"Biomarkers for cetuximab-based neoadjuvant radiochemotherapy in locally advanced rectal cancer"

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Abstract

PURPOSE: Phase II trials in locally advanced rectal cancer have shown that cetuximab-based neoadjuvant radiochemotherapy is feasible but without an improvement in complete pathologic response rates. Our goal was to identify patients who would benefit from cetuximab-based neoadjuvant chemoradiation measuring gene expression levels of proteins involved in tumor growth [endothelial growth factor receptor (EGFR)], angiogenesis [VEGF, VEGF receptors 1 and 2 (VEGFR1, VEGFR2)], DNA repair [excision repair cross-complementing 1 (ERCC1)], and drug metabolism [thymidylate synthetase (TS)]. We also determined mutation status of KRAS and BRAF. EXPERIMENTAL DESIGN: This study was carried out on 130 patients with locally advanced rectal cancer who were enrolled in 4 different phase II clinical trials, using cetuximab-based chemoradiation. Tumor tissues were obtained before neoadjuvant and at surgical therapy. After microdissection, intratumoral gene expression levels and KRAS/BRAF mutation status ...

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Biomarkers for Cetuximab-Based Neoadjuvant Radiochemotherapy in Locally Advanced Rectal Cancer

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Abstract

Purpose: Phase II trials in locally advanced rectal cancer have shown that cetuximab-based neoadjuvant radiochemotherapy is feasible but without an improvement in complete pathologic response rates. Our goal was to identify patients who would benefit from cetuximab-based neoadjuvant chemoradiation and measuring gene expression levels of proteins involved in tumor growth and drug metabolism.

Experimental Design: This study was carried out on 130 patients with locally advanced rectal cancer who were enrolled in 4 different phase II clinical trials, using cetuximab-based chemoradiation. Tumor tissues were obtained before neoadjuvant and at surgical therapy. After microdissection, intratumoral gene expression levels and KRAS/BRAF mutation status were analyzed.

Results: A significant decrease of TS, VEGFR1, and VEGFR2 gene expression was seen following neoadjuvant therapy (P < 0.03). High pretreatment VEGF gene expression was associated with nonresponse (P = 0.070). KRAS mutations were found in 42% and mutant KRAS (KRAS mt) was significantly associated with pathologic nonresponse (P = 0.037). In patients with wild-type KRAS (KRAS wt), low EGFR was significantly associated with higher nonresponse and VEGF mRNA expressions were associated with complete pathologic response (P = 0.012; P = 0.06). KRAS transversion (KRAS tv) was associated with tumor regression: nonresponse was more common in patients with KRAS wt than with KRAS mt (P = 0.007). BRAF V600E mutations were not detected in any of the patients.

Conclusion: This study suggests that pretreatment intratumoral EGFR and VEGF expression levels as well as KRAS mutation status are predictive markers of pathologic response to neoadjuvant cetuximab-based chemoradiation in locally advanced rectal cancer. Clin Cancer Res; 17(10); 3469–77. ©2011 AACR.

Introduction

Neoadjuvant radiation with concurrent 5-fluorouracil (5-FU)-based chemotherapy, followed by surgical resection including total mesorectal excision, is considered to be the standard treatment of locally advanced rectal cancer (1–3). However, high rates of distant metastasis, evaluated using multimodality therapy, in approximately 35% to 40% of patients remain a major problem in these patients. Targeted therapies have been incorporated to improve local response and possibly preventing metastatic disease in patients with locally advanced rectal cancer.

The monoclonal antibody cetuximab (Erbitux; Merck) is a chimeric immunoglobulin G1 directed against the ligand-binding domain of the epidermal growth factor receptor (EGFR). In metastatic colorectal cancer, cetuximab has shown to increase response rates, progression free survival, and overall survival in wild-type KRAS (KRAS wt) patients (4, 5). A phase III trial by Bonner and colleagues in patients with locally advanced head and neck cancer revealed that cetuximab in combination with radiotherapy (RT) significantly increases the median survival compared with using radiation alone, suggesting this EGFR antibody to be a clinically active radiosensitizer (6).
These data prompted the initiation of phase I/II trials to evaluate the effect of cetuximab in the neoadjuvant therapy of locally advanced rectal cancer (7–10). Unexpectedly, the results of these trials were disappointing. In fact, the rate of complete histopathologic response rates ranged only between 5% and 12% compared with 16% of complete response reported in phase II trials with oxaliplatin-containing neoadjuvant protocols without a targeted drug (11). Nevertheless, it remains possible that a subset of patients will benefit from cetuximab-based chemoradiation (CRT).

The goal of this project was to determine whether gene expression levels of proteins involved in tumor growth (EGFR), angiogenesis (VEGF, VEGFR1, VEGFR2), DNA repair (ERCC1), and drug metabolism (TS), as well as intratumoral mutation status of KRAS and BRAF, significantly correlate with histopathologic response to identify rectal cancer patients who will benefit from cetuximab-based neoadjuvant chemoradiation.

Patients and Methods

Eligibility criteria

To be eligible for this retrospective translational study, patients with locally advanced rectal cancer [uT3/4 and/or N1/N2; one study allowed M1 status] had to be enrolled in 1 of 4 phase I/II clinical trials assessing cetuximab-based CRT in European Cancer Centers (Brussels/Leuven, Belgium; Ljubljana, Slovenia; Halle/Erlangen/Göttingen/Regensburg, Germany; and Cologne, Germany). Details of the eligibility criteria, pretreatment evaluation, RT, chemotherapy, cetuximab administration, and surgery have already been published and are briefly specified later (9, 10, 12, 13). Altogether, 130 patients (57% men; 43% women; median age 61 years) from these 4 phase I/II clinical trials were enrolled in the present study.

The study was approved by the Institutional Review Boards of all the participating centers.

Study objectives

The primary aim of this study was to determine whether gene expression levels of proteins involved in tumor growth (EGFR), angiogenesis (VEGF, VEGFR1, VEGFR2), DNA repair (ERCC1), and drug metabolism (TS), as well as intratumoral mutation status of KRAS and BRAF, significantly correlate with histopathologic response to identify rectal cancer patients who will benefit from cetuximab-based neoadjuvant chemoradiation.

Staging

Clinical staging was based on results from endoscopy, endoscopic ultrasonography, and computed tomography/MRI of the chest and abdomen.

Treatment protocols

Brussels/Leuven, Belgium: Patients (n = 41) received capcitabine (4 patients at first dose level of 650 mg/m² twice a day; 37 patients at second dose level of 825 mg/m² twice a day) and intravenous cetuximab (400 mg/m² as initial dose 1 week before CRT followed by 250 mg/m²/week for 5 weeks) and 45 Gy (i.e., 25 x 1.8 Gy) of radiation (9).

Ljubljana, Slovenia: Patients (n = 31) received capcitabine 1,250 mg/m² twice daily for 2 weeks, followed by intravenous cetuximab 400 mg/m² at week 3, and then weekly intravenous 250 mg/m² cetuximab plus CRT including capcitabine 825 mg/m² twice daily (including weekends during RT) with RT of 45 Gy (25 x 1.8 Gy), 5 days a week for 5 weeks (12).

Halle/Erlangen/Göttingen/Regensburg, Germany: Patients (n = 42) received cetuximab, given as an initial dose of 400 mg/m² 7 days before the start of RT, and then at 250 mg/m² once weekly during RT (50.4 Gy; 28 x 1.8 Gy). Capecitabine and oxaliplatin were administered according to an established schedule of oxaliplatin (50 mg/m² on days 1, 8, 22, and 29) and capcitabine (days 1–14 and 22–35) at 3 dose levels: 1,000, 1,300, and 1,650 mg/m²/d (10).

Cologne, Germany: Patients (n = 16) were treated with 45 Gy (25 x 1.8 Gy) of radiation and cetuximab (400 mg/m² as initial dose 1 week before CRT followed by 250 mg/m²/wk for 5 weeks) in combination with 5-FU (13).

Surgical resection

Patients underwent radical resection within 4 to 8 weeks after completion of chemoradiation by either performing a
(low) anterior resection or an abdominoperineal rectal amputation with (total) partial mesorectal excision.

Tissue samples
Tumor biopsies from the study patients were obtained prior to neoadjuvant therapy, at the time of diagnosis (pretherapeutic biopsy), and after therapy, at the time of surgery (posttherapeutic biopsy). One hundred nine (84%) rectal cancer pretreatment biopsies and 117 (90%) post-treatment biopsies could be collected and analyzed in this study. All samples were reviewed by a local pathologist of each study center, and the tissue was evaluated for its tumor content. Seventy-eight (71.5%) of the pretherapeutic biopsies and 61 (52%) of the posttherapeutic biopsies contained enough tumor tissue for gene expression and mutation analyses.

Pathologic evaluation
For the determination of histopathologic response, a 5-point tumor regression grading system established by Dworak and colleagues (grade 0, no regression; grade 1, minimal regression; grade 2, moderate regression; grade 3, good regression; and grade 4, total regression) was used in all tumor samples to ensure that mutation analyses.

Microdissection
Formalin-fixed paraffin-embedded tissues from the included rectal patients were dissected as previously described (15). In patients with complete pathologic response, the ulcer region of the former primary tumor was microdissected. Ten-micrometer-thick slides were obtained from the identified areas with the highest tumor concentration and were mounted on uncoated glass slides. For histologic diagnosis, 3 sections representative of the beginning, middle, and end of the tissue were stained with H&E, using the standard method. Before microdissection, sections were deparaffinized in xylene for 10 minutes, hydrated with 100%, 95%, and 70% ethanol, and then washed in H$_2$O for 30 seconds. Following this, the sections were stained with hematoxylin and eosin (H&E) and used for both histopathologic staging and histomorphologic evaluation of the effect of CRT. Besides analyzing all 5 regression grades with each other, grades 0–1 were defined as nonresponse and grade IV as complete pathologic response for additional statistical analysis.

mRNA/DNA isolation
RNA and DNA isolation from paraffin-embedded samples was done according to a proprietary procedure defined by Response Genetics, Inc. (U.S. patent no. 6,248,535). After RNA isolation, cDNA was prepared from each sample as described previously (17, 18).

Real-time PCR quantification of mRNA expression
Quantitation of the gene mRNA expressions and an internal reference (β-actin) cDNA was done using a fluorescence-based real-time detection method [ABI PRISM 7900 Sequence detection System (TaqMan); Perkin-Elmer Applied Biosystem] as previously described (19). The PCR reaction mixture consisted of 1,200 nmol/L of each primer, a 200 nmol/L probe, 0.4 U of AmpliTaq Gold Polymerase, 200 nmol/L of dATP, dCTP, dGTP, dTTP; 3.5 mmol/L MgCl$_2$, and 1× TaqMan Buffer A containing a reference dye added to a final volume of 20 µL (all reagents from PE Applied Biosystems). Cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 46 cycles at 92°C for 15 seconds and 60°C for 1 minute.

The TS and probe sequences used were as follows: forward primer, GCCCTCGGTGTGGCTTTCA; reverse primer, CCGTGATGTGGCGCAAT; probe, 6FAM-TGCGCAGGAGGCAGT; the ERCC1 primers and probe sequences used were as follows: forward primer, GGGAATCTTGCCGAGCTG; reverse primer, GGGGATCTGGCCGAGCC; probe, 6FAM-ACACCGCTGCGCGCCGG; the EGFR primers and probe sequences used were as follows: forward primer, TGCGTCTCTTGCGCGG; reverse primer, CACCTTGCTTCGGAA; probe, 6FAM-TGGTCTGCGCGG;

KRAS and BRAF mutation analysis
KRAS analysis of 7 KRAS mutations (codon 12 and codon 13) and the BRAF V600E mutation was done according to a propriatory procedure defined by Response Genetics Inc. (U.S. patent no. 6,248,535).
Statistical analysis

Tumor response to neoadjuvant CRT evaluated by Dworak criteria was the primary endpoint. Patients with grade IV Dworak response were classified as having complete pathologic response. Patients having grade 0–III Dworak response were categorized as having complete pathologic response. Dworak response was also grouped into response (grades II–IV) or not (grades 0–I). Recurrence-free survival, the secondary endpoint, was defined as the period from the start of neoadjuvant therapy to first observation of tumor recurrence or death, whichever came first. It was censored at the time of last follow-up.

Intratumoral gene expression values measured prior to the and after the neoadjuvant therapy using TaqMan analyses were expressed as ratios between the continuous absolute measurements for the gene of interest and the internal reference gene. The gene expression values were not normally distributed, and nonparametric methods were used to examine the associations whenever appropriate. The difference in the expression values of the genes of interest before and after the neoadjuvant therapy was tested after log transformation (log expression value after the therapy – log expression value before the therapy), using a paired Wilcoxon signed-rank test. The difference in the gene expression values between patients carrying KRAS wt and mt tumors was examined using the Mann–Whitney U test. The correlations among the expression levels of the genes were examined by calculating Spearman correlation coefficient and its P values. Patients with missing gene expression values were deleted in each analysis (complete case analysis).

Finally, the maximal χ² approach of Miller and Sigmund and Halpern was used to determine the optimal cutoff value of gene expression levels associated with response to the neoadjuvant therapy (20, 21). The adjusted P value was calculated using 2,000 simulated samples randomly drawn from the original data, with replacement for the gene expression value and outcome variable separately. The maximal χ² statistics was calculated in each simulation sample. The adjusted P value was the proportion of the 2,000 simulated maximal χ² statistics that was greater than the one from the original data. The area under receiver operating characteristic (ROC) curves, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy were calculated to describe the associations between gene expression values and outcome. SPSS Statistics version 17.0 (SPSS Inc.) was used for all statistical analyses. All tests were 2-sided, with the level of significance set at 0.05.

Results

Patient characteristics are shown in Table 1 and are additionally described elsewhere (9, 10, 12, 13).

Histopathologic response

According to the 5-point tumor regression grading system established by Dworak and colleagues, the following histopathologic response rates were found in the 125 evaluable study patients: grade 0/1, 21 (17%) patients; grade 2, 55 (44%) patients; grade 3, 30 (24%) patients; and grade 4, 19 (15%) patients (Table 1).

Pre- and posttherapeutic mRNA expression

A significantly decrease in median TS, VEGFR1, and VEGFR2 mRNA expression was detected following neoadjuvant therapy (P = 0.015, P = 0.001, and P < 0.001, paired signed-rank test after log transformation; Table 2, Fig. 1 A–C). The median intratumoral gene expression of EGFR, ERCC1, and VEGF mRNA was not significantly changed after multimodality therapy (Table 2).

KRAS and BRAF mutation analysis

In pretherapeutic biopsies, KRAS mutations were found in 42 (42%) patients of 101 KRAS mutation analysis. BRAF V600E mutation was not found in any of the analyzed samples.

KRAS mutation status and mRNA expression

In KRAS wt tumors, the VEGFR2 mRNA expression was significantly higher than mutant KRAS (KRAS mt)
tumor tissue (Mann–Whitney U test, P = 0.004; Fig. 2).

In KRAS wt patients with gene expression values measured (n = 42), complete pathologic response (Dworak grade 4) was associated with higher VEGF mRNA expression (n = 2) and nonresponse (Dworak grades 0–1) was significantly associated with lower EGFR mRNA expression (n = 6; P = 0.062, P = 0.012; Mann–Whitney U test). Cutoff levels could be calculated using bootstrap estimations. High VEGF mRNA expressors (VEGF > 10.05; n = 6 of 42; 14.2%) were associated with complete pathologic response (cutoff adjusted P = 0.066) and low EGFR mRNA expressors (EGFR < 0.69; n = 6 of 42; 14%) were associated with pathologic nonresponse (cutoff adjusted P = 0.009) in KRAS wt tumors. For VEGF, we found an area under the ROC of 0.91 (95% CI: 0.82–1.00), a PPV of 33%, an NPV of 100%, sensitivity of 100%, specificity of 90%, and accuracy of 14.3%. For EGFR, we found an area under the ROC of 0.84 (95% CI: 0.67–1.00), a PPV of 67%, an NPV of 94%, sensitivity of 67%, specificity of 94%, and accuracy of 14.3%. When combining the groups with grades III and IV, no significant difference about the VEGF/EGFR mRNA expression compared with patients with grades 0–II was detected.

In 32 patients with both pre- and post-CRT treatment gene expressions available, 18 patients showed a KRAS mt and 14 were KRAS wt.

**Gene expression and response to multimodality therapy, recurrence-free survival**

High pretherapeutic intratumoral VEGF expression was correlated with nonresponse (Dworak grades 0–1; P = 0.069, Mann–Whitney U test). Cutoff levels could be calculated using bootstrap estimations. High VEGF mRNA expressors (VEGF > 8.92; n = 13 of 74; 18%) were significantly associated with nonresponse (cutoff-adjusted p-value p = 0.026). In patients independent from the KRAS status, the following values were found for VEGF: the area under ROC = 0.66 (95%CI: 0.49–0.82), PPV = 46%, NPV = 13%, sensitivity = 10%, and specificity = 53%. ERCC1, TS, EGFR, VEGFR1, and VEGFR2 were not associated with either complete pathologic response or nonresponse. Recurrence-free survival of all patients was not associated with any of the 6 analyzed genes.

**KRAS mutation analysis and response to multimodality therapy, recurrence-free survival**

The mutation status (wt vs. mt) was significantly correlated with histomorphologic response. KRAS mt patients showed a higher rate of nonresponse (12 of 39; 30.8%; Dworak grades 0–1) to neoadjuvant CRT compared with KRAS wt patients with 7 of 57 patients (12.3%) with nonresponse (P = 0.037, Fisher’s exact test). KRAS transversion (KRAS tv) was found in 31% of the KRAS mt (n = 13) and KRAS transition (KRAS ti) was found in 69% of the KRAS mutations (n = 29). KRAS tv was associated with nonresponse compared with patients with KRAS wt (P = 0.007, Fischer’s exact test), but KRAS ti showed no significant association with response or nonresponse compared with KRAS wt (P = 0.195, Fischer’s Exact Test). When combining the groups with grades III and IV, no significant difference relating to the KRAS status compared with patients with grades 0–II was detected.

Patients with KRAS tv (n = 13) showed a reduced recurrence-free survival compared with patients with KRAS ti (n = 29) or KRAS wt (n = 54); however, this was not statistically significant (Fig. 3). A Cox regression model adjusted for age, T- and N-status, and KRAS status was also not significantly associated with recurrence-free survival.

**Association between VEGF and EGFR gene expression and KRAS mutations status**

No significant correlation was found between EGFR and VEGF mRNA expression prior to the neoadjuvant therapy (Spearman’s ρ: P = 0.122, correlation coefficient = 0.18). In addition, no significant associations between KRAS status and VEGF/EGFR mRNA expression (P = 0.38 and P = 0.70, Mann–Whitney U test) were detected.
Association between VEGF and EGFR gene expression and ypT/N category and age

Measured gene expression of VEGF and EGFR was not associated with ypT or ypN category. Also, in patients older than 60 compared with patients with 60 or younger, no correlation with any of the investigated markers was found (data not shown).

Discussion

Our study results showed that intratumoral EGFR and VEGF mRNA expression levels and KRAS mutation status were associated with complete pathologic response to neoadjuvant cetuximab-based chemoradiation in patients with locally advanced rectal cancer. To the best of our knowledge, this is the largest translational study in the multimodality treatment with cetuximab-based chemoradiation of rectal cancer patients to date, suggesting that these molecular markers may be important predictive markers to select patients who benefit the most from cetuximab-based neoadjuvant chemoradiation therapy.

Recent phase II trials evaluating the effect of cetuximab in the neoadjuvant therapy of locally advanced rectal cancer revealed disappointing results in terms of histopathologic responses as an early endpoint (8–10). In fact, the rate of complete histopathologic response ranged only between 5% and 10%. Moreover, a current pooled analysis of 3 prospective trials of preoperative chemoradiation for rectal cancer, using oxaliplatin and capecitabine with or without cetuximab, suggested subadditive interactions between chemoradiation and cetuximab (22). Nonetheless, it remains unclear whether a subset of patients may benefit from a cetuximab-based neoadjuvant therapies.

Our quantitative real-time PCR data show that high pretherapeutic VEGF expression was correlated with

Figure 2. Boxplots showing the VEGFR2 mRNA expression in tumor tissue of KRAS wt and KRAS mt tumors. The VEGFR2 mRNA expression between KRAS wt (n = 44) and KRAS mt (n = 33) was statistically significantly higher in KRAS wt tumors (P = 0.004, Mann–Whitney U test).

Association between VEGF and EGFR gene expression and ypT/N category and age

Measured gene expression of VEGF and EGFR was not associated with ypT or ypN category. Also, in patients older than 60 compared with patients with 60 or younger, no correlation with any of the investigated markers was found (data not shown).

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Our quantitative real-time PCR data show that high pretherapeutic VEGF expression was correlated with
nonresponse to cetuximab-based neoadjuvant CRT. These findings are consistent with recent studies suggesting that EGFR signaling pathways are intimately involved in tumor angiogenesis, especially via the upregulation of VEGF (23). In fact, preclinical studies point out that inhibition of EGFR by cetuximab downregulates VEGF expression (24–26). Moreover, Vincenzi and colleagues revealed, in advanced colorectal cancer patients undergoing therapy with a weekly combination of cetuximab plus irinotecan, that the reduction of VEGF serum levels was a sudden and long-lasting phenomenon (27).

Recently, KRAS mutation as a marker for resistance to cetuximab in patients with metastatic colorectal cancer has been validated (28). A current meta-analysis including 2,188 patients with metastatic colorectal cancer from 22 studies revealed that patients with intratumoral KRAS mt are more likely to have a worse response and survival when treated with cetuximab compared with patients having a wild-type status (29). To our knowledge, there are only 2 studies that addressed the role of KRAS mutation status for cetuximab-based multimodality treatment of rectal cancer patients. Debucquoy and colleagues showed in a recently published translational analysis of 41 rectal cancer undergoing neoadjuvant RT in combination with capecitabine and cetuximab that tumors with KRAS mutations had similar response to preoperative treatment compared with patients having a wild-type status (30). As the number of patients was small, the authors could not exclude the likelihood that a study population with more KRAS wt tumors would have shown a statistical effect. In addition, Bengala and colleagues found in 41 rectal cancer patients receiving cetuximab-based neoadjuvant chemoradiation that intratumoral KRAS wt was significantly associated with a high EGFR gene copy number that was itself predictive for histopathologic response (31). In fact, in our study, we did find the mutation status to be significantly correlated with response: patients with a wild-type status showed more frequently a complete response to neoadjuvant treatment than in patients showing a KRAS mutation status. These findings are consistent with the recent studies about metastatic colorectal cancer describing KRAS mutation as a marker for resistance to cetuximab.

Interestingly, we found that KRAS wild-type status and high intratumoral EGFR and VEGF mRNA expressions were significantly associated with complete response. Similar results have been reported recently by Yen and colleagues, who determined the predictive values of KRAS mutation status and EGFR expression in metastatic colorectal cancer patients treated with cetuximab plus chemotherapy (32). The authors showed that patients with a KRAS wild-type status and a high intratumoral EGFR protein expression were more likely to have a better survival than patients showing a low EGFR expression and KRAS mutation status. In addition, Li and colleagues revealed in 30 metastatic colorectal cancer patients treated with cetuximab that an increased EGFR copy number was significantly associated with an objective tumor response to this anti-EGFR monoclonal antibody (33). These data suggest that there is a major role for the EGFR pathway in especially those tumors with high EGFR expression or amplification. Thus, patients with high EGFR mRNA expression or copy number may be more likely to respond to cetuximab-based therapy, particularly when the tumors do not have a KRAS mutation status.

Besides high intratumoral EGFR expression, high VEGF mRNA expression was also significantly associated with
complete response in patients with a KRAS wild-type status. As it is well documented that the EGFR pathway is involved in tumor angiogenesis, especially via the upregulation of VEGF, this finding may suggest an indirect effect induced by high EGFR expression levels (23).

Some possible limitations of our study are its retrospective design, pooled study patient, and missing pathologic central review. Therefore, our data are hypothesis generating and should be validated in prospective clinical trials. Also, we have missing data on EGFR immunohistochemistry from our pretreatment biopsies and posttreatment tissues, which would have clarified several details and should be addressed in future studies. Finally, it is still highly debated whether gene expression analysis can be adequately done in paraffin-embedded tissue. Indeed, there are some technical challenges but recently several studies have shown a good concordance of gene expression data in fresh-frozen and paraffin-embedded tissue (34).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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