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# The Evolution of Next Generation Sequencing in Colorectal Cancer: Advances, Applications, and Outcomes

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**Cover Page Footnote**

As a sub-investigator for clinical trials at Tennessee Oncology, the author has a personal interest in next generation sequencing for colorectal cancer care wherein patients in the practice are linked to ongoing or planned clinical trials as determined by the presence of specific actionable genetic defects. As the author is committed to the highest caliber of cancer care for all patients, next generation sequencing will not be offered solely to study candidates but to all patients who fail to achieve cancer remission with first line standard of care therapy. The goal of this practicum is to provide information to all members of the cancer team on implementing next generation sequencing in an effort to maximize benefits with a targeted approach to colorectal cancer management regardless of the patient's potential to be part of clinical research.

NEXT GENERATION SEQUENCING

# The Evolution of Next Generation Sequencing in Colorectal Cancer: Advances, Applications, and Outcomes

Colorectal cancer is not a single homogeneous entity but rather a complex disease process with dichotomous embryologic, anatomic, histologic, genetic, and immunologic differences between left and right sided disease.[1] The differences in cancers of the right and left sides of the colon are so unique that researchers and clinicians actually refer to colon cancers as RCC (right colon cancer) and LCC (left colon cancer.) Nitsche et al (2016) defined tumors between the ileocecal valve and the hepatic flexure as RCC, whereas LCC included tumors between the splenic flexure and rectum (Figure 1.) [2]

**Figure 1. Schematic: RCC v. LCC**

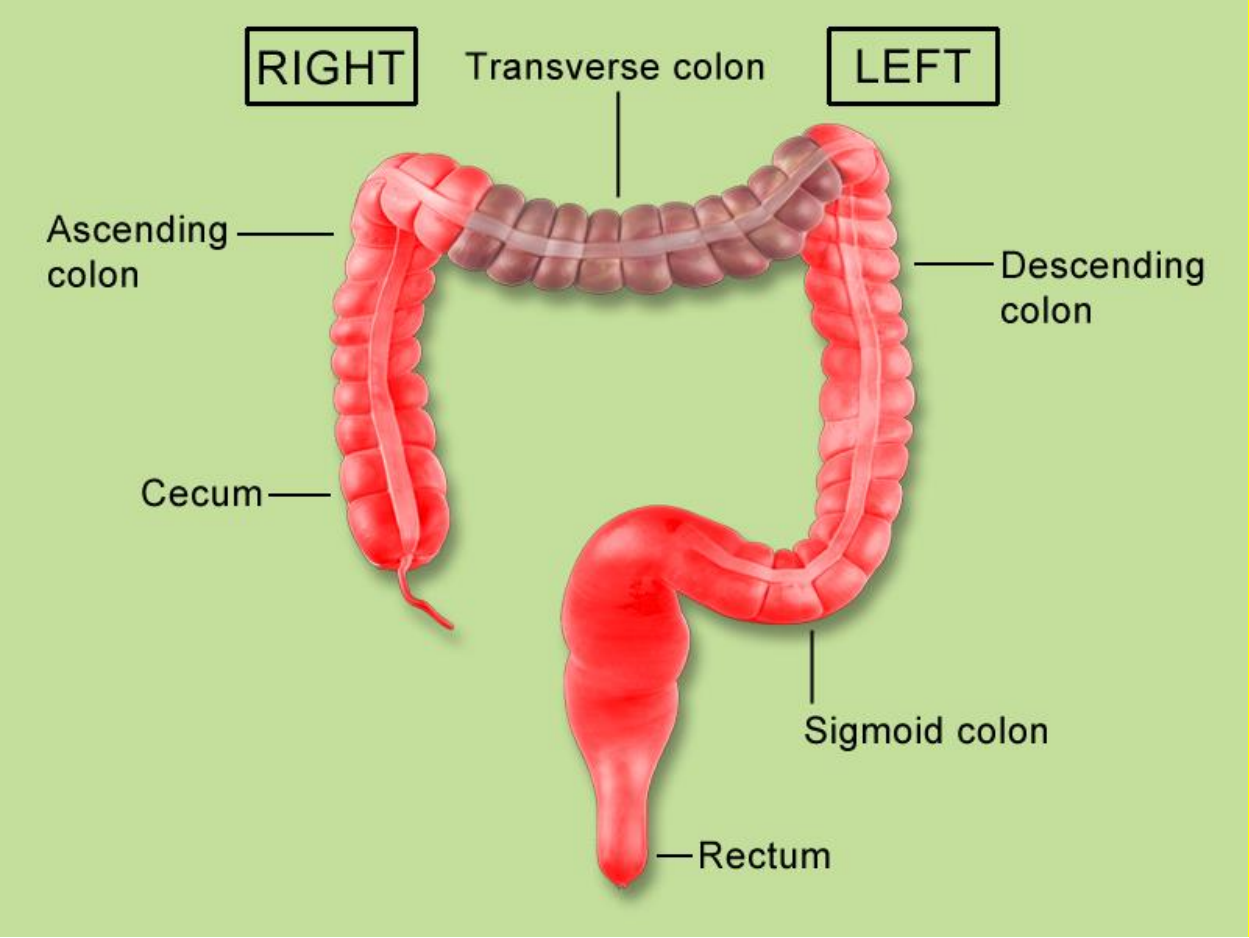


**Figure 1 explores the organs involved in right vs left sided colorectal cancer.**

Expanding on the concept of RCC and LCC, Shen et al (2015) published a compelling article in the *World Journal of Gastroenterology* detailing the anatomic differences of RCC in comparison to LCC.[3] These include unique blood supplies, nerve innervation, and lymphatic drainage. More importantly, they detailed the embryologic and molecular genetic differences between LCC and RCC, understanding that cancers developing in specific locations within the bowel result in differences in disease outcome, progression, development, and response to therapies. Because RCC presents with poorer histologies, higher incidences of associated anemia, and bowel perforation or obstruction, they tend to have poorer outcomes. They tend to be associated with risk factors such as insulin resistance, female gender, older age, and previous cancer history. LCC tends to be related more to low fiber diets, smoking history, and alcoholism. They are diagnosed more frequently than RCC but are lower grade tumors with more favorable histologies. RCC tends to involve "bulky, exophytic polypoid lesions" growing into the bowel lumen whereas LCC tend to include "infiltrating, constricting lesions encircling the bowel lumen" and often cause obstruction.[1]

## Molecular Aberrations Associated with Colorectal Cancers

Developing an understanding of the molecular aspects of colorectal cancers is paramount to disease management. Genetic mutations have been uniquely associated with both RCC and LCC, as have specific protein expressions, relapse paths, and prognostic biomarkers.[4] Carcinogenic pathways leading to RCC include MMR, KRAS, and BRAF. In contrast, pathways leading to LCC include NRAS and p53. A working knowledge of oncogenes involved in the carcinogenesis of colorectal cancer is critical to the science of next generation sequencing.

NEXT GENERATION SEQUENCING

Thirty percent of all human cancers have RAS mutations, making it one of the most commonly mutated genes in cancer.[5] The KRAS protein, otherwise known as p21, is a member of the RAS family of proteins. Anatomically located on human chromosome 12 and widely expressed in most human cells, KRAS is primarily responsible for EGFR-signaling activation.[6] Most KRAS mutations are single point mutations, and as many as 35-45% of all colorectal cancers involve a KRAS mutation. A pivotal paper by Andreyev et al (1998) detailed the RASCAL study, which involved 2721 patients from 13 nations.[7] This trial confirmed that the presence of a KRAS mutations in colorectal cancer patients increases both the risk of recurrence and death from disease. Other trials have not affirmed this association, including the broadly acknowledged PETACC phase III trial.[8]

KRAS is the most frequently mutated factor downstream of the EGFR pathway, making it an excellent molecular biomarker for anti-EGFR therapy for colorectal cancers. The National Comprehensive Cancer Network (NCCN) recommends routine testing for KRAS mutations in advanced stage colorectal cancers, having officially added it to their recommended treatment guidelines in 2009.[9]

BRAF is a protein kinase that also plays an important role in EGFR-signaling, with as many as 8-12% of colorectal cancers having BRAF mutations.[10] BRAF mutations are thought to be the catalysts that shift normal epithelium into adenomatous and serrated polyps, both of which are known to be precursors to colorectal cancer. [11] BRAF mutated colorectal tumors tend to have poorer histologies, are more common in women, and generally associated with microsatellite instability (MSI.)[12] MSI is a genetic predisposition toward mutation involving the MLH1, MSH2, MSH6, or PMS2 repair genes. Of note, microsatellite stable colorectal cancers have higher incidences of BRAF mutations and therefore poorer prognoses (Figure 3.)

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**Figure 3.**

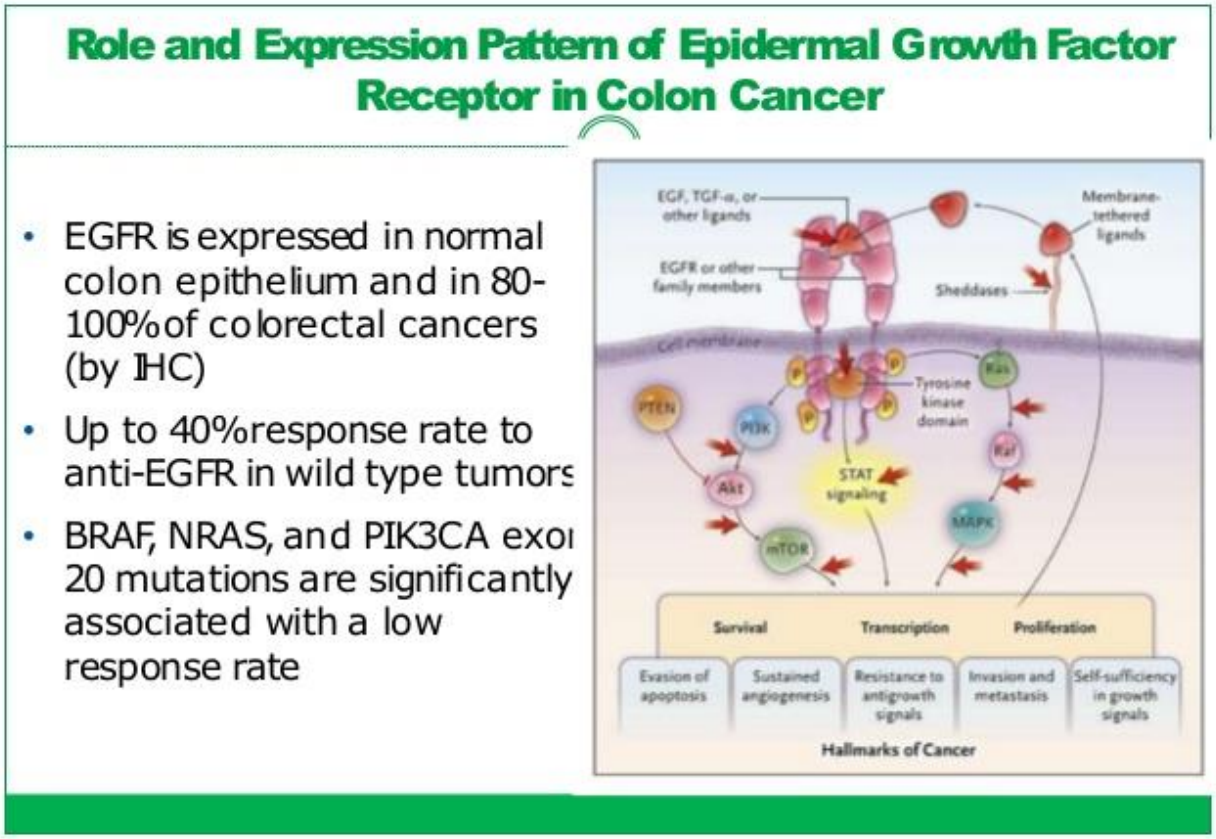


**Figure 3 is a schematic showing mutations commonly detected in colorectal cancer, especially those seen when next generation sequencing is performed.**

NRAS is also a member of the RAS family of proteins, and though it is reported in only 2-7% of all colorectal cancers, it plays an important role in the regulation of EGFR pathways.[13] NRAS mutated colorectal cancers are seen most commonly with left colon cancer, and like other RAS family mutations, accurately predict poorer disease prognoses. Dysregulation of p53 is also associated with poorer prognosis colorectal cancers. The p53 gene is located on human chromosome 17p, and functions to enhance cell apoptosis. As a tumor suppressor gene, its role is to promote normal cell death. If the gene is suppressed, cells are free to grow haphazardly until

cancers develop. Colorectal cancers with p53 defects have a greater propensity to treatment resistance.[14]

Single gene biomarkers have prognostic significance for RCC and LCC. The markers are unique to the anatomic side of colon cancer, much like the genetic mutations previously discussed. Higher integrin alpha a (ITGA3) expression levels can be predictive of relapse in right sided colon cancers, just as higher levels of NADPH oxidase 4 (NOX4) are in left sided colon cancer. ITGA3 is a gene on chromosome 17 that encodes for an alpha chain integrin protein. Because this protein plays a critical role in the development and differentiation of organs and tissue, aberrations in this protein can serve as the catalyst that promotes development of malignancies, including those arising in the colon.[15]

NOX4 is a gene located on chromosome 11, and its primary function is the production of ROS. ROS levels have been linked to both inflammatory and carcinogenic responses that lead to negative responses within the cells. These include angiogenesis, proliferation, and DNA damage responses.[15] Cells that have been damaged or altered such that they are no longer cycling through normal cell death cycles known as apoptosis become malignancies. Both NOX4 and ITGA3 contribute to cell migration and ROS production, enabling the development of more aggressive primary and metastatic colorectal cancers.

CDX2 is a gene located on chromosome 13. Because the gene plays a role in the early embryologic development of the intestinal tract, any aberrancy can lead to the development of colorectal cancers. It also has predictive value in the risk of recurrence and in the prediction of disease free interval in colorectal cancers. Bae et al (2015) published an article showing the significance of the loss of CDX2 expression in cells of the gastrointestinal tract.[16] They found a direct correlation with this loss and higher grade, poorer histology colorectal malignancies.

## NEXT GENERATION SEQUENCING

Microsatellite instability (MSI) is a "hypermutable phenotype caused by the loss of DNA mismatch repair (MMR) activity."[17] This is especially important in the management of colorectal cancers, as it is associated with as many as 15% of all colorectal cancers (Figure 2.) Further, 3% of these patients have Lynch syndrome, a hereditary risk for the development of colorectal cancer. The remaining 12% have sporadic mutations of the MLH1 gene.[17] These colorectal cancers have a slightly better prognosis than tumors without MSI, and do not respond to traditional chemotherapy drugs in the same manner as cancers that are microsatellite stable.

Microsatellite status can be tested through immunohistochemistry, pcr, and via next generation sequencing. All three assess for the presence of abnormalities in the proteins associated with MMR, which include MLH1, MSH2, MSH6 and PMS2. Absence of any one of these four proteins is considered abnormal, or "MSI-high." These can be tested with tissue, blood, or via stool specimens. The presence of MSI guides the management of colorectal cancers that have not been treated with curative intent with surgery alone. Colorectal cancers that test positive for MSI have been shown to respond to immunotherapy, a more tailored form of adjuvant therapy that may help avoid some of the toxicities related to traditional chemotherapy. Having a complete molecular profile, i.e. next generation sequencing, provides information to the oncology team not only about the biology of the tumor, but the potential benefit of traditional chemotherapy and newer forms of immunotherapy. This translates to better care, and potentially, a better response to care.

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**Figure 2.**

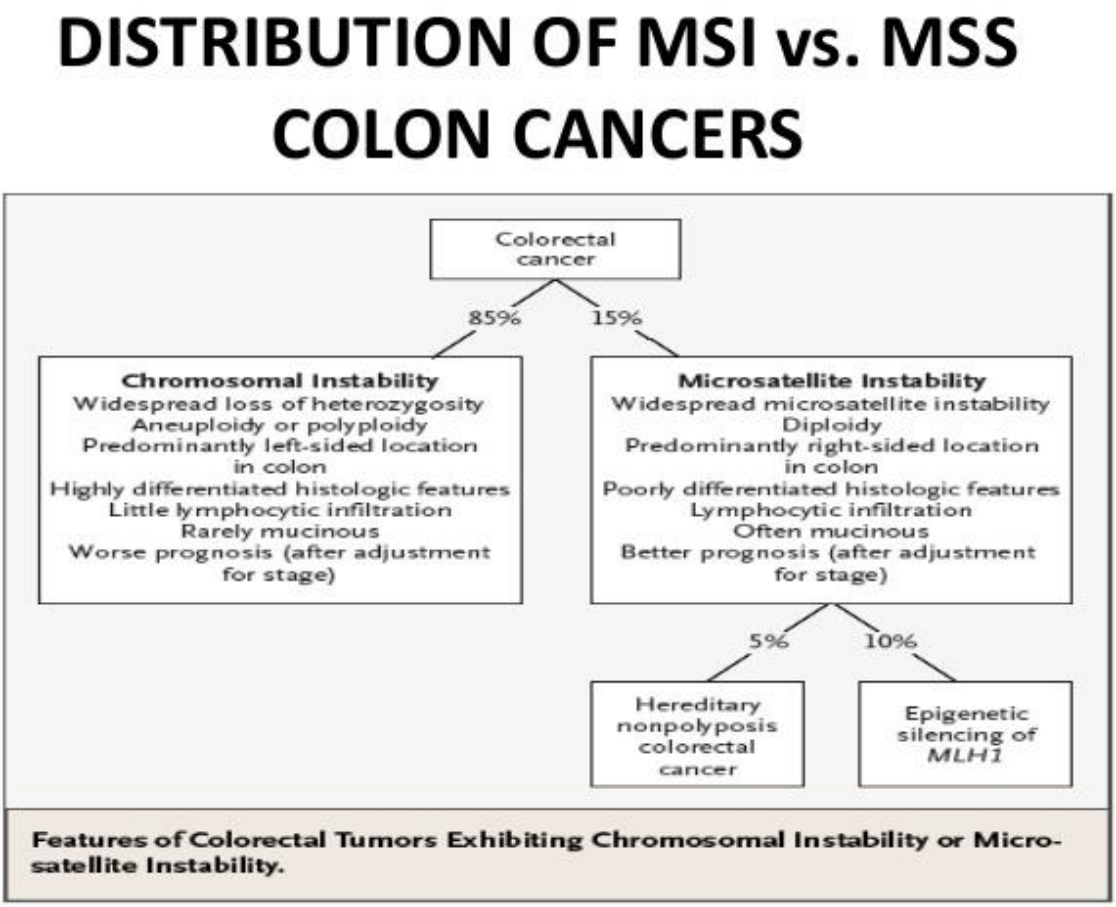


**Figure 2 depicts colorectal cancers with specific chromosomal instability or microsatellite instability.**

Emerging evidence suggests that some late stage, refractory colorectal cancers may be her 2 neu driven, and may therefore respond to anti-her 2 therapies. Though breast and gastric cancers have been studied extensively with respect to her 2 neu over-expression, colorectal cancers have only recently been targeted for investigation of the same genetic overexpression of the human epidermal growth factor 2 protein. At the recent ASCO meeting (2018), investigators participating in the phase 2 HERACLES trial presented data revealing that the combination of trastuzumab (Herceptin) plus lapatinib (Tykerb) achieved positive results in patients with heavily pretreated, HER2-positive metastatic colorectal cancer.[18] This two-pronged, HER2-directed approach

achieved clinical benefit in 70% of patients and an overall response rate of 30%. These patients typically do not respond well to available therapies for colorectal cancer, suggesting that her2 neu expression should be considered for colorectal cancers when making treatment decisions, especially in refractory disease.

## Management of Colorectal Cancers

Traditional management of colorectal cancers requiring therapy beyond surgery has included the same cytotoxic regimen first developed in the 1960s. This regimen, known to the oncology world as "FOLFOX," targets cells that are in the process of replicating their DNA. The regimen cannot differentiate between malignant and non-malignant cells, leading to cytotoxicity and a great number or secondary side effects.[19] The side effects include, but are not limited to: mucositis, pancytopenia, risk of fevers/infections, hand/foot/mouth desquamation, diarrhea, alopecia, and sensory peripheral neuropathy. While most of the side effects resolve post therapy, elements of any of these toxicities may remain for a lifetime. The most frightening element of all chemotherapy for patients is the known risk of developing secondary malignancies associated with many cytotoxic drugs.

In the 1980s, the first targeted therapies for cancer were developed.[20] Targeted therapies, according to the National Cancer Institute, focus on the cancer-specific molecular pathways that influence the growth, division, and spread of cancer cells. Other targeted therapies work by either boosting the body's natural immune system to kill cancer cells, blocking hormone receptor sites, or promoting oncogene death. Targeted therapies include hormonal therapies, immunotherapy, apoptosis (programmed cell death) inhibitors, and gene expression modulators.[4] Because targeted therapies do not produce uniform results in all patients, there is a distinct need to have a full panel of biomarkers that are unique to individuals in order to maximize the benefit

of these innovative therapies. Next generation sequencing provides the data to allow for the creation of a targeted therapy "recipe" that is tailored to the individual with the ultimate goal of maximizing benefits and minimizing toxicity.

Most of the current targeted therapies that are available commercially are monoclonal antibodies. By definition, a monoclonal antibody only binds to one substance. They can be used to carry drugs, toxins, or radioactive substances directly to cancer cells. Bevacizumab is one of the first monoclonal antibodies studied in colorectal cancer. It functions by shutting down angiogenesis, the mechanism by which cells develop blood supplies to procure a nutrition source.[21] This process, called VEGF inhibition, does not cure advanced stage colorectal cancer, but it has been shown to extend life by five months when used with chemotherapy.

Similarly, Cetuximab and Panitumumab target EGFR. EGFR, also known as epidermal growth factor receptors, are found on the surfaces of many cancer cells. They are especially abundant on colorectal cancer cells. When epidermal growth factor attaches to its receptor site, it causes cells to multiply, thus promoting cancer growth. Applying this information to specific genetic aberrations that would be discovered during next generation sequencing, Lievre et al (2006) the first to determine colorectal cancers with KRAS mutations have poor responses to the monoclonal antibody Cetuximab.[22] Subsequent research extended this knowledge base to include a similar lack of positive treatment response in colorectal cancers having NRAS mutations.[23]

## Next Generation Sequencing: The Process

Sanger sequencing, the first generation of DNA sequencing methods, was developed in the 1970s. Next generation sequencing is conceptually similar but allows the entire human genome to be sequenced in a single day at a cost of about $1000 (Muzzey et al, 2015.) Both methods repurpose the DNA replication machinery that copies DNA during every cell division. As Muzzey et al

(2015) point out, DNA replication only requires three types of molecules: "a template strand, free bases, and a polymerase enzyme that links the free bases together one by one into a new strand that is complementary to the template."[24] Both methods of sequencing use extendable fluorescently labeled modified bases: A, T, G, and C. With the Sanger method, only a small number of these bases are modified, but with NGS, all of the bases are modified. NGS uses positional separation of millions of DNA template strands, which then bind to discrete positions on a slide and remain fixed at that location through the entire sequencing process.

## NGS: Clinical Applications and Limitations

Next generation sequencing became available at the turn of the century, providing information about targeted "hot spots" in the genome of cancers. This genetic data then allowed for the development of prognostic and therapeutic molecular biomarkers. These biomarkers were subsequently incorporated into clinical trials that later confirmed the heterogeneity of colorectal cancer. Whole genome sequencing is not routinely recommended for colorectal cancer care planning, but rather a targeted panel specific to known colorectal cancer genetic mutations. Sequencing the entire genome is not cost effective and provides a vast body of data without practical utility. Limiting the sequencing to KRAS, NRAS, BRAF, EGFR, Her 2 Neu, and MMR allows for assessment of potentially actionable mutations.[4]

Limitations in next generation sequencing extend beyond the cost of testing. Larger panels will take more time to result, potentially resulting in delay of therapy initiation. Panels limited specifically to colorectal cancer are available and are the most practical for routine clinical practice. Tissue specimens are most reliable, though current panels provide accurate results with minimal DNA requirements. Implementation of targeted next generation sequencing allows for

reliable identification of the most common mutations known for colorectal cancers, which can guide therapeutic decision making.

## Conclusions

Targeted next generation sequencing allows providers to target cancer related mutations and create treatment plans specific to individuals. Though the technology may be limited by a two to four-week turnaround time for results and the expenses related to the testing, the information gained by NGS expands treatment options and may help extend the lives of patients affected by colorectal cancers. Improving the quality and quantity of life enjoyed by cancer patients is the summative goal of cancer care with hopes of expanding the utility to NGS to future curative intent.

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