The most important methods of colorectal cancer prognosis are TNM and Dukes staging, which have remained basic CRC classification systems for many years. In view of increasing incidence of this type of cancer, scientists are still looking for more advanced methods, allowing for early detection and better classification aimed toward personalized therapy. Some of such methods (diagnostic markers) could be implemented for screening of groups at risk. In parallel, new prognostic markers are also sought, which compared with the standard markers (invasion of cancer cells to lymph nodes, tumour size or histological malignancy) could have a greater ability to determine the prognosis of patients with colorectal cancer and facilitate the selection of optimal chemotherapy. In this article we will focus on the state and prospects of modern molecular diagnostics of colorectal cancer.

Key words: colorectal cancer, molecular markers, classification, DNA, mutation, diagnostic markers.

The state of contemporary molecular diagnostics of colorectal cancer

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Despite the progress in characterisation of colorectal tumours in terms of molecular biology, the prognosis of an individual patient with colorectal cancer (CRC) still depends on the grade of the disease at diagnosis. In consequence, some of the most developed countries have adopted screening programmes for CRC, whose purpose is to diagnose precancerous lesions and find asymptomatic cancers [1]. The most widely used tests are faecal occult blood test (FOBT), colonoscopy and the detection of genetic changes in cells isolated from faeces. According to the Fearon and Vogelstein model, benign colorectal adenomas gradually transform into an invasive form [2]; therefore removing the adenoma should prevent cancer progression. Molecular probes are useful in detection of changes in the patient’s genomic DNA isolated from faeces. The reason for the current disagreement with the widespread use of genetic methods for the diagnosis and prognosis is the necessity to meet several conditions [3]: the biomarker must show differential expression in normal tissues, premalignant changes and malignant tissue; the marker and the test in which it is assessed must both provide an acceptable level of accuracy in predicting the risk of developing or presence of the cancer; and the variability of the tests and intra- and interlaboratory variability must be clearly defined.

Diagnostic markers

Molecular diagnosis of CRC is likely to be developed mainly based on tests of DNA isolated from stool. Previously evaluated tests based on the determination of the level of carcinoembryonic antigen (CEA) in blood were found to have too low sensitivity and specificity, according to standardisation evaluated between different laboratories. Detecting changes in genomic DNA isolated from stool is based mainly on so-called defoliating markers (markers for colonocytes released from the outer layers of developing cancer). These include gene mutations in KRAS [4], TP53 [5], or APC, and microsatellite instability marker BAT26 [6]. Specificity of genetic tests is very high (95%), but there is still quite large variation in sensitivity of the tests (from 60 to 90%). Moreover, currently these tests are still relatively expensive and for that reason they are not being widely used in screening. CEA belongs to a family of glycoproteins originally detected in embryonic tissues and CRC. The level of CEA in the plasma can be relatively simply determined by radioimmunoassay. The usefulness of this test in the diagnosis of CRC is questioned, because high levels of CEA are also found in patients with other cancers (breast, pancreas, stomach, or lung [7]) and also in heavy smokers, or chronic diseases such as inflammatory bowel disease (IBD), and liver inflammation in alcohol addiction. Moreover, the clinical usefulness of this test for screening is limited by the fact that increasing levels of CEA usually occur only when the tumour penetrates the serosa; therefore CEA is useless in detection of tumours at an early stage [8].

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Prognostic markers

Prediction and prognosis of CRC patients and the option of chemotherapeutics with an optimal response are carried out mainly on the basis of molecular changes found between the tumour and normal mucosa. All of the abovementioned markers were evaluated by expert groups in the USA [9] and Europe [10]. Currently, none of them is recommended for clinical use. Progress in research on these markers and the standardization of techniques for their determination should contribute to their recommendation.

Chromosomal aberrations

Somatic mutations leading to chromosomal aberrations occur in approximately 85% of sporadic colorectal tumours [8]. Many of these changes have been described for the region 18q. Among the genes most frequently affected in this chromosomal region are DCC, DPC4 and genes of the TGF-β pathway. Loss of heterozygosity (LOH) at the 18q region may be tested by PCR or the genetic method can be substituted by immunohistochemical testing of proteins coded by genes from that region. LOH in the 18q region [11] or the region occupied by the DCC gene [12] is a predictor of survival of patients with stage II CRC. Loss of an 18q fragment containing at least two tumour suppressor genes (DCC, DPC4) is associated with the acquisition of a malignant phenotype by the tumour [13]. LOH within the 18q region was also associated with approximately 25% shorter overall survival (OS) in patients with stage III disease and high risk patients with grade II tumours. Generally, patients with a favourable history of the disease had no observable LOH in this region and showed no microsatellite instability [14]. For these reasons, changes in the 18q region are a promising predictive marker in patients with CRC.

P53 (TP53) gene

This gene, which is a central regulator of responses to DNA damage in the cell, is absent or mutated in approximately 50% of all cancers [2]. Most mutations lead to tumour progression, but some do not bring any substantial change in the functioning of the protein. Therefore, many studies have been conducted to clarify the relationship of P53 mutations with the results of CRC patients’ treatment, including the response to chemo- and/or radiotherapy, or aggressiveness of tumours, depending on their location. However, the results of these studies show large discrepancies, both in the methodology used (ranging from analysis of mutations to immunohistochemical methods) and the results obtained [15].

Microsatellite instability

The most commonly used test in the evaluation of microsatellite instability (MSI) is the length testing of five standard microsatellite sequences, none of which lies within a gene (BAT25, BAT26, D5S346, D2S123, and D17S250 [16]). There are also studies in which immunohistochemical detection of MMR proteins was used, although they are less frequent. In most of the reports, tumours classified as MSI-H give a worse prognosis of survival [8]. Determination of MSI status was also used to predict the response to chemotherapeutic treatment [17]. Some studies indicated a correlation between MSI-H phenotype and worse prognosis (compared with MSI-L/MSS) for grade II tumours [18], but there are also studies where the statistical analysis indicates an opposite correlation [19].

DNA content of tumour cells

Normal human cells, which are quiescent or active in the cell cycle (out of S phase), contain a set of 23 pairs of chromosomes, known as the diploid state; cells ready for division (in G2 phase) are tetraploid. The determination of DNA content during its synthesis (S-phase percentage) has a great diagnostic value and can be accomplished with a microscope and fluorescent probes or flow cytometry. Most studies on DNA content as a predictor for CRC patients use cytometry measurement. Cancer cells do not divide properly (they are aneuploid: with an abnormal number of chromosomes: one or more than two copies of the same chromosome), and are significantly different from normal. This test is not yet widely used in predicting CRC, because there are no conclusive literature data confirming the relationship between prognosis and tumour DNA ploidy status in this type of tumour [20, 21]. At the moment, ploidy is a recognized predictor only for neuroblastoma [22].

KRAS

Activation of this oncogene in tumour is achieved by gene amplification and selective mutations in tumour cells. Initial studies on the relationship between KRAS mutations and increased CRC aggressiveness showed the existence of such a correlation in many cases [23]. However, subsequent studies showed a lack of coherence and a large degree of variability of results [24]. There are also studies aiming at assessing the impact of KRAS mutations in metastatic lumps from patients without successful fluoropyrimidine/oxaliplatin/bevacizumab chemotherapy, and when use of FOLFIRI or FOLFOX4 therapy is planned [8].

New methods of molecular biology in the search for prognostic markers in CRC

With the advent of molecular techniques we are observing attempts to use them for diagnosis of various diseases, including CRC. In addition to the abovementioned “classical” tests, researchers have begun to evaluate the usefulness of such techniques as the detection of circulating tumour cells in the peripheral blood, microarray analysis of gene expression, tissue microarrays and proteomic analysis.

Circulating tumour cells

Detection of specific antigens of tumour cells circulating in peripheral blood of patients is based on the assumption that before the clinical appearance of metastasis, circulating tumour cells (CTCs) exfoliated from the primary tumour can be detected in peripheral blood. At this relatively early stage of cancer progression it is possible to detect up to
5 cells in 1 ml of peripheral blood and to detect the presence of specific mRNAs by RT-PCR or its more accurate version, real-time RT-PCR. Up to now the usefulness of this method for such genes as CEA, hTERT (telomerase), cytokeratin CK-19 and CK-20, or uPA (plasminogen activator, urokinase; PLAU) has been tested [25-27]. The best performing tests have implemented as many as six markers measured simultaneously with high sensitivity and specificity [25]. Despite these promising results and the fact that technology is becoming cheaper and more standardized, now there is a lack of studies on a larger number of patients (cited works were carried out at on a maximum of 200 patients [26], and the majority on less than 80).

**Real-time RT-PCR-based commercial assays**

The breast cancer OncotypeDX® test, approved by the FDA and recommended by two American cancer associations, is also based on real-time RT-PCR technique and the determination of expression levels of 21 genes in tumour tissues [28]. It is used to predict the success of chemotherapy and the likelihood of relapse in patients in early stages (I and II) of breast cancer without lymph node metastasis. In January 2010, Genomic Health announced a new test, OncotypeDX Colon Cancer Test®, which is based on measuring the expression levels of 12 genes and is used to determine the risk of recurrence in patients with CRC stage II. At the moment however, there is a lack of publications showing its true predictive value in CRC – a few citations in the company press release are only conference proceedings, and the test itself was assessed quite poorly by the experts from the online journal “PLoS Currents: Evidence on Genomic Tests” [29]. The test is based on the results of a study published by Genomic Health in 2007 [30].

cDNA microarrays

cDNA microarrays allow for examination of the expression of tens of thousands of genes in one experiment and obtaining a kind of molecular ‘signature’, which can be used for diagnosis and prognosis of patients with various forms of cancer [31]. So far, studies of CRC using this technique have failed to achieve effects similar as for breast cancer, namely the development of the test used by clinicians, the MammaPrint® test, based on a panel of 70 genes used to identify the risk of metastasis at a very early stage of tumour [32]. This does not mean, however, that the use of microarrays did not give promising results in CRC studies. This technique is also used for in vitro studies, especially for studies of genes involved in metastasis, and resistance to standard chemotherapeutics used in the treatment of patients with CRC [33].

**Tissue microarrays/proteomics**

Tissue microarrays (TMA) allow for examination of thousands of specimens of tumours in one experiment. Cylindrical biopsies of tumours are arranged in a single paraffin block, from which sections can then be obtained using a standard microtome. In the next stage the sections are subjected to standard immunohistochemical procedures to evaluate the expression of specific proteins. For subsequent sections, one can apply different antibodies, which greatly accelerates the pace of testing for a large group of patients and several markers [34]. TMA is used for evaluation of new antigens or antibodies using large sets of tumours of different stages simultaneously. The undeniable advantage of TMA over standard histopathological studies is that they can be automated and have standardised result analysis. This technique is still developing, but the number of studies in which it was applied to study CRC is already quite large and is growing fast. Recently, several studies were published on the expression of proteins associated with carcinogenesis in the colon and its role in survival prognosis of patients: a reduction of expression of Bax-interacting factor, Bif-1, paralleled the progression of CRC [35].

Another fast evolving branch of medical analysis is proteomics. Although, strictly speaking, proteomic techniques also include standard TMA and immunohistochemistry (they both examine protein levels), recently the meaning of that term is narrowing to mass spectrometry analysis and its two main techniques: SELDI (surface-enhanced laser desorption/ionization) and MALDI-TOF (matrix-assisted laser desorption/ionization – time of flight). They are based on the ionization of molecules and subsequent detection of the number and relative weight of the resulting ions [36]. Several studies concerning the application of these techniques in CRC have shown very promising results, revealing the existence of new potential markers of the disease [37].

**Summary**

All above “-omic” methods allow one to obtain profiles with different subtypes of CRC and increase the level of biological understanding of cancer, which should also translate into the discovery of new prognostic and predictive markers. The increasing application of these techniques allows one to perform integrated analysis of molecular profiles of different stages/subtypes of the disease at three levels: genomic, proteomic, and transcriptomic [3]. New technology constantly provides revealing information about the potential opportunities to facilitate early detection or new personalized therapeutic purposes, and perhaps even preventing cancer disease. It is commonly believed that molecular diagnostic medicine is able to sooner or later complement or even in many cases fully substitute pathological or clinical classification systems such as TNM or Dukes staging.

At the moment, however, the methods described above cannot be directly transferred to the clinic (with the exception of the already described validated commercial tests). Too many newly discovered prognostic factors, very promising in the initial studies, make the pursuit of all information not only impossible, but harmful. Firstly, both scientists and clinicians may be overwhelmed by the enormity of data generated using such methods. This problem is best illustrated by the fact that until recently it was taken for granted that only 1.1% of the genome contains exons encoding proteins, another 24% consists of intron sequences and the remaining 75% of intergenic DNA had no known function.
in RNA transcription or translation [3]. This situation changed to some extent with the discovery of microRNAs [38, 39]. Secondly, although the molecular markers are relatively non-invasive and suitable for use in large-scale screenings, the implementation of such tools requires strict monitoring of each stage of this process, namely the evaluation of diagnostic accuracy and reliability, cost assessment and comparison of the effects of risk in relation to the benefits [3]. Thirdly, the cost of molecular medicine (especially the current patent costs) may increase disproportionately to the benefits achieved in the treatment of patients, which may prevail in the debate on the benefits of molecular medicine on a large scale.

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