Molecular markers of response and toxicity to FOLFOX chemotherapy in metastatic colorectal cancer

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BACKGROUND: To investigate three genetic alterations (TP53 mutation, Kras mutation and microsatellite instability (MSI)) and three polymorphisms (methylene tetrahydrofolate reductase (MTHFR) C677T, excision repair cross complementing group 1 (ERCC1)-118 and X-ray repair cross complementing group 1 (XRCC1)-399) for their ability to predict response, survival and toxicity to FOLFOX first line chemotherapy in the treatment of metastatic colorectal cancer (mCRC).

METHODS: Tumour tissues from 118 mCRC patients who underwent FOLFOX treatment from three successive phase II trials were evaluated for mutations in TP53 (exons 5–8) and Kras (codons 12 and 13) and for MSI using PCR-based analysis. Genotyping for common single nucleotide polymorphisms in the MTHFR (codon 677), ERCC1 (codon 118) and XRCC1 (codon 399) genes was also carried out using PCR techniques. These genetic markers were correlated with clinical response, survival and toxicity to treatment.

RESULTS: Patients with the T allele of ERCC1-118 showed significantly worse progression-free survival in univariate analysis (HR = 2.62; 95% CI: 1.14–6.02; P = 0.02). None of the genetic alterations or polymorphisms showed significant association with clinical response to FOLFOX. The MTHFR, ERCC1 and XRCC1 polymorphisms showed no associations with overall haematological, gastrointestinal or neurological toxicity to FOLFOX.

CONCLUSIONS: The ERCC1-118 and MTHFR C677T polymorphisms were associated with progression and severe diarrhoea, respectively, after FOLFOX treatment in mCRC. Although our findings require confirmation in large prospective studies, they reinforce the concept that individual genetic variation may allow personalized selection of chemotherapy to optimize clinical outcomes.

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There have been significant developments in chemotherapy regimes for the treatment of metastatic colorectal cancer (mCRC) over the past decade with the introduction of new cytotoxic drugs including oxaliplatin, irinotecan, capcitabine and biological agents. Oxaliplatin in combination with a fluoropyrimidine has become one of the most common first line chemotherapy regimens used for mCRC and its efficacy has been confirmed in this setting (Giachetti et al, 2000; de Gramont et al, 2000; Cassidy et al, 2004; Colucci et al, 2005). The rapidly evolving disciplines of molecular oncology and pharmacogenetics aim to correlate gene mutations and polymorphisms, respectively, with drug efficacy and toxicity. Such information might allow treatments to be tailored to suit individual patients, thus sparing them from unnecessary toxicity and expense while still achieving the best response. Numerous studies have investigated novel predictive factors in tumour tissue and blood that could allow such individualised therapy. To date, however, none of these markers has been introduced into routine clinical practice for the treatment of CRC, with the exception of recent and consistent evidence for Kras mutation being predictive for non-response to anti-EGFR treatment (Amado et al, 2008; Bokemeyer et al, 2008; Karapetis et al, 2008; de Roock et al, 2008; van Cutsem et al, 2008).

Because of its role in DNA repair, the TP53 gene has been widely investigated as a possible predictor of response to chemotherapy. The results of a large international collaborative study indicate that wild-type TP53 status is predictive of good response to 5-fluorouracil (5-FU)-based therapies in CRC (Russo et al, 2005). Mutation of the Kras oncogene has also been widely investigated as a prognostic factor in CRC (Andreyev et al, 1998); however, the predictive value of this marker for response to 5-FU is less well known. Another genetic alteration that has received considerable attention is the microsatellite instability (MSI) phenotype. Although some studies indicate that MSI is associated with poor
response to 5-FU (Ribic et al, 2003), others suggest the contrary (Elsaleh et al, 2000; Kim et al, 2007). In addition to somatic genetic alterations found in tumour DNA, polymorphisms within the germline DNA may also have predictive value for response and toxicity to chemotherapy. This is because single nucleotide polymorphisms can alter enzyme activity and expression levels. One of the most well-studied polymorphisms in relation to the prediction of response and toxicity to 5-FU-based treatments is the C677T polymorphism in the methylene tetrahydrofolate reductase (MTHFR) gene (Etienne-Grimaldi et al, 2007). The mutant form of this gene is associated with lower MTHFR enzymatic activity leading to changes in the distribution of tissue folates. Similarly, tumour response to oxaliplatin may be influenced by polymorphisms in genes involved in the nucleotide excision repair pathway (Marsh and McLeod, 2004; Reed, 2005). These include polymorphisms in codon 118 (AAC to AAT) of the excision repair cross complementing group 1 (ERCC1) gene and codon 399 (CGG to CAG) of the X-ray repair cross complementing group 1 (XRCC1). Several groups have published on the predictive value of the ERCC1-118 polymorphism for response to oxaliplatin treatment in mCRC (Stoehlmacher et al, 2004; Viguier et al, 2005; Moreno et al, 2006; Ruzzo et al, 2007; Martinez-Balibrea et al, 2008; Pare et al, 2008), although the results have not been consistent. Less work has been published on the XRCC1-399 polymorphism and again the results have not been concordant (Stoehlmacher et al, 2004; Ruzzo et al, 2007; Pare et al, 2008).

The aim of this study was to evaluate three genetic alterations (TP53 mutation, Kras mutation and MSI) and three polymorphisms (MTHFR C677T, ERCC1-118 and XRCC1-399) for their ability to predict response and toxicity to FOLFOX first line chemotherapy in the treatment of mCRC. Tissue samples were obtained from patients enrolled in three successive and prospective phase II trials investigating modifications of the FOLFOX4 regimen and the utility of gabapentin for reduction of oxaliplatin-based neuropathy (Goldstein et al, 2005; Michael et al, 2006; Mitchell et al, 2006).

MATERIALS AND METHODS

The eligibility criteria for inclusion in the trials have been described earlier (Goldstein et al, 2005; Michael et al, 2006; Mitchell et al, 2006). Patients with a histologically confirmed diagnosis of advanced stage adenocarcinoma of the colon or rectum, who were chemotherapy naive were eligible to enter these trials. Patients were required to have measurable disease, adequate organ function, good performance status (ECOG performance status 0–2) and to have completed adjuvant treatment at least 6 months before entry into the trial. Patients were excluded if they had received earlier adjuvant chemotherapy with oxaliplatin. This subsample of molecular markers was performed with the approval of the individual institutional ethics committees where patients received treatment. A total of 134 patients were enrolled in the three trials and of these, primary tumour tissue samples were available for 118 (88%) patients.

Molecular analyses

Formalin fixed and paraffin-embedded tumour tissue blocks of surgical resection or biopsy specimens were retrieved from pathology archives. After histological confirmation of tumour cell content, several 10-μm sections were cut and the DNA extracted for PCR amplification as described earlier (Soong and Iacopetta, 1997). Fluorescent single strand conformation polymorphism analysis (SSCP) was used to screen for mutations in exons 5–8 of TP53 (Iacopetta et al, 2000a), codons 12 and 13 of Kras (Wang et al, 2003) and for MSI in the BAT-26 mononucleotide repeat (Iacopetta and Grieu, 2000b). Fluorescent SSCP was also used to determine the MTHFR C677T genotype (Grieu et al, 2004). Genotyping for the ERCC1 codon 118 (C>T) and XRCC1 codon 399 (G>A) polymorphisms was carried out using the BsrDI (Biolab, Australia) and MspI (Promega, Australia) restriction enzymes and the PCR primers and conditions described earlier (Stoehlmacher et al, 2004).

Chemotherapy response, toxicity and survival

Chemotherapy cycles were administered every 2 weeks until disease progression or the development of unacceptable toxicity. Radiological response was assessed as per the World Health Organisation Criteria (Miller et al, 1981). All toxicity was graded according to the National Cancer Institute Common Toxicity Criteria and toxicity assessments performed at day 1 of every cycle until the end of treatment. For this study, patients with complete or partial response were classified as responders, and patients with stable disease or progressive disease were classified as non-responders. Patients were also analysed according to disease stabilisation (complete response, partial response and stable disease) and progressive disease. Progression-free survival (PFS) was defined as the time from patient registration on clinical trial until the first documented tumour progression or death from any cause. Overall survival (OS) was defined as the time from patient registration to death from any cause.

Statistical analysis

Fisher’s exact tests were used to access association between genotypes and polymorphisms with response or toxicity outcome. Odds ratio and 95% confidence intervals (CIs) were reported on the basis of univariate logistic regression models. Time to event data were analysed using the Kaplan–Meier method. Hazard ratio (HR) and 95% CI were reported on the basis of univariate Cox proportion hazards regression analyses. No adjustment to the P-value was performed for multiple testing. Multivariate models were also constructed to evaluate the effect of genotypes and polymorphisms on PFS and OS after adjustment for other prognostic factors.

RESULTS

Clinical characteristics for the 118 patients evaluated in this study are shown in Table 1. Patients were predominantly male (68%) and the median age at diagnosis was 61 years (range, 31–75 years). A total of 102 patients (86%) had an ECOG performance status of 0 or 1. There was no evidence of heterogeneity in patient characteristics across the three trials. Information on clinical response to treatment was available for 106 patients, of whom 58 (55%) showed complete or partial response. The median PFS was 7.5 months (95% CI = 6.0–8.6 months) and median OS was 16.3 months (95% CI = 13.7–21.1 months). Major adverse events (grade 3 or 4 toxicity) were neutropenia (36% of patients), neurological toxicity (17%) and diarrhoea (9%).

Tumour samples for the 118 patients were analyzed at a single institution for the presence of mutations and for genotype status. No results for any marker were obtained for 1 patient, for MSI and MTHFR genotype in 2 patients, and for TP53 mutation, ERCC1 and XRCC1 genotypes in 3 patients.

TP53 mutation was found in 43 out of 115 (37%) cases, Kras mutation in 37 out of 117 (32%) cases and MSI + in 2 out of 116 (2%) cases. TP53 and Kras mutations showed no significant associations with clinical response to FOLFOX treatment. Only two cases showed MSI, of which one was associated with clinical response and the other was not. Genotype frequencies for MTHFR C677T were 37% (CC), 47% (CT) and 16% (TT), for ERCC1-118 they were 9% (CC), 56% (CT) and 35% (TT), whereas for XRCC1-
399 they were 34% (GG), 53% (GA) and 13% (AA). No significant associations were seen between these polymorphisms and clinical response (Table 2) or disease stabilization (data not shown).

The ERCC1-118 polymorphism was significantly linked to PFS in univariate analysis (Table 3; Figure 1). Patients carrying a C/T or T/T genotype showed significantly worse PFS compared with those with the CC genotype (HR = 2.62; 95% CI = 1.14–6.02; P = 0.02), but not for OS (P = 0.2). The median PFS for patients with the ERCC1-118 CC genotype was 8.7 months compared with 7.5 months for those with at least one T allele. In multivariate analysis, the association between ERCC1-118 genotype and PFS approached statistical significance (P = 0.07; Table 4). TP53 mutation, Kras mutation, MTHFR genotype and XRCC1 genotype were not associated with either PFS or OS (Table 3).

Somatic mutations are restricted to tumour tissue and were, therefore, not analyzed in relation to the prediction of systemic toxicity to treatment. The MTHFR-C677T, ERCC1-118 and XRCC1-399 polymorphisms showed no significant associations with overall haematological, gastrointestinal or neurological toxicity to treatment (Table 5). There were no associations when the results were analysed for toxicity after 3 or 6 months on treatment or the entire course of chemotherapy. Although only 11 patients experienced grade 3 or 4 diarrhoea, the MTHFR TT genotype was over-represented in this group (Table 6). The percentage of cases who suffered this severe toxicity was significantly higher for TT genotype patients (5 out of 19, 26%) than for CC or CT genotype patients (6 out of 97, 6%; P = 0.02, Fisher’s exact test).

**DISCUSSION**

The aims of this study were to investigate the associations of TP53 mutation, Kras mutation and MSI and response to FOLFOX in mCRC, as well as the predictive values of polymorphisms in MTHFR, ERCC1 and XRCC1 for both response and toxicity to this treatment. These genetic markers were evaluated in 118 patients, of which clinical response data were available for 106 patients. The frequencies of TP53 mutation (37%) and Kras mutation (32%) observed here are similar to those reported in other large studies of CRC (Andreyev et al., 1998; Russo et al., 2003). The very low frequency of MSI + (2%) is in keeping with the low propensity for these tumours to metastasise (Jass, 2006) and prevented investigation of the predictive value of this molecular marker in the current study of mCRC. The distribution of ERCC1 and XRCC1 polymorphisms were similar to those described in other Caucasian populations (Ruzzo et al., 2008) with a slightly higher frequency of the MTHFR 677 CC genotype in our population.

The TP53 gene has important functions in DNA damage repair and apoptosis (Royds and Iacopetti, 2006). Its lack of association with response and survival to FOLFOX in this study (Tables 2 and 3) was therefore surprising and contrary to a large study reporting the impact of TP53 on patients with Duke’s C tumours treated with 5-fluorouracil as adjuvant chemotherapy (Russo et al., 2005). The current finding that Kras mutation is not associated with response to FOLFOX supports an earlier study with 5-FU monotherapy (Etienne-Grimaldi et al., 2008). However, the strong predictive value of Kras mutation for response to anti-EGFR therapies has now been clearly established for mCRC (Amado et al., 2008; Bokemeyer et al., 2008; de Roock et al., 2008; Karapetis et al., 2008; van Cutsem et al., 2008). The MTHFR enzyme has a central function in regulating the pool of intracellular folates available for nucleic acid synthesis and DNA methylation. The common C677T polymorphism in MTHFR shows reduced enzyme activity that is hypothesized to increase intracellular folate concentrations and therefore increase sensitivity to fluoropyrimidines (Sohn et al., 2004). In support of this, human cancer cell lines with the MTHFR 677 T allele show greater sensitivity to 5-FU compared with those with the C allele (Sohn et al., 2004) and this was confirmed in a study of 98 CRC patients treated with 5-FU-based chemotherapy.
(Etienne et al., 2004). Similarly, patients with the MTHFR T allele are postulated to experience greater toxicity from fluoropyrimidine-based chemotherapy (Sharma et al., 2008).

**Table 3** Univariate Cox proportional hazards regression for progression-free survival (PFS) and overall survival (OS)

| Marker                  | n   | %  | HR   | 95% CI     | P  | HR   | 95% CI     | P  |
|-------------------------|-----|----|------|------------|----|------|------------|----|
| TP53 mutation           |     |    |      |            |    |      |            |    |
| Absent                  | 72  | 63 | 1.00 |            | 1.00 |      |            |    |
| Present                 | 43  | 37 | 0.85 | 0.58–1.26  | 0.4 | 0.88 | 0.56–1.37  | 0.6 |
| Kras mutation           |     |    |      |            |    |      |            |    |
| Absent                  | 80  | 68 | 1.00 |            | 1.00 |      |            |    |
| Present                 | 37  | 32 | 0.91 | 0.60–1.36  | 0.6 | 0.76 | 0.48–1.21  | 0.2 |
| MSI                     |     |    |      |            |    |      |            |    |
| Absent                  | 114 | 98 | 1.00 |            | 1.00 |      |            |    |
| Present                 | 2   | 2  | 0.36 | 0.05–2.62  | 0.3 | 0.40 | 0.06–2.92  | 0.4 |
| MTHFR 677               |     |    |      |            |    |      |            |    |
| CC                      | 43  | 37 | 1.00 |            | 1.00 |      |            |    |
| CT                      | 54  | 47 | 0.81 | 0.53–1.24  | 0.3 | 1.10 | 0.68–1.76  | 0.7 |
| TT                      | 19  | 16 | 1.05 | 0.61–1.81  | 0.9 | 1.35 | 0.75–2.45  | 0.3 |
| CT and TT               | 73  | 63 | 0.87 | 0.59–1.29  | 0.5 | 1.16 | 0.75–1.81  | 0.5 |
| ERCC1-118                |     |    |      |            |    |      |            |    |
| CC                      | 10  | 9  | 1.00 |            | 1.00 |      |            |    |
| CT                      | 64  | 56 | 2.68 | 1.15–6.23  | 0.02 | 1.88 | 0.75–4.71  | 0.2 |
| TT                      | 41  | 35 | 2.54 | 1.07–6.04  | 0.04 | 1.55 | 0.60–4.00  | 0.4 |
| CT and TT               | 105 | 91 | 2.62 | 1.14–6.02  | 0.02 | 1.74 | 0.70–4.30  | 0.2 |
| XRCC1-399               |     |    |      |            |    |      |            |    |
| GG                      | 39  | 34 | 1.00 |            | 1.00 |      |            |    |
| AG                      | 61  | 53 | 0.57 | 0.28–1.19  | 0.1 | 0.92 | 0.58–1.45  | 0.7 |
| AA                      | 15  | 13 | 1.01 | 0.39–2.60  | 1.0 | 0.52 | 0.24–1.14  | 0.1 |
| AG and AA               | 76  | 66 | 0.66 | 0.34–1.28  | 0.2 | 0.83 | 0.53–1.29  | 0.4 |

**Table 4** Multivariable analysis of clinical and molecular factors and PFS

| Variable | Hazard ratio | 95% CI | P  |
|----------|--------------|--------|----|
| Number of organs involved | 1.00 |       |    |
| >1       | 1.62         | 1.21–2.15 | 0.001 |
| Absolute neutrophil count at baseline | 1.00 |       |    |
| < ULN⁴   | 1.12         | 1.03–1.21 | 0.009 |
| ≥ULN     |              |         |    |
| ERCC1-118 | 1.00 |       |    |
| CC       | 2.16         | 0.94–4.97 | 0.07 |
| CT and TT|              |         |    |

*The upper limit of normal (ULN) for neutrophils was 7 x 10⁸ per litre.*

**Progression-free survival**

Figure 1  Progression-free survival and ERCC1-118 polymorphism.
However, two groups reported the T allele was associated with worse survival (Park et al, 2002; Ruzzo et al, 2007).

Stoehlmacher et al (2004) reported a two-fold increase risk of dying for patients with the CT or TT genotype compared with those with a C/C genotype (Stoehlmacher et al, 2004). Consistent with the results by Ruzzo et al (2007) and Stoehlmacher et al (2004), patients in this study with an ERCC1-118 CT or TT genotype had a 2.6-fold greater risk of progression with FOLFOX chemotherapy compared with those with the CC genotype. In multivariate analysis, this result approached statistical significance (HR = 2.16; 95% CI = 0.94 – 4.97; P = 0.07), with organ involvement and baseline neutrophil count being significant for PFS (Table 3). Our multivariate analysis replicated a recent report highlighting the value of clinical factors in predicting risk (Sanoff et al, 2008). ERCCI protein expression was not assessed in this; however, two earlier studies reported an association between low expression of ERCCI (mRNA and protein) and improved overall survival in CRC (Shirota et al, 2001; Kim et al, 2009).

Park et al (2002) earlier showed a trend towards higher mRNA levels with increasing numbers of ERCCI-118 T alleles (Park et al, 2002). ERCCI is important for the removal of DNA adducts caused by platinum compounds and hence the increased gene expression in CT and TT individuals may lead to treatment resistance. This could explain the worse PFS seen for these individuals in this study of FOLFOX treatment (Table 3). However, contrary results have been found in studies of ovarian cell lines, where the ERCCI codon 118 C–T substitution was associated with reduced levels of ERCCI mRNA and protein expression (Yu et al, 2000). The functional consequences of the silent ERCCI-118 polymorphism, therefore, remain unclear and may vary according to tissue type. The contrasting results from clinical studies of this ERCC-1 polymorphism may be due to small sample sizes and type I (false positive) rates, with more definitive results likely to be achieved through meta-analysis (Pajak et al, 2000).

Fewer studies have been carried out on polymorphisms in XRCC1 as an important factor for response to oxaliplatin-based chemotherapy. An initial report on the XRCC1-399 polymorphism suggested an association with response (Stoehlmacher et al, 2001); however, subsequent studies including one by the same group failed to confirm this finding (Stoehlmacher et al, 2004; Ruzzo et al, 2007; Pare et al, 2008). This study also failed to confirm an association between XRCC1-399 polymorphism and response or survival to FOLFOX (Tables 2 and 3). The lack of association with overall toxicity (Table 5) also suggests that ERCCI and XRCC1 are not involved in adverse reactions to FOLFOX treatment, consistent with results from other studies (Ruzzo et al, 2007).

In our study, there was also no correlation between neurotoxicity and response.

### Table 5 Associations between polymorphisms and overall haematological, gastrointestinal and neurological toxicity

| Genotype | Haematological | Gastrointestinal | Neurological |
|----------|----------------|-----------------|--------------|
|          | 0–2            | 3–4             | 0–2          | 3–4          | 0–2          | 3–4          | P          |
| MTHFR 677 |                 |                 |              |              |              |              |
| CC       | 24 (56)         | 19 (44)         | 37 (86)      | 6 (14)       | 33 (77)      | 10 (23)      | 0.17       |
| CT       | 40 (74)         | 14 (26)         | 50 (93)      | 4 (7)        | 49 (91)      | 5 (9)        | 0.10       |
| TT       | 12 (63)         | 7 (37)          | 14 (74)      | 5 (26)       | 14 (74)      | 5 (26)       | 0.10       |
| ERCC1 – 118 |               |                 |              |              |              |              |
| CC       | 7 (70)          | 3 (30)          | 10 (100)     | 0 (0)        | 9 (90)       | 1 (10)       |            |
| CT       | 43 (67)         | 21 (33)         | 56 (88)      | 8 (12)       | 55 (86)      | 9 (14)       |            |
| TT       | 25 (61)         | 16 (39)         | 35 (85)      | 6 (15)       | 33 (81)      | 8 (19)       | 0.66       |
| XRCC1 – 399 |              |                 |              |              |              |              |
| GG       | 9 (60)          | 6 (40)          | 13 (87)      | 2 (13)       | 15 (100)     | 0 (0)        |            |
| AG       | 41 (67)         | 20 (33)         | 53 (87)      | 8 (13)       | 49 (80)      | 12 (20)      |            |
| AA       | 25 (64)         | 14 (36)         | 35 (90)      | 4 (10)       | 33 (85)      | 6 (15)       | 0.17       |

*Fisher’s exact test (TT vs CT/CT genotype).

(Tables 5). However, patients with the TT genotype suffered a significantly higher incidence of grades 3–4 diarrhoea (5 out of 19, 26%) compared with those with the CC or CT genotype (6 out of 97, 6%; Table 6). Interestingly, an earlier study with UFT/leucovorin found that 1 of 2 patients with the MTHFR TT genotype developed grade 3 diarrhoea at the first dose level (Veronese et al, 2004). Preliminary data published in abstract form suggest that 75% of patients receiving adjuvant 5-FU-based chemotherapy who had combined MTHFR 677 TT and 1298 AA genotypes predicted toxicity (Adamo et al). However, a large German study found that neither the MTHFR C677T nor the A1298C polymorphisms were associated with toxicity to 5-FU in cancer patients (Schwab et al, 2008), supporting earlier observations in patients treated with 5-FU monotherapy (Cohen et al, 2003), FOLFOSX (Ruzzo et al, 2007) or FOLFRx (Ruzzo et al, 2008).

Capitan et al (2008) reported the A1298C polymorphism, but not C677T, was predictive of toxicity to 5-FU (Capitan et al, 2008). Clearly, more work in larger cohorts that includes analysis of both MTHFR polymorphisms is required to determine whether these genetic variants are associated with 5-FU toxicity, particularly for diarrhoea. The collection of additional information on blood or tissue folate status would also be very useful in helping to clarify the predictive significance of MTHFR polymorphisms.

ERCCI and XRCC1 are both involved in the repair of DNA damage and hence functional variants of these genes are candidate predictive markers for response to oxaliplatin. Unfortunately, results to date on the predictive value of the ERCCI-118 polymorphism for response to oxaliplatin-based chemotherapy have been inconsistent. Several groups have reported that mCRC patients with the ERCCI-118 TT or CT genotype had better tumour response or survival compared with CC patients (Viguier et al, 2005; Moreno et al, 2006; Martinez-Balibrea et al, 2008; Pare et al, 2008). However, two groups reported the T allele was associated with worse survival (Park et al, 2002; Ruzzo et al, 2007).
mutation and MSI are unlikely to be clinically useful molecular markers for the prediction of response to FOLFOX chemotherapy in mCRC. Similarly, the MTHFR C677T, ERCC1-118 and XRCC1-399 polymorphisms were not associated with clinical response to FOLFOX. Our observations on the association of ERCC1-118 and MTHFR C677T polymorphisms for response and toxicity, respectively, to FOLFOX in mCRC require confirmation in large prospective studies. Emerging information in this area based on prospective trials should lead to clinically useful information becoming available in the future.

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