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ORIGINAL ARTICLE

Matrix metalloproteinases and proangiogenic factors in testicular germ cell tumors

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Summary

Purpose: *Testicular cancer is the most frequent solid tumor in young male adults and a disease with elusive pathogenesis. The purpose of this study was to determine the role of matrix metalloproteinases and angiogenic factors in the pathogenesis of testicular germ cell tumors (GCTs).*

Methods: *Between 2003 and 2006 we measured the serum levels of matrix metalloproteinase 2 (MMP-2), matrix metalloproteinase 9 (MMP-9), tissue inhibitor of matrix metalloproteinase 2 (TIMP-2), vascular endothelial growth factor A (VEGF-A), basic fibroblast growth factor (bFGF), platelet derived growth factor BB (PDGF-BB) and angiopoietin 2 (Ang-2) in 50 patients with testicular GCTs, at baseline, one month after the completion of the second cycle of chemotherapy and one year after the completion of chemotherapy, and in 16 male age-matched controls at baseline.*

Results: *At baseline, mean TIMP-2 value was lower in patients than controls, mean MMP-2/TIMP-2 ratio was higher in patients than controls and MMP9/TIMP-2 ratio was also higher. Ang-2 value was higher in patients than controls and bFGF value was also higher. Comparisons of the same parameters were also made among the 3 consecutive serum samples of the patients. All parameters normalized after chemotherapy except Ang-2 which remained elevated.*

Conclusion: *The present study supports the hypothesis that tumor invasion and angiogenesis play a role in testicular GCTs pathogenesis. Also an interesting hypothesis was formed, concerning the role of elevated levels of Ang-2 found in testicular GCTs patients in the pathogenesis of the increased long term cardiovascular morbidity of these patients. Larger prospective studies are needed to confirm our results.*

Key words: angiopoietin, germ cell tumors, matrix metalloproteinases, proangiogenic factors, testicular cancer

Introduction

Testicular GCTs are the most frequent solid tumors in young male adults with increasing incidence over the last decades. Overexpression of cyclin D2, loss of regulators of germ cell totipotentiality and genomic imprinting are important pathways in GCTs development [1], but despite advances in understanding molecular biology of testicular tumors, their pathogenesis remains obscure.

Angiogenesis, the recruitment of new blood vessels from pre-existing ones, and local tissue invasion are key steps in tumor growth, progression, invasion and metastasis [2-4]. MMPs may play an important role in these steps by providing the capability of extracellular matrix (ECM) proteolysis [5-7], and the most extended studied members of this protease family are gelatinases MMP-2 and MMP-9 [8,9]. The activities of MMPs are controlled by tissue inhibitors of metalloproteinases (TIMPs) [10]. Also several angiogenic factors have proven activity in the establishment of neovascularization and among the better studied factors are VEGF-A, PDGF-BB, Ang-2 and bFGF [11,12]. Although serum levels of gelatinases (MMP-2, MMP-9) and their inhibitors, as well as serum levels of various angiogenic factors, have been studied extensively in

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other malignancies and have established prognostic and predictive role [13-17], only scant data are available for testicular GCTs [18-24].

Therefore, we determined the serum levels of the above factors in 50 patients with testicular GCT prior to the initiation of chemotherapy, one month after the completion of the second cycle of chemotherapy and one year after the last cycle of it and in 16 healthy age-matched male controls. We compared the levels of these factors between patients and controls, between the various patient prognostic groups and between the 3 consecutive serum samples, in an effort to investigate the role of these factors in GCTs pathogenesis [25].

Methods

Fifty men (mean age 35.1 ± 8.1 years) with histologically confirmed testicular GCTs bound to undergo chemotherapy in "Theagenio Cancer Hospital" were enrolled in the study. Blood samples were drawn between 08:00 and 10:00 h after overnight fasting and after 10 min of rest. Three serum samples were collected: the first prior to the initiation of chemotherapy, the second 20-30 d after completion of second cycle and the third 9-14 mo after completion of chemotherapy.

Serum samples from 16 healthy male, age-matched volunteers were also collected at baseline and used as controls.

Patients were classified according to 3 risk groups. Low risk patients were those with stage I disease or stage II-III disease low risk patients based on the International Germ Cell Cancer Collaborative Group (IGCCCG) risk stratification system [26,27]. Intermediate and poor risk patients were distributed based on IGCCCG stratification for stage II-III disease. Favorable outcome was considered a complete response of the disease without relapse, while failure to achieve this was regarded as unfavorable outcome. The protocol was approved by the local ethics committee and informed consent was obtained from all participants in the study.

We measured serum MMP-2, MMP-9, TIMP-2, VEGF-A, bFGF, PDGF-BB and Ang-2, using quantitative sandwich enzyme immunoassay technique (Quantikine assay-RandD Systems, Minneapolis, USA for all the above except bFGF where we used Ray Biotech, Inc, RayBio, USA assay). We also calculated MMP-2/TIMP-2 and MMP-9/TIMP-2 ratio. The intrassay and interassay coefficients of variations (CVs) were <10%. At baseline, height, weight, body mass index (BMI) and body surface area (BSA) were calculated and are summarized on Table 1.

Table 1. Demographic characteristics of patients and controls

Variables	*Patients n=50*	*Controls n=16*	*p- value*
Age (years)	35.1±8.08	34.3±6.43	NS
Height (m)	1.78±0.06	1.79±0.05	NS
Weight (kg)	75.8±14.2	80.1±13.3	NS
BMI (kg/ m^2)	23.8±4.03	25±3.75	NS
BSA (m^2)	1.93±0.19	1.99±0.17	NS
Good risk	32		
Intermediate risk	10		
Poor risk	8		
Favorable outcome	37		
Unfavorable outcome	13		
Seminomas	6		
Non-seminomatous GCTs	44		

BMI: body mass index, BSA: body surface area, GCTs: germ cell tumors, NS: non significant

Statistical analysis

Non-parametric tests were used to compare mean values, because values of the variables in patients did not follow normal distribution. All p-values were calculated with two-sided tests. Mean values between patients and controls and between patient risk groups were compared. Means between patients with favorable and unfavorable outcomes were also compared. The results were verified by applying logarithmic transformations to all non-normally distributed variables to obtain near normal distributions and using Student's t-test the results were virtually the same. All analyses were carried out using procedures available in the Statistical Package for Social Sciences software (SPSS version 16, Chicago, IL, USA).

Results

Patients and controls had similar baseline demographic characteristics, including age, height, weight, BMI, and BSA, as shown in Table 1.

As shown in Table 2, at baseline, the serum levels of MMP-2, MMP-9, VEGF-A and PDGF-BB were similar between patients and controls. Mean TIMP-2 value was lower in patients than controls (82.53±38.1 vs. 106.94±27.9 ng/ml; p=0.01), mean MMP-2/TIMP-2 ratio was higher in patients than controls (4.036±2.7 vs. 2.755±2.76; p=0.04) and MMP9/TIMP-2 ratio was also higher (15.55±11.93 vs. 8.39±5.95; p=0.036). Ang-2 value was higher at baseline in patients than controls (4138.42±2987.2 vs. 2647.19±1186.1 pg/ml; p=0.045) and bFGF value was also higher (666.47±1720.46 vs. 551.98±1373.98; p=0.035), although the latter result was not confirmed with Student's t-test after logarithmic transformation of bFGF values.

Table 2. Comparison of MMP-2, MMP-9, TIMP-2, MMP-2/TIMP-2 ratio, MMP-9/TIMP-2 ratio, VEGF-A, bFGF, PDGF-BB and Ang-2, between controls and patients at baseline

Variable	*Patients (n=50)*	*Controls (n=16)*	*Mann-Whitney U-test p-value*	*Student's t-test p-value*
MMP-2 (ng/ml) (mean values±SD)	295.4±163.39	261.18±230.28	0.12	0.246
MMP-9 (ng/ml) (mean values±SD)	1132.1±875.1	892.56±722.42	0.338	0.383
TIMP-2 (ng/ml) (mean values±SD)	82.53±38.1	106.94±27.9	0.01	0.02
MMP-2/TIMP-2 (mean values±SD)	4.036±2.7	2.755±2.76	0.04	0.02
MMP-9/TIMP-2 (mean values±SD)	15.55±11.93	8.39±5.95	0.036	0.026
VEGF (pg/ml) (mean values±SD)	228.33±641.45	158.94±179.53	0.94	0.81
bFGF (pg/ml) (mean values±SD)	666.47±1720.46	551.98±1373.98	0.035	0.22
PDGF-BB (pg/ml) (mean values±SD)	10623.8±6375.98	8726.13±4318.16	0.342	0.309
Ang-2 (pg/ml) (mean values±SD)	4138.42±2987.2	2647.19±1186.1	0.045	0.013

For abbreviations see text

Comparisons of the same parameters were also made among the 3 consecutive serum samples of the patients (Table 3), prior to onset of chemotherapy (sample 1), after cycle 2 (sample 2) and 1 year after the completion of chemotherapy (sample 3). MMP-2 and MMP-9 values were similar for all serum samples. TIMP-2 values normalized gradually between first and second sample (85.25±39 vs. 109.38±56.3 ng/ml; p=0.013), between second and third sample (108.5±60.4 vs. 171.7±88.4 ng/ml; p=0.03) and between first and third serum sample (83.61±32.1 vs. 171.7±88.4 ng/ml; $p < 0.001$). MMP-9/TIMP-2 ratio normalized shortly after the beginning of chemotherapy, i.e. between the first and second sample (14.37±11.48 vs. 8.67±8.99; p=0.007). Thereafter, the results were similar between the second and third serum sample. The difference was also evident between baseline and one year after the completion of chemotherapy (13.7±9.33 vs. 8.54±8.61; p=0.014).

MMP-2/TIMP-2 normalized slower, as the results were similar between the first and second sample. The ratio normalized between the second and third sample (3.99±3.1 vs. 1.84±1.4; $p < 0.001$).

VEGF-A and bFGF values were similar for all serum samples. PDGF-BB values normalized shortly after the beginning of chemotherapy, between the first and second sample (11304±6426 vs. 7428±3519 pg/ml: p=0.001) and later remained stable between the second and third serum sample. Ang-2 values were similar for all serum samples, retaining the higher values in patients than controls, even one year after the completion of chemotherapy.

We also compared mean values of the same parameters between the various risk groups and between patients with favorable and unfavorable outcome (Table 4). There was a trend for lower TIMP-2, higher MMP-2 and higher MMP-2/TIMP-2 ratio in patients with worse prognosis and also a trend for higher bFGF and Ang-2 values in these patients, but to get reliable statistical results a larger population is required.

Discussion

MMP are a family of zinc-containing proteases that have an established role in tumor invasion, angiogenesis and metastasis [28,29]. MMP-2 and MMP-9, called gelatinases, are among the better studied members of this family with established prognostic and predictive role in a variety of tumors [30-33]. The activity of this family of proteases is regulated at many levels. The activity of MMPs in the extracellular space is specifically inhibited by TIMPs, which bind to the highly conserved zinc binding site of active MMPs at molar equivalence. The TIMP gene family consists of 4 structurally related members, TIMP-1, -2, -3, and -4 [34]. Although MMPs have been extensively studied in other urological tumors there are

Table 3. Comparison of MMP-2, MMP-9, TIMP-2, MMP-2/TIMP-2 ratio, MMP-9/TIMP-2 ratio, VEGF-A, bFGF, PDGF-BB and Ang-2, between the three serum samples of patients

Variable	*Sample 1/ /Sample 2 (n=44)*	*Wilcoxon test (p1) Student's t-test (p2) p-values*	*Sample 1/ /Sample 3 (n=33)*	*Wilcoxon test (p1) Student's t-test (p2) p-values*	*Sample 2/ /Sample 3 (n=33)*	*Wilcoxon test (p1) Student's t-test (p2) p-values*
MMP-2 (ng/ml) (mean values±SD)	298.32±162/ /336.6±236.3	p1: 0.092 p2: 0.324	319±170.8/ /258.3±142	p1: 0.057 p2: 0.093	359.9±251.2/ /258.3±142	p1: 0.088 p2: 0.052
MMP-9 (ng/ml) (mean values±SD)	1083.3±839/ /760.1±562.3	p1: 0.056 p2: 0.077	1089±799/ /1231±1017	p1: 0.675 p2: 0.971	813.5±613/ /1231±1017	p1: 0.122 p2: 0.254
TIMP-2 (ng/ml) (mean values±SD)	85.25±39/ /109.38±56.3	p1: 0.013 p2: 0.012	83.61±32.1/ /171.7±88.4	p1<0.001 p2<0.001	108.5±60.4/ /171.7±88.4	p1: 0.03 p2: 0.01
MMP-2/TIMP-2 (mean values±SD)	3.99±2.7/ /3.8±3.1	p1: 0.455 p2: 0.119	4.25±2.9/ /1.84±1.4	p1<0.001 p2<0.001	3.99±3.1/ /1.84±1.4	p1<0.001 p2<0.001
MMP-9/TIMP-2 (mean values±SD)	14.37±11.48/ /8.67±8.99	p1: 0.007 p2: 0.01	13.7±9.33/ /8.54±8.61	p1: 0.014 p2: 0.05	9.55±10.36/ /8.54±8.61	p1: 0.41 p2: 0.527
VEGF (pg/ml) (mean values±SD)	249.5±681.8/ /184.7±210.7	p1: 0.852 p2: 0.907	295.7±784/ /139±134.7	p1: 0.33 p2: 0.198	186.6±223.8/ /139±134.7	p1: 0.367 p2: 0.232
bFGF (pg/ml) (mean values±SD)	721.6±1827/ /322.5±569	p1: 0.294 p2: 0.149	915.3±2080/ /310.8±397	p1: 0.131 p2: 0.044	381.2±643.3/ /310.8±397	p1: 0.893 p2: 0.558
PDGF-BB (pg/ml) (mean values±S.D)	11304±6426/ /7428±3519	p1: 0.001 p2<0.001	11316±7084/ /6862±3889	p1: 0.004 p2: 0.003	6906±3294/ /6862±3889	p1: 0.979 p2: 0.587
Ang-2 (pg/ml) (mean values±S.D)	4259±3075/ /3966±1910	p1: 0.304 p2: 0.827	4455±3292/ /4404±854	p1: 0.228 p2: 0.341	4048±2075/ /4404±854	p1: 0.249 p2: 0.053

For abbreviations see text

no studies investigating the circulating levels of MMPs and TIMPs in testicular tumors [35,36].

In our study we found that mean TIMP-2 level was lower in patients than controls and that mean MMP-2/TIMP-2 and MMP-9/TIMP-2 ratios were higher in patients than controls. The serum level of these parameters normalized gradually during the course of chemotherapy and the 3 consecutive serum samples, with MMP-9/TIMP-2 ratio normalizing earlier than MMP-2/TIMP-2 ratio. Also there was a trend for higher TIMP-2 value in poor risk patients, although the relatively low number of patients included in our study preclude any subgroup analysis. These results are in accordance with the role of MMPs system in tumor initiation, invasion, angiogenesis and metastasis.

Angiogenesis, the formation of new microvessels from preexisting ones, is normally under tight control of angiogenic mediators [37,38]. Although the serum level of angiogenic mediators have been extensively studied in other malignancies [39-42] there are only scant reports for patients with testicular GCTs [21-25].

In these studies PDGF expression correlated with tumor progression in Leydig cell tumors, microvascular density of the tumor correlated with VEGF mRNA expression and occult metastasis in retroperitoneal lymph nodes and bFGF levels reflected disseminated disease in patients with testicular GCTs.

In this study mean VEGF-A serum level was similar between patients and controls, did not change during the 3 consecutive measurements in patients and was similar in all patient subgroups. PDGF-BB level was similar between patient and controls and between patient subgroups, but declined gradually during chemotherapy. The serum level of bFGF was higher in patients than controls and also higher in poor risk patient group. Ang-2 serum level was higher in patients than controls and in poor risk patient group and had not normalized even one year after the completion of chemotherapy.

Considering the role of angiopoietin pathway in ischemia [43,44] and the elevated long term cardiovascular morbidity of testicular cancer patients [45], we can make the assumption that these two facts are related, an assumption that needs large and long term prospective epidemiological studies to be proven.

Our data support the hypothesis that tumor invasion and angiogenesis, as reflected by serum concentrations of MMP-2, MMP-9, TIMP-2, VEGF-A, bFGF, PDGF-BB and Ang-2, play a role in testicular GCT pathogenesis. TIMP-2, bFGF and Ang-2 seem to have the greater impact. The small size of our study and the fact that all blood samples from the patients were drawn after orchiectomy, are potential limitations and make necessary the design and implementation of large prospective studies in order to elucidate the role of events at

Table 4. Comparison of MMP-2, MMP-9, TIMP-2, MMP-2/TIMP-2 ratio, MMP-9/TIMP-2 ratio, VEGF-A, bFGF, PDGF-BB and Ang-2, between the various subgroups of patients*

Variable (Mean values±SD)	*Sample*	*Good*	*Intermediate*	*Poor*	*Favorable*	*Unfavorable*
MMP-2 (ng/ml)	1	288.34±170.6	321±171.4	291.62±153.7	296.19±167	293.16±168
MMP-2 (ng/ml)	2*	281.58±204.9	462.15±286.4	369.56±232.4	301.77±205	441.16±299
MMP-2 (ng/ml)	3	227.99±51.2	229.86±62.46	368.8±278.55	232.3±54.5	327.69±254
MMP-9 (ng/ml)	1	1105.5±786.7	952.9±467.8	1462.4±1471	1055.5±750	1350±1170
MMP-9 (ng/ml)	2	828.6±652.4	743.9±389.6	518.97±323	810.45±612	609.1±354.2
MMP-9 (ng/ml)	3	1367.5±957.7	741.75±711.1	1438.5±1381	1192.7±871	1332.7±1394
TIMP-2 (ng/ml)	1*	74.78±37.24	85.6±28.5	109.69±42.92	84.14±40.7	77.96±30.59
TIMP-2 (ng/ml)	2	112.26±62.78	91.45±30.1	123.86±58.7	110.4±58.9	106.45±50.2
TIMP-2 (ng/ml)	3*	184.56±79.8	129.37±79.54	188.7±113.3	190.9±88.6	113.8±60.9
MMP-2/TIMP-2	1	4.299±2.88	4.153±2.78	2.86±1.59	3.96±2.7	4.26±2.8
MMP-2/TIMP-2	2*	3.15±2.72	5.48±3.58	3.9±2.97	3.5±3.1	4.69±2.87
MMP-2/TIMP-2	3*	1.33±0.34	2.23±1.1	2.62±2.6	1.36±0.42	3.26±2.23
MMP-9/TIMP-2	1	17.26±12.67	11.85±6.48	13.38±13.89	14.35±10.9	18.97±14.51
MMP-9/TIMP-2	2	9.73±8.55	8.55±5.1	4.75±2.95	9.34±10	6.65±4.4
MMP-9/TIMP-2	3	8.59±8.4	7.3±6.95	9.84±11.58	7.69±7.42	10.97±11.65
VEGF (pg/ml)	1	271.7±795.5	114.2±88.97	197.6±197.3	262.8±741.5	130.1±127
VEGF (pg/ml)	2	170.52±206	217.8±244.3	191.8±204.49	169.6±220	229.8±181
VEGF (pg/ml)	3	125.1±138.8	178.32±153.35	129.86±109.6	133.7±138	153±132.45
bFGF (pg/ml)	1*	582.2±1919.9	971.4±1722.2	622.58±643.6	623.3±1899	789.4±1112
bFGF (pg/ml)	2	379.26±648.2	172.53±223.9	317.61±609.5	352.4±597	232.5±488
bFGF (pg/ml)	3*	258.19±293.8	126.16±233.7	657.3±582.26	221.9±268	547.8±580
PDGF-BB (pg/ml)	1	9969.9±6558	11605.2±6815	12012.5±5369	10584±7023	10734±4242
PDGF-BB (pg/ml)	2	8202±3712.68	6606±2912.43	5616±2941.68	7533.1±3434	7112.2±3920
PDGF-BB (pg/ml)	3	7679.78±4160	6937±3777.75	4675.43±2718	7187±4087.5	5997±3361.5
Ang-2 (pg/ml)	1*	3413.9±2793	5549.6±3309	5272.5±2667	3981.2±3171	4585.7±2441
Ang-2 (pg/ml)	2*	3441.89±1277	4747±2410.5	4870.43±2672	3420.6±1252	5601±2596
Ang-2 (pg/ml)	3*	4297.33±618	4283.2±1029	4816.43±1149	4306±804.75	4665.3±975

*p: significant for these comparisons (Student's t-test+Mann-Whitney U-test)

molecular level in the pathogenesis of testicular GCTs.

Despite these limitations, clarifying the molecular profile of testicular tumors may provide useful information with respect to understanding tumor biology and may help in the design of specific targeted therapies. Moreover the level of these factors may have potential use as a measure of tumor aggressiveness and treatment response.

Also, the correlation of elevated levels of Ang-2 and possibly of bFGF with long term cardiovascular morbidity of these patients is an appealing idea, which merits further evaluation.

Conclusions

Testicular cancer is the most frequent solid tumor in young male adults and a disease with elusive pathogenesis. The present study supports the hypothesis that tumor invasion and angiogenesis, as reflected by serum concentrations of MMP-2, MMP-9, TIMP-2, VEGF-A, bFGF, PDGF-BB and Ang-2, play a role in testicular GCT pathogenesis. Also, an interesting hypothesis was formed, concerning the role of elevated levels of angiopoietin-2 in testicular GCT patients in the pathogenesis of the increased long term cardiovascular morbidity of these patients. Larger prospective studies are needed to confirm our results.

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