Original paper

# Histopathological assessment of residual retroperitoneal mass removed in patients after chemotherapy for non-seminomatous germ cell tumours of the testis

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Between 1990 and 1999, 182 men were treated for non-seminomatous germ cell testicular tumours. In 24 of them after chemotherapy a residual retroperitoneal mass was removed. In 14 of them additional immunohistochemical (IHC) examinations using antibodies against cytokeratins, vimentin, PLAP, CD30, AFP, hCG, p53, and MIB-1 were performed. We compared the results of those additional studies with the results of routine histopathological examination. Histological assessment revealed most frequently (ca. 54% of cases) non-neoplastic lesions, i.e. fibro-cystic, necrotic or inflammatory tumours and lymphatic tissue. In about 33% of cases, surviving live neoplastic cells were found.

**Key words:** germ cell tumours, retroperitoneal mass, immunohistochemistry.

## Introduction

Incidence of testicular cancer in Poland is 2.4 new cases in 100 000 men per year [1]. Analyzing the incidence in relation to age, three different groups of the highest incidence may be established: infants, 25 to 40 year olds, and about 60 year olds. It is important that in young males (20-35 years old) this cancer is the most frequent one [2]. Mean age of non-seminomatous germ cell tumour of the testis (NSGCTT) patients is 25 years. The highest incidence is noted in the Scandinavian countries, Switzerland, Germany and New Zealand, and the lowest in Africa and Asia. In other European countries and in the USA incidence levels are average.

More and more precise understanding of cancer natural history is being gained from numerous multi-specialist studies. New algorithms of different sub-types of testicular cancer management are being developed. The key problem is to identify risk and prognostic factors. Recognition of these factors allows one to manage more properly the disease at different stages.

Germ cell tumours of the testis comprise a group of morphologically diverse neoplasms. The classification most frequently used by practitioners and recommended by the WHO is shown in Table I.

Non-seminomatous germ cell tumours (NSGCTs) are a group constituting about 50% of all germ cell tumours of the testis. The majority of them have two or more histological components, being mixed non-seminomas. In such cases the non-seminomatous component is crucial for prognosis and treatment. Precise, unequivocal and reliable histopathological diagnosis allows proper staging and employment of proper systemic therapy. It is very important in diagnosis of morphology of both primary tumour of the testis and the residual retroperitoneal mass after chemotherapy removed by retroperitoneal lymphadenectomy. Use of modern immunohistochemical (IHC) markers in histological diagnosis may improve its accuracy. The most frequently used IHC markers are cytokeratins, vimentin, PLAP, CD30 antigen, AFP, βhCG, p-53 protein, and Ki-67 antigen.

Cytokeratins are structural proteins of epidermal cells. In malignant testicular tumours expression of cytokeratins is typical in embryonal carcinoma cells. Among different cytokeratins a positive reaction to cytokeratin 8 (CK8), cytokeratin 18 (CK18) and cytokeratin 19 (CK19) is noted. Expression of CK19 is specific for embryonal carcinoma because it is not seen in other malignant germ cell tumours of the testis. Otherwise, a positive reaction to CK8 and CK18 is observed in seminomas, on average in about 40% in the literature of the subject. In different articles frequency of expression of CK8 and CK18 in seminomas varies from 0% to 73% [3].

Vimentin is a protein isolated from murine fibroblasts [4]. It is positive in almost all early, fetal cell lines [5]. It is not a cell-specific marker but in correlation with other markers it may be clinically helpful. It is especially useful in diagnosis of renal, endometrial, follicular thyroid and salivary gland cancer metastases [6].

CD30 antigen is one of the cluster of differentiation group of antigens and is seen most frequently on the surface of leukocytes [7]. The majority of seminomas do not show expression of CD30, in contrast to embryonal carcinoma [8]. There is a theory that if a seminoma is CD30 positive, it had transformed clinically into embryonal carcinoma. CD30 is a useful tool to distinguish between embryonal carcinoma and other germinal tumours, in which it is always negative [9].

PLAP (placental alkaline phosphatase) physiologically is produced by the placenta after 12 weeks of pregnancy and is responsible for cellular transport, proliferation and differentiation of cells, and metabolism and gene transcription [10, 11]. In the human body it may also be produced by cancer cells [12]. PLAP is not a specific marker of testicular tumours but it is also present in digestive system, pulmonary, breast and female reproductive organ cancers [13, 14]. Activity of this enzyme is noted in 98% of seminomas and *"carcinoma in situ (CIS)"* lesions, in 86-97% of embryonal carcinomas and in 85% of yolk sac tumours. Normal testicular tissues, with no CIS lesions, testicle dysgenesis, cryptorchidism or testicular neoplasm evidence, have no PLAP activity. This activity in choriocarcinoma is less than 50% [15].

**Table I.** WHO histological classification of testis tumours

| |
|---|
| **I. Tumours of one histological type (pure forms):** |
| 1. Seminoma |
| Seminoma with syncytiotrophoblastic cells |
| 2. Spermatocytic seminoma |
| Spermatocytic seminoma with sarcoma |
| 3. Embryonal carcinoma |
| 4. Yolk sac tumour (endodermal sinus tumour) |
| 5. Poliembryoma |
| 6. Choriocarcinoma |
| 7. Teratoma |
| Dermoid cyst |
| Monodermal teratoma |
| Teratoma with somatic type malignancies |
| **II. Tumours of more than one histological type (mixed forms):** |
| 1. Mixed embryonal carcinoma and teratoma |
| 2. Mixed teratoma and seminoma |
| 3. Choriocarcinoma and teratoma/embryonal carcinoma |
| 4. Others |

βhCG – β subunit of human chorionic gonadotropin – is synthesized by trophoblastic cells. βhCG serum level is elevated in the majority of choriocarcinoma patients and in about 10% of patients suffering from seminoma. During an IHC examination a positive reaction to βhCG helps to identify multinuclear trophoblastic cells in choriocarcinomas, embryonal carcinomas and seminomas.

AFP – α-fetoprotein – is a protein secreted mostly by the yolk sac and fetal digestive system epithelial cells. In adults it may be produced by regenerating hepatic cells. AFP serum level is elevated in nearly 75% of patients with nonseminomatous germinal tumours, unlike in patients suffering from seminomas, in which we never observe AFP secretion, so the expression of AFP in germinal tumour cells indicates its nonseminomatous character. AFP is also a marker of hepatocellular carcinoma.

The p53 suppressor gene is located on the short arm of chromosome 17 (17p13). It is responsible for the cell replication cycle; it stops progression to the S-phase. Mutations or inactivations of the p53 gene are frequently associated with neoplasms in humans

and lead to dysfunction of cell growth regulation [16]. Mutations in the p53 gene also lead to prolongation of the half-life of p53 protein. This fact allows p53 protein to be identified in the cell nucleus using IHC methods [17]. Overexpression of p53 protein is associated with poor prognosis in different kinds of cancer [18].

Ki-67 (MIB-1) is a marker of proliferative activity. The percentage of MIB-1-positive nuclear cells (index) is used in uropathology, especially in bladder and prostate cancer evaluation. Particularly in the former, together with p53 protein expression, grading and cell anaplasia may be predictive factors of recurrence or progression of disease [19]. This index can also be sometimes useful in uncertain clinical situations in cancer of the testis.

Objective: histopathological re-evaluation of removed residual retroperitoneal mass in patients suffering from non-seminomatous cancers of the testis after chemotherapy including immunohistochemical markers.

## Material and methods

Between 1990 and 1999, 182 men suffering from nonseminomatous testicular cancers were treated in the Maria Skłodowska-Curie Memorial Institute of Oncology, Cracow Branch. This group was retrospectively analyzed.

Mean age of patients was 28.9 years.

Clinical staging was assessed using the TNM classification (UICC 1997) and the American Joint Committee on Cancer classification. 79.68% of patients were classified as IA to IIC grade. 82.22% of patients had pT1 to pT2 tumours, and 71.98% of patients did not have distant metastases – M0.

Histological diagnosis was performed according to criteria in conformity with the WHO classification. Mixed nonseminomas were diagnosed in as many as 69.06% of those treated.

The mean follow-up period was 52.2 months.

In the group of 182 patients, 24 of them were qualified for surgery because of persistence of a retroperitoneal mass after chemotherapy. In all patients retroperitoneal lymphadenectomy with routine microscopic assessment was performed.

In 14 of them removed specimens were re-assessed on average 61 months later, and additionally IHC staining using cytokeratins, vimentin, PLAP, CD30, AFP, βhCG p53, and MIB1 markers was performed. We compared the results of routine histology and IHC staining examinations.

Specimens for the additional histological and IHC studies were obtained from paraffin blocks containing original tissue of retroperitoneal masses excised from the 14 operated men and stored in the Archive of the Department of Pathology. Microtomic samples of 4 $\mu$m from the blocks were taken, H&E stained and assessed by a consultant pathologist to choose representatives blocks to perform IHC staining. Representatives samples of 4 $\mu$m were put on 'SuperFrost plus' type microscopic slides and heated in 60°C for 24 hours.

Slides were then deparaffinized in xylene (30 minutes twice), placed in absolute alcohol for 5 minutes, twice for 5 minutes in 96% alcohol and rinsed in running water. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide solution for 20 minutes. Before staining for cytokeratin AE1/AE3 expression slides were initially digested with pronase (DAKO, pronase cat. no S2013) for 15 minutes at room temperature. Samples prepared for AFP, hCG, CD30 antigen, MIB-1, PLAP and two epitopes of the p53 gene product (P53-BP and P53-1801) assessment were placed in citrate buffer and subjected to microwaves twice for 10 minutes. After cooling down to room temperature samples were incubated in normal serum. Then they were incubated with primary antibody (Table II) overnight at 4°C followed by a peroxidase-conjugated polymer (DAKO En-Vision/HRP, Rabbit/Mouse, K5007). Finally, the sections were incubated with diaminobenzidine (DAB,

**Table II.** Primary antibodies used in the study

| Antigen | Clone | Source | Dilution | Retrieval |
|---|---|---|---|---|
| Cytokeratins | AE1/AE3 | Cell Marque | 1 : 100 | Citrate buffer pH 6.0 |
| Vimentin | V9 | Cell Marque | 1 : 250 | Citrate buffer pH 6.0 |
| PLAP | 8A9 | Novocastra | 1 : 40 | Citrate buffer pH 6.0 |
| CD30 | BerH2 | Cell Marque | 1 : 30 | Citrate buffer pH 6.0 |
| AFP | Polyclonal | DAKO | 1 : 200 | – |
| hCG | – | DAKO | 1 : 600 | Citrate buffer pH 6.0 |
| P53 | 1801 | Novocastra | 1 : 40 | Citrate buffer pH 6.0 |
| P53 | BP-53-12 | Novocastra | 1 : 50 | Citrate buffer pH 6.0 |
| Ki-67 | MiB1 | DAKO | 1 : 100 | Citrate buffer pH 6.0 |

DAKO S 3000) solution, followed by counterstaining with hematoxylin, then dehydration and mounting.

The slides stained immunohistochemically were assessed by a consulting pathologist using an Olympus optical microscope. Reaction to cytokeratin, vimentin, PLAP, AFP and βhCG was considered as positive when it was positive in tumour cell cytoplasm, and for CD30 and p53 protein cell membrane and nuclear reaction respectively. Estimating the reaction to MIB-1 (Ki-67) proliferation antigen, the proportion of stained cell nuclei was counted among 500 cells regardless of the intensity of staining in five high power fields (HPFs).

## Results

A group of 24 men were qualified for retroperitoneal lymphadenectomy and operated on for the residual mass after chemotherapy. All surgical specimens were routinely histologically examined. For the purposes of our work teratoma has been separated from other malignancies.

The most frequent pathological lesions (in about 54% of cases) were non-neoplastic lesions, i.e.: fibro-cystic, necrotic or inflammatory tumours, and lymphatic tissue. However, in nearly 33% of cases, surviving live neoplastic cells were found (Table III). In 12.5% of cases teratoma was found.

Results of the assessment of chosen tissue antigens (cytokeratin, vimentin, PLAP, CD30, α-fetoprotein-AFP and βhCG) and p53 suppressor gene product and Ki-67-1 (MiB1) antigen expression are shown in Table IV.

In examined group A – patients with surviving retroperitoneal malignant residual lesions diagnosed in routine pathological examination – IHC staining was performed in seven cases. In four of them the second histological and IHC assessment confirmed the diagnosis of a malignancy: embryonal carcinoma, squamous cell carcinoma and choriocarcinoma, twice. In the next three cases teratomas were diagnosed. Embryonal carcinoma texture was recognized by co-expression of cytokeratins, CD30 antigen and AFP. Cells of choriocarcinoma were identified by positive reaction to cytokeratin and βhCG and in one case also to vimentin and PLAP presence. In the cells of squamous carcinoma co-expression of cytokeratin, vimentin and CD30 antigen was observed. The index of proliferation MIB-1 (Ki-67) in all these cases was high and ranged from 14.4 to 78.0%.

In group B – patients with teratoma – histological and IHC evaluation confirmed the diagnosis.

In group C – patients with non-neoplastic lesions in routine microscopic examination – histological reassessment and IHC staining of specimens of six patients allowed teratoma to be diagnosed and the previous diagnosis to be changed.

Cells of benign teratoma in all these IHC examined cases were characterized by a low index of proliferation MIB-1 (Ki-67) between 1.4% and 7.6%.

## Discussion

Testicular cancer is one of the most promising cancers. This is due to routine combination therapy combining surgery, chemotherapy, and radiotherapy. Postoperative assessment of removed residual tumour of the retroperitoneal space is one of the factors contributing to this effect. The practical question is the real influence of modern chemotherapy on the histological texture of retroperitoneal metastases in comparison with the microscopic picture of primary tumour of the testis.

The segregation of patients based on the evaluation of the surgical specimen into three different groups (group A with persistent malignant cancer tissue, group B with teratocarcinoma and group C patient with nonmalignant changes) influences the patient's prognosis. Our results showed that additional immunohistochemical studies changed the results of routine microscopic examination in some patients in groups A and C, and confirmed all results in group B.

Searching the literature we could hardly find a paper concerning a similar matter. What is more, the data that describe the changes in the biology of metastases after chemotherapy as compared to the primary tumour are also limited.

Ito and others described a case of testicular cancer with malignant transformation in a residual retroperitoneal mature teratoma 8 years after the initial chemotherapy (cisplatin, vinblastine, and bleomycin combination – PVB) for a mixed germ cell tumour

**Table III.** Routine histopathological post-operation evaluation of excised retroperitoneal residual tumour persisting after chemotherapy

| Histological diagnosis | Number of patients | Percentage |
|---|---|---|
| A<br>surviving live neoplastic non-teratomatous cells | 8 | 33.3 |
| B<br>Teratoma | 3 | 12.5 |
| C<br>necrosis, fibro-cystic or inflammatory lesions, lymph nodes | 13 | 54.2 |
| Total | 24 | 100 |

**Table IV.** IHC assessment of chosen neoplastic markers' expression in tissue specimen after surgery for persistent retroperitoneal mass after chemotherapy

| Group | Sample no. | Cytokeratin | Vimentin | PLAP | CD30 | AFP | βhCG | P-53 | MIB-1% |
|---|---|---|---|---|---|---|---|---|---|
| A | 264.043 | + | – | – | – | + | + | + | 14.4 |
| A | 207.199 | + | + | – | + | – | – | – | 35.0 |
| A | 233.090 | + | – | – | + | +/– | – | + | 78.0 |
| A | 215.754 | + | + | + | – | – | + | – | 36.8 |
| A | 198.636 | + | + | – | – | –+ | – | – | 7.6 |
| A | 210.660 | + | + | + | – | –/+ | – | – | 4.0 |
| A | 229.979 | + | + | – | – | – | – | – | 1.0 |
| B | 258.237 | + | + | + | + | – | – | – | 6.3 |
| C | 269.388 | + | + | – | – | – | – | – | 6.4 |
| C | 257.447 | + | + | + | – | – | – | – | 4.2 |
| C | 200.681 | + | + | + | – | –/+ | – | – | 4.8 |
| C | 210.097 | + | + | + | – | – | – | – | 7.2 |
| C | 232.407 | + | + | + | – | – | – | – | 3.3 |
| C | 250.263 | + | + | – | – | –/+ | – | – | 1.4 |

*+ positive reaction, – negative reaction, –/+ poor reaction, +/– focal or single-cell reaction*
*PLAP – placental alkaline phosphatase; CD-30 – cluster of differentiation 30 antigen; p53 – p53 suppressor gene protein product; MIB-1 – mitotic index; AFP – α-fetoprotein; βhCG – β subunit of human chorionic gonadotropin*

composed of immature teratoma, embryonal carcinoma and seminoma. In retroperitoneal lymph node dissection (RPLND) massive necrosis and immature teratoma were found. One cause of combination chemotherapy with cisplatin and etoposide was given following the surgery. In the follow-up, enlargement of the residual lymph nodes around the left renal artery was detected by CT scanning. Since both PVB chemotherapy and the combination of cisplatin with etoposide did not achieve remission, RPLND was performed again. Histological examination revealed a mature teratoma which was far more differentiated than that resected earlier. Next, several enlarged para-aortic lymph nodes were found without the elevation of tumour markers, and en-bloc RPLND was performed. Microscopically, mature teratoma was observed in almost all specimens and, unexpectedly adenocarcinoma was also observed in enteric elements of the mature teratoma [20]. Undoubtedly, chemotherapy can modify the histological picture of metastatic tissues. It was confirmed by Mayer *et al*. in their paper published in 2011. The authors compared the histological picture of primary tumours (n = 9) and relapses (n = 9) which occurred after chemotherapy of patients with non-seminomatous malignant germ cell neoplasms. They found that not only teratoma can be found in post-chemotherapy residual tumours. The histology in relapse was pure yolk sac tumour in four of the nine patients analysed. Three had a non-germ cell malignancy, one was a mixed non-seminoma, and one was a pure mature teratoma [21].

There is a hypothesis that in nonseminomatous germ cell tumour (NSGCT) the pluripotent embryonal carcinoma (EC) cells are the precursors of the manifold differentiated structures but also drive the malignant growth. Mueller et al. believe that lack of OCT4 expression indicates cisplatin resistance. They found that post-chemotherapy residual metastatic tumours can be composed of exclusively OCT4-negative EC [22].

What is more, embryonal carcinoma after chemotherapy can give metastases of different histology to a different location. Such a case was described by Japanese researchers. Fujimura *et al*. recognized mature teratoma in a residual tumour and several years later they found a relapse in the retroperitoneal space and simulta-

neous metastases to supraclavicular lymph nodes in the form of mucinous cystadenocarcinoma [23].

## Conclusions

Additional immunohistochemical staining for cytokeratin, CD30, AFP and βhCG is helpful in pathological evaluation of the kind of neoplasm in retroperitoneal residual masses of germinal tumours after chemotherapy.

*The authors declare no conflict of interest.*

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