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Original Study

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Tumor Heterogeneity as a Predictor of Response to Neoadjuvant Chemotherapy in Locally Advanced Rectal Cancer

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Abstract

A standard therapy for locally advanced cancers includes neoadjuvant chemoradiation; however, currently there is no way of knowing which patients have disease that will respond to such therapy. We analyzed 21 pretreatment rectal cancer biopsy samples and found a positive correlation of the response to therapy with a quantitative mutant-allele tumor heterogeneity (MATH) score. This next-generation sequencing derived score may serve as a biomarker for response to therapy.

Background: Neoadjuvant chemoradiotherapy (nCRT) is the standard of care for locally advanced adenocarcinoma of the rectum, but it is currently unknown which patients have disease that will respond. This study tested the correlation between response to nCRT and intratumoral heterogeneity using next-generation sequencing assays. **Patients and Methods:** DNA was extracted from formalin-fixed, paraffin-embedded biopsy samples from a cohort of patients with locally advanced rectal adenocarcinoma (T3/4 or N1/2 disease) who received nCRT. High read-depth sequencing of $>$ 400 cancer-relevant genes was performed. Tumor mutations and variant allele frequencies were used to calculate mutant-allele tumor heterogeneity (MATH) scores as measures of intratumoral heterogeneity. Response to nCRT was pathologically scored after surgical resection. **Results:** Biopsy samples from 21 patient tumors were analyzed. Eight patients had disease noted to have complete response, 2 moderate, 4 minimal, and 7 poor. Higher MATH scores correlated with poorer response to treatment, demonstrating significantly increased tumor heterogeneity compared to complete response ($P = .039$). **Conclusion:** The application of MATH scores as a measure of tumor heterogeneity may provide a useful biomarker for treatment response in locally advanced rectal cancer.

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Keywords: Bioinformatics, Colorectal cancer, Personalized medicine, Targeted sequencing, Translational research

Introduction

Colorectal cancer (CRC) is the third most common and lethal cancer in the United States. Rectal cancers account for over one third of CRC cases, with approximately 39,000 new cases diagnosed each year.[1] Treatment of locally advanced rectal cancer is different from equivalent-stage colon cancer. Neoadjuvant chemoradiotherapy (nCRT) became the standard of care for locally advanced disease after a number of landmark randomized controlled trials found nCRT decreased rates of local tumor recurrence, increased rates of sphincter preservation, and decreased both acute and long-term treatment toxicity.[2-5] Since 2003, nCRT has been rapidly adopted and is now extensively used.[6] However, the disease demonstrates a spectrum of response to nCRT, ranging from complete to poor or no response. Complete response is associated with decreased local recurrence and improved overall survival (OS),[7-9] while poor response is associated with worse outcomes. Studies have explored mechanisms to explain resistance to nCRT,[10,11] but no biomarker or single somatic mutation is

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routinely used to predict which patients have disease that will respond to nCRT.

Intratumoral heterogeneity (ITH) has been proposed to be a mechanism of resistance to both chemotherapy and radiotherapy. Neoplastic cells are thought to proliferate from a singular common ancestor.[12,13] Tumor cells can undergo additional sequential mutations, resulting in subclones within the same tumor. The presence of ITH correlates with more rapid cell proliferation, increased metastasis, resistance to therapy,[14] and worse clinical outcomes. Although ITH is studied in research laboratories, a reproducible method to quantify ITH in the clinical setting is not available.

The mutant-allele tumor heterogeneity (MATH) score is a novel next-generation sequencing method to quantitatively measure ITH.[15] The MATH score is the width of the distribution of mutant-allele fractions among tumor loci. A locus that mutated early in the clonal evolution of the tumor will have a high mutant-allele fraction, while later, lower frequency loci mutations demonstrate a lower mutant-allele fraction.[16] In head and neck squamous-cell carcinoma, higher MATH scores, and therefore increased ITH, correlate with shorter OS and worse clinical outcomes.[16] We previously showed that higher MATH scores correlated with worse outcome in colon cancer.[17] One previous study applied MATH scores specifically to rectal cancer, demonstrating highly variable degrees of tumor heterogeneity in 6 tumors.[18] However, the correlation of MATH scores to clinical treatment or outcomes in rectal cancer is unknown.

The purpose of this study was to determine if MATH scores can predict response to nCRT in locally advanced rectal adenocarcinoma.

Patients and Methods

Twenty-three patients with locally advanced rectal cancer who underwent surgical resection of their tumors after nCRT were identified in a prospective database. Locally advanced tumors were defined as American Joint Commission on Cancer Staging, 7th edition, T3 or T4 tumors, node-positive (N1/N2) or T2 disease, with the possibility of sphincter-sparing surgery.[19] After institutional review board approval, formalin-fixed, paraffin-embedded blocks of rectal pretreatment tumor biopsy samples were obtained. Disease was categorized according to nCRT response, as described by histologic neoinduced regression in the surgical pathologic specimen (complete 0, minimal +1, moderate +2, poor +3) after surgical resection. Pretreatment rectal biopsy samples were used for patient DNA analysis.

DNA was extracted using standard techniques. Samples were analyzed as previously described[17] using the Ion AmpliSeq Comprehensive Cancer panel assay, which targets more than 400 cancer-relevant genes. Sequencing was performed on the Ion Proton instrument as described by the manufacturer (Thermo Fisher Scientific, Waltham, MA), generating average read depths of $> 1200\times$ across the targeted regions. As a quality control, only samples that yielded $> 500\times$ average coverage for all 4 multiplexed primer pools were used for downstream analyses. Variants were called by the Torrent Server Variant Caller (versions 4.21, 4.421, 4.607, or 5.021, with interchangeable results). The resulting variant caller format (VCF) files were compared using customized bioinformatics scripts, as described previously.[17] Analyses were limited to single nucleotide variants (no indels) with a read depth greater than 100 and at least 5 reads in each direction.

Variant allele frequencies (VAF) were calculated as the ratio of alternate allele observations to the read depth at each position. The MATH score is calculated as 100 × (median absolute deviation/median of VAF). It describes the ratio of the width of the data to the center of the distribution—effectively a score describing the spread in the data. We applied the MATH score[12] to quantify the tumor heterogeneity in our rectal tumor samples, as well as a modification to include both tumor and germline heterozygous (genotype 0/1) variants with VAF between 0.05 and 0.75. The comparisons of MATH score between response categories were conducted using Student t test statistics after checking the required assumptions of the distribution and equal variance. $P < .05$ was considered statistically significant.

Results

Targeted next-generation DNA sequencing was used to identify the somatic tumor variants in a cohort of 23 retrospective rectal cancer biopsy samples that included disease with response to nCRT treatment that was judged to be complete, moderate, minimal, or poor (Table 1). Two samples failed to pass quality-control criteria, so results from 21 samples were used for analyses.

Because no samples of normal tissue were available for comparison, we identified tumor mutations on the basis of their exclusion from databases of common germ-line polymorphisms (dbSNP 137), with VAF below 0.50. The most common mutations that were also listed in the Catalog of Somatic Mutations in Cancer (COSMIC) (PMID 27899578) are shown in Figure 1A. Most common mutations were in the *APC*, *TP53*, and *KRAS* genes, which is similar but not identical to the mutations most commonly observed by others.[20] The differences could suggest alternative genetic or environmental mechanisms in our cohort of patients from New Mexico. Figure 1B shows a plot of the observed VAF for each of the COSMIC mutations shown in Figure 1A. Some mutations (eg, *APC*, *KMT2C*, *KRAS*, *TP53*) were observed in a broad range of allele frequencies in different tumors, suggesting that these mutations were acquired early in the evolution of the tumors. However, many of the mutations that occurred less frequently (eg, *EGFR*, *FBXW7*, *LIFR*, *MSH6*) were only observed at low allele frequencies, suggesting that they were acquired later and were only present in a subset of tumor cells.

The correlation between the presence of specific mutations with either good or poor response to nCRT treatment was attempted. Figure 2 shows a heat map indicating the presence of COSMIC mutations in all samples, which were sorted by either complete or moderate response to nCRT, and minimal or poor response. There was no correlation between response and either the presence of specific mutations or the number of acquired mutations. For example, acquired mutations in *APC*, *KRAS*, or *TP53* were observed in samples from both groups. Thus, there appears to be no simple way of predicting response to nCRT solely on the basis of the use of targeted gene panel sequencing in these types of rectal cancer samples.

We next determined whether quantitative traits in the sequencing information could be used to gain additional insight into the biology of response to nCRT. We previously showed that the

Table 1 Characteristics of Rectal Cancer Patient Samples

Sample No.	Response	Sex	*APC* Mutation	*KRAS* Mutation	*TP53* Mutation	MATH Score
06	Complete	F	Yes	No	No	11.8
08	Complete	M	No	No	Yes	17.24
09	Complete	M	Yes	No	No	11.06
10	Complete	M	No	Yes	Yes	10.89
30	Complete	F	Yes	No	No	15.64
43	Complete	F	No	No	No	12.73
45	Complete	F	No	No	No	7.88
47	Complete	F	No	No	No	14.4
16	Moderate	M	Yes	No	No	6.62
17	Moderate	F	Yes	Yes	No	19.84
12	Minimal	F	No	No	No	8.62
13	Minimal	F	No	No	No	25.23
14	Minimal	M	No	No	Yes	28.69
15	Minimal	M	Yes	Yes	No	15.35
20	Poor	F	No	No	Yes	26.67
21	Poor	F	No	No	No	19.75
36	Poor	U	No	No	No	13.26
40	Poor	M	No	No	No	13.55
44	Poor	U	Yes	No	No	27.95
46	Poor	U	No	No	No	14.31
48	Poor	M	Yes	No	No	17.05

Abbreviations: MATH = mutant-allele tumor heterogeneity; U = sex information unavailable or not provided.

MATH score as a measure of tumor heterogeneity correlated with tumor stage in colon cancer.[17] We tested whether the MATH score could be used as a measure of tumor heterogeneity in rectal cancers. Figure 3 compares the distribution of VAF. Sample 16 shows peaks of allele frequencies at 0.5 and 1.0, which are the heterozygous and homozygous variants, with very few somatic mutations at other frequencies. In contrast, sample 44 shows a broad distribution of allele frequencies and has a much higher MATH score of 27.95, compared to 6.62 for sample 16. Thus, the higher MATH score quantifies the spread in the VAF observed in sample 44, which reflects increased tumor heterogeneity.

Next, we tested whether the MATH scores correlated with response to nCRT. As shown in Figure 4A, the samples from patients whose disease had complete response to nCRT had significantly ($P = .039$) lower MATH scores than samples from patients with disease that had poor response to nCRT. A similar difference was observed when comparing complete response to samples in all other categories (moderate, minimal, or poor, $P = .026$; Figure 4B) or when comparing complete + moderate response to minimal + poor response ($P = .02$; Figure 4C). In contrast, there was no difference in MATH scores when comparing samples that did or did not harbor mutations in *APC* (Figure 4D) or other common mutations (data not shown).

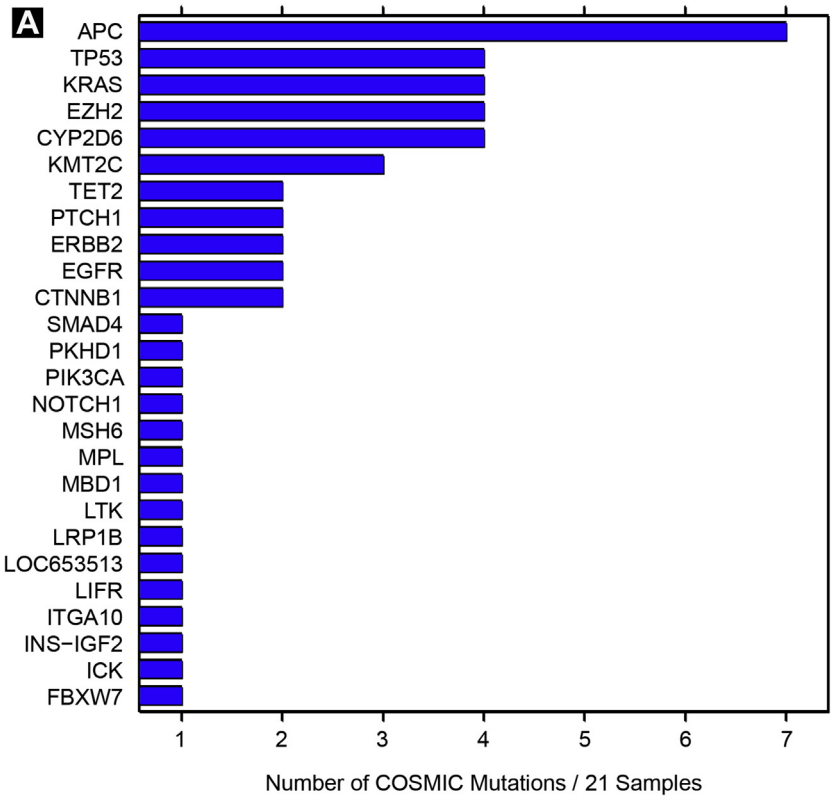
Discussion

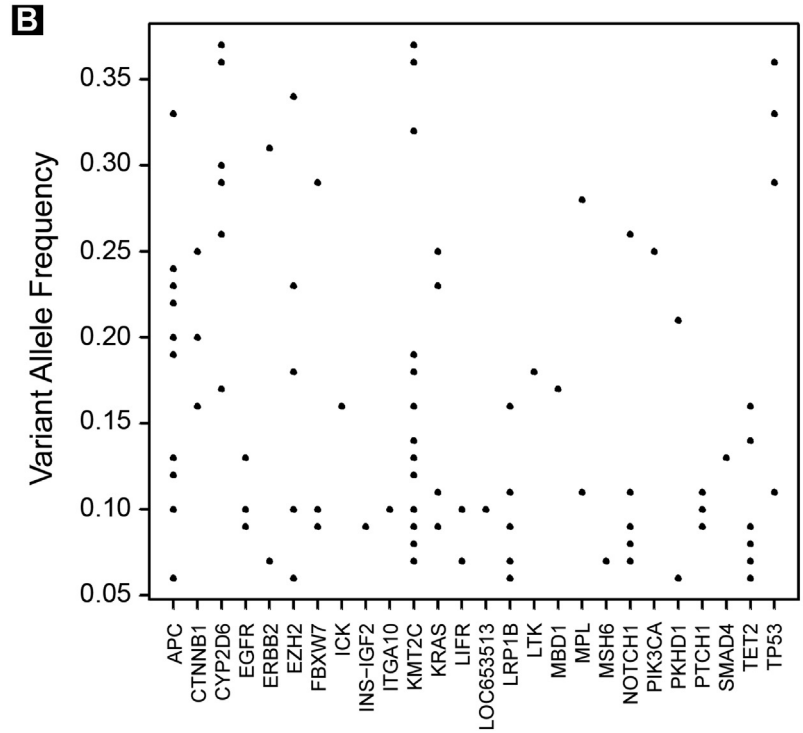
Preoperative nCRT is the standard of care for locally advanced rectal adenocarcinoma. MATH scores, a novel bioinformatics tool allowing quantitative measurement of tumor genetic heterogeneity, appears to have relevance in the study of rectal cancer. We observed higher MATH scores, and thus increased tumor genetic heterogeneity, to be associated with poorer response to nCRT. Although these results will need to be validated in a larger group of samples, it appears that measures of tumor heterogeneity such as the MATH score have the potential to predict treatment response.

Some studies examining biomarkers to predict nCRT response in rectal cancer have been promising; others have presented contradictory results. In 2001, Ki-67 expression was found to correlate with nCRT response, with a higher Ki-67 labeling index seen in patients with complete response.[10] In contrast, a study in 2008 demonstrated that lower Ki-67 expression was associated with higher level of tumor regression.[11] Increased expression of epidermal growth factor receptor (EGFR) and p21 have been observed in tumors with poor nCRT response,[21,22] while high thymidylate synthase levels and lower pretreatment DNA methylation are associated with higher response rates.[23] These studies suggest that a number of molecular markers may predict nCRT response, although confirmatory studies are ongoing, and their routine use in clinical practice is currently unavailable.

Although the effect of tumor heterogeneity on response to nCRT in rectal cancer is unknown, genetic heterogeneity is thought to be a major contributor to monoclonal antibody treatment resistance in CRC. A number of factors may explain how increased tumor heterogeneity contributes to treatment resistance in cancer. A tumor subclone may possess a Darwinian advantage in its ability to survive the stresses of hypoxia and cytotoxic chemotherapy,[13] or it may harbor a subclone with a mutation that renders the tumor resistant to targeted molecular therapy. CRC is considered to be a highly heterogeneous disease, with mounting evidence revealing various genetic aberrations in both colon and rectal tumors.[24]

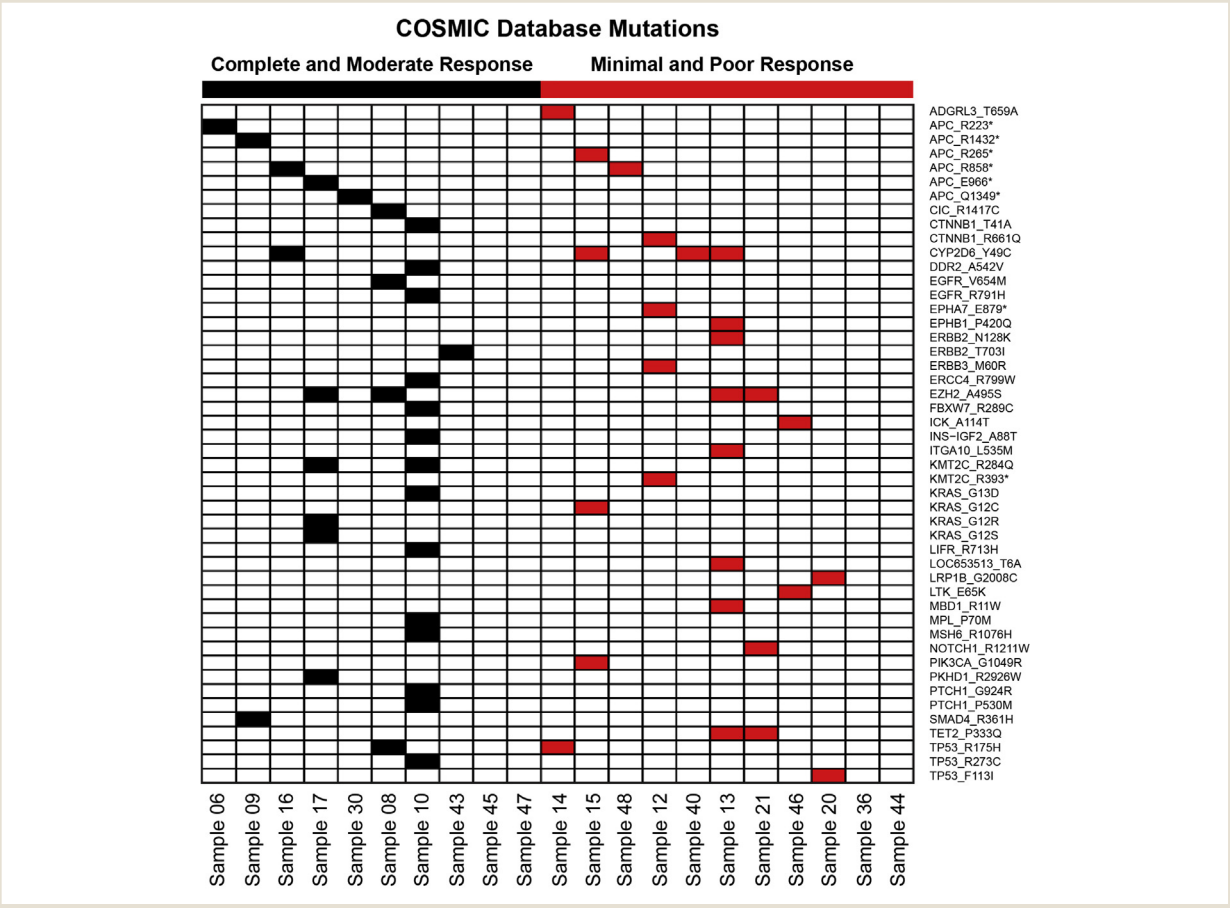
Figure 1 Acquired COSMIC Mutations in Rectal Cancer Samples. Frequencies of Occurrence (A) and Observed Variant Allele Frequencies (B) for Somatic Mutations Listed in COSMIC Database





Abbreviation: COSMIC = Catalog of Somatic Mutations in Cancer.

Figure 2 Distribution of COSMIC Mutations in Rectal Cancer Samples. Heat Map Shows Presence of Mutations Listed in COSMIC Database in Rectal Cancer Samples Analyzed. Samples at Left (Black) Showed Complete or Moderate Response to nCRT; Samples at Right (Red) Showed Minimal or Poor Response



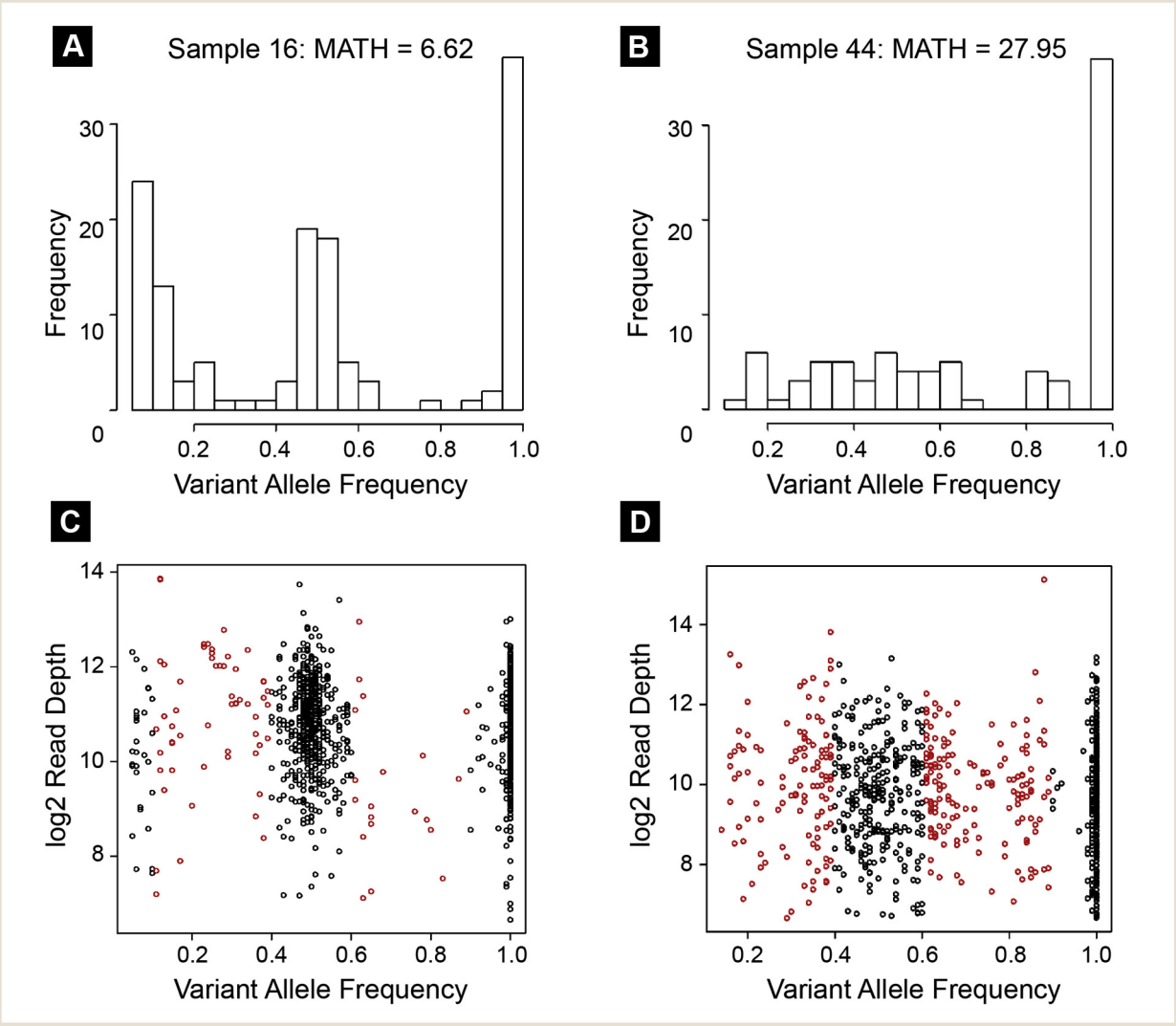
Abbreviations: COSMIC = Catalog of Somatic Mutations in Cancer; nCRT = neoadjuvant chemoradiotherapy.

The MATH score offers a global assessment of tumor heterogeneity, compared to the single-gene assessments currently used in CRC. *KRAS* mutations are present in nearly 40% of CRC tumors[25] and are known to drive resistance to anti-EGFR monoclonal antibody treatment. Patients with *KRAS* wild-type tumors should benefit from anti-EGFR therapy, though nearly 35% of such tumors will not respond to treatment.[25,26] It has been hypothesized that this treatment resistance is the result of the presence of subclones possessing *KRAS* mutations, many times with concurrent *BRAF* and *PIK3CA* mutations. The current standard method of tumor biopsy in both clinical and research settings cannot detect the presence or extent of such genetic heterogeneity, as only one area of the tumor is sampled. In fact, nearly one third of CRC tumors demonstrate ITH by analyzing *RAS* mutations in multiple tumor areas.[27] Similar rates of intertumoral heterogeneity (39%) are seen between primary tumors and either lymph node or distant metastases.[27] While detection of mutations such as *KRAS* has helped to guide targeted molecular therapy, the extreme degree of genetic heterogeneity in CRC presents a major challenge to precision medicine.[28]

Predicting which patients have disease that will respond to nCRT holds important clinical implications in rectal cancer. Complete pathologic response, defined as ypT0N0M0 after nCRT and total mesorectal excision, is seen in 15% to 20% of patients with rectal cancer. Complete response is associated with decreased local recurrence and improved OS.[7-9] Conversely, patients with disease with little or no response to nCRT experience poorer outcomes and survival. Although high ITH is known to contribute to cytotoxic and targeted molecular therapy resistance in CRC, breast, esophageal, and lung cancers,[29,30] no studies to date have directly examined the relationship of rectal cancer tumor heterogeneity and response to nCRT. By stratifying the biologic profiles of locally advanced rectal cancer, providers could have the ability to discuss an individual patient's prognosis. Recommendations could be tailored toward more aggressive adjuvant treatment or toward observation according to their predicted response to nCRT.

The MATH score, originally developed in 2013 and studied in head and neck squamous-cell carcinoma,[12] now presents a novel potential biomarker in CRC. We previously demonstrated that higher MATH scores are associated with more advanced stages of

Figure 3 Tumor Heterogeneity and MATH Scores. Observed VAFs Are Displayed as Histograms (Top, A and B) or Plots of VAF Versus Read Depth (Bottom, C and D) for Samples 16 (Left, MATH = 6.62) or 44 (Right, MATH = 27.95). Note Spread in Observed VAF in Sample With Higher MATH Score



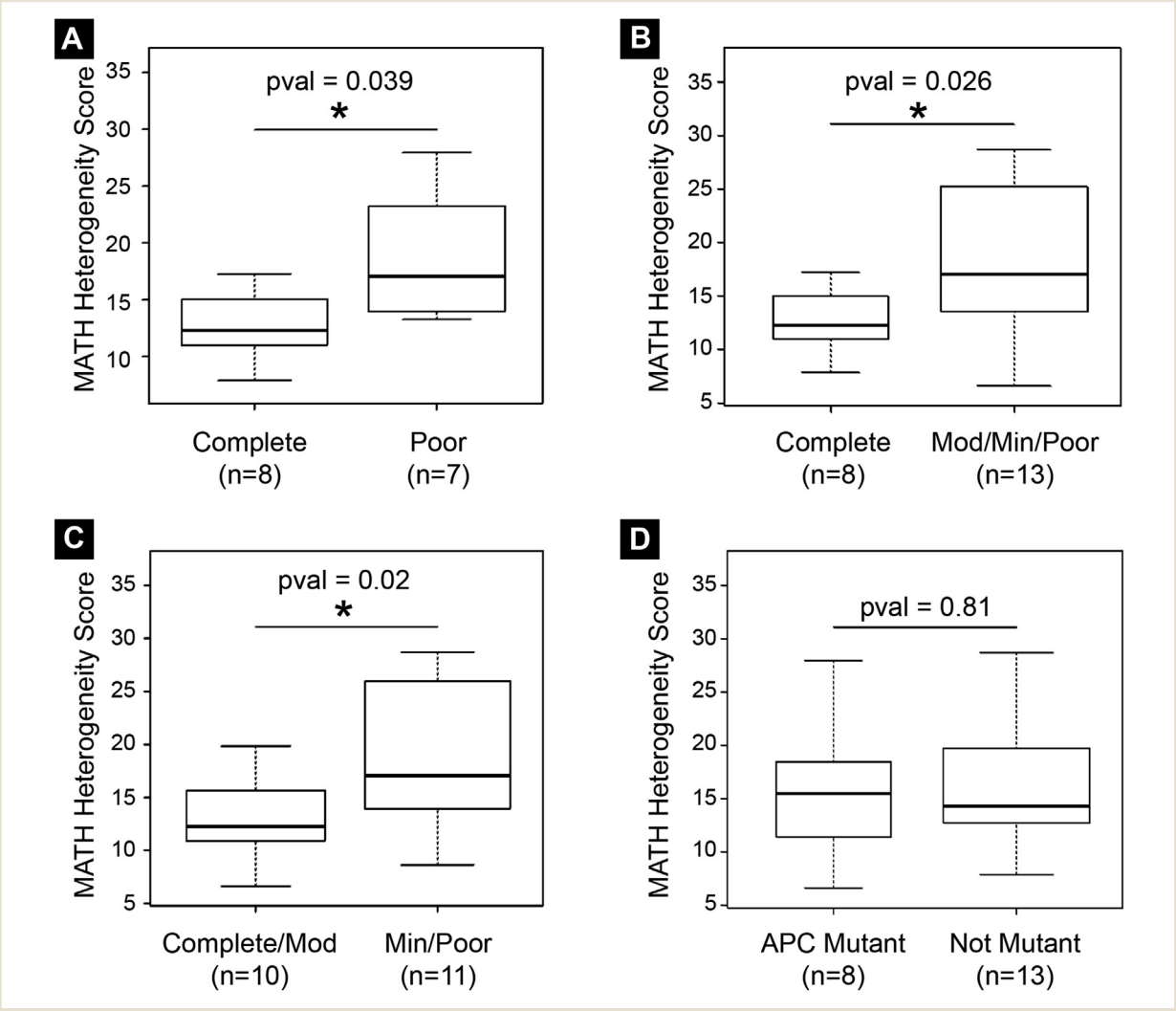
Abbreviations: MATH = mutant-allele tumor heterogeneity; VAF = variant allele frequency.

disease and increased metastatic capability.[17] One previous study to date has specifically applied MATH scores to rectal cancer.[18] Next-generation sequencing of 2 to 3 separate areas of 6 rectal tumors found that although all patients demonstrated heterogeneity, the degree varied significantly. Our study is consistent with these findings, with the added correlation of tumor heterogeneity to treatment response. The MATH score offers a promising method to identify patients at high risk of treatment failure, both in practice and in clinical trial settings. It should be mentioned, however, that although computation of the MATH score may allow for patient stratification and prediction of nCRT response, it does not address the complex issue of how to improve treatment for patients with increased tumor heterogeneity.

One limitation of this study is its small sample size of 21 rectal tumors. However, this is the largest analysis using MATH scores in rectal cancer to date, and the comparison of MATH scores by nCRT response categories was striking enough to yield significant results. Second, the rectal cancer samples and MATH scores were retrospectively obtained. A prospectively designed study must be conducted to further investigate MATH score as a clinical biomarker. Third, DNA sampling and analysis was performed from one area of the tumor. Although future studies will sample multiple tumor areas, whole-tumor sequencing is not yet possible. Future studies in rectal cancer should also examine MATH scores in relation to oncologic outcome data including OS, disease-free survival, and local recurrence.

Tumor heterogeneity presents a challenge to the developing field of precision oncology. In this pilot study, we applied a novel bioinformatics approach, the MATH score, to quantitatively measure tumor genetic heterogeneity in locally advanced rectal cancer.

Figure 4 MATH Scores Correlate With Poor Response to nCRT. Box Plots Compare MATH Scores for Patients Who Demonstrated Complete Versus Poor Response to nCRT (A), Complete Response Versus Moderate, Minimal, or Poor Response (B), and Complete or Moderate Versus Minimal or Poor Response (C). There Was No Correlation Between MATH Scores and Presence of Mutations in *APC* Gene (D)



Abbreviations: MATH = mutant-allele tumor heterogeneity; nCRT = neoadjuvant chemoradiotherapy.

Higher MATH scores were associated with poorer response to nCRT. MATH scores present a potential biomarker to predict nCRT response in rectal cancer in both clinical and research settings.

Conclusion

The MATH score is a quantitative measure of tumor heterogeneity. This technique does not rely on a single gene mutation or a unique signaling pathway. The MATH score uses the entire 400+ gene data set to look at the shape of the data. MATH scores generated from pretreatment rectal cancer biopsy samples correlate with response to nCRT. Thus, knowing a patient's MATH score may allow for customizing treatment protocols.

Clinical Practice Points

- Rectal cancer remains a leading cause of cancer-related morbidity and mortality.
- Locally advanced tumors are treated with chemotherapy and radiotherapy before surgical resection. This approach leads to decreased local recurrence rates, perioperative complications, and possibly increases the rate of sphincter preservation. Currently it is not known which patients have disease that will have a favorable response to chemoradiation.
- By using next-generation sequencing techniques, we found that the quantitative MATH score, a reflection of tumor heterogeneity, correlates with response to neoadjuvant chemoradiation treatment.

- Higher MATH scores correlate with a poorer response to neoadjuvant chemoradiation. This is important information to know, as patients with disease with less favorable response fare worse compared to patients with disease with a good response to such treatments.
- Knowing a patient's MATH score and thus the degree of heterogeneity may allow for a more practical and economic surveillance strategy, and possibly better personally designed treatment algorithms.

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Disclosure

The authors have stated that they have no conflict of interest.

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