INVITATION LECTURES

3. MODERN PATHOLOGY

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Molecular genetics of cancer in practice

KEYWORDS: Prognosis, Gene alterations, Genetics

INTRODUCTION

The progression of a tumor through preneoplasia to frank neoplasia and then to invasion and metastasis is the consequence of mutations in genes that control cellular proliferation and death. Mutations result either in the activation of oncogenes, which promote cellular proliferation or inhibit cell death, or in the inactivation of tumor suppressor genes, which inhibit proliferation or promote cell death. The consequences of many of the point mutations, deletions, transpositions, amplifications, insertions, rearrangements and translocations that can comprise these DNA alterations, represent the gene dysregulation which contributes to malignant transformation. In this paper some of the most common gene alterations in cancer, that are partly studied in our laboratory, are discussed, primarily from the aspect of their clinical utility.

CANCER AS AN INHERITED DISEASE

About 1% of all cancers arise in individuals with an unmistakable hereditary cancer syndrome. These individuals carry a particular germline mutation in every cell of their body. Although rare, the inherited cancer syndromes are of vast biological importance because they provide insights into molecular origins and pathogenesis of inherited and sporadic forms of cancer. Early diagnosis can usually reduce mortality and morbidity. Familial adenomatous polyposis of the colon (FAP) is a good example. Once the specific predisposing mutation has been identified in a given family, because FAP is dominantly inherited, genetic testing will identify those who have not inherited the mutant gene, who can therefore be spared screening by colonoscopy, and will target those who are at high risk. Similar benefits are likely in other inherited cancer syndromes, e.g., the multiple endocrine neoplasia (MEN), Von Hippel-Lindau disease, and retinoblastoma. Familial breast and ovarian cancer have got a great publicity following the identification of specific predisposing genes (BRCA1 and BRCA2). The common mutations in BRCA1 and BRCA2 confer a roughly 80% lifetime risk of either breast or ovarian cancer.

EARLY DIAGNOSIS OF CANCER

The findings that mutations, genetic instability and presence of cancerogenic viruses (HCV, HPV, HTLV2) are responsible for biological characteristics of tumor progression make it easy to appreciate why their detection in populations of cells may become a useful diagnostic tool. The ability to amplify DNA fragments about million times by the polymerase chain reaction allows the study of very small amounts of tissue, even when the tissue is formalin fixed and paraffin embedded. The DNA can then be analyzed for mutations, deletions, gene rearrangements, or microsatellite instability. The methods of molecular genetics enable us to detect the presence of mutant genes in small lesions or from small numbers of cancer cells shed into clinical samples. Because of their sensitivity/specificity, the tests have great potential for early diagnosis and detection of minimal residual disease (MRD). The successful detection of mutations in *ras* family and p53 genes in several clinical samples (urine, stool, pancreatic juice, blood, sputum, saliva), not only from patients with cancer (pancreatic, bladder, colorectal, head and neck, lung cancer) but also from those who later develop cancer and people at increased risk of developing cancer, illustrates the potential of molecular methods in the early diagnosis of cancer. Tandem repeat DNA sequences (usually the dinucleotide or trinucleotide) known as microsatellites represent a very common and highly polymorphic class of genetic elements within human genome. A more widespread microsatellite instability, demonstrated by expansion or deletion of repeated elements was reported in colorectal tumors and later in several tumor types. Microsatellite changes matching those in the transitional cell carcinoma of the bladder were detected in the urine sediment of 19 of 20 patients with the diagnosis of bladder cancer, whereas urine cytology detected cancer cells in only nine out of 18 of the samples. Conventional laboratory diagnosis of B cell proliferation's is based on histology or cytology, while clonality of B or T cell populations is established mainly by immunophenotyping. However, lymphomas, especially at early stages of development, can be very difficult to resolve from reactive lesions, even with the aid of immunophenotyping. The discovery of immunoglobulin (Ig) gene rearrangements and the application of molecular probes for these genes has been shown to be of value as it can demonstrate monoclonality, assign a disorder to either the B- or T-lymphocyte lineage, and enable the detection of small number of neoplastic cells. The complementarity determining region III (CDR3) of VH gene segment formed by VDJ junction can be amplified by PCR with primers that bind to consensus gene regions in IgH V framework regions. Primers have been designed to bind and amplify the most constant sequences or consensus regions within framework (FR) I, FRII or FRIII to the J regions. Only complete VDJ rearrangements with the V and J sequences in the right orientation are amplified. Monoclonal populations yield one or two dominant products, whereas polyclonal samples yield a wide range of product sizes that appears on gel. In addition, this technique has great potential in tracking minimal residual disease in lymphomas and leukemias, for monitoring clonal evolution in acute and chronic lymphoblastic leukemias and lymphomas as well as by detecting clonal rearrangements of T-cell receptor for differential diagnosis and monitoring of T-cell lymphoma. Minimal residual disease in patients with CML can be detected on molecular level by sensitive reverse transcription polymerase chain reaction (RT-PCR) of *bcr-abl* transcript which is a consequence of chromosomal translocation. In this way minimal residual disease or early relapse in patients after treatment or bone marrow transplantation can be detected before appearance of any clinical or laboratory signs of the disease.

MOLECULAR STAGING, PROGNOSIS AND PREDICTION OF THERAPEUTIC RESPONSE

Enormous accumulation of data concerning incidence of mutations in different oncogenes and tumor suppressor genes in human cancers led to increased efforts to investigate the usefulness of these findings in molecular staging, prognosis and prediction of therapeutic response. In that manner one group had designed a molecular pathologic substaging system in stage I non-small-lung cancer concerning mutations in K-*ras*, H-*ras* and p53 genes. Molecular assessment of head and neck cancer staging showed that at least 50% of patients with complete resection as presented by histopathological

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evaluation had at least one tumor margin positive for a p53 mutation identical to the primary tumor. These patients had significantly increased risk of local recurrence when compared with patients with molecularly assessed negative margins. Very encouraging results in assessment of tumor margins were obtained when DNA microsatellite alterations were used. The value of molecular markers as prognostic factors was first demonstrated by a report that amplification of protooncogene HER-2/neu in breast cancer correlated with increased rate of relapse and poorer survival. In another study the presence of p53 mutations was correlated with prognosis in a high number of breast cancer patients, strongly suggesting a relatively high risk of recurrence associated with p53 mutations. Several studies have assessed mutations of p53 as a prognostic factor for non-small-cells lung carcinoma (NSCLC), but these results still remain controversial. Multicentric "RASCAL" study of patients with colorectal carcinoma showed that K-ras mutations are associated with increased risk of relapse and death, but some mutations are more aggressive than others. The disregulation of bcl-2 and its partners like bax and bcl-x can protect cancer cells from apoptosis upon anticancer drug treatment. Although clinical studies paradoxically suggest an association between bcl-2 expression and a good prognosis for cancer patients, the clinical relevance of bcl-2 family is still under analysis.

CONCLUSION

In clinical oncology the recombinant DNA technology is already in use for the diagnosis, prognosis, prediction and therapy of many malignant tumors. It is necessary that clinical doctors become familiar with the basic terms of molecular genetic research and to be aware of clinical molecular diagnostic test that are becoming available for patients with malignant diseases. Soon, thanks to microarray technology, it will be possible to define molecular profiles of individuals and of their cancers and use them clinically to assess susceptibility, and to provide markers of preneoplasia, early diagnosis of cancer, staging, prognostication, and selection of treatment.

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Carcinogenesis in the large intestine

KEYWORDS: Colorectal carcinogenesis; Adenoma polyposis coli gene; Dysplasia

INTRODUCTION

The question of whether colorectal adenocarcinomas arise from adenomas or flat mucosa has generated controversy and confusion for years. The former hypothesis has been widely accepted in European countries, and the latter in Japan. Recently, small flat cancers without accompanying adenomas have been detected following the development of endoscopical technology. Shimoda et al (1989) (1) proposed that flat lesions may be "de novo cancer" arising directly from normal mucosa.

PRECURSOR LESIONS

During the past decade the natural history of colorectal carcinomas has been extensively studied in correlation with the underlying accumulation of generic alteration. This article will highlight both morphological and molecular features of the following precursor lesions: Aberrant crypt foci (ACF): the earliest morphological precursor of epithelial neoplasia is the ACF, ACFs have crypts of enlarged calibre thickened epithelium with reduced mucin content. Microscopy show two main types: ACFs with features of hyperplastic polyps and a high frequency of *ras* protooncogene mutations and dysplastic ACFs (micro-adenoma) associated with mutation of the APC (Adenomatous Polyposis Coli) gene (2). Progression from ACF through adenoma to carcinoma characterizes carcinogenesis in the large intestine. Transitional mucosa (TM) occurs in flat normal appearing mucosa of patients with carcinomas and adenomas and in polyposis coli. It has a significant increase in sialomucin production, crypt depth and diameter of the crypt. There seems to be a close correlation to proliferation activity, since an increase in mitotic activity and an extension of the proliferative zone could be observed in similar mucosal regions in the same conditions. The mucus alterations in the TM resemble the mucus secretion pattern of the human fetal colon. This may indicate that this kind of mucosa is immature and thus more sensible to neoplastic stimulation and transformation. The very close relationship to earliest neoplastic lesions as *oligotubular adenomas* (OTA - when the number of crypts showing dysplastic changes is less than 20) favors the view that this kind of TM may be the first step in the development of neoplasia (3). Hyperplastic poliposis: the genetic changes and the presence of serrated adenoma and *mixed hyperplastic polyp/adenoma* offer further evidence for a direct relationship between

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hyperplastic polyposis and colorectal carcinoma, and support the concept of a hyperplastic polyp-adenoma-carcinoma sequence (4). Adenomas: inactivation of the APC gene initiates the process and results in extension of epithelial proliferation in dysplastic epithelium from the base to the crypts where it normally occurs, toward or into the luminal surface (5). Polyps appear to grow as a consequence of accelerated crypt fission resulting from APC gene mutation. *Macroscopy*: colorectal adenomas can be classified into: *elevated*, *flat* and *depressed* group. *Elevated* adenomas range from pedunculated polyps with a long stalk or non-neoplastic mucosa to those that are sessile. *Flat* and *depressed* adenomas are recognized by mucosal reddening. Depressed adenomas are usually smaller than flat or protruding ones and tend to give rise to adenocarcinoma. These adenomas have a lower frequency of ras mutation than polypoid adenomas. Flat tumor is roughly defined as the tumor with a flat surface (or bottom) and is thick twice thickness of normal mucosa (1-2mm for the colorectal lesions). The flat neoplastic lesions have been designated by Japanese gastroenterologists as type II, with three subtypes: IIa (*en plateau* elevated); IIb (completely flat) and IIc (*en plateau* depressed). The depressed lesions have, despite a smaller diameter (mean diameter 11mm), a poor prognosis with prompt penetration in the submucosa. The rate of carcinoma or carcinoma containing lesion in the flat tumor (adenoma+carcinoma) was 15%, slightly higher than that for the polypoid tumor (12%). Histopathology: tubular adenomas are usually protruding, spherical and pedunculated, or flat (not protruding). Dysplastic glandular structures occupy at least 80% of the luminal surface. Villous adenomas are typically sessile with a hairy-appearing surface. Dysplastic glandular comprise more than 80% of the luminal surface. Villous architecture is defined by the length of the glands exceeding twice the thickness of normal colorectal mucosa. *Tubulovillous* adenomas have a mixture of tubular and villous structures with a ratio 80%/20% and 20%/80%. *Serrated* adenomas were diagnosed when polyps were formed of neoplastic epithelium with the cytological changes associated with adenoma (elongated irregular nuclei with higher nuclear/cytoplasmic ratios and serrated configuration typical of hyperplastic polyp). These adenomas are characterized by the saw-tooth configuration of a hyperplastic (metaplastic) polyp, but the epithelium lining the upper portion of the crypts and luminal surface is displastic. They can also have tubular or villous component, but low-levels of microsatellite instability (MSI-L) and altered mucin are characteristic for these serrated lesions. Mixed hyperplastic polyp /adenoma contains separate identifiable areas of each histopathological type. Some villous adenomas show in the slopes of the villi closely packed small glands, referred to as villo-microglandular adenomas. Intraepithelial neoplasia can also occur in the absence of an adenoma, in a preexisting lesion of another type. Hyperplastic (metaplastic polyps) have elongated, serrated crypts lined by proliferative epithelium in the bases with enfolded epithelial tufts and enlarged goblet cells in the upper crypts and on the luminal surface, imparting a saw-tooth outline. Neoplasia in chronic inflammatory bowel disease - Ulcerative colitis (UC): development of carcinoma is apparently metachronous to the development of intraepithelial neoplasia (classified as low-grade and high-grade) complicating chronic colitis. It may be flat or present as a *Dysplasia associated lesion or mass* (DALM); the latter is often associated with a synchronous carcinoma arising beneath the dysplastic surface. Diagnosis of DALM and high-grade flat dysplasia leads to total colectomy. Mutation of TP53 and p16 represents early event, specific only for adenocarcinoma associated with UC, leading to loss of other genes, such as APC and DCC. These changes are not usually seen in sporadic adenomas. This indicates that dysplasia in UC and sporadic adenoma may follow different genetic pathways. Crohn disease: intraepithelial neoplasia, classified as low-grade or high-grade is associated with a high proportion of Crohn carcinomas, either adjacent to the invasive lesion or at distance from it. Similar to UC, polypoid dysplastic lesions are diagnosed as DALM in Crohn`s disease. Mucinous adenocarcinoma are seen in Crohn disease more frequently than in sporadic colorectal carcinomas. TP53 and c-K-RAS mutations are observed earlier in Crohn-associated intraepithelial neoplasia than in the *adenoma-carcinoma sequence* of sporadic colorectal cancer.

CONCLUSION

Colorectal carcinogenesis is a multistep process. To decide from which level of the structural and cellular atypia the tumor should be diagnosed as a carcinoma, immunostaining to p53, Ki67, MUC1, MUC2 and MUC5 is useful, but still the orthodox HE staining-based diagnosis seems to be most important.

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Prognostic significance of molecular and immunohistochemical markers in colorectal carcinomas

KEYWORDS: Colorectal neoplasms; Carcinogenesis; K-ras gene; Angiogenesis

INTRODUCTION

The most frequently investigated genetic alterations in colorectal carcinomas (CC) are mutations in the K-*ras* gen and p53 tumor suppressor gene, present in about half of the patients with CC. However, there is little agreement on how those mutations relate to other clinico-pathological factors and survival. According to the multicenter "RASCAL" study (1), which accepted our preliminary data, at least nine groups have suggested that the presence of the K-*ras* mutation conveys prognostic significance, but 14 groups reached the opposite conclusion. There is no consensus regarding the frequency of some types of mutations as well as their prognostic implications. Some authors have reported that the p53 protein expression was associated with the depth of invasion of CC and with the shorter survival of patients. The other authors showed improved prognostic influence of p53 protein expression (2). CD44 expression has been shown to be associated with metastasis and poor prognosis in CC. Howe showed (2) that the loss or heterogeneous expression of E-cadherin in CC correlated closely with the advanced clinical stage, advanced depth of invasion, and with reduced overall survival rates. The published findings on CC angiogenesis are conflicting, ranging from strong prognostic relevance to none (3). The aim of this paper was to correlate mutations in the K-*ras* oncogene, expression of p53 protein, altered immunohistochemical expression of CD44 and E-cadherin and the quantitative estimation of angiogensis in CC tissue with clinicopathological features and survival of patients after colorectal surgery.

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MATERIAL AND METHODS

There was analyzed formalin fixed paraffin-embedded tumor tissue from 81 patients operated on at the Military Medical Academy between 1989 and 1993. All cases of CC were graded into Dukes' stages A - 21 patients, B - 24 patients and C+D - 36 patients (Dukes, 1932). The mean follow up time was 31.4 months (ranging from 2 to 66 months). Mutations in the 12th and 13th codon of the K-*ras* gene were determined by polymerase chain reaction (PCR) on 10 μm thick deparaffinized tumor sections. The type of mutation was identified by autoradiography. The expression of p53 protein, altered expression of E-cadherin, CD44 as well as the presence of CD31 positive endothelial cells in order to estimate mean microvessel count in tumor tisue were investigated immunohistochemically on paraffin embedded tumor tissue by applying Sreptavidin-biotin-peroxidase method according to LSAB + procedure (DAKO) and AEC substrate. Appropriate positive and negative controls were used. Immunostaining (nuclear for p53 protein and cytoplasmic for the other antigens) was calculated as the percentage of positive tumor cells in relation to the total number of tumor cells in representative fields. Statistical analysis was performed with the Statistica 5.0 PC program for Windows 95, using chi squared tests to compare categorical data and the log rank test in order to evaluate differences in failure-free survival and overall survival curves. The results were considered to be statistically significant when Pearson's correlation coefficient showed $p \leq 0.05$ and highly significant when $p \leq 0.01$.

RESULTS

K-*ras* mutation was found in 40.7% of CC, but the overall frequency of mutation was 43.2% because 2 patients had two K-ras gene mutations. The most frequent type of K-ras mutation, G-T transversion at the second base of the codon 12 and 13 was found in 45.7% of overall mutations or in 19.8% of analyzed CC, but there was not relation between this type of mutation and the patient survival. Among the patients with any mutation, 45,7% were in Dukes' stage C+D compared with 20% in stage A and 34,3% in stage B ($p \leq 0.05$). The mean survival time calculated by Kaplan Meier method was significantly shorter in K-*ras* positive compared with K-*ras* negative patients (20 and 37 months, respectively). The presence of nuclear p53 protein was found in tumor tissue of 58% of CC and didn't correlate with Dukes' stage, histologic and nuclear grade, but showed highly significant correlation with survival of patients. Both the K-*ras* mutation and the p53 protein expression were found in 90.9% of patients and they had significantly shorter time of survival than patients without any of mutations. Moderate or extensive expression of CD44 was present in 81.5% of CC and heterogeneous or loss of E-cadherin expression in 74,1% of tumors. There was a highly significant relationship between altered expression of CD44 but not E-cadherin and K-*ras* mutation. Survival was significantly shorter in patients with altered expression of analyzed adhesive molecules. The patients were dichotomized at the cutoff of a vessel count >34.17 per x200 field into: 39 hipovascular tumors (<34.17 vessels per visual field) and 42 hypervascular tumors (>34.17 vessels per visual field). Hypervascularity significantly correlated with advanced Dukes' stages, as well as with shorter survival of patients but not with the K-*ras* mutation and the p53 protein expression.

DISCUSSION AND CONCLUSION

Our study has shown that mutations in the K-*ras* gene in tissue of CC are associated with poorer prognosis. This finding is in agreement with the multicenter RASCAL study (1) that included the data on 4268 worldwide patients. The high rate of G-T transversion in a Yugoslav population compared with data from the other geographic area indicates that environmental factors might be included in colorectal carcinogenesis. However, we did not find adverse outcome for those patients contrary to the RASCAL study. The reason of difference might be due to small number of patients in our group. The finding that the decreased survival is in correlation with the presence of p53 protein,

altered expression of the adhesive molecules and tumor hypervascularity is closely similar with the findings of many other authors. Our results allow us to conclude that adequate combination of molecular and immunohistochemical markers might be of a clinical value in CC and may help to predict the biological behavior of the tumor. At the same time it give us a chance to select appropriately a susbset of patients with more aggressive neoplasm in order to apply the optimal postoperative therapy.

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The expression and interaction bcl 2 and bax proteins as prognostic parameter in chronic lymphocytic leukemia

KEYWORDS: Leukemia; Apoptosis; bcl-2; bax

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a neoplastic disease characterized by the accumulation of morphologically mature but immunologically dysfunctioned CD 5+ lymphocytes in the blood, bone marrow and lymphatic organs. B-CLL is a disease primarily caused by defects in the apoptosis mechanism. Apoptosis is a special type of cell death essentially different from necrosis in nature and by biological significance. A great number of genes are known today whose protein products take part in the regulation of apoptosis. The known cellular modulators of this process are the proteins of the Bcl 2 family. It is known that the protein product of the Bcl 2 gene plays a role in the promotion of cell survival and in the inhibition of apoptosis. Bcl 2 is the founding member of a growing family composed of both suppressors and promoters of apoptosis. First proapoptotic homologue as a co-immunoprecipitate of Bcl 2 protein was Bax protein. Korsmayer and his associates showed that Bcl 2 and Bax proteins can make homo and heterodimers. The antiapoptotic effect of Bcl 2 protein is based on its possibility to bind Bax protein in the heterodimer form, and in that way blocks making Bax/Bax proapoptotic homodimers because of its significant ratio of autonomic "cell rheostat", which determinates the type of cell reaction to apoptotic stimulation (1).

MATERIAL AND METHODS

Our study includes 20 patients with B-CLL (4 untreated and 16 previously treated) and 20 healthy persons (as a control group). B-CLL was diagnosed according to standard clinical and laboratory criteria. The patients with CLL

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were treated by high dose - chlorambucil. The samples of peripheral blood mononuclear cells from all patients and healthy controls homogenized in 10 volumes (wt/vol) of 10mM HEPES (pH 7.20) containing 0.25% Nonidet P-40, 142.5mM KCl, 5mM $MgCl_2$, 1mM EGTA, and one tablet of protease inhibitor cocktail. (Complete Mini; Boehringer Mannheim, Indianapolis, IN, U.S.A.) and processed for immunoblot as described (2). The following primary antibodies were used: 1:500 anti Bcl - 2 (DAKO A/S Denmark); 1:1000 anti - Bax (DAKO A/S Denmark). At the same time, 250mg of protein was incubated (2h, 4^0C) with 3μg of anti-Bcl 2 or anti - Bax antibody and further processed for imunoprecipitation and immunoblotting as described (2). Here, the blots were immunostained with anti-Bax (DAKO A/S Denmark) or anti - Bcl 2 antibody (DAKO A/S Denmark). The films were scanned on a HP-4C Scan Jet, and the bands quantified by using the NIH Image 1.62 software. The data obtained in these experiments were evaluated using the ANOVA test. When ANOVA showed significant differences, pair wise comparisons between the means were tested by Student's t-test. In all analyses, the null hypothesis was rejected at the 0.05 level. All statistical analyses were performed using Sigma Stat for Windows (version 2.0, Jandel Corp., San Rafael, CA, U.S.A.).

RESULTS AND DISCUSSION

Statistically significant increased level of Bcl 2 protein expression, compared to its expression in the peripheral blood mononuclears of healthy volunteers, is found in all CLL patients regardless the stage of CLL ($p=3.68 \times 10^{-10}$). There were no statistically significant differences in Bcl 2 protein expression between CLL patients in the analyzed group. The increased level of Bcl 2 protein expression in the peripheral blood samples is followed by an increased expression of Bax protein. The increased level of Bax protein has been detected in most of the peripheral blood samples (17/20) in CLL patients compared to the average expression of the same protein in the control group samples of healthy individuals. There is a statistically significant difference with p=0,019. Differences considering the level of Bax protein expression in analysed patients had no statistical significance. We used the co-immunoprecipitate method to determinate the level of interaction beetween these two proteins. Simultaneosly we analyzed the level of binding Bcl 2 protein to Bax protein and reversed. The intensity of Bcl 2 and Bax protein binding compared to the control samples of the peripheral blood of healthy individuals, is increased in CLL cells. In the group of patients with untreated CLL, the methods IP:Bax/WB:Bcl 2 and IP:Bcl 2/WB:Bax were done on the peripheral blood total protein samples gained during the first and third treatment day. Results IP: Bax/WB:Bcl 2 showed a high level of "free" Bcl 2 protein which is not bound in the heterodimer form to Bax protein. Simultaneously IP: Bcl 2 /WB: Bax has showed that a higher quantity of Bax protein is bound in the heterodimer form to Bcl 2 protein, than the rest of the potential homodimer is bound to Bax protein. In all four cases the difference between "free" Bax protein and Bax protein that is bound to Bcl 2 protein is reduced during the third day of therapy by increasing the quantity of "free" Bax protein which is found in the homodimer form, or it is bound other cell proteins. A reduced quantity Bcl/Bax heterodimer in these samples is in correlation with successful clinical reaction of the patients to the applied therapy. In the peripheral blood samples of the patients whose condition was determined as stabile or it is the condition after remission maintained without applying the so called maintenance therapy, we detected balanced results of "free" Bax protein, and bound Bax protein that is in the heterodimer form with Bcl 2 protein. In the patients who were in a partial remission, the findings of IP:Bcl 2/WB:Bax showed that Bax protein was predominantly present in the heterodimer form with Bcl 2 protein. Results of the peripheral blood analysis by the method IP:Bax/WB:Bcl 2 showed that besides the quantity of Bcl 2 protein that was in the heterodimer form bound to Bax protein, a great amount of "free" Bcl 2 protein was also found in the cells. With determination of the level of Bcl 2/Bax interaction we confirmed our opinion that the Bcl 2/Bax ratio could be used as a prognostic parameter in CLL treatment.

CONCLUSION

The levels of Bcl 2 and Bad protein were increased in all analyzed patients. The results of this study showed that the ratio between Bcl 2 and Bax protein was in correlation with the sensitivity of CLL cells to the antineoplastic treatment. Determination of the ratio between Bcl 2 and Bax protein could be a prognostic parameter in CLL treatment.

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Dedifferentiated chondrosarcoma arising from enchondromatosis

KEYWORDS: Chondrosarcoma; Bone Neoplasms; Immunohistochemistry

INTRODUCTION

Dedifferentiated chondrosarcoma (DChSa) is a primary malignant bone tumor with a focal transformation of the low-grade conventional chondrosarcoma into the high-grade sarcoma. DChSa often arises secondarily from enchondromatosis and rarely is *de novo* in origin (1). The low-grade tumors component usually corresponds to stage I chondrosarcoma and it could sometimes be very difficult to differentiate it from primary enchondromatosis (2). The high-grade sarcomatous component can resemble many different malignant mesenchymal tumors: malignant fibrous histiocytoma (MFH), osteosarcoma, fibrosarcoma, rhabdomyosarcoma etc. The imunohistochemical staining (IH) can enlighten the histogenesis of the high- grade component.

MATERIAL AND METHODS

Patients with clinical and radiological signs of bone tumors were operated at the Institute of Orthopedics and Traumatology "Banjica", Belgrade, and the sampled material sent for histopathology to the Institute of Pathology, School of Medicine, Belgrade. All preparations were stained classically with hematoxylin- eosin; in 3 cases IH: vimentin, cytokeratin, S-100 protein and CD68 were performed. Immunohistochemistry was necessary to determine the high-grade component.

RESULTS

From 1994 to 2001 (the last 8 years) eight cases of dedifferentiated chondrosarcoma were diagnosed at the Institute of Pathology, School of Medicine, Belgrade. The tumor was equally involving both sexes (4 males and 4 females). All the patients were in older ages (the youngest patient was 44 and the oldest 75 years old, the mean age 60.5 yr.). The main clinical symptoms were unspecific pain and swelling, and a pathologic fracture, a common sign of the bone tumor, was present in 4 patients (50%). Duration of the symptoms varied from 1 month to 1 year, but in the disease generally had a rapid progress. The most commonly involved bones were the long tubular bones (femur 5, humerus 2); in only one case the tumor was localized in the sacrum. In 6 patients (75%) a radiographic examination disclosed intramedullary osteolytic lesions with focal calcifications of "pop-corn" densities in the diaphysis and metaphysis of the long bones. This radiographic feature pointed to the chondral tumor. The definite diagnosis was established after pathologic analyses of the operative material. In all patients the first biopsy was followed by a radical surgery. In the biopsy material the following diagnoses were confirmed: dedifferentiated chondrosarcoma (6), chondrosarcoma grade I (1), MFH (1). The macroscopical, histological and IH analysis of the material after the therapeutic surgery (4 amputations, 4 radical partial resections) confirmed dedifferentiated chondrosarcoma in all cases. The chondral tissue was grossly visible in the operative material from all patients, even in the patient with a primary diagnosis of MFH. It was histologically confirmed. Different tumors contained the following components: enchondroma (6), chondrosarcoma grade I (8), MFH (7), fibrosarcoma (2), osteosarcoma (1), schwannoma malignum (1). These findings show that 75% dedifferentiated chondrosarcomas arose from enchondroma. The low-grade component was chondrosarcoma grade I in all patients. MFH was the most frequent high-grade component, associated with fibrosarcoma in one case and malignant schwannoma in the other. The single tumor with osteosarcoma for the high-grade component didn't contain MFH. In the cases stained with IH antibodies, the analyses showed clear positivity for vimentin and S-100 protein in the low-grade component, and negativity for cytokeratin and CD68 - typical for chondrosarcoma. The high-grade component was MFH in these cases and it was confirmed by strongly positive vimentin and CD68, and the expected negative S-100 and cytokeratin stains.

CONCLUSION

The diagnostics of the dedifferentiated chondrosarcoma demand a good correlation between clinical, radiological and morphological characteristics. The histopathological examination includes serious tissue analysis and description of all tumor components: enchondroma, the low-grade chondrosarcoma in juxtaposition with the high- grade component. IH stains are useful to determine histogenesis of the high-grade component.

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Lung pathology with presentation of unusual cases

KEYWORDS: Lung Neoplasms; Immunohistochemistry; Sclerosing haemangioma

INTRODUCTION

The aim of this presentation is the analysis of lung biopsies in regard to the last WHO lung tumors' classification. Moreover, some interesting and rare cases are presented, and synchronous multiple lung pathology is emphasized.

MATERIAL AND METHODS

Surgically resected pulmonary specimens from the period 1999-2000 were analyzed for synchronous multiple pathologic changes. Moreover, surgical tumor specimens were classified according WHO recommendations. Furthermore, interesting cases from the same period and from previous years are included in this presentation.

RESULTS AND PRESENTATION OF PATIENT

Among 409 pulmonary specimens, multiple pathologic changes appeared in 14%, one fifth of the latter as only no-tumors lesions. Among 358 resected lung tumors, squamous, adeno-, small-cell, large-cell, adenosquamous carcinomas and carcinoids presented 35.2%, 22.1%, 1.1%, 9.7%, 1.7%, and 4,7%, respectively. 1.9% of adenocarcinomas were bronchioloalveolar carcinomas, and 0.8% of carcinoids were atypical. Recently introduced histologic types, pleomorphic and combined large cell neuroendocrine carcinomas presented another 5.6% and 4.5%. Mesothelioma were found in 1.4%, soft tissue tumors in 9.8%, presented mostly by hamartomas in 7.3% and benign fibrous tumors in 1.9%. Metastatic pulmonary lesions were resected in 4.2% of cases. Interesting lung pathology cases included: generalized hyperplasia of neuroendocrine cells, unusual carcinoids and mesotheliomas, sclerosing hemangioma, paraganglioma, pulmonary malakoplakia, pulmonary lymphangioleiomyomatosis, and very rare combination of Langerhans' cell histiocytosis with Erdheim-Chester disease.

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Patient 1. Left upper lobectomy was performed in a 79-years old woman. She had been already operated because of breast cancer 6 years before lobectomy. Lung specimens revealed papillary adenocarcinoma as second primary with negative reactions for estrogen and progesterone receptors, minute meningothelioid nodule (chromogranine A and keratin negative, EMA and vimentin positive), and over 50 focuses of diffuse idiopathic neuroendocrine cells' hyperplasia, and multiple tumourlets in both left lung lobes.

Patient 2. In the last years, pulmonary carcinoids presented almost 5% of all lung tumors and were usually found in lobar bronchi. In the lung periphery, they usually appeared as spindle cell or oncocytic tumors.

Patient 3. Laser resection of endobronchial paraganglioma was performed in a 25-years old woman. The tumor recurred after 8 years. Similarly to typical carcinoids, this tumor contained S-100 positive sustentacular cells, yielded positive reactions for neuroendocrine markers, but negative for cytokeratins.

Patient 4. Almost encapsulated sclerosing hemangioma with papillary structures and sclerosing areas, both covered with cuboid epithelium, was resected in a 67-years old woman. In another patient, beside the tumourlet, similar mostly sclerosing papillary structures were found with differential diagnosis of fibromatous hamartoma.

Patient 5. Left pleuropneumonectomy was performed in a 52-years male patient because of malignant mesothelioma. The patient's brother died from malignant mesothelioma, too. They were both from the region with possible professional or vicinal exposure to asbestos dusts. Tumor disclosed focal areas of squamous metaplasia and areas with osteoclastic-like giant cells.

Patient 6. Pulmonary malakoplakia is a rare event in the lung, associated with emaciation, alcohol abuse, renal or heart transplantation or AIDS as in our patient with a fatal progression of the disease. Multiple nodules in the lung parenchyma consisted of macrophages with diagnostic cytoplasmic Michaelis-Gutmann bodies.

Patient 7. Pulmonary lymphangioleiomyomatosis in a woman in the reproductive age was confirmed by smooth-muscle actin and HMB-45 positive markers.

Patient 8. Diabetes insipidus was confirmed in a 27-years old male patient with polydipsia and polyuria and diffuse reticulonodular infiltrates in the lung. Open lung biopsy revealed interstitial infiltrates with macrophages, lymphocytes, scarce eosinophil granulocytes, few S-100 positive cells, and clusters of CD1a positive cells. Electron microscopy finding of Birbeck granules confirmed the diagnosis of Langerhans' cell histiocytosis. Eight years later, the bone pains brought the patient to the bone biopsy and x-ray of the skeleton. Diffuse sclerotic and focal lytic lesions were found simmetrically in both humerus, femur and tibia. Biopsy disclosed mostly thickened, focally thinned bone trabeculae with fibrous tissue, plasma cells, neutrophil and few eosinophil granulocytes, abundant clusters of foamy lipid-laden macrophages. Immunohistologically, there were some S-100 and scarce CD1a positive cells. Histology and x-ray suggested the diagnosis of Erdheim-Chester disease. Both diseases may affect the same organs, such as the bones, pituitary, lung, skin, etc.

DISCUSSION

Pleomorphism is the morphologic phenotype connected with worse prognosis. More than 10% of pleomorphic tumor cells were found in 32% adenocarcinomas and 26% squamous carcinoma (1), which are now included in the group of pleomorphic carcinoma according WHO classification. Multiple pathologic changes in the lung are frequently found in surgically resected pulmonary tissue. In patients with history of malignoma in the lung or elsewhere, they suggest the diagnosis of recurrent or metastatic malignoma. Histologic examination of surgical specimens in patients with multiple pulmonary lesions reveals a wide spectrum and combinations of other pathologic lesions, which should be elucidated by cytology or histology to avoid unadequate treatment. Sclerosing hemangiomas may be differentiated from other tumors by membranous and cytoplasmatic MIB-1 positive reaction, although we could not

confirm it definitely in our two cases (2). Incidental finding of minute mengingothelioid bodies has no clinical significance, but it should be differentiated from tumourlet or even from carcinoid, assessed by biopsy only on the periphery. Oncocytic lung lesions should confirm oncocytic carcinoid and exclude metastatic processes from thyroid (3). Both our cases of typical carcinoid with Cushing' s syndrome caused by ectopic ACTH secretion metastasized to regional lymphnodes (4). This fact confirms at least to some extent the opinion that paraneoplastic syndromes, unusual in majority of carcinoids, may be connected with carcinoid metastases outside the lung. Moreover, the lungs are known to metabolize biologic active substances quickly. Erdheim-Chester disease and Langerhans' cell granulomatosis have been regarded as two different entities of monocyte macrophage lineage. Considering our patient and based also on some recent reports, the question arises, if there are really two autonomous entities or possible metachronous expression of the same disease (5).

CONCLUSIONS

Although WHO classification of lung tumors is based mostly on slides, stained by hematoxylin and eosin, in many cases additional immunohistology is required for correct and definitive diagnosis. Multiple pulmonary lesions with all differential diagnostic possibilities should be kept in mind to choose appropriate treatment.

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HER2/neu expression in breast cancer patients - Correlation with estrogen and progesterone receptor status, p53 and Ki-67 immunoreactivity and clinicopathological parameters

KEYWORDS: Breast Neoplasms; , HER2/neu; Immunohistochemistry

Although the role of HER2/neu status is still unsettled, its determination is valuable in selecting breast carcinoma patients for adequate Herceptin® therapy. The purpose of this study was to investigate the association between HER2/neu expression with estrogen (ER) and progesterone (PgR) receptor status, p53 and Ki-67 immunoreactivity, as well as with other clinicopathological parameters in breast cancer patients. HER2/neu, ER/PgR status, p53 and Ki-67 expression was determined in 169 postoperative stage I-III (UICC, 1997) breast cancer patients using the standardized DAKO HercepsTest® and by the immunoperoxidase technique, respectively. The results were evaluated by performing the standardized scoring system. The values of HER2/neu expression were correlated to ER/PgR status, p53 and Ki-67 immunoreactivity and to clinicopathological parameters (tumor size, histopathologic grade, nuclear grade, tumor type, and lymph node status and patients' age). The statistical significance was determined with $\chi 2$ and Fisher's exact test. HER2/neu expression was positive in 66 patients (37%). There was no significant association between the values of HER2/neu and ER/PgR status, p53 or Ki-67 immunoreactivity, neither with any other clinicopathological parameter. ER is associated with PgR, tumor size, tumor type and lymph node status ($p<0.01$); PgR with histopathologic grade, tumor type and lymph node status ($p<0.01$) and Ki-67 with p53 immunoreactivity, tumor size and patients' age ($p<0.01$). The results of the current study indicate that HER2/neu is an independent prognostic marker in differentiating a subgroup of high-risk breast cancer patients. Additional studies are required to adjust HER2/neu testing results to the clinical outcome.

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Diagnostic and therapeutic "splitting" in breast oncopathology - through globalization to individualization

KEYWORDS: Breast carcinoma; Immunohistochemistry; Diagnosis

INTRODUCTION

THE GLOBALIZATION OF KNOWLEDGE: Doctors of a renaissance type, with gargantuan cognitive and reminiscent potentials, able to cure and diagnose completely on their own, are already part of history? Numerous synchronous prospective clinical-pathological researches on a worldwide level, performed on huge and stratified samples of patients, with a long follow up period, applying an up-to-date technology which measures and evaluates the results accurately - produce an abundance of information in the field of oncology. This knowledge is of a significant size, divergent, with a short period of half-life and a need for constant evaluation. It is being applied in the diagnostic and therapeutic oncological practice by a team: the success of diagnostics and treatment of malignoma according to the recent oncological doctrine implies the work of various doctors subspecialists (surgeons, radiologists, pathologists, radiotherapists, hemotherapists . . . .). If they act promptly, highly professionally and synchronously, they can be named an oncological consilium, whose decisions imply both the individual and collective responsibility of the members. The team embodies the global oncological knowledge, but also paramedical factors (cost-benefit principle), and its recurrent wrong (inefficient) decisions show that the 'embodiment' can remind of an ugly and multiple-headed dragon.

THE INDIVIDUALIZATION OF DISEASE: Pathologists baptize about 70% of all breast cancers by the diagnosis "Carcinoma ductale invasivum", in the same time wondering which is truer - "Nomen est omen" or "Nomina sunt odiosa". The question is rhetorical: the clinical "omen" of this diagnosis, the most frequent in the oncopathology of females, is practically non existent. After all, could more be expected from an entity defined per exclusione "carcinoma not falling into any of the other categories of invasive mammary carcinoma" (1)? On the other hand, in the past two decades we have seen unprecedented advances in our understanding of what makes cancer grow: the central roles of hormones (e.g. estrogen and progesterone, insulin-like growth factors) and their signaling pathways, and of important genes involved in the genesis and progression of breast cancers (e.g. HER-2, p53, PTEN, BRCA). However, the precision of the current definition of ductal breast cancer still reminds of defining a banana as a fruit "not at all like a plum". Thus the tendency of replacing general, common morphological diagnoses with more specific ones, based on the synthesis of morphological, functional (phenotypic) and genetic properties of malignant cells of individual tumors (patients) is not surprising at all (2-5). These properties are often quantified, therefore objective and reproducible. Diagnosis of the type "Carcinoma ductale invasivum mammae - poorly differentiated" are part of the past, and soon in the future this diagnosis will be "Breast carcinoma of Annabelle Barrenwoman ; NOS ; histological and nuclear grade (HG, NG) 2 ; T2 N1 M0 ; estrogen receptors (ER) low positive (H score 68) ; progesterone receptors (PR) negative (H score 17) ; HER-2 status = 3+ ; Ki-67 and p53 high positive ; bcl 2 negative ; mean nuclear area 52 μm^2 ; clonogenic assay =28 % ; BRCA1 + (comparative genomic hybridization).

MATERIAL AND RESULTS

Next table shows high phenotypic diversities of 20 consecutive diagnosed invasive ductal breast carcinoma HG and NG 2, $T_2N_{0,1}M_0$ stage (Table 1):

Table 1. High phenotypic diversities of 20 consecutive diagnosed invasive ductal breast carcinoma HG and NG 2, T2N0,1M0 stage

Antigen	negative		low positive		medium positive		high positive	
	n	%	n	%	n	%	n	%
ER	11	55	4	20	2	10	3	15
PR	10	50	3	15	6	30	1	5
HER-2	1	5	6	30	6	30	7	35
p53	10	50	4	20	4	20	2	10
Ki-67	8	40	5	25	6	30	1	5
bcl 2	11	55	4	20	5	25	0	0

CONCLUSION

Immunohistochemical and genetic methods give a significant contribution to the individualization of the diagnostics and therapy of malignoma: visualization and quantification of clinically (therapeutically and prognostically) important membranous, cytoplasmic and nuclear antigens of the cells belonging to the concrete tumor (a pathologist "sees" the antigen and the mutated gene on a tissue slide - morpho-functional borders disappear) make the individually specific treatment possible. If created like this, therapeutic protocols become less impersonal, they carry the personal mark both of the patient and the diagnostic therapeutic team (6,7) The specter of the specific oncotherapy broadens constantly, and numerous therapeutic decisions are based directly on the results of the immunohistochemical analysis of the tumor tissue: the immunotherapy with specific antibodies (Herceptin - for the treatment of HER-2 high positive mammary carcinoma) hormonal and/or cytostatic therapy? It seems that in the long-lasting struggle of the 'lumpers' and 'splitters' in the domain of diagnostics and therapeutic systems of classification of malignant diseases the latter win. Hippocrates is right once more - there are no diseases, there are only patients.

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Clinical value of immunohistochemical detection of proteins encoded by genes involved in chromosomal alterations in lymphoma and leukemia

KEYWORDS: Chromosomal alteration; Lymphoma; Leukemia; Immunohistochemistry

Acquired chromosomal alterations (mainly translocations) in hematological neoplasms result in both activation of quiescent gene (by its localization near some active promoter) and expression of an intact protein product, or formation of a fusion gene encoding chimeric protein. The result of these events is the expression of these proteins, which can be detected in the tissue by immunohistochemistry. Translocation t(14;18), present in 80%-90% of follicular lymphomas and in 20% of diffuse large B-cell lymphomas, leads to deregulated expression of BCL-2. Expression of BCL-2 distinguishes follicular lymphoma from reactive germinal centers and appears to be an independent prognostic factor in the diffuse large B-cell lymphoma and Hodgkin's disease. Expression of cyclin D1 as a consequence of t(11;14) and rearrangement of the CCND1(BCL-1) gene is a specific marker of the mantle cell lymphoma. This protein is over expressed in the multiple myeloma and hairy cell leukemia. BCL-6 is a marker of the maturation stage (for germinal center and activated B-cells). Rearrangement of BCL-6 is present in 40% of diffuse large B-cell lymphomas and in follicular lymphoma. Translocation t(1;14) is often present in MALT lymphoma and leads to over expression of BCL-10 protein. The protein products of other genes, activated by chromosomal rearrangement are markers of the lineage TAL-1(SCL) in acute T lymphoblastic leukemia and PAX-5 in B-cell neoplasms. Antibodies for detection of chimeric proteins are few. Staining for promyelocytic leukemia (PML) protein will detect acute PML with t(15;17). ALK protein (anaplastic lymphoma tyrosine kinase) is highly specific for CD30 positive large T-cell lymphomas with t(2;5) because wild type ALK is not found in normal lymphoid tissue. Immunohistochemical detection of the products of rearranged genes in lymphoma and leukemia is of diagnostic and prognostic value and provide information on cellular and subcellular protein expression that cannot be inferred by molecular analysis.

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Histomorphological and immunohistochemical features of pheochromocytomas

KEYWORDS: Pheochromocytomas, Immunohistochemistry, MEN syndrome

INTRODUCTION

Pheochromocytoma originates in chromaffin cells of adrenal medulla. Its incidence is similar in both sexes and most frequent between the ages of thirty and fifty (1). Multiple and bilateral pheochromocytomas constitute 5 to 10 per cent of all cases (2-4). Pheochromocytoma occurs sporadically or related to family syndromes such as the syndrome of multiple endocrine neoplasia - MEN IIA and IIB (5). The weight of pheochromocytoma is usually up to 100gr, although cases measuring 4kg are described in literature (6). Histological appearance of pheochromocytomas shows significant irregularities in shapes and dimensions of the cells and their patterns [6]. Malignant pheochromocytomas metastasize to regional lymph nodes, liver, lungs and bones, respectively (7). Tumour cells show immunopositivity to chromogranin A and NSE, whereas sustentacular cells are S-100 protein positive (8). Incidence frequency of S-100 protein positive sustentacular cells is high in pheochromocytomas related to family syndromes and low in sporadic pheochromocytomas (11).

MATERIAL AND METHODS

The aim of the study was to make histomorphological and immunohistochemical analysis of 20 pheochromocytomas. These cases represent the surgical material from the Centre of Endocrine Surgery, Institute of Endocrinology, Diabetes, and Metabolism Disorders, Clinical Centre of Serbia, Belgrade in the 2000 to 2002. period. Sixteen patients had unilateral pheochromocytoma, and 2 patients had bilateral pheochromocytomas, as the part of MEN IIA syndrome. Frozen and fixed sections, which were cut from paraffin-embedded material and stained by both hematoxylin-eosin and PAS, were used in order to make histopathological diagnoses. The expression of chromogranin A, NSE, S-100 protein and ACTH was investigated using the PAP method, with appropriate antibodies (DAKO) being applied.

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RESULTS

The patients were between 24 and 68 years of age, their average age being 44.4, and they were 11 females (61%) and 7 males (39%). The weight of the pheochromocytomas in question was from 6.2 to 163 grams, the average weight being 50.6 grams, and the diameter varied from 2.2cm up to 8cm (the average diameter was 5cm). On gross examination, the tumours proved to be well-defined, either by a fibrous capsule, or by adrenocortical tissue. The cross-sections of tumours were mainly of pale red-grayish colour, and showed numerous foci of necrosis, hemorrhage and cystic softening. Histological appearance of pheochromocytomas was with significant irregularities in shapes and dimensions of the cells and their patterns. The pheochromocytes were mostly of polygonal shape (in 17 cases, 85%), whereas in 3 cases (15%) fusiform cells were evident. The cells were arranged either in trabeculae intermingled with thin-walled sinusoids, or in small alveolae circumferenced by fibrovascular stroma. PAS positive hyaline globules were often present in the cell cytoplasm and also extracellularly. Cellular and nuclear pleomorphism was present in 14 cases (70%). Binuclear and multinuclear cells, as well as giant cells were evident in 10 (50%) pheochromocytomas. Mitotic figures were infrequent in 18 cases (90%). Seven patients (38%) had pheochromocytoma and the concomitant adrenocortical hyperplasia. All these pheochromocytomas were ACTH-immunonegative, so this association was incidental. All the studied pheochromocytomas showed diffuse and intense chromogranin A and NSE immunopositivity. Incidence frequency of S-100 protein positive sustentacular cells was high in pheochromocytomas related to family syndromes and low in malignant pheochromocytomas.

DISCUSSION

Numerous features of the described pheochromocytomas appear in the already published data, namely: the age of occurence, tumour weight, histological and immunohistochemical characteristics, frequent association of bilateral pheochromocytoma and MEN IIA (1,4,8,9,13). Immunohistochemical research has confirmed the importance of pan-neuroendocrine markers (chromogranin A, NSE) in pheochromocytoma diagnosing. High incidence frequency of S-100 protein positive sustentacular cells in pheochromocytomas related to family syndromes was also noted by other authors (11,14).

CONCLUSION

Histological appearance of pheochromocytomas reveals significant irregularities in shapes and dimensions of the cells and their patterns, with predominance of alveolae and trabeculae of cells. Cellular and nucelar pleomorphism, binuclear and multinuclear cells, as well as giant cells are evident in many pheochromocytomas. Mitotic figures are infrequent. Pan-neuroendocrine markers (chromogranin A, NSE, synaptophysin) may be useful in diagnosing of pheochromocytoma. Incidence frequency of S-100 protein positive sustentacular cells is high in pheochromocytomas related to family syndromes and low in malignant sporadic pheochromocytoma.

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Role of some immunohistochemical markers in the prognostic evaluation dysplasia grade in colorectal adenomas

KEYWORDS: Immunohistochemistry; Colorectal Adenoma; Dysplasia

INTRODUCTION

In colon carcinogenesis, a theory of the adenoma-carcinoma sequence is generally well accepted (1). Clinical and pathological parameters such as size, histological type of adenomas and grade of dysplasia are risk factors for malignant transformation from adenomas to adenocarcinomas (1). The mechanisms of dysplasia progression in adenomas are of special interest, because the prevention and early detection of carcinoma is of crucial importance for a patient. Classically, neoplasia has been considered a disturbance in the regulation of proliferation. In the search of prognostic discriminant, the expression of PCNA and Ki-67 as markers of cell proliferation and the expression of EMA have emerged as useful in determining the biological potential of the tumor (2,3). The nuclear antigen of cellular proliferation (PCNA) is a 36 kDa protein which correlates with DNA delta polymerase activity and is related to the proliferative state of the cell, because of its close association to the components of the cell cycle. PCNA is found in the cell nucleus mainly during the synthesis phase (S-phase) of the cell cycle (2). Ki-67,a large nuclear protein, possibly associated with the nucleolus and/or fibrilar component, probably plays an important role in the regulation of cell proliferation. Ki-67 antigen expression increases with the cell progression rising during the second half of the S-phase and reaching a peak in the G_2 and M-phase (3). Epithelial Membrane Antigen (EMA) represents a complex of high-molecular-weight cytokeratins isolated from the human milk fat globule (HMFG) membrane. The purpose of this study was to provide an immunohistochemical evaluation of the expression of PCNA, Ki-67 and EMA in colorectal adenomas with different grades of dysplasia, and to evaluate the prognostic significance of their expression and its correlation to clinicopathological findings.

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MATERIAL AND METHODS

Formalin-fixed and paraffin-embedded tissue blocks from 30 cases of colorectal adenomas (7 adenomas with mild dysplasia, 9 adenomas with moderate dysplasia and 14 adenomas with severe dysplasia) were used in this study. All selected slides of adenomas included the adjacent non-dysplastic mucosa as an internal control. The cases were selected from the files of the Department of Pathology, School of Medicine of Niš. The immunohistochemical studies were performed by using LSAB (labeled streptavidin-biotin) method. Paraffin sections (4 μm thick) were deparaffinized, rehydrated and incubated with monoclonal antibodies against PCNA, Ki-67 and EMA (Dako, Copenhagen). The immnunostaining findings were ranged as negative, positive and strongly positive.

RESULTS

Thirty colorectal adenomas were studied. The normal mucosa, included in all specimens, was constantly negative for these antigens. PCNA was positive in the nuclei in a granulated or diffuse pattern, but the nucleoli remained unstained. In contrast, Ki-67 antigen was mainly and strongly expressed in the nucleoli. EMA immunostaining was localized predominantly to the adenoma cell membrane, including the luminal aspect of the glands. Immunoreaction for PCNA was demonstrated in 63% adenomas. It was significantly higher in the adenomas with severe dysplasia (78%) than in adenomas with mild dysplasia (12%). Positive staining for PCNA correlated positively with increasing grade of dysplasia. Strong Ki-67 reactivity was seen in adenomas with severe dysplasia. Reaction for Ki-67 were strong in 83% adenomas with high grade atypia compared to 8% low grade adenomas. EMA positivity was present in 78% adenomas. EMA staining intensity correlated directly with increasing dysplasia.

DISCUSSION

Colorectal cancer represents the final stage of a slow, multistep process whose evaluation involves adenoma (1,4). An increase in epithelial proliferation and changes in proteins controlling the cycle has been described as possible mechanisms of colorectal oncogenesis (2,3). These practical considerations have led to the development of antibodies against proliferation-associated antigens as a means for a rapid and easy determination of the adenoma proliferation status, which may be routinely used with processed formalin-fixed paraffin-embedded sections. Among these antigens, PCNA and Ki-67 are well known. In this study, PCNA, Ki-67 and EMA immunoreactivity was positively correlated with dysplasia grade in colorectal adenomas. The strong reactions of these markers in adenomas with severe dysplasia show a close association with colorectal carcinoma (5,6). Our data suggest, that the difference in the expression of PCNA, Ki-67 and EMA in colorectal adenomas with diverse dysplasia grades could point at the tendency of the lesion progression.

CONCLUSION

The expression of PCNA, Ki-67 and EMA clearly shows differences between adenomas with different grades of dysplasia and it is especially useful as a prognostic factor in colorectal adenomas. Immunohistochemical analysis of a large spectrum of the markers in colorectal adenomas may be included as a part in routine pathological evaluation with conventional prognostic factors.

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Immunohistochemistry in detection of minimal residual disease in cases with gastrointestinal carcinoma

KEYWORDS: Minimal residual disese; Immunohistochemistry; Gastrointestinal neoplasms

INTRODUCTION

This concept emerged more than 15 years ago after a series of carefully controled clinical studies which revealed a group of patients at the high risk of cancer reccurrence after a curative surgery. Macroscopic tumor deposits (R2 category of TNM classification) or microscopic detectable tumoural residua (R1 category of TNM classification), as well as the finding of lymph node metastases (stage III of TNM classification) are considered to be the main prognostic factors in patients at high risk of the disease relapse and indication for adjuvant therapy. However, up to 40% of patients in stage I and II develop local reccurrences and/or metastases. It is thought to be due to the presence of a minimal residual disease at the time of surgery, which is not detected by conventional histological or imaging techniques. In those cases further and more detailed histological examination and the use of more sensitive and specific techniques such as immunohistochemistry and PCR very often reveal the cancer microinvolvement, including occult micrometastases. Detection of microscopic cancer residua means searching every nodular or suspect infiltration less than 2 mm in diameter in the whole peritumoral resection and peritoneal surface, a possible vascular infiltration, all radial unperitonealized as well as proximal and distal resection margins and especially detailed examination of the lymph nodes, which remains to be a gold standard in diagnostic and therapeutic approach. In practice, the oncopathological attention is focused on distinguishing the stage II from stage III, especially in pT3 pN0 cases of colorectal and oesophageal carcinoma and pT2 pN0 cases of gastric carcinoma, including detection of carcinomatous microinvolvement of the blood marrow and samples from local or systemic circulation. Many laboratories improve it by routine using of fat clearance techniques and immunohistochemistry combining it with multiple serial sectioning. As there is no consensus about sentinel lymph nodes in the gastrointestinal tract, in most cases searching of any microinfiltration is necessary for all regional lymph nodes. The optimal protocolar examination is strongly recommended with the minimum of 21 lymph nodes in gastric cancer cases and 12 lymph nodes in colorectal cancer cases, but it seems that highly significance is reached with more than 17 examined lymph nodes and more than 10-20 sections per lymph node. Microinvolvement is defined by the presence of cancer cells or proliferates within lymph node sinuses or pulp or other peritumoral tissues without stromal reaction and less than 2 mm in diameter. Microcarcinosis or the so-called occult microinfiltration is sometimes subdivided as follows: single cell (G_1) microinvolvement, cluster-type (G_2) microinvolvement with no more than 200 microns in diameter and largest, often histologically overt (G_3) micrometastasis (Kikuchi et al., 1999). The potential clinical use is under extensive investigation after highlighting the importance of adjuvant therapy in proven lymph node positive disease (NIH consensus conference, 1990), and since 1997 micrometastases are included as prognostic indicators in TNM stage disease classification as pN1mi category. In addition, micrometastases are considered to be a probable significant prognostic factor in high IIa category for colorectal cancer prognostification (College of American Pathologists Conference XXV, 2000).

MATERIALS AND METHODS

Materials comprised all isolated lymph nodes and other macroscopically uncertain nodulations in peritumoral fat larger than 1 mm of 66 curative resections with gastrointestinal carcinomas: 41 cases of colorectal carcinomas with primary pT3 pN0 status (19 lymph nodes examined in average), 24 cases of gastric and gastroesophageal junction carcinomas with primary pT2 pN0 status (27 lymph nodes examined in average) and 1 case of oesophageal carcinoma with primary pT3 pN0 status (17 lymph nodes examined). Fat clearance was performed in 31% of the cases and the number of slides varied between 6 and 20 per lymph node examined with succesive H&E and immunohistochemical stainings using monoclonal pancytokeratin and pandigestive cytokeratin 8/18 antibodies, excluding the use of anti-EMA and anti-cytokeratin in one case of gastric carcinoma and one case of colonic carcinoma, respectively. In 72% of the cases anti-CD31 and anti-vWF antibodies were simultaneously used as an aid in vascular infiltration examination.

RESULTS

Following previously mentioned criteria (Kikuchi et al, 1999) disseminated tumor cells were found in 19.5% of all cases: 2.4% of G_1 type, 9.8% of G_2 type, 7.3% of G_3 type and vascular infiltration in 22%. There were no micrometastases in cases with oesophageal carcinoma and in cases with gastric carcinoma they were found in 53.2% of lymph nodes: 4.2% of G_1 type, 33.3% of G_2 type, 16.7% of G_3 type and vascular infiltration in only 4.2%. The possible clinical implication and significant prognostic value carried at least 3 tumor cells per lymph node section in more than 10% of sampled lymph nodes per case (Fellbaum et al., 1997), so TNM recategorization with up-staging was done in 17.1% of cases with colorectal carcinoma and in 49% of cases with gastric carcinoma, mostly poorly differentiated carcinomas, concerning Lauren's classification with diffuse tumor type.

CONCLUSION

The relevance and probable clinical utility of detection of the minimal residual disease are not yet established and can be reached after the long term follow-up of great series of these patients, as the current standpoint implies the importance of effective adjuvant therapy in lymph node positive diseases. It seems that a detailed and careful searching for micrometastasis can ensure and even improve a reliable determination of tumor stage.

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Immunohistochemical statement of cyclin A, cyclin E, p16 and bcl-x in low grade and high grade non-Hodgkin's lymphoma

KEYWORDS: Non-Hodgkin lymphomas; Cyclin A; Immunohistochemistry

In order to investigate the possible role of cyclin A, cyclin E, p16 and bcl-x in the pathogenesis and progression of non-Hodgkin's lymphomas (NHL), we examined the statement of these cell cycle markers in follicular lymphomas (FCC), as representatives of low grade NHL, and in diffuse large B cell lymphoma (DLBCL), the subtype of high grade NHL. Immunohistochemistry was performed on sections of the paraffin embedded lymph node tissue from 20 patients with NHL (10FCC and 10DLBCL) and 5 reactive lymph nodes. Immunoreactivity was semiquantitatively analyzed (low statement <25% of positive cells, moderate 25-50%, high >50% of positive cells). Cyclin A, which controls the S phase of cell cycle, was moderately expressed in the majority of DLBCL (8/10), except 2/10 DLBCL which displayed a low statement. The statement of cyclin A was not seen in any FCC case. A product of the tumor suppressor gene, p16 (agonist in controlling late G1 cell cycle phase) was weakly positive in 7/10 DLBCL and it was absent in 3/10 DLBCL. A high p16 statement was observed in 8/10 FCC and a moderate one in 2/10 FCC. The statement of cyclin E (regulator of G1-S transition) was absent both in DLBCL and FCC. A high statement of bcl-x (which inhibits apoptosis) was found in all FCC, but bcl-x was absent in DLBCL. A loss of the p16 statement associated with a moderate statement of cyclin A in DLBCL and quite opposite results in the group of FCC may suggest the significance of these cell cycle regulators in the progression of NHL.

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Overvivew of recent and emerging advances in information technology planned for implementation at the Institute of Pathology and Forensic medicine of Military Medical Academy

KEYWORDS: Information technology; Medical Imaging; Internet technologies

INTRODACTION

Computer systems, especially in hospital practice, have been for many years the preserve of enthusiasts, probably because the computers were technically challenging, moderately unreliable and often only accessible with pernickety, typed, syntax. Icon-based interfaces, although common on personal computers in the home, have been slow to find there way into the laboratory information systems as they did not fit the mainframe server-client architecture that these computers frequently use. Even where they have been introduced, they use technology that is at least 5 years and frequently 10-years older than the currently available "state of the art" systems. With the introduction of Internet-based technologies, such as those used by the WWW, this is about to change. These have provided the technology that can be applied and integrated with relative ease to existing so-called 'legacy' systems. Pathology is one of the most computer intensive areas of medicine and as a result diagnostic pathologists in histopathology have often been at the cutting edge of computer literacy. The majority of laboratories use laboratory information systems to issue and store pathology reports. Many of these systems provide the diagnostician with the ability to retrieve reports and cases using coding systems such as SNOMED and ICD, but more advanced computer facilities that might assist the pathologist in the diagnosis or interpretation of a case are often lacking. In recent years advances in computer technology have begun to have a much wider impact on the practice of medicine and newer technologies are beginning to find their way into the reporting room. In this overview, we will cover some of the recent and emerging advances in IT that have the potential to revolutionize the practice of diagnostic histopathology in near future.

INTERNET TECHNOLOGIES have developed as an open architecture over the last 5 years by thousands of developers across the world. The user-base is large (currently about 500 million) and consequently new developments remain compatible with existing technologies. In addition, numerous bulletin boards and user-groups provide help on practical issues associated with the technologies. Internet technology is a based upon client-server architecture. The client, the web browser, is available for most computer types and provides common functionality and 'look and feel' to the interface, regardless of the computer architecture. This has important implications for cost-effective staff training. Content providers can run server software to deliver information without worrying about the make or specification of the client machine. Powerful client and server software is available free of charge and is often included with the purchase of many computer systems. Web technologies require only limited IT skills to implement and maintain, as such it should be possible to create a number of local experts distributed throughout the region who will be able to provide local in-house advice. Web technologies easily incorporate multimedia (images, sound video, virtual reality) and are therefore ideal for an image intensive specialty such as histopathology. Fundamental to Internet-technologies is the ability to link resources together on disparate servers using hypertext. This minimizes duplication of data and resources, allows each resource to be maintained and updated by those responsible for them and reduces the need for central data storage. Apart from the obvious cost effectiveness of purchasing industry standard equipment, the global acceptance of the technology facilitates collaboration across the public and private sectors. Internet technologies can support sophisticated security systems allowing data encryption, authentication and confidentiality.

DIGITAL MEDICAL IMAGING

The past 20 years have seen tremendous changes in medical imaging techniques. New modalities and protocols are expanding the available digital image data at a rapid rate. Medicine is increasingly image-intensive. The central importance of imaging technologies such as computerized tomography and magnetic resonance imaging in clinical decision making, combined with the trend to store many "traditional" clinical images such as conventional radiographs, microscopic pathology and dermatology images in digital format present both challenges and an opportunities for the designers of clinical information systems. Digital medical imaging is beginning important for medical informatics, computerized learning, and especially for the growing field of telemedicine. At MMA, Belgrade, we are developing and implementing an Picture Archival and Communicating System (PACS) integrated with Pathology Information System (PIS) and Hospital Information System (HIS). Two immediate issues conforming the building of medical image database systems are: lack of supporting infrastructure and inability to index images by contest. To circumvent these problems, the evolutionary medical image database system being implemented at PIS is based on a three-tiered client-server architecture: client medical workstations, database application servers, and a hospital-integrated picture archiving and communication system. PACS - viewer, console and report are a World Wide Web (WWW) clients for the Image Engine. PACS clients use advanced DHTML features such as frames, forms, tables and inline DICOM image display to provide an easy to use system for retrieving and viewing diagnostic images and reports generated by clinical procedures such as surgical pathology, radiology and gastrointestinal endoscopy. PACS client implements a number of WWW client-side features, such as DHTML forms data entry verification and makes extensive use of the ASP programming language. The PACS system uses a number of approaches for ensuring the confidentiality and security of patient data transmitted over the Internet.

DECISION SUPPORT SYSTEM

Diagnostic pathology is well placed to benefit from advances in database

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technologies that have taken place due to the rapid increase in biomedical knowledge. This has led to the need to develop new tools to allow close integration of separate information resources, PubMed, On-Line Mendelian Inheritance in Man (OMIM), Human Genome Mutation Database (HGMD), Cancer Genome Anatomy Project (CGAP), Online image atlases, Decision support systems, etc. A decision support system is the term used to describe any number of a range of resources that provide the user with assistance reaching a conclusion. There is no need for it to be electronic, books, paper guidelines and protocols are all forms of decision support. Expert systems are one specific subgroup of decision support environments that use computer programs and algorithms to analyze and interpret information in an intelligent way often based upon a collection of rules programmed into the computer (DXplain, PAPNET, PATHFINDER AND NORTESS). The four diagnostic support systems notificated all aim to help in reaching a diagnosis and yet none have been incorporated in widespread routine practice. In considering where the impact of information technology in the future it is perhaps useful to assess why this is so. Perhaps the most obvious answer is that for the majority of pathologists most diagnoses are straight forward and can be reached without resorting to a computer-based support system.

DISCUSSION

The benefit of IT for most pathologists will come from enhancements to the reporting process, improving efficiency and accuracy, ensuring that there is good automatic coding of the diagnosis, and that if there is new information available on a clinical condition that the pathologist or clinician should be alerted to it as they make the diagnosis. Computer-based speech recognition, either to enter the text of a report or for supporting up prewritten reports has the potential greatly to enhance practice, but at present all the systems require prolonged training for both users and computers and none of the programs are as accurate as a good secretary. It could be that the place for specialist decision support systems such as Pathfinder is in the analysis of complex cases. However, it is clear even from the published assessment of Pathfinder that as new diagnostic methods are introduced we will move even further away from simple recognition of morphological patterns, which are inevitably subjective, although have been the gold-standard for several centuries. Until we reach the stage of purely molecular diagnosis, seeking expert opinion through telepathology systems is likely to be of more value, than lengthy automated decision support.

CONCLUSION

Information technology will have a profound impact on all our lives over the next five to 10 years and this new technology has the potential to transform the way we practice diagnostic pathology. It will require vision and leadership from within the profession to ensure that all pathologists can benefit from these advances. Training programs in pathology should take into account the need for expertise in informatics and develop fellowships in this area to adequately prepare junior faculty members for their future professional role.

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Karyometric analysis of fine needle thyroid aspirates

KEYWORDS: Thyroid gland; Fine needle aspiration; Karyometry

INTRODUCTION

Thyroid nodules are a common occurrence, the reported incidence being 4 per 100 population. The majority of these (roughly 90%) are benign (1). Without the use of fine needle biopsy and histopathology the distinction between benign and malignant lesions in not possible either clinically, on radionuclide imaging, or on ultrasonography. However, diagnostic dilemmas are encountered because of overlapping cytologic features between neoplastic and non-neoplastic diseases, coexistence of non-neoplastic and neoplastic disease processes, multiple malignancies in the same gland, poor cellularity of the aspirated sample, degenerative changes masking and mimicking malignancy, and inexperience (2). In the last decades, objective morphometric methods are widely used in diagnostic pathology (2). The aim of this study was to estimate karyiometric parameters of epithelial cell nuclei in fine needle aspirates of the thyroid gland.

MATERIAL AND METHODS

At the Institute of Pathology, University of Niš, from January 2000 to December 2001, karyometric analysis of thyroid epithelial cells from patients with colloid goiter (n=7) and patients with thyroid carcinoma (n=10) was done. Smears were air dried and stained by routine hematoxyline-eosin method. For planimetric image analysis, the software package MicroImage 3.0 (Olympus, Tokyo, Japan) was used. Under the objective 40x of Swift microscope (Swift Instruments, Tokyo, Japan) (N.A.=0.60), the binary images were manually edited, and nuclear area, mean density, major and minor axis, diameter, and perimeter were estimated. For stereological estimation of the mean volume-weighted nuclear volume, original test grid was placed in objective 10x of NU-1 microscope (Carl Zeiss, Jena, Germany), and objective 100x with oil immersion was used. The cell nuclei were sampled by intercept method, with equation:

$$V_v = l_0^3 \text{-x } \pi/3 \quad (1)$$

were l_0 is intercept length and π is 3.14...(3).

Statistical significance of obtained differences was analyzed by two-tailed Student's t-test and MANOVA.

RESULTS

The mean nuclear area was significantly greater in malignant cells (84,36±12,25 μm^2) than in benign cases (37.84±9,25 μm^2)($p<0,001$). Similarly, major and minor axis, diameter, and perimeter were significantly greater and the mean volume-weighted nuclear volume was significantly higher in thyroid carcinoma patients (589.54±79.8 μm^3) compared with colloid goiter (347.59±96.47 μm^3)($p<0.05$).

DISCUSSION

Diagnostic dilemmas in thyroid gland fine needle aspiration cytology require additional, objective and quantitative parameters. In this study two different karyometric methods were used, with very similar results. By image analysis method many karyometric variables can be obtained, but same of them are highly correlated, e.g. area, major and minor axis, diameter, and perimeter. In a previous study of Mattfeld et al. (1987), the differences between benign and malignant cells were not statistically significant. On the other hand, DNA image cytometry may be helpful in the specific identification of neoplastic follicular cells (5). Our results are in accordance with findings from Eldar et al. (1999)(6), and Tseleni-Balafouta et al. (2000).

CONCLUSION

Estimation of nuclear size can be used in routine cytopathological practice.

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Region-based segmentation as the next step in medical image analysis

KEYWORDS: Pathology; Image analysis; Artificial intelligence

The CAMIA (Computer-Aided Medical Images Analyzer) is specialized software tool for morphometry and quantitative analysis in various medical, research and diagnostic, applications ranging from pathology microscopic imaging to neuro MRI. CAMIA utilizes novel image segmentation method based on simplified mathematical model of the human visual system, combining the classical image processing techniques and advanced artificial neural network methods. This way, CAMIA improves analysis consistency, objectivity and reproducibility, overcoming main weaknesses of most widely used analytic techniques: It offers easy and efficient but high-precision way of definition of ROIs (Regions of Interest) minimizing a need for tedious mouse-virtuosity needed by manual segmentation tools, It overcomes the subjectivity and noise-sensitivity of classical pixel-based thresholding technique, It insures a high reproducibility based on retina-like edge-detection model. CAMIA segmentation is superb in ROIs edge preservation than any classical region-growing algorithm. Additionally, it is more robust on hue and contrast variations as result of inconsistent lighting and contrast/coloring over single image or set of images. Principles of classic image processing are based on unnatural rectangular pixel geometry. Our novel segmentation algorithm, utilizing a artificial intelligence, extends pixel-based machine vision to next level of region-based image interpretation. That way, combining classical and AI-regionalized image interpretation, CAMIA offers easy-to-use methods and procedures for segmenting and selecting regions of interest (ROIs). Simple user interface still allows powerful control over complete process. Output results are more accurate and subjectively better accepted using less effort compared to manual approach. Semi-automatic procedure, on the other hand, allows more flexibility and user control than fully automatic tools making CAMIA more creative and suitable for wide range of research applications. High reproducibility of results is insured using robust image segmentation methods with full user control over the critical parameters. Thus, method sensitivity on subjective parameter variations is suppressed. Finally, manual retouching of segmented regions allows fine-tuning and near-perfect segmentation. Following this methodology, image is segmented semi-automatically through little iteration varying algorithm parameters. When satisfying result is achieved, minor manual corrections could be performed resulting in subjectively acceptable results with minimal effort. In order to support image segmentation and analysis, included are pre-processing filters and algorithms (color correction and transformation, noise removal, image enhancement, etc.). Also, basic ROIs statistics and output calculations are derived through number of application modules (i.e. volume estimation). By exporting that data, more complex mathematical analysis and calculations could be performed externally using specialized software like MS Excel, Matlab, SPSS or so.

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Computer-assisted image analysis for quantification of central adrenergic immunoreactivity in a rabbit model

KEYWORDS: Subarachnoid hemorrhage; Image analysis; Densitometry

Cerebral circulation is mostly controlled by autoregulation mechanisms. Some experimental evidence suggests the possibility of sympathetic involvement in the pathogenesis of cerebral vasospasm after subarachnoid hemorrhage. We intended to explore an influence of central sympathetic system in a physiological resting condition and in circumstances of post-subarachnoid hemorrhage (SAH). To examine the level of functional response in central adrenergic neurons a new technique was introduced by means of optical density (OD) and transmission (T) measuring. Brain samples were fixed in 10% neutral buffered formalin, than processed for paraffin sectioning. 5μm thick tissue sections were stained with the anti-dopamine β-hydroxylase by ICH using ABC staining method. Tissue sections were viewed using brightfield illumination on a Nikon microscope. Images were captured through consecutive red, green and blue separation filters on a digital camera. The color subtraction sequence removed background colors by replacing them with white. A "calibration" command allowed to transform pixel values directly from a scale which was linear with respect to T and into a scale correlated to OD. The average cytoplasmatic density for the control group compared to the group with SAH showed significant differences. On the basis of statistical analysis (variances, mean values, F-testing, t-test), the highest degree of optical density was discovered in the SAH group. A computer-assisted densitometric method has been found to be relatively easier and very accurate method for evaluating the level of functional response in cells labeled by IHC method.