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Pregledni članak
Review paper

SIGNIFICANCE OF THE »TISSUE MICROARRAY« TECHNIQUE IN DIAGNOSIS AND PROGNOSIS OF B NON HODGKIN'S LYMPHOMAS

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Summary: The novel technology of tissue microarray (TMA) allows rapid and cost-effective analysis of hundreds of markers on the same set of specimens. Limited amounts of tissue that could be analyzed and the problem of tissue heterogeneity are the major drawbacks of the TMA technique for immunohistochemical characterization of lymphomas. These problems do not outweigh the enormous advantages of TMA, namely the cost- and time-saving, and the mostly homogeneous results of immunohistochemistry. In Non Hodgkin's lymphomas (NHL), TMA detection of Oct1 and Oct2 immunoglobulin transcription factors and their coactivator BOB1 showed particularly useful in distinguishing T-cell-rich B-cell lymphomas from classical Hodgkin's disease, nodular lymphocyte predominant Hodgkin's lymphoma and plasmablastic lymphomas. Outcome prediction for subtypes of diffuse large B-cell lymphomas, using a TMA panel with CD10, Bcl-6 and MUM1, was comparable to results of cDNA microarray analysis. Detection of p53 tumor suppressor gene and Ki-67 proliferation antigen is important for the prognosis of many NHL sybtypes. In spite of their heterogeneity in expression, TMA showed 90 and 92% concordance rate, with conventional tissue sections for p53 and Ki-67 respectively, and that could be sufficient for routine practice. There is no doubt that the widespread use of TMAs will become an integral part of daily practice in research and routine clinical laboratories.

Key words: tissue microarray, proteomics, B-cell lymphoma

Introduction

Cost-effective parallel analyses of large numbers of samples are valuable in many scientific disciplines. Whereas high-throughput methods exist for parallel analyses of thousands of independent liquid samples, technologies for parallel analyses of solid or solidified samples have lagged behind. The novel technology of tissue microarray (TMA) preparation for high--throughput profiling of tumor specimens was originally described in 1998 by Kononen et al (1). These authors detailed the preparation of paraffin blocks containing up to 1000 cylindrical, 2 mm-diameter core biopsies from archived paraffin blocks of various tumors and normal tissue specimens. The tissue microarray block can be sectioned for morphologic review as well as for standard immunohistochemistry, 1- or 2-color fluorescence *in situ* hybridization, or mRNA *in situ* hybridization on consecutive sections. This novel methodology allows for rapid analysis of hundreds of markers on the same set of specimens: up to 200 sections can be cut from each block.

TMA construction

The construction of a successful TMA starts with the careful selection of donor tissues and precise recording of their localization details (1). The slides have to be reviewed so that suitable donor blocks can be selected and the region of interest defined on a selected paraffin wax block. All those aspects of tumor classification, staging, and grading that cannot be extracted from the evaluation of an extremely small tumor sample have to be re-evaluated. Nevertheless, there are clear differences in the distribution of the workup time required for individual steps in the conventional slide by slide and the TMA approach.

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With the use of TMAs, most of the work time is now focused on the preparation of TMA, in contrast to the conventional approach where a large proportion of time and materials is spent on sectioning and labeling. Some authors prefer the use of proprietory techniques and instruments for TMA production, but one can find many commercial devices created for this purpose. Many authors argue that those rather simple, mechanical tools have a quite remarkable price.

Costs and quality control

Several technical issues apparently compensate for some loss of information due to the small tissue size. The staining of a single TMA slide provides a much greater degree of consistency and standardization than the immunostaining of hundreds of individual slides and reduces the amount of antibodies. This significantly reduces high variability of intralaboratory and interlaboratory results, mainly because of interlaboratory differences in antigen retrieval, staining protocols, antibodies used, and in the interpretation of staining results (2). Furthermore, quantitation of immunostainings is markedly easier on arrayed samples than on large sections. Also, this facilitates a reproducible application of the selected scoring criteria because the entire tissue is always used for interpretation, and the subjective selection of one tumor area for decision making is avoided. In the future, the TMA technology may help to optimize and standardize the interpretation of immunostainings, which is currently subjective and poorly reproducible and often leads to major discrepancies in studies investigating clinical associations for novel biomarkers (2).

Advantages and drawbacks of punches with varying diameter

A potential caveat in TMA technology is the limited amount of analysed tissue. The TMA approach has been criticized for its use of small punches of usually only 0.6 mm diameter from tumors with an original size of up to several centimeters in diameter, comprising areas of increased proliferation, apoptosis, matrix remodeling, necrosis, etc. The problem of tissue heterogeneity is maybe the most pronounced in lymphomas. Tumor cells, e.g. in Hodgkin's lymphoma or T-cell-rich B-cell lymphoma, are outnumbered by non-neoplastic background infiltrates and may only be present in very low numbers in TMA punch biopsies. In addition, lymphoma growth may follow lymphoid structures, such as follicles in Follicular lymphoma (FL) or mantle zone infiltration in Mantle cell lymphoma (MCL), and thus TMA punch biopsies, in contrast to many solid tumors, may not contain relevant tumor areas. Furthermore, prognostic markers such as Ki67 or p53 are not homogeneously expressed, so that the lymphoma punch biopsy may not be representative of the whole neoplasm (3–5). Several experimental and clinicopathological efforts have been made to reduce and even eliminate these concerns. A recent paper of Hedavat et al clearly showed that the TMA technique can be used reliably in lymphomas to characterize protein and mRNA expression levels. In spite of the drawbacks mentioned above, lymphomas can be reproducibly evaluated on TMA. Nevertheless, criteria for the evaluation of quantitatively expressed markers can strongly influence the rate of concordance. The fact that the maximum expression of heterogeneously expressed markers cannot be reliably determined on TMA due to tissue heterogeneity and the limited amount of tissue in punch biopsies do not outweigh the enormous advantages of TMA, namely the cost- and time-saving and the mostly homogeneous results of immunohistochemistry (6). Of course, in this context it cannot be overemphasized that care in the composition of an array and a certain degree of redundancy are essential for minimizing TMA sampling drawbacks, because the selection of different tumor areas should be oriented towards the requirements of the investigated tumor entity.

Among alternatives to circumvent these problems is the use of larger punch needles of up to 2-mm diameter. Nevertheless, for the use of TMA in cancer research no obvious advantage can be seen, because when compared with the original size of a tumor with a diameter of up to several centimeters, an area of about 3 mm^2 (2 mm-diameter) is hardly more »representative« than 0.27 mm^2 (0.6-mm diameter). In addition, the obvious disadvantage is that instead of several hundreds of tumors on a single slide/section, far fewer than a 100 samples can be investigated at the same time. These large punches also cause considerable damage to the donor and acceptor block using conventional paraffin wax blocks. Despite the fact that these arrays might be suboptimal for cancer research, large punch arrays may be preferable for distinct areas of research and perhaps routine practice. Authors of this paper, in a recent study, used a 2-mm punch needle (7) *(Figure 1)*.

The number of core samples per case is also an important issue. The TMA immunostaining results agree with whole tissue section staining in 86% to 100% of cases, and as the number of core samples increases, the level of agreement also increases (8). In lymphomas it was found that added redundancy of 3 cores/case reduced the numbers of cases placed on TMA, but that did not increase the accuracy of a particular stain if tumor cells were present (6). Moreover, in situations with careful core sampling, especially in large studies, a single sample from each tumor may often be sufficient to derive information on clinical associations (2).

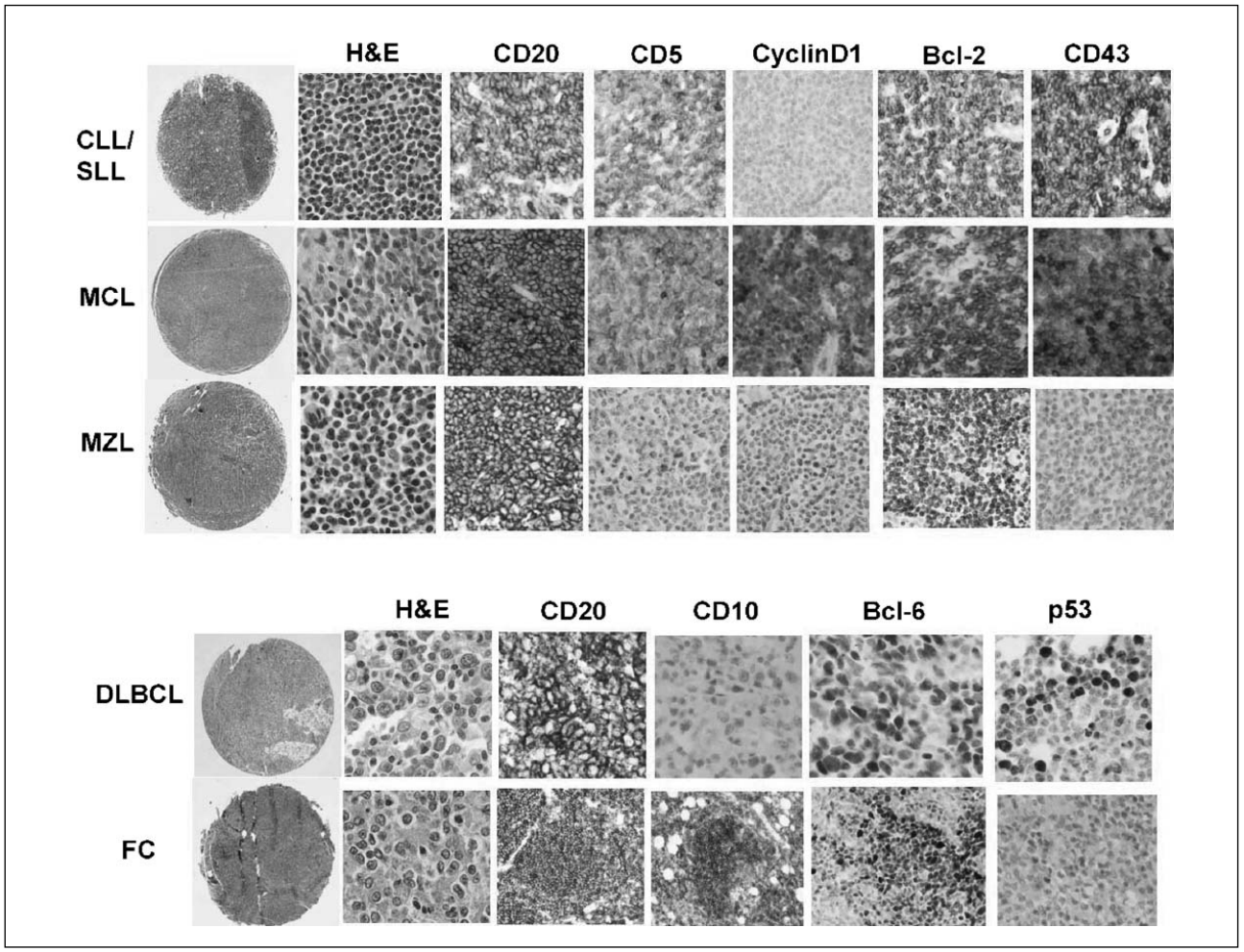

Figure 1 Example of diagnosed subtypes of B non Hodgkin's lymphoma, after Hematoxylin & Eosin (H&E) or immunohistochemistry staining using TMA. *Abbreviations:* CLL/SLL-Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma, MCL-Mantle Cell Lymphoma, MZL-Marginal Zone Lymphoma, DLBCL-Difuse Large B-cell Lymphoma, FC-Follicular lymphoma.

Application of TMA in analysis of B lymphomas

Validation of gene expression array with TMA in Diffuse Large B-cell Lymphoma (DLBCL)

Markers defining subgroups of diffuse large B--cell lymphoma (DLBCL) with a particularly aggressive course could be rapidly identified using TMA. Rosenwald et al (9) have recently identified 3 different DLBCL subgroups (germinal center B-cell-like--GCB, activated B-cell-like-ABC, and type-3 DLBCL) through the use of gene expression profiling. The validation of these findings at the post-transcriptional level was greatly enhanced by combining TMA with immunohistochemistry. In a recent paper by Hans et al TMA proved to be useful and accurate in defining the more favorable germinal center subtype of DLBCL from the activated subtype by using only Bcl-6, CD10 and MUM1 antigens (10). Using TMA, Hans and co--workers showed an outcome similar to that predicted

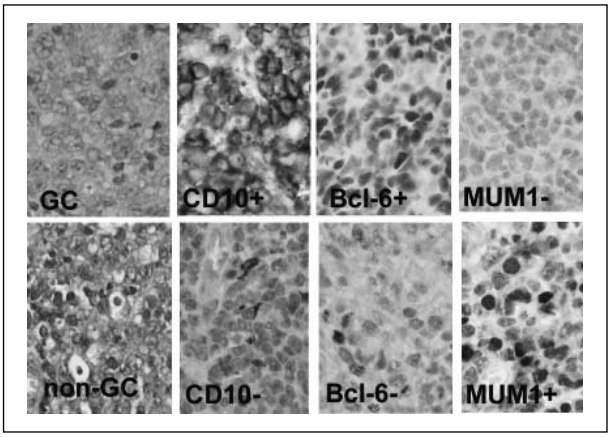

Figure 2 Subtypes of Diffuse Large B-cell Lymphoma diagnosed on TMA. (7) *Abbreviations:* GC-germinative center subtype: non-GC non-germinative center sybtype. GC-subtype is CD10+ or -, BCL-6+ and never MUM1+. Non-GC has immunophenotype that is opposite to GC and corresponds to activated and type-3 forms of DLBCL as detected with gene expression profiling (3).

by cDNA microarray analysis. In fact, this latter panel of immunostains predicted the cDNA classification in 71% of GCB and 88% of ABC or type-3 cases (10). The same panel was used in a recent study, published by the authors of this review (7) *(Figure 2)*.

The increased expression of Bcl-6 was one of the features that made it possible to define a subset of germinal center B-cell-like DLBCLs, characterized by lower aggressivity. Most studies coincide in showing that a high level of Bcl-6 expression, as determined by real-time polymerase chain reaction (PCR) or immunohistochemistry, is a favorable prognostic marker (11, 12). The existence of a large group of double Bcl-6+ MUM1+ cases, reaching as high as 47% cases, demonstrates that the mutual exclusion of these markers, as observed in reactive germinal centers, is not preserved in DLBCLs (13). Although these cases express Bcl-6, the outcome is most likely to be that of the ABC subtype, and this may explain why there are discrepancies in outcome prediction when using Bcl-6 expression alone. Gene expression profiling analysis discovered high correlation of IRF/MUM1 expression and activated DLBCL immunophenotype (9). This could be explained with the fact that constant activation of NFκB pathway is tightly connected with the presence of MUM1 protein.

TMA and lineage restricted transcription factors in diagnostics of specific B cell lymphoma subtypes

Morphological characteristics of a particular subset of DLBCL, called T-cell-rich B-cell lymphoma, are very similar to classical subtypes, or nodular lymphocyte predominant Hodgkin's lymphoma. Majority of cases of T-cell-rich B-cell lymphoma are strongly positive to Oct2 and BOB1, in addition to other B-cell lineage markers. In the majority of cases Reed Sternberg cells (RS) are both $BOB1^-$ and $Oct2^-$ and, when positive, expression is very weak. They are particularly important when RS cells appear CD20+ and lack expression of CD15 (14) *(Figure 3)*.

BOB1 is a coactivator of the transcription factors Oct1 and Oct 2 which regulate the transcription of immunoglobulins by binding to the octamer motif of the immunoglobulin gene (both H an L) promoter. Oct 2 also participates in the expression of other differentiation and proliferation genes of B-cells including CD20. BOB1 is a co-factor acting as a »clamp« fixing Oct2 to the promoter site (14). Oct2 and BOB1 are of potential use in identifying other CD20- B-cell lymphomas such as plasmablastic lymphoma or plasmacytoma/myeloma. In a recent paper, MacCune et al have elegantly showed the value of TMA technique in distinguishing B-cell lineage Non Hodgkin's lymphomas from classical Hodgkin's disease, nodular lymphocyte predominant Hodgkin's lymphoma, and anaplastic large cell lymphoma and precursor T-cell acute lymphoblastic lymphoma (15). In this paper, authors used a transcription factor Pax-5, which is also know as B-cell specific activator protein (BSAP), that regulates the downstream transcription of Oct1, Oct2 and BOB1. This transcription factor proved to be useful in separating anaplastic large cell lymphoma from classical Hodgkin's lymphoma (15).

TMA in detection of cell cycle regulators and prognosis of B Non Hodgkin's lymphomas

Markers of proliferation such as Ki 67 have significant prognostic value in mantle cell lymphomas (MCL). Patients with blastoid subtype of MCL might have Ki 67 index over 60% on TMA (7) *(Figure 4)*.

This is in agreement with Ratty et al, who found increased risk for transformation from classic to blastoid subtype in 25% of Ki-67+ tumor cells (5, 16). This prognostic cut of point is compatible to results of gene expression profiling analysis of mantle cell lymphomas (17). In a similar manner, evidence of transformation to more aggressive forms of B--Chronic Lymphocytic Leukemia/ Small Lymphocytic

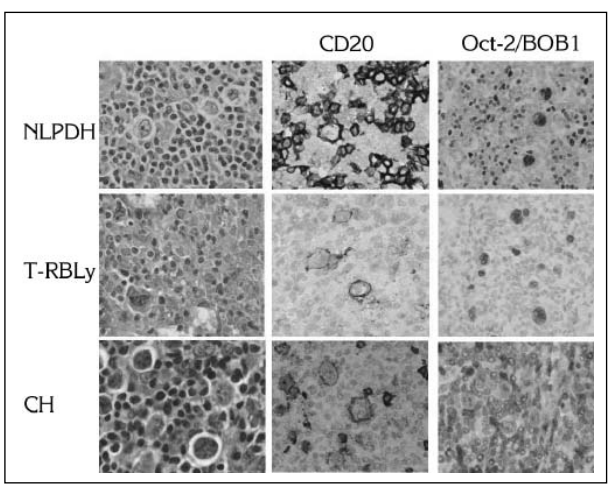

Figure 3 Determination of Nodular Lymphocyte Predominant Hodgkin's disease(NLPDH), from Classical Hodgkin's lymphoma (CH) and Subtype of Diffuse Large B-cell lymphoma called T-cell-rich B-cell lymphoma (T-RBLy) (7).

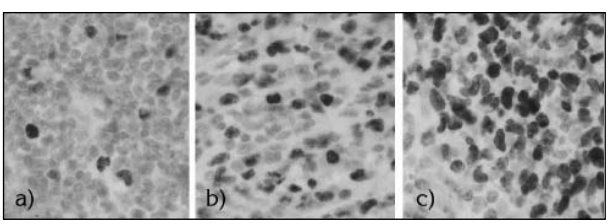

Figure 4 Difference in expression of Ki-67 proliferative antigen in various subtypes of mantle cell lymphoma (MCL) detected on TMA: a) classic sybtype of MCL with only 10% of Ki-67+ cells; b) and c) blastoid subtypes of MCL with 60% and 90% of Ki-67+cells, respectively (7).

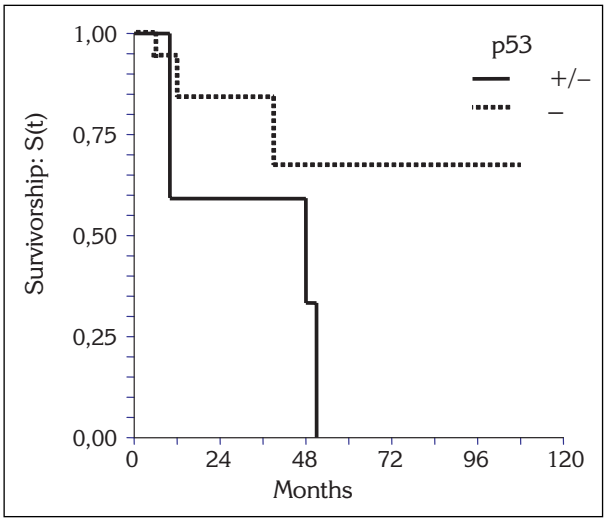

Figure 5 Survivorship of patients with indolent lymphomas according to presence of p53. In this heterogeneous group of patients Kaplan-Meier, 4-year survival estimate in patients with $p53^{+/-}$ was 25%, while in $p53^{-}$ group it was 80% (log rank p=0.0475) (7).

Lymphoma (B-CLL/SLL) could be found in cases with more than 16% Ki-67 positive cells (18). Expression of MUM1 is connected with the constant activation of NFκB pathway, and is already identified as an adverse prognostic factor in CLL/SLL (19).

Furthermore, p53 over-expression predicts a worse outcome in patients with B-cell chronic lymphocytic leukemia (B-CLL), certain low grade lymphomas and mantle cell lymphoma (MCL), irrespective of the stage of disease (20). Mutations and stabilization of p53 are reported to be related with more aggressive behavior of indolent lymphomas and resistance to chemotherapy, especially in B-CLL, FL and marginal zone lymphomas (21). According to recently published results, in a small series of indolent lymphomas using TMA method, authors of this review also found p53 positivity to be followed by adverse prognosis, as shown in *Figure 5* (7).

It is important to realize that, due to the heterogeneous expression of markers such as Ki-67 and p53, TMA could not reliably assess the maximal expression. Nevertheless, in case of mean KI-67 and p53 expression, TMA showed a 90 and 92% concordance rate with conventional tissue sections, and that could be sufficient for routine practice (22).

Conclusion and further perspectives

In summary, the TMA methodology is highly advantageous for the diagnosis and biological characterization of lymphomas in general. It is useful for the validation of gene expression profiling results, at the post-transcriptional level, in many subtypes of B Non Hodgkin's lymphomas. In spite of the drawbacks and limitations mentioned above, the TMA technology provides an important tool to accelerate the process of gaining knowledge of the molecular biology of B Non Hodgkin's lymphomas.

The era of tissue arrays has just begun. A multitude of different possibilities seem realistic, and some are already in use. For example, the use of »paraffin wax tissue banks« in pathology departments, for the retrospective evaluation of new tumor markers for individual patients.

It will only be a question of time before TMAs find their role in educational purposes. Nevertheless, the major focus of TMAs at the present time is in the fields of cancer and non-cancer research.

There is no doubt that the widespread use of TMAs will become an integral part of daily practice in research and routine clinical laboratories. With this clear perspective, »pathology« as an old, largely morphology-based medical speciality will find itself in the central position within these new developments. With the background of archives of well-characterised tumor cases, pathologists will be in the position to use the potential of TMA technology to present their well-defined historical and current archives in an arrayed manner to the scientific community.

ZNAČAJ »TISSUE MICROARRAY« TEHNIKE U DIJAGNOSTICI I PROGNOSTICI NE-HODGINSKIH LIMFOMA, B ĆELIJSKOG POREKLA

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Kratak sadržaj: Nova tehnologija »tissue microarray« (TMA) omogućava brzu i ekonomičnu analizu stotine markera na istom setu uzoraka. Najznačajniji nedostatak TMA tehnologije je mala količina tkiva koje se analizira, što je nepogodno kod heterogenih tkiva kao što su limfomi. Ovaj nedostatak je nadmašen velikim prednostima koje se ogledaju u homogenosti, efikasnosti i ekonomičnosti imunohistohemijske analize TMA uzoraka. U Ne-Hodginskim limfomima TMA detekcija transkripcionih faktora imunoglobulinskog gena Oct1 i Oct2 i njihovog koaktivatora BOB1 je naročito značajna za razlikovanje T-ćelijama bogatog B-ćelijskog limfoma od klasi ne--Hodgkinove bolesti, nodularno limfocitno predominantnog Hodgkinovog limfoma i plazmablastnih limfoma. Predviđanje preživljavanja obolelih od pojedinih podtipova difuznog B limfoma krupnih ćelija upotrebom TMA bilo je kompatibilno sa rezultatima cDNK »microarray« analiza. Detekcija p53 tumor supresorskog gena i Ki--67 proliferacionog antigena od velikog je prognostičkog značaja za brojne podtipove ne-Hodginskih limfoma. Uprkos činjenici da je ekspresija p53 i Ki-67 veoma heterogena, stepen poklapanja na TMA u odnosu na klasične imunohistohemijske isečke iznosila je 90% za p53 i 92% za Ki-67 što je dovoljno za svakodnevni rad. Nesumnjivo je da će široka upotreba TMA postati integralni deo svakodnevne kliničke ali i istraživačke prakse u laboratorijama.

Ključne reči: »microarray« tkiva, proteomika, B-ćelijski limfom

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