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Review Article

Laboratory Diagnostics of Colorectal Cancer

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Background

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the fourth leading cause of cancer death in the world.[1] Within Asia, the incidence of CRC is lower in South Asian developing countries and higher in developed Asian countries.[2] Prevalence of CRC is 6.5/100,000 in male and 2.7/100,000 in female in Bangladesh.[3] Colorectal cancer is a multifactorial disease process. Etiology contributing from environmental factors including dietary factors, obesity, alcohol intake, smoking, life style and genetic and epigenetic abnormalities. Early diagnosis and combined modality treatment can reduces CRC related morbidity and mortality. The limited response to conventional therapies for this aggressive tumor has been improved by introducing targeted therapies with monoclonal antibodies such as anti-EGFR (cetuximab and panitumumab).[4,5] *KRAS, NRAS, BRAF, PIK3CA* and *AKT1* mutation of these genes were negative predictors of response to targeted therapies with anti-EGFR antibodies.[6] Therefore, determining the mutational status of tumor samples has become an essential tool for managing patients with colorectal cancers.

Diagnostics of CRC

Colorectal cancer commonly develops slowly over many years. Screening and early detection are excellent measures for the secondary prevention of colorectal cancer and associated death.

Occult blood test

For screening purpose most widely used methods are gFOBTsguaiac fecal occult blood tests (gFOBTs) and fecal immunochemical tests for hemoglobin (FITs). These tests detect microscopic amounts of blood by targeting either heme (gFOBTs) or human globin (FITs).[7] The limited impact of gFOBT testing is due to its limited sensitivity for advanced adenoma with 10−15% and cancer with 30−35%. In contrast to gFOBTs, FITs are specific for human globin and have a higher sensitivity for advanced adenoma and cancer.[8]

Colonoscopy

Colonoscopy is the gold standard as a diagnostic tool for the colon and serves as the method of choice for the further work-up of positive stool tests and sigmoidoscopies. Colonoscopy was able to reduce the risk of dying from colorectal cancer by 68%.[9] Colonoscopy allows biopsy samples to be taken for definitive diagnosis with a simultaneous opportunity for a therapeutic polypectomy, therefore improving a long-term prevention of CRC deaths.[10] However, patients with tumor related stenosis, older patients and those with comorbidities are more likely to have an incomplete or difficult OC.[11,12]

Fecal calprotectin

Since 1992 fecalcalprotectin has been claimed to be a valuable parameter in screening andsurveillance of CRC risk subject. Calprotectin is a marker for inflammatory as well as neoplastic processes in the gut[13,14,15], and thus gives added information to occult blood testing in the diagnosis of CRC. Calprotectin levels were significantly increased in symptomatic as well as asymptomatic CRC patient. A cut-off limit of 50 mg/g resulted in a sensitivity of 87%-98% for symptomatic and 64%-82% for asymptomatic CRC. Within 3 months of CRC treatment, calprotectin levels are significantly decreased. Fecal calprotectin measurement can reduce the number of invasive procedures necessary in screening and post treatment surveillance programs for CRC.[16]

Imaging techniques

Recent developments in imaging technologies and validation of newer imaging techniques may lead to significant improvements in the management of patients

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with CRC. Currently applied imaging modalities such as virtual computed tomography colonoscopy, endorectal ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI), X-ray are used in primary diagnosis, initial clinical staging, selection of patients for neoadjuvant therapy and therapeutic response evaluation.[17] The accurate diagnosis of local tumor extension, location, T stage, potential circumferential resection margins, mesorectal fascial involvement and extramural or venous invasion is essential for defining the treatment strategy.[18]

Tumor markers

Tumor markers are applicable in screening tests, differential diagnostics, monitoring treatment and detection of recurrences. They may differentiate malignant from benign tumor in case of unspecific histological image.[19] Markers may be assayed in blood, urine and other body fluids.[20] For CRC most commonly used markers are carcinoembryonic antigen (CEA), cancer antigen (CA) 19-9, tumor antigen of colorectal cancer (tumor-associated glycoprotein, TAG-72), tissue polypeptide specific antigen (TPS) and TAG-72. Increase of tumor markers rarely occurs in early stages of the disease; usually it is observed in severe tumors.An increased level of CEA (> 5μg/ml) before the operation may correlate with adverse prognosis.[20] Tumor markers that used for CRC diagnosis have less sensitivity and organ specificity. Sensitivity and specificity increase along with the simultaneous assessment of several markers.[21]

Molecular diagnostics

Molecular testing can detect sporadic and inherited colorectal cancers that arise through the micro-satellite instability pathway and can determine the efficacy of targeted drug therapy. Considering that the field of medical diagnostics is moving towards molecular diagnostics, CRC can now be effectively screened and diagnosed with high accuracy and sensitivity.

Detection of oncogene mutations

Accumulation of several mutations in the tumor suppressor genes and proto-oncogene plays key role in promoting CRC. Among these, mutations of *KRAS, NRAS, BRAF, PIK3CA* and *AKT1* genes are more prevalent.[22] Worldwide prevalence of *KRAS, NRAS, BRAF* and *PIK3CA* somatic mutations in CRC are 36-40%,1-6%, 5-10% and 10-30%respectively.[23,24,25,26]

Sanger sequencing

Sanger sequencing is the earliest form of first-generation direct sequencing. Sanger sequencing was developed in 1975 and relies on the chain-termination sequencing of amplified DNA by polymerase chain reaction (PCR) and detection through electrophoresis.[27] Among the wide range of mutation detection techniques sequencing has been the gold standard.[28] Direct sequencing has a reported a limit of detection of approximately 10-30% mutant alleles.[29] Sanger sequencing is 10 times less sensitive than pyrosequencing and also it requires 18-19 hours for processing. Due to its limited sensitivity, high costs and long turnaround time have prompted the development of alternative methods for routine clinical testing that have greater diagnostic practicality for somatic mutation detection.[30]

Pyrosequencing

Pyrosequencing is the first alternative to the conventional Sanger method for de novo DNA sequencing. This method is based on the luminometric detection of pyrophosphate that is released during primer-directed DNA polymerase-catalyzed nucleotide incorporation.[31] Advantages of pyrosequencing is accuracy, flexibility, parallel processing and it can be easily automated. The method has been proven highly suitable for detection of *KRAS, NRAS* and *BRAF* gene mutation. Pyrosequencing was able to clearly identify *KRAS* and *BRAF* gene mutation containing 5% mutant alleles.[32]

High-resolution melting analysis (HRMA)

is a recently developed methodology that has enormous potential for the detection of DNA sequence changes.[30] Mutation scanning with HRMA is based on the dissociation behavior of DNA when exposed to an increasing temperature, in the presence of intercalating fluorescent dyes. The HRMA melting profile gives a sequence-related pattern, allowing discrimination between wild sequences and homozygote−heterozygote variants.[33] Owing to its high sensitivity, HRMA seems to present a more sensitive approach, allowing rapid, accurate and reliable detection of a minimal fraction of mutated cells in tumor tissue.[33,34] Limit of detection of this method is as low as 5% mutant alleles.[32]

PCR RFLP

To perform PCR-RFLP, the region surrounding the mutation is amplified and the mutation is detected by cutting the amplicon with the appropriate restriction enzyme. Mutations can inactivate a naturally occurring restriction site or generate a new restriction site so that digestion of the PCR product results in cutting of the mutant amplicon but not the normal control amplicon or vice versa.[35] PCR-RFLP method recommended in cases where large tumor samples are available, with rich cell content and where the DNA concentration is higher than 100 ng/μl.

Real Time PCR

Real-time PCR methods are becoming popular in molecular diagnostics and may replace the gold standard of Sanger sequencing or other methods with an insufficient limit of somatic mutation detection in the present quantitative PCR era.[36] This assays can detect less than one-percent of mutant in a background of wild-type genomic DNA and have limits of detection of 5 to 10 copies.[37,38] The molecular method is chosen for somatic mutation screening, not only costs but also several other factors should be taken into consideration such as the best limit of detection, sensitivity (false negatives) and specificity (false positives), reproducibility and reasonable turn-around time.[32] This real time PCR is only able to detect mutations targeted by the designed primers.

COLD PCR (co-amplification at lower denaturation temperature PCR)

In COLD-PCR, the critical denaturation temperature is lowered to 80°C (vs 94°C in conventional PCR). The sensitivity of COLD-PCR was determined by assessing serial dilutions. COLD-PCR is up to four times more sensitive than the conventional PCR method, able to detect 1.5% of tumor cells with KRAS mutation.[39]

Immunohistochemistry

One of the most common detection methods used in clinical laboratories is immunohistochemistry (IHC), which detects cellular markers and phenotypes specific to certain diseases through staining with highly specific antibodies.[40] Since IHC can be done on both fresh and formalin-fixed tissues, it serves as a convenient, simple, and cost-effective platform for clinical diagnosis. Immunohistochemistry can be performed within 24 hours. It is possible to assess diagnosis of malignant tumors only by the presence of scattered cells in the specimen. However, its validity relies on the specificity of antibodies which must recognize only the mutated protein.[41]

MSI (Micro satellite instability)

About 15% of CRCs arise through the MSI pathway and most of these tumors are sporadic. A small percentage of CRCs that arise via the MSI pathway are inherited as the result of a germline mutation in one of the mismatch repair (MMR) genes (Lynch syndrome/hereditary nonpolyposis colon cancer [HNPCC]; about 2% to 5% of all cases of CRCs). The most common mechanism of MMR inactivation is through an acquired methylation of the MLH1, MSH2, MSH6 and PMS2 genes promoter[42], and in particular hMLH1 was not expressed in most of sporadic CRCs. On this basis, of how many genes promoter are mutated by the degree of MSI can be categorized as high MSI (MSI-H) when two or more genes are involved, low MSI(MSI-L) if only one marker is involved, and MSS if none.[43] There are several methods in which MSI can be detected. Immunohistochemistry, Next-generation sequencing (NGS), Fragment analysis, Gene expression assay and miRNA microarray.[44,45,46,47]

Other molecular biomarkers

The complex genetic nature and heterogeneity of CRC necessitate the detection of a combination of biomarkers for a more accurate diagnosis. Others molecular markers includingMethylated Septin9 (mSEPT9), Programmed Death-Ligand 1 (PD-L1), Phosphatase and Tensin Homolog (PTEN), Human Epidermal Growth Factor Receptor 2 (HER2) and Micro-RNA (miRNA). These markers can be detected by real time PCR and immunohistochemistry. MethylatedSeptin 9 (mSEPT9) DNA is only molecular marker that used for the screening of CRC through the detection of cfDNA in the serum of CRC patients.[48,49] The sensitivity and specificity of mSEOT9 in CRC stages is 90% and 88% respectively which is greater than traditional screening methods such as fecal occult blood test (FOBT) and fecal immunochemical test (FIT).[50] Micro RNA is a prognostic factors and PD-L1, PTEN, HER2 are predictors of targeted therapies.

Conclusion

CRC is the most prevalent cancers in the world and mostly diagnosed in advanced stages. Early diagnosis, combined modality treatment and post treatment surveillances is important to reduces CRC related morbidity and mortality. Molecular methods can early diagnose CRC and the variety of molecular technologies available to map out the individual's unique molecular profile for efficient treatment strategies. Molecular methods for biomarker detection require sophisticated equipment, facilities, and trained personnel, resulting in the need for centralization. This necessitates the development of simple, automated and robust biomarker detection platforms with smaller footprints.

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