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Molecular Diagnosis of Response to Neoadjuvant Chemoradiation Therapy in Patients with Locally Advanced Rectal Cancer

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Abstract

Background—Pathologic complete response (pCR) to neoadjuvant chemoradiation (CRT) is an important prognostic factor in locally advanced rectal cancer. However, it is uncertain whether histopathological techniques accurately detect pCR. We tested a novel molecular approach for detecting pCR and compared it to current histopathological approaches.

Study design—Pre-treatment tumor biopsies and surgical specimens were collected from 96 patients with locally advanced rectal cancer treated with neoadjuvant CRT and surgery. Tumor response was categorized by tumor regression grade (TRG). Tumor DNA from pre-CRT tumor biopsies was screened for K-*ras* and *p53* mutations. DNA from paired surgical specimens was then screened for the same mutations using highly sensitive polymerase chain reaction (PCR)-based techniques.

Results—Sixty-eight out of 96 (71%) pre-treatment biopsies harbored K-*ras* and/or *p53* mutation; 36 (38%) had K-*ras* mutations, 52 (54%) had *p53* mutations and 20 (21%) carried both mutations. Of 70 patients with TRG 1–3, 66 (94%) had a concordant K-*ras* and *p53* mutation profile in pre- and post-treatment tissues. Of 26 patients with TRG 0 (pCR), 12 had K-*ras* or *p53* mutations in pre-treatment biopsies. Of these, 2 (17%) patients had the same K-*ras* (n=1) or *p53* (n=1) mutation detected in post-treatment tissue.

Conclusions—Sensitive molecular techniques detect K-*ras* and *p53* mutations in post-CRT surgical specimens in some patients with a pCR. This suggests histopathological techniques may not be completely accurate, and that some patients diagnosed with a pCR to CRT may indeed have occult cancers cells in their surgical specimens with K-*ras* and *p53* mutations serving as reliable surrogates for residual disease.

Keywords

chemoradiation; surgery; rectal cancer; molecular analysis; K-*ras*; *p53*

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Introduction

Pre-operative neoadjuvant chemoradiation (CRT) has an established role in the management of patients with rectal adenocarcinoma because it provides the opportunity to downstage tumors, increase sphincter preservation, and decrease the risk of local disease recurrence. (1–3) Rectal cancer response to CRT varies from minimal tumor downsizing to an apparent complete disappearance of all viable cancer cells, known as a pathologic complete response (pCR). Rectal cancer patients with a pCR after CRT have lower local recurrence rates and improved overall survival compared to patients with residual cancer cells in their surgical specimens. (4–10) These findings have clinical implications, as surgeons are cautiously proposing less extensive surgical resections, or even no surgery at all in patients who achieve a pCR to CRT. (11) However, before these approaches can be widely accepted, it is important to ensure that a diagnosis of pCR accurately represents the complete eradication of all tumor cells. Otherwise, the oncologic outcomes of these patients could be significantly compromised.

A pCR is currently diagnosed using histopathology. Representative sections of the tumor and lymph nodes are embedded in paraffin. A number of 5–10 μm sections are then stained with hematoxylin and eosin (H&E) and analyzed microscopically for the presence of cancer cells. However, given that a small number of rectal cancer patients with a pCR develop a local relapse after 5 years (10) it is currently unclear whether this method can accurately detect complete disease eradication or whether it can precisely detect the presence of minimal residual disease, information which is critical to predict oncologic control.

Previous studies have proven that molecular techniques able to identify tumor-specific molecular-genetic alterations can be more accurate than conventional histopathology in identifying cancer cells in surgical resection margins and regional lymph nodes of a variety of tumors. (12–16) Using this rationale we considered whether similar molecular techniques could be used to verify pCR or the presence of minimal residual disease in rectal cancer patients who undergo CRT. We used standard polymerase chain reaction (PCR) as well as the highly-sensitive PCR-based techniques, peptide nucleic acid (PNA) clamp PCR and pyrophosphorolysis activated polymerization-allele specific (PAP-A) PCR to determine the mutation status of K-*ras* and *p53*, two of the genes more frequently mutated in rectal cancer patients. (17–27) We prospectively analyzed mutation status in pre- and post-treatment samples from 96 rectal cancer patients who received CRT followed by surgery and compared the results of molecular and histopathological analysis of the surgical specimen to determine the accuracy of these techniques for verifying pCR.

Methods

Patients and treatment

Ninety-six rectal cancer patients with Stage II (uT3–4, uN0) or Stage III (any T, uN1–2) tumors were enrolled in the Timing of Rectal Cancer Response to Chemoradiation study, a multi-institutional clinical trial investigating the effect of increasing the CRT-to-surgery interval, and adding chemotherapy, modified FOLFOX-6 (mFOLFOX-6), during the waiting period (ClinicalTrials.org Identifier: NCT00335816). This trial was designed as a series of sequential Phase II trials or study groups (SGs), each with a progressively longer CRT-to-surgery interval and increasing cycles of pre-operative mFOLFOX-6. This study was approved by an Institutional Review Board (IRB) at each participating institution as well as a central IRB, and informed written consent was obtained from each patient prior to enrollment in the trial. Further details of patient eligibility for this trial are presented elsewhere. (28)

Patients in both SGs were treated with CRT; 5-FU 225 mg/m^2/day for 7 days in continuous infusion and a total of 50.4 Gy of radiation. Patients in SG1 underwent total mesorectal excision (TME) an average of 6 weeks after completing CRT (standard of care). Following CRT, patients in SG2 with no evidence of stable disease received 2 cycles of additional chemotherapy (mFOLFOX-6); leucovorin 200 mg/m^2 or 400 mg/m^2 plus oxaliplatin 85 mg/m^2 by 2h infusion, followed by bolus of 5-FU 400 mg/m^2 and a 46h infusion of 5-FU 2,400 mg/m^2. Patients in SG2 underwent TME an average of 11 weeks after completing CRT. The clinical outcomes for these patients are presented elsewhere. (28)

Assessment of response

Pathologic response after CRT was determined according to the AJCC tumor regression grading (TRG) classification. (29) Briefly, TRG 0 - complete response: no viable cancer cells; TRG 1 - moderate response: single cancer cells or small groups of cancer cells; TRG 2 - minimal response: residual cancer outgrown by fibrosis; TRG 3 - poor response: extensive residual cancer. TRG 0 was scored as a pCR. Surgical tissues were evaluated and graded by pathologists using standard histopathological techniques and TRG was determined for each tumor by two independent blinded pathologists.

Sample preparation and DNA extraction

Pre-treatment biopsies and post-treatment surgical tissues were collected prospectively and 5–10 μm tissue sections were taken from formalin-fixed, paraffin-embedded (FFPE) blocks, placed on microscope slides, and de-paraffinized. Slides were stained with 0.2% methylene blue and tumor cells were micro-dissected manually from pre- and post treatment samples under inverted microscopy.

For all post-treatment samples (TRG 0–3), representative tissue sections from throughout each tumor block were assessed to ensure sampling of the entire surgical specimen. Slide sections were pooled and used for DNA extraction. Pre- and post-treatment DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen; Valencia, CA) with the following modifications to the manufacturer's protocol: an extension of digestion time at 56°C from 1 hour to 48 hours and the addition of three 20 μl aliquots of Proteinase-K at 4, 20, and 28 hours during digestion. DNA was quantified by measuring absorbance (NanoDrop, Thermo Fisher Scientific, Inc.; Wilmington, DE).

Mutation analysis of pre-treatment biopsies and post-treatment surgical specimens

Tumor cells from pre-treatment biopsies and post-treatment surgical specimens were micro-dissected and tumor DNA was extracted as indicated above. Standard PCR analysis was performed to detect mutations in *p53* and K-*ras*. Primers were used to amplify genomic sequences in exons 4–8 of *p53* and exons 2–3 of K-*ras* with the following cycling conditions; 98°C for 10 seconds, 60°C for 20 seconds, and 72°C for 30 seconds, for 45 cycles. Primer sequences are listed in Supplementary Table 1 (online only). All sequencing reactions were performed in both the sense and antisense directions with PCR primers, and all mutations were confirmed by two independently derived PCR products.

Identification of rare mutations in post-treatment surgical specimens

In patients with TRG 0, FFPE tissue sections obtained from the treated tumor bed were examined and DNA was extracted from areas with ulceration or fibrosis. Since these tissues were scored as having no residual cancer cells, we employed more sensitive PCR techniques to detect minimal residual or occult disease.

PNA clamp PCR was performed to identify K-*ras* mutations at codons 12 and 13 in TRG 0 DNA extracted from post-treatment specimens. This technique was established to identify a

single mutant allele in the presence of over 10,000 copies of wild-type DNA. (30–34) PNA clamp PCR reactions were performed using Phusion HS II high fidelity polymerase (Thermo Fisher Scientific, Lafayette, CO). Reactions consisted of 1 X Phusion HF buffer, 0.2 mM dNTP, 0.15 μM primers, 0.25 μM PNA, 1U Phusion HS II DNA polymerase, and 200 ng of template DNA in a final volume of 25 μl. The cycling conditions were 98°C for 10 seconds, 76°C for 10 seconds, 60°C for 20 seconds, and 72°C for 30 seconds, for 60 cycles. PNA clamp information and PCR primer sequences are presented in Supplementary Table 1 (online only). All PCR products were sequenced to verify the presence or absence of K-*ras* mutations and the reactions were repeated (5 X) for each specimen.

In contrast to K-*ras*, mutations in *p53* can occur at several locations in exons 4–8. (22, 25–27) Therefore, a single PNA clamp PCR-based assay could not be used to detect the multiple *p53* mutations that may exist in the hundreds of codons in exons 4–8. Instead, we utilized a PAP-A PCR assay for the detection of known *p53* mutations. This technique can detect *p53* mutation with high sensitivity in the presence of more than 10,000 copies of wild-type *p53* DNA. (35) Post-treatment TRG 0 tissues with *p53* mutation in the paired/corresponding pre-treatment biopsies were assessed by PAP-A as follows: PAP-A primers were designed to detect the specific *p53* mutation identified in the pre-treatment biopsy (Supplementary Table 1, online only). A 100–200 bp region within the *p53* gene was amplified by PAP-A with mutant-specific primers with a 3′ dideoxy block. The blocked primers were synthesized by adding a specific dideoxynucleotide to the oligodeoxynucleotide by terminal transferase. (36) Each reaction contained 50ng of template DNA plus 50 mM Tris-HCl (pH: 7.8, 25uC), 16 mM $(NH4)_2SO_4$, 1 mM DTT, 1.5 mM $MgCl_2$, 90 mM PPi, 100 mM blocked primers (P*), 4% DMSO, 25 mM dNTP, and 4 U KlenTaq S in a total volume of 25 μl plus 20 ng fish DNA-carrier in sensitivity assays and negative controls. The cycling conditions were as follows: denaturation at 94°C for 2 minutes, then 94°C for 20 seconds, 58–61°C for 30 seconds, 64°C for 30 seconds, 68°C for 30 seconds, and 72°C for 30 seconds, for 50–55 cycles, and 72°C for 7 minutes for the last extension. 5 μl of PAP-A product was checked by agarose gel electrophoresis (2–4%) and reactions were repeated (5 X) to determine the presence or absence of *p53* mutations.

To internally validate the sensitivity of the PNA clamp PCR and PAP-A assays, tissue sections from surgical-specimen FFPE tissue blocks from TRG 1–3 patients were also analyzed by PNA clamp PCR (K-*ras* mutations) and PAP-A (*p53* mutations) as described above, to verify the K-*ras* and *p53* mutation status obtained for TRG 1–3 patients using standard PCR and sequencing.

Results

Patient demographics and clinical characteristics

Patient demographics and clinical characteristics for the 96 patients included in the analysis are summarized in Table 1. A total of 73 patients (76%) were diagnosed with Stage III rectal cancer. Most patients had T3 disease (92%) and N1 disease (71%). All patients completed the full course of CRT and all patients underwent TME.

Histopathological examination of surgical tissues was performed and response was categorized by TRG. A total of 26 patients (27%) had TRG 0, 21 (22%) had TRG 1, 34 (35%) had TRG 2 and 15 (16%) had TRG 3.

Pre-treatment mutation profile

Standard PCR of the pre-treatment patient biopsies identified K-*ras* mutation in 36 (38%) and *p53* mutation in 52 (54%) out of 96 specimens. The K-*ras* and *p53* mutations stratified by TRG are presented in Table 2. K-*ras* and *p53* mutations were less common in patients

with TRG 0 compared to TRG 1–3, but the differences were not statistically significant (p = 0.19). The majority of K-*ras* mutations (27 out of 36; 82%) were single point mutations in codon 12. The most common mutation was *G12D*. Other mutations included *G12S*, *G12V*, and *G13D*. In contrast, there was a broad spectrum of *p53* mutations including point mutations, base-pair substitutions, insertions, deletions, and nonsense mutations. Point mutations were the predominant *p53* aberrancy, identified in 39 out of 52 (75%) patients. The pre-treatment K-*ras* and *p53* genotypes for all patients are presented in Tables 3 and 4.

Mutation profile for pre- and post-treatment tissues (TRG 1–3)

Standard PCR of the post-treatment specimens in patients with residual disease (TRG 1–3) revealed that all patients with pre-treatment K-*ras* mutation (n = 31) had K-*ras* mutation in the paired post-treatment surgical tissue. K-*ras* mutation was concordant in 30 out of 31 (97%) patients (Table 3). The one discordant patient (TRG 1) had *G12S* in the pre-treatment biopsy and *G12D* in the post-treatment tissue (Table 3, Figure 1a). In the remaining 39 patients with no pre-treatment biopsy K-*ras* mutation, no K-*ras* mutation was detected in the paired surgical tissues.

There were 45 patients with *p53* mutation in pre-treatment biopsies (TRG 1–3) and 43 of them (96%) had *p53* mutation in the corresponding post-treatment surgical tissues. Discordance occurred in 2 patients who each carried a *p53* mutation in the pre-treatment biopsy and no *p53* mutation in the post-treatment surgical tissues (Table 3). Interestingly, both of these patients had K-*ras* mutation in both pre- and post-treatment tissues. In the remaining patients with *p53* mutation, *p53* genotype was unchanged between pre- and post-treatment tissues. One of the 25 patients with no *p53* mutation in the pre-treatment biopsy harbored a new *p53* mutation in the surgical specimen (Table 3, Figure 1b).

To ensure that mutations did not arise secondary to radiation and that there were no field defects in normal mucosa outside the tumor which had these mutations, we extracted DNA from normal proximal resection margin tissue and screened it for K-*ras* and *p53* mutations. No K-*ras* or *p53* mutations were detected in any TRG 1–3 patients (data not shown).

Internal validation of PAP-A and PNA clamp PCR assays

To assess the sensitivity of our PCR assays, we first tested PAP-A and PNA clamp PCR in FFPE tissue sections from patients with gross or microscopic residual disease (TRG 1–3). For PAP-A, we correctly detected all *p53* mutations in post-treatment tissues corresponding to the standard PCR results. Similarly, we detected the same K-*ras* mutations in both pre- and post-treatment tissues with the PNA clamp PCR assay. No new mutations were detected in tissues from TRG 1–3 specimens using the PNA clamp PCR technique (data not shown).

Detection of K-*ras* mutation in TRG 0 patients using PNA clamp PCR

In the TRG 0 patient cohort, K-*ras* mutation was detected in pre-treatment biopsies in 5 out of 26 (19%) patients (Table 4). Despite the expectation that no mutation would be detected in any post-treatment tissues, K-*ras* mutation was detected in the post-treatment tissue of 1 patient by PNA clamp PCR (Table 4, Figure 1c). The genotype of the mutation was the same as the pre-treatment biopsy. No new mutations were detected by PNA clamp PCR. None of the remaining patients who harbored K-*ras* gene mutation in the pre-treatment biopsies (n = 4) had mutant K-*ras* detected in the post-treatment tissues, and no K-*ras* mutations were detected in patient DNA from proximal resection margin tissue (data not shown).

Detection of p53 mutation in TRG 0 patients using PAP-A

In the TRG 0 patient group, *p53* mutation was detected in pre-treatment biopsies in 7 out of 26 (27%) patients (Table 4). PAP-A analysis detected a *p53* mutation in 1 post-treatment specimen, matching the *p53* genotype of the paired pre-treatment biopsy (Table 4). No new *p53* mutations were detected in the post-treatment surgical specimens. None of the remaining patients who harbored *p53* mutation in the pre-treatment biopsies (n=6), had *p53* mutation detected in the post-treatment tissues and no *p53* mutations were detected in patient DNA from proximal resection margin tissue (data not shown).

Discussion

Our study shows that overall *p53* and K-*ras* mutations in rectal cancer rarely change as a result of CRT. We found that for nearly all patients analyzed, the genotype of *p53* or K-*ras* mutation remained consistent between pre-and post-treatment tissues despite CRT. Importantly, using the highly sensitive PCR-based PAP-A and PNA clamp techniques, we were able to detect gene mutations in two post-treatment patient specimens that were determined to be TRG 0 by histopathology. Given that detection of these mutations may be a surrogate for residual or occult cancer cells this is a significant finding as it suggests that current histopathological techniques may miss occult cancer cells present in the surgical specimen and that the application of sensitive molecular-genetic techniques may improve the accuracy for diagnosing pCR in response to CRT in rectal cancer.

We focused on mutations in K-*ras* and *p53*, as these are two of the most common genetic aberrations found in colorectal cancer. (17–27, 37–41) Nearly 35–55% of colorectal cancer patients harbor a mutation in the *p53* gene and 35–45% of patients harbor a mutation in the K-*ras* gene. Overall, the prevalence of these mutations in our patient population is consistent with what has been reported previously, (22, 25–27, 38). However, it is important to note that these mutations were only present in sixty-eight (71%) patients in our study. The remaining twenty-eight (29%) patients could therefore not be assessed using molecular approaches. To fully determine the effectiveness of using mutation-screening-based molecular techniques to assess pCR, it will be important to screen a larger panel of colorectal genes for mutations so that all patients can be assessed. Imperiale, et al. adopted this approach in their study to identify abnormal DNA in stool samples from colorectal cancer patients (42). Their fecal DNA panel consisted of 21 mutations present in the K-*ras*, *p53*, and *APC* genes as well as the micro-satellite instability (MSI) marker BAT-26. Screening for a similar panel of mutations may detect additional mutations in our patient cohort, which may be informative for assessing pCR.

With the exception of three patients, *p53* and K-*ras* mutations detected in pre-treatment biopsies could also be detected by conventional PCR in paired post-treatment surgical tissues. Although a small number of studies have compared the mutation profiles between primary tumors and their corresponding metastasis after chemotherapy, ours is the first study comparing mutation profiles pre- and post-treatment in rectal cancer using neoadjuvant CRT and additional chemotherapy (mFOLFOX-6) before surgery. In general, our study is in agreement with prior published data and demonstrates concordance in the mutation profiles of the primary tumor pre- and post-treatment. (41, 43, 44) However, we found discordance in the *p53* genotype between pre- and post-treatment tissues in three patients and in one patient we found a change in the K-*ras* genotype. Of the three patients with a genotype change in *p53* from pre- to post-treatment, two had loss of *p53* mutation and one patient had a gain of *p53* mutation. The two patients with loss of *p53* mutation initially harbored both K-*ras* and *p53* mutations in their pre-treatment biopsies; and only mutant *p53*, not K-*ras*, was lost in the post-treatment tissues, suggesting that the loss of *p53* mutation is probably real and not likely a sequencing artifact. There are no other reported cases of a genotypic change

in the same mutant K-*ras* codon following chemotherapy or radiotherapy. These discordances have some plausible explanations. Solid tumors, and in particular colorectal tumors, are intrinsically heterogeneous and different regions have cell populations with different gene mutations. Therefore sampling different regions may yield a different mutation profile.(15, 45, 46) It is also possible that the genotoxic stress of chemotherapy and radiation may either select clones of cells with a genotype that might contribute to CRT resistance or introduce new mutations in oncogenes and tumor suppressor genes.

We found that patients with a pCR overall had fewer mutations in *p53* and K-*ras* compared to non-pCR patients. This is an important finding because for the current approach to be successful, it is critical that these mutations are detected in pCR patients. The concordance in mutation profiles in tumors before and after CRT also has important clinical implications. Detection of specific mutation profiles in plasma has been used to monitor tumor response and diagnose early relapse after treatment. (45, 46) However, the accuracy of this method requires that the mutation profile remain consistent throughout treatment. Our data suggest that mutations in two of the genes more commonly mutated in rectal cancer do remain largely stable after treatment with CRT and that for the most part the detection of K-*ras* and *p53* can be used to screen patients for residual or recurrent disease after treatment.

The development of local tumor recurrence after apparent complete surgical resection with negative microscopic surgical margins, and systemic disease in patients with node negative disease, has questioned the sensitivity of standard histopathological techniques to detect occult cancer cells or minimal residual disease. (14) Over the past decade, a number of sensitive molecular-genetic techniques have been developed for the purpose of detecting tumor-specific genetic alterations as indicators of the presence of occult cancer cells in the tissue, body fluids, or peripheral blood of cancer patients. (12–16, 30, 33, 47, 48) PAP-A and PNA clamp PCR are two molecular techniques that have the sensitivity to detect single copies of mutant DNA in a background of abundant wild-type DNA. The utility of PNA clamp PCR in detecting single copies of mutant K-*ras* alleles has been well established (30–33) however, its use is impractical to detect *p53* mutations because of the diverse locations of these mutations. To detect *p53* mutations, we utilized the sensitive PAP-A technique. (35, 36) Using these sensitive PCR assays, we detected tumor-specific genetic alterations in 2 out of 12 (17%) of the TRG 0 surgical specimens. This suggests that, similar to what occurs in histologically negative resection margins and lymph nodes, there may be occult cancer cells in some rectal cancer specimens diagnosed with pCR based on histopathological techniques. Our finding may provide a potential explanation as to why some rectal cancer patients develop local recurrence after an apparent complete response after CRT. (10) However, the clinical implications of finding tumor DNA in a surgical specimen with a histopathological diagnosis of pCR are still unknown. To prove that patients with a pCR and mutant DNA behave in a similar fashion to non-pCR patients with respect to local recurrence and oncologic outcomes, a long follow-up and a larger series of patients will be required.

In summary, we have demonstrated that sensitive molecular-genetic techniques can detect tumor-specific gene mutations in rectal cancer specimens diagnosed by standard histopathology as having a pCR in response to CRT. Given the high concordance of K-*ras* and *p53* genes mutations in pre- and post-treatment specimens, the detection of these gene mutation signatures may act as a surrogate of residual or occult disease. This has significant clinical implications for diagnosis of pCR in transrectal biopsies of the tumor bed and when considering a non-operative approach for patients with clinical complete response. Our studies suggest that molecular diagnosis may provide further verification beyond the resolution of histopathology that complete response has in fact been achieved. Future studies are planned to improve the accuracy of pre-operative biopsy such that it may be used to help select therapy after CRT, to follow these patients and look at their long term outcomes, and

to extend our study to include additional patients to further strengthen and validate our findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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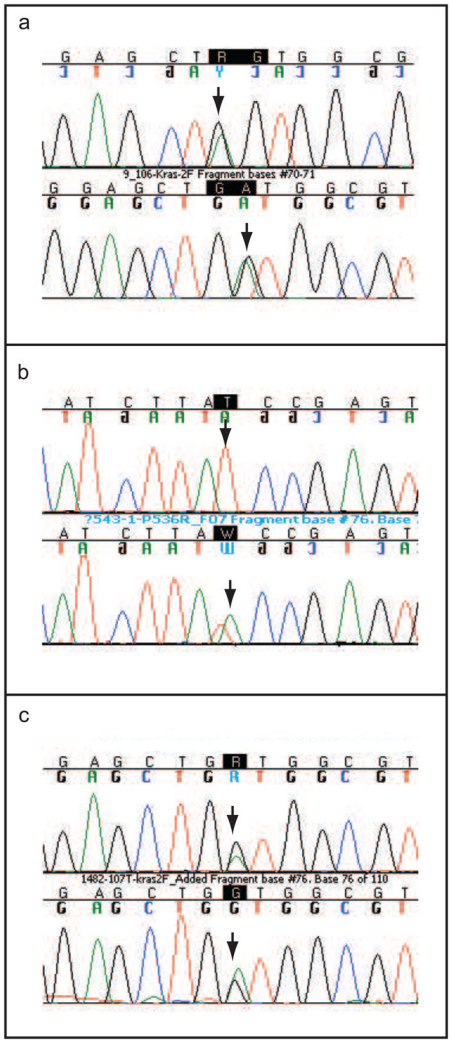

Figure 1.

Mutation analysis of pre-and post treatment patient specimens. (a): One patient (TRG 1) had a discordant *K-ras* mutation profile pre-and post treatment. The mutation is within codon 12. The upper panel shows K-*ras* mutation in the first position of codon 12 (GGT > AGT) in the pre-treatment biopsy specimen. The lower panel shows K-*ras* mutation in the second position of codon 12 (GGT>GAT) in the surgical specimen following treatment. (b): One patient (TRG 3) harbored a new *p53* mutation in the surgical specimen that was not present in the pre-treatment biopsy. The upper panel shows a wild-type genotype following sequencing exon 6 of the p53 gene in the pre-treatment biopsy. The lower panel shows a new mutation in codon 195 of exon 6 (ATC > AAC) in the surgical specimen following treatment. (c): One patient who was TRG 0 after treatment had a mutation in the K-*ras* gene. The upper panel shows mutation in codon 12 GGC > GAC of the K-*ras* gene in the pre-treatment biopsy specimen. The lower panel shows the same mutation in codon 12 GGC > GAC in the surgical sample detected by PNA clamp PCR.

Table 1

Pre-treatment Patient Demographics and Clinical And Pathologic Characteristics

Characteristics	Patients (n=96)
Sex, n (%)	
Male	56 (58)
Female	40 (42)
Age, y, median (range)	57 (32–85)
Clinical stage, n (%)	
II	24 (25)
III	73 (76)
T Stage, n (%)	
T2	7 (7)
T3	88 (92)
T4	1 (1)
Lymph node stage, n (%)	
N0	24 (25)
N1	68 (71)
N2	4 (4)

Table 2

Frequency of *p53* and K-*ras* Mutations Detected in Pre-Treatment Patient Biopsies

Gene mutation	Patients screened, n=96*		TRG 0 (n=26)		TRG 1 (n=21)		TRG 2 (n=34)		TRG 3 (n=15)	
	n	%	n	%	n	%	n	%	n	%
p53	52	54	7	27	12	57	25	74	8	53
K-*ras*	36	38	5	19	11	52	16	47	4	27

* Of 96 patients screened, 68 carried K-*ras* or *p53* mutations. Percentages for these patients are presented using the total number of patients screened (n=96) as the denominator.

TRG, tumor regression grade.

Table 3

Pre- and Post-Treatment K-*ras* and *p53* Mutation Analysis in Patients with Residual Disease (TRG 1–3) using Standard Polymerase Chain Reaction

Patients	Genotype of K-*ras*		Genotype of *p53*	
	Pre-T	**Post-T**	**Pre-T**	**Post-T**
TRG-1				
1	wt	wt	E298Stop	E298Stop
2	G12D	G12D	R175H	R175H
3	G12D	G12D	C242F	C242F
4	wt	wt	wt	wt
5	wt	wt	Y163N	Y163N
6	wt	wt	E5p.149InsT	E5p.149InsT
7	wt	wt	wt	wt
8	wt	wt	wt	wt
9	wt	wt	wt	wt
10	wt	wt	G245S	G245S
11	G13D	G13D	R181Y	R181Y
12	G13D	G13D	C176R	C176R
13 *	G12S	G12D	wt	wt
14	G12D	G12D	wt	wt
15	G12D	G12D	E285K	E285K
16	G12D	G12D	wt	wt
17	G13D	G13D	wt	wt
18	wt	wt	wt	wt
19 *	G13D	G13D	M237I	wt
20	G13D	G13D	R306Stop	R306Stop
21	wt	wt	R175H	R175H
TRG-2				
22	wt	wt	wt	wt
23	G12D	G12D	wt	wt
24	G12V	G12V	wt	wt
25	wt	wt	R282W	R282W
26	G12V	G12V	wt	wt
27	G12D	G12D	wt	wt
28	wt	wt	R282W	R282W
29	G12D	G12D	E298Stop	E298Stop
30	G12D	G12D	wt	wt
31	G13D	G13D	M237I	M237I
32	wt	wt	wt	wt
33	G12D	G12D	R175H	R175H

Patients	Genotype of K-*ras*		Genotype of *p53*	
	Pre-T	**Post-T**	**Pre-T**	**Post-T**
34	wt	wt	R175H	R175H
35	wt	wt	R196Stop	R196Stop
36	wt	wt	R282W	R282W
37 *	G13D	G13D	R273C	wt
38	wt	wt	R273P	R273P
39	wt	wt	E286K	E286K
40	G12D	G12D	R175H	R175H
41	wt	wt	R181C	R181C
42	wt	wt	E8p.269delGCT	E8p.269delGCT
43	G12D	G12D	R273H	R273H
44	wt	wt	R248W	R248W
45	G12V	G12V	Y205H	Y205H
46	wt	wt	wt	wt
47	wt	wt	R213Q	R213Q
48	G12D	G12D	Q167Stop	Q167Stop
49	wt	wt	R273C	R273C
50	wt	wt	R273C	R273C
51	G13D	G13D	R213L	R213L
52	G12D	G12D	R248W	R248W
53	wt	wt	Q144Stop	Q144Stop
54	wt	wt	wt	wt
55	G12V	G12V	E6p.768-769delTTT211-F212	E6p.768-769delTTT211-F212
TRG-3				
56 *	wt	wt	wt	L130H
57	G12D	G12D	wt	wt
58	wt	wt	R248Q	R248Q
59	wt	wt	R175H	R175H
60	G12V	G12V	M237I	M237I
61	wt	wt	R306Stop	R306Stop
62	G12D	G12D	G244D	G244D
63	wt	wt	R209Stop	R209Stop
64	wt	wt	E285K	E285K
65	wt	wt	wt	wt
66	wt	wt	wt	wt
67	wt	wt	wt	wt
68	G13D	G13D	wt	wt
69	wt	wt	A138T	A138T
70	wt	wt	wt	wt

*Patient had a discordant mutation profile between pre-treatment biopsy and post-treatment surgical specimen.

TRG, tumor regression grade; wt, wild-type; Pre-T, pre-treatment; Post-T, post-treatment.

Table 4

Molecular Analysis of K-*ras* and *p53* in Patients with a pCR (TRG 0) using PAP-A and PNA Clamp Polymerase Chain Reaction

Patients	Genotype of K-*ras*		Genotype of *p53*	
	Pre-T	Post-T PNA Clamp	Pre-T	Post-T PAP-A
TRG 0				
1	wt	wt	wt	wt
2	wt	wt	wt	wt
3	G12D	wt	wt	wt
4	wt	wt	R175H	wt
5	wt	wt	R175H	wt
6	wt	wt	wt	wt
7	wt	wt	wt	wt
8	wt	wt	wt	wt
9	wt	wt	wt	wt
10	wt	wt	wt	wt
11	G12D	wt	wt	wt
12	wt	wt	wt	wt
13 *	wt	wt	R213L	R213L
14	wt	wt	Exon8/Intron G>A Splice Site	wt
15	wt	wt	E7p.252del CTCACCATC	wt
16	wt	wt	wt	wt
17	G12D	wt	wt	wt
18 *	G12D	G12D	wt	wt
19	wt	wt	C176R	wt
20	wt	wt	wt	wt
21	wt	wt	R175H	wt
22	wt	wt	wt	wt
23	wt	wt	wt	wt
24	G12D	wt	wt	wt
25	wt	wt	wt	wt
26	wt	wt	wt	wt

* Patient had mutation in pre-treatment biopsy and post-treatment surgical specimen.

TRG, tumor regression grade; wt, wild-type; Pre-T, pre-treatment; Post-T, post-treatment.