Correlation between *DPYD* gene variation and *KRAS* wild type status in colorectal cancer

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ABSTRACT

Aims Failure and side effects of combined cytotoxic therapy are challenges in the treatment of metastatic colorectal cancer (CRC). *DPYD* gene variations can potentially predict toxicity to 5-fluorouracil (FU)-based therapy and *KRAS*-, *NRAS*-, *BRAF*-, *PIK3CA*-wild type status is a known prerequisite for epidermal growth factor receptor (EGFR) inhibitor therapy. This study was performed to search for a possible link between these therapeutic markers.

Methods The *DPYD* gene variations c.496A>G, c.1679T>G, c.2846A>T and *KRAS/NRAS/BRAF/PIK3CA* mutational status were determined in non-neoplastic, primary CRC and metastatic CRC tissue from 115 patients.

Results The polymorphism c.496A>G was the *DPYD* gene variant with the highest detection rate (12.9%), occurred predominantly in females (86.7%, p=0.0044) and was exclusively seen in *KRAS* wild type primary CRC (15/65 (23.1%) vs 0/51 (0%) in *KRAS*-mutated primary CRC, respectively, p=0.0001).

Conclusions This genetic profile could define a patient group requiring alternative combined therapeutic approaches. Global testing of large patient cohorts is necessary to prove this concept.

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the fourth leading cause of cancer-related death worldwide.[1] Up to 50% of patients show recurrence despite curative surgery and 20% of all patients present with metastases at the time of diagnosis.[2] 5-fluorouracil (FU)-based chemotherapy has become a fundamental tool to reduce recurrence in patients with stage III CRC.[3] Furthermore, combination of 5-FU and leucovorin (FLV) with oxaliplatin and irinotecan as well as additional blocking of epidermal growth factor receptor (EGFR) have been proven to increase overall survival of patients with metastatic CRC.[3] However, a proportion of patients gain little or even no benefit from these therapies.[4] Furthermore, 10%–40% of patients develop severe to life-threatening toxicity from 5-FU.[5] These clinical and health economic challenges released a wide spectrum of research to detect predictive biomarkers. New biomolecular approaches include genetic testing for *KRAS*-, *NRAS*-, *BRAF*- and *PIK3CA*-mutations as markers of resistance to EGFR-inhibitor therapy[6] and risk assessment of 5-FU-toxicity or 5-FU therapy failure by dihydropyrimidine dehydrogenase (*DPYD*) gene variation or expression analysis.[7 8] Parallel testing of *KRAS*-, *BRAF*-, *PIK3CA*-mutation status and DPYD expression has already been performed to identify prognostic genetic and protein markers in CRC.[7] Furthermore, a frequent *DPYD*-mutation has been included in a previously developed screening test for the simultaneous detection of *KRAS*- and *BRAF*-mutations.[9]

To extend knowledge about genetic profiles in the context of 5-FU based and EGFR-inhibitor therapy, we searched for a possible link between three *DPYD* gene variants with relatively high population frequency[10] and possible importance for 5-FU metabolism[5] and *KRAS*-, *NRAS*-, *BRAF*- and *PIK3CA*-mutation status.

MATERIAL AND METHODS

Tissue sampling and selection

Formalin-fixed paraffin-embedded (FFPE) CRC samples (116 primary tumours, 42 distant metastases, 109 lymph node metastases sample mixes, comprising between one and eight lymph node metastases per case) and 115 non-neoplastic FFPE samples from 115 patients were collected from the tissue archive (1999–2005) at Department of Pathology, Southern Norwegian Hospital Trust, Kristiansand. The material was partly included in a previous study.[11] Tissue and patient data were obtained and used after approval of the Regional Ethics Committee (REK) of Southern Norway in accordance with the Declaration of Helsinki and the International Conference of Harmonization—Good Clinical Practice. The anonymity of the patients investigated was preserved corresponding to rules of data protection of the National Data Protection Commission (NSD) of Norway and the institutional guidelines of our hospital. All tumour samples underwent histopathological review (BK). Only material containing <20% necrosis and <20% non-neoplastic adherent tissue was included in this study. Tumour response to treatment was classified according to the Response Evaluation Criteria in Solid Tumours (RECIST).[12]

Molecular genetic analysis

Mutation status of *KRAS*, *NRAS*, *BRAF* and *PIK3CA* of the tumour tissue has already been determined in a previous study.[11] Description of DNA isolation and molecular genetic analysis of these four genes is added as online supplementary text and table S1. Three *DPYD* variants, c.496A>G (rs2297595, Met166Val), c.2846A>T (rs67376798, Asp949Val) and c.1679T>G (rs55886062, Ile560Ser), were selected for this study, because of documented minor allele frequency >1%,[10] proven amino acid change and possible impact on 5-FU chemotherapy.[5] Assessment of allele frequencies in *DPYD* variants was done in a multiplex PCR in 12.5 µL using the GeneAmp PCR system 9700 (Applied

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Biosystems, Weiterstadt, Germany) with 0.3–1 ng of DNA as template in 15 mM Tris–HCl, 50 mM KCl, with 200 μM dNTPs (deoxyribonucleotide triphosphates), 1.5 mM $MgCl_2$, 0.1 μM each primer (primer sequences see online supplementary table S2) and 1.5 Units AmpliTaq Gold Polymerase (Applied Biosystems) for 30 cycles with an annealing temperature of 55°C. SNaPshot analyses were performed with the SNaPshot Multiplex kit (Applied Biosystems) (primer sequences see online supplementary table S2) in accordance with the manufacturer's instructions and evaluated on an ABI310 Genetic Analyzer (Weiterstadt, Germany). Electrophoresis results were analysed using the GeneMapper ID Software V.3.2 with self-designed panels and bins sets.

Statistical analysis

Data were analysed using the χ^2 test and the Fisher's exact test (Graph Pad Quickcalcs).[13] A p value of <0.05 was considered as statistically significant.

RESULTS

Clinical and histopathological data

Clinical data of the patients, histopathological characteristics of the primary tumours and distant metastatic sites are listed in table 1.Two patients, both <50 years old at the time of CRC diagnosis, presented a family history or clinical course, which could be suspicious for hereditary non-polyposis CRC. A third patient showed successive colorectal, urinary bladder and pancreatic cancer (table 1), but was already 78 years old at the time of first cancer diagnosis (CRC). Neither microsatellite instability in the tumours nor mutation status of mismatch repair (MMR) genes was determined for these patients.

DPYD variation analysis

The proportion of patients carrying *DPYD* gene variants is displayed in table 1. All variants occurred heterozygous. No differences of *DPYD* genotype were found between non-neoplastic tissue, primary tumours, lymph node metastases and distant metastases (ie, germline=somatic genotype). Variant c.496A>G occurred together with c.1679T>G in one patient and with c.2846A>T in another patient. The relationship between *DPYD* gene variants and clinicopathological parameters is displayed in table 1. Variant c.496A>G occurred significantly more frequently in females than in males ($p=0.0044$).

Chemotherapy response, side effects and liver function status among *DPYD* variant carriers are displayed in online supplementary table S3. Side effects were mainly seen in patients, who were treated with 5-FU-based combined therapy.

Mutational data of all patients and *DPYD* gene variant carriers

A case was regarded as mutated, if at least one sample (primary tumour, lymph node metastasis or distant metastasis) showed a mutation in one of the genes *KRAS*, *NRAS*, *BRAF* or *PIK3CA*. All investigated variants were point mutations, which resulted in amino acid changes or frameshift. Copy number changes have not been analysed in any of the genes. More detailed, primary tumours (n=116), lymph node metastases (sample mix/case, n=109) and distant metastases (n=42) showed *KRAS*-mutations in 44%, 51.4% and 61.9%, respectively, *NRAS*-mutations in 5.2%, 5.5% and 2.4%, respectively, *BRAF*-mutations in 12.9%, 12.8% and 0%, respectively, *PIK3CA*-mutations in 6.9%, 11% and 0%, respectively. The number of primary tumours with mutations in these genes is displayed in table 2.

DPYD gene variant c.496A>G correlated significantly with *KRAS* wild type status of the primary tumours (p=0.0001, table 2). All distant metastases of c.496A>G carriers showed *KRAS* wild type. Only one patient with both, c.496A>G and c.1679T>G, was tested positive for *KRAS* mutation in a lymph node metastasis. Three patients carrying c.496A>G showed mutations in other EGFR pathway regulating genes (*BRAF* and *PIK3CA*).

DPYD gene variant c.2846A>T was significantly associated with occurrence of *BRAF* mutations in the tumour tissue ($p=0.028$, table 2). We could not identify pairs or groups of individual cases showing exactly the same alterations in the investigated genes.

DISCUSSION

Investigations of adverse events and limited therapeutic effects following 5-FU administration focus mainly on altered function of DPYD, the key enzyme in the catabolism of 5-FU.[5] *DPYD* gene variants account for at least 20% of cases with severe 5-FU-related toxicity and are of even greater importance in 5-FU-based combined therapies than in 5-FU monotherapy.[5] Alternative or first-line EGFR-inhibitor therapy could be considered for patients carrying *DPYD* risk alleles.[14] This therapeutic strategy depends on wild type *KRAS*-, *NRAS*-, *BRAF*- and *PIK3CA*-status.[6] A link between these EGFR-pathway regulating genes and *DPYD* genotype has not been investigated so far. Therefore, this study combined analysis of tumour-related factors as *KRAS/NRAS/BRAF/PIK3CA* mutation status and host-related factors as the allelic status of the *DPYD* variants c.496A>G, c.2846A>T and c.1679T>G. We found a mutation rate within the published range for primary tumours in the case of *KRAS* (32%–45%),[15 16] *NRAS* (2.9%–5%)[16 17] and *BRAF* (7%–17.6%)[16 18 19] and a only slightly lower mutation rate than the published rates in the case of *PIK3CA* mutations (9%–21%).[16 18] The frequency of the minor allele of all three *DPYD* variants in our patient group did not differ significantly from that published for Europeans.[10] Despite a large variety of investigated distant metastatic sites, which are regarded to cause heterogeneous molecular genetic results in primary tumours and metastases,[20] all three *DPYD* variants were concordant in primary and metastatic tumour tissue.

The *DPYD* variants c.496A>G and c.2846A>T could be differently linked to genes of the EGFR signalling pathway: the variant c.496A>G correlated significantly with *KRAS* wild type status, whereas c.2846A>T was associated with *BRAF*-mutated tumour tissue. To the best of our knowledge, these correlations have not been reported previously. However, polymorphisms and low mRNA expression of thymidylate synthase (TS), which is another enzyme with known impact on response and toxicity to 5-FU chemotherapy in patients with CRC,[21–23] were found to be associated with mutant *KRAS*.[24] This finding and our results point to a possible interaction between tumour-specific markers and host-specific factors. The functional *DPYD* variants c.496A>G and c.2846A>T occur at significantly conserved sites close to the Fe-S motif and may disrupt electron transport.[25 26] Further biomolecular studies are necessary to evaluate, whether *DPYD* variant-related impairment of electron transport and dNTP pool imbalances due to altered DPYD activity have mutagenic or protective effects on EGFR pathway regulating genes.

The study of Maus *et al*,[24] which is comparable to our study, revealed higher TS expression levels in rectal compared with distal colon cancer. Therefore, we searched for a possible association between *DPYD* variants and clinicopathological parameters. In contrast to the data of Maus *et al*,[24] none of the

Table 1 Clinicopathological parameters of all patients and of *DPYD* gene variant carriers

Parameter	All* Patients (*n*=115) Tumours (*n*=116)	*DPYD* gene variant c.496A>G (*n*=15)	c.2846A>T (*n*=6)	c.1679T>G (*n*=3)
Age (years)				
Mean (range)	66 (32–88)	62 (32–88)	63 (42–77)	55 (32–65)
Gender				
Male	56 (48.7%)	2 (13.3%)	4 (66.7%)	1 (33.3%)
Female	59 (51.3%)	13 (86.7%)	2 (33.3%)	2 (66.7%)
Clinical stage				
III	68 (59.1%)	10 (66.7%)	2 (33.3%)	1 (33.3%)
IV	47 (40.9%)	5 (33.3%)	4 (66.7%)	2 (66.7%)
5-FU chemotherapy				
Yes	92 (80%)	12 (80%)	3 (50%)	3 (100%)
No	23 (20%)	3 (20%)	3 (50%)	0 (0%)
Anatomic site				
Caecum	22 (19%)	2 (13.3%)	1 (16.7%)	1 (33.3%)
Ascending	17 (14.6%)	3 (20%)	2 (33.3%)	0 (0%)
Transverse	11 (9.5%)	2 (13.3%)	0 (0%)	0 (0%)
Descending	19 (16.4%)	3 (20%)	1 (16.7%)	0 (0%)
Sigmoid	19 (16.4%)	0 (0%)	0 (0%)	1 (33.3%)
Rectum	28 (24.1%)	5 (33.4%)	2 (33.3%)	1 (33.3%)
pT stage				
≤2	5 (4.3%)	0 (0%)	0 (0%)	0 (0%)
3	90 (78.3%)	15 (100%)	4 (66.7%)	3 (100%)
4	20 (17.4%)	0 (0%)	2 (33.3%)	0 (0%)
pN stage				
0	6 (5.2%)	0 (0%)	0 (0%)	0 (0%)
1	68 (59.1%)	14 (93.3%)	0 (0%)	2 (66.7%)
2	41 (35.7%)	1 (6.7%)	6 (100%)	1 (33.3%)
Histological grade				
High	1 (0.9%)	0 (0%)	0 (0%)	0 (0%)
Moderate	89 (76.7%)	14 (93.3%)	6 (100%)	3 (100%)
Poor	26 (22.4%)	1 (6.7%)	0 (0%)	0 (0%)
Distant metastatic site†				
Liver	10 (23.8%)	1 (25%)†	0 (0%)†	1 (33.3%)†
Non-liver	32 (76.2%)	3 (75%)†	1 (100%)†	2 (66.7%)†
Other malignant tumour‡				
No	92 (80%)	11 (73.3%)	4 (66.7%)	3 (100%)
Skin tumours (BCC, SCC, MM)	13 (11.3%)	3 (20%)	1 (16.7%)	0 (0%)
Urinary bladder cancer	2 (1.7%)	0 (0%)	0 (0%)	0 (0%)
Urinary bladder and pancreatic cancer	1 (0.9%)	0 (0%)	0 (0%)	0 (0%)
Breast cancer	2 (1.7%)	0 (0%)	0 (0%)	0 (0%)
Lung cancer	2 (1.7%)	0 (0%)	1 (16.7%)	0 (0%)
Malignant mesothelioma	1 (0.9%)	0 (0%)	0 (0%)	0 (0%)
Renal cell carcinoma	1 (0.9%)	1 (6.7%)	0 (0%)	0 (0%)
Prostate carcinoma	1 (0.9%)	0 (0%)	0 (0%)	0 (0%)

*116 primary tumours of 115 patients were investigated.
†Number of investigated metastatic sites=42 in all patients, four in c.496A>G carriers, one in c.2846A>T carriers, three in c.1679T>G carriers.
‡Not analysed in this study.
BCC,basal cell carcinoma; 5-FU, 5-fluorouracil; MM, malignant melanoma; SCC,squamous cell carcinoma.

investigated *DPYD* variants in our study was related to a specific anatomic tumour site. However, the variant c.496A>G was significantly more frequently seen in female than in male patients. This female predominance is in line with a previous study, which detected *DPYD* c.1905+1G>A exclusively in women.[27] To the best of our knowledge, there is currently only one publication reporting an association between heterozygosity of a *DPYD* gene variant (*DPYD**2A) and increased FU-related toxicity in male.[21] Detailed genotype–phenotype analysis is necessary to evaluate, whether female predominance of several *DPYD* variants could be the genetic background for previously described lower DPYD expression levels in tumour tissue and plasma of females compared with males.[28–30]

The genotypic correlations found in this study could have different clinical importance. Considering *DPYD* variant c.496A>G as possible toxicity marker, its association with *KRAS* wild type status could define a patient group, which might be considered for first-line EGFR inhibitor monotherapy or eventually combined 5-FU/

Table 2 Primary tumour *KRAS/NRAS/BRAF/PIK3CA* mutation status of *DPYD* gene variant carriers

	DPYD gene variants		
	c.496A>G (*n*=15)	c.2846A>T (*n*=6)	c.1679T>G (*n*=3)
KRAS wild type (*n*=65)	15 (23.1%)	4 (6.1%)	3 (4.6%)
KRAS mutation (*n*=51)	0 (0%)	2 (3.9%)	0 (0%)
	p=0.0001	n.s.	n.s.
NRAS wild type (*n*=110)	15 (13.6%)	6 (5.4%)	3 (2.7%)
NRAS mutation (*n*=6)	0 (0%)	0 (0%)	0 (0%)
	n.s.	n.s.	n.s.
BRAF wild type (*n*=101)	13 (12.9%)	3 (3%)	3 (3%)
BRAF mutation (*n*=15)	2 (13.3%)	3 (20%)	0 (0%)
	n.s.	*p*=0.028	n.s.
PIK3CA wild type (*n*=108)	14 (13%)	6 (5.6%)	3 (2.8%)
PIK3CA mutation (*n*=8)	1 (12.5%)	0 (0%)	0 (0%)
	n.s.	n.s.	n.s.

n.s., not significant.

EGFR antibody therapy without other cytotoxic drugs. A possible limiting influence of concomitant *BRAF*- and *PIK3CA*-mutations has to be evaluated by large cohort studies. In contrast, the association between *DPYD* c.2846A>T and *BRAF*-mutations could point to a patient group with limited therapeutic options at all. However, it has not been clearly determined in the literature, whether *DPYD* variant c.496A>G predict toxicity to 5-FU-based chemotherapy or protection against adverse effects from this therapy[5] and due to the small sample size, we could not prove or exclude an impact of c.496A>G, c.2846A>T and c.1679T>G on 5-FU-based chemotherapy. Furthermore, we did not determine the MMR status of the tumours, which is probably associated with DPYD expression and might influence therapy response.[7] Another limitation of this study is that it considered only three out of approximately 33 000 recorded *DPYD* variants,[10] even if the vast majority of these mostly intronic variants can be expected to be non-functional.[5] Therefore, anticancer therapy should be increasingly based on results of high throughput sequencing technologies as recently published[8 31] and simultaneous testing of predictive biomarkers for several therapies[7 9] which are able to precisely define the clinical relevance of *DPYD* variants and their association to other tumour-related markers.

Take home messages

- *DPYD* gene variations can potentially predict toxicity to 5-fluorouracil (FU)-based therapy and *KRAS*-, *NRAS*-, *BRAF*-, *PIK3CA*-wild type status is a known prerequisite for epidermal growth factor receptor (EGFR) inhibitor therapy.
- The authors could demonstrate a correlation between occurrence of *DPYD* gene variant c.496A>G and *KRAS* wild type status of colorectal cancer tissue.
- This genetic profile could define a patient group requiring alternative combined therapeutic approaches.

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