

## COLORECTAL CANCER, NOVEL BIOMARKERS AND IMMUNOHISTOCHEMISTRY – AN OVERVIEW

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### Summary

Colorectal cancer (CRC) is the most common cancer in Europe and the leading cause of death. A combination of therapy with targeted agents and the knowledge of many biomarkers is significantly increasing to better guide the selection of treatment. Biomarkers that are currently used as predictive and prognostic, as well as factors for therapy selection, are described in this overview. It refers to microsatellite instability (MSI), RAS-family of oncogenes, BRAF, TP53, Ki-67, Oncotype DX<sub>+</sub>, phosphatidylinositol 3-kinase (PI3K)/AKT, 18q LOH, and CpG island methylator phenotype.

Only a few biomarkers are currently used and in routine reported by pathologists. Future studies need to consider the combination of markers, standardising protocols and, if possible, simple and standardised assays for the detection of molecular markers.

**Keywords:** colorectal cancer; biomarkers; microsatellite instability; KRAS; BRAF.

### INTRODUCTION

Colorectal cancer (CRC) is the most commonly diagnosed cancer in Europe and one of the leading causes of cancer death worldwide [1,2]. In 2008, 436,000 new cases of CRC were diagnosed in Europe and it was responsible for 212,000 (12.2%) deaths representing the second most common cause of cancer death after lung cancer (19.9%) [1]. In Croatia, combined, colon, rectum, rectosigmoid and anal cancers represented 15% in the male and 13% in the female cancer incidence in 2011 [3].

In the past years treatment and outcome of early and advanced disease has steadily improved. Currently, a broad variety of trials and retrospective analyses gave further insights into clinical questions like selection and duration of treatment, combinations with targeted agents and also knowledge of prognostic as well as predictive biomarkers is significantly increasing to better guide selection of treatment.

Therefore pathology report is becoming more complex and in the field of newfound and offered biomarkers it is becoming hard to identify and standardize those with truly predictive and prognostic value.

There are some factors definitively proven to be of prognostic importance based on evidence from multiple published trials and generally used in patient management. These are: the local extent of tumor assessed pathologically (the pT category of the TNM staging system of the American Joint Committee on Cancer and the Union Internationale Contre le Cancer [AJCC/UICC]); regional lymph node metastasis (the pN category of the TNM staging system); blood or lymphatic vessel invasion; residual tumor following surgery with curative intent (the R classification of the AJCC/UICC staging system), especially positive surgical margins [4].

Some factors biologically and clinically shown to have prognostic value for outcome and/or predictive value for therapy are also reported by pathologist, although it remains to be validated in comprehensive studies. It includes tumor grade, radial margin status and residual tumor in the resection specimen following neoadjuvant therapy (the ypTNM category of the TNM staging system) [4].

Factors shown to be promising in multiple studies are histologic type, histologic features associated with microsatellite instability (MSI) (ie, host lymphoid response to tumor and medullary or mucinous histologic type), high degree of MSI (MSI-H), loss of heterozygosity at 18q (DCC gene allelic loss), tumor border configuration (infiltrating vs pushing border), DNA content and all other molecular markers, perineural invasion, microvessel density, tumor cell-associated proteins or carbohydrates, peritumoral fibrosis, peritumoral inflammatory response, focal neuroendocrine differentiation, nuclear organizing regions and proliferation indices [4,5].

In recent years, colorectal cancer (CRC) has been divided into different subgroups with distinct precursor lesions, pathways of carcinogenesis, morphological, and molecular characteristics [6]. In spite of a tremendous amount of available literature on biomarkers only a few are nowadays used in da-

ily clinical practice, such as KRAS, BRAF, MSI and the Oncotype DX\_ Colon Cancer Assay [7].

## **BIOMARKERS**

### **Microsatellite instability**

There are two forms of genomic instability that reflect different genetic pathways of tumorigenesis. One refers to a clonal change in the number of repeated DNA nucleotide units in microsatellites caused by deletions or insertions, and appears in tumors with deficient mismatch repair (MMR) [8].

The biochemical basis of this phenotype is explained by strand-specific mismatch repair defects and linked to germline mutations of the MMR gene hMSH2 and hMLH1. MSI phenotype is also found in Lynch Syndrome as mutations in PMS2 and hMSH6. If there is a clinical suspicion of Lynch Syndrome (Bethesda Guidelines), MSI testing with molecular screening and/or immunohistochemistry is recommended by the ESMO Consensus [9].

Different mechanism causes the sporadic type of MSI to develop in CRC and it is associated with hMLH1 promoter hypermethylation and lack of hMLH1 expression and subsequently loss of mismatch repair system function. This sporadic type of MSI could be investigated through testing for a BRAF V600E mutation that is strongly associated with a sporadic origin or by analysis of hMLH1 promoter hypermethylation [8,10].

It has been shown that MSI CRC is associated with a better prognosis than non-MSI CRC, but appears to be more pronounced for Lynch Syndrome [8,11,12]. MSI testing in molecular pathology laboratories is becoming increasingly available, but requires expertise and experience in testing and interpretation. Nowadays, immunohistochemistry (IHC) shows high sensitivity and specificity in detecting MSI and could therefore offer a relatively cheap, easy to perform and universally available test for MSI, instead of a more complex polymerase chain reaction (PCR)-based MSI test [13,14].

### **KRAS**

The RAS-family of oncogenes consists of three members involved in tumor development, KRAS, HRAS and NRAS. Active KRAS mutations are found in 35–42% of CRCs and are thought to occur early in CRC carcinogenesis [15]. KRAS is part of the EGFR-signaling pathway downstream to EGFR, a

receptor tyrosine kinase which is activated through extracellular ligand binding. Activation of the pathway ultimately leads to the modulation of angiogenesis, cell migration, proliferation, cell adhesion, metastasis formation, and survival [16, 17]. Differences in KRAS mutations at codon 12 and 13 may result in different biological and functional consequences that could influence the prognosis of CRC. Initially, KRAS was found to be a strong prognostic factor in CRC, but this finding was later restricted to a codon 12 mutation, leading to a glycine to valine substitution (G12V).

American Society of Clinical Oncology recommends that all patients with metastatic colorectal carcinoma who are candidates for anti-EGFR antibody therapy should have their tumor tested for KRAS mutations. If KRAS mutation in codon 12 or 13 is detected, then patients should not receive anti-EGFR antibody therapy as part of their treatment [18,19]. The attempt to predict response to EGFR treatment by assessing EGFR expression by immunohistochemistry in analogy to HER2/new in breast cancer turned out to be unsuccessful [6]. Mutation of the KRAS gene results in a constitutively active KRAS protein and mitogen activated protein kinase (MAPK) pathway signaling independent from EGFR [6].

In general, KRAS mutational analyses concentrate on mutations in codon 12 and 13 with commercially available kits such as the 'Therascreen KRAS Mutation Test kit', which make up for 96% of all observed mutations. Other activating mutations have been identified in these codons and, additionally, in codon 61 and 146 of the KRAS gene.

Approximately 1% of tumors with wild type at codons 12 and 13 will have mutations in codon 146 and an additional 7% of these will be mutated in codon 61 (6,8,18,19). These mutations may very well predict resistance to anti-EGFR treatment as may mutations of the Neuroblastoma RAS viral oncogene homolog (NRAS) gene. It remains to be seen, whether expanded mutational analyses of KRAS and NRAS adds substantial additional predictive value [6,8].

## **BRAF**

The BRAF gene encodes a serine/threonine protein kinase belonging to the RAS-RAF-MEK-ERK kinase pathway regulated by KRAS protein activity and involved in CRC development. Nearly all oncogenic transformations of BRAF are the V600E mutations (8,20,21). The frequency of BRAF mutations

in CRC decreases with advancing UICC stage, approximately 8% of all CRC carry a BRAF mutation which is mutually exclusive to KRAS mutations [5,8,22].

After being primarily discussed as a potent predictive marker for resistance to anti-EGFR treatment, BRAF mutation has meanwhile been reported as a marker for poor prognosis in CRC in a number of retrospective analyses of large clinical trials.

The prognostic value of BRAF mutation is obviously influenced by the MSI status. In fact, patients with BRAF mutation and MSI had a favorable prognosis when compared with microsatellite stable (MSS)/ BRAF wild-type patients [5,8,22,23].

Immunohistochemistry (IHC) can be used in detecting BRAF mutation; it shows high sensitivity and specificity for BRAF V600E mutation.

### **Other potential biomarkers**

TP53 is a tumor suppressor gene on the short arm of chromosome 17, encoding a protein important in regulating cell division. It is aborting growth of potentially malignant cells. Mutations of the TP53 gene are detected in up to 85% of CRCs, usually occurring during the adenoma to adenocarcinoma transition (24,25,26). Lack of consensus on antibodies and scoring methods in immunohistochemical staining, lack of correlation between immunohistochemical overexpression and clinical data and discrepancies between immunohistochemistry and mutation analysis are responsible for conflicting results and are therefore important reasons for not justifying the use of TP53 in clinical practice [8,26,27].

Proliferation and ability to evade apoptosis is one of the most important attributes tumor cells must acquire for tumorigenesis. Ki-67 is used to determine proliferation in tumor cells but lack of uniformity in methodological approach and variations in the interpretation and reporting of pathologic findings are currently the most problematic issues associated with this factor. Further research should focus on combined analysis of proliferation and apoptosis, as a balance might exist between these two hallmarks of cancer [6,8,28-30].

Genomic signatures potentially have a high prognostic value and some are already in use in clinical practice, like Oncotype DX. Other genomic signatures need to be validated before introducing them in clinical practice, preferably using tissues from randomized clinical trials [6,8,31-33].

Activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway has been associated with the development of a human CRC, when stratified by KRAS status, a worse colon cancer-specific mortality associated with a PIK-3CA mutation was only found in KRAS wildtype tumors [8,34].

The prognostic value of 18q LOH also remains unclear and validation is necessary to draw further conclusions [35].

The existence of a new pathway for CRC pathogenesis which involves the transcriptional silencing of tumor suppressor genes by hypermethylation of CpG islands of the promoter region of various genes is increasingly studying. These tumors are classified as having the CpG island methylator phenotype (CIMP). CIMP could be used as a prognostic marker, but further research is necessary to confirm and validate these data [35,36,37].

## CONCLUSION

Our knowledge of the process of tumorigenesis has been increasing in the past decades and it affects the development of new treatment modalities in human cancer. Only a few biomarkers are currently used and in routine reported by pathologist. Future studies need to consider the combination of markers, standardising protocols and if possible simple, cheap, automated and standardized assays for the detection of molecular markers. Most importantly, results need to be validated in larger studies, followed by prospective trials.

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### Sažetak

#### **Kolorektalni karcinom, novi biomarkeri i uloga imunohistokemije**

Kolorektalni karcinom najčešći je maligni tumor u zemljama Europe te jedan od vodećih uzroka smrti od raka. Kombinirana terapija s lijekovima koji djeluju ciljano (tzv. pametni lijekovi) te sve više novih saznanja i novootkrivenih biomarkera koje tumor eksprimira značajno je povećala mogućnost selektivne terapije. Biomarkeri koji se trenutno sve više koriste kao prognostički i prediktivni, kao i oni koji su važni za izbor terapije opisani su u ovom pregledu. Među spomenute se najčešće ubraja mikrosatelitska nestabilnost zbog pogreške u popravku gena, RAS-obitelj onkogeni, BRAF, TP53, Ki-67, Onkotip DX, fosfatidilinozitol-3 kinaza (PI3K)/AKT, 18q LOH i CpG metilacijski fenotip. Trenutno je u široj upotrebi svega nekoliko markera te se rutinski spominju u patološkom izvještaju.

Buduće studije bi centar istraživanja trebale usmjeriti prema kombinacijama različitih markera, uspostavi standardiziranih protokola te jednostavnih i dostupnih analiza za otkrivanje ekspresije molekularnih markera.

**Ključne riječi:** kolorektalni karcinom; biomarker; mikrosatelitska nestabilnost; KRAS; BRAF.

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