Unfavourable expression of pharmacologic markers in mucinous colorectal cancer

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Patients with mucinous colorectal cancer generally have worse prognoses than those with the nonmucinous variety. The reason for this disparity is unclear, but may result from a differential response to adjuvant chemotherapy. We examined known molecular markers for response to common chemotherapy in these two histological subtypes. In all, 21 patients with mucinous and 30 with nonmucinous Dukes C colorectal cancer were reviewed for demographic data and outcome. Total RNA from the tumours and adjacent normal mucosa was isolated and reverse transcribed. Quantitative expression levels of drug pathway genes were determined using TaqMan RT–PCR (5-fluorouracil (5-FU); TYMS, DPYD, ECGF1; oxaliplatin: GSTP1 (glutathione S-transferase p), ERCC1 and 2; irinotecan: ABCB1, ABCG2, CYP3A4, UGT1A1, CES2, TOP1). Mucinous tumours significantly overexpressed both TYMS and GSTP1 relative to nonmucinous tumours and patient-matched normal mucosa. No significant differences in expression of the remaining markers were found. Mean follow-up was 20 months; 17 patients had recurrent disease. Among patients receiving 5-FU, those with mucinous tumours experienced shorter disease-free survival (DFS) than those with nonmucinous tumours (median DFS 13.8 vs 46.5 months, P = 0.053). Mucinous colorectal cancer overexpresses markers of resistance to 5-FU and oxaliplatin. Likewise, DFS may be decreased in patients with mucinous tumours who receive 5-FU. The presence of mucin should be carefully evaluated in developmental trials of new agents for treating colorectal cancer.

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Most evidence suggests that colorectal adenocarcinomas that produce an abundance of the glycoprotein mucin portend a worse clinical prognosis (Umpleby et al, 1985; Green et al, 1993; Nozoe et al, 2000), although this finding has not been consistently established (Sasaki et al, 1998; Halvorsen and Seim, 1988). Comprising between 10 and 15% of all colorectal adenocarcinomas, these tumours have a mucin content of at least 60% of tumour volume, typically at the advancing edge. The reasons for the worse prognosis with mucinous tumours are not clear, but possibly include difficulty obtaining complete resections (Umpleby et al, 1985), propensity for early spread to regional lymph nodes (Nozoe et al, 2000), and detection at more advanced stages of disease (Halvorsen and Seim, 1988). There may be a higher proportion of mucinous tumours in familial cancer syndromes; approximately 30% of mucinous tumours demonstrate microsatellite instability, a defining trait of hereditary nonpolyposis colorectal cancer (Kakar et al, 2004). The responsiveness to adjuvant chemotherapy of mucinous adenocarcinoma specifically has not been examined.

Various genes are known to influence tumour response or patient survival following chemotherapy (McLeod and Murray, 1999). Their products consist of intracellular drug targets, cell-membrane transporters, components that detoxify or metabolise, and DNA repair enzymes. TYMS encodes thymidylate synthase, a key enzyme in pyrimidine metabolism. Numerous studies have demonstrated that overexpression of TYMS correlates with poor response to 5-fluorouracil (5-FU) (Johnston et al, 1993; Leichman et al, 1997; Salonga et al, 2000; Iacopetta et al, 2001; Kornmann et al, 2002). Likewise, elevated levels of other enzymes in the 5-FU pathway (namely, DPYD (dihydropyrimidine dehydrogenase) and ECGF1 (thymidine phosphorylase)) have been shown to affect epithelial tumour sensitivity (Etienne et al, 1995; Nita et al, 1998), clinical response (Metzger et al, 1998a), and time to recurrence (Salonga et al, 2000) following treatment with 5-FU. Additionally, expression levels of DPYD and ECGF1 appear to be coregulated, particularly in metastatic colorectal cancer (Collie-Duguid et al, 2001).

Platinum agents such as oxaliplatin rely on the inability of nucleotide excision repair genes within the tumour to successfully remove bulky DNA adducts. Polymorphisms in ERCC1 (excision repair crosscomplementing isoform 1) have been related to
decreased survival in patients with colorectal cancer (Park et al., 2001). Similarly, expression of ERCC2 (excision repair cross-complementing isofrom 2) in gastric cancers predicts response and survival (Metzger et al., 1998b). GSTP1 (glutathione S-transferase pi) is a major route of detoxification of platinum agents. It is highly overexpressed in colon cancer, and drug-resistant tumours have elevated levels (Stoehlmacher et al., 2002).

Irinotecan is bioactivated to a more potent form via CES2 (carboxylesterase 2), and colorectal adenocarcinomas overexpressing CES2 are more sensitive to the drug (Wu et al., 2002). Irinotecan causes double-strand DNA breaks by binding to topoisomerase-I (TOP1) (Xu and Villalona-Calero, 2002), making it a key intracellular target. Members of the ATP-binding cassette superfamily of proteins (ABCB1 (Patel and Mitra, 2001; Gottesman et al., 2002) and ABCG2 (Wierdl et al., 2003)) function to export irinotecan from tumour cells, while drug metabolism via the cytochrome P450 system (CYP3A4 (Kehrer et al., 2002; Xu and Villalona-Calero, 2002)) or glucuronidation (UGT1A1 (Caruso et al., 1999, 2002)) can also occur.

In the current study, we examined expression of the above 12 genes in colorectal cancer in order to characterise possible differences in response between the mucinous and nonmucinous subtypes of colorectal cancer. These genes were selected as likely candidates affecting clinical response to common adjuvant chemotherapy (i.e. S-FU, irinotecan, and oxaliplatin) administered following resection of locally advanced tumours. A secondary goal was to correlate gene expression with disease-free survival (DFS), in the context of histological subtype.

MATERIALS AND METHODS

In all, 51 Dukes stage C colorectal cancer specimens were selected from the Tissue Procurement Core of the Siteman Cancer Center, Washington University, St Louis, MO, USA for genetic evaluation. No tumours from patients with known inflammatory bowel disease or familial cancer syndromes (familial adenomatous polyposis or hereditary nonpolyposis colon cancer) were included. In total, 21 mucinous adenocarcinomas were selected, histologically defined by a mucin content exceeding 60% of the total tumour volume. The remaining 30 nonmucinous tumours were randomly chosen from a contemporary selection of 145 available Dukes’ C colorectal cancer specimens. Adjacent normal mucosa was available for all tumours (Park et al., 2001). Similarly, expression of ERCC2 (excision repair cross-complementing isofrom 2) in gastric cancers predicts response and survival (Metzger et al., 1998b). GSTP1 (glutathione S-transferase pi) is a major route of detoxification of platinum agents. It is highly overexpressed in colon cancer, and drug-resistant tumours have elevated levels (Stoehlmacher et al., 2002).

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| Gene (NCBI LocusLink ID) | Sequence | Reporter dye |
|-------------------------|----------|--------------|
| ABCB1 (5243)            | GCTGGCACAGAAGAGGACACTCT | TET       |
| ABCG2 (9429)            | CAGGTCTTGTTGTCAATCCTACA | TET       |
| CES2 (8824)             | AATCCCACTGATGTTGAGAAGA | TET       |
| CYP3A4 (1576)           | TTGCTTTTATATTTCTGGAGAGA | TET       |
| DPYD (1806)             | ACCGTACTGCTGATCTACGATAGA | TET       |
| EGF1 (1890)             | GGTTCCTGGCGACGAGAT | TET       |
| ERCC1 (2067)            | TACCCCTGACAGAGGTAAG | TET       |
| ERCC2 (2068)            | GTTGGCTGGCTCTATGCTAT | TET       |
| GSTP1 (2950)            | CCTCACCCGTGATCACTCAAT | FAM       |
| TOP1 (7150)             | GCCCAGTGGATTCACGGATAG | TET       |
| TYMS (7298)             | GCCCTGTCTGCTTCTCTAG | TET       |
| UGT1A1 (7361)           | TTGGGATTGGGAGATAT | TET       |

Sequences are listed as forward, reverse, and probe oligonucleotides, in the 5’ to 3’ direction.

ROX passive reference dye 100 × (0.1 μl), and molecular-grade water for a total reaction volume of 10 μl. RT–PCR was performed with the following parameters: 50°C for 2 min to activate UNG enzyme, 95°C for 10 min to denature UNG and activate DNA polymerase, 40 cycles at 95°C for 20 s and 60°C for 1 min. All specimen reactions were performed in triplicate with appropriate nontemplate controls, and the coefficient of variation (CV) was less than 5% for all replicates. Relative gene expression was calculated using a modified comparative threshold cycle (Ct) method, as described previously (Pfaffl, 2001; Yu et al., 2003). This method accounts for varying RT – PCR amplification efficiencies between different primer/probe sets, rather than assuming all genes of interest amplify at an equal rate. Amplification efficiency (E) was determined from standard dilution curves for each primer/probe set. β-actin was used as the internal reference gene, as it had no significant variation between tumour and normal samples, and less than three-fold variation among all samples. Expression is given as a unit-less measure relative to the calibrator specimen that had the highest Ct (i.e. the 1 × sample), using the following equation:

Expression = \( \frac{\Delta\Delta Ct (\text{calibrator} - \text{sample})}{\text{E}_{\text{calibrator}} (\text{calibrator} - \text{reference})} \)

All statistical analyses were performed with GraphPad InStat (GraphPad Software, San Diego, CA, USA). THE Mann–Whitney
and Fisher’s exact tests were utilised as appropriate. Comparisons between tumour samples and patient-matched normal mucosa were made using the Wilcoxon test for matched pairs. Survival curves were generated using the Kaplan–Meier method, with significance assessed by the log-rank test. A P-value less than 0.05 was considered significant. Values are expressed as mean ± 95% confidence interval (CI).

**RESULTS**

Patient characteristics are depicted in Table 2. All patients underwent resection with curative intent. As previously mentioned, all patients had positive lymph node metastases (AJCC stage III). There were no significant differences between the mucinous and nonmucinous groups for age at diagnosis, gender, tumour size, histologic grade, or number of positive lymph nodes. No mucinous tumours occurred in the rectum, whereas 20% of the nonmucinous tumours were rectal. However, differences in tumour location did not reach statistical significance. A total of 13 mucinous tumours occurred proximal to the splenic flexure, compared to 14 nonmucinous tumours (P = 0.58).

Relative gene expression of **DPYD**, **ECGF1**, **ERCC1**, **ERCC2**, **ABCB1**, **ABCG2**, **CYP3A4**, **UGT1A1**, **CES2**, and **TOP1** did not differ significantly between mucinous and nonmucinous tumours, although the difference in **ERCC2** expression approached statistical significance (P = 0.083), with higher expression in the mucinous subgroup. However, as depicted in Figure 1, **TYMS** expression was significantly higher in mucinous tumours compared to nonmucinous tumours (17.7 arbitrary units [95% CI 5.8, 30.0] vs 9.6 [5.0, 14.3]) respectively, P = 0.013). Overexpression of **TYMS** was also observed in both subtypes of adenocarcinoma compared to normal mucosa. Relative to patient-matched mucosa, nonmucinous tumours demonstrated less overexpression (tumour mean 9.6 [5.0, 14.3] vs mucosal mean 4.9 [3.5, 6.3], P = 0.01) than the mucinous variant (tumour mean 17.7 [5.8, 30.0] vs mucosal mean 4.1 [3.2, 5.1], P < 0.001). There was no difference in nonmalignant mucosal expression of **TYMS** between patients with mucinous and nonmucinous cancers.

**GSTP1** expression was also significantly greater in mucinous tumours (193.0 [108.8, 277.3]) than in nonmucinous tumours (139.0 [72.2, 205.8], P = 0.029). Both histologic tumour subtypes had higher expression of **GSTP1** than the matched normal mucosa. The degree of overexpression in mucinous tumours (tumour mean 193.0 [108.8, 277.3] vs mucosal mean 60.0 [39.9, 80.0], P < 0.001) was greater than in nonmucinous tumours (tumour mean 139.0 [72.2, 205.8] vs mucosal mean 51.0 [36.4, 65.6], P < 0.001). There was no difference in nonmalignant mucosal expression of **GSTP1** in patients with mucinous and nonmucinous cancers.

Clinical follow-up data were available for 44 of the 51 patients (86%); those without complete data were excluded from further analyses. Of the 44 patients, 17 experienced recurrence. Time to disease recurrence was evaluated for mucinous and nonmucinous tumours. Disease-free survival between the two groups was not significantly different, with median times to recurrence of 54.2 and

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**Table 2** Patient characteristics

|                     | Mucinous | Nonmucinous | P-value |
|---------------------|----------|-------------|---------|
| N                   | 21       | 30          | —       |
| Age (years)         | 68.9     | 69.2        | 0.98    |
| Male:female         | 12:9     | 16:14       | 1.00    |
| Tumour diameter (cm)| 5.3      | 5.1         | 0.31    |
| Location            |          |             | 0.16    |
| Right               | 9 (43%)  | 12 (40%)    | —       |
| Transverse          | 4 (19%)  | 3 (10%)     | —       |
| Left/sigmoid        | 8 (38%)  | 9 (30%)     | —       |
| Rectum              | 0 (0%)   | 6 (20%)     | —       |
| Median no. LNs evaluated | 19  | 16.5       | 0.52    |
| Median no. positive LNs | 2  | 2          | 0.78    |
| Tumour differentiation|        |             | 0.71    |
| Poor                | 5 (24%)  | 7 (23%)     | —       |
| Moderate            | 13 (62%) | 16 (53%)    | —       |
| Well                | 3 (14%)  | 7 (23%)     | —       |

All patients are stage III.

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**Figure 1** Differential expression of **TYMS** and **GSTP1** in mucinous and nonmucinous colorectal adenocarcinoma. *P < 0.001 relative to patient-matched normal mucosa; †P < 0.05 relative to nonmucinous adenocarcinoma. Error bars represent the 95% CI of the mean.
46.5 months ($P = 0.5$) for patients with mucinous and nonmucinous adenocarcinoma, respectively. In all, 14 patients either did not complete a course of primary 5-FU-based chemotherapy ($N = 8$) or were treated with other first-line treatment ($N = 6$, irinotecan or neoadjuvant radiation only for rectal cancer). The median DFS for all 14 patients did not differ significantly from the entire cohort (52 months), nor was there a detectable difference between those with mucinous or nonmucinous tumours. However, subset analysis of the 30 patients who received 5-FU as primary adjuvant treatment revealed a shorter time to recurrence in patients with mucinous tumours (Figure 2). The median DFS was 13.8 months in the population of patients with mucinous adenocarcinoma ($N = 11$) who received 5-FU chemotherapy. Conversely, patients with nonmucinous tumours ($N = 19$) who underwent 5-FU treatment had a median DFS of 46.5 months; the trend of shorter time to recurrence in these patients approached statistical significance ($P = 0.053$). Among patients treated with 5-FU, those with mucinous tumours had significantly higher TYMS expression (mean 20.8 vs 7.4 for patients with nonmucinous tumours who received 5-FU, $P = 0.014$). Owing to limited patient numbers and clinical follow-up, subset analysis of patients receiving other chemotherapeutic agents (i.e. oxaliplatin or irinotecan) could not be performed. There were no differences in recurrence or survival when patients who were treated with adjuvant chemotherapy were stratified based on whether their tumour expressed greater than or less than the median level of either TYMS or GSTP1.

**DISCUSSION**

It is generally recognised that mucinous adenocarcinomas of the colon and rectum have a worse prognosis following resection than nonmucinous tumours (Symonds and Vickery, 1976; Upleby et al, 1985; Green et al, 1993; Consorti et al, 2000; Nozoe et al, 2000). Compared to the more common nonmucinous variety, mucinous tumours metastasise to lymph nodes with increased frequency (Nozoe et al, 2000), are more prone to local recurrence (Upleby et al, 1985; Connelly et al, 1991) and peritoneal carcinomatosis (Nozoe et al, 2000), are less likely to be resected with negative margins (Upleby et al, 1985), and are typically diagnosed at an advanced stage (Younes et al, 1993). They also have altered p53 expression (Hanski et al, 1996) and increased microsatellite instability (Messerini et al, 1997). Although mucinous tumours occur more frequently in the proximal colon, left-sided and rectal mucinous tumours have an overall worse prognosis relative to location-matched nonmucinous tumours (Green et al, 1993). This finding may be at least partially explained by over-expression of both p53 (Lenz et al, 1996) and thymidylate synthase (Allegra et al, 2002) in left-sided tumours, although no previous study has examined expression in the mucinous subset alone. Based on these genetic differences and its more aggressive clinical behaviour, some authors have suggested that mucinous adenocarcinomas should be categorised and treated as a biological entity distinct from other colorectal adenocarcinomas (Consorti et al, 2000). The current study confirms the biological difference between mucinous and nonmucinous tumours. We found significantly higher expression of thymidylate synthase (TYMS) and GSTP1 in mucinous colorectal adenocarcinomas than in equivalent-stage nonmucinous tumours. Both mucinous and nonmucinous tumours also overexpressed these genes relative to normal colonic mucosa.

The association between high levels of TYMS in tumours and poor clinical response following 5-FU treatment of colorectal cancer is well documented (Johnston et al, 1995; Leichman et al, 1997; Salonga et al, 2000; Iacopetta et al, 2001). Elevated expression has also been correlated with time to recurrence in patients with stage II or III disease (Kornmann et al, 2002). The finding of overexpression of TYMS in mucinous tumours in our study offers a possible explanation as to why patients with these tumours tend to do worse. To our knowledge, response of mucinous tumours to 5-FU treatment has not been specifically investigated. In the recent literature regarding mucinous adenocarcinoma, the large majority of patients with locally advanced colorectal cancer would have received 5-FU. Perhaps decreased response to 5-FU due to overexpression of TYMS contributes to the relatively poorer prognosis for patients with mucinous adenocarcinoma. Among patients in our study who received 5-FU following initial surgical resection, there was a trend towards increased DFS only in the nonmucinous tumour group. Although the $P$-value did not reach the significance threshold of 0.05, this finding intuitively makes sense, given the fact that tumours in the nonmucinous group had significantly lower expression of TYMS. Owing to the small number of patients who either did not complete 5-FU-based chemotherapy or received other adjuvant treatment, no definitive conclusions can be drawn.

Oxaliplatin causes cell death by forming bulky, DNA helix-distorting adducts via crosslinking between guanine bases. GSTP1 belongs to the glutathione S-transferase superfamily and is a major mechanism of detoxification of platinum agents. It is highly overexpressed in colorectal cancer, with increased levels in drug-resistant tumours (Stoehlmacher et al, 2002). In the current study, GSTP1 expression was found to be similarly elevated in malignant tissue relative to normal colonic mucosa. Interestingly, expression was significantly greater in mucinous tumours. As our cohort of tumours was assembled in a retrospective manner from historically collected samples, none had been treated with oxaliplatin. However, the finding of elevated GSTP1 expression within mucin-producing tumours suggests that diminished clinical response may be expected from oxaliplatin-treated tumours. Further research examining this hypothesis is warranted.

Regarding the remaining ten markers, there were no significant differences in gene expression between mucinous and nonmucinous colorectal cancers. This negative finding does not imply they do not have a role in determining outcome following chemotherapy. Rather, they may not contribute to the differing clinical responses seen in the treatment of mucinous adenocarcinoma relative to the nonmucinous variant. In fact, it would be expected that altered expression of some genes contributes to the general drug-resistant phenotype seen in all colorectal adenocarcinomas, while expression of others distinguishes individual biologically distinct subtypes and determines their relative clinical responsiveness.
The intent of the current study was to examine the relative expression in colorectal cancer of certain genes believed to influence clinical response to adjuvant chemotherapy. It was not designed to characterise extensively the relationships between gene expression, response to chemotherapy, and patient outcome. The clinical follow-up data were limited, with a median follow-up of only 17.2 months. Since this is a retrospective study, chemotherapy selection and dosing were not standardised. Additionally, rectal cancers were somewhat under-represented, comprising 20% of the nonmucinous cohort vs approximately 30% of all colorectal adenocarcinomas. Nonetheless, several significant associations were found that may lead to further investigations. Longer patient follow-up and standardised treatment will be required to fully explore the role of TYMS and GSTP1 overexpression in mucinous adenocarcinoma. Protein analysis of these enzymes may validate our findings and provide further insight into their complex intracellular interactions. However, much of the data supporting the negative influence of overexpression of both TYMS and GSTP1 on patient outcome has been generated by examining either germline polymorphisms or transcriptional levels (Metzger et al., 1998a; Salonga et al., 2000; Stoehlmacher et al., 2002).

In addition to pharmacogenetic markers for outcome following specific drug treatment, there are a variety of patient characteristics (e.g. lymph node metastases, age), histologic findings (e.g. neurovascular invasion, differentiation), and genetic factors (e.g. p53 expression, microsatellite instability), which appear to influence patient prognosis in colorectal cancer. We chose not to perform an exhaustive examination of all such prognostic factors, but instead to examine the heterogeneity and differential expression of known pharmacogenetic markers in mucinous adenocarcinomas. Whether other markers of prognosis such as microsatellite instability can be utilised clinically to predict outcome in mucinous colorectal cancer remains to be determined.

In conclusion, mucinous tumours significantly overexpressed TYMS and GSTP1 relative to both normal mucosa and to nonmucinous adenocarcinomas. Both these genes are known markers for resistance to chemotherapy. Among those who received adjuvant 5-FU, DFS was shorter in patients with mucinous tumours. It is unclear whether the overexpression of TYMS contributed to these earlier recurrences. Clinical response may be improved for mucinous colorectal cancer if treated with combination chemotherapy or perhaps agents such as irinotecan, where no differences in drug pathway markers were identified. The mucin status of colorectal tumours should be carefully addressed in future trials of novel treatments.

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