The Prognosis Value of the hTERT Gene in the Evaluation of Pulmonary Metastasized Testicular Carcinomas on a Reduced Number of Cases

MILENA ADINA MAN[1], DANA ALEXANDRESCU, IOANA NEAGOE[1], ANTIGONA TROFOR[3],

[1]University of Medicine and Farmacy „Iuliu Hatieganu" Cluj-Napoca, Romania
[2]University "Transilvania", Faculty of Medicine, Brasov, Romania
[3]University of Medicine and Farmacy Iasi, Romania
Cluj Napoca, Street Hasdeu 6
ROMANIA
manmilenaadina@yahoo.com

Abstract: Pulmonary metastases are frequently met in testicular cancers. Determining the pulmonary relapse model, studying prognosis factors for defining risk groups and applying different therapeutic strategies with the evaluation of survival represent the study's main objectives. We've taken into our study patients diagnosed with testicular cancer all presenting pulmonary, pleural or mediastinal metastases. We evaluated the risk factors correlated with survival: the average age is 29.63 (p=0.613), place of origin (p=0.895), histology (p=0.078 >0, 05); tumor markers for our batch: Beta HCG, AFP (alpha-fetoprotein); LDH does not influence survival (p=0.786), (p= 0.345) respectively (p= 0.153). Types of pulmonary metastases: (p=0.08 >0, 05). The presence of other metastases: does not influence survival (p= 0.439). The number of metastatic locations (p= 0.465 > >0, 05). Risk groups (p= 0.0254 <0, 0). Because the risk factors usually available are not sufficient to identify the subgroups of patients with an unfavorable prognosis, we tried to evaluate new genetic markers which could prove their prognosis value. The expression of the hTERT gene and the increase of telomerase's activity strongly correlate with the emphasis of certain malignant tumors as well as with the presence of metastases.

.Key-Words: hTERT Gene, testicular carcinomas, pulmonary metastasis, prognosis

1 Introduction Testicular cancer is a rare disease reaching only 1% of all cancer types characteristic to men with ages between 15 and 34 years (4, 6). Therapeutic improvements emerged at the same time with the introduction of chemotherapy in the 70's make it so that 95% of patients with testicular cancer (4) and 70-80% of patients with metastases become treatable (4). The etiology of germinal tumors is not known. An increase in the frequency among patients with development anomalies and testicle descent was described (2) and this signaled the existence of genetic components (5). The histology of testicular tumors divides these cancers into seminomas (50%), teratomas or non-seminomas and mixed tumors (6). The metastasis pattern is predictable. The first metastases to appear are retroperitoneal adenopathies (regional metastases). Hematogen metastases are responsible for the apparition of pulmonary nodules and left side over-clavicle ganglion metastases. Hepatic bony metastases are rare and represent an unfavorable prognosis factor.

2 Problem Formulation

THE WORK HYPOTHESIS Pulmonary metastases are frequently met among cases of testicular cancers. Determining the pulmonary relapse model, studying prognosis factors for defining risk groups and applying different therapeutic strategies with the evaluation of survival represent the study's main objectives. Knowing these prognosis factors and evaluating them allows us to identify metastasizing risk factors

(vascular invasion, histology of the primary tumor, precocious relapse). Identification of patients with risk relapse and metastasizing risk factors may change the treatment, surveillance and monitoring strategies for these patients with a substantial improvement of the survival rate.

MATERIAL Between 2000 and 2006 we studied 17 patients diagnosed with testicular cancer who presented pulmonary, pleural and mediastinal metastases. The patients were admitted at the "Leon Daniello" Clinical Pneumonology Hospital, the Oncology Institute from Cluj or the Oncology Department of the County Clinical Hospital. **Inclusion criteria**: patients with testicular cancer (tumors with germinal cells diagnosed from a histological point of view and/or serological one) which presented pulmonary metastases on the chest – pleural pulmonary x-ray and/or CT as well as the presence of Beta HCG and AFP markers. The histopathologic exam of pulmonary metastases was not necessary for the inclusion in the study.

METHOD Evaluation of the patients was made: trough anamnesis, clinical exam, biological samples and imagistic explorations. The histopathologic exam of the testicular tumor was made before acceptance into the study as it was established as an inclusion criterion. We analyzed the main prognosis factors: age, histology, position of the primary tumor, localization of metastases (regional or remote), the number of metastatic localizations, the type of pulmonary metastasis, the time span between the apparition of symptoms and diagnosis, the free interval the diagnosis of the primary tumor and metastasis, and value of tumor markers at the time of diagnosis. Based on these prognosis factors, the patients were divided into a good prognosis category (good) and unfavorable (poor) (4):

-Good risk: HCG and ATP increase, metastasized cervical ganglions, minimal pulmonary metastases (less than 5 on the pulmonary field and under 3 cm), mediastinal masses under 50% of the intra-thoracic diameter, solitary over 2 cm metastases with no abdominal masses;

-Poor risk: extended pulmonary metastases (more than 10 on the pulmonary field), pulmonary metastases bigger than 3 cm with/without abdominal masses, mediastinal masses greater than 50% of the intra/thoracic diameter, abdominal masses, non/pulmonary visceral metastases (hepatic, bone, cerebral).

The Multidisciplinary Board (oncologist, radiotherapist, surgeon, thoracic surgeon) decided the best therapy adapted to every case in part. The type of treatment applied was orchiectomy (before start of chemotherapy) associated with chemotherapy. The initial chemotherapy used Cisplatin in various combinations (bleomycin, vinblastin, etoposid, ifosfamid) with curative intent. Evaluation of treatment was made at 28 days after the first cycle and the last chemotherapeutic cycle. In case of remission we performed radiotherapy (20 Gy with 2.0 Gy for each session, 5 days a week).

We assessed within a single variant analysis different prognosis factors using the Kaplan Meyer method (type 1 standard error, 0.5 probability error, 95% confidence interval). Survival was calculated from the date the diagnose was established until the time of death. Because the risk factors usually available are not sufficient to identify the subgroups of patients with an unfavorable prognosis, we tried to evaluate new genetic markers which could prove their prognosis value. The expression of the hTERT gene and the increase of telomerase's activity strongly correlate with the emphasis of certain malignant tumors and it was detected also in pre-cancer lesions. We also sought to evaluate the involvement of the hTERT gene in testicular pathology. We analyzed the HTERT gene on 2 patients who had testicles with tumors that presented pulmonary metastases and one patient with a normal testicle. To this end we accomplished a quantitative determination (evaluation of the messenger RNA transcript) by the reaction of quantitative PCR (real time PCR) of the hTERT gene. The biologic products used for the evaluation of the gene evaluation level were both tumor tissue as well as normal testicle tissue. The stages necessary for the quantitative evaluation consisted in:

-Extraction of total RNA using the phenol-chloroform method;

-Purification of the RNA extracted through the Qiagen column;

-RNA's quantitative evaluation (NanoDrop ND 1000 spectral-photometer)

-Qualitative evaluation of the extracted RNA (bio-analyzer 2100);

-Synthesis of complementary DNA (DNAc) trough the reverse transcriptase reaction;

-Quantitative evaluation through the RT-PCR of the DNAc

3 Results

The diagnosis of testicular cancer was made before entering the study due to the apparition of symptoms on a local level in 15 from the 17 patients included in the batch. The pulmonary metastasis diagnosis was initially made only for two patients and we tried to find the starting point. In the hereditary collateral antecedents of 2 patients we emphasized first degree relatives with neoplasia (mother and grand mother with cancer without finding any men with testicular cancer). We only emphasized the presence of an ectopic testicle as risk factor in one patient. From the survival table for testicular cancer one can notice the 6 months survival rate was 0.93 (93%), the 13 months survival rate was 0.78 (78%), at 26 months it was 0.64 (64%), that at 39 months was 0.55 (55%) and the survival rate at 72 months equaled 0.38 (38%). These survival rates can also be seen in the diagram below:

The clinical pathological characteristics of patients with testicular tumors and evaluation of risk factors associated to the host.

The average age of the patients included in the study was 29.76 years (between 16 and 45 years of age). We cannot state if age is a prognosis factor for patients with testicular cancer (p=0.613). The average survival rates for patients younger than 30 years, respectively patients older than 30 are: 22.29 months, respectively 10.89 months.

The life environment: 65% of the patients included in the batch come from urban environments. Likewise, we could not state if the life environment is a prognosis factor for patients with testicular cancer in the case of the patients under study (p=0.895). The average survival rates for patients from an urban environment compared to those of patients from a rural environment are: 21.83 respectively 11.18 months.

If the time interval between the apparition of symptoms and diagnosis is shorter (respectively longer) than 6 months does not influence survival (p=0.622 >>0, 05) and this also applies if the reference time interval is 12 months (p=0.275).

Prognosis factors associated with the primary tumor (testicular tumor).

The histology of primary testicular tumors was established before admission into the study using orchiectomy. In the case of 4 patients (26%) it could not be confirmed (administrative reasons). In one case we could only emphasize an extended necrosis without being able to establish it into the type of testicular tumor (seminoma, non-seminoma, or mixed).

Our batch comprises cases of mixed tumors (65%) and non-seminomas (6%) but lacks cases of pure seminomas. This can explain the favorable evolution at a reduced number of patients (compared to data from literature). The histology of our batch does not influence survival, p= 0.078 >>0, 05.

The tumor markers for our batch: Beta HCG, AFP (alpha-fetoprotein), LDH does not influence survival (p= 0.786), (p= 0.345) respectively (p= 0.153).

Prognosis factors associated with the degree of tumor progression

All the patients in our study presented pulmonary metastases (2 unilateral, 15 bilateral, 5 other types of metastases). The types of pulmonary metastases: (unique 6%, micro-opacities 6%, multiple 7%, multiple + mediastinal adenopathies 6%, mediastinal adenopathies + pleuresy 6%) do not influence survival (p=0.08 >0.05). The micro and macro opacities do not influence survival (p= 0.171 >>0.05). The unilateral, respectively bilateral pulmonary metastases: statistically from out batch (2 patients could benefit from metastasistectomy) the types of pulmonary metastases (unique 12% or multiple 88%) do not influence survival: (p=0.149 >>0. 05).

The presence of other metastases: within our batch we encountered retroperitoneal metastases in 60% of cases, hepatic + retroperitoneal in 10% of cases, hepatic + peritoneal carcinomatosis 105, cerebral 105, and pelvic 10%. The presence or lack of ganglion retroperitoneal metastases or of other metastases does not influence survival (p=0.439, respectively p=0.115). The number of metastasis localizations does not influence survival in testicular cancer (p= 0.465 >>0.05).

Risk groups: if we divide patients into two groups as follows: the first group will contain patients with increased HCG and ACG and with multiple pulmonary metastases and the second group will consist of patients with unique metastasis and with retroperitoneal metastases and we found out that patients belonging to the first category have “high

risk" marking whereas the second group has a "poor risk" marking" (p=0.0254).

Treatment and response to treatment

Response to treatment influences survival: p =0.0064 < 0.05. It is noted that there is a favorable survival rate only in the case of 35% of patients. 24% only had a partial remission under treatment followed subsequently by evolution and death. Only 6 patients are still alive and have registered complete remission. Of the patients with an unfavorable evolution, the longest survival period was 18 months and the shortest was 3 months. For the patient who survives 3 months we tried to evaluate a genetic factor trough the presence of the hTERT gene. Extracting, purifying and making a quantitative evaluation of total RNAs from biological samples. From the qualitative analysis we noted the existence of a sample with degraded RNA (sample 2404) which did not correspond to quality requirements (heterogeneous base line and species ratio 28S/18S below the value of 1.5). From the data obtained so far we ascertained a grouping of samples into two subgroups specific to the type of pathology: a group of two samples were we noted an expression of the hTERT gene increased at the level of testicular seminomas respectively a group of two carcinomas compared to the adult teratoma considered as a reference. According to the data obtained we note that in the case of seminomas there is an increase in the level o gene expression equaling 1.215 E3 respectively 1.45E2 compared to the reference value taken into consideration for the adult teratoma. In the case of carcinomas, the level of expression is 2.93E3 for the embryonic one respectively 2.41E3 in the case of the epidermoide one compared to reference 1 for the adult teratoma. As we noted before in the evaluation of the hTERT gene, its level in aggressive testicular tumors with pulmonary metastases is way bigger than in the case of testicular tumors without dissemination.

4 Discussion

The incidence of testicular cancer is reduced and although the number of patients with testicular cancer is small, more than half of these cases present metastases. In Japan, for a population of 120 million inhabitants there are less than 50 new patients diagnosed every year compared to the USA where there are 7200. The evaluation of their evolution and identification of the prognosis factors made the object of several studies because the rate of survival of patients with a metastatic disease is very variable even with the new cytostatic treatments. There are descriptions of survival rates of only 45-55% among patients with metastasized testicular tumors compared to an 80-90% survival rate among patients without the disseminated disease. Identifying the subgroups of patients with a less favorable prognosis is the purpose of numerous prognostic models.

Bower and collaborators reported an average age between 27.3 (between 14-72%) (3). Kenneth Kester reported (in a study of 421 patients) an average age of 26.8 years. Stuart Kinton described in his study an average age of 32 years with ages between 19 and 52 years (7). Schmoll reported at the "European Congress for the Diagnosis and treatment of Germinal Tumors from 2004" an age of 34 years which he considered irrelevant as a prognosis factor in the evolution of patients (9). Aass reported an age over 35 years as an unfavorable prognosis factor. Within our batch we noticed an average age of 29.63 years (between 16 and 45) with a predominance in the 30 to 40 years age group (7 patients) without considering age a statistically significant prognosis factor (p=0.613).

The life environment and social economic level can influence the act of appearing before a doctor. In our study tough, this has not been a significant prognosis factor (p=0.895).

The time between the apparition of symptoms and the establishment of a diagnosis can represent a factor that may influence survival due to the increased stage of presentation. In our study, the average was 6.1 months and this ensues form the high stage of presentation of all patients from the batch (stage III and IV). Considering the delay is establishing a diagnosis, this did not influence survival (p=0.622). Other studies reported much smaller time intervals. In his study, Collette reported an average of 10 days from the date the disease made its appearance and the date diagnosis was established (4).Early metastasis did not always giverise in the limph nodes (13).Serum tumor markers play an important role, vital even, in the evolution of patients with testicular cancer (5). The 5 years survival rate for patients with testicular cancer is 96%. Tumor markers contribute to this favorable evolution due to the completion of the diagnosis on one hand and the monitoring of

follow-ups after orchiectomy on the other hand. They are important prognosis factors and their values, before the start of therapy help identification into various risk groups and indirectly help establish the therapeutic conduct. Also, their monitoring allows early identification of relapse cases after the start of treatment. Tumor markers are considered to be independent prognosis factors on one hand by participating in establishing the diagnosis as well as for their role in the early discovery of reappearance. Aass reported in a multivariate analysis that AFP greater than 1000 µg/l and HCG higher than 10.000units/l are unfavorable prognosis factors. Generally, the level of tumor markers after the 10^{th} day of the first chemotherapy cycle reflects the evolution under treatment without being highly selective (11). In our study (as in many other analyses) we have not assessed the discriminative value of these markers' decline. How quickly they decrease, in what percentage and how sensitive the modification of their value is. We haven't calculated the markers' standardization interval which in some studies from literature was defined as an independent prognosis factor (11). In our study the value of LDH, beta HGC and AFP did not represent a significant statistic prognosis factor (0.153; 0.786 and 0.345).

The performance status of patients influences survival in numerous neoplasias.

This was not a significant prognosis factor for the testicular tumor cases with metastases from our studies. Collette reports the influence of the performance status on survival (4). Patients with pulmonary metastases were included into different risk groups and depending on the other prognosis factors (number of metastatic localizations, position of the primary tumor, values of tumor markers). Although all patients had pulmonary metastases, in our study not all were included in the "poor risk" category. The chemotherapy treatment was made with various combinations of Cisplatin followed by the surgical resection of the residual tumor. The response to treatment was 35% complete response, 24% partial response and 41% progressive response. Motzer (12) reported a ratio of complete response among "good risk" patients (treated with four cycles of Etoposid + Cisplatin or three cycles of BEP) of 90%.

20 to 30% of patients with advanced testicular cancer suffered either from relapse or had an incomplete response to chemotherapy. These patients can be identified however during the initial presentation into "poor risk" groups (histology non-seminoma, pre-therapeutic high levels of markers, hepatic, bone, cerebral metastases, mediastinal localizations of primary tumors). Half of all "poor risk" patients will die. Patients who obtained only a partial response with the first BEP line and who were progressive were considered to be refractory and a second line of treatment was administered. Few of these patients will have a complete response with the rescue therapy (vinblastine, ifosfamide, cisplatin). These patients have a low chance of survival despite the high performance treatments (12).

The survival rate of patients with testicular cancer is generally very good as this is considered a type of curable cancer with a 5 year survival rate of approximately 90%. In the US and Europe this survival rate can reach even 95%. In Japan, although the survival rate has improved it could not be reported statistically due to the rarity of the disease (1, 11). Bower reported in a study conducted on 339 a rate of survival at 5 years of 82%. For patients with an unfavorable prognosis, the 3 year survival period was 75% (3). Schmoll and Kollmannsberger (Eupean Consensus) reported a 90% survival rate for patients with a favorable prognosis (56% of them with a favorable prognosis), 80% for patients with intermediary risk (28% of them) and a 50% survival rate at 5 years for patients with an unfavorable prognosis (16% of them) (5). Kenneth Kesler noted in a study of mediastinal metastases a survival rate at 5 years of 86% +/- 2% and a 74% +/- 4% survival rate at 10 years. De Vita reported survival within localized stages at 95.1% in cases with regional invasion 69.4% and with remote invasion 33.1 % (4). Stuart Kinton published in his study (trial of the Eastern cooperative oncology group) the favorable response of 80% of cases and a lasting response among 73% of patients with tumors of the germinal cells (8). In his study, Motzer published a complete response at 77%, incomplete response at 20%, partial response but with a standardization of tumor markers at 3% and relapse at 6%. He also reported a complete response based on prognosis factors 86%, 50% and 25%. Of the patients who had an unfavorable response and received rescue therapy with increased doses of chemotherapy medicines

associated with autolog Stem cell transplant, 57% had a complete response and 35% a durable response for more than 18 months. We observe the significant differences of evolution and survival between various reported studies (2, 7, 13, 14). This is explained on one hand by the different experience that various oncology centers have, by the diagnosis conducts and non-standardized therapeutic conducts, varied access to last generation medicines, application of chemotherapies with large dosages followed by autolog bone marrow transplant only in few centers and on a reduced number of patients.

Generally, patients with metastases will have a different prognosis depending on the relapse model and will require individual strategies selected based on the risk factors by combining available therapeutic modalities (irradiation, chemotherapy, surgery of residual tumor masses). This entire arsenal can lead to a very high healing rate. Nevertheless, testicular cancer cases without a complete remission are some of the most disseminative cancers. Early diagnosis and treatment of metastasis may lead to an improvement in the survival rate of cancer patients. The diagnosis of metastasis using molecular biological techniques has been attempted with various tissues including blood, pancreatic juice, ascites, lymph nodes, but the methods is still controversial. Micrometastasis, which is not detectable by routine histological examinations, can now be identified by genetic methods (10).

5.CONCLUSIONS

The presence of pulmonary metastases does not necessarily imply an unfavorable risk group if the patients have not presented a previous therapeutic failure and if they are not accompanied by other visceral metastases (liver, brain, bone). The evaluation of prognosis factors among patients with testicular tumors and pulmonary metastases emphasized the relatively reduced value of clinical prognosis factors: age (p=0.613), environment (p=0.895), performance indexes (p=0.096), the time interval between the apparition of symptoms and appearing before a medic (p=0.275), LDH (p=0.153), AFP (p=0.345), beta HCG (p=0.786) do not represent statistically significant prognosis factors which could influence survival. After the apparition of pulmonary metastases, the type and number of pulmonary metastases do not influence survival from a statistically significant point of view (p=0.08; p= 0.149). Knowing the prognosis factors and how they are used to identify patients into different risk groups is of vital importance of the management of testicular cancer therapy. Identification into good risk and poor risk groups has a statistical significance as a prognosis factor (p=0.0254). The data and results of the studies on clinical – imagistic bases are limited due to the small number of patients included into the study (low incidence). The evaluation of other prognosis factors at an immune-histological, genetic or molecular level allows the redefinition of the prognosis and improvement of the germinal tumors' management. We emphasized the presence of the hTERT more expressed from a quantitative point of view at the level of testicular tumors with pulmonary metastases compared with tumors without pulmonary metastases. Until the identification of new prognosis factors (for example hTERT), validated by future studies, treatment and conduct will be based on the predictive value of other classical prognosis factors. Long term survival (over 5 years) and the curability rate of patients with testicular cancer, although in literature is above 90%, our study revealed only a 35.29% survival rate.

Presently, survival among patients is low although improved compared to the last few years before the discovery of chemotherapy with Cisplatin. This justifies the increase of investigations regarding patients with unfavorable risk factors. Understanding the biology and tumor cell genetic can become research therapeutic targets.

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