TISSUE CYTOMETRY AS A DIAGNOSTIC TOOL IN TUMOR DETECTION

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Summary. Tissue cytometry allows histopathological analysis of tumors originating from various tissues. TissueFaxs is a diagnostic microscope-based cell analysis system for accurate quantitative evaluation of cells in tissue sections and smears. The system consists of inverted microscope with fluorescence module and TissueFaxs software. It is used for brightfield and fluorescence mode analysis of slides. For a differentiated analysis, Regions of Interest (ROI) can be drawn on the slide. The images are of high quality and resolution.

The purpose of study was to detect gene expression and compare histopathological changes in the sample sections from patients with squamous cell carcinoma of the larynx and healthy patients using TissueFaxs and HistoQuest software.

The slides obtained from sections of squamous cell carcinoma of the larynx were evaluated. The sections were topographically and immunohistochemically stained to help identify individual cell components as well as morphological structure. The slides were photographed using software TissueFaxs and quantitatively analysed by HistoQuest.

Immunohistochemical staining of the slides by TissueFaxs allowed for the identification of cellular gene expression and detection of potential pathologies.

Immunohistochemical staining with anti-XAF1 antibody allows to observe gene expression in the cell. HistoQuest software provides images of the sampled structure through the segmentation of morphological elements. The results are shown as a univariate histograms based on the mean color intensity.

Key words: immunohistochemistry, *XAF1*, laryngeal carcinoma, TissueFaxs, HistoQuest

INTRODUCTION

TOPOGRAPHIC STAINING

Microscopic examination is a valuable diagnostic tool as it provides a lot of histopathological information about disease. Pathomorphological investigation can pinpoint neoplastic cells. Various staining modalities are used to better visualize cellular organelles under microscope. Hematoxylin and eosin stain (H+E stain) is the gold standard stain in histology. It helps differentiate the nucleus and cytoplasm. Hematoxylin is a basic dye. It colors the cell nucleus blue as a result of reaction with negatively charged anions. Eosin is an acidic dye which counterstains the cytoplasm red as a result of reaction with positively charged cations [Smith, 2006].

IMMUNOHISTOCHEMICAL STAINING

Immunohistochemical reaction (IHC) is another basic diagnostic tool. The method involves indirect immunoenzymatic specific antigen-antibody reaction (Ag-Ab) and is used to detect an antigen by specific antibodies that have been either dye or enzyme labeled. The antibody is antigen-

specific and binds with antigen determinant, which is a fragment of the antigen. To detect antigens IHC technique requires proper tissue fixation in paraformaldehyde. Later, paraffin embedded sections are sliced at a range of 3-4μm. The slices are mounted on macroscopic slides and incubated with antibodies. IHC reaction is also accomplished using tissue microarray technique (TMA). The method allows to detect a greater number of antigens in the examined sample compared to microscopic evaluation. Immunohistochemistry is a semi-quantitative method as it does not allow to measure precise quantity of a protein. However, the technique is used to identify tumor type and grading and is widely used in clinical practice, especially in oncological diagnoses, as it facilitates the choice of treatment [True, 2008].

Immunohistochemical staining of E-cadherin and vimentin was successfully used to evaluate the expression of proteins in prostate carcinoma and HistoQuest software evaluated the percentage of stained cells [Puhr et al., 2012].

IHC method was also applied in research into tumor growth suppression by enhanced CDK6 activity. Immunohistochemically-based analysis of proliferation marker Ki-67 was used to detect changes in CDK6 activity in B lymphocytes [Kollmann et al., 2013].

Moreover, IHC technique was used to determine protein immunoreactivity in the cells of lymphoma. The evaluation was based on the percentage of cell staining intensity [Belaund-Rotureau et al., 2002].

Preparation of the sample is critical to avoid protein denaturation. This requires proper tissue fixation in formaldehyde, washing it in ethanol and xylene and embedding the sections in paraffin. Staining techniques are an important stage in histopathology as they allow for early detection of morphological changes and facilitate further examination [Puhr et al., 2012].

In the case of grade III and IV gastric tumor a considerably lower expression of XAF1 was observed in comparison with the early stage of cancer [Ling et al., 2013].

ADVANTAGES OF AN AUTOMATED SYSTEM

Tissue cytometry involves microscopic analysis of tissue cells. It allows to analyze intact or damaged cells on a microscopic slide. The analysis results of large tissue areas are subjected to statistical analysis. TissueFaxs system is an interesting research tool as it provides quantitative valuable information to diagnose progressive diseases. It is widely used to detect antigens in immunohistochemically stained (IHC) tissue slices [Tàrnok, 2006; http://www. tissuegnostics.com/EN/Systems/tissuefaxs.php 2013].

To detect macromolecules under microscope the stains called fluorochromes are used. The method uses either the affinity of negatively charged anionic groups present in macromolecules to positively charged alkaline fluorescent dyes or reverse relationship between positively charged cationic groups and negatively charged acidic dyes. This allows to locate and distinguish individual organelles in cells or tissues [Lakowicz, 1999].

In addition, IHC is used to describe and locate cellular organelles, which significantly facilitates structural analysis and histopathological diagnosis [Alberts et al., 2005].

TissueFaxs system is often considered the equivalent to flow cytometry in tissue analysis. This system consists of an inverted microscope with fluorescence module, producing high-quality images of individual tissues on a microscopic slide. TissueFaxs is successfully used to detect and determine the location of various cells in the analyzed object. It is also possible to draw on the slide and differentiate a specific region called region of interest (ROI) or a single cell [http://www.tissuegnostics.com/EN/Systems/tissuefaxs.php 2013].

TissueFaxs is a combination of high-quality hardware module and two software modules which allow image acquisition, data management and analysis of light and immunofluorescence stainings. It provides a smooth, automated workflow from image acquisition to publication quality, high resolution of output of graphs and images, as well as customizable data export for further processing. TissueGnostics system is a convenient tool because it allows to track microscope images on a computer's monitor. One of important features of this system is inverted microscope where lenses are arranged upside down, which provides good contact with the analyzed slide and automatic identification of cells. Standard lighting is by 12V 100W halogen lamp [http://tissuegnostics.com/EN/Systems/tissuefaxs.php 2013].

At the beginning, the automated system rapidly previews a microscopic slide, detects ROIs or whole tissue and determines its location using adequate magnification. The system is equipped with Autofocus, Acquisition and Stitching module. Autofocus makes easy adjustment to focus on the viewing object. Acquisition module captures the

object. The microscope is equipped with fluorescent filters that facilitate observation of the fluorochrome-labeled material. Stitching module allows high-quality matching of cells which were damaged. Captured images of tissue slices or cytological biopsy material are exported to the HistoQuest for further analysis [http://tissuegnostics.com/EN/Systems/tissuefaxs.php 2013].

HistoQuest software uses patented algorithms to detect the intensity of antigens in the cell nucleus, membrane and cytoplasm. The greatest advantage of this program is accurate cell counting. The program records and automatically detects tissue which is subjected to Color Separation module to differentiate between cells. The camera provides an image segmentation through the use of algorithms to separate overlapping morphological elements. This program can also measure parameters of a cell such as surface area (in μm^2), minimum and maximum intensity, circumference, diameter, length and width (in μm) [http://tissuegnostics.com/EN/Systems/tissuefaxs.php 2013].

The results are presented in the form of a two-dimensional diagram showing scattered cells in relation to two parameters and histogram representing the intensity of color. Another advantage of the system is the function that allows to locate a selected point on the graph which corresponds to a particular cell in the image [http://tissuegnostics.com/EN/Systems/tissuefaxs.php 2013].

MATERIALS AND METHODS

The subject of study were 12 microscopic slides prepared from squamous cells of the larynx: 6 samples were taken from patients with squamous cell carcinoma and 6 from healthy controls. In each group 3 preparations were H+E stained and the other 3 were histochemically labeled. The slides were photographed using TissueFaxs software and quantitatively analysed by HistoQuest. The investigation design complied with the protocol of The Bioethical Committee and was approved by the head of the clinic; each of the participants signed informed consent.

MICROSCOPIC PREPARATIONS (H + E STAINING)

A piece of tissue sample was fixed in 10% formaldehyde and embedded in paraffin. Next, the preparations were dehydrated in 99.8% ethanol and xylene wash. Then, the material was embedded in paraffin with xylene and sliced. The sections where placed onto glass slides and put in xylene to dewax, them. The preparations were stained with hematoxylin and eosin. Finally, they were dehydrated in ethanol and xylene and closed by cover glass. Six microscopic preparations of laryngeal cancer were H+E stained.

IMMUNOHISTOCHEMISTRY

Microscopic slides were immunohistochemically labeled using antibody XAF1.

Immunohistochemical examination was performed on formalin-fixed, paraffin-embedded sections. After deparaffinization and antigen unmasking, the sections were incubated with 3% H_20_2 (for 5min) to inhibit endogenous activity of peroxidase. They were incubated with primary antibodies against XAF1 (1:200, Abcam, UK) for 24 hours at 4°C. The sections were washed with PBS and incubated for 5 min with DAB at room temperature.

TISSUEFAXS AND HISTOQUEST ANALYSIS

Zeiss microscope Axio Observer.Z1 with TissueFaxs system was used to analyze the microscopic slides made from sections of squamous cell carcinoma of the larynx. The pictures were taken by a Zeiss microscope equipped with a Pixelink camera and TissueFaxs software at the magnification of 5 and 20 times. The quantitative analysis was performed by HistoQuest software (TissueGnostics).

RESULTS

In the sections that were immunohistochemically labeled, the analysis found lower expression in the cancer cells in comparison to the normal ones. Figure 1 shows XAF1 protein in laryngeal carcinoma.

In the case of immunohistochemical labeling by XAF1 antibody the expression was downregulated in cancer cells. HistoQuest produced quantitative analysis and presentation of results in the form of graphs. Two-dimensional diagram presents dispersed cells and the staining intensity (Fig. 2) and one-dimensional histogram shows the intensity of color (Fig. 3).

Weaker expression of XAF1 in squamous cell carcinoma of the larynx was observed, which indicated lower expression of genes in cancer cells. TissueFaxs system is a useful tool in the evaluation of gene expression, since it allows identifying

changes in the morphology of the analyzed tissues and provides information about their pathology. The use of monoclonal antibodies as markers in immunohistochemistry allows for accurate detection of antigens in histological material, therefore and it facilitates the diagnosis of cancer.

DISCUSSION

In histopathological technique the prepared slides are colorless due to low refractive index. Therefore, tissue staining is a valuable stage as it produces better contrast and allows much better observation of the various cell organelles [Misdorp et al., 1999]. Topographic H+E staining allows to determine the type and grade of cancer by analyzing the intensity of proliferation and level of differentiation of individual cancer cells [Misdorp et al., 1999].

Due to monoclonal antibodies that are used for analysis it is possible to precisely detect abnormalities. There are reports on early detection of myocardial infarction by IHC method [Ribeiro-Silva et al., 2002].

The immunohistochemical method allows to evaluate progress of cancer, e.g. squamous cell carcinoma of the larynx. Nijkamp reported that low expression of AKT (protein kinase B) indicated metastases to the lymph nodes. Tumors with lymph node metastases had approximately 10 times lower AKT activity than tumors without lymph node metastases [Nijkamp et al., 2013]. A diagnostic advantage of IHC is its ability to detect erbB protein, which is a prognostic factor for colorectal cancer. Osako observed that in the case of colon cancer, the expression of proteins was present in the cytoplasm [Osako et al., 1998].

IHC reaction can be evaluated in terms of the frequency and intensity of color. The staining intensity is analyzed on a scale of 0 to 3, where 0 represents no staining, 1 - weak staining, 2 - moderate staining, and 3 - is a strong staining [Ling et al., 2013].

In the case of prostate cancer, some scientists found the relationship between the index of proliferation (IP) and the process of carcinogenesis and histopathological grading. It was observed that mean IP increased with the degree of malignancy according to Gleason's scale [Kubben et al., 1994].

Analysis of the microscopic slides of squamous cell carcinoma of the larynx showed altered height of the epithelium compared to the healthy tissue. Haffner observed similarly altered endothelial height, however it was in the case of prostate cancer. He found correlation between high expression of cyclooxygenase (COX2) and squamous PSMA antigen present in the oral cavity [Haffner et al., 2012].

Mountzios used IGF1R-alpha and IGF1R-beta markers in immunohistochemical method. IGF1R-alpha stained the cell membranes whereas IGF1R-beta stained the cytoplasm. He observed that high levels of IGF1R-alpha expression occurred in the case of III or IV grade of tumor. In immunohistochemical analysis IGF1R is a prognostic biomarker of laryngeal cancer [Mountzois et al., 2013].

Miranda et al. observed high expression 3-Galectin in cytoplasm, which allowed to evaluate aggressiveness of laryngeal carcinoma. 3-Galectin was employed as biological marker to estimate invasive laryngeal carcinoma but had no diagnostic value in case of metastasis [Miranda et al., 2009].

Another example of immunohistochemistry applied into research is the invesigation by Du et al., in which specific antibodies were used to detect increase of expression of tumor suppressor proteins p53 and p65 in the nuclei of laryngeal cancer cells [Du et al., 2003].

Another IHC study, which used mouse anti-TGM2 monoclonal antibodies, demonstrated positive expression of TGM2, both in the nucleus and in the cytoplasm of squamous cell carcinoma of the larynx. Dardik et al. pointed out that the expression of TGM2 in the endothelium of blood vessels contributed to the metastasis and proliferation of cancer cells [Jin et al., 2012; Dadrik and Inbal, 2006].

It has to be emphasized that the quality of prepared slide is very important because it is used for further analysis by TissueGnostics system. Another important aspect is to take high quality pictures of histopathological specimens, with optimal lighting and focus. Then it is possible to calculate precisely the number of cells with expression in the nuclei. The nuclei that have positive expression of receptors or antigens are brown stained, while the nuclei with no expression stain blue (by hematoxylin). A common problem is the overlap of morphological elements which hinders the ability to count cells. However, HistoQuest analysis allows for the separation with algorithm dividing nuclei [Khan et al., 2011].

Lv used HistoQuest to study apoptosis in cancer cells in ROIs. He found that the chimeric human-murine antibody (Adimad) inhibited the proliferation of tumor cells by inducing apoptosis in colon cancer cells [Lv et al., 2011].

It is worth noting that HistoQuest system is used in many studies, since it allows to locate the

T-cell proliferation in the lymphoid organs. To ensure easier location of used antibodies, they are conjugated with fluorescent stains [Florian et al., 2010].

CONCLUSIONS

We investigated microscope slides of normal laryngeal tissue and squamous cell carcinoma of the larynx by TissueFax system. Weaker expression of XAF1 in squamous cell carcinoma of the larynx was observed which indicated lower expression of genes in cancer cells. TissueFax system is a useful tool in the evaluation of gene expression, since it allows identifying changes in the morphology of the analyzed tissues providing information about their pathology. The use of monoclonal antibodies as immunohistochemical markers allows accurate detection of antigens in histological material, therefore, facilitating diagnosis of cancer.

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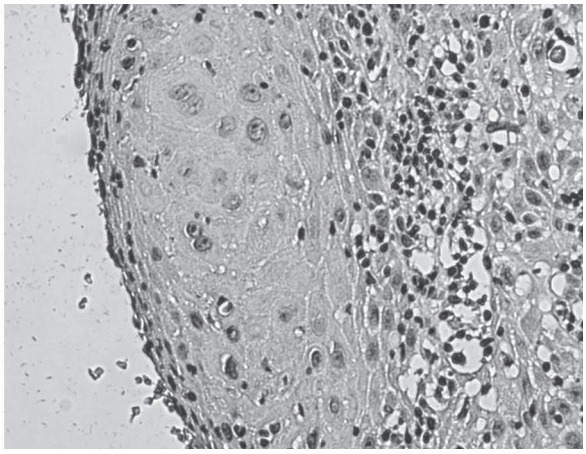

Figure 1. The XAF1 protein in laryngeal carcinoma cells

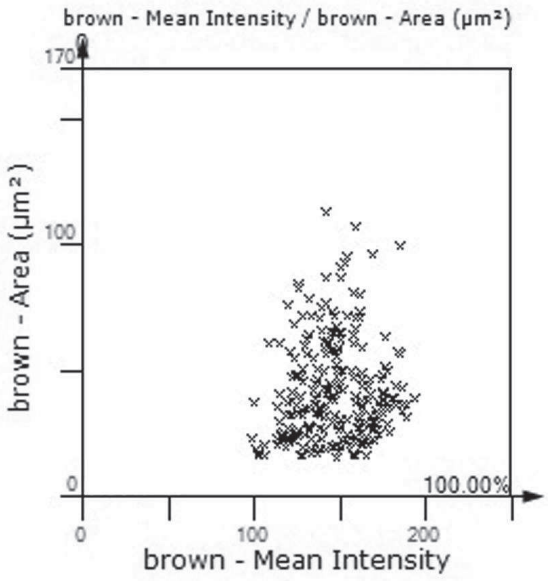

Figure 2. Two-dimensional diagram presenting stained mean intensity.

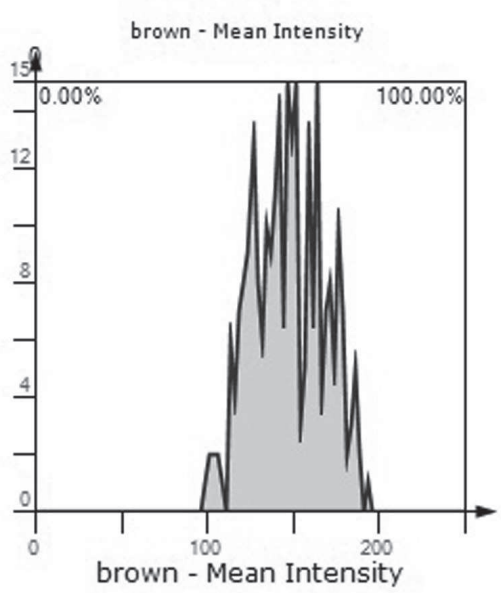

Figure 3. Histogram - one-dimensional diagram showing the intensity of color.

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