Today the role of pathologists is increasingly focused on finding more accurate prognostic and predictive parameters that will be necessary for targeted treatment of patients. Improving understanding of colorectal carcinogenesis allow us to consider incorporation of these new knowledges in molecular classification of colorectal cancer. There are different ways of molecular classification, but most of them are based on: 1. type of genetic instability; 2. methylator phenotype and 3. single molecular events such are KRAS and BRAF mutations. This review considers a new molecular classification of colorectal carcinoma proposed by J. Jass in 2007 which is based on the correlation of molecular and morphological features. We would also like to point out to the new role of pathologists in the era of personalized medicine in diagnosis and prognosis of colorectal carcinomas as well as in selection of patients for some modalities of targeted therapy.

Key words: colorectal carcinoma, molecular classification, microsatellite instability, KRAS, BRAF

INTRODUCTION

Before 2000, the pathological diagnosis of tumors was mainly based on morphological analysis (including sometimes immunohistochemistry). In the last decade, the knowledge on pathogenesis and molecular background of colorectal carcinoma (CRC), as well as the many other tumors has increased dramatically. As a result, pathological diagnosis of CRC is now more complex and requires molecular tests, for example assessment of microsatellite instability (MSI) for identification of patients with increased risk for Lynch syndrome. The main driver of changes in this diagnostic process has been the arrival of drug therapies targeting specific molecular aspects of colorectal carcinoma as well as directed uses of old therapies. These specific targeted therapies require implementation of results of additional and more complex molecular genotypic tests, such as analysis of the Epidermal Growth Factor Receptor (EGFR) and KRAS genes mutations in pathohistological reports of patients with colorectal carcinoma.

Until 10 years ago we did not know which genes are involved in colorectal carcinogenesis. Today, we can interpret what we see in terms of molecular mechanisms. Because of that, there are increasing efforts to establish more accurate prognostic and predictive factors by combining both clinico-pathological and molecular data.

The role of pathology in colorectal carcinoma diagnosis and therapy

Currently, tumor-node-metastasis staging remains the gold standard for prognostic classification of CRC. But, during the last 20 years, there have been significant advances in our understanding of colorectal cancer pathogenesis. It is now clear that these tumors include a heterogenous complex of disease that reflects different underlying mechanisms of carcinogenesis. Despite radical surgery, we have often seen that patients at the same stage of tumour disease have a completely different course and outcome. For CRC, for a long time it has been known that standard histopathological criteria is not precise enough to determine the appropriate therapy. Therefore, we are constantly looking for such parameters that will provide a reliable prognosis of the disease and for finding out such predictive parameters that will provide optimal therapy for each patient.

CRC is a surgical disease, with radical resection as a method of choice in the most cases. Resected specimen is the most important pool of informations regarding prognosis of disease, and recently regarding prediction of therapy response. Today we know that stage of CRC described the disease at the operating time and doesn’t say...
anything about biology of CRC. Despite all, TNM staging system is still the most important for prognosis of CRC. According to the 7th edition of TNM staging system, there are several changes (Error! Bookmark not defined.). The definition of T3 has been expanded because it now includes the adverse impact of peritumoral deposits named "satellite nodules" (SN) on outcome (see later). T4 tumors are subcategorized as T4a (penetrates visceral peritoneum) and pT4b (directly invades or adhere to other organs or structures). Stage II is subdivided into IIA (T3a/bN0), IIB (T4aN0) and IIC (T4bN). Precise determination of stage II disease is important because it determines the needs for administration of adjuvant therapy. The definition of stage III has been also revisited. Namely, pN1 is subdivided into pN1a (metastasis in 1 regional lymph node) and pN1b (metastasis in 2-3 nodes), pN2 is subdivided into pN2a (metastasis in 4-6 nodes) and pN2b (metastasis in 7 or more nodes).

According to the International Union against Cancer’s (UICC’s) publication "Prognostic Factors in Cancer" a several new site-specific factors have been defined for routine assessment: the presence of satellite nodules (peritumoral deposits of tumor that lacks of evidence of residual lymph node that were before classified as metastatic lymph nodes and N category); estimation of tumor regression grade (TRG) for neoadjuvantly treated tumors; perineural invasion (PN) as an adverse prognostic factor; the status of circumferential resection margin (CRM). The importance of quality of surgery is well recognized today and have to be monitored by the pathologist: the quality of total mesorectal excision surgery can be assessed by estimation of status of CRM and macroscopic appearances of the mesorectum.

Recently, it has been shown that a new site specific factor, "tumor budding" is associated with lymph node metastasis and other adverse outcomes. Tumor budding reflects a detachment of tumor cells at the invasive front of CRC into single cells or clusters up to five cells. Many studies suggest that tumor budding is reliable marker of molecular prognostic and predictive factors

Today, it became clear that the most accurate prognostic information will be achieved by combining both clinicopathological and molecular data. Molecular classification of CRC has become important with development of personalized anticancer therapy. In current practice, the majority of CRC patients receive treatment unnecessarily, either because they were cured or because they will relapse despite treatment. It is important to identify patients who will benefit from adjuvant therapy, and escape for others needless toxicity.

Molecular genetics of colorectal cancer

Development of CRC is multistep process which includes accumulation of genetic and epigenetic alterations. These alterations have some morphological correlates and vice versa. The goal of studying molecular

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Drug selection</th>
<th>Mutation frequency</th>
<th>Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRAS</td>
<td>Predicts resistance to anti-EGFR Th</td>
<td>40%</td>
<td>Yes</td>
</tr>
<tr>
<td>BRAF</td>
<td>Predict resistance to anti-EGFR Th</td>
<td>10%</td>
<td>Probable</td>
</tr>
<tr>
<td>PI3KCA</td>
<td>Resistsence to anti-EGFR Th</td>
<td>20%</td>
<td>Possible</td>
</tr>
<tr>
<td>PTEN</td>
<td>Resistsence to anti-EGFR Th</td>
<td>30%</td>
<td>Probable</td>
</tr>
<tr>
<td>Microsatellite instability</td>
<td>May predict adverse outcome with 5FU and improved outcome with Irinotecan</td>
<td>15%</td>
<td>Possible</td>
</tr>
<tr>
<td>18qLOH/SMAD4 loss</td>
<td>May predict resistance to 5FU</td>
<td>50%</td>
<td>Probable</td>
</tr>
<tr>
<td>Topo 1 low</td>
<td>May predict resistance to Irinotecan</td>
<td>50%</td>
<td>Probable</td>
</tr>
</tbody>
</table>

5FU- 5-fluorouracil

![FIGURE 1. ADENOMA-CARCINOMA PROGRESSION SEQUENCE. MICROSATELLITE AND CIN PATHWAYS IN COLORECTAL CANCER PROGRESSION. (VOGELSTEIN B. tumor progression and bad outcome even indipendently of used evaluation system)](32 S. Knezevic-Usaj et al. ACI Vol. LIX)
correlates is to identify clinically useful biomarkers which could be associated not only with patients outcome but with treatment response also, as well as to help in treatment decision. Molecular correlates can suggest surrogate markers in clinical and research settings.

Originally, Vogelstein and Fearon thought that all of CRC developed by the progressive accumulation of genetic alterations in the same, linear manner to several key genes:

1. Inactivation (by mutation and loss) of both copies of the tumor suppressor gene APC resulting in the initiation of a small adenoma;
2. Mutation of the KRAS oncogene giving a larger adenoma;
3. Reduced expression of SMAD4 and
4. Mutation and loss of additional tumor suppressor gene, p53, driving the transition from adenoma to carcinoma.

According to recent concept, CRC can develop as:

1. Part of hereditary cancer syndromes i.e. Hereditary NonPolyposis Colon Cancer-HNPCC (< 10% of patients);
2. Sporadic CRC (about 85%) and
3. In patients with long lasting inflammatory bowel disease.

Today, it became clear that CRC are more heterogeneous regarding molecular events. At least four types of genetic alterations are described in CRC:

1. Chromosomal instability (CIN);
2. Microsatellite instability (MSI);

CIN pathway: approximately 85% of CRC are evolving through chromosomal instability pathways or microsatellite stability (MSS) pathway and they are characterized by allelic losses, chromosomal amplifications and translocations. In these tumors are frequent deletions at 1p and 8p, and loss of heterozigosity (LOH) of 17p and 18q.

Mechanisms underlying of this type of genomic stability are still poorly understood. Large metaanalyses have demonstrated that CIN is marker of poor prognosis in CRC.

MSI pathway: the second pathway, present in about 15% of CRC, is referred as microsatellite instability pathway and these tumors display mutations in short repetitive uncoded sequences of DNA known as microsatellites. MSI is defined as more than 30% unstable loci in the NCI consensus panel or more than 40% unstable loci in a panel of mononucleotide microsatellite repeats. MSI is developed as a result of defects (mutation of genes) in the mis-
match repair mechanisms (MMR). In HNPCC there are germline mutations in MSH2 and MLH1 MMR genes, whereas in sporadic CRC, MSI is usually due to loss of expression of the MMR by epigenetic mechanisms\textsuperscript{27}. Defective MMR facilitates malignant transformation by allowing the rapid accumulation of mutations that inactivate genes with some of the key function in the cell. CIN and MSI pathways are mutually exclusive. For the purposes of detecting individuals with Lynch syndrome (HNPPC), MMR testing is currently recommended for all cases of colorectal cancer arising in individuals less than 50 years of age\textsuperscript{28}.

Epigenetic instability pathway: it is apparent that cancer is also a disease that is caused by epigenetic changes, i.e. by such defected genes expression that are mediated by mechanisms that do not affect the primary DNA sequence\textsuperscript{18}. One of them is global DNA hypomethylation. But, the best understood, is the transcriptional inactivation of the tumor suppressor genes by abnormal methylation of DNA at the promoter regions of these genes rich in cytosine and guanin, so-called CpG island and CpG island methylator phenotype (CIMP). Normally, these regions are nonmethylated. Hypermethylation is a manner of silencing genes by which many tumor suppressor genes can be reduced or eliminated and it can be CIMP high (CIMP-H) and CIMP low (CIMP-L)\textsuperscript{19}. Epigenetic silencing through DNA methylation can begin very early in tumor progression and may affect multiple genes involved in different cellular pathways including cell cycle control, DNA repair many others\textsuperscript{30}. Strong association between BRAF V600E mutations and CIMP in CRC suggests potential role for BRAF in the pathogenesis of methylator phenotype, as well as a link between sporadic MSI and CIMP\textsuperscript{31}. BRAF and KRAS are present in the same signal transduction pathways, and CIMP-low phenotype with KRAS mutation probably represent a new molecular subtype of CRC\textsuperscript{32,19,16}.

Molecular classification of CRC and morphological correlates

There are several attempts of classifying CRC according to molecular events\textsuperscript{17,33,34}. Jass has been proposed five molecular subtype of CRC based on:

1. Underlying type of genetic instability,
2. DNA methylation status,
3. KRAS or BRAF mutation and
4. Ploidy\textsuperscript{17}.

-Group 1: CIMP-H, methylation of MLH1, BRAF mutation, chromosomal stable, MSI-H, origin in serrated polyps. This type, known as sporadic MSI-H type, is present in about 12% of patients.
-Group 2: CIMP-H, partial methylation of MLH1, BRAF mutation, chromosomal stable, MSS or MSI-L, origin in serrated polyps (8%)
-Group 3: CIMP-low, KRAS mutation, MGMT methylation, CIN, MSS or MSI-L, origin in adenoma or serrated polyps (20%)

-Group 4: CIMP negative, CIN, mostly MSS, origin in adenomas, sporadic or Familial Adenoma Polyposis (FAP) associated or MUTYH polyposis associated (57%).
-Group 5: Lynch syndrome: CIMP negative, BRAF mutation negative, chromosomally stable, MSI-H, origin in adenomas (3%).

Each of these groups has a different morphological correlates that can be identified by the pathologist in tumor tissue. Often, there are some overlapping between two or more groups regarding some morphological features. But, particular histopathological characteristics are more specific for one molecular type than for the others and pathologist must be familiar with these correlations and to be able, based on morphological characteristics, to suggest an additional immunohistochemical or molecular tests\textsuperscript{19}.

For example, a number of pathological features have been linked with MSI-H: mucinous differentiation (Figure 2a), medullary histological type, signet ring cell morphology, as well as Crohn-like lymphoid reaction (Figure 2b), abundant tumor-infiltrating lymphocytes (Figure 2c) and poor differentiation\textsuperscript{27}. The presence of MSI-H correlated with an older age, the presence of tumor in the proximal colon, and female sex\textsuperscript{19}. Mucinous differentiation in CRC associated with group 1 (sporadic MSI-H), comprise intestinal (MUC2) and gastric type (MUC5AC) of mucin, whereas secretory mucus in serrated adenocarcinoma comprise small intestine mucin type (non-0-acetylated sialic acid substituents)\textsuperscript{32}. Patients with MSI-H are usually resistant to chemotherapy with 5FU, but have a better prognosis\textsuperscript{36-38}. It is also now recognized that sporadic MSI tumours are associated with the serrated neoplasia pathway (Figure 2d) and frequently carry BRAF V600E mutations, while cancers resulting from germline mutations in MMR genes (Lynch syndrome) do not have mutated BRAF. The presence of a BRAF mutation in an MSI tumour practically excludes the possibility that the tumour is developed as a consequence of Lynch syndrome\textsuperscript{39,40}. MSI status can be identified by immunohistochemistry using panel of five monoclonal antibodies with high sensitivity and specificity of 94 and 97% respectively in comparison with molecular tests\textsuperscript{41}.

CIMP high CRCs (Group 2) have a distinct clinical, pathological and molecular profile such as proximal tumor location, female sex, poor differentiation, MSI or MSS, high BRAF mutation rate and rarely p53 mutation\textsuperscript{39,42,19,29}.

In the group 3. tumors are CIMP-low and chromosomal stable, KRAS positive, usually in left colon and more often in male patients. There are no specific morphological features.

Tumors classified as group 4. are more often in left colon and in male gender. Sometimes in malignant lumina of CRC there is no mucinous but eosinophilic material admixed with necrotic cell debris, so-called "dirty necrosis". Tumor budding is closely correlated with lymphovascular and perineural invasion and discontinuous mesenteric deposits and it is usually present in group 4\textsuperscript{42}. Budding cells have the properties of malignant stem cells, including po-
tential for re-differentiation and immunophenotype that include upregulation of β catenin, lamin, matrix metalloproteinase-7, p16, CD133, CD44, COX2 and down-regulation of E-cadherin and CDX2. All of these markers we are able to identify by immunohistochemistry in tumor tissue by using commercial antibodies. Unexpectedly, this type of tumor growth has low Ki-67 proliferative rate.

Serrated morphology: In 1996 Torlakovic and Snover, in a review of a series of patients with so-called hyperplastic polyposis, suggested that this was a condition with a high propensity or the development of adenocarcinoma, despite the consensus at that time that this syndrome is not associated with an increased risk. This finding subsequently was confirmed by several other groups and today is generally accepted. Serrated adenocarcinoma are recognized by the presence of several additional features: cribriform and lace-like structures, mucinous intra and extracellular secretions, low nuclear:cytoplasmic ratio, vesicular ovoid or round nuclei with chromatin condensed at the nuclear membrane, large nucleolus and "pink" appearance due to abundant eosinophilic cytoplasm. Tumors within group 1 and 2 are developed through serrated pathways and they have been linked with MSI; more often with MSI-L than MSI-H. Bookmark not defined. Also, many features in serrated morphology are linked with DNA methylation. BRAF mutation is often in this type of morphology.

Predictive biomarkers in CRC

The promise of personalised medicine is now a clinical reality, with colorectal cancer genetics at the forefront of this next major advance in clinical medicine. The potential of genetic and epigenetic alterations to be effective predictive molecular markers has received considerable attention lately and has lead to the use of some of these markers (Table 1) in the routine care of patients with colorectal cancer. Because of that ancillary studies are being increasingly used to indicate the likelihood of patient response to specific biologic therapies.

Testing for the presence of mutations in the KRAS gene is typically requested by the clinician when metastatic disease is present. In this situation, the result should be appended to the initial pathology report. More recently KRAS mutation status has been shown to predict response to drugs, such as cetuximab and panitumumab, that specifically target the epidermal growth factor receptor (EGFR). KRAS has central role in the EGFR signalling pathway and mutated KRAS activate the EGFR signalling pathway independently of stimulation of receptor by ligand. Tumours that harbour mutations in KRAS (~15% of patients) are resistant to the effects of these medications. Thus, testing for KRAS mutations will become increasingly important in determining the optimal targeted therapy. KRAS mutation status is currently determined by a variety of genetic methods that are not routine in most diagnostic laboratory settings. In some patients with metastatic colorectal carcinoma (mCRC) which does not react to anti-EGFR targeted therapy, BRAF mutations have been reported to be associated with poor prognosis and lack of response to EGFR targeted agents. Mutations of the BRAF gene are rare in tumours arising from a Lynch syndrome background. As a consequence, when present, BRAF mutations can be useful in helping to distinguish between sporadic tumours arising through hypermethylation, and Lynch syndrome associated tumours arising from a germline mutation.

In several small studies published to date, phosphatidylinositol-3 kinase (PI3K) mutations or phosphatase and tensin homologue (PTEN) loss have been associated with lack of response to cetuximab. However, the relationship of oncogenic alterations in PI3K signalling and cetuximab or panitumumab response is much less clear than that of KRAS and BRAF mutations. In fact, recent data demonstrate that when PIK3CA mutations and PTEN loss of expression are combined with KRAS and BRAF mutational analysis, up to 70% of patients are unlikely to respond to cetuximab or panitumumab may be identified.

The majority of molecular tests can be performed on formalin fixed paraffin embedded tumor tissue and requests for blocks containing tumour with a high proportion (preferably over 70%) of cancer cells. The central role in selection of tumor tissue has pathologist. Selection must be done on formalin fixed paraffin embedded tumor tissue stained by hematoxylin & eosin. Handling with resected specimen or biopsy tissue must be careful. Tissue should be placed in formalin within 20-30 minutes after removing from the body.

There are increasing evidences to support the observations that CRCs with MMR defect are less responsive to 5FU-based adjuvant chemotherapy although this has not been shown conclusively in all studies. MSI-H tumors appear to be more responsive to irinotecan-based adjuvant chemotherapy. Results from a large randomized trial of stage III CRC demonstrated improved outcome in MSI positive patients treated with an irinotecan-containing regime that included 5-FU compared with 5-FU-leucovorin alone. Finding that MSI is a predictive biomarker for irinotecan suggests that MSI could be useful for adjusting adjuvant therapy for patients with colorectal cancer.

Conclusion and future directions

Molecular classification and correlates in CRC are still evolving, and current proposal of molecular-morphologic classification represent our best understanding at this moment. The future for colorectal cancer prognostication and therapeutics is very promising, but we must concentrate our mind on optimising current methods, above all histopathology and discovering new subtypes of CRC. The recognition of molecular subtypes of CRC represents the future of personalized oncology and will guide drug-development strategies. Pathologists play an increasingly important role in the diagnosis and management of colorectal cancer because of the advent of new targeted therapies. Pathologists have an obligation to reestablish their role in everyday practice as well as in general from "looking into microscope" towards "integrating the results
of both histology and molecular pathology diagnostics" for the benefit of patients and their science itself.

SUMMARY

KOLOREKTALNI KARCINOM POD MIKROSKOPOM: PATOHISTOLOGIJA ILI MNOGO VIŠE?


Ključne reči: kolorektalni karcinom, molekularna klasifikacija, mikrosatelitna nestabilnost, KRAS, BRAF

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List of abbreviations:
CRC- colorectal carcinoma
HNPCC- Hereditary NonPolyposis Colon Cancer
EGFR- Epidermal Growth Factor Receptor
TRG- tumor regression grade
MSI- Microsatellite instability
PTEN- Phosphatase and tensin homologue
CIN- Chromosome instability
PI3K- Phosphatidylinositol-3 kinase
MSS- Microsatellite Stable
CIMP- Cpg island methylator phenotype
LOH- Loss of Heterozigosity
MMR- Mismatch repair mechanisms