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SHORT COMMUNICATION

Value of gene polymorphisms as markers of 5-FU therapy response in stage III colon carcinoma: a pilot study

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Abstract
Purpose The role of pharmacogenetics in chemotherapy response in colon carcinoma is controversial. We studied the value of known SNPs in genes involved in 5-FU metabolism as biomarkers of chemotherapy response in patients with stage III colon carcinoma.
Methods DNA was isolated from normal colonic tissue of 60 patients with stage III colon carcinoma treated adjuvantly with 5-FU combined with leucovorin. The tested SNPs were validated SNPs on the OPRT, TYMS and DPYD genes and a synonymous SNP on the TYMP gene. Real-time PCR, sequencing and RFLP were used for genotyping.
Results None of the studied genotypes was associated with any of the tumor or patient characteristics. Moreover, none of the genotypes studied had effect on patient survival.
Conclusion In conclusion, the tested SNPs are not biomarkers of chemotherapy response in our stage III colon cancer patients group.

Keywords 5-FU · Colon carcinoma · OPRT · DPYD · TYMS · TYMP · SNP

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Introduction

In colon cancer, the role of pharmacogenetics for drug toxicity and efficacy is still under discussion [1].

5-Fluorouracil (5-FU) is the main drug of choice in the treatment of stage III colon carcinoma. Several proteins are involved in the metabolism of 5-FU, and many of the genes coding for these proteins have been shown to be polymorphic.

Orotate phosphoribosyltransferase (OPRT) and thymidine phosphorylase (TP) activate 5-FU by phosphorylation into active metabolites, which, respectively, incorporate into RNA or inhibit thymidylate synthase (TS). Dihydropyrimidine dehydrogenase (DPD) inactivates 5-FU in the liver [2, 3]. The genes encoding for these proteins harbor functional polymorphisms.

The OPRT gene contains several polymorphisms; among those, the G638C SNP causes a $Gly^{213}Ala$ substitution. This SNP has been associated with a higher expression and activity of the protein and with an increased toxicity of 5-FU therapy [4].

There are several polymorphisms described in the TP gene (TYMP); however, there are no confirmed polymorphisms in coding regions causing changes in aminoacid sequence. The value of these polymorphisms as markers of response to 5-FU therapy is, to our knowledge, unknown.

The polymorphisms in the enhancer region of the TS gene (TYMS) have been widely studied in their relation to response to 5-FU therapy and with protein expression and activity. The studied polymorphisms consist in a 28-bp repeat at the 5′ untranslated region of the gene and a G > C SNP in the second repeat of the three repeat allele. In the Caucasian population, the variants with two (2R) or three (3R) repeats are the most frequent alleles found. On the basis of the effects of the SNP in the second repeat of the

3R allele on TS protein expression, patients could be classified as high TS protein producers when carrying the G allele and low TS protein producers when carrying the C allele [5, 6]. However, up-to-date, the results concerning the effect of these polymorphisms in 5-FU response remain inconclusive [7].

Finally, the DPYD gene has been shown to play a very important role in toxicity of 5-FU. The polymorphism in the exon/intron boundary at exon 14 is responsible for severe toxicity in these patients [8]. However, little is known about the value of these and other polymorphisms as markers of response.

We aimed to study the value of known polymorphisms in the OPRT, TYMP, TYMS and DPYD genes as markers of response in patients with stage III colon carcinoma treated with 5-FU chemotherapy in combination with leucovorin.

Materials and methods

Patient material

Sixty patients with stage III colon carcinoma treated with surgery and 5-FU chemotherapy following the Mayo regime were studied.

All diagnoses were made at a central laboratory for pathology between 2003 and 2004. Population data were obtained from the cancer registry database of the Comprehensive Cancer Centre South. Follow-up information was obtained from medical records.

The use of clinical material for this retrospective study was approved by the institutional review board according to the guidelines of the Dutch Federation of Research Associations.

DNA was isolated from normal colonic mucosa from formalin-fixed, paraffin-embedded (FFPE) material after proteinase K digestion and purification using the HPTTP kit (Roche, Almere, the Netherlands).

Target genes and polymorphisms

Polymorphisms must be non synonymous and confirmed by independent research groups. However, in the case of the TYMP gene, there were no confirmed non synonymous polymorphisms, and therefore we chose one confirmed synonymous polymorphism.

OPRT

The G638C SNP (rs1801019) causing a Gly213Ala substitution was tested by real-time PCR with the following primers and probes: forward 5′ GCT GAG ACA GTT GGG AGA GTG A 3′, reverse 5′ TGA GTT CTT TGG GTG CTT CCT T 3′, probe for G allele 6FAM 5′CGA ATC ATA ATG GTT C3′and probe for C allele 6FAM 5′AGC GAA TCA TAA TGC TT3′. Reactions were performed using Roche chemistry in a final volume of 20 µl in the light cycler v2 (Roche, Almere, the Netherlands).

TYMP

The rs470119 SNP was assessed by restriction fragment length polymorphism (RFLP). PCR was performed using the following primers: forward 6FAM-5′TCC AGA GCC CAG GTA3′ and reverse 5′CTG GCC AGG GTC TCC ATC A3′. The 71-bp-long PCR product was then digested with MboI restriction enzyme (New England Biolabs, Ipswich, United Kingdom). After digestion, fragment length analysis was carried out by capillary electrophoresis. The following fragment length was expected for homozygous GG, 40 and 30 bp, for AA 71 bp and for heterozygous AG 71, 40 and 30 bp.

TYMS

The two polymorphisms in the enhancer region of this gene were typed using PCR and RFLP as described elsewhere [9]. Briefly, the 28-bp repeat was typed by PCR means followed by electrophoresis on 2% agarose gel. The G > C SNP was typed by digestion of the PCR product with HaeIII restriction enzyme. The G–C substitution abrogates the restriction site for this enzyme. Subsequently, products were separated by agarose gel electrophoresis.

DYPD

The SNP A1627G (rs1801159) causing Ile543Val was tested by PCR followed by sequencing using the following primers: forward 5′GCA GTC ACA ATA TGG AGC3′ and reverse 5′TTA CCT TAT CAA GAG AGA AAG TT3′. The expected length of the product was 225 bp. Subsequently, PCR products were purified using enzymatic purification with ExoSapIT (USB, USA), and the sequencing reaction was performed using the same primers as for the PCR reaction and Big Dye chemistry (Applied Biosystems, Nieuwerkerk aan den Ijssel, the Netherlands). Sequences were analyzed using the sequencing analysis 5.3.3 software (Applied Biosystems).

Statistical analysis

SPSS software package for Windows (Chicago, Il., U.S.A.) was used for statistical analysis. Categorical data were analyzed by means of a chi-square or Fischer's exact test. The end point of this study was progression-free survival (PFS, time between surgery and disease progression).

Univariate survival analysis was performed by Kaplan–Meier analysis, and differences were analyzed using the log-rank method. Hazard ratios and multivariate analysis were calculated using the Cox proportional hazard model.

All tests are two tailed, and a result was considered significant when $P = 0.05$.

Results

Briefly, characteristics of the sixty patients studied were as follows: median age at diagnosis was 64(range 30–81), 52% ($n = 31$) of the tumors were located on the right side of the colon and 53% ($n = 32$) of the patients were men. The majority 70% ($n = 42$) had a T3 tumor. Median follow-up was 39 months (range 2–57). Forty percentage was still alive without evidence of disease at the end of the follow-up, 24% had developed a local recidive or a distant metastasis, 31% was dead because of cancer-related causes and 5.2% was dead because of non cancer related causes as specified in their medical records. Median time to progression was 15 months (range 6–47).

Frequencies of the different alleles are shown in Table 1. All frequencies followed Hardy–Weinberg equilibrium and did not differ significantly from frequencies published on the Hap-Map database for the Caucasian population.

In this group of patients, there were no significant associations between any of the genotypes found and any of the clinical and histopathological variables tested including gender, tumor location, T stage and N stage.

Survival analysis

For the survival analysis, TYMS SNP genotypes were grouped as putative high TS expression (genotypes 2R/3G, 3C/3G and 3G/3G) and putative low TS expression (2R/2R, 2R/3C. 3C/3C). No effect on progression-free survival of the several genotypes found was seen in a univariate (Fig. 1) or in a multivariate survival analysis, containing other known prognostic variables for colon carcinoma such as T stage and N stage of the tumor.

Table 1 Frequencies of the different alleles

SNPs	Frequencies *N* (%)
OPRT	
GG	44 (75)
GC	15 (25)
CC	0 (0)
TYMP	
GG	25 (42)
AG	29 (48)
AA	6 (10)
TYMS	
2 repeats	10 (17)
2 and 3 repeats	35 (58)
3 repeats	15 (25)
TYMS SNP	
2R/2R	10 (16.7)
2R/3RC	16 (26.7)
2R/3RG	19 (31.7)
3RC/3RC	7 (11.7)
3RG/3RC	4 (6.7)
3RG/3RG	4 (6.7)
DPYD	
AA	37 (67)
AG	14 (26)
GG	4 (7)

Discussion

We aimed to study whether known SNPs in genes involved in 5-FU metabolism were good markers of therapy response in patients with stage III colon carcinoma.

According to our results, we conclude that the SNPs tested in the OPRT, TYMP, TYMS and DPYD genes are not good markers of therapy response in the present cohort. No effect on survival of the different genotypes was seen; however, results should be considered with caution due to the small number of patients analyzed.

Although increased expression of OPRT mRNA and protein activity have been related to a shorter survival of patients with colorectal carcinoma treated with 5-FU [10–12] together with the fact that the studied SNP has been proven to be associated with a higher protein expression and activity [13, 4], no effects of the different genotypes in DFS were seen in the present cohort. The OPRT CC variant is very rare in the general population. Our results reflect the low frequency of this genotype in the Caucasian population. If the CC variant would have an effect on survival, the fact that it is such a rare variant makes it very difficult to prove since numbers of patients needed would be very large.

The existing literature is more conclusive about TP protein expression, which seems to have no influence in survival of patients with colorectal [14–19]. Accordingly, the SNP in the TYMP gene was not a good marker for therapy response in our group.

The role of the typed polymorphisms on the TYMS gene remains controversial. It has been widely studied as protein and mRNA expression as well as DNA genotyping; still the results are inconclusive as reviewed by Popat et al. [7]. These contradictory results are probably due to differences

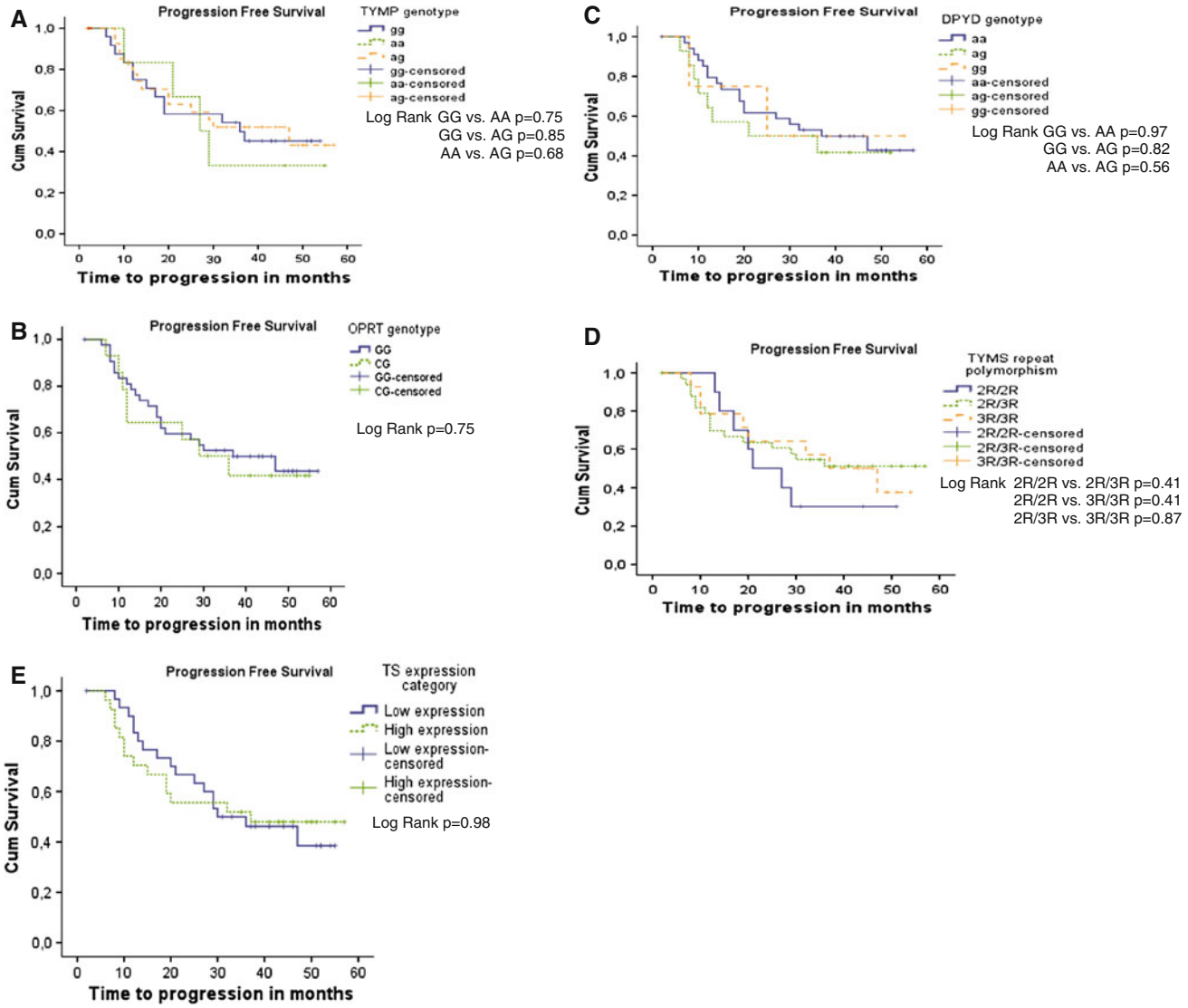

Fig. 1 Kaplan–Meier Curves for PFS according to all genotypes tested; **a** TYMP genotypes, **b** OPRT, **c** DPYD, **d** TYMS repeat **e** TYMS expression category determined by SNP genotypes (Low expression 2R/2R, 2R/3C, 3C/3C; High expression 2R/3G and 3G/3G)

in methodology, technology and patients' selection. In the present patient group, TYMS polymorphisms in the enhancer region are not good markers of therapy response, even when grouping patient by category of putative TS expression no difference in survival was found between putative low and high producers. These results agree with previous results in a larger cohort of patients [20].

Finally, although DPYD has been widely studied in relation to 5-FU toxicity, it also seems to have a role in outcome [15, 19, 21–23]. However, whether this effect is due to the enzyme itself or due to high toxicity and subsequent therapy, interruption is not clear. In the present cohort of patients, the studied SNP is not a good marker of therapy response.

In conclusion, polymorphisms in genes involved in 5-FU metabolism are not valuable as markers of response in the present cohort of patients with colon cancer. Recently, the results of a clinical trial over the value of some polymorphisms in predicting toxicity in a large group of patients with colon carcinoma failed to find any relation between the tested SNPs and toxicity [1]. In that study, the value of several SNPs involved in 5-FU metabolism was tested in relation to toxicity. In the same way, we cannot show any value of these SNPs as markers of response to 5-FU in our group of patients.

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