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Concordance of genotype for polymorphisms in DNA isolated from peripheral blood and colorectal cancer tumor samples

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Abstract

Background & aim: Results from different pharmacogenetic association studies in colorectal cancer are often conflicting. Both peripheral blood and formalin-fixed, paraffin-embedded (FFPE) tissue are routinely used as DNA source. This could cause bias due to somatic alterations in tumor tissue, such as loss of heterozygosity. We therefore compared genotypes in DNA from peripheral blood and FFPE colorectal tumor samples for SNPs with putative influence on the cytotoxicity of chemotherapy.

Materials & methods: Eleven SNPs in nine genes involved in anticancer drug metabolism or efficacy were determined in matched samples from blood and FFPE tissue of colorectal tumors by pyrosequencing and TaqMan® techniques. The k-statistic was calculated to assess concordance.

Results: A total of 149 paired FFPE tissue and EDTA blood DNA samples were available for comparison. Overall, 20 out of 1418 genotypes were discordant (1.4%); in ten cases, loss of heterozygosity could not be ruled out. Only *GSTP1* showed significant discordance between FFPE tissue and blood genotype (k = 0.947; 95% CI: 0.896–0.998).

Conclusion: FFPE tissue-derived DNA can be used as a valid proxy for germline DNA for a selection of SNPs in (retrospective) pharmacogenetic association studies in colorectal cancer. However, for future studies, genotyping of blood-derived DNA is preferred.

Introduction

The field of pharmacogenetics has developed rapidly over the last decade[1;2], as have techniques for DNA isolation and SNP genotyping. Pharmacogenetic association studies have shown inconsistent results for candidate genes in many pharmacologic pathways. Among other variables, the source of DNA could be a confounding factor explaining these inconsistencies. The majority of studies use whole blood as a source of DNA, since it supplies genomic DNA and is easy to obtain in a clinical setting. Alternative sample types include buccal swabs or saliva, both of which are ideal for use in nonclinical settings and for shipping by mail.[1;3]

Tumor tissue as a source of DNA is of particular interest, as tumor biopsies and resection specimens of large patient populations have been archived and are potentially useful for retrospective pharmacogenetic studies.[4;5] However, the quality of DNA isolated from formalin-fixed, paraffin-embedded (FFPE) material is often inferior to DNA from peripheral blood leukocytes.[5;6] As a result of chromosomal damage, DNA amplification and primer recognition may be hampered. Indeed, unpublished pilot experiments showed that genetic variations in the *TYMS* 28-bp repeat and *UGT1A1**28, both spanning a larger number of base pairs, could not be determined in most of our FFPE samples due to low DNA quality.

Although these arguments make peripheral blood the preferred DNA source for most pharmacogenetic studies, blood samples are not always available, especially in retrospective studies. Indeed, many pharmacogenetic association studies in colorectal cancer (CRC) have used FFPE tissue specimens as the primary source of DNA.[7–19] However, the use of FFPE tissue may lead to genotyping results that differ from the germline genotype, because tumor DNA harbors somatic alterations that are associated with carcinogenesis on top of germline characteristics. These may involve point mutations, copy number variations or, more frequently, loss of heterozygosity (LOH).

Concordance of germline drug metabolism pathway polymorphisms in DNA from peripheral blood and FFPE tissue has not been extensively investigated in colorectal carcinomas. It is therefore unknown whether results from studies using archived tumor samples as the primary source of DNA can be assessed alongside those from studies using blood-derived DNA. Therefore, the aim of the present study was to compare the genotypes of a large set of paired blood and FFPE samples in CRC patients for a selection of SNPs with putative influence on the cytotoxicity of commonly used chemotherapeutic drugs for CRC.[20]

Materials and methods

DNA was obtained from previously untreated metastatic CRC patients participating in the multicenter CAIRO trial of the Dutch Colorectal Cancer Group. The inclusion criteria and clinical results of this study have been published elsewhere.[21] All included patients gave written informed consent before inclusion. EDTA–blood was stored at -20°C before DNA

isolation. FFPE colorectal tumor resection samples were collected from multiple pathology laboratories in The Netherlands and stored under standard conditions. Procedures for tissue collection and fixation are uniform across Dutch hospitals. Germline DNA was isolated from EDTA–blood with the MagNA Pure LC® (Roche Diagnostics, The Netherlands) according to the manufacturer's instructions. Tissue DNA was obtained by macrodissection from FFPE samples of areas optically containing predominantly tumor tissue with QIAamp® DNA Mini Kit columns (Qiagen, The Netherlands).

SNP selection and genotyping assays

We selected polymorphisms in genes with putative influence on the pharmacokinetics or pharmacodynamics of fluoropyrimidines, oxaliplatin and irinotecan. SNPs in the following genes were included in our analyses: *ABCB1*, *ABCG2*, *MTHFR*, *SLC91A1*, *ERCC1*, *ERCC2*, *XRCC1*, *GSTP1* and *TP53*. In addition to *MTHFR*, other genes have been associated with fluoropyrimidine cytotoxicity, including the genes *TYMS*, *DPYD* and *TYMP*. However, polymorphisms in these genes were not included in the present study. The *TSER* polymorphisms (rs34743033 and rs11540151) were not included because, in pilot experiments, genotyping for these SNPs failed in the majority of FFPE samples, presumably due to the extended length of the polymorphism (data not shown). *DPYD**2A (rs3918290) was also excluded, because the very low minor allele frequency and expected low number of heterozygotes in our population (1–2%[9;22]) would preclude statistical analyses. Although *TYMP* expression has been linked to fluoropyrimidine cytotoxicity, very few studies have addressed the effect of genetic variation in this gene. It was therefore not included in this study. Regarding irinotecan pharmacogenetics, it would have been interesting to test UDP-glucuronosyltransferase polymorphisms, particularly *UGT1A1**28. Unfortunately, genotyping was not successful in most FFPE samples, which led us to exclude this polymorphism from further analyses.

In order to maintain statistical power, we chose to limit the number of polymorphisms to be studied per gene. Because our intention was to validate results from earlier publications, we aimed to include the polymorphism that was cited most frequently in publications to date for each gene. Therefore, in these cases, we searched PubMed for the respective polymorphisms and their rs numbers and included the SNP with the highest number of citations.

The following SNPs were determined using the TaqMan® 7500 real-time PCR system (Applied Biosystems, The Netherlands) according to the manufacturer's protocol: *ABCB1* rs1128503 and rs1045642; *ABCG2* rs2231142; *ERCC1* rs11615; *MTHFR* rs1801133; *SLC19A1* rs1051266; and *ERCC2* rs1799793 and rs13181. To genotype *ABCB1*, *ABCG2* and *SLC19A1*, we used custom-designed assays. To genotype *ERCC1*, *MTHFR* and *ERCC2*, we used predesigned assays. Additionally, *GSTP1* rs1695, *TP53* rs1042522 and *XRCC1* rs25487 were determined using the Pyrosequencer 96MA™ (Isogen, The Netherlands). The pyrosequencing reactions were performed according to the manufacturer's protocol. PCR primers, target sequences and the calculated dispensation orders for each SNP are listed in Table 1. Note that the lowercase nucleotides in the dispensation order are negative controls, which would not be incorporated

into the target DNA and consequently should not appear in the pyrogram.

The technicians performing the analyses were blinded with respect to sample identity. As a quality control in the pyrosequencing and TaqMan assays, at least 5% of samples were genotyped in duplicate, and water was used as a negative control. No inconsistencies were observed. In case of discrepancies between genotypes in tumor and blood DNA, the discordant pair was reanalyzed in one run. In this run, we included five randomly selected samples and water as controls.

SNP	Target#	Sequence 5'-3'	Modification
GSTP1 rs1695	PCR-f	AGGACCTCCGCTGCAAATAC	Biotin
	PCR-r	CTGGTGCAGATGCTCACATAGTT	
	Sequence primer-f	CTCCGCTGCAAATAC	
	Target Sequence	A/GTCTCCCTCAT	
	Dispensation order	cAGaTCTCT	
TP53 rs1042522	PCR-f	GAAGACCCAGGTCCAGATGAAG	Biotin
	PCR-r	CCGGTGTAGGAGCTGCTGG	
	Sequence primer-r	GGTGCAGGGGCCACG	
	Target Sequence	C/GGGGGAGCAGCCT	
	Dispensation order	tGCGcAGCAG	
***XRCC1* rs25487**	PCR-f	TAAGGAGTGGGTGCTGGACTGTC	Biotin
	PCR-r	CAGGGTTGGCGTGTGAGG	
	Sequence primer-r	CGTGTGAGGCCTTACC	
	Target Sequence	TCC/TGGGAGGGCA	
	Dispensation order	gTCTcGAGC	

Table 1. Primers and probes for pyrosequence analysis and fragment length analysis
f = forward orientated, r = reverse orientated

Statistical analysis

Concordance of genotypes determined in DNA from EDTA–blood and FFPE samples was tested using the k-statistic, which tests the agreement between two paired results. A k-value larger than 0.95 was considered good agreement. We calculated 95% confidence intervals (95% CI) of the k-statistic. The null hypothesis was that there is no difference in genotyping results due to source of DNA, corresponding to a k of 1.00. The two-sided significance level was set at $p < 0.05$. All statistical analyses were performed using SPPS software, version 20.0 (SPSS, Inc., IL, USA).

Gene	Genetic variation	rs No.	Chrom	% success in blood	Genotype distribution in blood (%)	MAF in blood	% success in FFPE	Genotype distribution in FFPE (%)	MAF in FFPE
ABCB1	C1236T, Gly412Gly	1128503	7q1	95.3%	17.6-50.0-32.4	0.426	91.9%	18.2-48.9-32.8	0.427
ABCB1	T3435C, Ile1145Ile	1045642	7q1	96.0%	28.0-48.3-23.8	0.479	96.0%	28.7-46.2-25.2	0.483
ABCG2	C421A, Lys141Gln	2231142	4q21	94.6%	66.7-32.6-0.7	0.170	95.3%	69.7-30.3-0.0	0.154
ERCC1	T496C, Asn118Asn	11615	19q13.3	91.9%	39.4-48.9-11.7	0.361	96.6%	40.3-47.9-11.8	0.358
ERCC2	A2251C, Lys751Gln	13181	19q13.3	97.3%	35.9-49.7-14.5	0.393	85.9%	41.4-41.4-17.2	0.379
ERCC2	G965A, Asp312Asn	1799793	19q13.3	91.9%	41.6-48.2-10.2	0.343	91.3%	44.1-42.6-13.2	0.346
GSTP1	G342A, Ile105Val	1695	11q13	100%	38.3-53.0-8.7	0.352	89.9%	44.0-48.5-7.5	0.317
MTHFR	C677T, Val222Ala	1801133	1p36.3	87.2%	42.3-50.8-6.9	0.382	91.9%	43.1-47.4-9.5	0.332
TP53	G466C, Arg72 Pro	1042522	17p13	100%	45.6-48.3-6.0	0.302	77.2%	49.6-44.3-6.1	0.283
SLC19A1*	G80A, Arg27His	1051266	21q22	99.3%	33.8-50.0-16.2	0.412	85.2%	31.5-51.2-17.3	0.429
XRCC1	G1301A, Arg399Gln	25487	19q13.3	100%	34.2-55.7-10.1	0.379	94.6%	35.5-54.6-9.9	0.372

Table 2. Genotype results

* Also denominated reduced folate carrier (RFC). MAF, minor allele frequency; FFPE, formalin-fixed paraffin embedded tissue.
Genotype distributions are shown as: homozygous wildtype-heterozygous-homozygous mutant.

Results

Genotyping results

Both EDTA–blood and FFPE tissue samples were available for 149 patients with metastatic CRC who participated in the CAIRO trial. Paraffin tissue and peripheral blood genotypes were determined for a total of 11 SNPs from nine chromosomal regions. Depending on genotype, results were obtained in 87–100% of peripheral blood samples; FFPE tissue-derived genotypes were obtained in 77–97% of samples. Paired results for FFPE tissue and peripheral blood genotype were obtained for 77–95% of patients, depending on the studied SNP. Genotype distributions were in accordance with previously published results.[9;23–25](Table 2) All genotypes were in Hardy–Weinberg equilibrium, with the exception of *XRCC1* (both in EDTA–blood and FFPE tissue; $\chi^2 = 4.999$, p = 0.025 and $\chi^2 = 3.997$, p = 0.046, respectively), *GSTP1* (EDTA–blood only; $\chi^2 = 3.896$, p = 0.048) and *ABCG2* (FFPE tissue only; $\chi^2 = 4.520$, p = 0.034).

Gene	rs No	Number of evaluable pairs (% of 149 pairs)	No of discordant pairs (%[#])	κ statistic	95% Confidence interval[$]
ABCB1	1128503	131 (88%)	3 (2.3%)	0.963	0.921-1.00
ABCB1	1045642	137 (92%)	0 (0%)	1.00	-
ABCG2	2231142	134 (90%)	0 (0%)	1.00	-
ERCC1	11615	132 (89%)	2 (1.5%)	0.975	0.940-1.00
ERCC2	13181	124 (83%)	2 (1.6%)	0.974	0.914-1.00
ERCC2	1799793	124 (83%)	3 (2.4%)	0.960	0.915-1.00
GSTP1	1695	134 (90%)	4 (3.0%)	0.947	0.896-0.998
MTHFR	1801133	120 (81%)	2 (1.7%)	0.971	0.932-1.00
TP53	1042522	115 (77%)	2 (1.7%)	0.969	0.926-1.00
SLC19A1*	1051266	126 (85%)	1 (0.8%)	0.987	0.961-1.00
XRCC1	25487	141 (95%)	1 (0.7%)	0.987	0.961-1.00

Table 3. Concordance of genotypes between peripheral blood and formalin-fixed paraffin-embedded tissue

* Also denominated: Reduced folate carrier (RFC). [#] Percentage of evaluable pairs that is discordant.
[$] Ninety-five percent confidence interval for κ statistic.

Concordance of EDTA–blood & FFPE genotype

We found an excellent agreement between the peripheral blood genotypes and the genotypes determined in corresponding FFPE samples.(Table 3) With the exception of *GTSP1* rs1695 (95% CI for k-statistic: 0.896–0.998), all SNPs showed an agreement between samples that was not significantly different from 100%. The 95% confidence levels of genotype concordance ranged from 0.961–1.000 (*XRCC1* rs25487) to 0.914–1.000 (*ERCC2* rs1799793).

Description of discordant results

In total, 20 out of 1418 (1.4%) genotype pairs in 18 patients were discordant in our sample set.

For each individual SNP, no more than 3.0% of pairs showed dissimilar results. Table 4 shows the genotypes found in peripheral blood and FFPE tissue for all discordant pairs. A potential source of discordance is LOH. We assessed LOH using heterozygous genotypes from adjacent loci. Because LOH involves larger stretches of DNA, loss of an allele for one SNP should be accompanied by loss of an allele for neighboring loci.[26] Using this approach, the number of mismatches potentially due to LOH was reduced to ten, including four sample pairs for *GSTP1* in which LOH could not be ruled out.

SNP	Subject No.	Genotype blood	Genotype FFPE	FFPE genotype in adjacent locus		Difference caused by LOH?
				Adjacent locus		
***ABCB1* rs1128503**	1	CC	TT	-	-	No
	2	CC	CT	-	-	No
	3	CT	CC	*ABCB1* rs1045642	missing	Possible
***ERCC1* rs11615**	4	TC	TT	*XRCC1* rs5487	heterozygous	No
	5	TC	CC	*ERCC2* rs13181	heterozygous	No
***ERCC2* rs13181**	6	AC	AA	*ERCC2* rs1799793	missing	Possible
	7	AC	AA	*XRCC1* rs5487	heterozygous	No
***ERCC2* rs1799793**	8	GA	GG	*ERCC2* rs13181	missing	Possible
	5	GA	AA	*ERCC2* rs13181	heterozygous	No
	9	GA	AA	*ERCC2* rs13181	heterozygous	No
***GSTP1* rs1695**	10	AG	AA	-	-	Possible
	11	AG	AA	-	-	Possible
	12	AG	AA	-	-	Possible
	13	AG	AA	-	-	Possible
***MTHFR* rs1801133**	2	CT	CC	-	-	Unlikely*
	14	CT	CC	-	-	Possible
***TP53* rs1042522**	15	GC	CC	-	-	Possible
	16	CC	GC	-	-	No
***SLC19A1* rs1051266**	17	AA	GA	-	-	No
***XRCC1* rs25487**	18	GA	AA	*ERCC2* rs13181	heterozygous	No

Table 4. Exploration of discordant pairs on the basis of genotype in adjacent loci

* Mix-up of samples is likely to have happened, given the genotyping results for ABCB1 rs1128503 for this patient.

LOH: loss of heterozygosity; Possible: loss of heterozygosity cannot be determined due to missing genotypes and/or no adjacent loci were genotyped. Other explanations for discordant pairs include patient mix-up, sample mix-up, genotype errors.

Discussion

Although concordance of genotypes between colorectal tumor and adjacent normal mucosa has been studied by others, this is the first study in which the results of an extended set of genotypes were compared between peripheral blood DNA and archived FFPE tissue DNA from patients with metastatic CRC. The results of our study show that genotyping using material from FFPE tissue and EDTA–blood yields highly concordant results. Consequently, this implies that the findings from retrospective trials using archived FFPE tissue can be reliably compared with studies using peripheral blood leukocytes as the DNA source for a considerable number of SNPs.

Although for most SNPs no significant discordance between DNA derived from EDTA–blood and FFPE tissue was found, a small discrepancy was found for *GSTP1*. This is of particular interest because of the possible role of *GSTP1* in carcinogenesis. Knockdown experiments with a CRC cell line showed that GSTP1 function is essential for *in vivo* growth of xenografts[27] and GSTP protein levels are frequently increased in colon cancer tissue.[28] The enzyme is part of the JNK pathway and as such is involved in cell cycle regulation and apoptosis.[29;30] The *GSTP1* rs1695 SNP leads to an amino acid substitution (Ile105Val) that lies within a JNK protein binding site, and may therefore have functional implications in carcinogenesis.[31] Despite the high concordance rate between blood-derived and FFPE tissue-derived DNA, we cannot exclude the possibility that LOH occurs for this gene. Therefore, use of FFPE tissue from resection specimens for genotyping this SNP in pharmacogenetic association studies is possibly not advisable. Additionally, genotyping for *TP53* failed in 23% of our FPPE tissue samples, thereby indicating that this SNP is also less suitable for genotyping in archived tumor tissue.

Only a few studies have compared genetic variations in malignant and normal tissues, and most did not include CRC patients. Marsh and coworkers compared genotypes of tumor and adjacent normal tissue in fresh-frozen tumor samples of 44 CRC patients for 28 polymorphisms in 13 genes.[32] Overall, 13 out of 1139 genotypes (1.1%) showed discordant results, similar to our findings. By contrast, Le Morvan and colleagues found considerable LOH in FFPE colorectal tumor samples for *GSTP1* (five out of 25 samples) and *ERCC2* (14 out of 32 samples).[16] These authors did not elaborate on the methods used for detecting LOH in their samples. A third study compared genotypes in EDTA–blood and FFPE rectal tumor samples for seven genes, including *GSTP1*, and found extensive discordance, with only 14 out of 65 sample pairs showing no discrepancies.[33] Explanations for these contrasting results include different rates of genotyping success and different tumor characteristics in colon and rectal tumors.

In a recent debate, the concordance between germline and tumor genotype has been questioned when using tumor samples in the determination of *CYP2D6* genotype for breast cancer patients in large association studies of tamoxifen efficacy.[34–37] Significant deviation from the Hardy–Weinberg equilibrium in tumor material was occasionally found for *CYP2D6* genotypes, possibly due to hemizygous chromosomal deletions. According to some authors,

this should preclude studies with pharmacodynamic end points using tumor material as a source of DNA.[34]

Indeed, LOH may explain discordance between genotype in DNA from EDTA–blood and FFPE tissue samples. However, in our study, we excluded LOH in ten out of 20 discordant cases, using heterozygous alleles in adjacent loci as a marker for chromosomal loss. This technique does not exclude hemizygous loss of very short chromosomal regions. Nevertheless, we believe that this is a valid method for evaluating the presence of LOH in our sample set, because LOH in colorectal carcinogenesis is thought to involve large stretches of chromosomal material in most cases.[38] Minimal presence of LOH was also expected, since most of these SNPs have not been convincingly associated with colorectal carcinogenesis and therefore selection of somatic mutations in these genes is unlikely.

For cases in which LOH could not be excluded, copy number gain of one allele could also explain discordant results, as the strong signal from the amplified allele would obscure the signal from the other allele. However, copy number amplification for the selected genes is not a common event in colorectal carcinogenesis, and is therefore a less likely cause of discordance. Alternative explanations for discordance include patient mix-up, sample mix-up or repeated genotyping errors. Reanalyzing all discordant pairs reduced the chance that disagreements were induced by genotyping errors or sample mix-up, but does not rule out patient mix-up or technical problems in earlier stages of sample preparation. However, assuming that only one result in every discordant pair is incorrect, we observed a maximum error rate of 0.7% for all samples (20 out of 2836 assays). This is likely to reflect the actual practice in clinical trials, which, in our opinion, is an acceptable level of inaccuracy.

The almost complete concordance of genotypes between blood-derived and FFPE tissue-derived DNA most likely reflects the actual absence of LOH in our tumor samples. However, our method may be hampered by the presence of stromal cells in our FFPE samples. In the presence of large amounts of non-cancerous stromal cells, FFPE tissue-derived genotypes may actually reflect germline genotypes, rather than tumor genotypes. Techniques for DNA extraction from FFPE tissues have been optimized in recent years and the use of microdissection instead of macrodissection would presumably have reduced the amount of stromal contamination in our samples. By contrast, most previously published articles have used macrodissection as the technique for DNA extraction.[7–19] It was our aim to confirm that results from these studies can be reliably compared with those from studies using blood-derived DNA for genotyping. Whether the concordance when using macrodissection is a reflection of a large stromal component or of actual agreement between the germline and tumor genotype is therefore only of theoretical importance.

We showed excellent concordance for all studied genetic variations. Unfortunately, it is unknown whether this concordance can be extrapolated to other genes or genetic variations of interest, or other types of cancer. The current study does not account for other genetic variations, such as amplification, methylation and copy number variation, which could be pharmacogenetically relevant. Taking these limitations into consideration, our results show

that FFPE tissue-derived DNA can be used as a valid proxy for germline DNA in CRC when blood samples are not available. However, even with the expanding possibilities of DNA collection from archived material, we believe peripheral blood should be the preferential source of DNA for future prospective pharmacogenetic studies.

Future perspectives

The field of pharmacogenomics is rapidly evolving. Most oncological clinical trials now include pharmacogenetic side-studies, for which peripheral blood samples are routinely collected. The use of archived FFPE tumor tissue will therefore be limited to retrospective studies and pharmacodynamic endpoints.

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