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ORIGINAL ARTICLE

Functional study of the 830C>G polymorphism of the human carboxylesterase 2 gene

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Abstract
Purpose Carboxylesterase 2 (CES2) is involved in the activation of the anticancer drug irinotecan to its active metabolite SN-38. We previously identified a single nucleotide polymorphism (SNP), with an allele frequency around 10%, as possibly involved in enzyme expression (Clin Pharmacol Ther 76:528–535, 2004), which could explain the large individual variation in SN-38 disposition.
Methods The 830C>G SNP, located in the 5′ untranslated region of the gene, was analysed in various DNA samples extracted from: (1) the National Cancer Institute NCI-60 panel of human tumour cell lines; (2) a collection of 104 samples of normal tissue from colorectal cancer patients; (3) blood samples from a population of 95 normal subjects; (4) a collection of 285 human livers. *CES2* genotypes were tentatively related to irinotecan cytotoxicity and CES2 expression in the NCI-60 panel; to response to treatment and event-free survival in colorectal cancer patients; and to CES2 expression and catalytic activity in subsets of the human liver collection.
Results No significant relationship was found in the NCI-60 panel between *CES2* 830C>G genotype and irinotecan cytotoxicity or CES2 expression. No significant relationship was found between *CES2* 830C>G genotype and the toxicity and therapeutic efficacy (tumour response, event-free survival) of irinotecan in colorectal cancer patients. There was no significant relationship between *CES2* 830C>G genotype and CES2 expression and catalytic activity determined in a subset of genotype-selected liver samples.
Conclusion The 830C>G SNP of *CES2* is unlikely to have significant functional consequences on CES2 expression, activity or function.

Keywords Carboxylesterase 2 · Irinotecan · NCI-60 · Colorectal cancer · Polymorphisms · Pharmacogenetics

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Introduction

Carboxylesterase 2 (CES2) is a drug-metabolising hepatic and intestinal enzyme that has been shown to be responsible for the activation of irinotecan, a major drug for the treatment of advanced colorectal cancer patients [1, 2]. There is a large interindividual variability in the plasma levels of SN-38, the activation product of irinotecan, in patients treated with irinotecan, which could be attributed, among other causes, to variability in CES2 activity. In a prior study on CES2 activity in human liver microsomes [3], a fourfold intersubject variation in the rate of SN-38 formation from irinotecan was noticed. In addition, irinotecan undergoes in vivo transformation to several species via the action of cytochrome P450 3A4 [4], which may also explain the interindividual variability of SN-38 formation via variations of irinotecan availability for activation by carboxylesterases.

Drug-metabolising enzymes are often subject to gene polymorphisms which alter protein expression or catalytic

activity. The question has been raised of whether common *CES2* gene polymorphisms could explain differences in enzyme availability or intrinsic activity, resulting in phenotypic differences in SN-38 formation and irinotecan efficiency in patients. We have recently explored all 12 exons of the gene as well as the 5′ and 3′ untranslated domains, and we identified a total of 11 single nucleotide polymorphisms (SNPs) which were present in a population consisting of 115 normal subjects mainly from Caucasian origin [5]. None of them altered the amino-acid sequence of the protein, but one of them, located at position 830 of the gene (171 nucleotides before the translation initiation codon), was associated with a trend to lower gene expression in heterozygous samples of a set of 60 human liver extracts obtained from a North-American population. This trend was, however, non-significant ($P = 0.2$), possibly because of the small size of the population studied and the relatively low allele frequency for this variation (around 10%), leading to the absence of variant homozygous samples in the population studied. This SNP is the one also identified by Wu et al. [6] and Marsh et al. [7] with the same allele frequency, and located 363 nucleotides before the translation initiation codon. According to the most recent release from the International Human Genome Sequencing Consortium, the translation initiation codon is now located 192 nucleotides upstream from the original one, which explains the difference in nomenclature.

In order to determine whether this SNP had functional consequences, we developed several approaches aimed at correlating the polymorphism with the phenotype. We first identified the 830C>G *CES2* genotype in the 60 human tumour cell lines of the panel of the National Cancer Institute (NCI-60) and tentatively correlated the presence of the polymorphism with the in vitro cytotoxicity of irinotecan as extracted from the NCI database. The intra-tumour activation of irinotecan remains uncertain, some authors finding that CES2 activity was predictive of irinotecan cytotoxicity [8] while others failed to detect CES2 activity in cultured cells [9]. We then identified this polymorphism in a total of 104 colorectal cancer patients, 46 of whom were treated with a drug combination containing irinotecan. Finally, we studied a collection of 285 human liver DNA extracts and, after selection of a subset of extracts with a given genotype, we evaluated CES2 gene expression and enzyme activity in the corresponding samples.

Materials and methods

Human tumour cell lines

Frozen cell pellets from 59 of the 60 NCI cell lines of the panel were kindly provided by Dr S. Holbeck, Cancer Therapeutic Branch, NCI, Bethesda, USA. One cell line, MDA-N, is no longer available in the NCI panel. Genomic DNA was extracted from cell pellets using QIAamp® DNA minikit from Qiagen and quantified by spectrophotometry.

Colorectal cancer patients

A total of 104 patients were selected from colorectal patients' files of Institut Bergonié, Hôpital Saint-André and Hôpital Haut-Lévêque (all from the Bordeaux area) upon the following criteria: palliative first-line treatment with oxaliplatin or irinotecan combined with folinic acid-modulated 5-FU or capecitabine; availability of all clinical data in the files; availability of paraffin-embedded non-tumour tissue in the pathological archives of the institutions where the tumour had been surgically removed. Patients had been diagnosed and treated between February 1999 and May 2004; all of them but seven were dead at the time of the study; none of them had made any reserve against the scientific use of the tumour specimens which had been obtained for pathological examinations. They had been treated every 2 weeks either with the FolFox regimen (5-fluorouracil 400 mg/m^2 i.v. bolus followed by 2,400 mg/m^2 infusion over 48 h + leucovorin 400 mg/m^2 mg and oxaliplatin 100 mg/m^2, 58 patients) or with the FolFiri regimen (5-fluorouracil + leucovorin, same as above, + irinotecan 180 mg/m^2, 46 patients). They had been followed regularly all along the evolution of the disease and CT scans had been performed every 2 months for evaluation of drug response and event-free survival. The study protocol, conducted in accordance with the Declaration of Helsinki, was submitted to and approved by the institutional ethics committee for clinical research.

Paraffin blocks obtained after surgical removal of the tumours and associated nodes were supplied for each patient by the Pathology department where they were archived; those containing only non-tumour tissue were selected for this study.

DNA extraction from paraffin embedded tissues

For paraffin elimination, each group of 10–20 tissue slices in paraffin were treated with xylene at 65°C under agitation and pelleted at 2,000 g for 5 min. Slices were then washed twice with absolute ethanol and treated five times with lithium carbonate to eliminate the fixative. Before digestion, the slices were washed in TNE 1×buffer (Tris–EDTA–NaCl 100 mM, pH 8) and kept in 500 µl of this buffer. Tissue digestion was then performed by adding 50 µl of proteinase K at 20 mg/ml to the 500 µl of TNE 1× buffer containing the paraffin-free slices. They were then incubated at 55°C overnight until complete digestion. Genomic DNA was then purified from the proteinase K digest on

GFX columns (Amersham-Biosciences). The amount and quality of the eluted genomic DNA was controlled on an agarose gel electrophoresis.

Control population

A blood sample from a population of 95 blood donors from the Bordeaux area was obtained from the Établissement Français du Sang Aquitaine-Limousin. DNA was extracted using the Qiagen kit following manufacturer's instructions.

Human liver samples

Liver tissue samples used in this study had been previously collected from patients of Caucasian European origin undergoing liver surgery for various reasons at the Department of General, Visceral and Transplantation Surgery, Campus Virchow, University Medical Center Charité, Humboldt University in Berlin, Germany (kindly provided by Drs Natascha Nüssler and Andreas K. Nüssler). Only normal liver tissue was collected, stored at −80°C, and absence of tumour material had been confirmed by histochemical analysis as previously described [10]. The study was approved by the ethics committee of the Medical Faculty of the Charité, Humboldt University, Berlin, and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient. Hepatic microsomal and cytosolic protein fractions were prepared according to standard procedures as described [11]. Genomic DNA was isolated from corresponding blood-samples of 285 liver donors by using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). RNA was isolated from corresponding liver tissue using RNeasy Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Following genotyping as described below, a subset of 26 samples with different genotypes was selected for further functional measurements.

Polymerase chain reaction

Polymerase chain reactions (PCR) were performed on genomic DNA in 50 µl reaction mixture containing 5 units of AmpliTaq Gold (Applied Biosystems), 10 mM of each dNTP, 1× *Taq* polymerase buffer, 100 ng genomic DNA and 10 µM of each primer. Amplification was done using a reverse primer with a biotin label in 5′. Sequences were as follows: forward primer 5′-CTCCTGGGGTCTCCAATTCT-3′; reverse primer 5′-Biotin GAAAGGTGGGTGTGGTAGGA-3′. After initial denaturation at 95°C for 5 min, 35 amplification cycles were performed, each consisting of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C, with a terminal elongation at 72°C for 2 min. The PCR products were purified and desalted using a Millipore PCR desalting plate (Millipore) according to manufacturer's protocol.

Genotyping

NanoChip™ cartridges harbouring microelectronic 100-test site arrays, obtained from Nanogen Europe BV (Helmond, The Netherlands), were processed on the Molecular Biology Workstation (Nanogen Europe BV). Reference DNA and test PCR products were both supplemented with 50 mM histidine and addressed for 120 s at 1.9 V on each pad and then denatured 180 s with NaOH 0.3 N.

For hybridisation, the complementary oligonucleotides were labelled with Cy3 for the wild type allele (CES2-WT discriminator: 5′-CTGAGTCCGAACATTGAGTTCCCTCCTATCGATC-3′) and with Cy5 for the variant allele (CES2-MUT discriminator: 5′-GCAGTATATCGCTTGACATTCCCTCCTATCGATG-3′). An oligonucleotide stabiliser was used to increase the specificity of the discrimination system (stabiliser: 5′-CCCCAGCGCGCTCATCGGGCCTG-3′). All oligonucleotides were diluted to 100 nM in 50 mM histidine. The mixtures of complementary and stabiliser oligonucleotides were incubated on the cartridge for 3 min at room temperature. The chip surface was washed with HSB buffer (Tris–HCl 20 mM, pH 7.6, KCl 50 mM, $MgCl_2$ 5 mM, Tween 20 0.1%). For SNP analysis, the fluorescence of Cy3 and Cy5 was scanned on each pad and data were determined in the fluorescence analyser.

Quantitative RT-PCR

We quantified *CES2* relative mRNA amounts in human liver RNA extracts. RNAs were first reverse-transcribed to cDNA using random primers and Superscript III reverse transcriptase (Invitrogen, La Jolla, CA, USA). A 20-µl reaction volume was used for 500 ng of total RNA, 50 ng of random primers, 10 mM of each dNTP, 1× first-strand buffer, 0.1 M dithiothreitol, 40 units RNase OUT Recombinant RNase Inhibitor and 200 units Superscript III reverse transcriptase. The reaction volume was incubated at 50°C for 60 min and inactivated by heating at 70°C for 15 min. The specific primers for the *CES2* promoter were designed with the Primer 3 software (forward 5′-TCTGGGGGATCCTGAACG-3′; reverse 5′-GACTGGGCAGGGAGTCAG-3′). Amplification was performed in a 25 µl reaction volume containing 5 units of AmpliTaq Gold (Applied Biosystems), 10 mM of each dNTP, 1× *Taq* polymerase buffer, 100 ng genomic DNA, 10 µM each primer supplemented with 1 µl Sybr Green I diluted to 1/1,000 (Interchim, Montluçon, France). The PCR reaction was performed using a real-time PCR device (Rotor Gene, Corbett Research, Australia).

Two independent experiments were performed using reference genes which were assumed to be expressed at constant levels in liver: adaptor-related protein complex 2 mu 1 subunit (*AP2M1*) and Interleukin enhancer binding factor 2 (*ILF2*) in the first experiment, and ribosomal protein L32 (*RPL32*) and *ILF2* in the second experiment. Data are expressed as ratios of mRNA expression in samples from variant genotype vs. mRNA expression in samples from common genotypes, using the REST software from Corbett Life Science [12].

Carboxylesterase 2 activity

Since CES2 appears as the principal enzyme for irinotecan activation into SN-38 [2], the rate of this conversion was chosen as representative of CES2 activity. It was evaluated on two human liver subcellular fractions, microsomes and cytosol. Incubations were performed with 310 µg proteins in 320 µl of reaction mixture. The reaction was initiated by the addition of 10 µl of 25-µmol/l irinotecan and was performed for 1 h at 37°C. Aliquots of 37.5 µl were taken every 15 min for SN-38 estimation, as previously described [13]. These aliquots were added to 75 µl of a methanol/acetonitrile (1:1) mixture containing 1% hydrochloric acid and 20 ng camptothecin (internal standard). After vortex blending and centrifugation, 100 µl of the supernatant was injected onto the chromatograph. An isocratic high performance liquid chromatography method was employed according to Escoriaza et al. [14] with a Nova-Pak C18 column (Waters Associates, Milford, MA, USA) and a mobile phase consisting of 0.1 M phosphate buffer (pH 4.2)/acetonitrile (70:30). Fluorometric detection was performed with excitation and emission wavelengths of 228 and 540 nm, respectively. The chromatographic system was from Thermo-Quest (Les Ulis, France). Results were expressed as picomoles of SN-38 formed per milligram of protein per minute.

Statistical analysis

The Fisher exact test was used for comparing genotype distributions in all the populations studied. Relative mRNA amounts were compared using the REST software [12]. Contingency tables and Fisher's exact test were used for descriptive analyses relating treatment toxicity (regrouped as grades 0–1–2 and grades 3–4 according to NCI-CTC criteria), treatment response (defined as objective response, stable disease or progression according to WHO criteria), and the *CES2* polymorphism as a function of the treatment received. Event-free survival (EFS) was calculated from the time that a patient started treatment until progression or treatment failure or toxicities or death; patients who still responded to first line treatment at last follow up were censored at that time. The log-rank test and Kaplan–Meier plots were used to evaluate association of EFS with *CES2* genotype. Median survival was calculated based on Kaplan–Meier estimator. All statistical testing was conducted at the $P < 0.05$ level and SPSS software (version 12.0) was used.

Results

Human tumour cell lines

Among the 59 human tumour cell lines available in the NCI panel, we identified six cell lines which were heterozygous for the 830C>G *CES2* variation and three which were variant homozygous, all the other one being common homozygous (Table 1). This corresponds to an allele frequency of 10%. The genotype distribution significantly differed from the Hardy–Weinberg distribution ($P = 0.0030$, Fisher exact test). It is remarkable that three of the six heterozygous and one of the three variant homozygous cell lines were of colorectal cancer origin, giving a variant allele frequency of 35% in this tumour type whereas this frequency varied between 0 and 16% in all other tumour types. This distortion appeared significant using the Fisher's exact test ($P = 0.0056$). When extracting the cytotoxicity data of irinotecan from the NCI data base (http://dtp.nci.nih.gov), no correlation appeared between the IC_{50} values of the cell lines according to their genotype. The mean IC_{50} of irinotecan, expressed as $-\log(IC_{50})$, was 4.91 ± 0.51 in the common homozygous cell lines, 4.67 ± 0.50 in the heterozygous cell lines and 4.75 ± 0.20 in the variant homozygous cell lines. The expression of *CES2* was also extracted from the NCI database, the results of two experiments of gene profiling being accessible in this database. No significant relationship between the presence of the polymorphism and *CES2* gene expression could be evidenced (Table 2).

Colorectal cancer patients and control population

Among the 104 patients studied, 58 had received a combination of folinic acid-modulated 5-fluorouracil and oxaliplatin (FolFox regimen), 5-fluorouracil being replaced in two cases by capecitabine and in six cases by raltitrexed, and 46 a combination of folinic acid-modulated 5-fluorouracil and irinotecan (FolFiri), irinotecan being administered alone in one case. The group of patients receiving oxaliplatin and no irinotecan was included as a control group in order to distinguish any effect of the polymorphism on response or survival which would not be related to irinotecan metabolism. When considered first all together, we found in the 104 colorectal cancer patients specimens four variant

Table 1 Polymorphism of the 830C>G *CES2* genes found in the NCI-60

Tumour type	Cell line	*CES2*
Leukaemia	CCRF-CEM	C
	HL-60	C
	K-562	C
	MOLT-4	C
	RPMI-8226	HT
	SR	C
Lung cancer	A549/ATCC	C
	EKVX	C
	HOP-62	C
	HOP-92	C
	NCI-H226	C
	NCI-H23	C
	NCI-H322M	C
	NCI-H460	C
	NCI-H522	V
Colon cancer	COLO-205	HT
	HCC-2998	HT
	HCT-116	C
	HCT-15	HT
	HT29	V
	KM12	C
	SW-620	C
Central nervous system	SF-268	C
	SF-295	C
	SF-539	C
	SNB-19	C
	SNB-75	C
	U251	C
Melanoma	LOXIMVI	C
	MALME-3M	HT
	M14	C
	SK-MEL-2	C
	SK-MEL-28	C
	SK-MEL-5	C
	UACC-257	C
	UACC-62	HT
Ovarian cancer	IGROV1	C
	OVCAR-3	C
	OVCAR-4	V
	OVCAR-5	C
	OVCAR-8	C
	SK-OV-3	C
Renal cancer	786-0	C
	A498	C
	ACHN	C
	CAKI-1	C
	RXF-393	C
	SN-12C	C
	TK-10	C
	UO-31	C
Prostate cancer	PC-3	C
	DU-145	C
Breast cancer	MCF-7	C
	NCI/ADR-RES	C
	MDA-MB-231	C
	HS578T	C
	MDA-MB-435	C
	BT-549	C
	T-47D	C

C common homozygous, *V* variant homozygous, *HT* heterozygous

homozygous and nine heterozygous samples for the 830C>G variation in the *CES2* gene (allele frequency = 8.2%). In comparison, in the control population of 95 healthy blood donors, we found one variant homozygous and 19 heterozygous subjects (allele frequency = 11.0%). Using the Fischer's exact test, we observed that the distribution of genotypes in the colorectal cancer patients samples was significantly different from what could be expected from the Hardy–Weinberg distribution ($P = 1 \times 10^{-4}$), whereas this was not the case for the control population. We then analysed separately the toxicity, response to treatment and event-free survival of patients receiving either treatment, as a function of their *CES2* genotype. There was a consistent difference in the severity of toxic symptoms in patients receiving oxaliplatin- or irinotecan-based treatments, but this was the same for all genotypes. No significant difference appeared in response rate or event-free survivals in either group of patients. We analysed matched tumour samples available in 78 patients (39 treated with oxaliplatin and 39 treated with irinotecan). No new mutation occurred in the tumour samples at the locus studied. In four cases, the tumours of patients identified as heterozygous in the normal tissue appeared to only bear the common allele. This can be interpreted as a loss of heterozygosity, with the special feature that the variant allele was the only one to be lost. The small number of discordances between normal and tumour tissue did not allow to conclude on the clinical outcome of this loss of heterozygosity.

Human liver samples

In the 285 DNA extracts of the human liver collection, we identified two variant homozygous and 44 heterozygous

Table 2 Response to treatment and event-free survival according to the 830C>G *CES2* genotype, as a function of the drug combination received

	TS inhibitor + Oxaliplatin (58 patients)	TS inhibitor + Irinotecan (46 patients)
Toxicity of the treatment (numbers of patients with grade 3–4/grade 0–1–2)	33/25	37/9
Common homozygous patients (91 patients)	30/21	32/8
Heterozygous patients (nine patients)	2/2	4/1
Variant homozygous patients (four patients)	1/2	1/0
Response to treatment (numbers of patients with progression/stable disease/objective response)	8/17/33	6/18/22
Common homozygous patients (91 patients)	7/17/27	5/16/19
Heterozygous patients (nine patients)	0/0/4	1/2/2
Variant homozygous patients (four patients)	1/0/2	0/0/1
Event-free survival (months)	9.14 (6.10–12.2)	11.0 (7.62–14.4)
Common homozygous patients	7.88 (5.03–10.8)	9.44 (5.96–12.9)
Heterozygous patients	9.93 (5.70–14.2)	12.4 (0–27.8)
Variant homozygous patients	13.1 (0–30.7)	11.6

Response to treatment and event-free survival were compared in the two groups of patients and as a function of genotype, using the Fisher's exact test and the log-rank test, respectively. No parameter was significantly different when comparing genotypes or when comparing treatment groups

samples for the 830C>G variation in the *CES2* gene (allele frequency = 8.4%). The distribution was in agreement with the Hardy–Weinberg distribution. We selected randomly 12 common homozygous and 12 heterozygous subjects, in addition to the two variant homozygous subjects, for quantitative analysis of CES2 expression and enzyme activity. Due to the fact that we had only two variant homozygous samples, they were combined with heterozygous samples as having at least one variant allele. Table 3, Table 4 shows that CES2 expression, evaluated by real-time RT-PCR in two independent experiments, was not significantly different in samples from common genotypes and samples from variant genotypes ($P = 0.639$ and 0.160 in the two experiments). The carboxylesterase-mediated catalytic activity of irinotecan biotransformation into SN-38 was studied in both microsomal and cytosolic fractions. No activity was observed in the cytosolic fractions, confirming the membrane localisation of CES2. The formation of SN-38 was not significantly different in microsomal fractions originating from common homozygous, heterozygous and variant homozygous samples. We did not observe on the chromatograms the presence of any metabolite other than SN-38; especially, there was no detectable formation of SN-38 glucuronide from SN-38 and no formation of APC or NPC from irinotecan in the conditions of incubation we have used.

Discussion

There is a substantial individual variability in SN-38 concentrations in human plasma after irinotecan administration. In an early study conducted in our laboratory [15], the relative extent of conversion of irinotecan to SN-38 varied from 0.01 to 0.11, and this was confirmed in subsequent studies [16]. Enzymes involved in drug metabolism often bear gene polymorphisms with functional consequences [17], which explain a large part of the individual variability of drug response and toxicity. Concerning the availability of SN-38 in patients treated with irinotecan, a first identified cause of variability lies in the gene polymorphism that affects the enzyme in charge of SN-38 detoxification, UDP-glucuronosyl transferase 1A1 (UGT1A1), as shown by Iyer et al. [18]. This polymorphism affects the TATA box of the *UGT1A1* gene, with reduced conjugation activity in about 20% of subjects, and is known as Gilbert's syndrome. Another cause of variability could be associated with the important individual differences that exist in CYP3A4 levels in liver, but this enzyme is affected by relatively rare polymorphisms and its variations are rather due to environmental causes. Finally, the gene polymorphisms affecting the enzyme in charge of SN-38 formation, CES2, could also explain the large interindividual variability in SN-38 disposition. Among the various SNPs we had identified in a previous study [3], only one could be possibly associated with reduced enzyme expression, and was localised in the 5′-untranscribed part of the gene, at position 830, i.e. 171 nucleotides before the translation initiation codon (363 in the earlier releases from the International Human Genome Sequencing Consortium). Several systems were used in this study to carefully evaluate the functional role of this SNP. We explored both normal tissues, as usual for constitutional genotypes, and tumour tissues, since direct irinotecan activation in tumour tissue has been shown to be predictive of drug cytotoxicity [8].

Table 3 mRNA expression of reference genes and *CES2* in RNA extracts of human liver samples

	Gene	Reaction efficiency	Relative expression	*P* value
First experiment	*AP2M1*	0.8125	1.024	0.949
	ILF2	0.8075	0.976	0.949
	CES2	0.7825	0.830	0.639
Second experiment	*RPL32*	0.6745	0.932	0.987
	ILF2	0.7310	1.073	0.972
	CES2	0.6856	0.514	0.160

Results were obtained from real-time RT-PCR experiments as described in Sect. "Materials and methods", using the software REST for data analysis (adapted from [4]). Relative expression indicates the mean expression ratio (mRNA expression in samples from variant genotype vs. mRNA expression in samples from common genotype). Due to the fact that we had only two variant homozygous samples, they were combined with heterozygous samples as having at least one variant allele. *P* indicates the probability that the difference between variant and common samples is only due to chance

Table 4 Carboxylesterase 2 catalytic activity in microsomal fractions from human liver samples, as evaluated by the conversion of irinotecan to SN-38

	SN-38 formation (pmol/mg proteins/min)
Common homozygous (12)	0.13 ± 0.05
Heterozygous (12)	0.13 ± 0.07
Variant homozygous (2)	0.15 ± 0.02

We first confirm that this SNP occurs with an allele frequency of about 10% in Caucasian populations. Using the NCI panel of 60 human tumour cell lines, we observed a significant aggregation of variant alleles in colon cancer cell lines, which was not found when comparing colon cancer patient samples and healthy controls. The variation studied cannot, therefore, be considered as associated with colon cancer. We observed a significant deviation from the Hardy–Weinberg distribution in the panel of human tumour cell lines, probably reflecting loss of heterozygosity at this chromosomal locus (16q22.1). As a consequence, some cell lines may be hemizygous rather than homozygous. This might be the case for at least two cell lines, OVCAR-3 and HL-60, which appear monosomic for the long arm of chromosome 16.

No relationship was found between the presence of the variation, at the heterozygous or homozygous state, and the cytotoxicity of irinotecan vis-à-vis the NCI-60 panel or the expression of *CES2* evaluated by gene expression profiling. The NCI panel has been used for the discovery of relationships between the expression of specific genes and the cytotoxicity of anticancer agents [19, 20], and we recently showed its interest for relating gene polymorphisms to anticancer drug activity [21]. One of the major advantages of this panel is the public availability of several databases, especially the growth inhibitory activity of several hundreds of potential anticancer agents towards the 60 cell lines as well as a large variety of 'molecular targets', including oncogenic mutations in relevant genes and gene expression profiles obtained from DNA microarray experiments. It must be emphasised that, using the values of the NCI databases, no relationship between CES2 expression and irinotecan cytotoxicity could be evidenced. This absence of relationship between CES2 expression and irinotecan cytotoxicity is puzzling, since it had been shown on a set of nine lung cancer cell lines that carboxylesterase activity was a significant determinant of irinotecan cytotoxicity [8]. The relatively poor reliability of gene expression profiling at the time these evaluations were done on the NCI panel may explain the apparent absence of relationship between irinotecan cytotoxicity and *CES2* gene expression. Concerning the 830C>G SNP, it clearly appears that it plays no role in irinotecan cytotoxicity as evaluated with the growth inhibition technique implemented by the NCI.

The availability of a panel of 285 human liver extracts of DNA and of a representative subset of RNA extracts and subcellular fractions from the different genotypes allowed us to determine the functionality of the SNP of interest. Using a technique of real-time RT-PCR, we could not detect a significant difference of CES2 expression according to CES2 830C>G genotypes. Furthermore, the conversion of irinotecan to SN-38 by liver microsomes was not different according to the presence of the SNP considered. Taken together, these results show that the relatively common polymorphism of the *CES2* gene, which was the more likely to affect CES2 function, is devoid of evaluable consequences at the level of CES2 expression, CES2 catalytic activity and irinotecan cytotoxicity. As a consequence, the high individual variability in irinotecan disposition is unlikely to be dependent upon a polymorphism affecting CES2, at least in the Caucasian population we studied. This might not be true in populations of other ethnic origin, since Kubo et al. [22] have described three functionally deficient rare CES2 SNPs in the Japanese population.

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