



# Predictive biomarkers for response to neoadjuvant chemoradiotherapy in colorectal cancer

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**rezime** Different types of biomarkers can be analyzed at various levels and by different techniques in order to assess the response to neoadjuvant chemoradiotherapy (nCRT) in colorectal cancer (CRC). The clinical practice currently relies mostly on imaging markers, carcinoembryonic antigen (CEA), and tumor histopathology. Molecules that have predictive potential in CRC patients subjected to nCRT are those involved in cellular pathways that metabolize specific chemotherapeutics and protect the cell from radiation, but none of them have been approved in clinical practice. Candidate molecules investigated so far can be sorted in the following groups based on their physiological functions: 5-FU metabolism pathways, cell cycle and DNA repair, and oxidative stress and inflammation. However, differences in schedules and doses of chemoradiotherapy regimens, variations in intervals between nCRT and surgery, and non-standardized tumor response evaluation make comparisons of results among studies and drawing conclusions extremely difficult.

Key words: biomarker, colorectal cancer, neoadjuvant chemoradiotherapy

## INTRODUCTION

Surgical removal of the primary tumor is the mainstay treatment for colorectal cancer (CRC), while other modalities of adjuvant and palliative therapy include chemotherapy, external irradiation and molecular targeted therapy in selected groups of patients<sup>1</sup>. The standard treatment protocol for patients with locally advanced CRC includes neoadjuvant chemoradiotherapy (nCRT) followed by total tumor excision<sup>2</sup>. In general, nCRT is based on infusional 5-fluorouracil (5-FU) combined with conventional radiation doses of 1.8–2Gy per fraction over 5-6 weeks (total dose of 45–50.4Gy)<sup>3</sup>. Trials have high-

lighted lower toxicity, reduced local recurrence rates, and prolonged disease-free survival (DFS) of preoperative vs. postoperative chemoradiotherapy<sup>4,5</sup>. The main benefit from nCRT is tumor downstaging, which improves tumor resectability in most patients<sup>6</sup>. In a significant number of patients (10-30%), nCRT leads to a pathological complete response (pCR), defined as no residual cancer found on histological examination of the specimen<sup>6,7</sup>.

Predictive biomarkers enable stratification of patients based on clinical outcome in response to a particular treatment<sup>8</sup>. In CRC, predictive biomarkers have been extensively studied in metastatic disease, and some of them were validated as indicators of poor response to biological therapies targeted against epidermal growth factor receptor (EGFR)<sup>9</sup>. However, predictive biomarkers are far less investigated in patients with non-metastatic CRC subjected to nCRT and none of the markers studied so far have been approved in clinical practice<sup>10</sup>. Identification and validation of predictive biomarkers for response to nCRT would help clinicians to identify CRC patients who would probably benefit from this multimodal treatment. The discovery of biomarkers and their evaluation in larger, prospective trials and in combined predictive models could potentially be used to define tailored therapeutic strategies.

## MEASUREMENT OF RESPONSE TO NCRT IN CRC

There are different types of biomarkers that can be analyzed at various levels and by different techniques for the purpose of assessment of response to nCRT in CRC. The clinical practice currently relies mostly on imaging markers, in particular on magnetic resonance imaging (MRI) and positron emission tomography/computed tomography (PET-CT)<sup>7</sup>. Molecular imaging is a promising technique for the early identification of responders to nCRT, but it has still not widely used in clinical practice<sup>11</sup>. Diffusion weighted MRI (DWI-MRI) appears to be the best imag-

ing tool to predict response to nCRT, since several studies have reported its accuracy in predicting tumor regression and, in particular, pCR, with high sensitivity and specificity<sup>7</sup>.

Carcinoembryonic antigen (CEA) is a widely recognized marker for evaluation of response to nCRT and routinely used in clinical practice<sup>12,13</sup>. It is inexpensive, reproducible, and rapidly available. However, its relatively low specificity and use of different cutoff values question usability of CEA for follow up of nCRT effect. Decrease in CEA levels after nCRT are considered useful for predicting pCR and better patient outcome, but these data require validation in larger prospective trials.

Tumor histopathology before and after nCRT remains the essential tool in assessment of the response to nCRT<sup>14</sup>. The most frequently used histological indicators of response to treatment are downstaging, tumor regression grade (TRG) an pathologic complete response (pCR) defined as no viable cancer cells in surgical specimens.

Response to nCRT is highly variable and patients with CRC present with different responses in terms of both treatment efficacy and toxicity. Tumor regression after nCRT varies substantially among individuals, ranging from tumor shrinkage to complete disappearance at the primary site. However, differences in schedules and doses of chemoradiotherapy regimens, variations in intervals between nCRT and surgery, and non-standardized tumor response evaluation make comparisons of results among studies and drawing conclusions extremely difficult.

#### *MOLECULAR BIOMARKERS FOR RESPONSE TO NCRT IN CRC*

Different molecules are being explored in order to find a biomarker or a combination of biomarkers that can predict response to 5-FU and radiation therapy. Most research on improving nCRT outcomes has focused on modulating 5-FU and radiation doses in order to improve treatment effect on cancer cells. However, some inherited characteristics and some characteristics of the tumor have pivotal roles in determining treatment outcomes. Inherited genetic alterations in the molecules involved in cellular pathways that metabolize 5-FU and/or protect from radiation effect can modify the cellular response to nCRT. Changes in the tumor microenvironment induced by prolonged exposure to chemotherapeutics and/or irradiation may promote chemoresistance/radioresistance and tumor recurrence<sup>15</sup>.

Most candidate biomarkers are selected based on the knowledge of a molecule's involvement in the specific treatment-related molecular pathways. Molecules that have predictive potential in CRC patients subjected to nCRT are those involved in cellular pathways that metabolize specific chemotherapeutics and protect the cell from radiation. Candidate molecules investigated so far as potential biomarkers for response to nCRT can be sorted in the following groups based on their physiological functions: 5-FU metabolism pathways, cell cycle and DNA repair, and oxidative stress and inflammation<sup>16,17</sup>.

#### *5-FU METABOLISM PATHWAYS*

As the main backbone of combination chemotherapy in both the adjuvant and metastatic disease settings, 5-FU is the most commonly used drug in the treatment of CRC<sup>18</sup>. Infusion of 5-FU in combination with radiotherapy is recommended as a standard preoperative treatment in patients with locally advanced rectal cancer<sup>19</sup>. The primary action of 5-FU is irreversible inhibition of thymidilate synthase<sup>18,20</sup>. This drug also works as antimetabolite, mimicking the structure of the metabolic pyrimidines. Genes involved in pharmacological actions of fluoropyrimidines confer variants that can modify molecular actions of 5-FU, and hence affect the response to treatment.

Thymidilate synthetase (TYMS) is an essential enzyme in metabolism of nucleic acids. It converts deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), thus providing thymidines for the synthesis of DNA. With inhibition of TYMS, an imbalance in levels of deoxynucleotides occurs, which causes DNA damage. Expression of TYMS in tumor tissue and several polymorphisms in the TYMS gene promoter are candidates for predictive markers in CRC patients following nCRT. Lack of TYMS expression in the tumor tissue evaluated by immunohistochemistry was associated with tumor downstaging after nCRT<sup>21</sup>. The homozygotes for three 28bp repeat sequences of the variable number tandem repeat (VNTR) polymorphism in the TYMS promoter who underwent nCRT had a better response, as well as increased DFS<sup>22-24</sup>. Tumor and nodal downstaging rate was also associated with the presence of G at the 12<sup>th</sup> nucleotide in the second repeat of the VNTR polymorphism<sup>23,24</sup>. Polymorphism 6-bp insertion/deletion at position 1494 in the 3'-untranslated region of the TYMS gene may also be an important predictor for histopathological tumour regression in CRC patients receiving nCRT, since 6-bp deletion was associated with better response to therapy<sup>25,26</sup>.

Methylenetetrahydrofolate reductase (MTHFR) is involved in folate metabolism and is necessary for homocysteine remethylation to methionine, thus affecting the metabolism of pyrimidines. Natural variation in this gene is common in healthy people, and two MTHFR polymorphisms were studied as predictive markers of tumor response in gastrointestinal cancer patients submitted to nCRT, 677C/T (rs1801133) and 1298A/C (rs1801131)<sup>27</sup>. The MTHFR haplotype 677T/-1298A was shown to have the potential as predictive biomarker for response to therapy<sup>28</sup>. Carriers of 677CC genotype were less likely to respond to treatment than patients carrying 677TT genotypes<sup>29</sup>. Also, the genotype 677TT appears to contribute to the radioresistance<sup>30</sup>.

Although clearly relevant for response to 5-FU based therapy in CRC patients, TYMS and MTHFR variants were not confirmed as predictive biomarkers. Their significance and role in chemoradioresistance remain unclear due to controversial results obtained by different studies. Genetic variability in folate-metabolizing enzymes was found to be associated only to a limited degree with clinical outcomes and characteristics among

patients with CRC treated with nCRT. Further epidemiological studies on larger cohorts are needed to establish the contribution of TYMS and MTHFR genotypes and their combinations to the response to nCRT in CRC patients.

#### CELL CYCLE AND DNA REPAIR

Abberations in structure and function of molecules involved in cell cycle and DNA repair have been long recognized as causal factors for cancer development<sup>31,32</sup>. Numerous genes encoding molecules involved in the cell cycle are known oncogenes and tumor suppressor genes, and their deregulation leads to malignant transformation resulting in cancerous growth. Defects in the cellular DNA repair systems lead to mutations or chromosomal aberrations that affect oncogenes and tumor suppressor genes, thus indirectly contributing to the malignant transformation. In addition to being involved in malignant process and determining the molecular characteristics of the tumor, cell cycle and DNA repair molecules have an important role in response to chemoradiotherapy.

Cyclin-D1 (CCND1) is involved in the regulation of the G1 phase of the cell cycle, which precedes the DNA duplication and the cell division. Considering that radiotherapy targets dividing cells, variants in CCND1 may alter the response to radiation due to modulation of cell cycle control. The variant G870A (rs9344) of the CCND1 gene appears to be relevant for the response to nCRT. Patients carrying genotype AA had tumors with significantly higher radiosensitivity and the presence of A allele correlated with a lower risk of local failure and recurrence<sup>33,34</sup>. Contradictory results were obtained in another study that has analyzed multiple markers and pCR, which has shown that AA genotype correlated with non-pCR and that patients with at least one 870G allele had a better outcome.<sup>30</sup>

X-ray repair cross-complementing protein 1 (XRCC1) is involved in the efficient repair of DNA single-strand breaks formed by exposure to ionizing radiation and alkylating agents. The variants of XRCC1 were indicated to be involved in genetic susceptibility, as well as predispose patients to therapy response in human cancers, including CRC<sup>29,35</sup>. For Arg399Gln (rs25487) polymorphism in XRCC1 a pCR was found more frequently and longer survival was observed in carriers of G allele<sup>22</sup>. This variant is also associated with a greater probability of the major histopathologic response<sup>24</sup>.

V-Ki-ras2 Kirsten rat sarcoma viral oncogene (KRAS) is one of the most commonly studied molecules in CRC. The protein product of the normal KRAS gene performs an essential function in normal cell signaling, while mutations cause its constitutive activity that represents an essential molecular event in the development of various cancers<sup>36</sup>. Also, most biological therapies for CRC are directed against EGFR, and since KRAS represents its downstream effector, the role of this molecule is of importance for therapeutical decisions as well. Mutations in the KRAS gene detected in tumor samples of CRC patients are the indicators of poor response to anti-EGFR

agents. However, the role of KRAS mutations as biomarkers for response to nCRT is far less studied. Tumors positive for KRAS codon 13 mutations seem to be resistant to nCRT and these patients are less likely to achieve a pCR<sup>37</sup>. However, KRAS status in tumor tissue may change after nCRT<sup>38</sup>. The expression of EGFR was also shown to alter in response to nCRT, which should be taken into consideration for therapeutic decisions<sup>39</sup>.

In addition to CCND1, XRCC1 and KRAS, several other molecules involved in cell cycle and DNA repair may influence the response to nCRT in CRC. Upregulation of molecules that stimulate proliferation, migration and invasion, as well as downregulation of molecules that induce apoptosis and autophagy are generally associated with malignant process<sup>40</sup>. These molecules are extensively studied in response to therapy in metastatic disease, but severely understudied in patients with primary tumors treated with nCRT. A better understanding of these molecules, their roles and interactions of the highly complex cellular machineries that control cell cycle and DNA repair will lead to important improvements in therapy of cancers, including CRC.

#### OXIDATIVE STRESS AND INFLAMMATION

There is a growing evidence supporting the concept that reactive oxygen species and inflammatory mediators, which are known to be implicated in a range of diseases, may be important progenitors in cancers, including CRC. Considering that various environmental and lifestyle factors significantly influence gastrointestinal homeostasis, oxidative stress and inflammation may be of special importance for the development and characteristics of CRC<sup>41,42</sup>.

Superoxide dismutase 2 (SOD2) is a mitochondrial enzyme, member of the iron/manganese superoxide dismutase family<sup>43</sup>. Its role is to transform the toxic superoxide, a byproduct of the mitochondrial electron transport chain, into hydrogen peroxide and diatomic oxygen. By performing this function, SOD2 clears mitochondrial reactive oxygen species and confers protection against oxidative stress, ionizing radiation, and inflammatory cytokines. The variant 16Val of the A16V polymorphism (rs4880) was associated with reduced enzyme activity and in patients with the 16ValVal genotype a better response of the tumor to chemoradiation was observed<sup>44</sup>.

Interleukin 13 (IL-13) is a mediator of allergic inflammation and disease, with an important role in tumor immune surveillance<sup>45</sup>. The polymorphism 1112C/T (rs1800925) in the IL-13 gene was investigated as biomarker of response to nCRT. One study has found increased probability to obtain a favourable response in 1112CC genotype carriers<sup>44</sup>. However, these results were not confirmed by another study<sup>46</sup>.

Many endogenous and exogenous factors determine control of oxidative status and inflammation, and can also influence response to treatment. Inflammation and protection from oxidative stress confer a variety of cellular and molecular mechanisms and their interactions.

Both processes rely on delicate balance of many different molecules, therefore multiple factors can influence an individual's response to chemoradiotherapy. Future studies should investigate multiple molecules involved in control of oxidative status and inflammation in larger cohorts in order to estimate the clinical relevance of their alterations for the response to nCRT in CRC.

#### *FUTURE PROSPECTS*

The growing need for biomarkers able to predict response to nCRT in CRC has been additionally increased by introduction of watch and wait approach in the management of primary CRC. The watch and wait policy avoids the morbidity associated with radical surgery, but it could be considered a therapeutic option only in patients with pCR. Biomarkers that would enable assessment of patient's status in addition to histopathological examination after nCRT would be highly beneficial to identify these patients. The interval for watch and wait approach from the completion of nCRT to surgical resection is still under discussion and different hospitals rely on different protocols. These variations complicate optimization of the watch and wait protocol and molecular biomarkers for follow up of response to nCRT would significantly facilitate treatment personalization and disease management.

Assessment of response in CRC patients with primary tumors treated with nCRT in clinical practice currently relies mostly on imaging, histopathology and CEA analysis. The markers currently in use are insufficiently sensitive and often produce unsatisfactory results. Evaluation of complete response in clinical terms according to currently adopted criteria based mostly on imaging markers and CEA has low sensitivity and pCR often does not correlate with clinical complete response. However, none of the molecular biomarkers studied for this purpose have been validated for use in clinical practice. All the studies that have investigated molecular biomarkers had relatively small sample size and can be defined as exploratory, therefore no firm conclusions are available. More comprehensive studies are necessary in order to identify molecular biomarkers that could be used in stratification of patients prior to nCRT. Future studies will have to include analysis of multiple determinants of response to chemoradiotherapy. There is a growing consensus that chemoradiosensitivity is a highly complex trait, dependent on the interaction of many molecules involved in multiple cell processes. For this reason, future studies will have to rely on advanced technologies that can offer high throughput molecular analyses, such as next generation sequencing and gene expression microarrays.

Strategies for molecular biomarkers identification and validation for future use need to consider several different aspects in terms of methodological approach. The ultimate goal is identification of the single marker that would be able to predict response before therapeutical treatment. However, more realistic expectation is to discover two or more biomarkers that combined would be able to provide information on which patients would

most likely respond to nCRT. The combination of two or more markers also offers increased predictive power, and decreases the possibility of obtaining false negative or false positive results. In analysis of molecular biomarkers, the sample that will be used for analysis should be carefully considered. Tumor samples and healthy tissues present many differences in terms of molecular characteristics and genetic determinants. The blood samples are increasingly used as a source of material for cancer biomarkers, since they contain different types of molecules that originate from the tumor tissue. They are termed liquid biopsies due to the fact that they reflect molecular profile of the tumor at the primary site. The role of these noninvasive markers is potentially of great importance, and their use in combination with PET-CT, MRI and CEA may provide basis for more effective assessment of response to treatment. Along with predicting the response to treatment, noninvasive biomarkers also provide the opportunity to easily monitor patients during nCRT.

Identification and validation of predictive biomarkers is necessary in support of treatment schemes that have attempted to broaden the horizons of standard therapy by use of nCRT and watch and wait approach in order to achieve better patient selection and the avoidance of overtreatment and unnecessary adverse effects.

#### *SUMMARY*

#### *PREDIKTIVNI BIOMARKERI ZA ODGOVOR NA NEOADJUVANTNU HEMORADIOTERAPIJU U KOLOREKTALNOM KANCERU*

Za procenu uspešnosti odgovora na neoadjuvantnu hemoradioterapiju u kolorektalnom kanceru mogu se koristiti različiti biomarkeri i različite tehnike za njihovu analizu. Kliničke procedure trenutno se uglavnom oslanjaju na "imidžing" markere, karcinoembrionalni antigen i histopatologiju tumora. Molekuli koji imaju prediktivni potencijal kod pacijenata sa kolorektalnim kancerom koji se podvrgavaju neoadjuvantnoj hemoradioterapiji učestvuju u ćelijskim putevima koji metabolišu određene hemoterapeutike i tako štite ćeliju od zračenja. Međutim, do sada nijedan od ovih molekularnih biomarkera nije validiran za upotrebu u kliničkoj praksi. Molekuli koji predstavljaju kandidate za biomarkere se na osnovu njihove fiziološke uloge mogu podeliti u sledeće grupe: molekuli koji učestvuju u obradi 5-fluorouracila, molekuli koji učestvuju u regulaciji ćelijskog ciklusa i popravke oštećenja na DNK i molekuli koji učestvuju u regulaciji inflamacije i zaštite od oksidativnog stresa. Razlike u hemoradioterapijskim protokolima, varijacije intervala između hemoradioterapije i operacije, kao i nestandardizovana procena odgovora značajno otežavaju poredjenje među različitim studijama i donošenje pouzdanih zaključaka.

Ključne reči: biomarker, kolorektalni kancer, neoadjuvantna hemoradioterapija

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