIF was 5.05 ng/ml in TGCT patients, 4.03 ng/ml in healthy men and 5.83 ng/ml in patients with benign testicular disease. Thus, MIF levels in serum were neither different in cancer patients and healthy male ($p=0.071$) nor in patients with benign testicular disease ($p=0.221$). However, MIF was increased in patients with benign testicular disease compared to healthy male ($p=0.019$). MIF levels were similar in seminoma and non-seminoma TGCT ($p=0.414$). Thus, serum MIF levels failed as a diagnostic biomarker: the area under curve was 0.541 (95% confidence interval 0.454-0.627) for cancer patients *vs*. healthy male and benign patients, and 0.587 (0.495-0.679) for cancer patients *vs*. healthy males, respectively (Figure 2). We also investigated whether MIF levels were correlated with clinical-pathological parameters: advanced pathological stage (pT1 *vs*. pT3 $p=0.045$; pT2 *vs*. pT3 $p=0.039$) was associated with MIF levels (mean: pT1 5.04 ng/ml, pT2 4.54 ng/ml, pT3 7.62 ng/ml). Clinical tumor stage, IGCCCG stage and serum tumor markers (AFP, HCG) were not correlated with serum MIF concentrations (all $p>0.1$).

Discussion

Novel non-invasive tumor markers with improved sensitivity compared to the classical markers AFP and hCG could improve the clinical management of TGCT patients. In former studies, several proteins including FGF-2 (fibroblast growth factor-2) (11), PTN (pleiotrophin) (11), MIF (macrophage migration inhibitory factor; Stefan Holdenrieder *et al*., unpublished data) and HFG (hepatocyte growth factor; Stefan Holdenrieder *et al*., unpublished data) markers have been investigated in small TGCT cohorts with promising results. However, all candidate markers still require validation in independent cohorts and therefore we determined the serum levels of these markers in an enlarged cohort of patients with TGCT, non-malignant testicular disease and healthy men.

We first determined FGF-2, HGF, MIF and PTN levels in a screening cohort. Surprisingly, PTN was not detected in most patients, and HFG and FGF-2 were not differentially expressed in TGCT patients and healthy men. Thus, our study does not confirm a role of these proteins for diagnostic

Table I. *Clinical-pathological parameters of patients investigated in the screening experiments.*

	TGCT (n=20)	Seminoma (n=10)	Non-seminoma (n=10)	Healthy (n=10)
Age				
mean	40.0	46.7	33.3	32.3
min-max	18-76	32-76	18-57	23-42
pT-stage				
pT1	12 (60%)	5 (50%)	7 (70%)	n.a.
pT2	6 (30%)	4 (40%)	2 (20%)	n.a.
pT3	2 (10%)	1 (10%)	1 (10.0%)	n.a.
Clinical stage (Lugano)				
Stage 1	15 (75%)	8 (80%)	7 (70%)	n.a.
Stage 2	4 (20%)	2 (20%)	2 (20%)	n.a.
Stage 3	1 (5%)	0 (0%)	1 (10%)	n.a.
Tumor marker				
AFP	3 (15%)	0 (0%)	3 (30%)	n.d.
HCG	10 (50%)	4 (40%)	6 (60%)	n.d.
Missing	0 (0%)	0 (0%)	0 (0%)	10 (100%)

TGCT: Testicular germ cell tumor; n.a.: not applicable; n.d.: not done.

Table II. *Clinical-pathological parameters of patients investigated in the validation experiment.*

	TGCT (n=84)	Seminoma (n=48)	Non-seminoma (n=35)	Healthy (n=64)	Benign (n=24)
Age					
Mean	34.9	38.3	30.1	31.6	36.9
Min-max	14-56	23-56	14-53	19-64	18-71
pT-stage					
pT1	59 (70.2%)	36 (75.0%)	23 (65.7%)	n.a.	n.a.
pT2	17 (20.2%)	7 (14.6%)	10 (28.6%)	n.a.	n.a.
pT3	4 (4.8%)	2 (4.2%)	2 (5.7%)	n.a.	n.a.
"burned out"	3 (3.6%)	3 (6.2%)	0 (0%)	n.a.	n.a.
Clinical stage (Lugano)					
Stage 1	54 (64.3%)	35 (72.9%)	19 (54.4%)	n.a.	n.a.
Stage 2	21 (25.0%)	11 (22.9%)		10 (28.6%)	n.a.n.a.
Stage 3	9 (10.7%)	3 (6.2%)	6 (17.1%)	n.a.	n.a.
IGCCCG classification					
Good prognosis	22 (28.6%)	11 (22.9%)	11 (31.4%)	n.a.	n.a.
Intermediate prognosis	6 (7.1%)	3 (6.2%)	3 (8.6%)	n.a.	n.a.
Poor prognosis	2 (0%)	0 (0%)	2 (5.7%)	n.a.	n.a.
Not applicable	54 (61.9%)	35 (72.9%)	19 (54.4%)	n.a.	n.a.
Tumor marker increase					
AFP	18 (21.4%)	0 (0%)	18 (51.4%)	n.d.	0 (0%)
HCG	36 (42.8%)	14 (29.2%)	22 (62.9%)	n.d.	0 (0%)
Missing	0 (0%)	0 (0%)	0 (0%)	64 (100%)	6 (25.0%)

n.a.: Not applicable; n.d.: not done; IGCCCG: International Germ Cell Cancer Collaboration Group.

purposes in patients with TGCT. It should be noted that Aigner *et al*. used a self-constructed ELISA using a monoclonal antibody to measure PTN levels in serum (11), whereas a commercial ELISA kit from Cloud-Clone was used in this study. Similar to Aigner *et al*., we used the R&D Quantikine ELISA assay to determine FGF-2 levels, thereby limiting the bias due to different detection techniques. It should also be noted that PTN [colorectal (12), lung (13), pancreatic (12)] and FGF-2 [colorectal (14), ovarian (15), lung (16)] levels were also increased in other tumor entities,

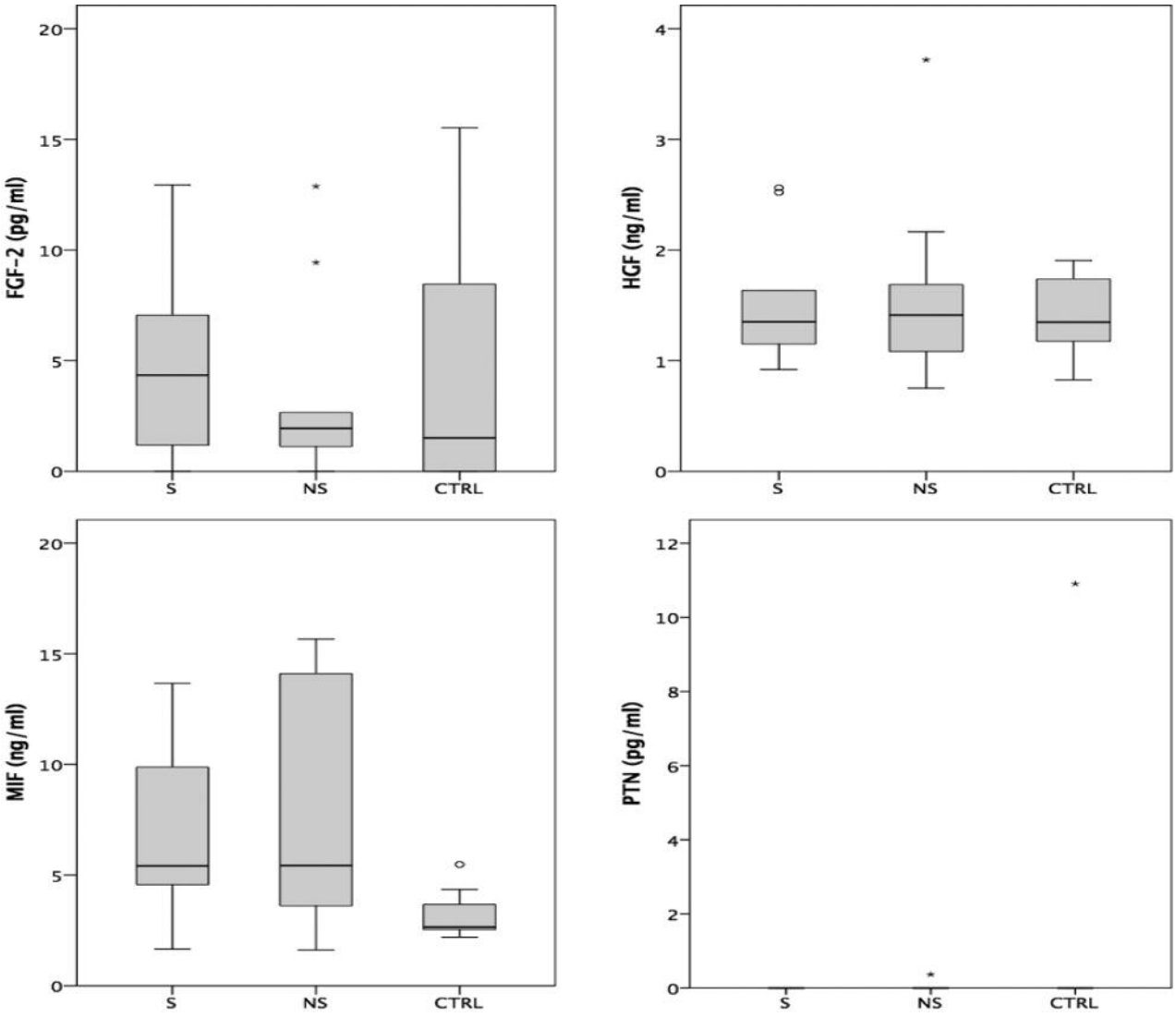

Figure 1. *Screening cohort: Analysis of serum levels of FGF-2, HGF, MIF and PTN in patients with seminoma (S, n=10) and non-seminoma (NS, n=10) testicular germ cell cancer and healthy men (CTRL, n=10). The concentration of MIF was significantly increased in cancer patients.*

thus the biomarker would lack specificity and would be of limited clinical value.

MIF levels were significantly increased in TGCT patients in the screening cohort of 30 study subjects, and were thus further investigated in an independent validation group consisting of 172 subjects. However, we did not observe a difference in MIF levels in TGCT patients, men with non-malignant testicular disease and healthy men. This finding highlights the need for validation of candidate biomarkers in independent and large-scaled cohorts which would also include relevant control subjects (*i.e.* non-malignant testicular tumors). Notably, we identified several expression profiling studies (17-20) showing increased MIF levels in seminoma TGCT compared to normal testis tissue using the NextBio database (21). A role for MIF as a diagnostic biomarker has been observed in other malignancies: *e.g.* serum MIF levels were increased in oral carcinoma (22), hepatocellular carcinoma (23), breast cancer (24), and ovarian cancer patients (25). Interestingly, a polymorphism in the *MIF* promoter region (-173G/C) was associated with an increased cancer risk (26).

Conclusion

Our study failed to confirm any diagnostic value for FGF-2, HGF, MIF and PTN in patients with TGCT.

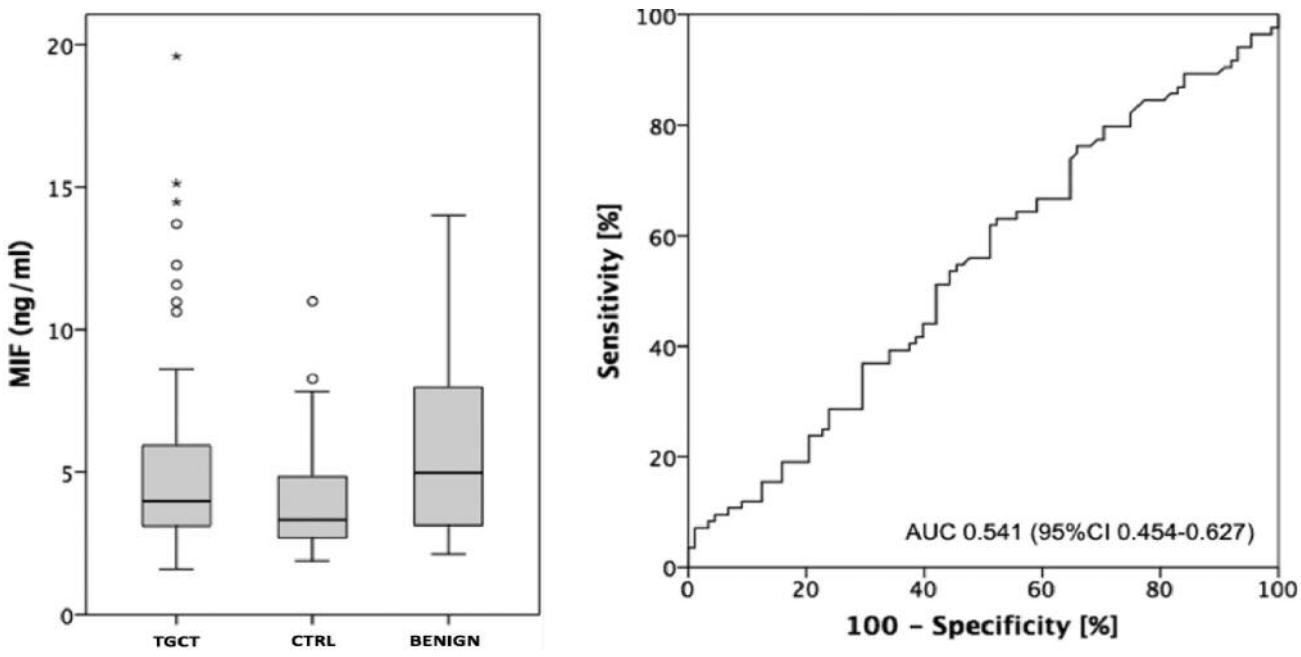

Figure 2. *Validation cohort: Analysis of MIF serum levels in patients with testicular germ cell cancer (TCGT, seminoma, n=48; non-seminoma, n=35), healthy men (CTRL, n=64) and patients with various benign testicular tumors/diseases (n=24). The concentration of MIF did not differ in cancer patients and control subjects. AUC: Area under curve; 95%CI:95% confidence interval.*

Conflicts of Interest

The Authors declare that they have no competing interests regarding this study.

Authors' Contributions

All Authors made substantial contributions to the conception, design, and acquisition of data. AK performed the experiments. AK, IS and JE analysed and interpreted the data. JE and SH designed the study. AK and JE drafted the manuscript. Samples were provided by KPD and SCM. IS, SH, KPD and SCM revised the manuscript. All Authors read and approved the final manuscript.

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