Geographic Heterogeneity for Mismatch Repair Proteins Is Associated with Defects in DNA Repair

Ariel Greenberg MD[1], Revital Kariv MD[2,3], Irit Solar PhD[1] and Dov Hershkovitz MD PhD[1,3]

Departments of [1]Pathology and [2]Gastroenterology, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel
[3]Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

ABSTRACT: **Background:** Evaluation of mismatch repair (MMR) deficiency is conducted via immunohistochemistry or by microsatellite instability (MSI) analysis. Heterogeneous immunohistochemistry staining for MMR proteins may show different patterns; however, according to current guidelines, all of those patterns should be interpreted as MMR proficient. This conclusion might lead to false negative results because although most cases of heterogeneity stem from technical factors and biological variability, other types of heterogeneity represent true MMR deficiency.

Objectives: To identify a unique heterogeneity pattern that is associated with true MMR loss.

Methods: We analyzed 145 cases of colorectal carcinoma. Immunohistochemistry staining for MLH1, PMS2, MSH2, and MSH6 were performed. We defined geographic heterogeneity as areas of tumor nuclear staining adjacent to areas of loss of tumor nuclear staining with intact staining in the surrounding stroma. All cases were evaluated for the presence of geographic heterogeneity. In addition, 24 cases were also evaluated by MSI testing.

Results: Of the 145 cases, 24 (16.5%) were MMR deficient. Of the 24 cases for which MSI analysis was also available, 10 cases (41.7%) demonstrated biological heterogeneity, 5 (20.8%) demonstrated technical heterogeneity, and 2 (8.3%) demonstrated geographic heterogeneity. Only the two cases with geographic heterogeneity were MSI-high via MSI analysis. In addition, a germline mutation in MSH-6 was identified in one of these cases.

Conclusions: Geographic heterogeneity may raise a suspicion for a MMR-deficient case, which should be further analyzed using additional methodologies such as MSI analysis.

IMAJ 2020; 22: 32–36

KEY WORDS: hereditary nonpolyposis colorectal cancer (HNPCC), immunohistochemistry, intra-tumor heterogeneity, microsatellite instability (MSI), mismatch repair (MMR)

Inactivation of the mismatch repair (MMR) system is found in a subset of various tumors, most notable of which is colorectal carcinoma (CRC). Any one of the major MMR genes may be inactivated by a germline mutation, a condition known as Lynch syndrome or hereditary nonpolyposis colorectal cancer (HNPCC) [1]. Although HNPCC is the most common cause of familial colorectal carcinoma worldwide, other familial causes have been reported in specific populations [2]. Non-hereditary causes of MMR deficiency that can be found in CRC include sporadic hyper-methylation related inactivation of an MMR gene promoter (most commonly, MLH-1), also known as CpG island methylator phenotype [1]. Biallelic somatic mutations of the MMR genes have also been reported [3].

MMR deficiency allows small base pair mistakes to accumulate, which results in a hyper-mutator phenotype that may affect oncogenes and tumor suppressor genes. Base pair substitutions also affect nucleotide repeat sequences known as microsatellites, which results in microsatellite instability (MSI), a marker of genomic instability [3].

MMR-deficient CRC has several characteristic clinicopathologic features such as a tendency to involve the right colon, tumor infiltrating lymphocytes and morphological heterogeneity including mucinous, and medullary or signet-ring components [3]. More importantly, the MMR deficient phenotype affects prognosis and treatment response. MMR deficient tumors tend to have a better prognosis and are resistant to treatment with 5-fluorouracil, yet are more susceptible to immunotherapy and Irinotecan [4]. MMR status, combined with genetic information such as tests for point mutation or RNA expression patterns, are all used to direct treatment of CRC [5].

MMR deficiency may be detected by use of immunohistochemistry for each of the four major MMR proteins or by MSI analysis. Current guidelines and practices classify MMR deficiency as a complete absence of staining for one or more MMR proteins, with preserved staining of the stroma. Any nuclear staining in the tumor cells is considered an intact (normal) result [6].

MMR immunohistochemistry is both sensitive (93%) and highly specific for predicting MSI when used correctly [3]. However, even under optimal conditions interpretation is subjected to many confounders such as aberrant staining patterns (e.g., cytoplasmic, dot-like), reduced staining intensity, intra-observer variation, and staining heterogeneity [7]. Heterogeneity in MMR immunohistochemistry may take several different forms. Most cases of heterogeneity are tech-

nical with weak or patchy staining (sometimes over distant areas of the mass) [8], are presented as a single focus without clear demarcation [9], or are lacking adequate internal control. Most of these cases represent truly MMR-proficient cases and the technical issues could usually be solved by repeating the immunohistochemistry on a different tissue block or with longer exposure time [10]. However, there have been reports of heterogeneous staining for MMR proteins that were associated with an actual defect in MMR function and microsatellite instability [10-12].

In the present article, we hypothesized that geographic heterogeneity, a unique pattern of heterogeneity for MMR staining, is associated with true MMR deficiency and MSI.

PATIENTS AND METHODS

Immunohistochemistry for MMR proteins was performed on 145 cases of CRC using the monoclonal antibodies: MSH-2 (clone G219-1129 Ventana, Roche Diagnostics, USA), MSH-6 (clone 44 Ventana), PMS-2 (clone EPR3947 Ventana), and MLH-1 (clone M1 Ventana). Staining was performed on automated stainer Benchmark XT/ Ultra (Ventana) using OptiView universal DAB detection and amplification kit (Ventana). All cases were microscopically evaluated for the presence of geographic heterogeneity, which was defined as areas of tumor nuclear staining adjacent to areas of loss of tumor nuclear staining with intact staining in the surrounding stroma. MSI status was performed by a fluorescent PCR-based assay using five mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24, and mono-27) for MSI determination according to the revised Bethesda guidelines for MSI detection (MSI Analysis System, Version 1.2, Promega Corporation, Madison, WI, USA). Tumor samples in which more than or equal to 40% of mononucleotide repeat markers are altered (more than or equal to 2 markers) were classified as MSI-H. Analysis was performed on DNA samples extracted from both tumor tissue and normal tissue.

The study and all methods used were approved by the local ethics committee.

RESULTS

Of the 145 cases, 24 (16.5%) demonstrated a deficiency in one or more MMR proteins. Nine (37.5%) of the MMR deficient cases were also tested for MSI and were all MSI-H. An additional 15 cases evaluated for MSI status were microsatellite stable (MSS). Of the cases for which both MMR staining and MSI testing were performed, three distinct patterns of heterogeneity were documented:

- Ten cases (41.7%) demonstrated variability in staining intensity and even lack of staining between adjacent cells in clusters or glands. This type of heterogeneity was defined as biological heterogeneity caused by different expression level of MMR proteins between cells [Figure 1A]
- Five cases (20.8%) demonstrated areas with strongly positive staining and adjacent areas negative for staining. However, the negative areas also lacked nuclear staining in the surrounding lymphocytes and stromal cells. This pattern was classified as technical heterogeneity, probably caused by technical pre-analytical problems with tissue fixation or during immunostaining [Figure 1B]
- Seven cases (29.2%) displayed no significant heterogeneity
- In two cases (8.3% of the MSI group, and 1.4% of all cases) we were able to identify geographic heterogeneity with clearly demarcated areas of positive staining, and adjacent negative areas with a positive internal control in stromal cells

The first case was a 34-year-old male of Russian descent with no family history of malignancy. He was referred for evaluation as an outpatient due to rectal bleeding and tenesmus. Colonoscopy revealed a 6-cm rectal polyp diagnosed as a tubulovillous adenoma with high-grade dysplasia. Immunohistochemical staining for MMR proteins showed intact nuclear staining for MLH-1, PMS2, and MSH-2, while the staining pattern of MSH-6 showed geographic heterogeneity [Figure 2]. Further analysis of MSI revealed the tumor to be MSI-H, with 4 out of 5 markers showing instability.

The second case was of a 40-year-old male of Syrian origin (non-consanguinity) with a family history of colorectal carcinoma. Colonoscopy revealed an obstructive, ulcerated mass, 70 cm from the anus. Histological examination of the surgical specimen revealed a heterogeneous tumor mass composed of a moderately differentiated adenocarcinoma and a trabecular pattern adenocarcinoma, both as separate components with a clear demarcation between them. In addition, a marked increase in tumor infiltrating lymphocytes and areas of Crohn's like reaction were noted [Figure 3].

Figure 1. Patterns of heterogeneity for MMR protein staining. Biological heterogeneity **[A]**, showing variability in staining intensity between different cells, attributable to differences in MMR protein expression. Technical heterogeneity **[B]** may demonstrate non-specific, cytoplasmic staining, yet lacks nuclear staining in either the tumor cells or the stromal cells indicating a false negative result. Both patterns are considered as intact staining under current criteria

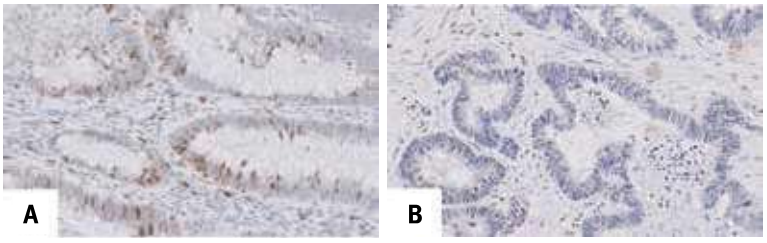
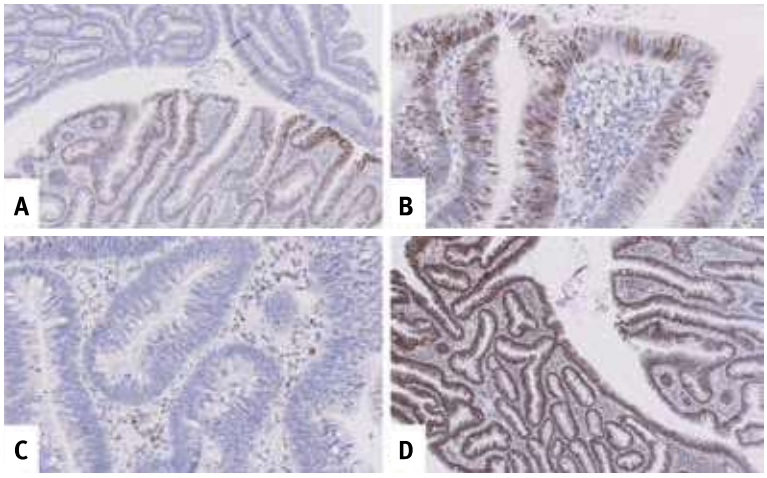


Figure 2. MMR staining for the four major MMR proteins in a single region of the adenoma presented in case 1. MSH-6 **[A]** demonstrates a heterogenic staining pattern, in a geographic distribution with areas showing intact nuclear staining **[B]**, clearly separated from areas with loss of nuclear staining and intact staining in the stroma **[C]** Intact staining for MLH-1 presented for comparison **[D]**



MMR = mismatch repair

Staining for MMR proteins mirrored the heterogeneity of the tumor mass. The moderately differentiated component demonstrated loss of MLH1 and PMS2 with intact nuclear expression of MSH2 and MSH6. Conversely, the trabecular component showed intact staining for MSH2 alone, with loss of MLH1, PMS2, and MSH6 [Figure 4]. MSI analysis revealed the tumor (as a whole) to be MSI-high, with 5 out of 5 markers showing instability. Germline gene panel analysis of all HNPCC related genes (MLH1, MSH2, MSH6, PMS3, and EPCAM 3'-UTR) identified a single heterozygous variant of unknown significance in the gene MSH6 (c.2006T>C; p.Ile669Thr NM _000179). This variant is graded 3 by the American College of Medical Genetics (ACMG) , and predicted as damaging by 4 in silico programs (PhyloP, SIFT, LRT, mutation tester). In addition, the variant is reported to be very rare and conserved, thus increasing the likelihood for pathogenicity.

DISCUSSION

Malignancies are evolving and dynamic entities, which may display considerable heterogeneity, with the emergence of many

Figure 3. H&E stain of case 2. At low magnification **[A]** two distinct histological patterns are evident. On higher magnification, the pattern on the right is compatible with moderately differentiated adenocarcinoma **[B]**, while the left hand pattern is compatible with trabecular variant adenocarcinoma **[C]**

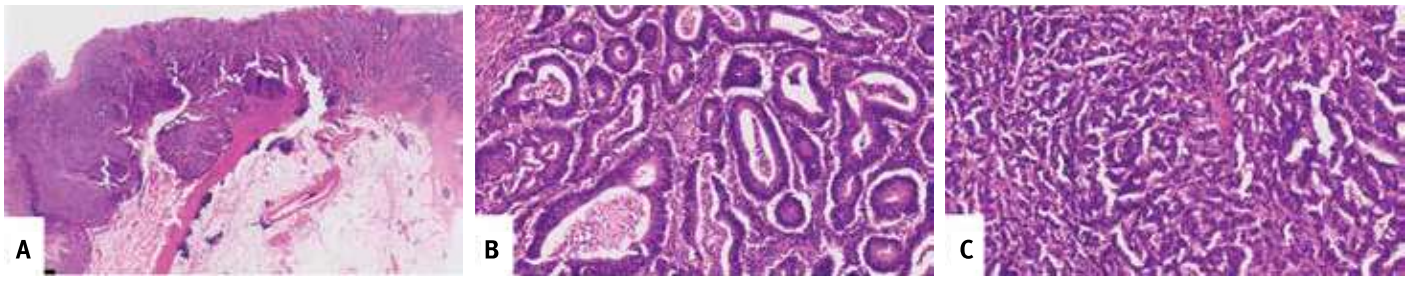
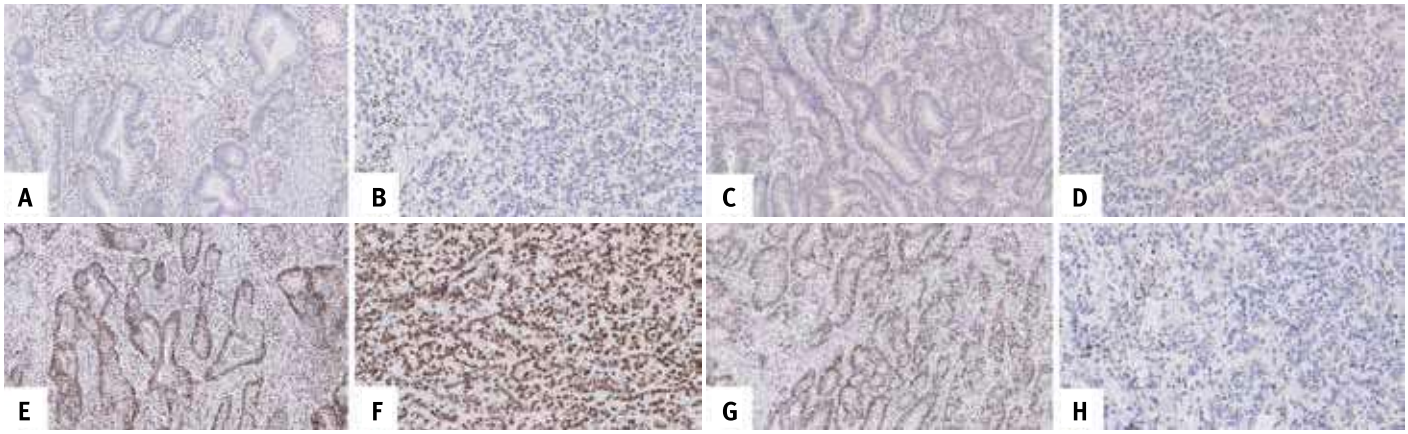


Figure 4. MMR staining of the two histological patterns identified in case 2 (left: moderately differentiated, right: trabecular). MLH-1 **[A] [B]**, PMS-2 **[C] [D]** were deficient in both regions. MSH-2 **[E] [F]** was found to be intact in both regions. MSH-6 **[G] [H]**, however, showed heterogeneity between both regions, with intact staining in the moderately differentiated component and loss of staining in the trabecular component



MMR = mismatch repair

different subclones, both over time as well as in different foci of the same tumor mass. Subclonality for driver mutations in cancer have been reported in several malignancies [13,14].

Heterogeneity in MMR protein staining may take several forms, most of which are due to technical issues or normal variance and are not indicative of an underlying defect in mismatch repair function. Technical reasons for heterogeneity in MMR staining do not seem to correlate to the antibodies specificity, but rather to the staining protocol used. The College of American Pathology (CAP) provides recommendations for the developments and validation of MMR staining protocols. These include careful empirical optimization of the staining protocol and subsequent validation with a requirement for at least 90% concordance with known competitor tests. However, up to 10% of the MMR deficient cases would sill demonstrate normal or equivocal staining necessitating addition validation, such as via MSI analysis [6]. In our analysis we were able to show that geographic heterogeneity represents a true loss of MMR function as proven by the MSI high status of these tumors. In previous studies [11,12], local loss of nuclear staining for MSH6 with intact staining in the stromal cells was shown to correlate with an underlying somatic mutation in the MSH6 gene. These reports might represent other cases of geographic heterogeneity and strengthen the association between this unique pattern and true MMR deficiency.

We defined geographic heterogeneity as areas of true positive staining, adjacent to areas of true negative staining (loss of staining in the tumor nuclei with intact staining in stromal cell nuclei). This pattern was quite uncommon in the examined cohort with only two cases (1.4%): one an adenoma, the other a carcinoma with two morphologically different components. We suggest that the identification of this pattern merits confirmation and further examination by MSI analysis as well as a genomic analysis for MMR gene mutation status.

Several mechanisms for heterogeneity of MMR protein expression have been described in the literature, including regional heterogeneity of MLH-1 promoter methylation [7,8] as well as a secondary mutation in a subclone of a MSI primary tumor [8,10-12]. Furthermore, intra-tumor heterogeneity has been described with other driver mutations and tumor types [15-18]. Specifically, in colorectal carcinoma development, subclonality was described both at the adenoma and the carcinoma stages [19].

The first case of geographic heterogeneity we identified was a tubulovillous adenoma. Walsh and colleagues [20] found that the majority (72%) of adenomas from Lynch syndrome patients showed an appropriate loss of MMR proteins via immunohistochemistry. These adenomas are also often associated with a villous component and a tendency toward high-grade dysplasia. The Walsh group encountered several adenomas that demonstrated heterogeneity in MMR staining. The majority, however, showed complete loss of expression, which the researchers attributed to a rapid clonal expansion secondary to the loss of mismatch repair functions.

Adenomas represent a relatively early stage of tumor development, during which emerging subclones compete. The results presented by Walsh et al. [20] suggested that, in most cases, a single subclone with a growth advantage will become the dominant clone at an early stage and consequently, occupy most, if not the entire adenoma. However, in a minority of cases adenomas may be resected before the loss of MMR proteins or when the loss is still subclonal. Previous studies have also shown that earlier lesions are associated with more subclonal driver events. In colonic lesions, the concordance of *KRAS* mutation between adenomas and carcinomas that developed was lower for earlier lesions [21]. However, more advanced adenocarcinoma were mostly homogeneous with regard to KRAS mutation [17] and even show a high degree of concordance in MMR protein deficiency between the primary mass and metasteses [22]. Together with our case, these findings support the concept that earlier lesions are associated with higher rate of intra-tumor heterogeneity for common mutations. A tumor subclone would be expected to first expand and proliferate in a localized focus, which would explain the relatively demarcated and geographic pattern and would be observed between subclones deficient or proficient in certain MMR proteins. However, the identification of geographic heterogeneity in this setting would require a specific timing both in terms of the creation of the relevant subclones and in terms of the timing of resection and is, therefore, expected to be rare.

The second case with geographic heterogeneity displayed two morphologically distinct components. Accordingly, the geographic nature of the tumor was far more pronounced. A geographic heterogeneity for MSH-6 protein was noted whereas staining for MLH-1 and PMS-2 was lost in both components. On molecular testing, both components were also MSI-H.

This pattern seems to correlate with the one previously reported [10-12]. However, in all previous reports, the MSH-6 mutation was somatic, while in our case a germline variant, most probably damaging, was identified.

The loss of the first allele of MSH-6 was germline, yet the protein was still expressed in the moderately differentiated component, indicating that it retained its second, functioning allele. Had the second, trabecular component not been tested as well, the patient's Lynch syndrome may have gone undiagnosed. Of note, no germline mutation in MLH-1 or PMS-2 was detected. The loss of MLH-1 and MSH-2 protein expression may have been due to other mechanisms such as MLH-1 promotor hypermethylation or a somatic mutation.

MSI analysis is also susceptible to the confounding effect of intra-tumor heterogeneity. The rate of intra-tumor heterogeneity was found to vary (7.7–41.7%) depending on the classification system used (Bethesda, Promega) and the test result [23]. In addition, MSI-H status may lead to intra-tumor heterogeneity

in the expression of other genes, notably, apoptosis related genes [24]. In both cases [23,24], the authors suggested that multiple biopsies might serve as a possible method to address intra-tumor heterogeneity in the evaluation of the tumor mass. In this study, MSI analysis was performed on a single sample from each case. However, supplemental data provide additional confidence in these results. Areas that lacked MMR protein staining in the tumor cells still demonstrated preserved staining in lymphocytes and other stromal cells, thus providing a suitable internal control. In addition, genetic information was available for one of the cases. We acknowledge however, that testing multiple biopsies for MSI can indeed provide further support of an MSI-H result as well as help prevent a false negative results in heterogenic cases. Whether this situation should be a common practice or one reserved for equivocal cases, merits further research. Accurate analysis of MMR deficient cases is clinically important both as a part of Lynch screening and for prediction of response to treatment with immune check-point inhibitors [25]. Heterogeneous cases, showing intact nuclear expression in some of the cells may be falsely interpreted as MMR-proficient.

CONCLUSIONS

Within this group, many patterns may exist, including rare forms such as geographic heterogeneity. As demonstrated, geographic heterogeneity may occur in many different contexts – in both adenomas and carcinomas, due to a somatic mutation or in the context of Lynch syndrome. We propose that geographic heterogeneity should raise a suspicion for a MMR-deficient case that should be further analyzed using other methodologies such as MSI analysis.

Correspondence
Dr. D. Hershkovitz
Dept. of Pathology, Tel Aviv Sourasky Medical Center, Tel Aviv 6423906, Israel
Phone: (972-3) 697-3530
Fax: (972-3) 697-4648
e-mail: dovh@tlvmc.gov.il

References

1. Druliner BR, Ruan X, Sicotte H, et al. Early genetic aberrations in patients with sporadic colorectal cancer. *Mol Carcinog* 2018; 57 (1): 114-24.
2. Schayek H, Laitman Y, Katz LH, et al. Colorectal and endometrial cancer risk and age at diagnosis in BLMAsh mutation carriers. *IMAJ* 2017; 19 (6): 365-7.
3. Shia J. Evolving approach and clinical significance of detecting DNA mismatch repair deficiency in colorectal carcinoma. *Semin Diagn Pathol* 2015; 32 (5): 352-61.
4. Ryan E, Sheahan K, Creavin B, et al. The current value of determining the mismatch repair status of colorectal cancer: a rationale for routine testing. *Crit Rev Oncol Hematol* 2017; 116: 38-57.
5. Fisher Y, Hershkovitz D. Molecular and morphometric tools for next-generation pathology diagnosis of colon carcinoma. *IMAJ* 2016; 18 (7): 426-32.
6. Sepulveda AR, Hamilton SR, Allegra CJ, et al. Molecular biomarkers for the evaluation of colorectal cancer: guideline from the American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and American Society of Clinical Oncology. *J Mol Diagn* 2017; 19 (2): 187-225.
7. Shia J, Holck S, Depetris G, et al. Lynch syndrome-associated neoplasms: a discussion on histopathology and immunohistochemistry. *Fam Cancer* 2013; 12 (2): 241-60.
8. Tachon G, Frouin E, Karayan-Tapon L, et al. Heterogeneity of mismatch repair defect in colorectal cancer and its implications in clinical practice. *Eur J Cancer* 2018; 95: 112-6.
9. Chapusot C, Martin L, Bouvier AM, et al. Microsatellite instability and intratumoural heterogeneity in 100 right-sided sporadic colon carcinomas. *Br J Cancer* 2002; 87 (4): 400-4.
10. Djordjevic B, Broaddus RR. Laboratory assays in evaluation of lynch syndrome in patients with endometrial carcinoma. *Surg Pathol Clin* 2016; 9 (2): 289-99.
11. Graham RP, Kerr SE, Butz ML, et al. Heterogenous MSH6 loss is a result of microsatellite instability within MSH6 and occurs in sporadic and hereditary colorectal and endometrial carcinomas. *Am J Surg Pathol* 2015; 39 (10): 1370-6.
12. Shia J, Zhang L, Shike M, et al. Secondary mutation in a coding mononucleotide tract in MSH6 causes loss of immunoexpression of MSH6 in colorectal carcinomas with MLH1/PMS2 deficiency. *Mod Pathol* 2013; 26 (1): 131-8.
13. Nagawkar SS, Abu-Funni S, Simon E, et al. Intratumor heterogeneity of KRAS mutation status in pancreatic ductal adenocarcinoma is associated with smaller lesions. *Pancreas*. 2016; 45 (6): 876-81.
14. Finkel A, Liba L, Simon E, et al. Subclonality for BRAF mutation in papillary thyroid carcinoma is associated with earlier disease stage. *J Clin Endocrinol Metab* 2016; 101 (4): 1407-13.
15. Liu J, Dang H, Wang XW. The significance of intertumor and intratumor heterogeneity in liver cancer. *Exp Mol Med* 2018; 50 (1): e416.
16. Yates LR. Intratumoral heterogeneity and subclonal diversification of early breast cancer. *Breast* 2017; 34 Suppl 1:S36-S42.
17. Farber L, Efrati E, Elkin H, et al. Molecular morphometric analysis shows relative intra-tumoural homogeneity for KRAS mutations in colorectal cancer. *Virchows Arch* 2011; 459 (5): 487-93.
18. Dzobo K, Senthebane DA, Thomford NE, et al. Not everyone fits the mold: intratumor and intertumor heterogeneity and innovative cancer drug design and development. *OMICS* 2018;22(1):17-34.
19. Wu H, Zhang XY, Hu Z, et al. Evolution and heterogeneity of non-hereditary colorectal cancer revealed by single-cell exome sequencing. *Oncogene* 2017; 36 (20): 2857-67.
20. Walsh MD, Buchanan DD, Pearson SA, et al. Immunohistochemical testing of conventional adenomas for loss of expression of mismatch repair proteins in Lynch syndrome mutation carriers: a case series from the Australasian site of the colon cancer family registry. *Mod Pathol* 2012; 25 (5): 722-30.
21. Hershkovitz D, Simon E, Bick T, et al. Adenoma and carcinoma components in colonic tumors show discordance for KRAS mutation. *Hum Pathol* 2014; 45 (9): 1866-71.
22. Haraldsdottir S, Roth R, Pearlman R, et al. Mismatch repair deficiency concordance between primary colorectal cancer and corresponding metastasis. *Fam Cancer* 2016; 15 (2): 253-60.
23. Choi YJ, Kim MS, An CH, et al. Regional bias of intratumoral genetic heterogeneity of nucleotide repeats in colon cancers with microsatellite instability. *Pathol Oncol Res* 2014; 20 (4): 965-71.
24. Choi MR, Gwak M, Yoo NJ, et al. Regional bias of intratumoral genetic heterogeneity of apoptosis-related genes BAX, APAF1, and FLASH in colon cancers with high microsatellite instability. *Dig Dis Sci* 2015; 60 (6): 1674-9.
25. Dudley JC, Lin MT, Le DT, et al. Microsatellite instability as a biomarker for PD-1 Blockade. *Clin Cancer Res* 2016; 22 (4): 813-20.

"Every creature is better alive than dead, men and moose and pine trees, and he who understands it aright will rather preserve its life than destroy it"

Henry David Thoreau (1817–1862), American essayist, poet, philosopher, abolitionist, naturalist, tax resister, development critic, surveyor, and historian