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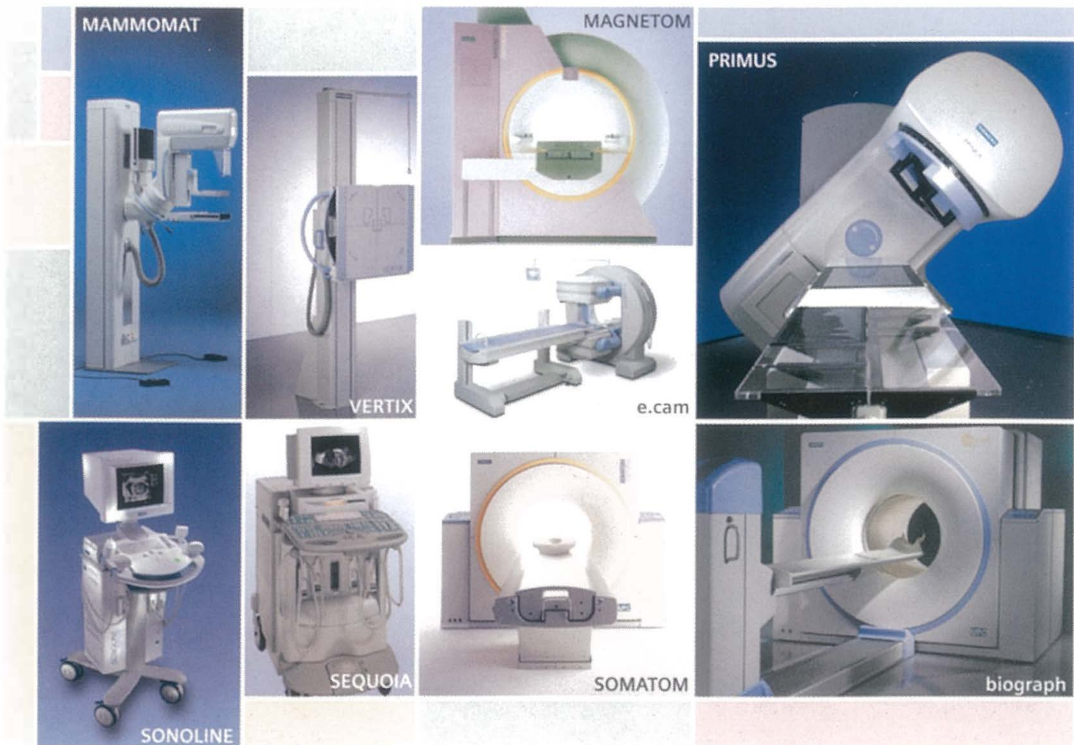

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review

Tumor markers in clinical oncology

Srdjan Novaković

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The subtle differences between normal and tumor cells are exploited in the detection and treatment of cancer. These differences are designated as tumor markers and can be either qualitative or quantitative in their nature. That means that both the structures that are produced by tumor cells as well as the structures that are produced in excessive amounts by host tissues under the influence of tumor cells can function as tumor markers. Speaking in general, the tumor markers are the specific molecules appearing in the blood or tissues and the occurrence of which is associated with cancer.

According to their application, tumor markers can be roughly divided as markers in clinical oncology and markers in pathology. In this review, only tumor markers in clinical oncology are going to be discussed. Current tumor markers in clinical oncology include (i) oncofetal antigens, (ii) placental proteins, (iii) hormones, (iv) enzymes, (v) tumor-associated antigens, (vi) special serum proteins, (vii) catecholamine metabolites, and (viii) miscellaneous markers.

As to the literature, an ideal tumor marker should fulfil certain criteria - when using it as a test for detection of cancer disease: (1) positive results should occur in the early stages of the disease, (2) positive results should occur only in the patients with a specific type of malignancy, (3) positive results should occur in all patients with the same malignancy, (4) the measured values should correlate with the stage of the disease, (5) the measured values should correlate to the response to treatment, (6) the marker should be easy to measure. Most tumor markers available today meet several, but not all criteria. As a consequence of that, some criteria were chosen for the validation and proper selection of the most appropriate marker in a particular malignancy, and these are: (1) markers' sensitivity, (2) specificity, and (3) predictive values. Sensitivity expresses the mean probability of determining an elevated tumor marker level (over the "cut-off value") in a tumor-bearing patient. Specificity expresses the mean probability that a normal tumor marker value derives from a tumor-free individual. The predictive value shows the applicability of a tumor marker in a mixed group of patients.

Many theoretical applications exist for tumor markers in clinical oncology. Clinically important utilization of markers includes (i) early detection of the tumor, (ii) differentiating benign from malignant conditions, (iii) evaluating the extent of the disease, (iv) monitoring the response of the tumor to therapy, and (v) predicting or detecting the recurrence of the tumor. Since no ideal tumor markers with adequate sensitivity and specificity currently exist, they are only exceptionally used in screening (prostate specific antigen - PSA). Nevertheless, tumor markers can play a crucial role in the detection of an early disease relapse and assessment of response to therapy in selected groups of patients. In monitoring the patients for disease recurrence, tumor marker levels should be determined only when meaningful treatment is possible.

Key words: neoplasms-diagnosis; tumor markers, biological

What exactly are tumor markers?

The result of malignant transformation is a malignant cell that, in each cycle of cell division, generates a new malignant cell. In this process, the malignantly transformed cells acquire some new properties through which they differ from nonmalignant cells of the same origin. The acquired properties can be either the changes in cellular morphology, physiology, or the changes in cell growth (behavior).[1] These subtle differences between normal and malignant cells are therefore being exploited in the detection of malignant cells (or malignancy in general), and the substances that are being determined in this process are termed tumor markers.

According to the fact that the differences between tumor cells and normal ones appear on various levels (see above), also tumor markers differ one from another and represent a rather broad conception that comprises both various substances and various cellular processes. Consequently, membrane antigens, hormones, enzymes, polyamines, nucleosides, products of oncogenes, products of tumor suppressor genes, or DNA ploidy and proportion of cells in S phase of the cell cycle (proliferative activity) can be considered as tumor markers.[2,3] Different fields in oncology utilize different tumor markers according to their needs and their techniques of follow-up: tumor markers in clinical oncology differ from the ones in molecular biology, and these differ from the ones in immunohistochemistry, in physiology, etc.

Table 1. Utilization of tumor markers in oncology

I) FOR FOLLOW-UP OF THE DISEASE
- **1) determination in body fluids**
  - a) to monitor the treatment response
  - b) to detect early the disease recurrence
  - c) to evaluate the extent of disease
  - d) to differentiate benign from malignant conditions
  - e) as screening method for some types of cancer
- **2) immunoscintigraphy and lymphoscintigraphy**
- **3) immunohistochemistry**
  - a) to set the diagnosis
  - b) to determine the prognosis
  - c) to predict the treatment response

II) FOR TREATMENT
- **1) direct cytotoxicity of specific monoclonal antibodies (MoAb)**
  - a) binding of complement to specific MoAb
  - b) binding of cytotoxic cells to specific markers-receptors
- **2) binding of drugs to specific MoAb**
- **3) binding of toxins to specific MoAb**
- **4) binding of radioactive isotopes to specific MoAb**
- **5) inhibition of growth factor receptors**

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The application of tumor markers

Theoretically, the possibilities for the application of tumor markers in oncology are numerous, but the utilization depends upon the sensitivity and specificity of the marker and upon reliability of other methods that are being used for the same purpose (Table 1).

Tumor markers in clinical oncology

The standard definition of tumor markers in clinical oncology comprises predominately the substances that are produced by malignant cells or the substances that are produced by other cells under the influence of malignant cells and that can be determined in body fluids. Tumor markers can be either newly synthesized substances (that are not commonly produced by normal healthy cells) or the substances that can be found in normal organisms in much lower concentrations.[2] The determination of tumor markers in clinical oncology is helpful in many processes: in the process of diagnose setting and prognose prediction, in determining the disease extent and planning the treatment, as well as in the early detection of disease recurrence or metastasis. However, the markers in clinical oncology are nowadays, due to their incompetences, only exceptionally applied as screening methods for the detection of malignant diseases.

Different markers are used for different purposes – namely, some of them are more appropriate for the follow-up of the disease and the others for the early detection of the disease recurrence.[3-5] In addition to the above applications, tumor markers can also serve as predictors of a disease outcome. The follow-up of malignant disease before, during and after treatment, and careful processing of the data, namely, gives us a valuable information about the nature of malignancy, and thus also about the patient's prognosis. In general, extremely high concentrations of tumor markers are predictors of poor outcome.[6]

Determination of tumor marker in patient's sera

In body fluids, tumor markers are found in low concentrations and for their determination highly sensitive technology is needed. The techniques that are being used are more or less based on the same principle – *i.e.* the determination of antigen-antibody complexes. Most widely used techniques are the radio-immune assay, the enzyme-immune assay, and the luminometric-immune assay, which differ in the compound bound to the detection antibodies, and in the method of detection of the formed complexes.[7]

Sensitivity and specificity of tumor markers

The ideal tumor marker should be (i) present only in tumor cells, (ii) specific for the organ and type of tumor, (iii) assessable in the sera of all patients with the same type of tumor, (iv) assessable in the serum at the very beginning of tumor development and its concentrations in the serum should correlate with the tumor burden. Besides, the serum concentrations of the marker should be regarded as a valid predictor of disease in patients with the specific type of tumor.[2]

Up till now, no antigenic structure is known that would be present only in tumor cells – and that means that the antibodies against certain tumor markers crossreact also with other antigenic structures. We can therefore conclude that no tumor marker and no methods of following up the presence of malignant cells are 100% specific. When assessing these results, we have to bear in mind that not only a malignant disease causes elevated levels of tumor markers, but that there are also other factors that affect their concentration. The most com-

mon incompetences of tumor markers are (i) inadequate specificity for the type of malignancy, (ii) production of markers in high concentrations in nonmalignant diseases (different inflammatory processes, benign tumors, nonmalignant diseases of the liver and pancreas), (iii) production of markers in different physiological conditions (pregnancy, menstruation, lactation), and (iv) production in perfectly healthy tissues.[2,3,7]

To determine as accurately as possible the role and applicability of a specific tumor marker and the method in a specific type of malignancy, some new terms were introduced – including the sensitivity and specificity. The sensitivity of a marker reports the proportion of patients bearing a specific type of tumor in which the serum (urine, plasma, cerebrospinal liquor) level of marker is elevated. The more patients with the same type of tumor have an elevated level, the more sensitive is the marker, and the lower is the expected number of false negative determinations. The specificity of a marker represents the proportion of patients who do not have a certain type of malignancy and in whom the marker level is normal. That means that the lower is the number of patients not bearing a certain type of tumor and having an elevated marker level, the higher is the specificity, and the lower is the expected number of false negative determinations.

In case there are more tumor markers elevated in one type of malignancy, it is possible to increase the sensitivity of detection by combining these markers' determinations, but we have to bear in mind that, in this way, the specificity of detection is decreased. By combining properly the marker determinations, the specificity of detection can be only slightly lowered, while the sensitivity is significantly increased. The prerequisite for a proper combination of different markers is their high specificity and complementarity for the type of tumor. An example of such proper combination is the determination of ßHCG (human chorionic-gonadotropin) and AFP (alpha fetoprotein) in non-seminoma germ tumors. Each of the above-mentioned markers has a specificity of more than 90% and the sensitivity of approximately 60% for this type of tumors. Due to their complementarity (meaning that they are elevated in different patients), the sensitivity of the combination of the two markers reaches approximately 95%.[8-10]

Classification of tumor markers

Tumor markers can be classified in several ways: according to their chemical structure, their tissue of origin, types of malignancies in which they are elevated, etc. The most common classification tries to combine their biochemical properties, tissue of origin, and functionality. According to this classification, we distinguish: oncofetal proteins, hormones and/or carcinoplacental antigens, enzymes, tumor-associated antigens, special serum proteins, and miscellaneous markers.[3,11]

Oncofetal proteins

Oncofetal proteins are antigens that are normally produced during the embrional development. In adults, their production is limited or completely absent (stopped). Elevated concentrations in adults result from reactivation of certain genes that control cellular growth and are directly connected to malignant process.

Carcinoembryonic antigen (CEA) is a typical representative of this group and one of the first known tumor markers. During embrional development, it is produced in epithelial cells of the gastrointestinal tract, liver, and pancreas. CEA is important for the follow-up of patients with colorectal cancer because 65% of all patients (including those with localized disease and stage I), and as much as 100% of patients with metastatic disease have elevated serum levels of CEA.[2,4]

Besides, this marker is convenient also for the follow-up of patients with other malignancies – especially breast, ovarian, pancreatic, lung, liver, and endometrial cancer.[12,13] Serum concentrations between 4 - 10 ng/ml can be found either in the patients with malignant or in the patients with benign diseases, and even in some heavy smokers, while concentrations above 10 ng/ml speak more in favor of a malignancy.[14] Elevated serum concentrations can be found also in the patients with bronchitis, gastritis, duodenal ulcer, liver diseases, pancreatitis, colorectal polyposis, etc.[14]

Alpha-fetoprotein (AFP) is known for approximately as long as CEA. It was discovered in 1963 in the sera of mice with hepatocellular carcinoma.[15] AFP is a glycoprotein produced in yolk sac, in the epithelial cells of the gastrointestinal tract and liver during embrional development.[16] In pregnancy, AFP enters amniotic fluid through fetal blood, and passes the placenta, thus going into maternal blood. In healthy adults, AFP can be found in the blood in very minute concentrations. Normal serum concentrations appear approximately 9 months after birth. Elevated serum AFP levels (above 10 ng/ml) in adults can be found in the patients with acute viral hepatitis, liver cirrhosis, obstructive icterus, and in some malignant diseases, as pancreatic cancer, lung cancer or gastric cancer. The main role of AFP is to follow up the patients with hepatocellular carcinoma (95 to 100% specificity and sensitivity) in whom the concentrations above 1200 ng/ml practically confirm the diagnosis of primary liver (hepatocellular) cancer, and the patients with non-seminoma germ tumors (specificity 60%).[16,17]

Hormones

Malignant formations can alter the synthesis and secretion of various hormones. Quantitative and qualitative alterations of the synthesis and hormone secretion can therefore be the indicators of a malignant process and can be monitored as tumor markers. Quantitative alterations occur when tumors develop in the tissue of endocrine glands, thus influencing the normal production of hormones by either increasing or decreasing it. This group comprises hormones of malignant endocrine tumors as *parathyroid hormone, insulin, prolactin, catecholamines* and others. Qualitative changes take place when malignantly transformed cells of some organs (lungs, breast, stomach, central nervous system, ovaries) start producing hormones – i.e. the so called ectopic hormone production (e.g. *calcitonin* and *parathyroid hormone* in breast cancer, *lipotropin* in carcinoid tumors, *calcitonin, insulin, parathyroid hormone* in thymic malignomas).[2,3]

Among all hormones, *human chorionic gonadotropin (ßHCG)* is one of the most applicable tumor markers. This is a protein with the molecular mass of 45 KD. It belongs to the group of carcinoplacental antigens – proteins that are synthesized in placenta during pregnancy (most early pregnancy tests are based on the detection of ßHCG) and can be found in adults only exceptionally.[18] Elevated serum concentrations of ßHCG can be found in almost all female patients with germ tumors with trophoblastic component (choriocarcinomas), hydatidiform moles, and in the majority of male patients with germ tumors. ßHCG has a very short serum half-life (36-48 hours) and is therefore functional to follow up treatment response, as well as to predict prognosis. In combination with AFP, ßHCG is an excellent marker for monitoring the patients with germ tumors. On the other hand, approximately 10% of germ tumors do not synthesize tumor markers; hence, they cannot be used for diagnostic purposes and in the follow-up of these patients. Slightly elevated levels of ßHCG can be found also in the patients with breast, gastric, lung, liver, or colorectal cancer, but are irrelevant in clinical monitoring of these patients.[19]

Enzymes

Certain enzymes that are produced more intensely if a malignant process is occurring in the organism can also be used as tumor markers.

Prostatic acid phosphatase is an enzyme that is produced in normal prostatic tissue. Elevated serum concentrations (above 3 ng/ml) can be observed in the patients with prostatic cancer and usually correlate with an advanced phase of the disease when the tumor penetrates the prostatic capsule. The determination of prostatic acid phosphatase is therefore convenient for the discrimination of benign (hypertrophy) from malignant processes.[20]

Alkaline phosphatase exists in the form of iso-enzymes that are synthesized in the liver, bones or placenta. Elevated serum concentrations in patients with malignant disease usually indicate a metastatic spread of the disease into the liver and/or bones, and/or the presence of primary bone tumors (osteosarcoma).[20]

Neuron specific enolase (NSE) is a cytoplasmic glycolytic enzyme that was primarily detected in the cells of neuroectodermal origin and in neuronal cells. The latter were proved to be in the tumor tissue of the tumors with neuroectodermal or neuroendocrine differentiation.[21-23]

Among other (nonspecific) markers in this group, we should not leave out *lactic dehydrogenase (LDH)* that is quite often elevated in the sera of patients with malignant lymphomas and germ tumors (seminomas), *γ glutamyl transpeptidase (GGT)* that indicates cholestasis (often elevated because of liver metastasis), and *thymidine kinase (TK)* that helps to evaluate the disease spread in the patients with leukemia, lymphoma, brain tumors, small cell lung cancer, and breast cancer.

Tumor-associated antigens

This is a heterogeneous group of markers that comprises various membrane structures of tumor cells. Updated technology exploits the possibility of producing specific monoclonal antibodies against certain antigenic structure that is most characteristic for the type of tumor cells. Therefore, the markers in this group are more specific for the type of malignancy than the others and quite often their serum concentrations reflect more accurately the growth or regression of the tumor mass.

Carcinomic antigen 15-3 (CA 15-3) is produced in the secretory epithelium (of the breast, lungs, gastrointestinal tract, uterus, etc.) and can be found frequently in the excretions of healthy adults. Elevated serum concentrations of this marker (above 30 U/ml) are detected predominately in the patients with breast cancer; however, raised levels of CA 15-3 can be observed also in other malignancies as lung, prostatic, ovarian, cervical, and gastrointestinal cancer.[13, 24] Hence, CA 15-3 is no specific marker either for the organ or for the type of tumor. Despite that, CA 15-3 is a good indicator of treatment response and disease course in breast cancer patients (whose tumors produce that antigen). The serum level of CA 15-3 can be influenced also by different non-tumoral diseases of the breast, by benign breast tumors, but the values rarely exceed 40 U/ml. Raised concentrations can be observed in approximately 8% of pregnant women between 38th and 40th week of gestation.[3]

Concomitant determination of CA 15-3 and CEA in breast cancer patients increases the sensitivity (reduces the number of false negative determinations), while retaining a substantial specificity of the procedure (rather low number of false positive determinations).

Carcinomic antigen 125 (CA 125) is a characteristic marker for ovarian cancer (it is elevated in more than 80% of patients with non-mucinous ovarian cancer). During embrional development, CA 125 is produced in celomic epithelium, Muellerian ducts, epithelial cells of the pleura, pericardium, and peritoneum.[25,26] In adults, CA 125 can be found

in mucosa of the cervix uteri and in the lung parenchyma, however, it is not produced by healthy ovarian tissue. Elevated concentrations of CA 125 (above 35 U/ml) can be found not only in the patients with ovarian cancer but also in the patients with benign or malignant gynecological diseases (endometriosis, ovarian cysts, endometrial cancer, cervical cancer) as well as in the patients with non-gynecological malignancies (lung cancer, prostatic cancer, peritoneal malignant mesothelioma). In the group of patients with ovarian cancer, CA 125 is most reliable and applicable in the follow-up of patients with epithelial and undifferentiated ovarian cancer.[27]

Carcinomic antigen 19-9 (CA19-9) is a glycolipid and actually represents a modified Lewis's hapten from the blood group system. CA 19-9 is frequently elevated in the serum of patients with gastrointestinal tumors. The marker is slightly more specific for pancreatic and liver cancer, yet quite often raised concentrations can be found in patients with colorectal, gastric, and ovarian cancer. In relatively high concentrations, it can be detected in healthy adults in the prostatic fluid, gastric fluid, amniotic fluid, and in the excretions of the pancreas and duodenum. Therefore, only determinations in the serum or plasma are rational because there the concentrations will be elevated only in case of disease.[28]

Prostate specific antigen (PSA) is a serine protease first isolated from the tissue extract of the prostate and sperm. It is produced in the prostate tissue and excreted in the prostate fluid where it can have very high concentrations. The role of this serine protease is to prevent coagulation of sperm. In healthy persons, very minute amounts of PSA enter the bloodstream so that its concentration in serum is rather low. In the patients with prostatic disease, the amounts of PSA that enter bloodstream increase significantly (especially in case of prostatic malignancy), thus generating high serum concentrations. This marker is substantially specific for prostatic cancer and its serum concentrations reflect very well the tumor burden. Due to its high sensitivity and extraordinary correlation with tumor burden we prefer applying PSA to prostatic acid phosphatase in the follow-up of prostatic cancer patients.[20,29] Besides, the method (together with other procedures) is being utilized in the screening of prostatic cancer in the group of males over 50 years old who have more risk factors (e.g. prostatic cancer in patient's father, breast cancer in patient's mother or sister, obesity, prostatic cancer in more than one generation). With the determination of different forms of PSA (*i.e.* total, bound or free), and with a proper evaluation of free to total PSA ratio, it is possible to predict quite confidently if the patient suffers from a benign or malignant prostatic disease.[30]

Special serum proteins

This group comprises various proteins. One of the best known is *feritin* that binds iron intracellularly and is responsible for detoxication (e.g. binding of free radicals). Under normal circumstances, high concentrations of feritin can be found in the liver, spleen, and bone marrow. Normal serum level of feritin ranges from 8 to 440 ng/ml. Raised concentrations can be observed in the patients with acute leukemia, lymphomas (especially Hodgkin's lymphomas), lung, liver, and prostatic cancer.[2]

Thyroglobulin is an intracellular glycoprotein responsible for the production and storage of thyroxine. In low concentrations, it can be found in the sera of most healthy persons (0-75 ng/ml), while extremely high concentrations can be detected in the patients with well differentiated follicular (rarely anaplastic) thyroid carcinoma. Conversely, in the patients with medullary thyroid carcinoma, the serum levels of thyroglobulin do not follow the development and course of malignancy.[2,3]

Beta-2-microglobulin is a protein that is identical with the HLA light chain and thus appears

on the cell membrane of almost all differentiated cells. Raised serum concentrations can be observed in the patients with lung, breast, pancreatic, colorectal cancer, as well as lymphomas and chronic lymphoid leukemia (CLL).[2,3]

S-100 protein was first isolated from bovine brain. Normal serum concentration of this marker is below 0.3 ng/ml. In addition to being a good indicator of traumas to CNS, S-100 can be applied as tumor marker in the patients with neurinoma, glioblastoma, astrocytoma, and meningeoma. It has a special role as prognostic factor and in the follow-up of the patients with malignant melanoma (<0.3 ng/ml - 85% 3 years' survival; 0.3-0.6 ng/ml - 50% 3 years' survival; >0.6 ng/ml - 10% 3 years' survival).[31,32]

Miscellaneous markers

This group involves markers the production of which correlates perfectly with the changes in velocity of cellular proliferation. It is a heterogeneous group of substances which are not specific for the type of tumor but generally indicate the presence of a malignant process. The group comprises polyamines, nucleosides and tissue polypeptide antigen (TPA).

Polyamines like spermine, spermidine and putrescine were detected in elevated concentrations in urine in cases of a rapid regeneration of cells of certain tissue.

Nucleosides as dimethylguanosine and pseudouridine are components of RNA that (like polyamines) enter the circulation in larger amounts in cases of enhanced cellular proliferation.

Tissue polypeptide antigen (TPA) is likewise a nonspecific marker of enhanced cellular proliferation. The molecular mass of this polypeptide is approximately 180 KD and the molecule is composed of different cytokeratin units – *i.e.* of cytokeratin 19 (44%), cytokeratin 18 (36%), and cytokeratin 8 (30%). During embrional development, it is produced in various embrional tissues as well as in the placenta. In adults, TPA is a part of cellular membranes (cytoskeleton) of normal and tumor cells. It is synthesized during S phase of the cell cycle and its concentrations reflect the velocity of cellular proliferation. A more rapid cellular proliferation demands a more rapid synthesis of TPA, and consequently, larger amounts of this polypeptide enter the circulation. Therefore, TPA is a common (universal) marker that goes together with pathological cellular proliferation (that is usually present in malignant transformation) regardless of the type or localization of the tissue. Unlike in other markers, the serum concentrations of TPA poorly reflect the tumor burden. Normal serum concentrations of TPA are below 90 U/ml and concentrations higher than 120 U/ml can be either a consequence of a malignant or of a benign process.[2]

Biological factors that affect serum concentrations of tumor markers

Regarding the facts, that no tumor marker is ideal and that the substances that are being applied as tumor markers are not synthesized exclusively as a consequence of malignancy, I present some of the most common factors that affect serum concentrations of tumor markers (Table 2).

When to determine tumor markers?

Tumor markers are determined in such a way that we gain as much as possible of clinically useful data. We have to prepare an approximate concept that includes the determination of markers (i) prior to surgery or any kind of treatment (chemotherapy, radiotherapy, biological therapy, or hormonal therapy); (ii) after the surgery, during the treatment, and after the treatment once in 3 to 6 months' period during the first and second year, then once yearly or at regular controls; (iii) in case of sus-

Table 2. Factors (in addition to malignant disease) that affect serum concentrations of tumor markers

False positive results	
	➢ presence of inflammatory processes;
	➢ benign liver diseases and consequential disturbancies in metabolism and excretion (AFP, TPA, CEA, CA 19-9, CA 15-3);
	➢ disturbancies of renal function (beta-2-microglobulin, calcitonin, PSA, CEA, CA 19-9, CA 15-3);
	➢ extensive tumor necrosis;
	➢ as a consequence of diagnostic and therapeutic procedures (digitorectal examination, mamography, surgery, radio and chemotherapy);
	➢ as a consequence of different physiological conditions (pregnancy - ßHCG, CA 125, CA 15-3, MCA, AFP, menstrual cycle - CA 125).
False negative results	
	➢ complete absence of production (e.g. CA 19-9 in $Le^{(a-b-)}$ persons);
	➢ insufficient expression of a certain antigenic determinant (or production in only some of tumor cells);
	➢ insufficient blood circulation in the tumor;
	➢ production of immune complexes with autoantibodies;
	➢ rapid degradation and clearance of antigens;

pected relapse or disease progression; (iv) prior to introduction of a novel treatment; (v) at least 3 weeks after the introduction of a novel treatment; (vi) 2 to 3 weeks after the determination of raised concentrations of the marker.

The proposed concept of tumor marker determinations is applicable in the majority of tumor markers. Certainly, the demands in clinical oncology are quite often different and we have to adjust the dynamics of determinations in accordance with the type and properties of the tumor, and with the planned methods of treatment. In other words, the above mentioned concept represents only an approximate model that has to be further modified in each patient specifically regarding the fact that each patient is a control to himself (monitoring of the dynamics of serum concentrations).

Conclusion

Determination of tumor markers for monitoring the course of malignant disease is an established and often an irreplaceable oncological laboratory method. Tumor markers are reliable predominately in monitoring the treatment response, as well as in early detection of disease recurrence (prior to development of clinically notable signs). Due to their incompetences, tumor markers' determinations can be only exceptionally applied as screening methods or as the sole diagnostic tool; however, in combination with other diagnostic methods, they play an important role in the diagnostic process and in treatment planning. Besides, by combining various tumor markers we can achieve a greater specificity and sensitivity in the follow up of one type of malignancy. The simplicity and noninvasiveness of the method for the determination of tumor markers enable monitoring the disease also in the patients not eligible for other types of diagnostic procedures.

On account of individual differences in the serum concentrations of each individual tumor marker, we recommend multiple determinations of tumor markers and monitoring the dynamics of serum concentrations (even in cases when serum concentrations are below the cut-off values). It would be ideal to determine the level of tumor markers in each patient before

treatment, several times between the treatment (depending upon the type of treatment, type of malignancy, and the sort of tumor marker), and after the treatment. Tumor markers should be monitored also for a certain period after the treatment has been finished, best at regular control examinations (once in six months or once yearly). This kind of follow up will enable a timely detection of disease recurrence even in asymptomatic patients. From a single determination of tumor markers we can find out whether the malignancy has developed or not and, if it has, what is its extent, but only if the concentrations are very high. In patients who are undergoing just symptomatic (palliative) therapy, the determination of tumor markers no longer makes sense. If the patient had normal concentrations of tumor markers prior to any kind of treatment, and if these concentrations did not change (increase or decrease) at disease progression or regression, it is very unlikely that concentrations will be elevated in case of disease recurrence. In these patients, tumor marker levels need not to be followed either during treatment or after treatment. Hence, it is important to make the first determination of tumor markers prior to any treatment in order to disclose the group of patients in whom tumor marker determinations are not sensible.

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review

Clinical utility of serine proteases in breast cancer

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The serine protease uPA and its inhibitor PAI-1 are involved in the degradation of tumor stroma and basement membrane. The independent prognostic value of serine protease urokinase-type plasminogen activator uPA and its inhibitor PAI-1 in breast cancer has been almost uniformly confirmed in numerous individual studies as well as in a meta-analysis, including 18 data sets of more than 8,000 patients. According to these observations, the risk of relapse in node negative patients with low levels of uPA and PAI-1 is less then 10%; these patients could be spared from toxic adjuvant systemic therapy. Clinically relevant and even more important is the information that uPA and its inhibitor PAI-1 may also have a predictive value for response to either hormonal or cytotoxic therapy in early breast cancer. According to our data obtained from altogether 460 operable breast cancer patients, uPA and PAI-1 may have a predictive value for the response to hormone therapy, but not to chemotherapy. The high PAI-1 levels were associated with a higher risk of relapse in the patients without adjuvant systemic therapy (HR 2.14; C.I. 95%= 0.48-9.56; p=0.321) and in the patients treated with chemotherapy (RR 2.48; C.I. 95%= 1.35-4.57; p=0.003). However, in the patients treated with adjuvant hormone therapy, either alone or in combination with chemotherapy, the prognostic value of uPA and PAI-1 was diminished. Moreover, high levels of both uPA and PAI-1 were associated with a lower risk of relapse (HR 0.79; p=0.693 and HR 0.26 p= 0.204, respectively). On the basis of currently available evidence, serine protease uPA and its inhibitor PAI-1 are certainly the markers that improve a proper selection of candidates for adjuvant systemic therapy and may also be the markers that could facilitate treatment decision in each individual patient, which is of utmost importance.

Key words: breast neoplasms; urinary plsminogen activator; plsminogen activator inhibitor 1; prognosis

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Introduction

Breast cancer is the most common malignancy in females all over the world; more than a million of women diagnosed with this disease each year. Tremendous improvements in the treatment of this disease have been made during the last decades and the survival rates improved from around 50% to more than 70% in operable disease. This improvement is mostly

due to early detection of the disease and to the introduction of adjuvant systemic therapy.[1] According to the meta-analysis, in which data obtained from more than 50,000 patients participating in different studies were included, adjuvant systemic therapy was found to reduce the risk of death by approximately one third in all operable breast cancer patients, irrespective of their individual risk of death based on traditional prognosticators, such as lymph node involvement, tumor stage, tumor grade, etc.[2,3] The absolute benefit is the same in all patient sub-groups and only the relative benefit is much higher in the high risk subgroup of patients. According to the so-called classical clinico-pathological data, the majority of patients are categorized into average and high-risk groups and treated by some kind of adjuvant therapy, although it is well known that less than 50% of all patients and only 30% of patients with node- negative disease are to develop metastases without any systemic treatment. Therefore, there is an urgent need to identify new prognostic markers that will enable us to categorize patients according to their individual risk of relapse more precisely. On the other hand, there is a continuous search for new biological markers, which could not only prognosticate the faith of the disease, but would rather predict the response of each individual patient to the particular therapy. This approach ensures that both the individual patient and the society as a whole benefit, by minimizing the treatment-related side effects and maximizing cure rates. There are a lot of new, prospective prognostic and predictive factors under investigation and serine proteases are among them.[4]

Prognostic factors in breast cancer

A prognostic factor for breast cancer is defined as any measurement available at the time of diagnosis or surgery and associated with disease-free or overall survival in the absence of systemic adjuvant therapy. Based on the knowledge of prognostic factors, the risk of relapse for each individual patient treated solely by local therapy could be estimated. Nowadays, tumor size, lymph node status, histological type and grade, mitotic figure count, and hormone receptors are considered the standard prognostic factors according to which the adjuvant therapy is planned. The prognostic value of these, so-called standard prognosticators in early breast cancer, has been confirmed in multiple studies and their value as category I prognostic factors has been recognized at a 1999 Consensus Conference held under the auspices of the College of American Pathologists.[5] According to these factors, only about 10% of patients are considered to have minimal, less than 10% risk of relapse and are therefore not offered adjuvant systemic therapy.[6,7]

There are additional new prognostic factors under investigation, which may better separate patients according to their own risk of relapse and spare the patients with low risk of relapse from unnecessary toxic treatment. Undoubtedly serine protease, urokinase-type plasminogen activator uPA and its inhibitor PAI-1 are among them. The prognostic value of both uPA and PAI-1 has already been confirmed at the highest level I evidence, in the meta-analysis and in the prospective randomized trial.[8,9]

Prognostic value of uPA and PAI-1

The serine protease uPA and its inhibitor PAI-1 are involved in the degradation of the tumor stroma and basement membrane. A critical balance of uPA, its cell surface receptor uPA-R, and PAI-1 are the prerequisites for efficient focal proteolysis, adhesion, and migration, and hence, the subsequent tumor cell invasion and metastases.[10] In 1988, Duffy with co-workers was the first to report that high primary tumor enzymatic activity of uPA

is associated with poor survival of breast cancer patients.[11] Shortly, the same authors did not only confirm this finding in a larger study, but also demonstrated the independent prognostic impact of uPA on the disease-free survival and overall survival.[12] This information was confirmed and even strengthened by the finding of the German group that not only the enzymatic activity, but to an even greater extend also the tumor tissue antigen level of uPA is of prognostic relevance.[13] The prognostic impact of uPA has since been confirmed by several investigators[14-24]; and PAI-1 was also found to be an independent prognostic marker in breast cancer (Table 1).[14-16,18-25] Surprisingly, high levels of PAI-1 were found to be associated with a higher risk of recurrence and a shorter overall survival in breast cancer. The data from basic research may help to explain the finding that PAI-1 does not act as a true uPA inhibitor, but rather as a proteolitic factor. PAI-1 was found to be indispensable for optimal focal proteolysis, adhesion, and migration, and subsequent tumor cell.[10]

The independent prognostic value of serine protease uPA and its inhibitor PAI-1 in breast cancer has been confirmed in a meta-analysis, including 18 data sets of 8,377 patients with a median follow-up of 6.5 years.[8] In this meta-analysis, uPA, PAI-1 as well as the combination of both were found to be the strongest prognostic factors in breast cancer, next to lymph node involvement, irrespective of the type of surgery, year of surgery, publication status of data sets, as well as menopausal status and lymph node status.[26] The independent prognostic value of uPA and PAI-1 was also confirmed in a prospective randomized multicenter therapy trial in node-negative breast cancer ("Chemo N_0"), randomizing high-uPA/PAI-1 patients to adjuvant CMF or observation only, which was performed in 13 German academic centers and in our center in Slovenia between 1993-1999.[9] In this study, uPA and PAI-1 were prospectively determined in detergent extracts of primary tumor tissue using commercially available ELISA assays (American Diagnostica Inc., Greenwich, CT). The first scheduled interim analysis of this

Table 1. Prognostic impact of uPA and PAI-1 in early breast cancer – overview of selected references

Author (year)	Country	Factors analyzed	Patients	(N0)	Follow up (median, months)	Reference
Duffy (1988)[11]	Ireland	uPA	52	(25)	17	Cancer 62:531
Jänicke (1993)[13]	Germany	uPA, PAI-1	247	(101)	30	BCRT 24:195
Grøhndahl-Hansen (1993)[15]	Denmark	uPA, PAI-1	119	(12)	102	Cancer Res 53: 2513
Foekens (1994)[16]	Netherlands	uPA, PAI-1	657	(273)	48	J Clin Oncol 12:1648
Fernö (1996)[17]	Sweden	uPA	688	(265)	42	Eur J Cancer 32:793
Kim (1998)[18]	Japan	uPA, PAI-1	130	(130)	53	Clin Cancer Res 4:177
Kute (1998)[19]	USA	uPA, PAI-1	168	(168)	58	BCRT 54:147
Knoop (1998)[20]	Denmark	uPA, PAI-1	429	(178)	61	Br J Cancer 77:932
Harbeck (1999)[21]	Germany	uPA, PAI-1	316	(147)	77	Breast Cancer Res Treat 54:147
Bouchet (1999)[22]	France	uPA, PAI-1	499	(233)	72	J Clin Oncol 17:3048
Foekens (2000)[23]	Netherlands	uPA, PAI-1	2780	(1405)	88	Cancer Res 60:636
Jänicke (2001)[9]	Germany	uPA, PAI-1	556	(556)	32	JNCI 93:913
Konecny (2001)[24]	USA / Germany	uPA, PAI-1	587	(283)	26	Clin Cancer Res 7:2448
Look (2002)[8]	Europe	uPA, PAI-1	8377	(4676)	79	JNCI 94:116

study confirmed the independent prognostic impact of both uPA/PAI-1 for the disease-free survival; within the frame of this study, the previously optimized cut-offs for uPA and PAI-1 used to distinguish between low and high uPA and PAI-1 were validated.[21] According to the data obtained in the frame of this prospective study, the low-risk group, identified by uPA/PAI, encompasses as much 50% of node-negative patients, which is much more than 10% of node-negative patients, categorized into the low-risk group according to the clinical-pathological criteria. The risk of relapse in the patients with low levels of uPA and PAI-1 was found to be only 6.7% at 3-years, and these patients could be spared from toxic adjuvant systemic therapy, especially chemotherapy.

The uniformly found prognostic value of uPA and PAI-1 in multiple individual studies as well as in a prospective randomized trial and meta-analysis is quite unique among prognostic markers. For most of the so-called established and very well recognized prognostic factors, such as tumor size, histological type and grade, hormone receptor status as well as mitotic index, the data are far from being so consistent. In addition, the standardization of the method of determining, interpreting, and reporting the uPA and PAI-1 levels was possible, which is a prerequisite for a clinically useful marker. The immunoenzymatic assays are standardized, international quality assurance of the kit is guaranteed, [27] and the optimal cut-off values have been validated.[21] In addition, the extracts, prepared from as little as 100 μg tumor tissue, corresponding to about 1 μg protein extract, suffice for testing. All these facts make a serine protease uPA and its inhibitor PAI-1 an ideal prognostic factor for routine clinical use.

Predictive factors in breast cancer

To optimize treatment approaches and to improve the results of treatment, new biological markers, which will help us not only to predict the course of disease, but will also be able to predict the response to specific therapy in each individual patient, the so-called predictive markers are urgently needed. A predictive factor is any measurement associated with response or lack of response to a particular therapy. If, in addition to prognostic value, a marker also has a predictive value for response to a particular therapy, its prognostic strength could be increased or diminished, which depends on the fact whether worse or better prognosis correlates with treatment efficacy.

After four decades of systemic treatment, we are still faced with only two established predictive factors in breast cancer: hormone receptor status for response to hormonal therapy and HER-2 status for response to HER-2 antibody trastuzumab. Thus, the patients whose tumors strongly express hormone receptors are likely to respond to tamoxifen or other hormonal manipulates, while the patients with receptor-negative disease do not benefit from hormonal therapy.[3] Similarly, HER-2 overexpression in primary tumors predict a better response to trastuzumab.[28] There are also some new, putative markers for the response to either hormonal therapy or different chemotherapeutic agents, such as HER-2, p53 and BCL-2, which are under investigation. However, the data on their predictive value are still insufficient and even contradictory[4], which enables us to use them as a guide in the selection of systemic therapy. According to our data,[29] as well as the data published by Munich and Rotterdam group,[30,31,32] serine protease uPA and its inhibitor PAI-1 may also have a predictive value for the response to either hormonal or cytotoxic chemotherapy in early breast cancer.

Predictive value of uPA and PAI-1

The latest observations, based on the data provided by the patients mostly treated by

some kind of adjuvant systemic treatment, showed a possible loss of the prognostic value of uPA and PAI-1[17,29-32], which indicates that the level of proteases in the primary tumor could also predict a response to systemic therapy. In addition, the data from meta-analysis show that the bad prognostic impact of high levels of either uPA or PAI-1 was most pronounced in the sub-groups of patients with node-negative disease and in the sub-groups of patients treated before nineties which are the sub-groups that did not receive adjuvant systemic treatment in such an extent as the sub-groups of patients with node-positive disease, and the sub-groups of patients treated in the last decade.

So far, findings on the predictive value of uPA and its inhibitors for response to hormonal therapy are limited and, to some extent, even contradictory. Preclinical data observations that estrogens as well as antiestrogens modulate the expression of uPA and tumor cell growth in vitro[33-35] suggested that the levels of serine proteases in the primary tumor could be predictive for the efficacy of hormonal manipulations in breast cancer. In a large group of 235 patients with metastatic breast cancer, high levels of both uPA and PAI-1 in primary tumor predicted a poor response to tamoxifen.[36] On the contrary, the observations made in the frame of the Munich group[30,31] and Swedish group[17] pointed out that high levels of uPA[17,30] and PAI-1[30] or the combination of uPA/PAI-1[31] in the primary tumor may predict a better response to the adjuvant tamoxifen treatment. However, in a large study conducted in another Swedish center, PAI-1 was not found to predict any benefit of adjuvant tamoxifen treatment.[37] Similarly, in the largest data set obtained from two different centers (Munich and Rotterdam), no significant interaction between the combination of uPA/PAI-1 and the efficacy of adjuvant tamoxifen was found in altogether 3,424 patients.[32] Our data, obtained from 460 early breast cancer patients, speak in favor of the predictive value of high uPA and PAI-1 for a good response to hormonal therapy in adjuvant setting.[29]

The data on the possible predictive value of serine proteases for the response to chemotherapy are even more scarce and maybe less contradictory. In the first published study no significant influence of PAI-1 levels on the response to the neoadjuvant anthracycline based chemotherapy in locally advanced breast cancer was found.[38] Recently, the results from Munich pointed out that high levels of PAI-1 alone[30] or the combination of uPA/ PAI-1[31] may predict for a better response to adjuvant chemotherapy. This observation was confirmed in a large data set obtained from two different centers (Munich and Rotterdam); in a collective of 3424 patients the benefit from the adjuvant chemotherapy was found to be significantly higher in the subgroup of patients with high levels of uPA/PAI-1.[32]

According to our data, obtained from 460 operable breast cancer patients, uPA and PAI-1 may have a predictive value for the response to hormone therapy, but not to chemotherapy.[29] In our study, the high uPA levels were found to be associated with a higher risk of relapse in the patients without any adjuvant systemic therapy and in the patients treated with adjuvant chemotherapy (HR 1.37 and HR 1.44, respectively; non-significant). The high PAI-1 levels were also associated with a higher risk of relapse in the patients without adjuvant systemic therapy (HR 2.14; C.I. 95%= 0.48-9.56; p=0.321) and in the patients treated with chemotherapy (RR 2.48; C.I. 95%= 1.35-4.57; p=0.003) (Table 2). However, in the patients treated with adjuvant hormone therapy, either alone or in combination with chemotherapy, the prognostic value of uPA and PAI-1 was diminished, even more, high levels of both uPA and/or PAI-1 were associated with a lower risk of relapse (HR 0.79; p=0.693 and HR 0.26 p= 0.204, respectively) (Table 2). The 3-

Table 2. Risk of relapse according to uPA and PAI-1 levels in sub-groups of patients with different adjuvant systemic therapies (adopted from reference 29).

Prognostic factor (in ng/mg protein)	Without ST (n=52)	HT (n=141)	HT or ChT&HT (n=252)	ChT (n=156)	ChT or ChT&HT (n=267)
uPA	p=0.71	p=0.693	p=0.914	p=0.381	p=0.194
(<3vs ≥3)	HR=1.37	HR=0.79	HR=1.04	HR=1.44	HR=1.50
	95%CI (0.27-7.1)	95%CI (0.24-2.56)	95%CI (0.50-2.18)	95%CI (0.64-3.25)	95%CI (0.81-2.77)
PAI-1	p=0.321	p=0.204	p=0.8294	p=0.003	p=0.002
(<14 vs ≥14)	HR=2.14	HR=0.26	HR=0.64	HR=2.48	HR=2.22
	95%CI (0.48-9.56)	95%CI (0.03-2.06)	95%CI (0.25-1.68)	95%CI (1.35-4.57)	95%CI (1.35-3.66)

ST=adjuvant systemic therapy; HT= adjuvant hormone therapy; ChT=adjuvant chemotherapy

year DFS rates were not found to be influenced by uPA and/or PAI-1 in the patients treated with hormonal therapy, whereas in the patients treated with adjuvant chemotherapy and in the small group of patients without any adjuvant therapy, the bad prognostic impact of high uPA or/and PAI-1 levels is obvious (Figure 1).

Unfortunately, the data on the predictive value of serine protease uPA and its inhibitor PAI-1 are not as univocal as are the data on the prognostic value of these two markers. According to their mechanism of action, it could be expected that uPA and PAI-1 does not play a major role in the prediction of the response to either hormonal therapy or chemotherapy in large tumors presented in the metastatic disease. However, serine proteases may have an impact on the growth and spread of micrometastatic disease under adjuvant systemic therapy. The above data, although obtained in the frame of the retrospective observations and contradictory in some way, are informative enough to strengthen our believes that serine protease uPA and its inhibitor PAI-1 may have predictive value for the response to either hormonal therapy or chemotherapy in early breast cancer. To make any firm conclusions on the predictive value of proteases, the data from a larger data sets need to be obtained and analyzed by means of different statistic tools, like interaction terms used, and the assessment of the predictive value of these markers in the frame of prospective clinical trial should be made. The ideal way to evaluate the predictive value of any marker is to set a prospective randomized study with no treatment arm. However, due to ethical reasons, such a study is not feasible in adjuvant breast cancer treatment any more.

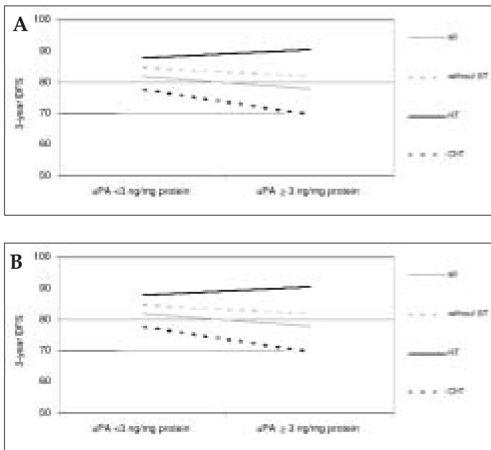

Figure 1. A: 3-year DFS of the patients with low and high values of uPA in the sub-groups of patients treated with different adjuvant systemic therapies (adopted from reference 29). B: 3-year DFS of the patients with low and high values of PAI-1 in the sub-groups of patients treated with different adjuvant systemic therapies (adopted from reference 29).

Conclusion

A part of breast cancer research is still focused on finding prognostic markers which

would help us to identify better the patients who could be spared adjuvant systemic therapy, but there is also an even more urgent need to identify new predictive markers that would help us to understand better which treatment option may be of benefit to each individual patient. On the basis of currently available data, serine protease uPA and its inhibitor PAI-1 are certainly the markers that improve a proper selection of patients for adjuvant systemic therapy and could be used as a new prognostic tool that will reduce the risk of over-treatment with better identification of patients who need adjuvant systemic therapy. In addition, according to the encouraging preliminary data, serine protease uPA and its inhibitor PAI-1 might also be the markers that will improve the treatment decision in each individual patient in the near future.

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review

Cathepsins and their inhibitors as tumor markers in head and neck cancer

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The invasion and metastasizing of tumor cells is closely connected with the disintegration of basement membranes and extracellular matrix. The carriers of these processes are different proteolytic enzymes, among them cysteine and aspartic cathepsins B, H, L and D as well, a group of ubiquitous lysosomal proteases, and endogenous inhibitors of the former, cystatins. The aim of the present review was to collect the current knowledge on the predictive and prognostic value of cathepsins and their inhibitors in squamous cell carcinoma of the head and neck. In this particular tumor type, the UICC/AJCC TNM-classification system and histopathological characteristics of the tumors were found inadequate to reliably predict either the response to therapy or patients' survival. Moreover, to date, no factor within the wide spectrum of biochemical and histological factors has yet been identified as reliably predicting the natural course of the disease or its response to therapy. To construct a prognostically meaningful tumor profile, new markers are intensively investigated.

Key words: head and neck neoplasms; carcinoma, squamous cell; cathepsins, cystatins; prognosis

Introduction

During the past decades it has been shown that proteases of various classes can act as tumor progression factors in all stages of malignant progression. They can create a local environment that is supportive to the growth, survival, and progression of a tumor through the modulation of growth factor pathways, cell-cell adhesions, and cell-matrix adhesions.[1]

Cathepsins are lysosomal proteolytic enzymes. With regard to their chemical composition, cathepsins are glycoproteins, and the majority of them belong to the group of endopeptidases. In cancer, the most studied cathepsins are those of the cysteine and aspartic classes, cathepsins B, H, L and cathepsin D. Endogenous inhibitors of cysteine cathepsins belong to cystatins, which are subdivided into three families, i.e. stefins, cystatins, and kininogens, and thyropins, whereas the naturally occurring inhibitor of aspartic protease, cathepsin D, has not been found yet in men.[2] Their contribution to the progression of breast, lung, and colorectal cancer has been most extensively investigated at a preclinical level and

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as markers predicting the response to various treatment regimens and prognosis.[3,4]

Head and neck cancer

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most prevalent cancer worldwide, with a global yearly incidence of 500,000.[5] Despite the evolution and refinement of multimodal treatments for the head and neck cancer in the last 20 years, 5-year survival rates have not improved significantly, remaining at the 50% level.[6] Patients grouping with the use of conventional UICC/AJCC TNM staging system and established histopathological characteristics of the primary tumor as well as its metastases on the neck in operated patients allow significant prognostic variation among the individuals within any of these groups.

Compared to the carcinoma of the breast, lung or colorectum, the SCCHN falls into a far less investigated group of cancers. Apart from the studies focused on the activity or level of cathepsins and their inhibitors as determined in matched pairs of tumor tissue and normal mucosa [7], there is only a limited number of reports in literature assessing their predictive or prognostic value in this particular type of cancer. In the present review, clinical data on the predictive and prognostic value of cathepsins B, H, L, and D, and their endogenous inhibitors stefin A, stefin B, and cystatin C in SCCHN have been assembled. The results of our own investigations are also presented and their applicability in routine clinical practice discussed.

Clinical relevance of cathepsins and their inhibitors in squamous cell carcinoma of the head and neck

Markers for diagnosis

The largest pertinent study was reported by Kręcicki and Siewiński[8], who measured serum cathepsin B-like activity in 110 samples from patients with laryngeal carcinoma. Enzyme activity was significantly higher in malignant samples compared to healthy controls, whereas no difference was found between the latter and the group with non-malignant, mainly infectious diseases. In cancer patients, no false-negative serum values of cathepsin B-like activity were obtained. The sensitivity of the assay was calculated to be 100% and the specificity 97.5%.[8] The methodology used in this study was criticized by Bongers *et al*.[9] They claimed that cathepsins B-like activity as determined by Kręcicki and Siewiński is better referred to as "serum-protease-activity". However, when serum-protease-activity was compared between patients with SCCHN and non-cancer controls using the same methodology, and after the adjustment for alcohol and tobacco consumption, no difference was observed between the two groups. We used enzyme-linked immunoassay (ELISA) and also found no alterations in serum cathepsin B between cancer patients and healthy controls, as was the case with cathepsin L.[10] When the same group of patients was tested for cathepsins H and D, a significant increase of both enzymes was found in the sera of patients with cancer.[11,12]

The diagnostic value of the inhibitors of cysteine proteases was first studied by Siewiński *et al*.[13] Using their method they were able to discriminate between total inhibitory activity (free molecules and enzyme-inhibitor complexes) and that of active (enzyme-free) and latent (enzyme-inhibitor complexes) forms of specific papain-like cysteine inhibitors; however, the method had no potential to assess the contribution of individual inhibitors. A significantly higher total inhibitory activity (and of latent fraction) and lower activity of active fraction of the inhibitors were found in cancer patients compared to healthy controls or patients with inflammatory diseases. On the contrary, our ELISA measurements allowed quantification

of specific inhibitors but were not able to recognize different forms of the inhibitor molecules. Significantly higher stefin A [10] and cystatin C [14] levels were measured in the patients' sera than in controls, whereas levels of stefin B were significantly lower.[10]

Due to accompanying alterations of cathepsins and their inhibitors in non-malignant conditions[1], it seems that their screening perspectives are quite limited. Furthermore, considerable overlap of their concentration ranges between patients and healthy controls or those with benign diseases further reduces their diagnostic strength.

Predictive markers for lymph node metastasis

The presence of lymph node metastases is the single most adverse independent prognostic factor in SCCHN that, in comparison to node negative patients, reduces a 5-year overall survival rate by about 50%.[15] The use of tumor-related histopathological factors in predicting lymph node metastases or advanced imaging techniques is not a reliable method. Furthermore, one should be aware that up to two-thirds of clinically N0 necks are classified as node-free on histopathological examination after surgery (pN0) and that some morbidity resulted even after most strictly limited dissections on the neck, and *vice versa*, a substantial proportion of clinically positive necks are actually pN0.[16]

The predictive value for lymph node metastasis most often correlated with increased Cathepsin D immunohistochemical staining. Grandour-Edwards *et al*.[17] reported on 34 patients with oral cavity, oropharyngeal and hypopharyngeal SCCs. In node negative group, 13/15 (87%) tumors were found to be cathepsin D negative, whereas 11/19 (58%) pN+ tumors stained positive for cathepsin D. When adjusted for tumor stage and grade, cathepsin D positivity was nearly twice as likely to be associated with node metastasis.[17] In two other studies, including exclusively oral cavity tumors, cathepsin D immunoreactivity correlated significantly with the pN-stage of the disease [18,19], whereas in the study of Resnick *et al*, limited to laryngeal tumors, no such relationship was found.[20] Of the other cathepsins, a statistically insignificant trend of higher levels of intensity of immunoreactivity in pN+ group compared to pN0 group of oral cavity tumors was also reported for cathepsin B [18] and cathepsin L.[21]

The cathepsin inhibitors were studied by our team only. In the subgroup of patients with operable tumors (various subsites) and clinically positive neck nodes at presentation, stefin A and stefin B concentrations emerged as significant predictors of lymph-node involvement with tumor cells, i.e. pN-stage (Figure 1).[22] Differentiating between the patients with nodes enlarged due to inflammation and those with metastatic nodes, a portion of patients could be spared more aggressive therapy and treatment-related side effects. On the other hand, in the patients with clinically undetectable nodes at diagnosis, stefins had no potential in predicting pN-stage of the disease. However, clinical relevance of this finding is limited if surgery is technically correctly performed because no adjuvant therapy is indicated in pN0 subgroup, whereas pN+ patients are highly curable with a moderate-dose of postoperative radiotherapy, i.e. ≥95% cure rate at a dose of 50 Gy.[23]

Predictive markers for response to therapy and for recurrent disease

When assessing the efficiency of particular therapy by monitoring the presence of tumor cells in the body, surgery-based therapies and non-surgical therapies should be evaluated separately regarding the differences in mechanisms of tumor cell eradication.

Unfortunately, no study was found to have analyzed the predictive value of cathepsins and their inhibitors for response and for disease recurrence to non-surgical therapies, i.e.

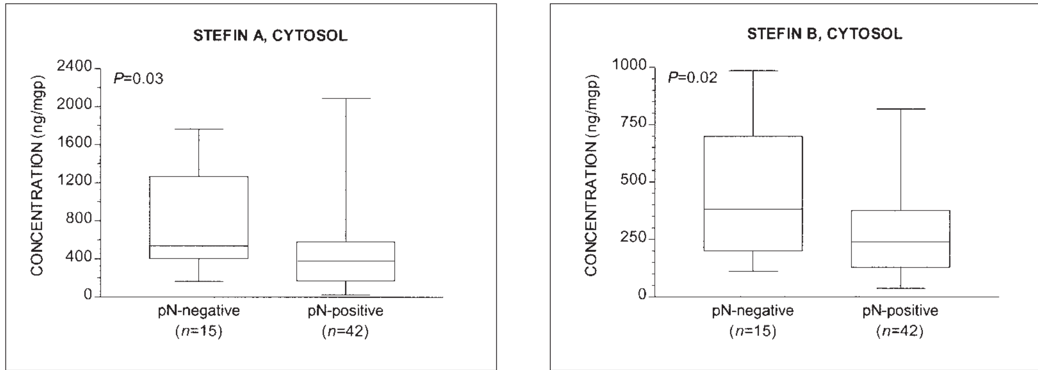

Figure 1. Distribution of tumor concentrations of stefins between patients with histopathologicaly determined negative and positive necks, as measured in a group with clinically palpable nodes at presentation. The top and the bottom of the box represent the 25th and 75th percentiles, respectively, and the ends of the bars represent the rang. The line in the box is the median value. *n*, number of samples. (Reproduced by kind permission of Radiology & Oncology from Strojan P *et al.*, *Radiol Oncol* 2002; **36:** 145-6.)

radiotherapy and/or chemotherapy. For the patients successfully treated with surgery for larynx carcinoma, Kręcicki and Siewiński reported a constant decline in serum cathepsin B-like activity, which normalized within four months of the operation.[8] In the subgroup of patients in whom the treatment failed, the mean serum values of cathepsin B-activity dropped in the first month after surgery, but rapidly increased in subsequent assays. The elevation occurred in all cases at least two months before clinical evidence of metastases or a recurrent tumor became apparent.[8]

According to our experience with more heterogeneous group of tumors treated with surgery and postoperative radiotherapy, cytosolic level of stefin A [24,25] and pretreatment serum level of cathepsin L [10] were found predictive for disease outcome, which, in turn, reflected the tumor response to applied therapy. In the same population the additional sample of serum was collected during regular follow-up visits 7 to 407 days (median, 59 days) after the completion of all therapies. No correlation was found between post-treatment concentrations of any of the studied cathepsins or inhibitors and the time of serum sampling. However, when only those patients with a time interval of more than 45 days (n = 14) from the completion of therapy to the serum sampling were considered, a trend of gradual decline in stefin B (unpublished data) and cystatin C concentrations was observed with the increasing time delay (Figure 2).[14] It appears that the decrease in enzyme and inhibitor activity in the treated area after the resection of the gross tumor burden was followed by a transient elevation of their serum levels, likely due to the inflammatory response of the tissues confined in the irradiation field during postoperative radiotherapy. The cutoff time of 45 days concurs well with the duration of radiomucositis as seen in clinics, gradually subsiding in 4–6 weeks following the end of a radiotherapy course.[26]

Markers for prognosis

The prognostic relevance of cathepsin D was studied most frequently (Table 1). It showed a universal trend of higher survival probability in the patients with low cytosolic or serum levels of the enzyme [24,27-29], and low intensity level of immunoreactivity.[19] The prognostic reliability of cathepsin D was proved in three [27,30,31] out of four [32] studies that utilized multivariate analysis. The same relation between enzyme expression and survival was ob-

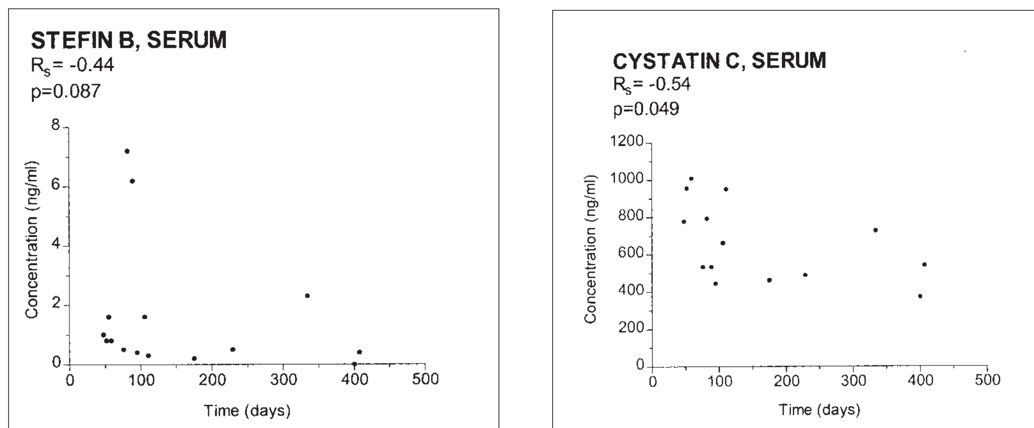

Figure 2. Relationship between post-therapy concentration of stefin B and cystatin C, and the time interval from the completion of therapy to serum sampling in non-relapsed patients with a time delay of more than 45 days (n = 14).

served for cathepsin B [10,25,33] and cytosolic levels of cathepsin L [24,25], whereas higher levels of cathepsin L in the serum was identified as prognostically superior [10], as was the case in cathepsin H.[11,24] However, due to univariate setting of survival analysis, the prognostic information collected from these studies leaves much to be desired.

Table 1. Prognostic relevance of cathepsins and their endogenous inhibitors in squamous cell carcinoma of the head and neck

Author (Ref.)	No.of patients	Tumor site	Method	Prognostic significance
Cathepsin D				
Maurizi *et a.*[27]	63	Larynx	IRA	↑, MVA
Lazaris *et al.*[30]	64	Larynx	IHC	↑, MVA
Seiwerth *et al.*[31]	61	Larynx	IHC	↑, MVA
Kawasaki *et al.*[19]	78	Oral cavity	IHC	↑, UVA
Cathepsin B				
Russo *et al.*[33]	68	Larynx	EA	↑, UVA
Cathepsin H				
Strojan *et al.*[11]	18	All sites	ELISA	↓, UVA
Cathepsin L				
Budihna *et al.*[24]	23	All sites	ELISA	↑, UVA
Strojan *et al.*[10]	35	All sites	ELISA	↓, UVA
Stefin A				
Strojan *et al.*[25]	90	All sites	ELISA	↓, MVA
Stefin B				
Strojan *et al.*[25]	90	All sites	ELISA	↓, MVA
Cystatin C	82			
Strojan *et al.*[34]		All sites	ELISA	↓, MVA

IRA, immunoradiometric assay; IHC, immunohistochemical analysis; EA, enzyme activity; ELISA, enzyme-linked immunosorbent assay; MVA, multivariate analysis; UVA, univariate analysis; ↑, correlation of high levels with poor prognosis; ↓, correlation of low levels with poor prognosis.

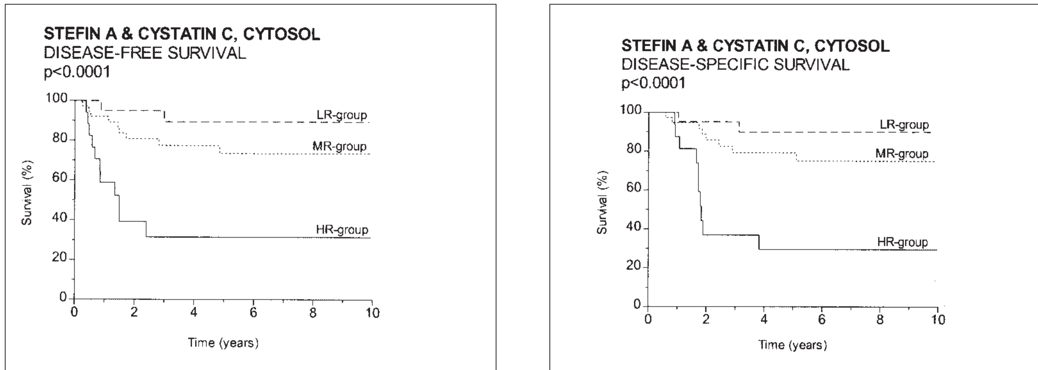

Figure 3. Prognostic significance of the combination of cystatin C and stefin A concentrations: disease-free survival and disease-specific survival. LR, Low-risk group (high stefin A and high cystatin C, *n*=23); MR, Medium-risk group (high stefin A and low cystatin C *or* low stefin A and high cystatin C, *n*=41); HR, High-risk group (low stefin A and low cystatin C, *n*=18).

The results on the prognostic value of cathepsin inhibitors were provided by our team only.[22,24,25,28,34] In operable SCCHN, higher cytosolic concentrations of stefin A, stefin B and cystatin C strongly correlated with longer survival probability in univariate survival analysis, which concurs with the concept of protective role of high levels of cysteine protease inhibitors in tissue homogenates. In multivariate analysis, only stefin A and cystatin C retained their independent prognostic information. However, when comparing the prognostic strength of stefin A with that of cystatin C, the latter lost its significant prognostic power. In addition, the combination of the two inhibitors, stefin A and cystatin C could further stratify the risk of adverse event (Figure 3).[34] No prognostic information was provided from the serum levels of any of the studied inhibitors.[10,14]

Conclusions

Although the clinical utility of cathepsins and their endogenous inhibitors in the management of SCCHN remains open to investigation, it is evident that they exhibit potential in the clinical setting, particularly as markers for lymph node metastasis, for monitoring the presence of tumor cells in the body, and for prognosis. In future studies, larger numbers of patients with clinically more homogenous characteristics should be included and a comparison between various analytical procedures should be focused on. It seems, however, that clinical value of tumor marker profiling in SCCHN patients would be further improved by combining the predictive information from several markers with unrelated or mutually opposing biological roles.

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Pharmacogenetics of thiopurines: can posology be guided by laboratory data?

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Background. *The purpose of this study was to investigate the relationships between the presence of mutations in the TPMT gene, the consequent reduced enzymatic activity, and the clinical toxicity of the treatment with thiopurine antimetabolite drugs.*
Materials and methods. *The study was performed on 44 patients with inflammatory bowel disease treated with AZA. DNA was extracted from blood samples collected from each patient, and genotyping was performed using specific polymerase chain reaction assays in order to detect the three more frequent mutations of the gene. Enzymatic activity was measured on red blood cell lysates by HPLC.*
Results. *Among the subjects, 4 (9.0%) were heterozygous for mutations in the TPMT gene; no subject was homozygous for mutations in the TPMT gene. A complete concordance between TPMT mutated genotype and reduced enzymatic activity could be determined. The incidence of toxicity in the subjects with a mutated genotype was not different from that observed in the patients with a normal TPMT gene.*
Conclusion. *Genotyping methods provide a simple and reliable DNA-based strategy to identify TPMT homozygotes that should avoid thiopurines administration. However, it seems that the most common, less dangerous forms of thiopurine toxicity could be caused by factors different from TPMT gene mutations examined.*

Key words: inflammatory bowel diseases – drug therapy; 6-mercaptopurine; azathioprine; genotype; phenotype

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Introduction

6-mercaptopurine (6MP) is an antimetabolite drug that oncologists have been using for more than 30 years to treat acute lymphoblastic leukemia in children. It is a prodrug that undergoes a complex metabolism[1]: it requires an intracellular activation to its thioguanine nucleotides (TGNs) in order to exert cytotoxic effects. Particularly relevant

for the cytotoxicity of 6MP, and thus of TGNs, is the interference with *de novo* purine biosynthesis, and the modification of DNA structure after incorporation of TGNs that produces an alteration of the function of DNA processing enzymes.[2,3]

In vivo biotransformation of thiopurines also leads to their metabolic inactivation, either by oxidation to thiouric acid catalyzed by xanthine oxidase (XO), or by methylation of the thiol moiety of the molecule by thiopurine-S-methyltransferase (TPMT).

The treatment of patients with thiopurines can cause various adverse effects that can be so severe (even life-threatening) to require the cessation of therapy. The most common adverse effects that have been described include nausea, bone marrow suppression, hepatitis and pancreatitis. The toxicity of the treatment with thiopurines, and the occurrence of bone marrow toxicity in particular, have been ascribed to a genetically determined deficiency of the enzyme TPMT which is held responsible for the metabolic deactivation of the drug.[4-6] TPMT exhibits genetic polymorphism in all large ethnic groups studied to date[7-10]; approximately one individual in 300 inherits two mutant TPMT alleles and is TPMT deficient, and about 10% are heterozygous at the TPMT gene locus and have intermediate enzyme activity. It has been reported that subjects who inherit a deficiency in TPMT exhibit intolerance to thiopurines medications, including 6MP and azathioprine (AZA). Unless TPMT-deficient patients are treated with 10- to 15- fold lower doses of these medications, they develop a profound haematopoietic toxicity that precludes the administration of thiopurines and also of other chemotherapeutic agents and that can be fatal.[4,5,10,11] Moreover, some reports indicate that, in ALL patients who are treated with 6MP carrying a mutant allele for TPMT and who receive intracranial irradiation, there is a greater risk of developing secondary fatal tumours.[12]

More than 10 non-functional mutant alleles for TPMT have been reported, and reliable polymerase chain reaction (PCR) based assays were developed to detect the three most prevalent mutant alleles: TPMT*2, TPMT*3A and TPMT*3C.[13] These variants result from the following point mutations in the TPMT open reading frame: G238C transversion for TPMT*2 alleles and the G460A and A719G transitions for TPMT*3 alleles. While variant alleles other than TPMT*2 and TPMT*3 may lead to a reduced enzyme activity, the frequency of these variant alleles is likely to be very low. Indeed, genotyping for TPMT*2 and TPMT*3 mutant alleles yielded 95% concordance between genotype and phenotype in different populations.[13-17]

Currently, there is a great debate about the opportunity of suggesting genetic testing for TPMT when prescribing 6MP aiming to identify, before treatment, the subjects with a higher risk of severe toxicity. The question of whether to add on an advisory gene testing to the 6MP package label is now before FDA; 6MP is the first drug to be evaluated as possibly requiring a gene test before use.[18]

It has to be noted that thiopurine medications are employed for some 'off label' uses, in particular to treat inflammatory disease like ulcerative colitis, Crohn's disease and rheumatoid arthritis.[19-21] There are indications that the number of prescriptions of thiopurines could be 10 times higher for these patients as compared to cancer patients, which constitutes the registered indication for the use of these agents.[18] Although thiopurines are used to treat inflammatory diseases at a dose lower than that employed for cancer therapy, the treatment is usually much more prolonged, providing a strong argument in favor of testing TPMT genotype, in order to prevent the appearance of serious adverse effects in both types of patients.

In the last two years, a collaborative study run by the Department of Biomedical Sciences of the University of Trieste and the Burlo Garofolo Children's Research Hospital

of Trieste is being implemented in order to examine the occurrence of thiopurine induced adverse effects and the presence of mutations in the TPMT genes. The samples are being obtained from the hemato-oncology and gastroenterology clinics that use thiopurines to treat leukaemia and inflammatory bowel disease (IBD), respectively. This paper reports the current results about the relationship between the outcome of the treatment with AZA used as an immunosuppressive drug to treat IBD, and TPMT genotype in children population or young adults.

Materials and methods

Patients

Between July 2002 and July 2003, 44 patients with IBD were enrolled. The average age at the time of analysis was 16.4 years (range 4-38); among these patients, 20 (45.4%) were female. They all received AZA at an average dose of 2 mg/kg/day (range 1-5 mg); the average length of the treatment with AZA was of 20.6 months (range 0.5-63).

Blood sample preparation

Blood samples of the patients were obtained in Vacutainer Tubes, using EDTA as an anticoagulant. Total genomic DNA was isolated using a commercial kit (Talent, Trieste Italy) according to the suppliers' instructions. Collected DNAs were dissolved in distilled water to a final concentration of 20 ng/μl, as determined by UV spectrophotometry; these solutions were used as template in the PCR reactions.

Erythrocyte lysates were prepared from the blood samples to measure TPMT activity according to the procedure already described.[22] Erythrocytes (RBC) were collected by centrifugation at 800xg for 10 minutes at 4˚C, washed twice with two volumes of an isotonic sodium chloride solution (0.9% w/v), and lysed with distilled water added to the final volume of 10 ml.

TPMT enzyme assay

TPMT activity was measured with the HPLC assay previously described.[22] This assay is based on the *in vitro* conversion of 6MP to 6-methylmercaptopurine (6MMP), using S-adenosyl-L-methionine (SAM) as the methyl donor. Briefly, RBC lysates were purified from bivalent cations that could interfere with the assay by an incubation of 1 hour at 4˚C with the chelating resin Chelex 100 (BIO-RAD, Richmond, CA, USA). The purified lysates were than incubated in the presence of 6-MP and SAM for one hour at 37˚C. 6-MMP produced during the reaction was separated by an HPLC system Hewlett Packard HP Agilent 1100 including a G1311A Quaternary Pump, G1315A Diode Array Detector, G1313A Autosampler, G1322A Vacuum Degasser. The analytical column was a C18 reverse phase 250 mm long (VARIAN, Palo Alto, CA, U.S.A.); the mobile phase consisted of a solution of acetic acid 0.1% and 15% acetonitrile, with a flow rate of 1 ml/min. The detection wavelength was 290 nm, and the quantitative determination was performed comparing the area of the 6MMP peak with a standard curve of the compound dissolved in water. All chemicals were obtained from Sigma-Aldrich (Milan, Italy).

TPMT genotyping

The genotype of each individual at the TPMT*2, TPMT*3A, TPMT*3B and TPMT*3C alleles was determined using previously described polymerase chain reaction (PCR)-based assays.[13,23,24] The mutations present in the variants of the alleles TPMT*3 (G460A and A719G) were assayed by restriction fragment length polymorphism (RFLP) analysis. TPMT exon 7 and exon 10 were amplified in two separate reactions by the use of primers that hybridized

with the sequences flanking each of these polymorphic nucleotides. The sequences of the primers employed to amplify exon 7 were P460F: AGG CAG CTA GGG AAA AAG AAA GGTG and P460R: CAA GCC TTA TAG CCT TAC ACC CAG G[23], for exon 10 were P719F: AAT CCC TGA TGT CAT TCT TCA TAG TAT TT and P719R: CAC ATC ATA ATC TCC TCT TCC.[24] The exon 7 amplicon was digested for 1 hour at 60 °C with MwoI restriction enzyme (New England Biotechnologies, Beverly, MA, U.S.A). MwoI restriction site was present in the wild type allele, but not in the mutant allele. The exon 10 amplicon was digested for 1 hour at 37 °C with Acc I (New England Biotechnologies, Beverly, MA, U.S.A). AccI restriction site was present in the mutant allele, but not in the wild-type allele. The unpurified products of the enzymatic reaction where recognized by electrophoresis in a 2-per cent agarose gel stained with ethidium bromide. An allele specific PCR was used for the analysis of the G238C mutation (TPMT*2). DNA was amplified in two specific reactions, one containing a forward primer wild-type specific (P2W: GTA TGA TTT TAT GCA GGT TTG) and one a mutant specific primer (P2M: GTA TGA TTT TAT GCA GGT TTC); the reverse primer (P2C: TAA ATA GGA ACC ATC GGA CAC) was the same in both reactions.[13] Unpurified PCR products were analyzed after electrophoresis in a 2-per cent agarose gel stained with ethidium bromide. A DNA fragment was amplified with P2M and P2C primers when C238 (mutant) was present, or with P2W and P2C primers when G238 (wild type) was present. The primers were purchased from Invitrogen (Milan, Italy).

Results

TPMT genotype distribution

The distribution of the TPMT genotype in 44 patients with IBD so far enrolled in the study is reported in Table 1. Among these subjects, 40 had a wild type TPMT genotype, while 4 subjects were heterozygous for a mutation in the TPMT gene. Three patients displayed a TPMT*3A mutated allele, and one patient displayed a TPMT*2 mutated allele; no patients were homozygous for TPMT variant alleles.

Table 1. TPMT genotype in 44 patients with IBD

Genotype	N
Normal (wild type/wild type)	40 (90.9 %)
Heterozygous (wild type/mutant)	4 (9.1%)
Homozygous (mutant/mutant)	0
Total	**44 (100.0 %)**

The considered mutations for the TPMT gene are G238C, G460A and A719G, identified as described in the experimental section.

Table 2. TPMT activity in patients with IBD according to TPMT genotype.

	TPMT activity (nmol h^{-1} ml^{-1} RBC)
Wild type (n=23)	11.2 ± 0.1
Heterozygous (n=4)	6.0 ± 0.4 *

TPMT activity is expressed as nmol of 6MMP produced during 1 hour of incubation by 1 ml of RBCs and is reported as mean ± SE. * Means significantly different, t student's test, $p<0.0001$.

TPMT activity

TPMT activity was measured in 27 out of these 44 subjects enrolled in the study, and the results obtained are reported in Table 2. The average activity among these patients was 10.4 nmol/hour/ml RBC; 23 of these subjects had a normal TPMT gene, whereas 4 had a mutated TPMT gene. The average TPMT activity in the subjects with a normal gene was 11.2 nmol/hour/ml RBC, whereas in those with a mutated gene was 6.0 nmol/hour/ml RBC ($p < 0.0001$ t student's test).

Clinical toxicity of AZA treatment and correlation with TPMT genotype

The toxicity of the treatment in the 44 subjects is reported in Table 3. During AZA ad-

ministration, 22 patients (50%) developed side effects. Because of this toxic event, the dosage was reduced in 8 patients and the treatment was suspended in 14 cases. In 13 subjects (29.5%), the bone marrow toxicity manifested as lymphopoenia or thrombocytopoenia. In 9 patients, other side effects were observed: pancreatitis, n = 2 (4.5%); hepatotoxicity, n = 2 (4.5%); infections, n = 3 (6.8%), neuropathy, n = 2 (4.5%). Among the 4 subjects with a mutated TPMT allele, 2 responded normally to therapy whereas 2 developed neuropathy.

Discussion

Thiopurine drugs play an important role in the treatment of leukemias and of some chronic inflammatory diseases. However, the use of these drugs is limited due to serious adverse effects, among which bone marrow suppression may require even the cessation of therapy. Individual differences in susceptibility to AZA/6MP have been observed, and they were attributed to variable intracellular concentrations of the cytotoxic metabolites of the drugs, TGNs. It is known that TPMT deficiency, caused by a frequent genetic polymorphism, might induce a profound bone marrow suppression in the patients receiving thiopurines because it causes a reduced methylation of 6MP and the consequent accumulation of TGNs toxic metabolites. About 10% of Caucasians inherit a mutant allele of the TPMT; they also have a reduced TPMT activity; on the other hand, the subjects homozygous for the variant alleles of TPMT are encountered at the much lower rate of about 1 per 300 Caucasians, and have no measurable TPMT activity. In the 44 subjects examined in the present study, no subject homozygous for TPMT mutated gene was identified, presumably because of the insufficient number of subjects genotyped; at the same time, 4 subjects (9.1%) were found to be heterozygous for a mutated allele of the TPMT gene. The overall frequency of defective alleles obtained in the present study is comparable with that reported in the Italian population[25] and also in other populations of Caucasian origin by other researchers.[13,24,26]

As far as phenotype is concerned, TPMT activity has been so far measured in 27 of the 44 subjects enrolled. The average value of the enzymatic activity measured in the patients with a mutated TPMT genotype was significantly lower than that determined in the remaining group not carrying the mutations considered. This finding is in agreement with the studies showing a strong correlation for TPMT between genotype and phenotype in patients with IBD.[13-17] This finding also confirms the view that genotyping for the identification of the considered TPMT mutations may allow an approach, effective in identifying the subjects with a reduced inherited TPMT activity.

Several studies have been recently published reporting the investigations about the relationship between TPMT reduced enzymatic activity, observed in IBD patients with inherited mutated alleles of TPMT gene, and bone marrow toxicity, which occurred after

Table 3. Correlation between TPMT genotype and the toxicity of AZA treatment in patients with IBD

		TPMT genotype		
	n	Wild type	Heterozygous	Homozygous
Without adverse effects	22 (50.0 %)	20 (45.5 %)	2 (4.5 %)	0
With adverse effects	22 (50.0 %)	20 (45.5 %)	2 (4.5 %)	0
Total	44	40 (90.9 %)	4 (9.1 %)	0

The adverse effects considered are bone marrow toxicity (lymphopoenia, thrombocytopoenia, hepatotoxicity, pancreatitis, infections and neuropathy).

thiopurine treatment.[27,28] The results reported in these studies show in some instances a significant increase in the occurrence of profound bone marrow toxicity in the subjects with a mutated TPMT gene; on the other hand, other side effects (such as hepatotoxicity, pancreatitis, mild lymphopenia and reduced platelet counts) appeared not to be related to TPMT mutations. In particular, in a group of 41 patients with Crohn's disease displaying significant myelotoxicity following the treatment with thiopurines, only 10% were homozygous for the considered TPMT mutations which lead to a severe enzymatic deficiency, and 17% were heterozygous for the same mutated alleles. These mutations, which are associated with the reduced TPMT activity, were overrepresented in this group as compared with a general population, although in most patients, the bone marrow toxicity was not associated with the genotype corresponding to low TPMT activity.[27] Similar results were published also in a further study, reporting the TPMT genotype of 50 patients with IBD treated with thiopurines who suffered from adverse effects that required to discontinue the drug administration: five patients (10%) were heterozygous for TPMT mutated alleles, and one (2%) was homozygous. Even if the single subject with the homozygous mutated genotype developed bone marrow suppression after AZA treatment, the treatment toxicity could not be related to a reduced TPMT activity in most of the patients.[28]

When the adverse effects of AZA in the IBD patients were examined in the group of 44 patients considered in the present investigation, the toxicity of the treatment was in general moderate (grade II or III). The most frequent toxicity encountered was bone marrow toxicity (13 subjects, 29.5%), and none of these patients had any of the considered mutated TPMT alleles. Four subjects were heterozygous for a TPMT mutation; among them, two responded normally to the therapy, whereas two developed an idiosyncratic form of myalgia and arthralgia, which required to discontinue the treatment. Myalgia and arthralgia are more associated with with Type I hypersensitivity reaction than direct drug toxicity, and are unlikely to be associated with TPMT mutated alleles.[29] These results thus appear to agree with those showing that the more common forms of myelosuppression during AZA administration to the IBD patients have causes different from genetic factors such as the considered mutations leading to a reduced TPMT activity.[27,28]

Drug toxicity is a multifactorial phenomenon involving multiple biological and environmental processes, including drug interactions. As far as AZA is concerned, almost all the subjects treated with this drug receive also other medications, which might be responsible for the interactions leading to adverse effects. Drugs like aminosalycilates are commonly used to treat IBD in combination with AZA; at the same time, they have been reported to reduce the metabolic inactivation of thiopurines, through TPMT inhibition.[30,31]

Moreover, genetic factors different from TPMT mutated alleles, such as the polymorphism of genes whose transcripts are involved in the detoxification of xenobiotics, could also influence the pharmacokinetics of thiopurines, and consequently, their clinical efficacy. In this connection, a study is being carried on by the authors aiming to examine the relationships between the clinical toxicity of the treatment with AZA and polymorphisms in the genes for P-glycoprotein[32], a transporter involved in the extrusion of xenobiotics from cells, and polymorphisms in the genes for glutathione-S-transferase, the enzyme responsible for the conjugation of drugs with glutathione.[33]

High throughput techniques are currently available for the investigation of the relationships between the response to drug treatment and the genetic characteristic of the patients. Microarray technology allows the study of the expression of thousands of genes in a single

sample, and has been employed to study the molecular basis of diseases, including various cancers [34], and the molecular mechanisms of drug action.[35] One of the authors (S.G.) spent a short period as visiting scholar at St.Jude Children's Hospital in Memphis (TN, USA) in order to learn the basis of microarray data analysis. These methods were applied to the study of thiopurine cytotoxicity and gene expression aiming to find new markers of drug action that could assist the clinician in determining the patients' individual sensitivity to the effectiveness and toxicity of a drug. In this study, the expression of genes involved in the small GTPase signaling pathways were determined in relation to the effectiveness of the drug, expressed as the magnitude of the decrease of white blood cell number in the 4 days following a single drug administration. Interestingly, the gene expression of Rac1 small guanosine triphosphatase seems to be related to the efficacy of thiopurines: indeed, 6MP is less effective in the subjects with a higher expression of this protein 24 hours after the treatment. This finding is in agreement with a recent observation, indicating a new possible mechanism for thiopurines toxicity, consisting of the inhibition of a Rac1, a critical regulator in mammalian T cells.[36]

These results allow to conclude that genotyping provide a simple and reliable DNA-based strategy to identify TPMT homozygotes who should avoid AZA/6MP administration at conventional dosages. However, it seems that the most frequent, even if less dangerous, forms of thiopurine toxicity in IBD patients could be attributable to factors different from a mutated TPMT genotype. Although TPMT genotyping may be useful to identify subjects with a risk of very severe toxicity, clinicians should still need to monitor carefully the patients treated with these toxic medications, in order to detect other common forms of toxicity. Further studies are needed to enlight the genetic characteristics of subjects experiencing toxic effects after thiopurine treatment, which may lead to the identification of additional genetic markers of toxicity that could assist the clinician in the treatment of patients with these toxic medications.

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Development of quantitative RT-PCR assays for wild-type urokinase receptor (uPAR-wt) and its splice variant uPAR-del5 *

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The receptor for the serine protease urokinase-type plasminogen activator, uPAR (CD 87), plays an important role in tumor cell invasion and metastasis of solid malignant tumors. uPAR is a highly glycosylated, glycan lipid-anchored membrane protein, consisting of three homologous domains. Each individual domain is encoded by two exons: DI by exons 2+3, DII by exons 4+5, and DIII by exons 6+7. Beside the wild-type (wt) uPAR mRNA, two splice variants either lacking exon 5 (uPAR-del5) or both exons 4 and 5 (uPAR-del4/5) have been described. Previously, we studied expression of the mRNA variant uPAR-del4/5 and uPAR mRNA encompassing exons 2, 3, and 4 (i.e. uPAR-wt plus uPAR-del5) applying real-time RT-PCR assays for quantification of the mRNA concentration.

In the present paper, we established two additional specific, robust and highly sensitive RT-PCR assays, based on the LightCycler technology, to specifically quantify either uPAR-wt or its splice variant, uPAR-del5. Expression of uPAR-wt and uPAR-del5 was analyzed in different human malignant cell lines (ovarian cancer cell lines OVMZ-6 and OVMZ-10; breast cancer cell lines MDA-MB 231, MDA-MB 231 BAG, MDA-MB 435, and aMCF-7; brain tumor cell line LN 18) as well as in a set of 174 breast cancer tissue samples. uPAR-del5 mRNA was found to be expressed very frequently at a rather low level (typically less than 1% of uPAR-wt mRNA). In tumor tissue from breast cancer patients, a statistically significant correlation between uPAR-del5 and uPAR-wt mRNA ($r = 0.779$; $P < 0.001$) was observed. There was no association between the expression level of either mRNA and clinical parameters such as nodal status, tumor size and grade. In estrogen receptor negative tumors, a significantly higher uPAR-del5 expression was found ($P = 0.023$).

The two developed quantitative RT-PCR assays described here may aid further analysis of the function and clinical relevance of uPAR-wt and one of its splice variants, uPAR-del5, in malignant tumors.

Key words: neoplasms; urinary plasminogen activator; RNA, messenger; reverse transcriptase polymerase chain reaction; RNA splicing

Introduction

Tumor cell dissemination and formation of metastases is facilitated by the interaction of diverse proteolytic systems, including serine proteases, cysteine proteases, and matrix metalloproteinases.[1] These proteases enable tumor cells to degrade the extracellular matrix and to cross natural boundaries.[2] The receptor for the serine protease urokinase-type plasminogen activator (uPAR, CD 87) is essentially involved in this process as it focuses the proteolytic activity of uPA to the cell surface. Furthermore, it interacts with a broad variety of other ligands, including vitronectin or integrins, and by this modulates proliferation, cell adhesion and migration, invasion, and angiogenesis.[3,4] High tumor levels of uPA and/or its inhibitor PAI-1 have been shown to be a predictor for poor prognosis of patients with solid tumors, including breast, gastric, esophageal, ovarian, colorectal or hepatocellular cancer.[4,5] Different therapeutic approaches have been employed to obstruct the uPA/uPAR system, using small molecules, such as antibodies or modified toxins.[6-9]

uPAR is a highly glycosylated, glycan lipid (GPI-)-anchored membrane protein, consisting of three structurally homologous domains (DI, DII, DIII).[10] In the past, a number of glycosylation variants and different molecular forms of uPAR antigen such as soluble uPAR, uPAR-DI, and uPAR-DII+III has been described and analyzed (for a summary see Luther *et al.*[11]). In some cases, certain (novel) functions or activities could be assigned to these variants: endoproteolytic processing of CD87 with removal of DI is, *e.g.*, a likely pathway for controlling cell adherence and migration [3,12,13], as the extent of glycosylation of DI strongly contributes to the affinity for its ligand uPA.[14] Furthermore, splice variants of uPAR have been identified, *i.e.* an uPAR mRNA splice variant lacking exon 5 (uPAR-del5) as well as a variant lacking exons 4 and 5 (uPAR-del4/5).[11,15] Since splice variants of genes often display a different expression pattern and biological role compared to that of the wild-type genes, especially in tumor tissue [16] , we previously studied the expression of the mRNA variant uPAR-del4/5 in a representative set of breast cancer tissues applying a real-time RT-PCR assay for quantification of the mRNA concentration.[11] The mRNA variant uPAR-del4/5 was, in fact, expressed very frequently in breast cancer tissue and, strikingly, higher uPAR-del4/5 expression was significantly associated with shorter disease-free survival of breast cancer patients. Thus, these results suggest that uPAR-del4/5 mRNA may serve as a prognostic marker in breast cancer. The aim of the present study was to establish a highly sensitive real-time RT-PCR assay based on LightCycler technology for the uPAR-del5 mRNA variant in order to be able to analyze the expression pattern of this alternatively spliced mRNA in solid malignant tumors.

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Material and methods

Cell lines and cell lysates, uPAR ELISA

Human ovarian cancer cell lines OVMZ-6 and OVMZ-10, human breast cancer cell lines MDA-MB 231, MDA-MB 231 BAG, MDA-MB 435, and aMCF-7 as well as the human brain tumor cell line LN 18 were cultured at 37°C in a humidified atmosphere of 5% CO_2 and

95% air in DMEM medium (Invitrogen, Karlsruhe, Germany), supplemented with 10% fetal calf serum (Invitrogen) and 1% penicilline-streptomycine (Biochrom, Berlin, Germany), 1% arginine-asparagine (Sigma, Deisenhofen, Germany) and 1% HEPES buffer (Invitrogen). Cells were harvested from monolayer dishes after two days. Total RNA from the cells was extracted using Trizol Reagent (Invitrogen). cDNA was synthesized using AMV cDNA First Strand Synthesis Kit (Roche Diagnostics, Penzberg, Germany). cDNAs from the cell lines were diluted 1:15 and aliquoted at -20 °C.

For uPAR antigen detection, 2 x 10^6 cells were cultured for two days on monolayer dishes, then harvested, resuspended in phosphate-buffered saline and sedimented by centrifugation (200 x *g*, 10 min, RT). Cells were disrupted by two freezing and thawing cycles, followed by a solubilization step (10 min, in 100 µl per 10^6 cells sample buffer containing 0.2% Triton X-100) and stored at -20 °C. uPAR antigen was determined in cell lysates by uPAR IIIF10 ELISA as described by Kotzsch and co-workers (2000). Protein content was determined using the Micro BCA protein assay kit (Pierce, Rockford, IL). uPAR antigen levels in cell lysates are expressed as ng per mg of total protein.

Patients - tissue selection

The study adhered to national regulations of The Netherlands on ethical issues and was approved by the local ethical committee. Tumor tissue was obtained from patients with unilateral breast cancer after surgical resection of the primary tumor. Patients who had received neo-adjuvant treatment, or who had a previous diagnosis of cancer or who had a carcinoma *in situ*, were excluded. Furthermore, patients with recurrent disease within one month after surgery or with distant metastases at time of diagnosis were excluded as well. After surgery, performed between November 1987 and December 1997 in participating hospitals of the Comprehensive Cancer Center East in The Netherlands, a representative part of the tumor was selected by a pathologist, frozen in liquid nitrogen, and sent to the Department of Chemical Endocrinology for routine determination of estrogen (ER) and progesterone (PgR) receptor status by ligand binding assay.[17,18] Remaining frozen tissue or tissue powder (after dismembration) prepared from this tumor was kept in liquid nitrogen. For the present study, samples were selected based on the availability of tissue stored in the tumor bank.

Patients- cDNA synthesis

Total RNA was isolated from approx. 20 mg of tissue powder using the RNeasy mini kit (Qiagen, Hilden, Germany) with on-column DNase-I treatment as previously described.[18] Reverse transcription was performed using the Reverse Transcription System (Promega Benelux BV, Leiden, The Netherlands) according to the manufacturer's protocol. After annealing of random hexamers for 10 min at 20 °C, cDNA synthesis was performed for 60 min at 42 °C, followed by an enzyme inactivation step for 5 min at 95 °C. cDNAs were diluted 1:3 and aliquoted.

Quantitative real-time RT-PCR of uPAR-del5, uPAR-wt, and the housekeeping gene G6PDH

RT-PCR primers and hybridization probes were obtained from TibMolBiol (Berlin, Germany). RT-PCR was performed using the LightCycler apparatus (Roche, software version 3.5). Assays for quantification of uPAR-wt and uPAR-del5 were established, with primer sequences as follows: Ex 2F: GAC CTC TGC AGG ACC ACG AT; Ex 6,4R: CAG ATT TTC AAG CTC CAG GAC TT; Ex 5A: GGT GGC GGT CAT CCT TTG. RT-PCR was performed with a master mix with 3.0 µM $MgCl_2$, 0.6 µM of the primers, 0.2 µM of each

of the hybridization probes and 2 µl of reagent mix, in a total volume of 20 µl. The amplification program started with pre-denaturation at 95 °C, followed by 45 cycles of amplification: denaturation 10 sec at 95 °C, annealing for 15 sec at 66 °C, and elongation at 72 °C for 15 sec. A standard curve was generated for each run using eight glass capillaries (Roboscreen, Leipzig, Germany) coated with a defined number of molecules of uPAR (wild type or del5) plasmid (range from 100,000 to 10 copies). The generation of the plasmids pRcRSV-GPI-uPAR-wt (encoding uPAR-wt) and pRcRSV-GPI-uPAR-del5 have been described previously.[11] A negative control containing buffer only was included in each run. For normalization of the data h-G6PDH (human glucose-6-phosphate-dehydrogenase) Housekeeping Gene Set (Roche) was used, according to the manufacturer´s protocol. All cDNA samples displaying less than 10,000 molecules of G6PDH were considered to be of non-optimal quality and were excluded from further analyses (18 of 192 cDNA samples = 9.4%). Relative expression levels were determined calculating the ratio between absolute template molecule and G6PDH housekeeping molecule numbers. The ratios of target genes and reference gene were multiplied with factor 1,000.

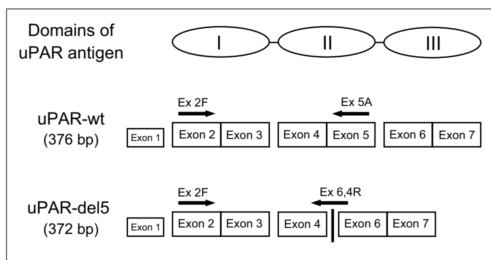

Figure 1. Detection of uPAR-wt and uPAR-del5 mRNA. uPAR consists of three homologous domains. Each individual domain is encoded by two exons: DI by exons 2+3, DII by exons 4+5, and DIII by exons 6+7 (exon 1 encodes the signal sequence of uPAR). For specific amplification of uPAR-wt mRNA a reverse-primer directed to exon 5 was designed. In order to specifically amplify uPAR-del5 a primer overlapping the unique boundary between exons 4 and 6 was chosen. Both RT-PCR assays use the same forward primer within exon 2. The amplicon length is 376 bp for uPAR-wt and 372 bp for uPAR-del5.

Statistics

The association of uPAR mRNA levels with histomorphological and clinical parameters was analyzed using non-parametric tests (Mann-Whitney U test; Kruskal-Wallis test). Statistical analyses were performed using the SPSS 11.5 software.

Results and discussion

Development of RT-PCR assays for uPAR-del5 and uPAR-wt

For quantification of uPAR-del5 mRNA, we established a highly sensitive real-time RT-PCR assay applying the LightCycler technology. In this assay, the 5′ amplification primer (Ex 2F) is identical to the 5′ primer used in the RT-PCR assays previously described [11] for detection of uPAR-2/3/4 mRNA (encompassing exons 2, 3, and 4) and uPAR-del4/5 mRNA (encompassing exons 2, 3, and 6 and lacking exons 4 and 5, respectively). The 3′ amplification primer (Ex 6,4R) overlaps the alternative splicing site of exons 4 and 6 and, therefore, selectively binds to uPAR-del5 mRNA (and not to uPAR-wt or uPAR-del4/5). For generation of standard curves, glass capillaries coated with defined numbers of uPAR-del5 plasmid - exactly determined by HPLC calibration [19] - were used. In addition to the uPAR-del5 assay, a quantitative RT-PCR assay for uPAR-wt mRNA was established, which does neither amplify uPAR-del5 nor uPAR-del4/5 mRNA, since the 3′ amplification primer (Ex 5A) is directed to exon 5 (Figure 1). Since in both assays the 5′ and 3′ amplification primers are directed to different exons, amplification of (possible contaminating) genomic uPAR-

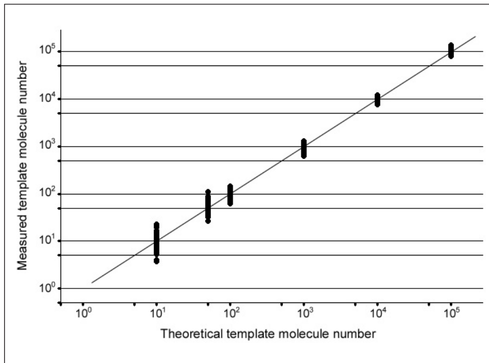

Figure 2. LightCycler PCR standard curves for quantification of uPAR-del5 mRNA. The plots of molecule numbers detected *versus* theoretical molecule numbers of uPAR-del5 were generated from 48 independent PCR runs. Correlation of the values is $r = 0.990$; $P < 0.0001$).

DNA is excluded due to the large amplicon size of > 10 kbp. With both assays, the detection limit was (at least) 10 copies of cDNA, which corresponds to the lowest glass capillary standard. Figure 2 depicts a plot of measured molecules *versus* theoretical molecule numbers of uPAR-del5 in 48 independent PCR runs for the standards ranging from 10 to 100,000 copies. In case of uPAR-wt, similar results were obtained (data not shown). To test for specificity of the uPAR-del5 and uPAR-wt assays, we analyzed samples containing large plasmid copy numbers (corresponding to about 1.5 pg DNA) of either uPAR-wt, -del5, and -del4/5 or included glass capillaries coated with 100,000 copies of the three different plasmids. In neither case, we observed an amplification signal above the buffer control for the control plasmids (data not shown). Thus, the target sequence (either uPAR-del5 or uPAR-wt) is selectively amplified with the two newly established RT-PCR assays.

Quantification of uPAR mRNA variants and uPAR antigen in cell lines

Seven different cell lines were selected and the uPAR antigen content determined applying the uPAR HU/IIIF10-ELISA.[20] As can be seen from Table 1, the uPAR antigen content ranged from undetectable (< 0.05 ng/mg, breast cancer cell line MDA-MB 435) to about 7 ng/mg total protein in the case of the ovarian cancer cell line OVMZ-10. uPAR-wt mRNA concentration (normalized to the expression of G6PDH) was determined and compared to the uPAR antigen values: the highest antigen values (OVMZ-10 and MDA-MB 231 BAG) corresponded to the highest mRNA levels, the cell line with undetectable uPAR protein levels displayed an extremely low uPAR-wt mRNA expression as well. The other cell lines displayed an intermediate expression both at the protein and mRNA level ($r = 0.786$; $P < 0.05$) (Table 1). uPAR-del5 expression was detected in all of the cell lines albeit to a low extent ranging from 0.23 to 1.53% of uPAR-wt expression.

Table 1. Detection of uPAR antigen, uPAR-wt and uPAR-del5 mRNA in seven different cancer cell lines. Cellular uPAR antigen content (expressed as ng per mg total protein) was measured using the HU/IIIF10 ELISA.[20] uPAR-wt and uPAR-del5 mRNA was quantified by the newly established RT-PCR assays and expression normalized to G6PDH mRNA .

Cell lines		ELISA [ng/mg]	RT-PCR measurements [relative to G6PDH]		
Name	**Origin**	**HU/IIIF10**	**uPAR-wt**	**uPAR-del5**	**uPAR-del5 / uPAR-wt [%]**
MDA 435	breast	< 0.05	21	0.2	0.95
MDA 231	breast	1.99	747	2.0	0.27
LN 18	brain	2.07	137	2.1	1.53
OVMZ-6	ovary	2.18	648	5.5	0.85
aMCF-7	breast	3.19	671	3.9	0.58
MDA 231 BAG	breast	5.28	1204	2.8	0.23
OVMZ-10	ovary	6.86	3588	28.0	0.78

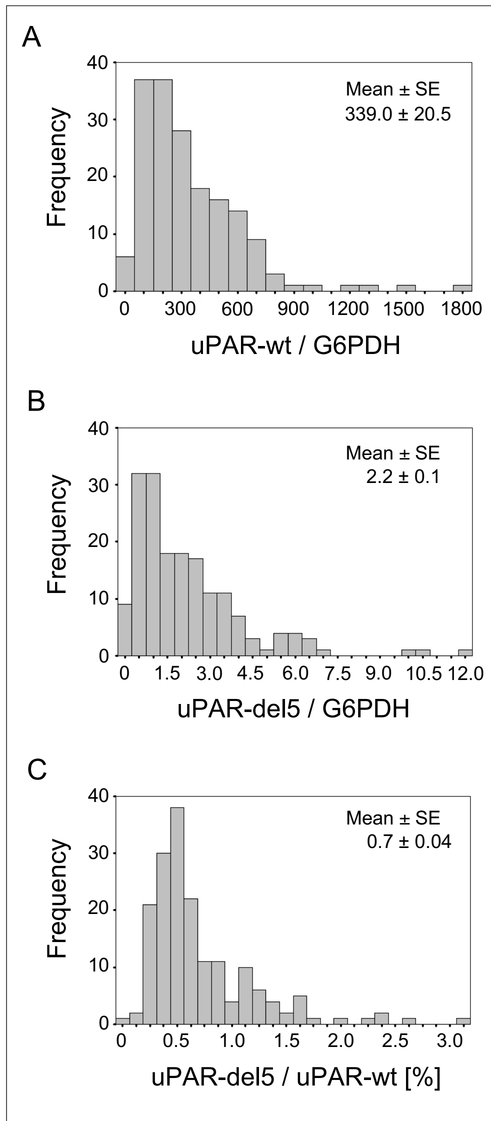

Figure 3. uPAR mRNA expression in human breast cancer tissue. 174 primary breast cancer samples were measured for uPAR-wt and uPAR-del5 mRNA with the newly established quantitative RT-PCR assays. The histograms depict the frequency distribution of **(A)** uPAR-wt expression (normalized to G6PDH), **(B)** uPAR-del5 expression (normalized to G6PDH), and **(C)** relative uPAR-del5 expression compared to uPAR-wt [%].

Quantification of uPAR mRNA variants in breast cancer tissue

In order to select an appropriate housekeeping gene for normalization of mRNA concentrations, we first analyzed expression of three different housekeeping genes (PBDG, G6PDH, and GAPDH) in a representative set (n=46) of breast cancer cDNA samples. The absolute mRNA concentrations of PBGD, G6PDH, and GAPDH strongly correlated with each other (ranging from r = 0.880 to 0.908, $P < 0.0001$) indicating that these genes are, in fact, expressed constitutively in breast cancer. As both genes, G6PDH and uPAR, are moderately high expressed, G6PDH was subsequently used for normalization.

We assessed the level of expression of uPAR-del5 and uPAR-wt mRNA in 174 cases of breast cancer patients (Figure 3). uPAR-wt expression was found in all cases (median relative expression level: 263). uPAR-del5 was detected in the breast cancer samples with high frequency but with a significantly lower expression level (median: 1.57). In one case, there was no uPAR-del5-amplification signal at all, in further 40 of the 174 cases (23.6%), the determined copy number was below the lowest standard included in the assay. The relative expression rate of uPAR-del5 to uPAR-wt was between 0 and 3.1% (median uPAR-del5$_{rel}$/uPAR-wt$_{rel}$: 0.53%). The mRNA concentrations of uPAR-wt and uPAR-del5 significantly correlated with each other (r = 0.779; $P < 0.001$). We observed no statistically significant association of uPAR-wt or uPAR-del5 expression with clinical parameters such as nodal status, tumor size or grade (Table 2). A significantly higher uPAR-del5 expression was found in estrogen receptor negative tumors (P = 0.023).

Conclusions

In the present study, we developed a quantitative real-time RT-PCR method to specifically quantify uPAR-wt mRNA (excluding both

Table 2. uPAR-wt and uPAR-del5 mRNA expression and clinical parameters. Associations of uPAR-wt and uPAR-del5 expression with clinical parameters were analyzed by using non-parametric tests. All tests were performed at significance level of a < 0.05.

174 breast cancer patients			uPAR-wt / G6PDH		uPAR-del5 / G6PDH	
Variable	**Total**	**No. of patients (%)**	**Mean ± SE**	***P* value**	**Mean ± SE**	***P* value**
Menopausal status [a]	174					
pre/peri		39 (22.4)	378 ± 53	**0.397** (ns)	2.2 ± 0.3	**0.785** (ns)
post		135 (77.6)	327 ± 22		2.1 ± 0.2	
Nodal status [a]	149					
negative		79 (53.0)	360 ± 31	**0.058** (ns)	2.5 ± 0.3	**0.070** (ns)
positive		70 (47.0)	291 ± 30		1.9 ± 0.2	
x		25				
Size (pT) [b]	171					
1		55 (32.2)	398 ± 38	**0.101** (ns)	2.6 ± 0.3	**0.240** (ns)
2		97 (56.7)	286 ± 20		1.9 ± 0.2	
3		15 (8.8)	405 ± 118		2.1 ± 0.4	
4		4 (2.3)	369 ± 133		1.5 ± 0.6	
x		3				
Grade [a]	123					
I/II		65 (52.8)	275 ± 23	**0.116** (ns)	1.7 ± 0.2	**0.112** (ns)
III		58 (47.2)	397 ± 47		2.4 ± 0.3	
x		51				
ER status [a]	174					
negative		56 (32.2)	427 ± 48	**0.066** (ns)	2.8 ± 0.3	**<u>0.023</u>**
positive		118 (67.8)	297 ± 19		1.9 ± 0.1	
PgR status [a]	174					
negative		67 (38.5)	390 ± 39	**0.274** (ns)	2.6 ± 0.3	**0.153** (ns)
positive		107 (61.5)	307 ± 22		1.9 ± 0.2	

a Mann-Whitney-U Test
b Kruskal-Wallis Test
x Unknown status

splice variants uPAR-del5 and uPAR-del4/5) and uPAR-del5 mRNA in breast cancer. The assays are rapid, robust, and sensitive. As these assays require only minute amounts of cDNA, they are well suited for studies when the amount of sample is limited. For validation of the uPAR-wt assay, we initially measured uPAR mRNA expression in cell lines of different origin and observed that the measured mRNA expression levels corresponded well to the respective antigen levels determined by a uPAR ELISA (HU/IIIF10). In the tumor cell lines, we also detected low expression of uPAR-del5 mRNA. *In vivo* expression of this uPAR splice variant was subsequently proven by analyzing breast tumor samples. Similar to uPAR-wt, we found no significant association of uPAR-del5 expression in rela-

tion to the nodal status, tumor size or grade in the analyzed patients cohort.

The cDNA sequence of the uPAR-del5 variant has been originally published by Casey *et al*. [15] Recently, we verified expression of uPAR-del5 in tumor cells by amplification of uPAR mRNA with primers directed to exon 1 and 6, respectively, followed by direct sequencing of the resulting PCR products.[11] Further evidence for the *in vivo* occurrence of uPAR-del5 comes from searching available nucleotide databases, in which a series of independent submissions of the cDNA sequence encoding uPAR-del5 obtained from various sources (*e.g.* accessions AX281707, AA481366, BM543893 or BM767461) is found. Thus, the uPAR-del5 splice variant seems to be often expressed in human cells. Previously, we have generated stably transfected Chinese hamster ovary cells, which harbor an expression plasmid encoding uPAR-del5. By ELISA, flow cytofluorometry, and Western blot analysis, we confirmed synthesis, secretion and cell surface-association of uPAR-del5. These experiments indicate that uPAR-del5 mRNA is translated and processed in a similar manner as wild-type uPAR. Therefore, experiments are on the way to search for (new) functions of uPAR-del5 (and the other expressed splice variant uPAR-del4/5) which may play a role for tumor invasion and metastasis.

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Ex vivo flow mammalian cell electropulsation

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Cell electropulsation brings a local and reversible permeabilization of cell membranes. This gives the technical possibility to introduce (load) exogenous compounds (drugs, proteins, DNA) into cells. Flow-through electropulsation allows to treat a large volume (number) of cells as requested for cell therapy. Cells are flowing through a pulsing chamber where they are submitted to a well-defined number of calibrated pulses. A proper setting of pulse frequency and flow rate controls the number of pulses. A large volume of cells can therefore be electrotreated in a small sized pulsing chamber. The viability of pulsed cells appears to be greatly preserved.

Key words: cell membrane permeability; electroporation

Introduction

Cell treatment with high intensity electric field pulses provokes a change in the membrane structure leading to a loss of its barrier function — phenomenon indicated as electropermeabilization or "electroporation".[1,2] By a proper choice of the parameters of the applied electric field, this change in the membrane permeability can be reversible or irreversible leading to leakage of cytoplasmic content and cell death.[3]

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When a short (microsecond) electric field pulse is applied to a cell, the resulting change in membrane potential difference may result in a localized long lived but reversible change in the membrane organization. This new state of the membrane is called « electropermeabilized » and can support the transfer of hydrophilic compounds into the cytoplasm and their leakage out of the cell. A key feature is that under controlled electrical conditions this membrane change is transient and the «normal» impermeable state can be recovered. The cell viability can therefore be preserved. This is obtained by a proper choice of electrical parameters (field strength, pulse duration and number of pulses) and buffers (pH, osmotic pressure and additives). This brought the technical possibility to introduce (load) exogenous compounds (drugs) into cells. A clinical development was proposed with big success (electrochimiotherapy).[4] This

transient membrane organization remains poorly characterized from a structural point of view. A peculiar associated property is fusogenicity. When cells, which are in the electropermeabilized state, are brought into contact, membrane coalescence occurs leading to the formation of viable polykaryons (electrofusion).[5] This is indicative that the repulsive hydration forces have been abolished. This was supported by the observation of an alteration of the interfacial layer of electropermeabilized cells. Another observation is that it was possible to get protein expression by electropulsing cells in a solution containing the relevant plasmid (electrotransformation, electrotransfection).[6] This again can be obtained in vivo in tumors (electrogenotherapy).[7]

Ex vivo treatment of cells appears as an interesting procedure for cell therapy. A present limit of electropulsation is that most protocols were designed for batch process. Only limited volumes can be treated due to the power limitations of most pulse generators. Furthermore safe conditions avoiding contamination (either microbial or electrochemical) need the use of rather expensive equipments (large laminar flow hood, sterilization of the cuvettes). Introduced in the 80's electric field treatment using a flow system seems nowadays a very promising technique for ex vivo cell therapy.[8-12] Our conclusions were reproduced on different cell systems by other groups [13] and very recently either on a large volume as we did in 1992 [14] or on microdevices.[15]

Recently we showed that application of series of electric pulses could provoke not only a drug loading but an important release of different cytoplasmic enzymes by a batch process. The efficiency of this process was dependent on the intensity, number and duration of pulses, on the growth phase and postpulse incubation media composition. This batch approach even very efficient was suitable only for treatment of micro volumes. Large volumes were successfully treated by the flow process.[16] A new aspect of flow-through electropermeabilization — the release of macromolecules from mammalian cells and its potential clinical application is validated in the present study.

Up-sizing of laboratory scale processes was always limited by the amount of energy to be delivered by the power generators. We presented a vast field of evidences that it is possible to utilize electropulsation using a flow system to work on a large volume of cells. Power specifications for the pulse generator are mostly driven by the required pulse frequencies. Nevertheless kHz trains can be delivered meaning that high flow rate (i.e. large volumes) can be treated (up to 1 l/min).[17,18]

All aspects of electropulsation can be obtained with the flow process: drug loading, protein and metabolite extraction, eradication, gene transfer and expression and hydrid production. A key advantage for clinical applications is that contamination can be avoided by using closed loop circuits.

The present paper described the systematic investigation of exogeneous compound loading and protein extraction from chinese hamster ovary cells by a flow electropulsation method by emphasing the good preservation of the viability of the treated cells.

Materials and methods

Cells

Chinese hamster ovary (CHO) cells were used as a model system. The WTT clone was selected for its ability to both grow in suspension and plate easily. They were grown in suspension in MEM medium as previously described.[11]

Electropermeabilization

Culture medium was removed and replaced by a pulsing buffer (10 mM phosphate, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.4) at a cell density of 10^6 per ml. This low volume fraction of

cells was chosen to avoid any viscosity effect on the flow. Penetration of propidium iodide PI (100 μM, in pulsing buffer) was used to monitor permeabilization. Analysis was performed with a cytofluorimeter (Facscan, BD) to evaluate both the percentage of fluorescent cells (i.e. percentage of PI positive cells) and the mean level of fluorescence of the cell population.

The protein release was monitored as follows. Cells are kept 10-20 min at room temperature after the electrical treatment and then on ice. The protein concentration in the supernatant of pulsed and control cells was assayed by the Biorad kit.

Cell viability was assayed 24h after the electrical treatment by the crystal violet method by taking advantage of the selectivity of viable cells to plate on a culture dish.

Electropulsation

Electric field treatment was performed on a CNRS high power cell electropulsator generating rectangular pulses with adjustable voltage up to 1.5 kV.

A flow through pulsing chamber with a 0.3 mL volume was used. Two stainless steel flat parallel electrodes at a distance of 0.3 cm were used to apply repetitive pulses. Pulse duration (T) and frequency (Ff) were triggered by a TTL pulse generator. All pulsing parameters were monitored on line with an oscilloscope connected to a PC computer when storage was needed. An ohmic behaviour was observed. Cells were treated at room temperature with series of pulses with controlled duration and frequencies. The flow rate was in the range from 1.2 to 60 mL/min and freely adjustable with a peristaltic pump (Gilson, France).

Flow electropulsation

The basic concept was to apply calibrated pulses at a delivery frequency which was linked to the flow rate (Figure 1). The desired number of pulses was actually delivered on each cell during its residency in the pulsing chamber. The geometry of the chamber (flat parallel electrodes) was chosen to give a homogeneous field distribution on a laminar flow. Therefore, the residency time T_{res} of a given cell in the chamber was:

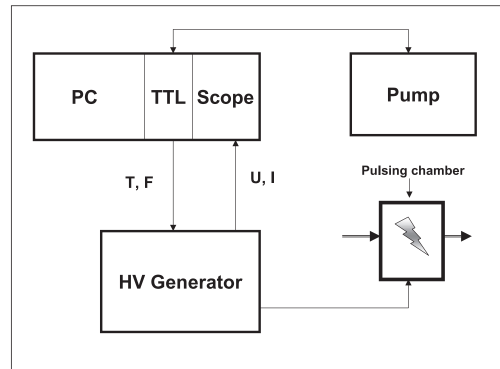

Figure 1. Configuration of a flow through electropulsator. The TTL trigger drives the delivery of the pulse with a preset duration and frequency. The voltage is set on the generator. All signals are monitored on line on the oscilloscope and the PC. The flow rate is adjusted with a peristaltic pump.

$$T_{res} = Vol / Q \qquad \text{(Eq. 1)}$$

where Vol was the volume of the pulsing flow chamber and Q , the flow rate. The number of pulses delivered per cell was:

$$N = Vol\ F\ f / Q \qquad \text{(Eq. 2)}$$

F f being the frequency of the pulses, therefore N was under experimental control. Nevertheless one should take into account that a parabolic distribution of the flow rate was present in the chamber. More pulses were applied on cells close to the walls than in the middle of the chamber

The field strength was taken as the voltage to electrode distance ratio

$$E = U/d \qquad \text{(Eq. 3)}$$

d being the width between the two electrodes, U, the voltage. The field distribution

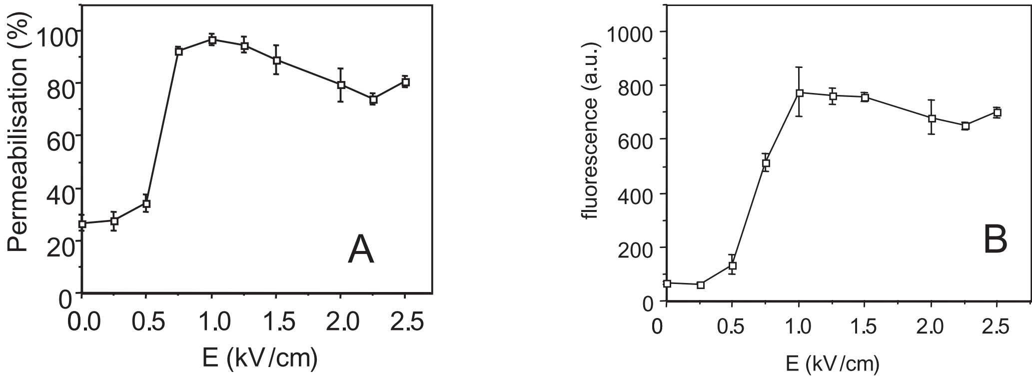

Figure 2. Control by the field on cell permeabilization. CHO cells (10^6 per ml) were treated by a train of 10 pulses lasting 1 ms at a frequency of 1 Hz with a flow rate of 1.2 ml/min. The pulsing buffer contains the hydrophilic dye PI. Permeabilization is assayed by the number of fluorescent cells (A) and by the mean fluorescence emission of the population (B) by use of a flow fluocytometer.

was homogeneous when taking into account the geometry of the chamber (parallel flat electrodes).

This average power associated to the train of pulses was:

$$\langle P \rangle = U\ I\ f\ T \qquad \text{(Eq. 4)}$$

T being the single pulse duration.

As the chamber resistance, when filled by the sample, could be approximated by

$$R = d / (\Lambda\ S) \qquad \text{(Eq. 5)}$$

where L is the conductance of the sample and S the section of the electrodes, then

$$\langle P \rangle = f\ T\ E^2\ \Lambda\ Vol \qquad \text{(Eq. 6).}$$

From Eq. 2, an increase in the flow rate Q while keeping the number of applied pulses N constant needs to increase FVolfVol, i.e. either F f or Vol (or both). Only a limited energy and current are delivered with each pulse. The power and current specifications are not requiring sophisticated designs. The only difficulty is to have a main power supply able to maintain the interpulse recharging of the internal capacitors when working at high frequencies.

Another important parameter is the nature of flow which is given by the Reynolds number Re

$$Re = (v\ d\ \rho\ )/\mu \qquad \text{(Eq. 7)}$$

Where v is the flow velocity, r, the volumic mass of the liquid, m its dynamic viscosity

In our experimental conditions, Re is always smaller than 2000, i.e. the flow is under a laminar condition. No tumbling is affecting the cell population.

Results

Effect of the field strength

A peculiar observation was that a rather high percentage of cells were PI positive just by flowing across the flow through chamber without any electrical treatment. This was much higher than the basal level in the native CHO cell population. This was associated with a decrease in the cell viability of about 30%.

A train of 10 pulses lasting each 1 ms was applied on the flow of cells at a frequency of 1 Hz (flow rate 1.2 ml/min). An increase in the number of PI positive cells and in PI stain-

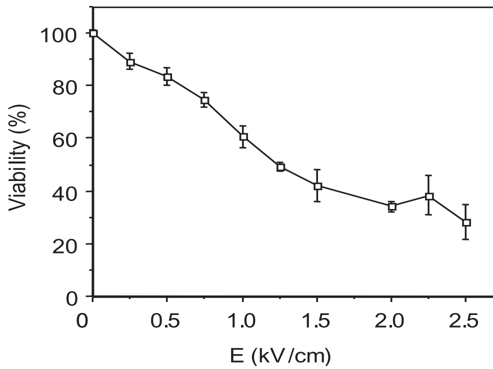

Figure 3. Viability of pulsed cells. CHO cells (10^6 per ml) were treated by a train of 10 pulses lasting 1 ms at a frequency of 1 Hz with a flow rate of 1.2 ml/min. Their viability was assayed 24 h after the treatment by the crystal violet test. The data were corrected from the effect of the flow on the control cells, which brought a 30% loss.

ing was clearly present as soon as the field strength of the applied pulses was a large as 0.5 kV/cm (Figure 2 A,B). A maximal effect was present around 1 kV/cm and a small decrease was observed for larger intensities.

The viability of the pulsed cells was affected in a field dependent way (all other parameters being kept constant). Cells in the flow process were apparently very sensitive to the field as shown by the effect of 0.25 kV/cm pulses. But cells were observed to be somehow resistant to very strong electric field (Figure 3). The loss in viability apparently leveled off above 1.5 kV/cm.

Effect of pulse duration

Cells were submitted to a train of 10 pulses at a frequency of 10 Hz and a magnitude of 1 kV/cm with a flow rate of 1.2 ml/min. These conditions were chosen by taking into account that when the pulse duration was 1 ms, all cells were permeabilized. We observed that even with a pulse duration as short as 0.1 ms the electrical treatment brought a permeabilization of all cells (Figure 4A). Increasing the pulse duration above this value did not induce any further change. PI staining was observed to increase continuously with the pulse duration (Figure 4B).

Cell viability was strongly dependent on the pulse duration. A sharp decrease was observed up to 0.2 ms followed by a slow decrease with a further increase of the pulse duration (Figure 4A).

Interestingly if the pulse duration was increased up to 5 ms, a much more limited lev-

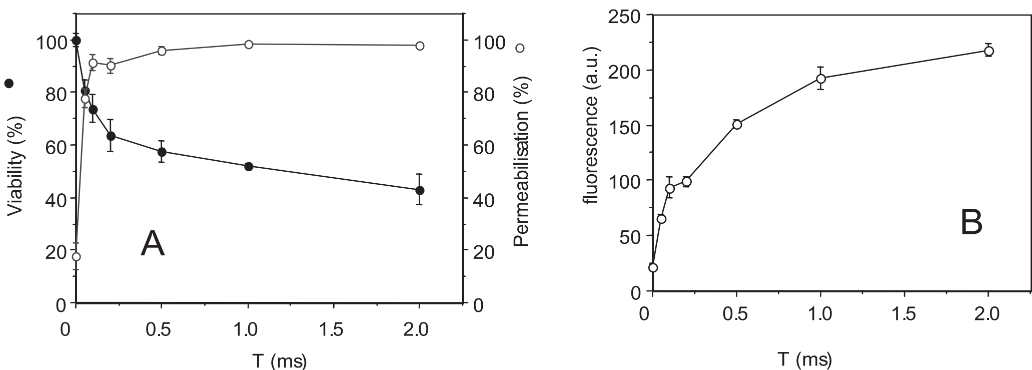

Figure 4. Effect of the pulse duration on cell permeabilization. CHO cells (10^6 per ml) were treated by a train of 10 pulses of 1 kV/cm at a frequency of 1 Hz with a flow rate of 1.2 ml/min. The pulsing buffer contains the hydrophilic dye PI. Permeabilization is assayed by the number of fluorescent cells (A) and by the mean fluorescence emission of the population (B) by use of a flow fluocytometer. Their viability was assayed 24 h after the treatment by the crystal violet test (A). The data were corrected from the effect of the flow on the control cells, which brought a 30% loss.

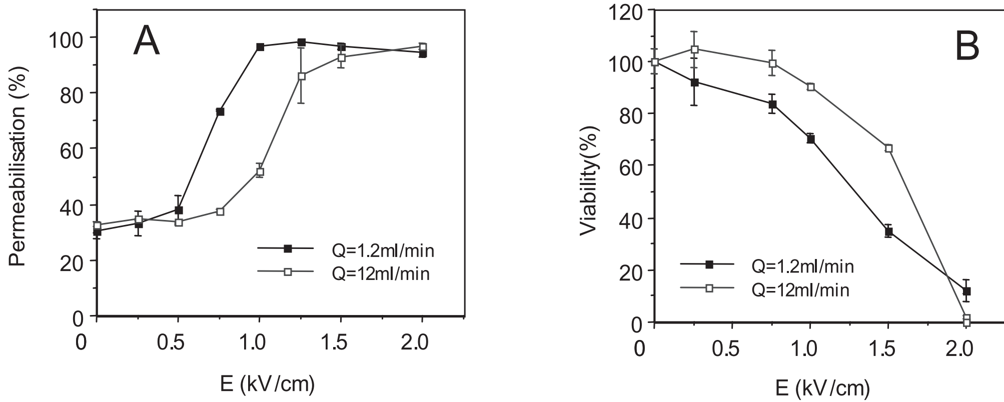

Figure 5. Effect of the flow rate on cell permeabilization. CHO cells (10^6 per ml) were treated by a train of 10 pulses of 1 kV/cm lasting 1 ms at a frequency of 1 Hz with a flow rate of 1.2 ml/min and 10 Hz with a flow rate of 12 ml/min. Permeabilization is assayed by the number of fluorescent cells (A). Their viability was assayed 24 h after the treatment by the crystal violet test (B). The data were corrected from the effect of the flow on the control cells, which brought a 30% loss.

el of permeabilization was observed and was observed to be associated to an irreversible process (cell death). No reversible permeabilization was observed.

Effect of the flow rate

Treatment of a large volume would take advantage of a high flow rate. We compared the behavior of cells when submitted to a train of 10 pulses of 1 ms. Their frequencies were adjusted to the flow rate, being 1 Hz at 1.2 ml/min and 10 Hz at 12 ml/min. The on-line monitoring of the signal on the oscilloscope showed that even under the 10 Hz procedure, the shape of the pulses remained square. The flow remained laminar under the two conditions.

Permeabilization was detected as soon as the field strength was larger than a critical value of 0.5 kV/cm. Its increase was sharper with a further increase in the field strength under the low flow rate conditions. All cells were permeabilized under the two conditions when a field as large as 1.5 kV/cm was applied (Figure 5 A).

Cell viability was less affected for a given field strength at the high flow rate (Figure 5 B). As a conclusion, reversible permeabilization was obtained under higher field conditions with the high flow rate.

Cytoplasmic protein release

We previously showed that flow-through electropulsation was an efficient approach for protein extraction from yeasts.[16] Clinical biotechnology is taking advantage of the bioproduction of proteins in mammalian cells.[19] CHO cells are one of the most successful cell factories. Results in the preceding part of this work was dealing with the loading of small (drug size like) molecules. In this part of the work we checked how effective was the flow-through electropulsation for the extraction of cytoplasmic proteins from CHO cells.

Cells were submitted to a train of 10 pulses lasting 1 ms at a frequency of 10 Hz with a flow rate of 1.2 ml/min. This pulse duration was chosen being shown in batch experiments to be needed to obtain macromolecule loading.[20] No protein release was observed with field strength up to 1 kV/mscm. High level of extraction was observed between 1.2 and 1.5 kV/cm (Figure 6). Interestingly as reported above the viability while affected by the electrical treatment remained larger than

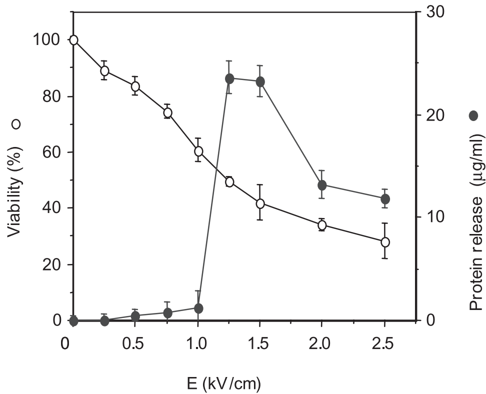

Figure 6. Electrorelease of cytoplasmic proteins. CHO cells (10^6 per ml) were treated by a train of 10 pulses of 1 kV/cm lasting 1 ms at a frequency of 1 Hz with a flow rate of 1.2 ml/min. Their viability was assayed 24 h after the treatment by the crystal violet test. The data were corrected from the effect of the flow on the control cells, which brought a 30% loss.

40% under the field conditions where the high electroextraction was obtained.

Discussion

The present study shows the flexibility and the effectiveness of the flow through electropulsation in the case of mammalian cells.

A high level of loading evaluated both in the number of PI positive cells and in the number of internalized polar molecules (as quantified by the fluorescence of PI in the present experiments) was obtained while preserving the viability of most cells. By using the 1 ms and 1 Hz conditions, we observed that up to 60% of the population could be loaded (permeabilized) and remained viable. This takes into account the fact that more than 20% of the population was killed by the flow. This clearly should be optimized for a clinical development of the method for cell therapytreatment. This can be optimized by taking into account that 0.1 ms pulses were effective to obtain a high level of loading. Short pulses may be less detrimental for cells.

Under laminar flow conditions (Re less than 2000), the maximal efficiency of loading was not affected by the flow rate up to 12 ml/min. Viability was sensitive to the flow rate. Higher flow rate can be obtained by an array of parallel chambers where all cells would be treated under the same conditions (electrical parameters, flow velocity).

The current which was delivered during the pulses under the optimized conditions (0.75 kV/cm) was only 1 Amp. The average power was 2.5 W under the high flow rate (12 ml/min, 10 Hz). This condition brought a temperature increase of only 2˚C of the cell suspension under the assumption that no heat dissipation occurred between the pulses. This conclusion further supports the safety of this approach for drug loading in cell therapy.

We observed a shift in the permeabilization /field strength plots when the flow rate was increased. This could result from the viscoelasticity of CHO cells. Their spherical shape observed under batch conditions would be altered by the drag of the flow. This drag increased with the flow rate. In simpler words, their shape turned in a more elongated one. It was well established that the sensitivity of a cell to an electric field was controlled by its shape and its orientation relative to the field lines.[21] Our observation that cell permeabilization needed higher field strength under the high flow rate where they were elongated in a direction perpendicular to the field was fully supported by the theoretical approach. This interpretation is further supported by the shift of the viability plot: elongated cells in a direction perpendicular to the field are less sensitive to the field

The protein extraction is clearly a very efficient new approach for clinical biotechnology. The observation that a large subpopulation was still viable after the train of 1 ms pulses suggested that it should be possible to recycle the pulsed population in a fermentor for a further growth of the cells. This is indicative that cell viability is not affected and

that electrochemical contamination if present is not harmful and furthermore can be decreased by a bipolar pulse. [22].

As a final conclusion, flow electropulsation offers a safe and powerful tool for the development of cell therapy

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Immunohistochemical expression of HER-2/neu in patients with lung carcinoma and its prognostic significance

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Background. *The HER-2 protein or p185her2 is a membrane receptor with tyrosine kinase activity encoded by HER-2/neu gene. Overexpression of HER-2/neu has been observed in many human cancers, including lung cancer. In the study, the expression of HER-2 protein is determined in the spectrum of lung cancer (adenocarcinoma, squamous cell carcinoma and small cell carcinoma).*

Patients and methods. *The study population consisted of two groups: 19 patients that had undergone surgical treatment and 10 patients that had undergone fiber-optic bronchoscopy and biopsy for primary diagnosis only. Tissue specimens were neutral formaldehyde-fixed and paraffin-embedded. Standard histochemical and immunohistochemical staining were used for diagnosis. Expression of HER-2/neu protein was determined by immunohistochemical staining with Hercep Test^{TM} (DAKO). The results were graded 0-1 as negative and 2-3 as positive.*

Results. *Overall incidence of HER-2/neu overexpression was 34.4% (10 of 29). Higher incidence was found in the patients with adenocarcinoma 45.4% (5 of 11). In squamous cell carcinoma and small cell carcinoma, the overexpression incidence was 30.7% (4 of 13) and 20% (1 of 5), respectively. No statistically significant difference was seen given the age and gender. HER-2/neu overexpression was more pronounced in the patients with advanced tumour: all patients with squamous cell carcinoma and HER-2/neu overexpression had stage IIIB and stage IV disease, while 80 % of adenocarcinoma patients with HER-2/neu overexpression had stage IIIA and IIIB disease.*

Conclusions. *These results are satisfactory and encourage us to continue this work in the follow-up study to evaluate HER-2/neu role as predictive and prognostic factor for the patients with lung cancer.*

Key words: lung neoplasms – pathology; receptors,, erb-2; prognosis; immunohistochemistry

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Introduction

Lung cancer is the leading cause of cancer mortality worldwide. In Macedonia, lung cancer is the cause of death in nearly 19.44% of all cancer deaths.[1] Most of the patients had

an advanced stage of the disease at the time of diagnosis. Even with early diagnosis and an adequate treatment, the 5-year survival rate of the patients with stage I disease is about 70%.[2]

We presume that there is an intrinsic factor that determines the clinical course of the disease. Advances in molecular pathology showed that expression of p53, c-myc, c-fos, c-erbB-1,[3] and Her-2/neu[4,5] is associated with lung cancer. The HER-2 protein or p185-her 2 is a membrane receptor with tyrosine kinase activity encoded by HER-2/neu gene.

HER-2/neu overexpression has been observed in many human cancers, including carcinoma of the breast, ovary, gastrointestinal tract, salivary gland and lung.[6] There are different reports about the expression of this protein in different lung cancers.

The aim of the study was to determine the expression of HER-2 protein in the adenocarcinoma, squamous cell carcinoma (squamous CC) and small cell carcinoma (small CC) of the lung, and to assess the correlation between HER-2/neu expression and clinical stage of the patients.

The evaluation of the expression of HER-2/neu is important because of its prognostic significance and possibility to treat the patients with trastuzumab (Herceptin™).

Material and methods

The study population consisted of two groups: 19 patients that had undergone surgical treatment and 10 patients that had undergone fiber-optic bronchoscopy and biopsy for primary diagnosis only.

The stage of the disease was classified according to the new international staging system for lung cancer, using the following clinical investigations: blood examination, biochemical studies, chest radiography, whole bone scan and computed topographic scan of the chest.

Tissue specimens from surgical and biopsy material were neutral formaldehyde-fixed and paraffin-embedded. Standard histological stainings (Hemalaun-Eosin, PAS, Alcian blue –PAS and reticulin – Gomori) were used for diagnosis.

HER-2/neu oncogene expression was determined by immunohistochemical staining with Hercep Test™ (DAKO). The slides were deparaffinized in xylene and rehydrated with graded ethanol. Following the incubation with the primary rabbit antibody to human HER-2 protein, ready-to-use Visualisation Reagent consisted of both secondary goat anti-rabbit immunoglobulin and horseradish peroxidase, was used. The enzymatic conver-

Table 1. Distribution of the patients according to the histological type of the tumour and clinical stage

Histological type	Clinical stage					Total
	I	II	IIIa	IIIb	IV	
Adenocarcinoma	0	5	3	3	0	11
Squamous CC	0	5	2	4	2	13
Small CC	0	2	3	0	0	5
Total	0	12	8	7	2	29

Table 2. Overexpression of HER2 according to histological type and clinical stage of disease

Histological type HER2+/total	Clinical stage					Total (%)
	I	II	IIIa	IIIb	IV	
Adenocarcinoma	0/0	1/5	2/3	2/3	0/0	5 (45.4%)
Squamous CC	0/0	0/5	0/2	3/4	1/2	4 (30.7%)
Small CC	0/0	0/2	1/3	0/0	0/0	1 (20%)
Total of HER2+	0	1	3	5	1	10 (34.4%)

sion of the subsequently added DAB chromogen resulted in formation of brown precipitation at the antigen site.

Hercep Test[TM] was interpreted as negative for HER-2 protein overexpression (0 and 1+ staining intensity), and positive for HER-2 protein overexpression (weakly positive 2+ staining intensity and strongly positive 3+ staining intensity).

Results

The mean age of the patients was 55.4 years, ranging from 41 to 72 years. Twenty-six patients were males and 3 patients were females. More tumours were found in the left lung (19 cases, 65.5%) than in the right lung (10 cases, 34.5%). In the patients who underwent surgical treatment, lobectomy and mediastinal lymphadenectomy were performed. Other patients received radiotherapy and/or chemotherapy, depending on clinical stage. The follow-up of the patients was from 3 to 24 months. In two patients, the outcome of surgery was fatal. Two patients died of advanced disease, and one died of cardiac failure. Table 1 shows the patients distribution according to the histological type and clinical stage of lung cancer.

Histological analysis revealed 11 cases of adenocarcinoma, 13 cases of squamous CC and 5 cases of small CC. The majority of patients had a clinical stage II disease (12 cases), 8 patients had stage IIIA, 7 patients IIIB, and two stage IV.

The overall incidence of HER-2 overexpression was 34.4% (10 of 29). Higher incidence was found in the patients with adenocarcinoma – 45.4% (5 of 11) (Figure 1), while in squamous CC patients, the incidence was 30.7% (4 of 13) (Figure 2), and in small CC patients, the incidence was 20% (1 of 5) (Figure 3) (Table 2).

There was no statistically significant difference given the age and gender.

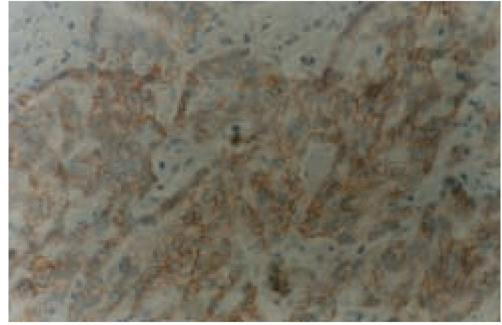

Figure 1. Positive immunostaining of HER-2 in pulmonary adenocarcinoma (DAKO Hercep Test [TM]).

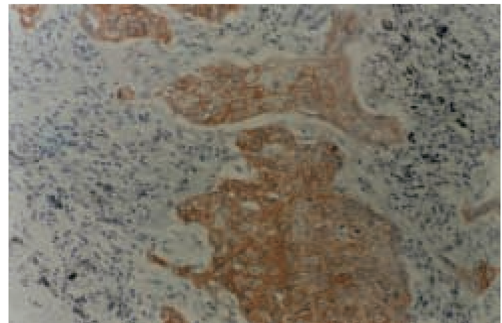

Figure 2. HER-2 overexpression in sqamous cell carcinoma of the lung (DAKO Hercep Test [TM]).

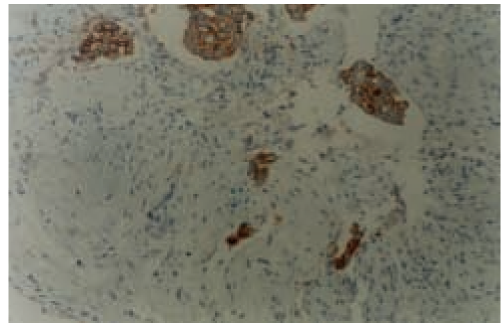

Figure 3. Positive immunostaining for HER-2 in small cell lung carcinoma (DAKO Hercep Test [TM]).

As shown in Table 2, HER-2 protein overexpression was more pronounced in the patients with advanced clinical stage: 4 of 5 patients (80%) with adenocarcinoma and HER-2 protein overexpression had stage IIIA and IIIB disease, and 3 of 4 patients (75%) with

squamous CC and HER-2 protein overexpression had stage IIIB disease, while of two patients with stage IV squamous CC, only one (50%) showed HER-2 protein overexpression. One patient with small CC and positive for HER-2 had in stage IIIA disease.

Discussion

Immunohistochemistry is a frequently used method for evaluating gene expression in tumours. However, a great variation in the interpretation of results has been noted. In the case of lung carcinoma, the positive rate of HER-2 expression ranged from 14 to 87.5%.[7] This variation is probably due to different methods, materials or subjective biases. There is also a possibility for heterogeneous expression of HER-2 in tumour cells. Hercep Test™ (DAKO) offers an objective and quantitative evaluation of immunohistochemical results. This is especially important when this immunohistochemically based marker is employed for assessing the therapy and prognosis.

In our study, the overall incidence of HER-2 overexpression was 34.4%, with a higher incidence found in the patients with adenocarcinoma – 45.4%. The patients with squamous CC overexpressed HER-2 in 30.7 % and those with small CC in 20%. These results are slightly higher than those presented in the review of Agus *et al*.[8] and could be due to the sensitivity of the antibody Hercep Test™ (DAKO).

There were many previous reports which have described the p185neu expression in lung cancer.[2-7] In some cases, the overexpression of HER-2/neu was shown to correlate also with the survival.[4,5] However, a limited number of cases, lack of a scoring system for HER-2/neu expression and conflicting results made the interpretation of these studies difficult. Harpole *et al*[9] suggested that, beside the other parameters (male, sex, presence of symptoms, tumour size, poor cell differentiation, vascular invasion, p53 expression and high Ki-67 index), the HER-2/neu expression could be an independent prognostic factor in the patients with stage I non-small cell lung cancer. They further propose that the outcome of the disease is the "dose response" of the additive effect of these parameters. It appeared that the disease progression could be dependent on several clinicopathologic parameters. The cause of early cancer deaths as well as the mechanism for the early relapse and metastasis remain to be determined.

Osaki *et al*[10] have shown that the patients with stage IIIB or T4 lung adenocarcinoma had an increased serum level of HER-2 and that it was correlated with the overexpression in tissue sections. Diez et al.[11], working on fresh samples of non-small cell lung cancer, did not find significant correlation between p185 level and TNM classification, but proved that shortened median time of tumour relapse was proportional to the rise of p185 in the tumour tissue.

The results presented in this paper demonstrate that the overexpression of HER-2/neu in the patients with lung carcinoma correlated with the advanced clinical stage. The follow-up of the patients in our study was short, so the determination of the relapse rate or disease-free period was limited. Two patients who died of the advanced disease had overexpression of HER-2 and short partial remission of 5 and 8 months.

In conclusion, our results are satisfactory and encourage us to continue this work in the follow-up study to evaluate HER-2/neu role as predictive and prognostic factor for the patients with lung cancer. However, the determination of this parameter is required in more patients and for longer period to confirm the value of HER-2 overexpression. The correlation with other oncoproteins is needed for the elucidation of a cause of early cancer death as well as of a mechanism for an early relapse and metastasising.

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New saccharide derivatives of indolo[2,3-b]quinoline as cytotoxic compounds and topoisomerase II inhibitors

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Some of alkyl- and alkylamino- derivatives of 6H-indolo[2,3-b]quinolines are known to be active antiproliferative and cell cycle modulating compounds. Their cytotoxic properties are, at least in part, due to DNA intercalation ability and topoisomerase II inhibition activity. To improve physicochemical and biological properties of 6H-indolo[2,3-b]quinolines the series of new, saccharide (C-2, C-9 or N-6) derivatives were designed and synthesized. The influence of different carbohydrate units (D-glucose, D-lactose, L-rhamnose, L-acosamine, L-daunosamine), position of attachment and linker size on cytotoxic properties and topoisomerase II inhibition activity were tested. Among compounds tested there were 2-deoxy-α-D-glucopyranoside (1-6), 2-deoxy-α-L-rhamnopyranoside (7-12) and 2-deoxy-α-D-lactopyranoside (13-18) derivatives in the group of saccharide moiety containing compounds and a-L-daunosaminide (19-24) and a-L-acosaminide (25-27) in the aminosaccharide derivatives series as well.

Key words: DNA topoisomerases, type II – antagonists and inhibitors; indols; quinones

Introduction

Indolo[2,3-b]quinoline derivatives are a group of compounds being the synthetic analogs of a plant alkaloid neocryptolepine (5-methyl-5H-indolo[2,3-b]quinoline), which (together with cryptolepine) is present in *Cryptolepis sanguinolenta* extracts used in natural medicine in Africa.

Cryptolepine (5-methyl-5H-indolo[3,2-b]quinoline) - major *Cryptolepsis sanguinolenta* alkaloid - displays a plenty of pharmacological effects, such as antimuscarinic, noradrenergic receptor antagonistic, antihypertensive, vasodilative, antithrombotic, antipyretic and antiinflammatory properties.

Neocryptolepine and cryptolepine derivatives reveal antiplasmodial and antitrypanosomal[1] and, first of all, cytotoxic activities.[2]

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Indolo[2,3-b]quinoline derivatives can also be considered as the analogs of the DNA intercalating anticancer drug, ellipticine, and its natural isomer, olivacine. The alkaloids of the pyrido[4,3-b]carbazole group are able to stabilize *in vitro* the topoisomerase II-DNA cleavable complexes. One of the cytotoxic olivacine derivatives - S 16020 - showing no immunogenicity in clinical phase I trial was qualified for phase II studies.[3]

Previous studies revealed that topoisomerase II is in fact a cellular target involved in the mechanism of cytotoxic action of 5,11-dimethyl-5H-indolo[2,3-b]quinoline (DiMIQ), first examined cytotoxic compound of the group.[4]

Further investigation into the impact of the substituents introduced into the indolo[2,3-b]quinoline was conducted. Consequently, the series of different derivatives (including various types of substituents and position of their attachment) of 5H- and 6H-indolo[2,3-b]quinolines were synthesized and their cytotoxic activity and ability to induce topoisomerase II-dependent DNA cleavage *in vitro* were tested.

It was initially stated that among methyl-substituted indolo[2,3-b]quinolines, only derivatives belonging to the 5H series (and none of the 6H series), display cytotoxicity against human cervix carcinoma KB cells - ID_{50} (inhibitory dose 50%) values were in the range of 2 to 9 μM - and against several human cancer cell lines of different origin (ID_{50} values varied from 0.6 to 1.4 μM), as well as stimulate the formation of calf thymus topoisomerase II-mediated DNA cleavage at concentrations between 0.2 and 10 μM.[5,6]

Further SAR (structure-activity relationship) studies conducted on 6H series showed that the introduction of an alkyl-amino-alkyl substituent at the N-6 position of indolo[2,3-b]quinoline accounts for the appearance of the cytotoxic properties against KB cell line. ID_{50} values obtained were in the range of 2.0 to 9.0 μM. These results indicate on a strong relation between 6H-indolo[2,3-b]quinoline derivatives structure and their cytotoxic activity, corresponding well with the ability to bind DNA and to inhibit topoisomerase II activity.[7]

To avoid problems connected with poor solubility and resulting inefficient bioavailability, another members of the cytotoxic indolo[2,3-b]quinoline family, well soluble in water in a non-pH-dependent manner, were synthesized and tested.[8]

Simultaneously, attempts at preparing liposomally-formulated 5H-indolo[2,3-b]quinolines[9] and obtaining cytotoxic 6H sugar and aminosugar bearing indolo[2,3-b]quinoline derivatives were undertaken. The results of research on the 6H series are the subject of present publication.

Material and methods

Compounds

All compounds tested were synthesized at Pharmaceutical Research Institute in Warsaw.[10]

Human topoisomerase II activity inhibition assay

Topoisomerase II Assay Kit (Catalog No. 1001-2) and Human Type II Topoisomerase (p170 Form) (Catalog No. 2000H-2); 2U/ml, used in the assays were purchased from TopoGEN, Inc. (Columbus, Ohio, USA).

The start solutions of compounds tested (10 mM) were prepared by dissolving the substances in DMSO (dimethyl sulfoxide, Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) and diluting in reaction buffer to the concentrations of 0.0005, 0.0025, 0.005, 0.025, 0.05, 0.25 and 0.5 mM.

Electrophoresis was run in 1% agarose gel (Roth, Karlsruhe, Germany) with ethidium bromide (0.5mg/ml, Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) at 150V. The same amount of ethidium bromide was added

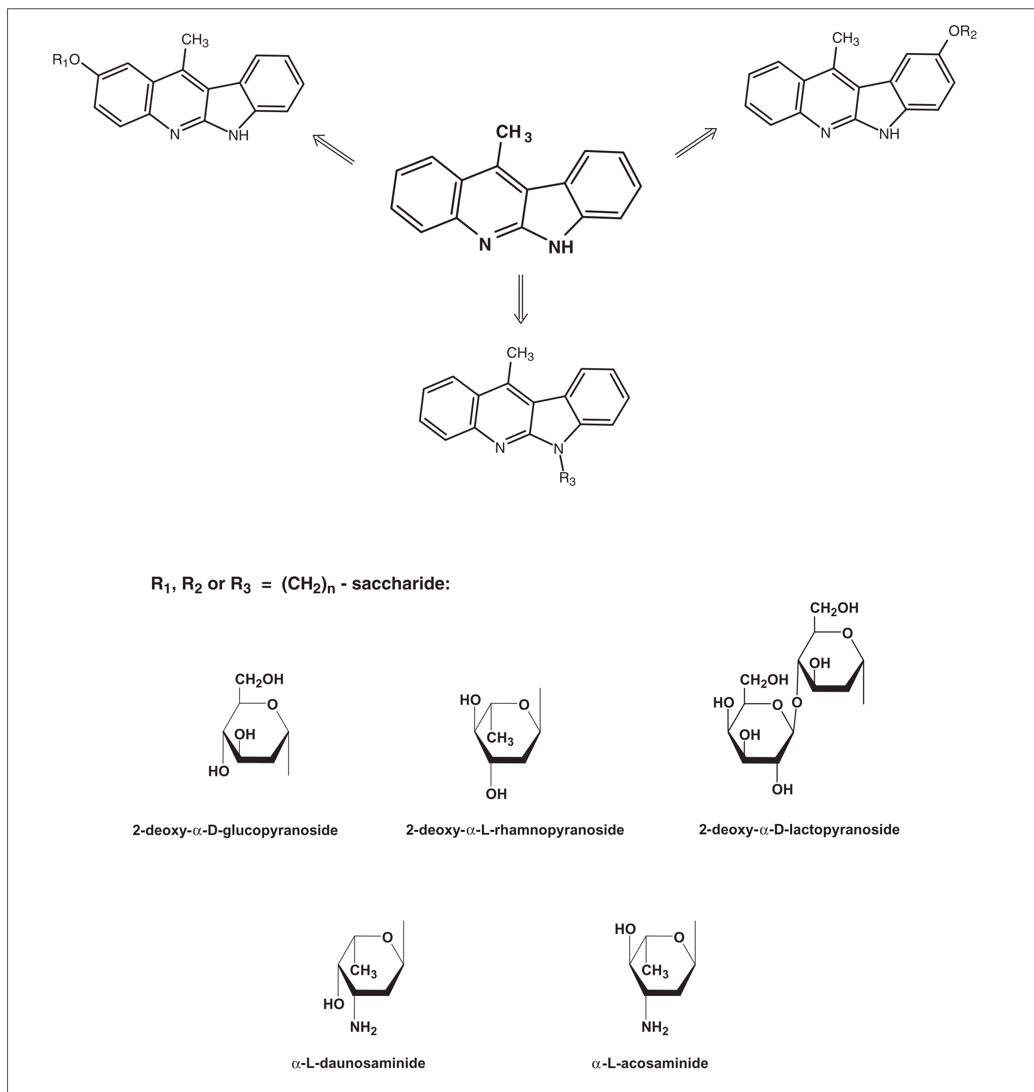


Figure 1. The structures of saccharide and aminosaccharide derivatives studied.

to electrophoresis buffer - 1x TBE (89mM Tris-borate, 1mM EDTA, pH 8.0).

For results acquisition Typhoon 8600 Variable Mode Imager (Molecular Dynamics Inc, Sunnyvale, CA, USA) was applied.

Inhibition of topoisomerase II activity by compounds tested was measured as a degree of inhibition of the kinetoplast DNA decatenation. It was assumed that total enzyme activity inhibition resulted in the same outcomes as achieved for sample with the substrate kinetoplast DNA only.[11,12]

Cell lines

Established *in vitro,* human cervix carcinoma (KB) and Jurkat (T-cell leukemia) cell lines were used. Both lines were obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.) and maintained in the Cell

Table 1. Cytotoxicity and inhibition of topoisomerase II activity revealed by D-glucose indolo[2,3-b]quinoline saccharide derivatives.

Compound tested	Cytotoxity - ID_{50} [μM]	Total topoisomerase II inhibition [mM]
(No. 1) **R_3** = 2-deoxy-α-D-glucopyranoside **n = 2 .**	98.30 ± 31.95	n.a.
(No. 2) **R_1** = 2-deoxy-α-D-glucopyranoside **n = 2**	n.a.	n.t.
(No. 3) **R_2** = 2-deoxy-α-D-glucopyranoside **n = 2**	15.40 ± 5.05	n.a.
(No. 4) **R_3** = 2-deoxy-α-D-glucopyranoside **n = 5**	68.20 ± 1.24	n.a.
(No. 5) **R_1** = 2-deoxy-α-D-glucopyranoside **n = 5**	46.30 ± 2.05	n.a.
(No. 6) **R_2**= 2-deoxy-α-D-glucopyranoside **n = 5**	57.60 ± 8.50	n.a.

ID_{50}[1)] – compound concentration leading to 50% inhibition of cell proliferation;
n – number of carbon atoms in linker moiety (as it is shown in Figure 1);
n.a. - not active;
n.t. - not tested.

Culture Collection of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. The cells were cultured in the RPMI 1640 + Opti-MEM (KB) or RPMI 1640 (Jurkat) medium supplemented with 2mM glutamine (Gibco, Warsaw, Poland), streptomycin (100 mg/ml), penicillin (100U/ml) (both antibiotics from Polfa, Tarchomin, Poland) and 5% (KB) or 10% (Jurkat) fetal calf serum (Gibco, Grand Island, U.S.A.). The cell cultures were maintained at 37°C in humid atmosphere saturated with 5% CO_2.

Anti-proliferative assay in vitro

Test solutions of the compounds (1 mg/ml) were prepared by dissolving the substances in 100 μl of DMSO completed with 900 μl of tissue culture medium. Afterwards, the tested compounds were diluted in culture medium to reach the final concentrations of 100, 10, 1, and 0.1 μg/ml. Results were converted into μM concentrations.

The details of the SRB assay were described by Skehan *et al.*[13] Twenty-four hours before addition of the tested agents, the cells were plated in 96-well plates (Sarstedt, U.S.A.) at a density of $1x10^4$ cells per well. The cytotoxicity assay was performed after 72-hour exposure of the cultured cells to varying concentrations (from 0.1 to 100 μg/ml) of the tested agents. The cells attached to the plastic were fixed by gently layering cold 50% TCA (trichloroacetic acid, Aldrich-Chemie, Germany) on the top of the culture medium in each well. The plates were incubated at 4˚C for 1 hour and then washed five times with tap water. The background optical density was measured in the wells filled with culture medium, without the cells. The cellular material fixed with TCA was stained with 0.4% sulforhodamine B (SRB, Sigma, Germany) and dissolved in 1% acetic acid (POCh, Gliwice, Poland) for 30 minutes. Unbound dye was removed by rinsing (4x) with 1% acetic acid. The protein-bound dye was extracted with 10mM unbuffered Tris base (POCh, Gliwice, Poland) for determination of

Table 2. Cytotoxicity and inhibition of topoisomerase II activity revealed by L-rhamnose indolo[2,3-b]quinoline saccharide derivatives.

Compound tested	Cytotoxicity - ID_{50} [μM]	Total topoisomerase II inhibition [mM]
(No. 7) R_3 = 2-deoxy-α-L-rhamnopyranoside **n = 2**	n.a.	n.t.
(No. 8) R_1 = 2-deoxy-α-L-rhamnopyranoside **n = 2**	n.a.	n.t.
(No. 9) R_2 = 2-deoxy-α-L-rhamnopyranoside **n = 2**	43.70 ± 8.41	n.a.
(No. 10) R_3 = 2-deoxy-α-L-rhamnopyranoside **n = 5**	70.80 ± 0.90	n.a.
(No. 11) R_1 = 2-deoxy-α-L-rhamnopyranoside **n = 5**	n.a.	n.t.
(No. 12) R_2 = 2-deoxy-α-L-rhamnopyranoside **n = 5**	39.60 ± 13.80	n.a.

ID_{50}[1)] – compound concentration leading to 50% inhibition of cell proliferation;
n- number of carbon atoms in linker moiety (as it is shown in Figure 1);
n.a. - not active;
n.t. - not tested.

optical density (at 540 nm) in a computer-interfaced, 96-well microtiter plate reader Multiskan RC photometer (Labsystems, Helsinki, Finland). Each compound in given concentration was tested in triplicates in each experiment. Every experiment was repeated 3-5 times.

Fluorescent microscopy studies

The cultured cells were washed twice in PBS (phosphate buffer saline, Ca^{2+}, Mg^{2+} - free) and centrifuged. Then the cells were resuspended in the same buffer and incubated for 10, 20 and 60 seconds with 1μM of compound 24. Axioskop 20 (Zeiss, Germany) was used for results acquisition and imaging.

Results and discussion

The results of cytotoxic activity *in vitro* were expressed as ID_{50} – the dose of compound (in mM) that inhibits proliferation rate of the tumour cells by 50% as compared to control untreated cells. The results are presented in Tables 1-4.

Inhibition of the topoisomerase II activity by compounds tested was measured as a degree of the inhibition of kinetoplast DNA decatenation. Some of the compounds tested inhibited the activity of topoisomerase II and the results are presented in Tables 1-4.

As it is shown in Table 1, D-glucose derivatives (1-6) revealed moderate cytotoxic but no topoisomerase inhibitory activity.

Among L-rhamnose containing compounds, only 3 out of 6 (9-11) revealed moderate antiproliferative activity - one with N-6 pentyl linker moiety and two (C-9 substituted) – containing pentyloxy and ethyloxy chains. No one compound from this group revealed any ability to inhibit activity of topoisomerase II.

D-lactose derivatives are the least active cytotoxic compounds (Table 3). Only one compound - N-6 pentyl derivative - shows moderate anti-proliferative activity (16, ID_{50} =

Table 3. Cytotoxicity and inhibition of topoisomerase II activity revealed by D-lactose indolo[2,3-b]quinoline saccharide derivatives.

Compound tested	Cytotoxity - ID_{50} [μM]	Total topoisomerase II inhibition [mM]
(No. 13) R_3 = 2-deoxy-α-D-lactopyranoside **n = 2**	n.a.	n.t.
(No. 14) R_1 = 2-deoxy-α-D-lactopyranoside **n = 2**	n.a.	n.t.
(No. 15) R_2 = 2-deoxy-α-D-lactopyranoside **n = 2**	n.a.	0.5
(No. 16) R_3 = 2-deoxy-α-D-lactopyranoside **n = 5**	45.90 ± 5.73	n.a.
(No. 17) R_1 = 2-deoxy-α-D-lactopyranoside **n = 5**	n.a.	n.t.
(No. 18) R_2 = 2-deoxy-α-D-lactopyranoside **n = 5**	n.a.	n.t.

ID_{50}[1)] – compound concentration leading to 50% inhibition of cell proliferation;
n - number of carbon atoms in linker moiety (as it is shown in Figure 1);
n.a. - not active;
n.t. - not tested.

45.90 ± 5.73 μM) but no topoisomerase inhibition is observed. Surprisingly, one of the compounds, which did not reveal antiproliferative activity, inhibited topoisomerase II activity in the highest concentration tested (compound 15, 0.5 mM). This derivative contains ethyloxy chain attached to indolo[2,3-b]quinoline C-9 carbon atom.

It is evident that for the saccharide containing compounds the crucial for cytotoxic and topoisomerase inhibition activities are C-9 and N-6 substitution positions, and that in the case of N-6 derivatives only five carbon units chain leads to the compounds with biological activity.

Interestingly, substitutions with aminosaccharide moieties give an important increase of cytotoxic properties. All compounds are active and their ID_{50} values are few times lower compared to "non-amino" monosaccharide derivatives. These compounds reveal also strong topoisomerase II inhibitory activity what confirms thesis that there is a third condition (apart from position of substituent and size of linker chain) essential for specific activities of indolo[2,3-b]quinolines derivatives. This condition concerns the type of sugar residue constituted.

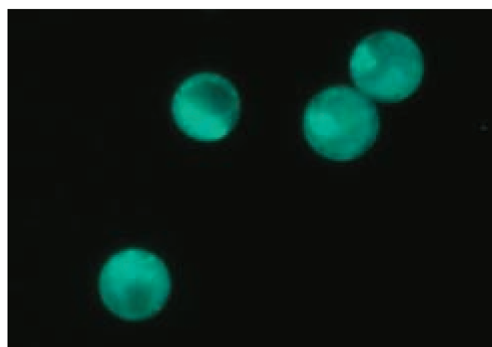
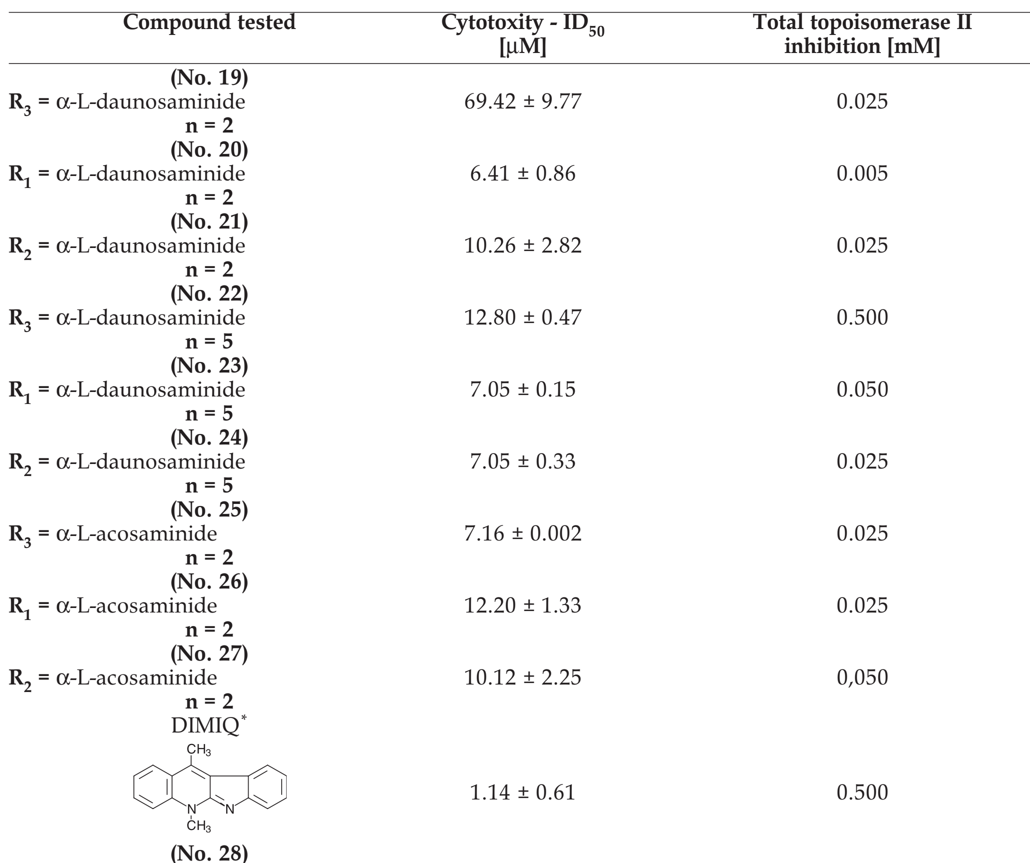


Figure 2. Jurkat cells after 10 seconds of incubation in 1.0μM of compound 24. The cultured cells were washed twice in PBS (phosphate buffer saline, Ca^{2+}, Mg^{2+} - free) and centrifuged. Then the cells were resuspended in the same buffer and incubated for 10, 20 and 60 seconds with 1μM of compound 24. This compound is a fluorophore, so lighting areas in the cells indicate places of its intake. Axioskop 20 (Zeiss, Germany) was used for results acquisition and imaging (Zeiss Filterset 02 - max excitation: λ = 365; emmission: λ > 420 nm – was applied).

Table 4. Cytotoxicity and inhibition of topoisomerase II activity revealed by L-daunosamine and L-acosamine indolo[2,3-b]quinoline saccharide derivatives.



Compound tested	Cytotoxity - ID_{50} [μM]	Total topoisomerase II inhibition [mM]
(No. 19) R_3 = α-L-daunosaminide **n = 2**	69.42 ± 9.77	0.025
(No. 20) R_1 = α-L-daunosaminide **n = 2**	6.41 ± 0.86	0.005
(No. 21) R_2 = α-L-daunosaminide **n = 2**	10.26 ± 2.82	0.025
(No. 22) R_3 = α-L-daunosaminide **n = 5**	12.80 ± 0.47	0.500
(No. 23) R_1 = α-L-daunosaminide **n = 5**	7.05 ± 0.15	0.050
(No. 24) R_2 = α-L-daunosaminide **n = 5**	7.05 ± 0.33	0.025
(No. 25) R_3 = α-L-acosaminide **n = 2**	7.16 ± 0.002	0.025
(No. 26) R_1 = α-L-acosaminide **n = 2**	12.20 ± 1.33	0.025
(No. 27) R_2 = α-L-acosaminide **n = 2**	10.12 ± 2.25	0,050
DIMIQ* CH_3 N N CH_3 **(No. 28)**	1.14 ± 0.61	0.500

ID_{50} [1)] – compound concentration leading to 50% inhibition of cell proliferation;
n - number of carbon atoms in linker moiety (as it is shown in Figure 1);
n.a. - not active;
n.t. - not tested;
*DiMIQ - referential compound - 5,11-dimethyl-indolo[2,3-b]quinoline;

Within range of aminosaccharide derivatives of indolo[2,3-b]quinolines, the most promising are compounds 20 and 21 – C-2 and C-9 substituted ethyloxy, as well as 23 and 24 - C-2 and C-9 substituted pentyloxy L-daunosamine derivatives (Table 4). In the series of L-acosamine derivatives, the best properties were revealed by compound 25 with five carbon linker chain attached to N-6 position of indolo[2,3-b]quinoline.

Cytotoxic activities observed for the active compounds in the series are comparable to values estimated previously for 5H and 6H derivatives.[5-7] The most cytostatic against KB cells and inhibiting activity of topoisomerase II are aminosaccharide substituted indolo[2,3-b]quinolines.

Since some of previously tested indolo[2,3-b]quinoline derivatives show poor solubility (data not shown) what causes the problems connected with bioavailability, next step of the studies was to examine the cell intake of the compound 24. These results are shown in Figure 2. It can be seen that the compound is

well distributed in cytosol and accumulated in some subcellular structures.

Although a great effort have been made to find an effective anticancer chemotherapeutics, the number of clinically active drugs remains quite small and their spectrum of antitumor activity is rather limited. Because of that, there are still many projects and research aiming to discover the new, more effective or more selective anticancer compounds.[14]

Beside the cytotoxic properties assessed in *in vitro* tests, the candidates for the new anticancer drugs should reveal some special features, as good solubility and bioavailability and of course low toxicity. Structure-activity relationship studies are one of the ways to select the compound of the best properties.

On the basis of presented here results, the most promising indolo[2,3-b]quinoline derivatives were chosen for further preclinical *in vitro* and *in vivo* studies.

Acknowledgements

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Regional comparison of cancer incidence

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Background. *Due to specific war and post-war situation in Balkan region, differences in the number, type, development, biological course, treatment of malignant tumours and its outcome are possible. In order to perceive the situation realistically, it is necessary to gather continuously exact data about malignant tumours and compare them with the data from other European and world countries.The aim of the study was to collect and analyse the data on cancer incidence in the region of Sarajevo city, which represents a symbol of difficult times in the recent past, and to compare it to the incidence in the neighbouring countries.*
Patients and methods. *Data on all newly diagnosed cancer cases, permanent residents of Sarajevo Canton, in the years 1999 and 2000 were collected. Crude incidence rate has been calculated according to the years observed, gender and localizations of the disease The data were compared to the cancer registries of Slovenia and Croatia and were observed in the light of specific local situation.*
Results. *The crude cancer incidence of all sites but skin was the highest in both years and by both genders in Croatia. The incidence of the most common tumours (lung and breast cancer) was similar in all three countries. The differences in the incidence between both genders in the Sarajevo canton were registered in laryngeal and urinary bladder cancer, as well as in bone and cartilage sarcoma. Cervical cancer had extremely high incidence and was high up on the incidence list in the Sarajevo canton, which correlates with the data in developing countries. The incidence of other tumours in the post-war period is reaching expected numbers.*
Conclusions. *It is difficult to identify whether the war and post-war stress, irregular and insufficient nutrition during and after the siege of the city of Sarajevo or some other factor influenced the cancer incidence among exposed population.*
The prevalence of smoking in the whole region is extremely high, in Bosnia and Herzegovina almost complete, which can influence not only the incidence of lung cancer but also laryngeal and urinary bladder cancer.

Key words: neoplasms- epidemiology; incidence; Bosnia - Hercegovina cancer incidence

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Introduction

Malignant tumours represent one of the major health problems today; their occurrence and severity are constantly increasing.

According to the available data, malignant tumours are the leading cause of death in most countries in Europe and rank second, after cardiovascular diseases.[1,2]

The war in Balkan (1991-1996) had dire consequences leading to a great number of victims, destroyed infrastructure, industry, environment, health capacities and equipment. Health situation in several countries has significantly worsened.[3,4] Due to specific war and post-war situation, the differences in the number, type, development, biological course, treatment of malignant tumours, and in the outcome are possible. Actual situation related to the use of ammunition containing depleted uranium has raised additional questions about its influence on human health. Contamination with the debris from depleted uranium shells could have increased the risk of developing cancer and kidney damage. The so-called Balkan-syndrome is often linked with depleted uranium contamination.[5-8] Consequently, eventual increase in cancer incidence in Bosnia and Herzegovina is possible.

It has been very often publicly mentioned that the number of malignant tumours in Bosnia and Herzegovina is enormously increasing and that we are faced with cancer epidemic. But the information on cancers, cancer rates and trends is incomplete in Bosnia and Herzegovina. Bosnia and Herzegovina does not have a proper cancer registry for the population nor does it have precise data on its incidence. The claims made about the increases in many types of cancers were based on clinical observations, but were not substantiated by the information on cancer rates relating the number of cases to the population these cancers come from.[9,10]

In order to perceive the situation realistically and to avoid panic and ignorant attitude, it is necessary to gather continuously exact data about malignant tumours, not only to detect risk factors, or follow them up and control them, but also to be able to compare them with the data from other European countries and countries worldwide.

The above observations, strong fear from malignant tumours, and frequent disinformation, motivated the authors to investigate and to define a true situation regarding malignant tumours at least in part of Bosnia and Herzegovina. We chose the canton of Sarajevo relying on the fact that it would be possible to obtain the complete data about malignant tumours there because patients had rarely been treated in other places or cities (diagnostics and treatment for cancer are available in Sarajevo; there are no political or organizational obstacles in sending patients to oncology departments). Beside that, the number of citizens in the observed period of time was stable and known. Those facts helped us, to conduct the study and to reach basic indicators about the incidence and type of malignant tumours during the period of 1999 - 2000.

Besides, Sarajevo went through difficult times. It was under siege that was the longest since World War II and suffered from ruthless horrors of war. During the war, approximately 3 tons of depleted uranium munitions were used against armoured targets, predominantly in the surroundings of Sarajevo.[8,11] Its citizens' health was jeopardized by numerous factors, some of which are:

Stressful situations during the war and after the war which could influence the appearance of certain diseases, including malignant tumours;

Difficult communication due to the siege and blockades which prevented early detection and treatment of diseases;

Quantitatively and qualitatively insufficient nutrition due to the siege, war conditions and difficult economic situation after the war;

Increasing trend of smoking and, after the war, increased consumption of alcoholic beverages;

Possible increase of radioactivity due to ammunition containing depleted uranium used in the wider region of the city and possible effect of chemical weapon;

A shift in the population's attitude toward their own health.

Material and methods

We collected the data on newly diagnosed cancer cases in the Canton of Sarajevo during the period from January 1st, 1999 until December 31st, 2000. All data relate to permanent residents of the 6 municipalities in Sarajevo at the time of diagnosis.

Cases have been confirmed microscopically or clinically. Intraepithelial tumours of cervix and urinary bladder have not been included in this analysis.

The main source of information was the reports filled in by doctors from hospitals or outpatient departments where disease had been diagnosed and/or treated. These reports, which were legally binding, were printed on the form no. 3-35-86 as "The report of malignant tumours". The collection of data was related to histological diagnoses of newly discovered tumours and was done at the Institute for Pathology at the Medical Faculty (Sarajevo University Hospital) and at the Department for Pathology at the Sarajevo General Hospital. The data on mortality (deaths caused by malignant tumours) were collected from obligatory reports written on DEM 2 death certificates.

Institute for Statistics of the Federation of Bosnia and Herzegovina provided the data of the number of citizens in the Sarajevo canton.[12] For the study, the population number was estimated in the middle of a certain year (Table 1).

Crude incidence (per 100 000 population of a certain gender and of all ages) of malignant tumours in the years 1999 and 2000 was calculated, regarding site and gender. The data were compared to those from the Cancer Registry of Slovenia 1999 and 2000, Cancer Registry of Croatia 1999 and 2000 and regional indicators and predictions.[13-17]

Results

During the period of 1999-2000, 2,897 new cases of malignant tumours (1496 among males and 1401 among females) were registered in the Sarajevo Canton. Fifteen cases (0.5 %) were registered only on the basis of death certificate. Pathohistologically or cytologically proven tumours were found in 2,571 patients (88.75 %). The number of new cancer cases by year of diagnosis and sex is presented in Table 2.

The crude incidence rate in males was 432/100 000 in 1999 and 384/100 000 in 2000 and in females 390/100 000 in 1999 and 354/100 000 in 2000. Compared to Slovenia, the incidence rate in our country was lower.

If non-melanoma skin cancer is excluded, the crude incidence rate in 2000 in Canton Sarajevo was 339/100 000 males and 286/100 000 females, respectively. The estimated incidence from GLOBOCAN 2000 is 309.3 new cases per 100.000 men and 267.9 cases per 100 000 women in Bosnia and Herzegovina.[13]

Crude incidence rates of most important cancer sites in males and females in Southern Europe, Slovenia, Croatia and Canton Sarajevo are presented in Tables 3 and 4.

Table 1. Estimated population by gender in the Sarajevo canton during the period 1999-2000

Year	Males	Females	Total
1999	178 951	201 932	380 883
2000	183 161	207 373	390 534

Table 2. Number of malignant tumours in the Sarajevo canton in the years 1999-2000: all sites; by gender

Years	Males	Females	Total
1999	791	766	1557
2000	705	645	1350
Total	1496	1401	2897

Table 3. Crude cancer incidence per 100,000 males of all ages in the years 1999 and 2000 according GLOBACON 2000,[13] Cancer Registry of Slovenia,[14,15] Cancer Registries of Croatia[16,17] and the Sarajevo canton

Cancer	**Southern**	**Slovenia**		**Croatia**		**Sarajevo canton**	
Primary site	**Europe 2000**	**1999**	**2000**	**1999**	**2000**	**1999**	**2000**
Oral cavity	13.7	11.8	13.0	17.1	15	7.3	9.27
Nasopharynx	1.2	0.4	0.4	0.8	1.0	0.6	0.0
Other pharynges	6.9	15	11.9	13.2	15.8	1.0	7.6
Oesophagus	7.3	8	7.3	9.1	8.2	1.1	1.1
Stomach	32.5	32.5	29.3	39.7	34.3	21	16.2
Colon / Rectum	55.1	59.2	60.5	71	73.0	40.8	41.5
Liver	16.5	7.8	7.4	12	11.0	9.5	12.6
Pancreas	11.5	11	12.1	15.3	14.2	2.8	4.9
Larynx	**13.78**	**9.2**	**11.5**	**20.2**	**17.9**	**46.9**	**27.3**
Lung	95.9	85.0	85.9	115.6	121.8	106.2	90.1
Melanoma of skin	5.2	11	12.3	9.9	11.1	18.6	14.5
Prostate	44.7	44,1	40.8	41.1	43.8	24.0	20.0
Testis	5.0	8.8	8.8	7.2	7.0	2.8	4.4
Bladder	**41.6**	**15.0**	**14.9**	**31.1**	**33.0**	**27.8**	**38.8**
Kidney	13.4	11	10.8	16.2	15.7	10.6	6.3
Brain/nervous system	8.9	8.4	5.4	15.1	15.4	10.4	13.9
Thyroid	1.5	3.0	2.8	3.5	3.7	2.8	1.1
Non-Hodgkin lymphoma	13.2	8	9.9	12.6	10.8	4.4	4.5
Hodgkin Disease	2.9	1.5	2.1	4.3	3.6	3.8	7.8
Multiple myeloma	4.7	4.3	3.3	5.3	4.4	1.1	2.8
Leukaemia	11.3	8.1	1.4	17.7	14.9	10.6	5.8
Bone/ cartilage		**1**	**0.9**	**5.9**	**4.9**	**8.4**	**7.9**
Soft tissue sarcoma		**1.3**	**2.4**	**3.5**	**2.9**	**1.68**	**0.54**
All sites but skin	443.4	**402.7**	**393.9**	**533.3**	**523.9**	**390**	**354**

Discussion

The aim of our study was to analyse the cancer incidence in Canton Sarajevo and compare it with neighbouring countries. When trying to explain the differences, it is important to emphasise, that they may be real, but also due to different practices in reporting.

Compared to Slovenia, in Canton Sarajevo the incidence of all cancer sites was rather lower.

If melanoma skin cancer is excluded, the incidence in Croatia was the highest in both years and both genders. According to the Cancer Register of Croatia, the crude incidence per 100 000 population in 1999 was 533.3 for men, and 432.1 for women. The same proportion was reported in the year 2000, i.e. 523.9 for men and 424.3 for women, whereas in Slovenia, it was 448.3 for men and 420.4 for women, and in Sarajevo, 384 and 311, respectively.[11,12,15,16] Therefore, the registered crude incidence of cancer in the Sarajevo Canton correlates with the estimations for the whole region of South Europe rather than with the one estimated for Bosnia and Herzegovina. In both genders, the crude incidence is comparable to the one registered in the Republic of Slovenia for the year of 1999 (390 men and 339 women in the Sarajevo Canton; 402 men and 359 women in Slovenia).

In Slovenia, the incidence of cancer of the upper respiratory and digestive organs in the years 1999 and 2000 was 36.4 and 36.8, respectively, in Croatia, 51.3 and 49.7 respec-

Table 4. Crude cancer incidence per 100,000 females of all ages in the years 1999 and 2000 according GLOBACON 2000,[13] Cancer Registry of Slovenia,[14,15] Cancer Registries of Croatia[16,17] and of the Sarajevo canton

Cancer	Southern	Slovenia		Croatia		Sarajevo canton	
Primary site	Europe 2000	1999	2000	1999	2000	1999	2000
Oral cavity	3.0	**3,1**	**2.6**	**5.7**	**3.4**	1.5	1.4
Nasopharynx	0.4	0,2	0.4	**0.4**	**0.2**	0.5	0.0
Other pharynges	0.7	0,8	1.2	**1.5**	**2.1**	0.5	0.5
Oesophagus	1.4	2.2	1.4	**1.9**	**2.3**	0.5	0.9
Stomach	20.3	**16.3**	**18.0**	**21.0**	**23.2**	8.9	5.8
Colon / Rectum	44.3	41.3	47.9	**53.0**	**54.0**	28.9	27.2
Liver	7.6	**3.5**	**2.4**	**7.2**	**8.0**	6.4	5.3
Pancreas	0.1	9.8	11.3	**11.6**	**12.2**	2.0	1.0
Larynx	0.9	0.7	1.3	**2.0**	**1.5**	**8.4**	**6.3**
Lung	15.2	**21.2**	**23.5**	**23.5**	**28.0**	18.3	22.4
Melanoma of skin	6.9	**13.1**	**11.1**	**9.1**	**9.7**	14.0	10.8
Brest	**88.5**	97.8	91.2	110.7	95.0	95.8	77.8
Cervix uteri	**13.7**	**19.9**	**19.6**	17.2	19.0	**36.6**	**35.2**
Corpus uteri	**23.6**	24.8	24.6	25.7	22.6	26.3	22.0
Ovary	**13.8**	**17.0**	**19.4**	22.9	24.6	12.5	14.5
Bladder	**8.8**	**5.1**	**4.4**	9.0	9.0	**15.9**	**8.6**
Kidney	6.4	**8.1**	**6.9**	**9.0**	**10.7**	6.0	5.9
Brain/ nervous system	6.8	**3.7**	**5.4**	**13.0**	**12.0**	8.9	5.3
Thyroid	6.7	**6.7**	**5.8**	**12.1**	**12.0**	6.9	6.7
Non-Hodgkin lymphoma	11.0	**8.8**	**11.8**	**9.8**	**9.6**	5.5	2.4
Hodgkin disease	2.5	**1.9**	**1.6**	**3.4**	**3.2**	4.5	1.9
Multiple myeloma	4.3	**7.1**	**4.2**	**6.2**	**3.4**	3.0	1.4
Leukaemia	8.4	**3.7**	**7.9**	**13.3**	**11.3**	4.9	5.8
Bone/ cartilage	**1.3**	**0.6**	**3.2**	**4.4**	8.4	3.8	
Soft tissue sarcoma	**2.2**	**3.2**	**2.6**	**2.5**	**2.4**	**2.8**	
All sites but skin	336.9	**358.4**	**363.6**	**432.1**	**424.3**	**339**	**286**

tively, and 56.6 and 44.73 in the respective years in the Sarajevo canton. The greatest difference is registered in cases of laryngeal cancer where incidence is 2-3 times greater in the Sarajevo Canton than in other countries in the region. The incidence of oral and pharyngeal cancer was lower. Even if we presume that a certain number of cancers of upper respiratory organs were wrongly assigned to advanced laryngeal cancers, this still could not explain extremely and constantly high incidence of this tumour in the Sarajevo canton.

Lung cancer is most common in all three countries, with the incidence comparable to the one predicted by GLOBCAN report. The lowest incidence was registered in Slovenia, where the prevalence of smokers is lowering.

The incidence of all digestive tract tumours was the highest in Croatia. The incidences in Slovenia and Sarajevo are comparable to the ones predicted by GLOBCAN report. Somewhat lower incidences of stomach and pancreatic cancer were registered in Sarajevo.

The incidence of urinary bladder cancer in Croatia and Sarajevo is 2 times higher than in Slovenia, but still correlates to GLOBCAN estimation for Southern Europe. The prostate cancer incidence in Croatia and Slovenia is at the level of expected values that correlates to GLOBCAN estimation, but in the Sarajevo

canton, it is lower. This can be explained by the fact that, in Bosnia and Herzegovina, no routine screening for PSA is being implemented, therefore only clinically apparent tumours are diagnosed.

In clinical practice, an unusually high number of histologically confirmed bone and cartilage sarcomas from different parts of Bosnia and Herzegovina have been observed, which is illustrated and presented through the data from the Sarajevo canton and for which we don't have an acceptable explanation.

From the given data, it is evident that other tumours of haematopoietic system are not more common in the Sarajevo canton in comparison to what has been expected and to the number of cases registered in Slovenia and Croatia.

In females, breast is the most common cancer site in all three countries. It is important to note that incidence of cervical cancer is the highest in Sarajevo Canton and is comparable to the incidence in undeveloped countries. It certainly reflects the insufficient opportunistic screening.

Among the women in the Sarajevo Canton, higher incidences of laryngeal and urinary bladder cancer were also registered as well as bone sarcomas.

Conclusions

Several factors may have contributed to cancer incidence in Canton Sarajevo: the war and post-war circumstances with irregular nutrition during and after the siege of the city, heavy smoking and drinking. The incidence of smoking in the whole region, especially in Bosnia and Herzegovina is extremely high, which can influence not only the incidence of lung cancer but also of laryngeal cancer and urinary bladder cancer.

Additional studies are needed to evaluate whether some other factors have been implicated in the aetiology of malignant tumours among exposed population.

It is important to emphasize the need for establishing a national population registry of malignant tumours for the whole Bosnia and Herzegovina, not only to detect, follow-up and control the risk factors but also to be able to compare our data with the data from other European countries and the countries worldwide

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Letter to the Editor

Chemotherapy for small-cell lung cancer with paraneoplastic nephrotic syndrome

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Platinum-containing chemotherapy has been commonly used as standard therapy for small cell lung cancer (SCLC). However, platinum causes renal dysfunction.[1,2] We report a SCLC patient with paraneoplastic nephrotic syndrome who was successfully treated with platinum-containing chemotherapy. A complete tumour response could be achieved; however, his proteinuria did not decrease and renal function got worse every time he received the chemotherapy.

A 74-year-old male was admitted to our hospital with the oedema of the lower extremities that developed during the last three months. On physical examination, oedema was still present. Laboratory results were as follows: haemoglobin 10.6 g/dl, potassium 4.3 mEq/l, serum creatinine 1.0 mg/dl, blood urea nitrogen 34.2 mg/dl. Creatinine clearance was 43.0 ml/min and the urine sediment was free of casts and erythrocytes. However, serum albumin was 2.1 g/dl, and the 24 h urine collection revealed proteinuria of 10 g daily. Renal biopsy disclosed membranous glomerulonephritis. A chest X-ray on admission revealed mediastinal widening and a right hilar mass. Chest CT scan showed a large mass in the right middle lobe with mediastinal lymph node swellings. Small lung nodules up to 10 mm in both lungs were also observed. Transbronchial biopsies revealed SCLC in an advanced desease stage. Paraneoplastic nephrotic syndrome was diagnosed to be associated with SCLC. Chemotherapy for SCLC was started with carboplatin (AUC 5 mg/ml per minute, Calvert formula, day 1) and etoposide (100 mg/m2, days 1, 2, and 3). After two courses complete remission was achieved, however, oedema of both extremities did not disappeare. Serum creatinine and blood urea nitrogen increased every time he received the chemotherapy, and proteinuria did not decrease in spite of the complete tumour response. Because of the impaired renal function, no additional chemotherapy was indicated. Thereafter, the patient received supportive care, and he died from brain metastasis 8 months after from the initial treatment.

Since the introduction of platinum in the early 1980s, commonly used combination chemotherapy regimens for SCLC have been platinum analogy combined with etoposide.[3,4] Despite the advantage of the plat-

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inum-containing chemotherapy, the cyclophosphamide, doxorubicin, and vincristine regimen has still been commonly used for SCLC patients with paraneoplastic nephritic syndrome.[5-6] In our case, carboplatin-contaning chemotherapy could achieve a complete tumour response; however, paraneoplastic nephrotic syndrome was not improved and the renal function was deteriorated. Platinum is nehprotoxic and, therefore, platinum itself can induce nephrotic syndrome.[1,2] In comparison with cisplatin, the nephrotoxicity of carboplatin is reduced but not completely eliminated. We cannot conclude by insisting on the disadvantage of platinum-containing regimens for SCLC patients with paraneoplastic nephrotic syndrome, but by recommending to be careful in indicating the platinum-containing regimens, especially for SCLC patients with paraneoplastic nephrotic syndrome.

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Radiol Oncol 2004; 38(2): 73-83.

Tumorski označevalci v klinični onkologiji

Novaković S

Majhne razlike med normalnimi in tumorsko spremenjenimi celicami izkoriščamo v diagnostiki in zdravljenju malignomov. Te razlike označujemo z imenom tumorski označevalci in so lahko kvalitativne ali kvantitativne po svoji naravi. To pomeni, da lahko kot tumorski označevalci služijo tako molekule (snovi), ki jih tvorijo maligne celice kot tudi molekule (snovi), ki nastajajo v povečanih količinah v normalnih gostiteljevih tkivih pod vplivom malignih celic. Na splošno so tumorski označevalci značilne molekule, ki se pojavijo v krvi ali tkivih v povezavi z maligno boleznijo.

Glede na uporabo delimo tumorske označevalce v grobem na označevalce v klinični onkologiji in označevalce v patologiji. V tem preglednem članku bomo obravnavali le označevalce v klinični onkologiji. Sedanja razdelitev tumorskih označevalcev v klinični onkologiji vključuje: onkofetalne antigene, placentalne proteine, hormone, encime, antigene, ki spremljajo tumor, posebne serumske proteine, kateholaminske metabolite ter skupino različnih označevalcev.

Na kratko lahko povzamemo, da mora idealni tumorski označevalec izpolnjevati določene pogoje: (1) povišane koncentracije označevalca se morajo pojaviti že na začetku razvoja malignoma, (2) koncentracije označevalca morajo biti povišane le pri bolnikih z določeno vrsto malignoma, (3) povišane koncentracije se morajo pojaviti pri vseh bolnikih z enako vrsto malignoma, (4) izmerjene koncentracije morajo odražati velikost tumorske mase, (5) izmerjene koncentracije morajo odražati učinek zdravljenja in (6) določanje označevalca mora biti enostavno. Marsikateri med tumorskimi označevalci, ki jih določamo vsakodnevno, izpolnjuje nekatere, ne pa vseh navedenih pogojev. Kot posledico tega so raziskovalci vpeljali nekaj pojmov, ki naj bi opredelili kvaliteto tumorskega označevalca in omogočili čim boljšo izbiro označevalca za spremljanje določene vrste malignoma. Ti pojmi vključujejo občutljivost (senzitivnost) označevalca, njegovo zanesljivost (specifičnost) in napovedno vrednost. Občutljivost pove, kakšna je srednja verjetnost, da bomo pri bolniku z malignomom določili povišane vrednosti (nad razmejitveno vrednostjo) tumorskega označevalca. Zanesljivost pove, kakšna je srednja verjetnost, da bomo pri preiskovancu, ki nima malignoma, ugotovili normalne vrednosti označevalca. Napovedna vrednost pa pove, kakšna je uporabna vrednost tumorskega označevalca v mešani skupini preiskovancev.

Določitve tumorskih označevalcev lahko v klinični onkologiji uporabljamo z različnim namenom. Pomagajo lahko pri (i) zgodnjem odkrivanju malignomov, (ii) razločevanju malignih in benignih procesov, (iii) ocenjevanju razširjenosti maligne bolezni, (iv) spremljanju odgovora na terapijo in (v) napovedovanju in dokazovanju ponovitve maligne bolezni. Ker žal ne poznamo idealnega označevalca, ki bi imel ustrezno visoko občutljivost in zanesljivost, določitve tumorskih označevalcev le izjemoma uporabljamo kot presejalne metode za odkrivanje malignoma (primer takšnega označevalca je prostatični specifični antigen - PSA). Kljub vsemu pa tumorski označevalci igrajo ključno vlogo pri odkrivanju zgodnjih ponovitev bolezni in spremljanju odgovora na terapijo pri izbranih bolnikih. Določanje tumorskih označevalcev z namenom odkrivanja ponovitev bolezni je smiselno le, kadar pri takšnem bolniku še obstaja možnost uspešnega zdravljenja.

Radiol Oncol 2004; 38(2): 155-60.

Radiol Oncol 2004; 38(2): 85-92.

Klinična uporabnost serinskih proteaz pri raku dojk

Čufer T

Serinska proteaza uPA in njen inhibitor PAI-1 sta udeležena pri razgradnji tumorske strome ter bazalne membrane. Neodvisni napovedni pomen serinske proteaze uPA in njenega inhibitorja PAI-1 pri raku dojke je bil enoznačno potrjen v številnih posameznih raziskavah kot tudi v metaanalizi, v katero je bilo vključenih 18 datotek s podatki več kot 8000 bolnic. Glede na te izsledke je tveganje za ponovitev bolezni pri bolnicah brez prizadetih pazdušnih bezgavk in nizkimi vrednostmi uPA in PAI-1 v prvotnem tumorju manjše od 10%. Tem bolnicam lahko prihranimo dopolnilno sistemsko zdravljenje, katerega pogosto spremljajo neželeni učinki. Za vsakodnevno klinično delo pa je še pomembnejše, da prvi izsledki potrjujejo tudi možen napovedni pomen uPA in PAI-1 za odgovor na dopolnilno sistemsko zdravljenje zgodnjega raka dojk. Naša opažanja pri 460 bolnicah z operabilnim rakom dojk potrjujejo napovedni pomen uPA in PAI-1 za odgovor na hormonsko zdravljenje, ne pa za odgovor na citostatsko zdravljenje. Bolnice z visokimi vrednostmi PAI-1 v prvotnem tumorju so imele več kot dvakrat višje tveganje ponovitve bolezni, če niso bile zdravljene z dopolnilno sistemsko terapijo (HR 2.14; C.I. 95%= 0.48-9.56; p=0.321) ali pa so bile zdravljene samo s kemoterapijo (RR 2.48; C.I. 95%= 1.35-4.57; p=0.003). Slab napovedni pomen visokih vrednosti uPA in PAI-1 pa se je povsem izničil pri bolnicah, ki so prejele dopolnilno hormonsko zdravljenje, samo ali v kombinaciji z kemoterapijo. Še več, pri bolnicah, ki so prejele hormonsko zdravljenje, je bilo tveganje za ponovitev bolezni celo manjše v primeru visokih vsebnosti uPA ali PAI-1 v prvotnem tumorju (HR 0.79; p=0.693 and HR 0.26 p= 0.204). Serinska proteaza uPA in njen inhibitor PAI-1 sta danes močna napovedna dejavnika poteka raka dojk, ki omogočata ustreznejši izbor bolnic za dopolnilno sistemsko zdravljenje. Kot kažejo prvi izsledki, pa sta tudi obetavna napovedna dejavnika odgovora na zdravljenje, na podlagi katerih bomo v prihodnosti lahko izbirali najučinkovitejše zdravljenje za vsako posamezno bolnico.

Radiol Oncol 2004; 38(2): 93-100.

Katepsini in njihovi inhibitorji kot tumorski označevalci pri raku glave in vratu

Strojan P

Invazija in zasevanje tumorskih celic sta tesno povezana z razgradnjo bazalnih membran in zunajceličnega matriksa. Nosilci teh procesov so različni proteolitični encimi, med njimi tudi skupina ubikvitarnih lizosomskih encimov, tj cisteinske proteaze katepsini B, H in L, aspartatna proteaza katepsin D in endogeni inhibitorji prvih, cistatini. Namen tega pregleda je bil zbrati dosedanje vedenje o napovedni in prognostični vrednosti katepsinov in njihovih inhibitorjev pri ploščatoceličnem karcinomu glave in vratu. Pri tej vrsti raka so se UICC/AJCC TNM-klasifikacijski sistem in histopatološke značilnosti tumorjev izkazali kot nezanesljivi kazalci za napoved bodisi odgovora na zdravljenje bodisi preživetja bolnikov. Poleg tega do sedaj še noben dejavnik iz širokega spektra biokemičnih in histoloških dejavnikov ni bil spoznan kot zanesljiv napovedovalec naravnega poteka bolezni ali odgovora na zdravljenje. Prav z namenom izdelati zanesljiv prognostični profil tumorja potekajo intenzivne raziskave številnih novih označevalcev.

Radiol Oncol 2004; 38(2): 101-9.

Farmakogenetika tiopurinov: Ali so laboratorijski podatki lahko odločilni v pozologiji?

Stocco G, Martelossi S, Decorti G, Ventura A, Malusa N, Bartoli F, Giraldi T

Izhodišče. Namen študije je bil ugotoviti odvisnost med mutacijami v genu TPMT, oslabljeno encimsko aktivnostjo zaradi teh mutacij in klinično toksičnostjo zdravljenja s tiopurinskimi antimetaboliti.
Material in metode. V študijo smo vključili 44 bolnikov z vnetnim obolenjem črevesja, ki so bili zdravljeni z azatioprinom. Iz vzorcev krvi vsakega bolnika smo izločili DNK in določili genotip s polimerazno verižno reakcijo, da bi tako našli tri najpogostejše genske mutacije. Encimsko aktivnost smo merili z visokoločljivostno tekočinsko kromatografijo na razgrajenih celičnih vsebinah rdečih krvničkah.
Rezultati. Med izbranimi bolniki so bili 4 (9,0%) pri mutacijah gena TPMT heterozigotni, medtem ko med njimi pri teh mutacijah nihče ni bil homozigoten. Z raziskavo lahko potrdimo popolno soodvisnost med mutiranim genotipom TPMT in oslabljeno encimsko aktivnostjo. Pri primerjanju posameznikov z mutiranim genotipom in normalnim genotipom TPMT nismo opazili sprememb v toksičnosti.
Zaključek. Z metodami genotipiziranja, ki temeljijo na strateški uporabnosti DNK, je mogoče enostavno in zanesljivo odkriti homozigote TPMT - posameznike, ki jih ne smemo zdraviti s tiopurini. Hkrati pa ugotavljamo, da običajnejše in manj nevarne oblike toksičnosti tiopurinov povzročajo drugi dejavniki, ki niso povezani z mutacijami omenjenega gena TPMT.

Radiol Oncol 2004; 38(2): 155-60.

Radiol Oncol 2004; 38(2): 111-9.

Razvoj kvantitativnih RT-PCR testov za nemutirani urokinazni receptor (uPAR-wt) in njegovo izrezovalno različico uPAR-del5*

Farthmann J, Holzscheiter L, Biermann J, Meye A, Luther T, Kotzsch M, Sweep F, Schmitt M, Span P, Magdolen V

Receptor serinske proteinaze plazminogenskega aktivatorja urokinaznega tipa, uPAR (CD 87) igra pomembno vlogo v invaziji tumorskih celic in pri metastaziranju čvrstih malignih tumorjev. uPAR je močno glikoziliran membranski protein, vsajen v glikan-lipidne membrane in sestavljen iz treh homolognih domen. Posamezna domena je kodirana z dvema eksonoma: D1 z eksonoma 2 + 3, DII z eksonoma 4 + 5 in DIII z eksonoma 6 + 7. Poleg nemutirane uPAR-wt RNA, sta bili opisani tudi dve izrezovalni različici, ki bodisi nimata eksona 5 (uPAR-del5) bodisi obeh eksonov 4 in 5 (uPAR-del4/5). Predhodno smo z metodo RT-PCR v realnem času za kvantitativno določevanje mRNA koncentracije proučevali izražanje mRNA različic uPAR-del4/5 in uPAR mRNA, ki vsebujeta eksone 2,3 in 4 (to je nemutirana uPAR-wt in uPAR-del5).

V tem prispevku smo dodatno uveljavili dva specifična, robustna in zelo občutljiva testa RT-PCR, ki temeljita na tehnologiji Light-Cyler, da bi specifično določevali vsebnosti uPAR-wt in njegovo izrezovalno različico uPAR-del5. Izražanje uPAR-wt in uPAR-del5 je bilo merjeno v različnih človeških malignih celičnih linijah (ovarijskih rakavih celicah OVMZ-6 in OVMZ-10; rakavih celic dojke MDA-MB 231, MDA-MB 231 BAG, MDA-MB 435 in aMCF-7; možganskih celičnih linijah LN18) kakor tudi v zbiru 174 vzorcev tkiva raka dojke. uPAR-del5 mRNA je bila zelo pogosto izražena v razmeroma nizkih vsebnostih (značilno manj kot 1% glede na uPAR-wt mRNA). V tumorskem tkivu rakavih bolnikov je bila opažena statistično značilna korelacija med uPAR-del5 in uPAR-wt mRNA ($r=0.779$; $P > 0.001$). Povezave med izražanjem obeh vrst mRNA in kliničnimi parametri, kakor so status bezgavk velikostjo tumorja in gradusa ni bilo. Značilno povišanje uPAR-del5 pa je bilo opaženo v mailgnih tumorjih z negativnim estrogenskim receptorjem.

Oba razvita kvantitativna testa RT-PCR, ki smo ju opisali tu, lahko v bodoče prispevata k funkcionalni analizi in klinični pomembnosti uPAR-wt in ene od njegovih izrezovalnih različic uPAR-del5 v malignih tumorjih.

Radiol Oncol 2004; 38(2): 121-9.

Ex vivo pretočna elektroporacija

Vernhes MC, Eynard N, Rols MP, Ganeva V, Teissié J

Elektroporacija celic povzroči lokalno in reverzibilno permeabilizacijo celične membrane. To omogoča vnašanje eksogenih snovi v celice, kot so zdravila proteini in DNA. Pretočna elektroporacija omogoča tretman velike količine celic, kar je potrebno v primeru celične tarpije. Celice tečejo skozi komoro za elektroporacijo, kjer so izpostavljene točno dolečenim pogojem elektroporacije. Potrebno je prilagoditi frekvenco električnih pulzov, hitrost pretoka celic in število dovedenih pulzov. Na ta način je mogoče z elektroporacijo vnesti v celice velik volumen celic v majhni komori za elektroporacijo. Tudi viabilnost celic po elektroporaciji je zelo dobro ohranjena.

Radiol Oncol 2004; 28(2): 131-5.

Napovedni pomen imunohistokemičnega določevanja HER-2/neu pri bolnikih s pljučnim rakom

Petrusevska G, Ilievska-Poposka B, Banev S, Smickova S, Spirovski Z

Izhodišča. Protein HER-2 ali p185her2 je membranski receptor s tirozinsko kinazno aktivnostjo, ki je določena z genom HER-2/neu. Prekomerno aktivnost HER-2/neu lahko zasledimo pri bolnikih z različnimi onkološkimi obolenji, tako tudi pri raku pljuč. Ker obstajajo različna poročila o stopnji aktivnosti tega gena pri pljučnem raku, smo v raziskavi določali protein HER-2 pri bolnikih z žleznim, skvamoznoceličnim in drobnoceličnim pljučnim rakom.

Bolniki in metode. Protein HER-2 smo določali pri tumorjih 29 bolnikov s pljučnim rakom; 19 bolnikov je bilo operiranih, pri 10 pa smo tumorsko tkivo dobili ob diagnostičnem postopku z upogljivim bronhoskopom preden smo jih zdravili s kemo/radioterapijo. Vzorce tkiva smo fiksirali v nevtralnem formalinu in naredili parafinske preparate. Za diagnosticiranje smo uporabljali standardna histokemična in imunohistokemična barvanja. Prisotnost proteina HER-2/neu smo potrjevali z imunohistokemičnim barvilom s pomočjo Hercep Testa (DAKO). Rezultate testiranja smo označili s stopnjami od 0 do 3. Stopnjo 0 in 1 smo ocenili kot negativno, s stopnjo 2 ali 3 pa kot pozitivno.

Rezultati. Prekomerno izraženost proteina HER-2/neu smo zasledili v 34,4% (pri 10 od 29 bolnikov). V večji meri smo ga ugotovili pri bolnikih z žleznim rakom in to v 45,4% (pri 5 od 11 bolnikov), pri bolnikih s skvamoznoceličnim rakom pa v 30,7% (4 od 13 bolnikov) in pri bolnikih z drobnoceličnim rakom le v 20% (1 od 5 bolnikov). Glede na starost in spol nismo našli statistično značilnih razlik. Pri napredovalih oblikah bolezni smo ugotovili večjo izraženost proteina HER-2/neu, saj so imeli vsi bolniki s skvamoznoceličnim rakom pljuč in stadijem bolezni IIIB in IV povišan protein, bolniki z žleznim karcinomom in stadijem IIIB in IV pa v 80%.

Zaključki. Obetajoči rezultati kažejo, da bi lahko imel HER-2/neu pri bolnikih s pljučnim rakom prediktivni in prognostični pomen, kar bomo poskusili potrditi po daljšem spremljanju bolnikov.

Radiol Oncol 2004; 38(2): 137-44.

Novi saharidovi derivati indolo[2,3-b]kinolina kot citotoksične spojine in inhibitorji topoizomeraze II

Godlewska J, Badowska-Rosłonek K, Ramza J, Kaczmarek Ł, Peczyńska-Czoch W, Opolski A

Nekateri alkilni in alkilaminski derivati 6H-indolo[2,3-b]kinolinov imajo antiproliferativen učinek in modulirajo celični cikel. Njihove citotoksične lastnosti deloma izvirajo iz sposobnosti interkaliranja DNK in sposobnosti inhibiranja topoizomeraze II. Ker smo želeli doseči boljše fizikalno-kemične in biološke lastnosti 6H-indolo[2,3-b]kinolinov, smo sintetizirali serijo novih saharidnih derivatov ((C-2, C-9 ali N-6). Testirali smo vpliv različnih karbohiratnih enot (D-glukoza, D-laktoza, L-ramnoza, L-akozamin, L-daunozamin), mesto vezave in velikosti linkerja na citotoksične lastnosti in inhibicijo topoizomeraze II. Testirali smo spojine iz skupine saharidov, ki vsebujejo derivate 2-deoksi-?-D-glukopiranozida (1-6), 2-deoksi-?-L-ramnopiranozida (7-12) in 2-deoksi-?-D-laktopiranozida (13-18) ter tudi serijo aminosaharidnih derivatov ?-L-daunozaminid (19-24) in ?-L-akozaminid (25-27).

Radiol Oncol 2004; 38(2): 145-51.

Primerjava incidence raka v Sarajevu z incidenco v Slovenijii in Hrvaški

Obralić N, Gavrankapetanović F, Dizdarević Z, Durić O, Šišić F, Selak S, Balta S, Nakaš B

Ozhodišča. Vojna in razmere v povojnem obdobju dela Balkana so lahko vplivali na incidenco različnih vrst raka pa tudi na potek bolezni, način in uspešnost zdravljenja. Zaradi točne ocene pojava rakastih obolenj, je potrebno kontinuirano zbirati podatke in jih primerjati s podatki iz drugih držav Evrope in sveta. Namen naše raziskave je bil tako zbrati in analizirati podatke o malignih obolenjih v Sarajevu ter jih primerjati s podatki iz registrov raka drugih držav.
Bolniki in metode. Zbarali smo podatke o rakastih obolenjih prebivalcev s stalnim prebivališčem v Sarajevu med leti 1999 in 2000. Grobo letno incidenco smo razvrstili po spolu in lokalizaciji bolezni ter plodatke primerjali s podatki iz Registra raka Slovenije in Registra raka Hrvaške.
Rezultati. Najvišja skupna groba letna incidenca vseh vrst rakastih obolenj (brez raka kože) je bila na Hrvaškem.. Incidenca najbolj pogostih rakov (pljučni rak in rak dojke) je bila v vseh treh državah enaka. V Sarajevo pa je bila višja incidenca raka grla, raka mehurja ter raka kosti in hrustanca pri obeh spolih. V Sarajevu je tudi močno izstopala visoka incidenca raka vratu maternice, ki je primerljiva z incidenco v razvijajočih deželah. Incidenca raka ostalih lokalizacij je bila enaka pričakovanim podatkom.
Zaključki. Iz zbranih podatkov je težko zaključiti, da bi vojna, povojni stress, neredno in nezadostno prehranjevanje med in po obleganju Sarajeva ali katerikoli drugi dejavniki okolja bistveno vplivali na opazovano incidenco raka. Incidenca kajenja pa je v celotni regiji izjemno visoka, kar bi lahko vplivalo ne samo na pogostnost raka pljuč, pač pa tudi na pogostnost raka grla in mehurja, ki sta zvišana v primerjavi s podatki iz Slovenije in Hrvaške.

Radiol Oncol 2004; 38(2): 155-60.

Notices

*Notices submitted for publication should contain a mailing address, phone and/or fax number and/or e-mail of a **Contact** person or department.*

Oncology

June 24-27, 2004
The 16th international symposium "Supportive Care in Cancer" will take place in Miami Beach, Florida, USA.

Contact Ms. Cynthia N. Rittenberg, RN, MN, AOCN, Executive Director, MASCC, %00 Rue St. Ann, Suite 223, Matairie, LA 70005 USA; or call +1 504 828 2184; or fax +1 504 828 2180; or e-mail cyndyrit@bellsouth.net; or see http://www.mascc.org

Oncology

July 3-6, 2004
The 18th EACR (European Association for Cancer Research) Congress will be held in Innsbruck, Austria.

See http://www.fecs.be/conferences/eacr18

Gynaecological cancer

August 26-28, 2004
The ESTRO advanced teaching course on "Brachyherapy for Gynaecological Cancer" will take place in Vienna, Austria.

Contact ESTRO office, Avenue E. Mounier, 83/12, B-1200 Brussels, Belgium; or call +32 775 93 40; or fax +32 2 779 54 94; or e-mail info@estro.be; or see http://www.estro.be

Medical physics

August 29 – September 2, 2004
The ESTRO course "Physics for Clinical Radiotherapy" will take place in Leuven, Belgium.

Contact ESTRO office, Avenue E. Mounier, 83/12, B-1200 Brussels, Belgium; or call +32 775 93 40; or fax +32 2 779 54 94; or e-mail info@estro.be; or see http://www.estro.be

Paediatric oncology

September, 2004
The International Society of Paediatric Oncology - SIOP Annual Meeting will be held in Oslo, Norway.

See http://www.siop.nl

Radiobiology

September 19-23, 2004
The ESTRO course "Basic Clinical Radiobiology" will take place in Lausanne, Switzerland.

Contact ESTRO office, Avenue E. Mounier, 83/12, B-1200 Brussels, Belgium; or call +32 775 93 40; or fax +32 2 779 54 94; or e-mail info@estro.be; or see http://www.estro.be

Lung cancer

September 23-25, 2004
The "9th Central European Lung Cancer Conference" will be offered in Gdansk, Poland.

Contact Conference Secretariat, "9th Central European Lung Cancer Conference", Via Medica, ul. Swietokrzyska 73, 80 180, Gdansk, Poland; or call/fax +48 58 349 2270; or e–mail celcc@amg.gda.pl; or see www.lungcancer.pl

Radiation therapy

October 3-7, 2004
ASTRO Annual meeting will be held in Atlanta, USA.

Contact American Society for Therapeutic Radiology and Oncology Office, 1891 Preston White Drive, Reston, VA 20191, USA; or see http:// www.astro.org

Oncology

October 7-9, 2004

The 3rd ASTRO Annual meeting will be held in Atlanta, USA.

Contact American Society for Therapeutic Radiology and Oncology Office, 1891 Preston White Drive, Reston, VA 20191, USA; or see http:// www.astro.org

Therapeutic radiology and oncology

October 24-28, 2004

The 23rd International Chicago Symposium on "Malignancies of the hest and Head & Neck" will be held in Chicago, USA.

Contact Center for Continuing Medical Education, 950 East 61st Street, Suite 101, Chicago, IL, 60637, or fax +1 773 702 1736; or e-mail mgoldber@uchicago.edu; or see http://www.uchicago.edu/bsd/cme/

Medical oncology

October 29 – November 2, 2004

The 28th ESMO Congress will be held in Vienna, Austria.

See http://www.esmo.org

Radiation oncology

November 7-12, 2004

The ESTRO course "Evidence-Based Radiation Oncology: Methodological Basis and Clinical Application" will take place in Cyprus.

Contact ESTRO office, Avenue E. Mounier, 83/12, B-1200 Brussels, Belgium; or call +32 775 93 40; or fax +32 2 779 54 94; or e-mail info@estro.be; or see http://www.estro.be

Radiation oncology

November 25-28, 2004

The ISRO international teaching course on "Practical Radiation and Molecular Biology with Mayor Emphasis on Clinical Application" will take place in Chiangmai Thailand.

See http://www.isro.be

Radiation oncology

March, 2005

The ISRO international teaching course on "Palliative Care in Cancer Treatment" will take place in Dar es Salaam, Tanzania.

See http://www.isro.be

Lung cancer

July 3-6, 2005

The "11th World Conference on Lung Cancer" will be offered in Barcelona, Spain.

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Radiation oncology

September - October, 2005

The ISRO international teaching course on "Rational Developments from developing to developed Countries" will take place in Lombok, Indonesia.

See http://www.isro.be

Oncology

October 30 – November 3, 2005

The ESTRO 24 / ECCO 13 Conference will take place in Paris, France.

Contact FECS office, Av. E. Mounier, 83/4, B-1200 Brussels, Belgium; or call +32 7759340; or fax +32 2 7795494; or e-mail info@estro.be; or see http://www.fecs.be

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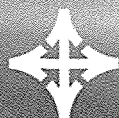

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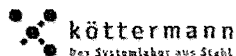

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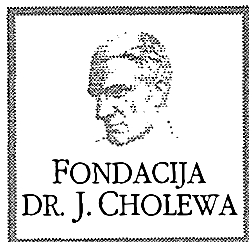

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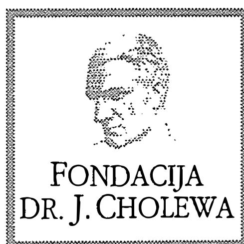

Activity of »Dr. J. Cholewa« Foundation for Cancer Research and Education - A Report for the First Quarter Of 2004

In the course of the year 2004 the Dr. J. Cholewa Foundation for Cancer Research and Education Foundation and the Health continues to support various activities associated with cancer research and education in Slovenia. Although it remains the fact that various public and privately owned enterprises find it more and more difficult to contribute financially to help running day to day operations of the Foundation and its many scopes of activity, several new initiatives and suggestions were discussed and evaluated during the recent meetings of the Foundation. The Supervising Board of the Dr. J. Cholewa Foundation for Cancer Research and Education Foundation at the last meeting in May of the year 2004 discussed several of the requests addressed to it. The requests included support for the publication of books of substantial professional interest, support for various research and educational activities and various study grants.

Financial reports of the Foundation's activity showed that despite several of the aforementioned constraints it achieved all the anticipated and stated goals for the year 2003 and in the first five months of 2004. The main goal still remains to support and sustain research in cancer in Slovenia, and various pathways are to be undertaken to achieve this goal. The Foundation will continue to support the regular publication of "Radiology and Oncology" international scientific journal, which is edited, published and printed in Ljubljana, Slovenia. The Dr. J. Cholewa Foundation for Cancer Research and Education remains optimistic about the prospects in the year 2004 and notices for the competitive for research and education grants will soon appear in all major dailies in Slovenia.

On several meetings it was discussed that the Foundation will put considerable efforts in the plan to organise the first "Prof. dr. Vinko Kambi? Memorial Meeting". The "Memorial Meeting" is planned to be held in about one and a half year's time and consequently to take place every two or three years. This opportunity may also help the Foundation to find the new ways to collaborate with institutions sharing the same goals all over Europe and beyond.

Tomaž Benulič, MD, MSc
Borut Štabuc, MD, PhD
Andrej Plesničar, MD, MSc

Glivične okužbe

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Datum priprave besedila: marec 2003

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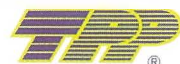

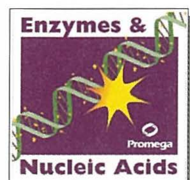

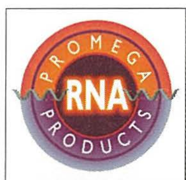

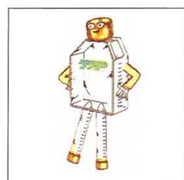

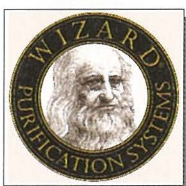

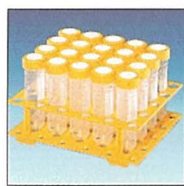

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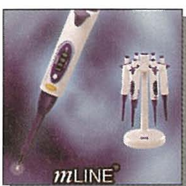

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Introduction should state the purpose of the article and summarize the rationale for the study or observation, citing only the essential references and stating the aim of the study.

Material and methods should provide enough information to enable experiments to be repeated. New methods should be described in detail. Reports on human and animal subjects should include a statement that ethical approval of the study was obtained.

Results should be presented clearly and concisely without repeating the data in the tables and figures. Emphasis should be on clear and precise presentation of results and their significance in relation to the aim of the investigation.

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Illustrations and tables must be numbered and referred to in the text, with appropriate location indicated in the text margin. Illustrations must be labelled on the back with the author's name, figure number and orientation, and should be accompanied by a descriptive legend on a separate page. Line drawings should be supplied in a form suitable for high-quality reproduction. Photographs should be glossy prints of high quality with as much contrast as the subject allows. They should be cropped as close as possible to the area of interest. In photographs mask the identities of the patients. Tables should be typed double spaced, with descriptive title and, if appropriate, units of numerical measurements included in column heading.

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Dent RAG, Cole P. *In vitro* maturation of monocytes in squamous carcinoma of the lung. *Br J Cancer* 1981; **43:** 486-95.

Chapman S, Nakielny R. *A guide to radiological procedures.* London: Bailliere Tindall; 1986.

Evans R, Alexander P. Mechanisms of extracellular killing of nucleated mammalian cells by macrophages. In: Nelson DS, editor. *Immunobiology of macrophage.* New York: Academic Press; 1976. p. 45-74.

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