**Chapter 24**

***Molecular Testing in Colorectal Cancer***

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**CASE 1**

**Introduction** - CRC is a heterogeneous and complex disorder that develops as a consequence of accumulation of both genetic and epigenetic genomic alterations.

**Primary Question** - ***At least three molecular pathways lead to development of CRC. Which of the following answers is correct?***

1. The conventional suppressor pathway is initiated by inactivation of the APC/β-catenin/Wnt signaling pathway.
2. The CpG island methylator pathway leads to development of both microsatellite stable and MSI-high CRC.
3. Microsatellite instability can be seen in both hereditary and sporadic CRCs.
4. All of the above are correct.

**Correct Response**: D

**Explanation of Correct/Incorrect Response:** Three main molecular pathways have been described for CRC development. The conventional suppressor pathway was originally proposed by Fearon and Vogelstein, and is characterized by mutations in *APC* and exemplified by Familial Adenomatous Polyposis (FAP). The CpG island methylator pathway contributes approximately 35% of total CRCs. CpG island methylation within promotor regions of some genes leads to transcriptional silencing of involved genes and loss of gene function. Depending on which genes are silenced by the methylation, the arising carcinoma may be microsatellite stable (methylation of tumor suppressor genes) or MSI-high (methylation of *MLH1*). Lynch syndrome-associated CRC is microsatellite instable due to inactive mutations in one of the 4 MMR genes. Therefore, the correct answer is D.

**First Reply Question – *Which of the following statements are correct?***

1. The conventional suppressor pathway is also called chromosomal instability pathway.
2. Sporadic CRCs are never microsatellite instable.
3. The CpG island methylator pathway only leads to development of MSI-high CRCs.
4. There are more Lynch syndrome-associated MSI-high CRCs than sporadic MSI-high CRCs.

**Correct Response:** A

**Explanation of Correct/Incorrect Response** - Chromosomal instability, including numeric and structural chromosomal abnormalities, is the most common type of genomic instability identified in CRCs, especially those resulting from the conventional suppressor pathway. Therefore, the conventional suppressor pathway is also called chromosomal instability pathway, in contrast to the microsatellite instability present in MSI-high CRCs. Therefore, A is correct. The CpG island methylator pathway contributes approximately 35% of total CRCs. CpG island methylation within promotor regions of genes leads to their transcriptional silencing and loss of gene function. Depending on which genes are silenced by the methylation, the arising carcinoma may be microsatellite stable (methylation of tumor suppressor genes) or MSI-high (methylation of *MLH1*). Therefore, B and C are incorrect. Approximately two-thirds of MSI-high CRCs are sporadic. Therefore, D is incorrect.

**Second Reply Question - *Which of the following statements are correct?***

1. CRCs in FAP patients are always MSI-high.
2. CRCs in Lynch syndrome patients are always MSI-stable.
3. The CpG island methylation pathway contributes development of MSI-stable CRCs only and the MSI pathway contributes to development of MSI CRC.
4. MSI-high CRCs can arise through the CpG island methylation pathway or the MSI pathway.

**Correct Response:** D

**Explanation of Correct/Incorrect Response** - Familial Adenomatous Polyposis (FAP) is the best model for CRC arising through the conventional suppressor pathway. Therefore, A is incorrect. Lynch syndrome patients have a mutation in one of the four MMR genes or *EPCAM;* and microsatellite instability is the diagnostic feature of Lynch syndrome-associated CRCs. Therefore, B is incorrect. Depending on which genes are silenced by the methylation, the arising carcinoma may be microsatellite stable (methylation of tumor suppressor genes) or MSI-high (methylation of *MLH1*). Therefore, C is incorrect, and D is correct.

**CASE 2**

**Introduction** - Several polyposis syndromes have been identified that are associated with a high risk of developing CRC. Which of the following statements is correct?

1. FAP is caused by biallelic germline mutations in the *APC* gene.
2. *MUTYH-*associated polyposis can mimic attenuated FAP.
3. Patients with Lynch syndrome have more colonic adenomas than the general population.
4. Patients with a germline mutation in *SKT11* or *SMAD4* always present with adenomatous polyposis.

**Correct Response:** B

**Explanation of Correct/Incorrect Response** - FAP is an autosomal dominant disease, caused by a germline mutation in *APC* in one allele. Therefore, A is incorrect. Compared to classic FAP, attenuated FAP present with less adenomas (<100) and at a later age, clinically similar to *MUTYH*-associated polyposis. Therefore, B is correct. C is incorrect, since patients with Lynch syndrome usually do not have more polyps than the general population. Peutz-Jeghers and juvenile polyposis syndromes are the two most common harmatomatous polyposis syndrome. Therefore, D is incorrect.

**First Reply Question – *Which of the following statements are correct?***

1. Atypical FAP is not caused by APC mutations.
2. Testing for *MUTYH*-associated polyposis is recommended in patients with more than 10 adenomatous polyps who have tested negative for *APC* mutations.
3. Lynch syndrome is one type of hereditary polyposis syndrome.
4. Peutz-Jeghers and Juvenile polyposis syndromes are featured by numerous colonic adenomas.

**Correct Response:** B

**Explanation of Correct/Incorrect Response** - Both classic and attenuated FAP is caused by a germline mutation in the *APC* gene. Attenuated FAP and *MUTYH*-associated polyposis share similar clinical features; therefore, testing for *MUTYH* mutations should be performed when a patient has more than 10 adenomas and a negative result for *APC* mutations. Therefore, B is correct. C is incorrect, since patients with Lynch syndrome usually do not have more polyps than the general population. Peutz-Jeghers and juvenile polyposis syndrome are the two most common harmatomatous polyposis syndromes. Therefore, D is incorrect.

**Second Reply Question – Which of the following statements is correct?**

1. Classic FAP typically shows the presence of >100 colonic adenomas, autosomal dominant inheritance, and an almost 100% lifetime risk of CRC, if colectomy is not performed.
2. *MUTYH*-associated polyposis displays autosomal dominant inheritance.
3. Mutations in one allele of one of the 4 MMR gene mutated are sufficient to develop Lynch syndrome-associated CRC.
4. Peutz-Jeghers and Juvenile polyposis syndromes are not associated with an increased risk for development of CRC.

**Correct Response:** A

**Explanation of Correct/Incorrect Response** - FAP patients have a germline mutation in one copy of the *APC* gene (autosomal dominant), followed by a second event leading to inactivation of the second allele. Classic FAP features the presence of >100 colonic adenomas with a 100% lifetime risk of CRC, and an increased risk for a number of other cancers. Therefore, A is correct. *MUTYH*-associated polyposis is an autosomal recessive disease, requiring germline mutations in both copies of *MUTYH*. Therefore B is incorrect. Although Lynch syndrome displays autosomal dominant inheritance (like FAP), both copies of one of the 4 MMR genes must be mutated before CRC develops. Therefore, C is incorrect. Although harmatomatous (non-adenomatous) polyps are the feature for both Peutz-Jeghers and Juvenile polyposis syndromes, these polyps can become dysplastic and eventually lead to the development of CRC. These patients do show an increased risk for developing CRC. Therefore, D is incorrect.

**CASE 3**

**Introduction** - MSI testing has been used to identify Lynch syndrome. Several organizations including the National Comprehensive Cancer Network (NCCN) provide guidelines for screening of Lynch syndrome. Different techniques have been used for identify MSI-high CRC and patients with Lynch Syndrome.

**Primary Question** - ***Which of the following statements is INCORRECT?***

1. Application of the revised Bethesda criteria as screening tool Lynch syndrome is sufficient for identification of all affected patients.
2. Microsatellite instability can be evaluated by a PCR-based MSI testing and/or immunohistochemical labeling for MLH1, MSH2, MSH6 and PMS2.
3. Identification of a *BRAF* mutation (V600E) in MSI-high CRCs essentially rules out Lynch syndrome.
4. A PCR-based molecular assay or immunohistochemistry can be used to assess *BRAF* mutational status.
5. MSI-high CRCs with MLH1 promotor methylation are sporadic.

**Correct Response:** A

**Explanation of Correct/Incorrect Response** - The sensitivity of the revised Bethesda criteria for screening for patients with Lynch syndrome is low. Therefore, A is the correct answer. We use either a PCR-based MSI assay or/and immunohistochemical labeling for MLH1, MSH2, MSH6 and PMS2 proteins to identify MSI-high CRCs. In addition, *BRAF* mutation is common in sporadic MSI-high CRC, but almost never occurs in a Lynch syndrome-associated CRC. A number of PCR-based assays can be used to detect *BRAF* mutations, including Sanger sequencing and allele specific PCR. Immunohistochemical labeling for *BRAF* V600E has recently developed and utilized in clinical samples. Therefore, B, C, D and E are correct statements, but incorrect answers.

**First Reply Question -** ***Which of the following statements is correct?***

1. The new NCCN guidelines recommend evaluation of MSI status by PCR-based or IHC methods on CRCs that meet the revised Bethesda criteria.
2. PCR-based MSI testing is more sensitive than immunohistochemical labeling for MLH1, MSH2, MSH6 and PMS2 proteins.
3. *BRAF* mutations do not have any role in differentiating sporadic CRCs from Lynch syndrome-associated CRCs.
4. Analysis of MLH1 promoter methylation does not help identify sporadic MSI-high colorectal cancers.
5. Germline mutation analysis is required in MSI-high CRCs that are *BRAF* wild-type and lack *MLH1* promoter methylation due to the high probability of Lynch syndrome.

**Correct Response:** E

**Explanation of Correct/Incorrect Response** - The current NCCN guidelines recommend evaluation of MSI status by molecular or IHC methods on all resected CRCs, or CRCs diagnosed in patients <70 years of age and in patients≥70 years of age who meet the Bethesda criteria. Therefore, A is incorrect. The sensitivity of PCR-based MSI testing and IHC labeling for the 4 MMR proteins is similar. Therefore, B is incorrect. *BRAF* mutations and *MLH1* promoter methylation are associated with MSI-high sporadic CRCs. Therefore, C and D are incorrect, and E is correct.

**Second Reply Question -** ***Which of the following statements is correct?***

1. A): The new NCCN guidelines recommend evaluation of MSI status by molecular or IHC methods on all resected CRCs, or CRCs diagnosed in patients <70 years of age and in patients≥70 years of age who meet the Bethesda criteria.
2. B) Loss of expression of MSH2/MSH6 and intact MLH1 expression favors a sporadic MSI-high CRC.
3. C) Every sporadic MSI-high CRC should have a mutation in *BRAF.*
4. D) Tumor samples are used to analyze germline mutations in the MMR genes.

**Correct Response:** A

**Explanation of Correct/Incorrect Response** - The new NCCN guidelines recommend evaluation of MSI status by molecular or IHC methods on all resected CRCs, or CRCs diagnosed in patients <70 years of age and in patients≥70 years of age who meet the Bethesda criteria. Therefore, A is correct. Loss of expression of MSH2/MSH6 strongly indicates Lynch syndrome, whereas loss of expression of MLH1 could be seen in both sporadic and Lynch syndrome-associated CRCs. Therefore, B is incorrect. A *BRAF* mutation is frequently detected in sporadic MSI-high CRCs, but some of sporadic MSI-high CRCs are *BRAF* wild type. Therefore, C is incorrect. Blood should be used to test for germline mutations in the MMR genes. Therefore, D is incorrect.

**CASE 4**

**Introduction** - Molecules in the EGFR signaling pathway have been employed as therapeutic targets for treatment of advanced CRCs. Downstream pathways in the EGFR pathway include the RAS/RAF/MAP kinase signaling pathway and PI3K/AKT/mTOR signaling pathway. Which of the following statements is correct?

1. *EGFR* mutations are not uncommon in CRCs.
2. Mutations in *KRAS* only occur in exon 2.
3. *BRAF* V600E is not the most common mutation of the *BRAF* gene seen in CRCs.
4. Genes in the PI3K/AKT/mTOR signaling pathways are rarely altered in CRCs.
5. Activating *KRAS* mutations are associated with resistance to anti-EGFR monoclonal antibodies.

**Correct Response:** E

**Explanation of Correct/Incorrect Response** - *EGFR* mutations are extremely rare in CRCs. Approximately 90% of *KRAS* mutations occur in exon 2. Rare cases have mutations in exons 3 and 4. The most common mutation of the *BRAF* gene in CRC is V600E. Mutations, deletions and promoter methylation commonly occur in genes in the PI3K/AKT/mTOR signaling pathways. Therefore, A, B, C, and D are incorrect. Anti-EGFR monoclonal antibodies are currently used only in advanced CRCs with no activating *KRAS* mutations. Therefore, E is correct.

**First Reply Question** - ***Which of the following statements is correct?***

1. Anti-EGFR monoclonal antibodies are only used in CRCs with *EGFR* mutations.
2. In CRCs, activating *KRAS* mutations are common, whereas activating *NRAS* mutations are rare, but are known to occur.
3. *PIK3CA* is only mutated in exon 20 in CRCs.
4. It has been proven that *BRAF* mutations confer resistance to anti-EGFR monoclonal antibodies.
5. The current NCCN guidelines recommend evaluation of *KRAS* mutations but not *NRAS* mutations in stage IV CRCs.

**Correct Response:** B

**Explanation of Correct/Incorrect Response** - EGFR mutations are rare in CRCs, and anti-EGFR monoclonal antibodies are currently used only in advanced CRCs with no activating *KRAS* mutations. Therefore, A is incorrect. While *KRAS* mutations are common (40-50%), mutations in *NRAS* are rare. Therefore, B is correct. The most common *PIK3CA* mutations occur in exon 9 and exon 20. Therefore, C is incorrect. While studies have shown that mutations in both *KRAS* and *NRAS* confer resistance to anti-EGFR monoclonal antibodies, the data regarding *BRAF* status are still controversial. Hence, the current NCCN guidelines recommend evaluation of *KRAS* and *NRAS* mutations and evaluation of *BRAF* mutations only if *KRAS* and *NRAS* are wild type. Therefore, D and E are incorrect.

**Second Reply Question - *Which of the following statements are correct?***

1. The current NCCN guidelines recommend *KRAS* and *NRAS* genotyping of tumor tissue for all patients with metastatic CRC.
2. *BRAF* mutations are not uncommon, with the most common mutation being V600E.
3. The clinical effect of *PIK3CA* mutations on anti-EGFR MoAb is still unclear.
4. All of the above is correct.

**Correct Response:** D

**Explanation of Correct/Incorrect Response** - The current NCCN guidelines recommend *KRAS* and *NRAS* genotyping of tumor tissue for all patients with metastatic CRC. *BRAF* mutational testing should be performed in certain situations, but is not currently required for treatment decisions. Approximately 15-20% of CRCs harbor a mutation in *BRAF*, more than 90% of which are V600E. The clinical significance of *PIK3CA* mutations on anti-EGFR MoAb is unknown but some data suggest that mutations in exon 20 may confer resistance to anti-EGFR MoAb. Therefore, all the above statements (D) are correct.

**CASE 5**

**Introduction -** Mutational status of *KRAS*, *BRAF*, *PIK3CA* and *PTEN* are now commonly analyzed in metastatic CRCs. Mutational analyses of many additional molecular biomarkers in CRCs will likely become increasingly recommended as new targeted therapies make their way into clinical practice. The vast majority of gene mutations identified in CRCs are point mutations. They can be detected by various molecular technologies, each of which has advantages and limitations.

**Primary Question** - ***Which of the following statements is correct?***

1. Sanger DNA sequencing, allele-specific PCR, melting-curve real-time PCR, pyrosequencing, and single base extension may all be used for detection of point mutations.
2. Allele-specific PCR, melting curve analysis, and pyrosequencing are more sensitive than Sanger sequencing.
3. Multigene assays for detection of gene mutations include single nucleotide extension assays.
4. Next generation sequencing technologies are increasingly utilized for molecular testing in clinical laboratories.
5. All of the above are correct.

**Correct Response:** E

**Explanation of Correct/Incorrect Response** - Point mutations can be detected by Sanger sequencing, allele-specific PCR, melting-curve real-time PCR and pyrosequencing methodologies. Sanger sequencing of DNA requires greater than 25% tumor cellularity, while other assays including allele-specific PCR, melting curve analysis and pyrosequencing require a much lower tumor cellularity for detection of mutations. The limitation of these assays is that they cannot be multiplexed. A number of platforms have been developed to simultaneously detect multiple point mutations, including single base extension. Next generation sequencing technologies, which enable high-throughput massively parallel sequencing of nucleic acids at a higher sensitivity, are increasingly used in clinical samples. Therefore, E is the correct answer.

**First Reply Question - *Which of the following statements is correct?***

1. Allele-specific PCR is used to specifically determine the presence of a single mutation.
2. Sanger sequencing, allele-specific PCR, melting-curve analysis, and pyrosequencing have high-throughput capabilities.
3. Multiple gene mutations cannot be identified using single base extension assays, such as the SnapShot platform.
4. All data generated from next generation sequencing of DNA are useful clinically.

**Correct Response:** A

**Explanation of Correct/Incorrect Response** - Allele-specific PCR was developed as a method to detect single nucleotide polymorphisms. Therefore, A is correct. B is incorrect, since a limitation of these assays is that they cannot be multiplexed. However, single base extension assays including SNaPSHOT platform and mass spectrometry are multiplex assays. Therefore, C is incorrect. Data analysis in next generation sequencing is complex, and many DNA polymorphisms identified by next generation sequencing have unknown clinical significance. Therefore, D is incorrect.

**Second Reply Question -** ***Which of the following statements is correct?***

1. Sanger sequencing, allele-specific PCR, melting-curve analysis, and pyrosequencing are able to simultaneously detect multiple mutations.
2. Sanger sequencing has a higher sensitivity than allele specific PCR in detection of point mutations.
3. Single base extension assays cannot be performed in multiplex.
4. Data analysis is complex for next generation sequencing of DNA and requires significant bioinformatics input. A major effort is required for annotation and variant classification.

**Correct Response:** D

**Explanation of Correct/Incorrect Response** - Sanger sequencing, allele-specific PCR, melting-curve analysis, and pyrosequencing are used to detect single nucleotide polymorphisms. Therefore, A is incorrect. Sanger sequencing requires tumor cellularity of >25%, whereas allele-specific PCR is able to detect <1% tumor cells. Therefore, B is incorrect. Single base extension assays, including the SNaPSHOT platform and mass spectrometry, are multiplex assays. Therefore, C is incorrect. Data analysis is a complex process in next generation sequencing, which requires significant bioinformatics input. Many variants identified by next generation sequencing have unknown clinical significance. Therefore, D is correct.