Molecular and Morphometric Tools for Next-Generation Pathology Diagnosis of Colon Carcinoma

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ABSTRACT: Colorectal carcinoma (CRC) is a common malignancy associated with considerable morbidity and mortality. While CRC affects a single organ, it is quite a heterogeneous disease. Good diagnostic tools are required to determine patients' prognosis and to choose the most effective treatment option. In the past, diagnosis was based solely on morphological features of the tumor which were evaluated by pathologists using light microscopy. These morphological parameters include architectural abnormalities, cytological and nuclear changes, invasion into vascular structures, tumor budding, and the presence of intra-epithelial inflammatory infiltrate. In the last decade, with the development of better DNA sequencing technologies and improved understanding of the molecular basis of cancer, genetic tools have been incorporated into the routine clinical and pathological practice. These include tests for point mutations in oncogenes such as *KRAS*, *NRAS*, *BRAF* and *PIK3CA*, which have prognostic and treatment decision implications, as well as tests for defects in DNA mismatch repair genes. More recently, it was demonstrated that molecular tests based on RNA expression patterns might provide useful information that can help guide patients' management. In the present review we discuss the morphologic and molecular parameters relevant to the management of CRC patients. Additionally, we suggest how a combination of both tools might be useful for addressing some newer concepts in cancer biology, such as intra-tumor heterogeneity and nuclear structure alterations.

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Colorectal cancer (CRC) is the third most common cause of cancer death in all age groups. In the last decade there has been a decline in CRC incidence and associated mortality, both attributed to CRC screening tests and risk factor modifications [1]. Screening colonoscopy, detecting both cancer and precancerous lesions (adenomatous polyps), played a major role in the reduction of CRC incidence and death rates [2].

Cancer is the result of mutation acquisition in the cells. Driver mutations may involve oncogenes or tumor suppressor genes, as well as genes encoding proteins that regulate apoptosis and DNA repair. Continuous acquisition of mutations leads to progression from normal, through benign neoplasms to malignant tumors. The malignant tumor may acquire more mutations that can cause further progression and metastasis. The presence of a continuous and progressive process, termed adenoma-carcinoma sequence, is supported by the fact that removal of adenomatous polyps by colonoscopy leads to reduction in CRC incidence [3]. Additionally, histologic examination often demonstrates adenomatous remnants around areas of colorectal adenocarcinomas in different stages, with reduced frequency in more advanced tumors [4].

The adenoma-carcinoma sequence in the colon is characterized by morphologic and molecular changes that have been studied extensively [4]. Morphological features have been the basis for pathological diagnosis of colon carcinoma for years. Morphology-based staging and grading systems were the only tools used to determine prognosis and therapy. In recent years, there has been a change in this approach with the identification of somatic mutations occurring at the early (*APC*, β-catenin pathway), middle (*KRAS*), and late (*TP53*, *SMAD2*, *SMAD4*) stages of the adenoma-carcinoma sequence [4], as well as a molecular pathway that is caused by defects in DNA mismatch repair genes. The molecular characterization of CRC initiation and progression has led to the development of molecular tests that provide information about CRC risk, prognosis, and response to therapy.

In this review we present the common morphologic and molecular features that are evaluated in the routine management of patients with colon carcinoma. Additionally, we will discuss some newer concepts in CRC biology and how molecular and morphologic tools can lead to more accurate and informative diagnoses.

MORPHOLOGICAL FEATURES IN CRC DIAGNOSIS

Current diagnosis of malignancy is principally based on examination of the morphology of tumor on a hematoxylin and eosin (H&E) stained tissue slide using light microscopy. Classical signs of malignancy include lack of differentiation (anaplasia), local invasion and distant metastasis. Anaplasia is characterized morphologically by cytologic changes such as cellular

pleomorphism, abnormal nuclear morphology and atypical mitoses, as well as architectural changes such as loss of polarity. These features are integrated by pathologists in the diagnosis of tumor grade and stage. In CRC, several morphological features have been associated with clinical features. These include tumor grade, vascular invasion, tumor budding and intra-tumoral lymphocytes, which will be discussed.

- **Tumor grade**

Traditionally, tumor grade was based on morphological features alone and classified as well, moderately and poorly differentiated. Currently, the terms low grade and high grade are preferred. Low grade refers to well or moderately differentiated adenocarcinomas that demonstrate more than 50% gland formation [Figure 1A] [5]. High grade refers to poorly differentiated tumors, with less than 50% gland formation [Figure 1B] [5]. Tumors that do not show any gland formation or other specific morphologic features are termed "undifferentiated carcinoma." Tumors with higher grade are associated with worse prognosis [5]. One exception is tumors with defective DNA mismatch repair proteins termed microsatellite instability high (MSI-H), which are regarded as low grade, irrespective of their degree of differentiation, because of their favorable clinical behavior [6].

- **Vascular invasion**

Vascular invasion in colorectal carcinoma was first recognized as a predictor of distant metastases in 1938. It refers to the presence of tumor cells in lymph or blood vessels (mainly veins). Venous invasion is associated with higher rates of disease progression and disease-related death [7]. Routinely, vascular invasion is detected in H&E stained sections. The use of a histochemical stain for elastic fibers may increase detection rates of vascular invasion [Figure 1 C&D]. In recent years the use of specific immunohistochemical stains for lymph endothelium (D2-40, CD34) has been investigated, but the prognostic value of this remains unknown [8].

- **Tumor budding**

Tumor budding refers to the detachment of single or small clusters of cells (usually a maximum of five cells per cluster) from the invading tumor glands [Figure 1E]. This morphologic feature is attributed to the biologic feature of epithelial-mesenchymal transition [9]. Tumor budding is associated with lymph node metastases in stage I disease, worse prognosis in patients with stage II-III disease, and predicts poor response to anti-epidermal growth factor receptor (EGFR) therapy in metastatic tumors [9]. Sporadic MSI-H tumors show no tumor budding, corresponding with the better prognosis of these tumors. In contrast, tumors associated with Lynch syndrome (HNPCC), which is also MSI-H due to a germline mutation in mismatch repair genes, do show tumor budding (up to 20%), probably because of associated somatic mutations in the Wnt pathway [9]. Tumor budding is divided into low and high grade, the cutoff level being 10 buds in 0.375 mm^2 (20x field), yet this cutoff has not been validated as a predictor of prognosis [9].

In the past, diagnostic pathology of tumors was based on morphological analysis of tissue slides alone

- **Tumor-infiltrating lymphocytes**

Tumor-infiltrating lymphocytes represent the immune system activity against the tumor [Figure 1F]. Studies on tumor-infiltrating lymphocytes reveal a favorable prognosis and better response to therapy in patients with high densities of T cytotoxic (CD8+) cells, and worse prognosis in patients with high densities of T regulatory cells [10]. Tumors that tend to

Figure 1. Morphologic features in the diagnosis of CRC. Tumor grade is determined by the percentage of gland formation in the tumor, with › 50% gland formation defined as low grade **[A]** and ‹ 50% gland formation defined as high grade tumor **[B]**. High grade tumors also show increased mitotic count (arrowhead). Identification of vascular invasion within the tumor can sometimes be challenging in H&E stained slides **[C]**. Histochemical staining for elastic fibers can highlight the vascular wall and increase detection of tumor cells within the vessel **[D]** (arrow). Tumor budding is the detachment of single or small groups of tumor cells (arrowheads) from the invasion front of the tumor **[E]**. Intraepithelial lymphocytes **[F]** are surrounded by a clear halo in many slide preparations.

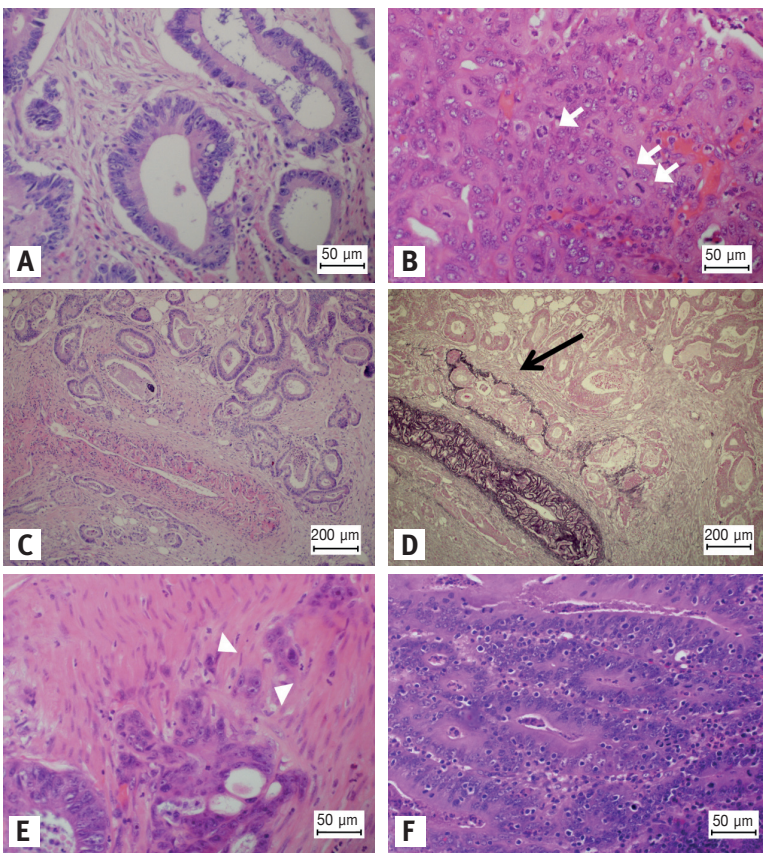


Figure 2. The epidermal growth factor receptor (EGFR) signaling pathway. Activation of the EGFR pathway is common in CRC, leading to cell proliferation, inhibition of apoptosis, angiogenesis and metastasis in malignant tumors. Following binding of ligand, EGFR initiates a signaling cascade that results in activation of two branches: the RAS-MAPK pathway and the PI3K pathway. Genes from this pathway carry mutations in different percent of CRC cases (indicated in brackets). Several biological treatments were shown to be effective in targeting different proteins in this pathway, including anti-EGFR monoclonal antibodies and *BRAF* kinase inhibitors. *PIK3CA*-mutated tumors show response to treatment with aspirin.

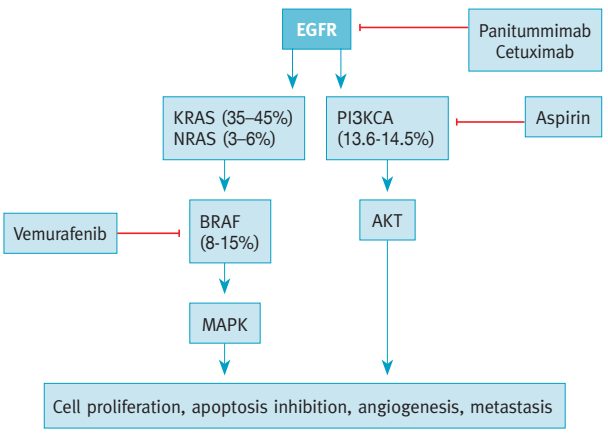
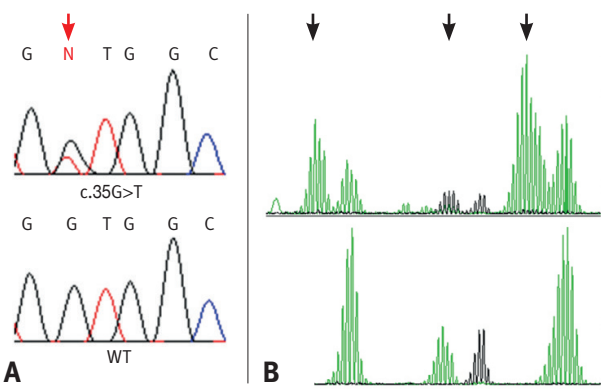
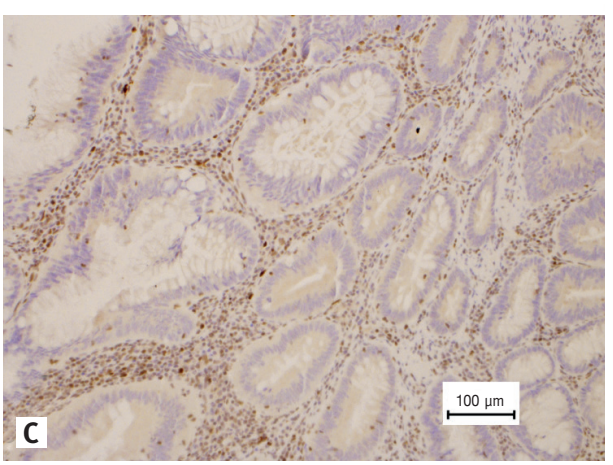


Figure 3. Molecular tests in the diagnosis of CRC. [A] Direct sequencing of *KRAS* shows a missense mutation, c.35G>T in codon 12 (upper panel, arrow). The wild-type sequence is given for reference (lower panel). **[B]** Genotyping analysis of microsatellite markers shows microsatellite instability (upper panel) in a case of colon carcinoma. This is characterized by additional peaks (arrowheads) compared to the normal tissue (lower panel). The additional peaks are the consequence of defect in DNA mismatch repair mechanisms. **[C]** Analysis of the same case by immunohistochemistry for the mismatched repair proteins showed loss of nuclear staining for MSH2, a mismatch repair protein, in the tumor gland nuclei. Note the positive nuclear staining in the non-tumor stromal cells that serve as an internal positive control.





recur show a lower level of immune reaction compared with tumors that do not recur, irrespective of tumor stage [11]. This may indicate that a stronger host immune response serves to control micrometastasis and reduce the risk of progression to frank metastasis.

MOLECULAR FEATURES IN CRC DIAGNOSIS

For many years the morphological parameters were the only tool used for diagnosis and classification of CRC. Recent advances in our understanding of the molecular basis of cancer helped move the field of oncology into the era of targeted biological therapy. The first targeted therapy used in CRC was bevacuzimab, an anti-vascular growth factor receptor (VEGF) agent, which inhibits tumor angiogenesis. While this treatment was targeting the angiogenesis pathway, which is ubiquitously essential in CRC, more recently developed drugs target specific molecular pathways that are activated only in a subset of tumors. This has led to the development of molecular diagnostic tools that can help select the most suitable treatment for each patient. Additionally, some molecular tools also provide information about disease prognosis and familial risk for CRC.

RAS FAMILY GENE MUTATIONS

The most important signaling pathway in CRC is the EGFR signaling pathway [Figure 2]. Mutations in different components of this pathway are seen in most cases, making this the focus of research of molecular testing and targeted therapy. The most commonly mutated gene in this pathway is *KRAS*, a member of the RAS gene family. The RAS family comprises GTP/GDP binding proteins downstream to growth factor receptor tyrosine kinases stimulating the MAPK signaling pathway. A mutation in *KRAS* is an early carcinogenic event, present in 35–45% of colon cancers [12]. The most common alteration is a missense mutation in codons 12/13 [Figure 3A] causing constant activation of downstream signaling pathways [12]. Tumors with the *KRAS* mutation respond poorly to targeted therapy with antibodies for epidermal growth factor receptors (EGFR) – cetuximab and panitumumab [12]. Recently, additional mutations in codons 61 and 146 were recognized

in approximately 2% of colorectal carcinomas, and have been shown to cause resistance to anti-EGFR therapy [13].

A subset of patients with wild-type *KRAS* also show poor response to anti-EGFR therapy. Some of these tumors were found to harbor mutations in *NRAS*, another member of the RAS family. Mutations in *NRAS* are found in approximately 3–6% of CRCs, up to 12% of patients with wild-type *KRAS*, a large enough proportion to justify routine testing and avoiding ineffective therapy [14]. First-line treatment of RAS-negative CRC with anti-EGFR antibodies is associated with improved response rate, increased resectability of liver metastases, and prolonged overall survival compared to *KRAS* or *NRAS* mutant tumors [15].

BRAF MUTATION

Another frequently mutated protein in CRC is *BRAF*, a serine/threonine kinase downstream from the RAS proteins, which activates the MAPK pathway. Eight to fifteen percent of tumors show the p.V600E mutation in *BRAF* [13,14,16]. Though effective in BRAF-mutated melanomas, a mutation-specific *BRAF* inhibitor (vemurafenib) was associated with low response rates in colon cancer [16]. This is probably due to activation of the EGFR signaling cascade via other pathways such as MAPK, RAS and CRAF [16]. Though not considered a contraindication for anti-EGFR antibody treatment, recent studies indicate reduced response of *BRAF* mutation-positive tumor to such therapies [14]. More recent studies suggest a beneficial effect of the combination of anti-EGFR antibodies and *BRAF* kinase inhibitors [14]. The addition of anti-EGFR antibodies to anti-*BRAF* therapy was shown to act in synergism, thus providing a new therapeutic opportunity for tumors that until recently had no targeted treatment options [16].

Today the morphology is supplemented by a molecular test, which can support diagnosis and provide additional predictive and prognostic information

BRAF mutational status is also of prognostic significance. Mutant *BRAF* is associated with shorter overall survival, especially in patients with MSI-low (MSI-L) or MSI-stable (MSS) status [17].

PIK3CA MUTATION

The other arm of the growth factor receptor signaling pathway is the Akt pathway. This pathway is activated by PI3K proteins, which are mutated in 10%–30% of CRCs [18]. Mutant *PIK3CA* is morphologically associated with mucinous tumors, and at the molecular level may be associated with *KRAS* mutation and alterations in p53 and β-catenin [18]. *PIK3CA* is associated with worse prognosis and inferior response to anti-EGFR treatment [18]. Interestingly, aspirin inhibits the PI3K pathway and improves survival in tumors harboring *PIK3CA* mutation [19].

- **Microsatellite instability (MSI)**

Genomic instability and mutator phenotype is one of the hallmarks of cancer. This is commonly the consequence of defects in DNA repair mechanisms. In 15–20% of CRC cases there is a deficient mismatch repair (dMMR) system as a result of either germline mutations (Lynch syndrome) or sporadic epigenetic silencing [20]. The dMMR system repairs "spell errors" that occur during DNA replication.

Tumors can be classified into MSI-H (high), MSI-L (low) and MSS (microsatellite stable). MSI-H tumors have a favorable prognosis, regardless of histologic grade, and are therefore referred to as low grade tumors [6]. They are associated with a lower frequency of distant metastasis [21]. The influence on prognosis is more significant in stage II patients compared to stage III patients. In terms of medical therapy, in the adjuvant setting dMMR tumors show resistance to 5-FU [22] and beneficial effects of combined treatment with 5-FU, leucovorin and irinotecan [22].

Due to the potential of identifying family members with risk for developing CRC and since molecular screening is more accurate than clinical criteria for diagnosis, a global screening program for Lynch syndrome is recommended [23]. If in the past screening was proposed for patients younger than 50 with a family history of colorectal or endometrial carcinoma or a personal history of multiple colorectal or endometrial carcinoma, today this has been extended to all patients younger than 70 or with a familial history of CRC [24]. Screening may be done by genotyping or by immunohistochemical staining for MMR proteins with similar results [20] [Figure 3 B&C]. The expression of four proteins is being tested routinely: MLH1, MSH2, MSH6 and PMS2. A negative immunohistochemical stain for one or more of these proteins calls for further analysis to determine genetic (mutational) or epigenetic nature for the lack of protein production [20].

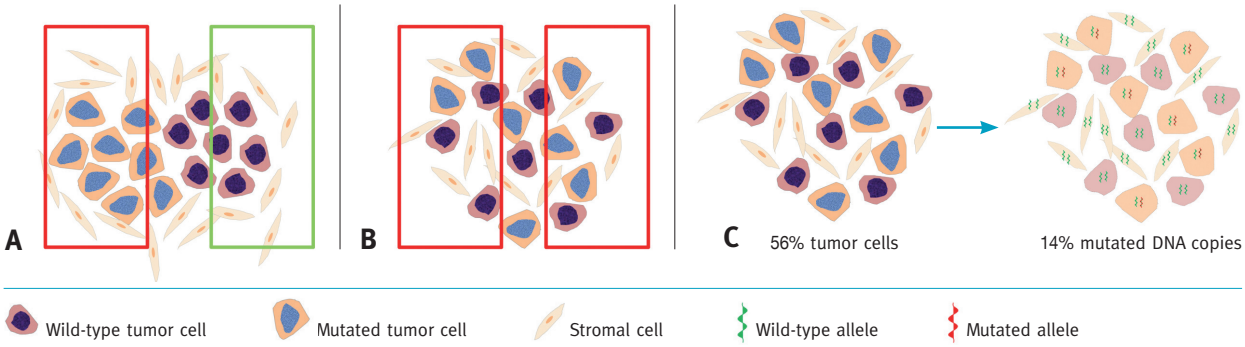
- **Gene expression patterns**

Apart from the genetic mutations, an additional mode for characterizing tumors is based on gene signatures. Apparently, RNA levels from certain genes are differentially expressed in different tumor subtypes, and RNA expression analysis might allow tumor sub-classification and even stratification of patients to different prognosis and treatment groups.

Specifically, in colorectal carcinoma, gene expression profiling can classify CRC into three to six molecular subtypes that are different in their histopathologic characteristics [25,26]; some are associated with shorter time to disease progression [25] and treatment resistance [26]. Interestingly, the gene expression data gave information beyond the routinely analyzed gene mutations (*KRAS*, MSI, *BRAF*). This is possibly because gene expression analysis can provide additional information regarding RNA expression in the tumor microenvironment, which has a central role in disease biology [27].

In patients undergoing surgery for stage II and III CRC, gene expression profiles can provide a useful tool for stratifying

Figure 4. Modes for identifying intra-tumor heterogeneity (ITH). Two different approaches are used to identify ITH. The multi-region sampling approach will be useful if the different tumor sub-clones are spatially distinct **[A]**. In this case, analysis of one locus (red box) will identify a mutation, and analysis of another locus (green box) will give a wild-type diagnosis, indicating ITH. On the other hand, if the sub-clonal populations of the tumor are intermixed **[B]**, analysis of both loci will give a mutation-positive result even though only a subset of the tumor cells carry the mutation. In contrast, the molecular morphometric approach compares the actual fraction of mutated alleles to the expected fraction, which is based on morphometric quantification of the percentage of tumor cells in the sample. In the provided example **[C]** there is an intermixed population of two different sub-clones: 56% of cells are tumor cells (left), while only 14% of DNA copies carry a mutation (right), indicating ITH.



patients into those who would benefit from adjuvant chemotherapy and those who can be safely managed without chemotherapy [28].

POTENTIAL APPLICATIONS FOR COMBINED MOLECULAR AND MORPHOLOGIC TOOLS

- **Intra-tumor heterogeneity**

In the last decade there have been major developments in the field of genetic sequencing technologies and computation analysis of data. This, in turn, improved our understanding of the molecular biology of cancer as well as the dynamics of cancer evolution and has led to some interesting insights. One such important example is intra-tumor heterogeneity (ITH). In the past, the hypothesis was that since tumors originate from the same progenitor cell the final cellular population of the tumor should be homogeneous. More recently, however, it has been shown that tumor cells are heterogeneous at the morphologic and the genetic level. Importantly, it seems that ITH might affect diagnostic accuracy, treatment efficiency and patients' prognosis [29].

Integration of morphometric and molecular tools by pathologists can increase our understanding of tumors and highlight novel cancer concepts such as intra-tumor heterogeneity

ITH can be demonstrated in gastrointestinal tumors. A recent study that examined various subtypes of gastrointestinal malignancies found that different allelic frequencies of different mutations were noted in the same tumors, suggesting some degree of ITH [30]. More specifically, in colon carcinoma there is some evidence of heterogeneity with different mutations in multifocal primary tumors [31], differences between the adenoma and carcinoma components of tumors [32], and the identification of additional mutations in metastatic versus primary tumors [33]. However, at least for the commonly mutated and targetable genes there seems to be a high degree of homogeneity in CRC [34]. This is also supported by the high degree of concordance for these mutations between primary tumors and distant metastasis [35].

Currently, there are two different approaches to identify ITH; multi-region sampling and the quantitative molecular-morphometric approach. With the multi-region sampling approach, different areas of the tumor are analyzed and a tumor is considered heterogeneous for a specific mutation if this mutation is present only in a subset of the areas examined. The quantitative molecular-morphometric approach is based on the ability of next-generation sequencing technology to quantify the fraction of mutant alleles in the sample, and the ability of computerized morphometry to calculate the fraction of tumor cells in the tissue sample. By combining the results of the molecular and morphometric analysis it is possible to determine whether a specific mutation is present in all the tumor cells or only in a subclone [Figure 4]. One possible disadvantage of the multi-region sampling method is that it assumes clustering of the different sub-clones and therefore might miss heterogeneity if the different sub-clones are intermixed [Figure 4]. With the advances in our understanding of cancer and acknowledgment of the role of ITH, methodologies for detection and quantification of ITH should be applied to provide a more in-depth characterization of tumors.

- **Nuclear morphometry**

It has been long established that the carcinogenesis process is accompanied by continuous changes in the nuclear morphol-

ogy of tumor cells [36]. Some of these changes are likely related to the recently identified role of the nuclear envelope in cancer development and progression [37]. Among these changes are increased nuclear size, higher nucleus to cytoplasm ratio, increased chromatin content in the cells (hyperchromasia), and changes in nuclear shape and texture. These changes are evaluated in a qualitative manner by the pathologist in routine clinical practice. The application of computerized image analysis algorithms to tissue slides allows objective quantification and identification of architectural and chromatin texture differences that are not readily recognized by the naked eye. In colon carcinoma, image cytometry could be used to quantify genomic instability [38]. Moreover, nuclear morphometric tool parameters were associated with prognosis [39] and with specific genetic alterations in the tumor cells [40]. The clinical role of nuclear morphology parameters and their association with molecular changes needs to be further evaluated.

CONCLUSIONS

Current diagnosis of CRC is based on the classical morphological changes in the tumor tissue as well as on more recently developed molecular tools. With continued development of sequencing technology and reduction in costs, these tools would, in the near future, become part of the clinical routine in many medical centers. The combination of molecular and morphometric tools would allow better classification of tumors and tailored and more effective therapy for more CRC patients. The utility and clinical significance of this should be studied in future clinical trials.

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