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Original article

Plasma ceramide, a real-time predictive marker of pulmonary and hepatic metastases response to stereotactic body radiation therapy combined with irinotecan

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ABSTRACT

Background and purposes: Early biomarkers of tumour response are needed to discriminate between responders and non-responders to radiotherapy. We evaluated the ability of ceramide, a bioactive sphingolipid, to predict tumour sensitivity in patients treated by hypofractionated stereotactic body radiation therapy (SBRT) combined with irinotecan chemotherapy.

Materials and methods: Plasma levels of total ceramide and of its subspecies were measured before and during treatment in 35 patients with liver and lung oligometastases of colorectal cancer included in a phase II trial. Cer levels were quantified by LC–ESI-MS/MS and compared to tumour volume response evaluated one year later by CT-scan.

Results: Pretreatment plasma ceramide levels were not indicative of tumour response. Nevertheless, the levels of total ceramide and of its 4 main subspecies were significantly higher at days 3 and 10 of treatment in objective responders than in non-responders. According to Kaplan–Meier curves, almost complete tumour control was achieved at 1 year in patients with increased total ceramide levels whereas 50% of patients with decreased levels experienced an increase in tumour volume.

Conclusions: Total plasma ceramide is a promising biomarker of tumour response to SBRT combined with irinotecan that should enable to segregate patients with high risk of tumour escape.

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Radiation therapy is a common palliative and curative anticancer treatment but its efficacy is limited by intrinsic tumour resistance to radiation [1]. The development of stereotactic body radiation therapy (SBRT) has led to better tumour targeting and to the delivery of higher radiation doses in a limited number of fractions [2]. However, the efficacy of SBRT in reducing oligometastases and small solid tumours needs to be assessed for all tumours depending upon its resistance, size and location, usually by measuring tumour volume by non-invasive imaging techniques, such as CT-scan, MRI, or PET-scan. Since the response of the tumour is generally detected long after the end of radiotherapy, with late radiological modifications after SBRT being particularly difficult to assess [3], there is a real risk of prolonged unnecessary and ineffective exposure to radiation and of delays in initiating alternative treatments.

The availability of biomarkers that distinguish between responding and refractory patients early during the course of radiotherapy would represent a major clinical advance to define patients with high risk of tumour escape to the treatment. A potentially interesting biomarker is ceramide (Cer), a pro-apoptotic sphingolipid generated rapidly after irradiation. Cer is produced in the outermost layer of the cell membrane on hydrolysis of sphingomyelin by acidic sphingomyelinase (ASM) or neutral sphingomyelinase (NSM) and is synthetized *de novo* in endoplasmic reticulum by Cer synthase [4]. Adding exogenous Cer to androgen-sensitive human prostate adenocarcinoma cells (LNCAP cells) enhanced cell radiosensitivity and tumour regression [5]. Increasing endogenous Cer in human T lymphocyte cells (Jurkat cells) by the action of inhibitors of glucosyl-Cer synthase and ceramidase inhibitors (DL-PDMP and D-MAPP, respectively) also enhanced cell radiosensitivity [6]. Cer induced by high dose

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irradiation provoked massive endothelial cell apoptosis via ASM activation, and regression of fibrosarcoma or melanoma transplanted in wild-type mice [7].

Elevated Cer levels have been observed in plasma and serum from patients with lung emphysema [8], Wilson disease [9] and multiple organ failure [10]. Plasma Cer levels increased during lipid infusion in humans and rats, and were correlated with insulin sensitivity, inflammation and atherosclerotic risk [11]. Increased Cer serum levels were recorded after 3 days of treatment in 5 of 7 patients who responded to spatial fractionated grid radiotherapy (SFGRT) including a first irradiation at 15 Gy followed by 30×2 Gy [12]. However, no robust correlation was established between Cer and radiotherapy efficacy because of the small number of patients (11 in total) and the diversity of tumours.

During a phase II trial of hypofractionated SBRT combined with irinotecan chemotherapy, we measured plasma Cer levels in patients with liver and lung oligometastases originating from primary colon tumours. The objective was to validate plasma Cer as an early biomarker of treatment efficacy by correlating variations in Cer levels during treatment with long-term tumour response.

Materials and methods

Patient eligibility

Biological samples were collected from an ancillary study of a phase II clinical trial (EudraCT 2006-005440-87; clinical trial NCT01220063) performed from 2008 to 2013 by 3 French oncology centres (Nantes, Lyon and Lille). The main objective of the phase II clinical study (Table 1) was to test the feasibility of SBRT in combination with irinotecan chemotherapy for colorectal adenocarcinoma lung and liver metastases. The clinical follow-up is still ongoing and will be published separately.

Treatment planning and delivery

A phase II clinical trial (EudraCT 2006-005440-87; clinical trial NCT01220063) of SBRT and concomitant irinotecan chemotherapy for colorectal adenocarcinoma lung and liver metastases was performed from 2008 to 2013 in 3 oncology centres in France (Nantes, Lyon and Lille). Patient inclusion criteria were inoperable or recurrent hepatic and/or lung metastases after surgery, relapse after fluorouracil treatment with or without eloxatin or irinotecan, life expectancy >6 months, measurable metastases (largest diameter $\leqslant$6 cm, sum of the maximum diameter of multiple metastases $\leqslant$6 cm), a clinical target volume (CTV) located more than 12 mm laterally or 15 mm in the cranio-caudal direction of the stomach, small intestine, oesophagus, trachea, and pulmonary arteries, and an adequate haematologic cell pool and adequate hepatic and renal functions to receive irinotecan. Exclusion criteria were a performance index >2 (World Health Organization scale), prior thoraco-abdominal irradiation, a contraindication to irinotecan, prior (within 5 years) or concomitant treatment of an invasive cancer, diffuse metastatic disease, or more than 3 metastases. The ethics committees of all 3 institutions approved the protocol, and signed informed consent was obtained from all patients.

Table 1
Patient characteristics and demography.

		Location	
		Lung	Liver
Patient (number)	Male	8	21
	Female	1	5
Age (year)	Median	65	66.5
	Youngest	32	33
	Oldest	77	84
Tumour diameter (mm)	Median	13	36
	Smallest	4	11
	Largest	26	100

Treatment protocol is described in Fig. S1. Radiotherapy consisted in 4 fractions of 10 Gy delivered on days (D) 1, 3, 8 and 10 using linear accelerator-based devices (Novalis, Brain Lab, Feldkirchen, Germany, or Cyberknife, Accuray, Sunnyvaley, CA). In each case, 99% of the CTV was encompassed by the 75–95% isodose lines corresponding to a 42–53 Gy dose at the target centre. Irinotecan (40 mg/m^2) (Pfizer, New York, NY) was intravenously injected 30–90 min before delivery of the first and third radiotherapy fractions.

Response criteria

As decided in 2008, tumour response was assessed, using RECIST 1.1 (Response Evaluation Criteria In Solid Tumors) on chest or liver CT-scans, 3, 6, and 12 months post-treatment [13]. A complete response (CR) was defined by the disappearance of all target lesions, a partial response (PR) as a >30% decrease in the sum of the largest diameter (LD) of target lesions, progressive disease (PD) as a >20% increase of the LD of each lesion, and stable disease (SD) as neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD.

Plasma biocollection

Blood samples (20 ml) were collected in sodium citrate tubes before the first (D0) and after 30 min the second (D3) and fourth (D10) fractions (Fig. S1), stored at 4 °C for 30 min, then centrifuged at 1000 g for 5 min at 4 °C. The plasma aliquots were stored at −80 °C until analysis.

Cer extraction, purification and analysis

Ultrapure standards of 12 Cer subspecies (C14:0, C16:1, C16:0, C18:1, DHC18:0, C18:0, C20:1, C20:0, C22:1, C22:0, C24:1 and C24:0) and non-natural C17:0 Cer used as an internal standard were purchased from Avanti Polar Lipids (Alabaster, AL). UPLC grade methanol and analytical grade organic solvents were purchased from Fisher Scientific (Pittsburgh, PA). Forty microlitres of 1 μM C17:0 Cer were added to each plasma sample. Lipids were extracted from 100 μl of plasma samples in two steps using a previously described procedure with minor modifications [14]: (1) addition of 1.5 ml of a acidified hexane/propan-2-ol mixture (60:40, v/v), vortexing, centrifugation at 3000*g* for 5 min at 4 °C and collection of the upper phase; (2) addition of 1.5 ml of acidified methanol, homogenization, centrifugation at 8000*g* for 5 min at 4 °C, and collection of the upper phase. The organic phases from steps 1 and 2 were combined, dried under nitrogen at room temperature and resuspended in 150 μl of hexane/propan-2-ol (60:40 v/v).

The lipid extract was purified using an optimized published method [15]. Briefly, samples were loaded on 100-mg LC-NH_2 cartridges (Interchim, Montluçon, France) preconditioned with 2 ml of hexane. The cartridges were washed first with 1.4 ml of ethyl acetate–hexane 15:85 (v/v) to elute neutral lipids in a single fraction, then with 1.6 ml of chloroform/methanol 23:1 (v/v) to elute free Cer. The Cer fraction was dried under nitrogen and dissolved in 300 μl of MeOH containing 10 mM highest grade ammonium acetate (Fluka, Buchs, Switzerland) and 0.2% formic acid. Samples were stored at −20 °C until analysis.

Purified Cer fractions were analysed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) on an Acquity H-Class UPLC system combined with a Xevo

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