original article

p53 status correlates with histopathological response in patients with soft tissue sarcomas treated using isolated limb perfusion with TNF-α and melphalan

J. Muret[1,8]*, M. Yacoub[2,8], P. Terrier[2], F. Drusch[7], A. Laplanche[3], C. Gaudin[8], C. Richon[8], C. Le Péchoux[4], A. Le Cesne[5], F. J. Lejeune[9], T. Tursz[5], P. Fouret[2,8†], S. Bonvalot[6†] & S. Chouaib[8]

[1]Department of Anesthesia; [2]Department of Pathology; [3]Department of Public Health; [4]Department of Radiotherapy; [5]Department of Medical Oncology; [6]Department of Surgery; [7]Laboratoire de Recherche Translationnelle, Module d'Histocytopathologie; [8]Institut National de la Santé et de la Recherche Médicale U753 Unit, Immunologie des Tumeurs Humaines: Interaction Effecteurs Cytotoxiques-système Tumoral, Institut Gustave-Roussy, Villejuif Cedex, France; [9]Multidisciplinary Oncology Centre, Centre Pluridisciplinaire d'Oncologie, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland

Background: Recombinant tumor necrosis factor-α (TNF-α) combined to melphalan is clinically administered through isolated limb perfusion (ILP) for regionally advanced soft tissue sarcomas of the limbs. In preclinical studies, wild-type *p53* gene is involved in the regulation of cytotoxic action of TNF-α and loss of p53 function contributes to the resistance of tumour cells to TNF-α. The relationship between p53 status and response to TNF-α and melphalan in patients undergoing ILP is unknown.

Patients and methods: We studied 110 cases of unresectable limbs sarcomas treated by ILP. Immunohistochemistry was carried out using DO7mAb, which reacts with an antigenic determinant from the N-terminal region of both the wild-type and mutant forms of the p53 protein, and PAb1620mAb, which reacts with the 1620 epitope characteristic of the wild-type native conformation of the p53 protein. The immunohistochemistry data were then correlated with various clinical parameters.

Results: P53DO7 was found expressed at high levels in 28 patients, whereas PAb1620 was negative in 20. The tumours with poor histological response to ILP with TNF-α and melphalan showed significantly higher levels of p53-mutated protein.

Conclusions: Our results might be a clue to a role of p53 protein status in TNF-α and melphalan response in clinical use.

Key words: isolated limb perfusion, limb sarcoma, melphalan, p53 status, tumour necrosis factor-alpha

introduction

Sarcomas are among the most resistant tumours to chemotherapy (CT). In these tumours, preoperative doxorubicin-based CT, at maximum tolerated drug doses, is not very effective [40% of radiological response and 6% of pathological complete response (CR)] [1] and does not permit limb salvage in patients who needed an amputation before CT [2].

Hyperthermic isolated limb perfusion (ILP) [3] combined with tumour necrosis factor-α (TNF-α) and melphalan [4] is currently one of the therapies available for patients with advanced soft tissue sarcomas (STSs) of the limbs, allowing conservative surgery. ILP requires isolation of the patients

involved limb, its connection to a heart–lung machine and the administration of TNF-α and melphalan at 39–40°C, followed by conservative surgery 2 months later. The rationale is to improve the response rate by increasing the drug concentration while avoiding systemic toxicity. In a multicentre European trial, ILP with high-dose TNF-α and melphalan resulted in a 76% response rate and 71% limb salvage in patients with limb-threatening STSs [5]. In a dose–response study [6], objective tumour response (OR) (65%) and limb salvage (71%) did not vary in the four different dose groups tested (TNF-α dose between 0.5 and 4 mg). The well-studied mechanisms involved in TNF-α activity are an early effect on tumour vascularisation that is an increase in endothelium permeability, leading to increased melphalan concentration within the tissue tumour. The apoptosis pathway mediates a late effect that induces a selective destruction of tumour vessels with vascular occlusion due to intravascular coagulation followed by haemorrhagic necrosis [7–9].

Correspondence to: Dr J. Muret, Service d'Anesthésie, Institut Gustave-Roussy, 39 rue Camille Desmoulins, 94805 Villejuif Cedex, France. Tel: +33 1 42 11 40 18; Fax: +33 1 42 11 52 09; E-mail: muret@igr.fr

†Both these authors contributed to this article on a par.

p53 is a suppressor gene located on chromosome 17p13 and is mutated in 17% of soft tissue tumours [10] (IARC TP53 mutation database, R11 release, October 2006). Evidence has been provided indicating that in sarcomas, p53 protein mutation correlated with a worse outcome especially if associated to an amplification of mdm2 gene [11]. *In vitro* studies revealed that the tumour resistance to TNF-α-induced cell death was associated with *p53* gene mutation and the subsequent loss of its transactivation activities [12, 13]. While the disruption of wild-type *p53* gene function by E6 gene transfection protects MCF7 cells from TNF-α-induced apoptosis, the adenovirus-mediated transfer of the wild-type *p53* gene sensitises TNF-α-resistant MCF7 cells to the cytotoxic effects of this cytokine [14]. The mechanisms by which the restoration of the wild-type *p53* gene function in *p53* gene mutant cells increases their susceptibility to the cytotoxic action of TNF-α include a restoration of caspase 8 cleavage and the re-establishment of mitochondrial signs of apoptosis [15]. This also correlated with a significant down-regulation of c-*myc* and a decrease in retinoblastoma protein [16].

Moreover, melphalan, as an alkylating agent, induces cell death through p53 that is activated by DNA damage [17, 18]. Mutated *p53* may prevent from this activation and could explain resistance of sarcomas to melphalan during ILP treatment.

These findings indicate that p53 protein status may be an important determinant of the efficacy of treatment protocols on the basis of the use of apoptosis inducers such as TNF-α and melphalan. In this context, wild-type p53 may be an essential component in promoting the dynamic of cell death and may help to achieve a significant therapeutic index of cytotoxic mechanisms and develop more effective therapeutic immune interventions.

These studies were designed to investigate whether treatment response and clinical outcome of patients treated with ILP were associated with p53 protein status. We demonstrated that p53 protein mutation correlates with histopathological response in patients with STS following ILP treatment with TNF-α and melphalan.

patients and methods

Patients with unresectable STS of the lower or upper limb were treated with ILP in the reporting centre. Cases undergoing such treatment in our institution from June 2000 to June 2005 were included when paraffin-embedded material of the tumour was available before (named preoperative specimens) and/or after ILP at the time of the residual tumour excision 2 months later (named postoperative specimens). All cases were reviewed by a pathologist before treatment to ascertain diagnosis and to verify the quality of the tissue samples submitted to the study. Our institutional ethics committee approved the study.

hyperthermic ILP

Under general anaesthesia, the main artery and vein of the affected limb were clamped and cannulated after heparinisation. Cannulae were then connected to a heart–lung machine and a pneumatic tourniquet was applied proximally to prevent leakage into the general circulation. ILP consisted of extracorporeal circulation with mild hyperthermia (38–40°C) obtained with a heat exchanger. TNF-α (TNF-α-1a, Beromum™,

Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany) was injected into the arterial line and 30 min later, 10-mg/l melphalan (Alkeran, GlaxoSmithKline, London, UK) per limb volume was added for the following 60 min. At the end of the procedure, a 4- to 6-l wash out of the limb, using a mixture of Hartmann's solution and macrodex, was carried out. A delayed excision of the residual tumour was planned 2 months after ILP excepted in the case of multiple tumours or metastatic progression. The aim was to carry out *en bloc* residual tumour excision with free margins whenever possible.

magnetic resonance imaging clinical response criteria

The clinical response was determined by magnetic resonance imaging (MRI) carried out before and 2 months after ILP. The MRI examinations included T1-weighted spin echo (SE) and fast SE T2-weighted fat-saturated sequences, as well as dynamic sequences (T1-weighted SE repeated six times every 40 s), displaying the maximum intensity slope in each pixel. CR was defined by complete necrosis (>90%) of the tumour or disappearance of all measurable disease. A partial response (PR) was defined as a regression of the tumour size >50% in the product of the bi-dimensional measurements. Stable disease (SD) was defined as no change in the percentage of necrosis and in the tumour size. Progressive disease (PD) was defined as a >25% disease progression or the appearance of any new lesion. OR included CR and PR and nonresponse (NR) included SD and PD.

histopathological response criteria

Histopathological response was defined as good if ≤10% identifiable residual tumour cells were present in the postoperative specimens obtained 8 weeks after ILP. If >10% residual tumour cells were identifiable, histopathological response was defined as poor.

immunohistochemistry analysis

Blocks of tumour embedded in paraffin were analysed by a pathologist and portions containing tumour were sectioned in freshly cut slides to carry out immunohistochemistry following standard protocols using an automated immunohistochemical processor [NexES® IHC (Ventana, Tucson, AZ)]. The murine monoclonal primary antibodies were DO7 (M 7001, DAKO, Glostrup, Denmark, 1 : 50, 20 min at 37°C), which react with an epitope which is localised in the amino-terminal region of both the wild-type and mutant forms of the p53 protein [19], and PAb1620 (Ab-5, Calbiochem, San Diego, CA, 1 : 320, overnight at 4°C), which recognises the wild-type native conformation of p53 protein [20]. After dewaxing, epitopes were retrieved using a temperature-controlled water bath at 98°C during 20 min in sodium citrate buffer (pH 7.3, 10 mM Microm Microtech, Francheville, France; Diapath, Bergamo, Italy, 30PA-0310) for DO7 and at 98°C during 30 min in sodium citrate buffer (pH 6.0, Zymed, San Francisco, CA, 00-5000) for PAb1620. After cooling at room temperature, the following steps were carried out according to the manufacturers' instructions using an iView DAB Detection Kit (Ventana) for DO7, and an ABC avidin–biotin kit (VECTASTAIN® ABC, Vector Laboratories, Burlingame, CA, PK-6200) for PAb1620. For DO7, diamino benzidine (Ventana for DO7 and NovaRed vector, SK-4800 for PAb1620) was used as chromogen with haematoxylin (Hemalun, Mayer, RAL reactives, Martillac, France) as counter stain. Positive controls for DO7 mAb included sections from a breast cancer showing genetically proven missense mutation of the *p53* gene (Figure 1A) and for PAb1620, paraffin-embedded MCF-7 cell pellets derived from a breast adenocarcinoma including an accumulation of wild-type p53 protein (Figure 1B). Negative controls included sections from another breast tumour with a nonsense *p53* mutation. Controls were included in each analysis with DO7 and PAb1620. PAb1620 antibody was used in those samples positive for DO7. The incubation conditions for PAb1620 were
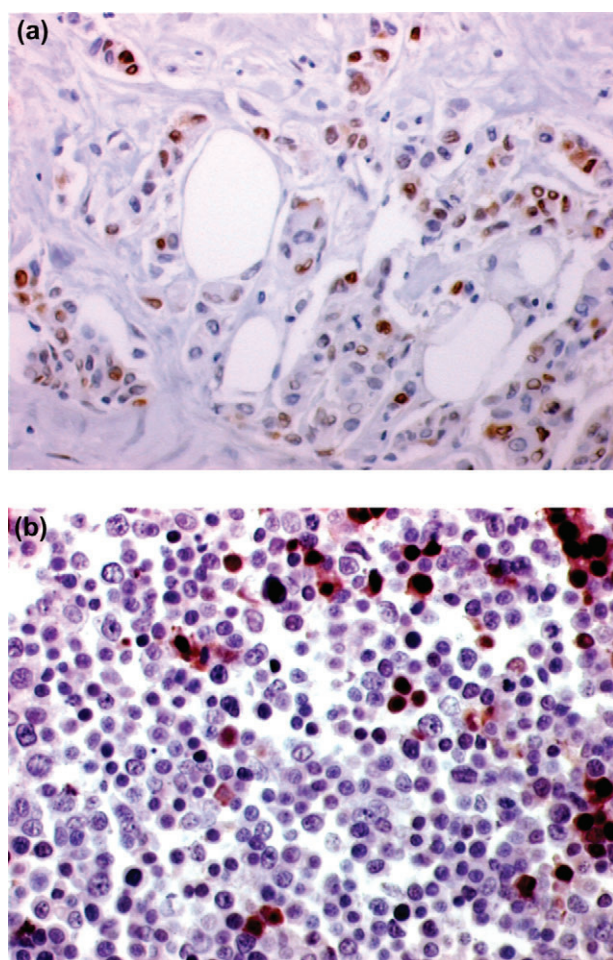
Figure 1. Immunohistochemical positive controls: sections of a breast cancer showing genetically proven missense mutation of the *p53* gene were used as a positive control for DO7 (A) and paraffin-embedded MCF-7 cell pellets deriving from a breast adenocarcinoma cell line including an accumulation of wild-type p53 protein were used as a positive control for PAb1620 (B).

adjusted so that it reacted with MCF-7 cells, but not with the p53-mutated tumour cells.

analysis of immunostaining

Two pathologists blindly evaluated specimens for p53 staining with DO7 and PAb1620. The percentage of positive cells was recorded and used to attribute a score on a scale 0–3 as follows: score 0 if <10% cells were positive; score 1 if 11%–25% of cells were positive; score 2, 26%–50% positive cells and score 3, >50% positive cells [21]. If the score was 2 or 3, samples were considered as positive for the antibody tested and negative under 2. Samples were considered to be mutated for p53 protein when they reacted with DO7, but not PAb1620. If a discordance was observed for p53 status between pre- and postoperative specimens in patients with both specimens available, p53 was considered to be mutated.

statistical design

Results are expressed as percentages or medians and ranges. Survival and disease-free survival (first recurrence to occur, i.e. metastases, second cancer, local relapse and death) were calculated using the Kaplan–Meier method [22] and Rothman's 95% confidence intervals (95% CIs) [23]. Survival curves were compared using the log-rank test [24].

results

patient characteristics

One hundred and six consecutive patients with a locally unresectable limb STS were treated by ILP and included in this study. Clinical characteristics of the patients are represented in Table 1. Four patients with a locally unresectable desmoid tumour were also treated by ILP and included in this study. Tumour concerned superior limb in 37% of patients and inferior limb in 63%. Size of the tumour was 50 and 80 mm, respectively. Histopathological subtypes are liposarcoma (*n* = 24, 22%), synovial sarcoma (*n* = 24, 22%), undifferentiated sarcoma (*n* = 19, 17%), malignant peripheral nerve sheath tumours (*n* = 13, 12%), epithelioid sarcoma (*n* = 9, 8%), leiomyosarcoma (*n* = 5, 5%), fibroblastic sarcoma (*n* = 3, 3%), desmoids tumours (*n* = 4, 4%), angiosarcoma (*n* = 3, 3%) and other (*n* = 6).

Before ILP, 30 patients (27%) had been treated with radiotherapy, 56 (51%) by CT, 1 had a previous ILP and 63 had undergone surgery (37 once, 11 twice, 15 three to nine).

tumour response and post-ILP evolution

Complete clinical response evaluated by MRI reached 45% and overall response 70%. Good histopathological response to ILP treatment was found up to 33% (Table 2).

Limb salvage was initially achieved in 90% of our patient population but this fell to 82% at a median time of 19 months after ILP.

By 1st December 2005, 66 patients of the 110 were still being followed, whereas follow-up was lost for 18 patients (16 had been followed until 2004 and 2 until 2003). In all, 26 (24%) patients had died. Of the patients in this study, nine had metastasis before treatment, seven of who died. In the remaining 101 patients, 18 patients (18%) had local recurrence [at a median time of 12 months (2.5–30)] and 30 (30%) had

Table 1. Clinical characteristics of the patients before ILP

	No. and %	Median (range)
Male/female	58/52	
Age (years)		45 (8–81)
Location and size		
Superior limb size (mm)	41 (37%)	50 (10–200)
Inferior limb size (mm)	69 (63%)	80 (10–208)
Histological grade[a]		
I	16 (16%)	
II	46 (46%)	
III	38 (38%)	
Recurrences before ILP		
0	43 (39%)	
1	42 (38%)	
2	11 (10%)	
3	9 (8%)	
≥3	5 (5%)	
Synchronous metastasis	9 (8%)	

[a]In 100 patients, histological grade could not be retrieved in 10 patients because initial histological diagnosis was made years before.
ILP, isolated limb perfusion.

Table 2. Responses to ILP according to p53 protein mutation

	No. and (%)	Wild-type p53 protein	Mutated p53 protein
TNF-α dose			
0.5 mg	18	15	3
1 mg	53	46	7
2 mg	20	13	7
3 mg	11	9	2
4 mg	8	7	1
MRI response			
CR	50 (45%)	43	7
PR	27 (25%)	18	9
OR	77 (70%)	61	16
NR	32 (29%)	29	3
Histopathological response[a]			
Good	32 (33%)	31	1
Poor	64 (67%)	47	17
Post-ILP surgery			
No surgery	13 (12%)	11	2
Conservative surgery	86 (78%)	69	17
Initial amputation	11 (10%)	10	1
Local recurrence[b]	18 (18%)	15	3
Metastatic recurrence[b]	30 (30%)	22	8

[a]In 96 patients, histopathological response was not carried out in 13 patients who were not operated on and in one patient amputated outside our institution.
[b]In 101 patients, 9 patients had a synchronous metastasis at the time of ILP. TNF-α, tumour necrosis factor-α; MRI, magnetic resonance imaging; CR, complete response, PR, partial response; OR, Objective tumour response; NR, nonresponse; ILP, isolated limb perfusion.

metastasis [at a median time of 10 months (2–40)], 10 had both and 2 deceased during follow-up without metastasis or recurrence.

After ILP (and eventually residual tumour excision), 57 patients (52%) received adjuvant therapy consisting of radiotherapy in 48 patients, systemic CT in 3 patients and a combination of both in 6 patients. In 40 patients (36%), surgery was the sole treatment after ILP.

p53 protein status

We analysed a total of 170 slides belonging to 110 different patients. Sixty patients had both pre- and postoperative specimens, 35 had preoperative specimens only and 15 had postoperative specimens only. Among the 35 patients without postoperative specimens, no residual tumour was found after ILP in 13, and 13 patients (12%) were not operated on after ILP (six for multiple tumour and seven for metastatic progression). Three patients were amputated outside our institution and specimens could not be retrieved. Six patients had surgery but histochemistry could not be carried out due to lack of tumour material. All the 15 patients without preoperative specimens had been treated before ILP in other hospitals and this material could not be retrieved.

Ninety-five preoperative specimens and 75 postoperative specimens were immunostained with p53 DO7 mAb. p53 protein was overexpressed in 39, which were immunostained with PAb1620 mAb (Figure 2). Afterwards, 10 preoperative

specimens and 14 postoperative specimens were p53 DO7 accumulated and PAb1620 negative corresponding to 20 patients with a mutated p53 protein. Among the 60 patients with both pre- and postoperative specimens available, we did not find the same result before and after ILP in nine (Table 3).

Patients' tumour characteristics in relation to p53 protein mutation are represented in Table 2.

p53 protein mutation and clinicopathological implications

As depicted in Figure 3, tumours of patients with poor histological response to ILP with TNF-α showed significantly higher levels of mutated p53 protein as 94% of patients with mutated p53 protein had a poor histopathological response. We, however, found a lack of correlation between mutated p53 protein and survival (Figure 4), disease-free survival (Figure 5) or MRI response. No correlation was observed between histopathological response and disease-free survival either (data not shown, $P = 0.65$).

With a median follow-up of 25 months, the 2-year overall survival rates (95% CI) were 88% (78%–93%) and 69% (43%–86%) in the p53 wild-type and mutated p53 protein groups ($P = 0.1$) (Figure 4). The 2-year disease-free survival rates were 62% (50%–73%) and 40% (19%–65%), respectively, ($P = 0.31$) (Figure 5).

discussion

In the course of these studies, we examined the immunohistochemical expression of p53 in STS patients and analysed correlations between the immunohistochemical findings and treatment response following ILP with TNF-α and melphalan.

Our current results by immunohistochemical detection of mutated p53 protein with two different antibodies support the notion that mutated p53 protein significantly correlates with poor histopathological response. A better control of the disease, at least by histopathological examination, may be due to a better sensitivity of tumour cells to TNF-α and melphalan when p53 protein is wild type. Since we had no control group (i.e. not treated with TNF-α and melphalan), we cannot make any final statement regarding this hypothesis. Preclinical studies on cell lines, however, indicate the role of *p53* gene mutation in tumour response to TNF-α since transfection of wild-type *p53* gene in cell lines with mutant *p53* gene sensitises cells to the cytotoxic effect of TNF-α [16]. Moreover, melphalan can be discussed as another aspect of drug resistance. It has been established that STSs do not respond to melphalan alone even in the context of ILP. It was demonstrated experimentally [25, 26] that TNF-α selectively increases melphalan penetration in tumour tissue and we postulate that it is the case in this clinical setting. STS are not necessarily resistant to melphalan, but rather there is a lack of drug penetration in the tumour in the absence of TNF-α. ILP uses two compounds that are synergistic but do not hit the same target. TNF-α has an early effect on tumour vascularisation that increases permeability. The late effect is to induce a strong apoptosis of
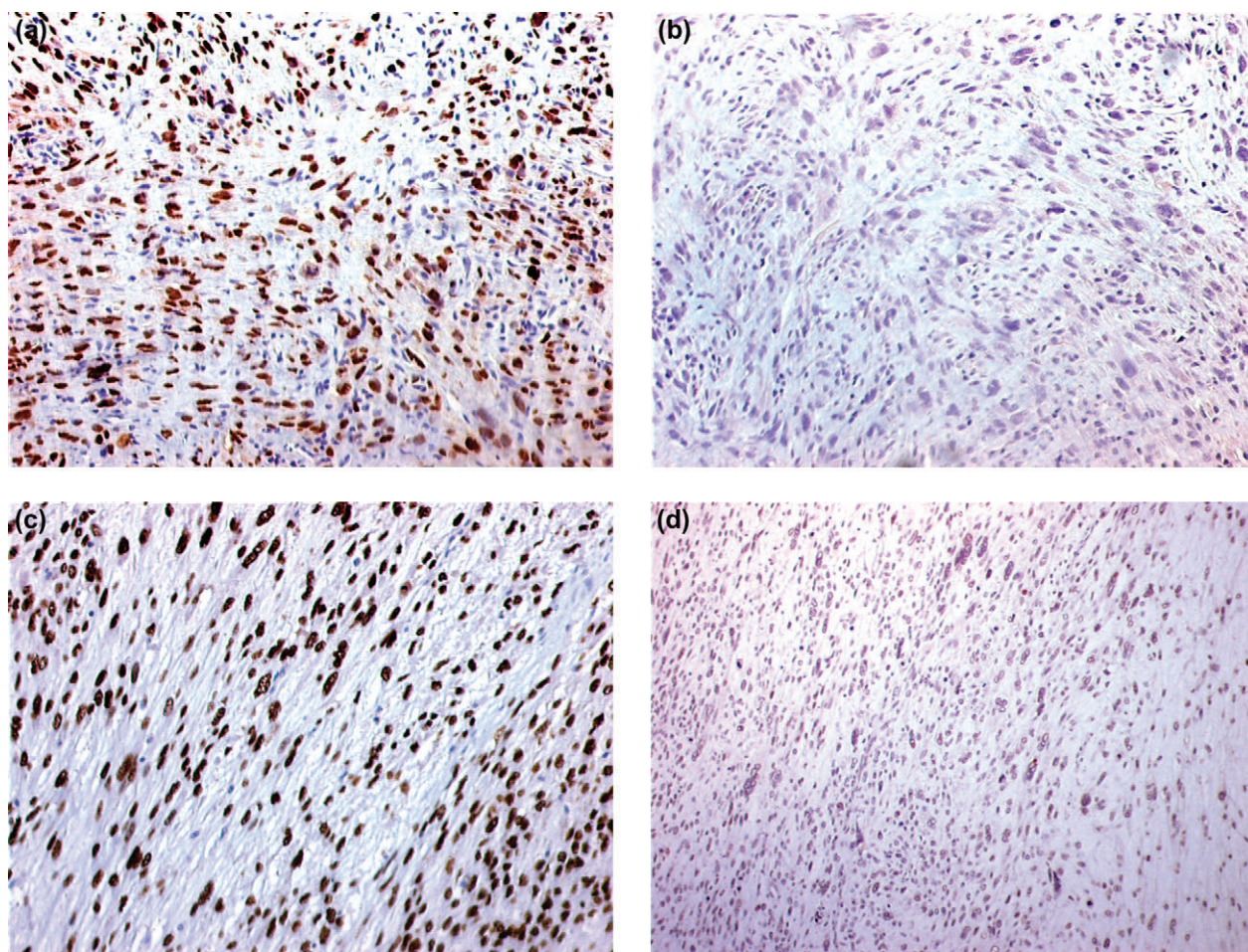
Figure 2. Immunohistochemical analysis of p53 (DO7, detecting both wild-type and mutant forms of p53 protein and PAb1620 detecting only wild-type p53 protein) in a sarcoma with p53 protein mutation (A and B) showing (A) nuclear immunostaining for DO7 and (B) negative staining for PAb1620 and in a sarcoma with nonmutational accumulation of wild-type p53 protein (C and D) showing (C) nuclear immunostaining for DO7 and (D) for PAb1620.

Table 3. Immunochemistry data using D07 and PAb1620mAb in the 60 patients with both pre-and postoperative specimens available

Patients (n)	p53 DO7 expression and negative PAb1620 staining in the preoperative specimens	p53 DO7 expression and negative PAb1620 staining in the postoperative specimens
47	No	No
4	Yes	Yes
4	Yes	No
5	No	Yes

Figure 3. Correlation between mutated p53 protein and poor histopathological response; * $P = 0.005$ (Fisher).

endothelial cells and the collapses of the tumour vessels. In contrast, as an alkylating agent, melphalan induces cell death through p53 that is activated by DNA damage [17, 18]. In our study, we observed an increased accumulation of p53 protein in tumours that did not respond histologically to ILP. p53 protein-increased expression indicates *p53* mutation and inactive protein accumulation. In other words, sarcomas that did not respond histologically to ILP may be resistant to TNF-α and also to melphalan because of the lack of *p53* activation.
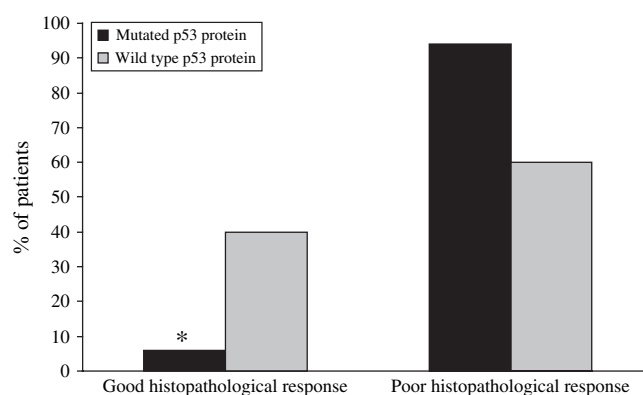
Our patients' population of 110 included 14 different histological subgroups of STS and different types of pretreatment (surgery, radiotherapy, CT and combinations). STS are very uncommon tumours, and in studies with STS patients treated by ILP, it was previously demonstrated that despite different histological subgroups are characterised by
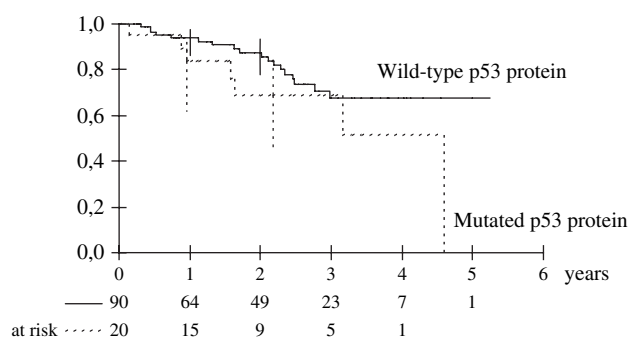
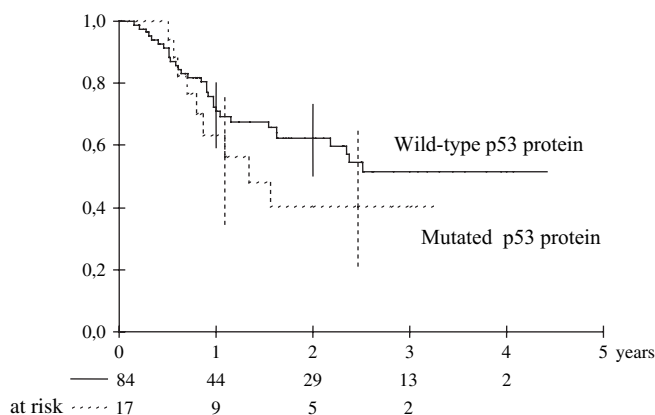**Figure 4.** Survival according to p53 protein mutation; $P = 0.10$ (log-rank).

Figure 5. Disease-free survival according to p53 protein mutation; $P = 0.31$ (log-rank).

different molecular backgrounds, TNF-α response in ILP treatment was independent of the type of STS [5, 6, 27]. Moreover, overall response to TNF-α and melphalan did not differ significantly according to pre-irradiation, preoperative CT and TNF-α doses [6, 28].

Although a good correlation (73%) between radiological and histopathological responses was observed in our study, no significant correlation between mutated p53 protein and clinical tumour response assessed by radiology was found as we found for histopathological response. This discordance may be attributable to the fact that a notable discrepancy exists between the clinical and the histopathological response assessment in ILP studies. The clinical evaluation of tumour regression is on the basis of the estimated size of the necrosis of the tumour, which is measured 2 months after ILP and can be operator dependant. The histopathological response, i.e. assessment of the percentage of viable cells, can be difficult to evaluate because it is on the basis of the residual volume of the tumour. If the tumour shrinks but still contains viable tumour cells, the percentage of residual tumour is often overestimated. Our series shows a higher rate of clinical CR (45%) than other series [9]. This may be due to the fact that we did not combine clinical and histopathological response to define the final outcome [5]. CR *per se*, however, is not crucial, whether the tumour responds completely or partially is irrelevant, as long as it becomes amenable to residual tumour excision without loss of limb function. Our overall response rate (70%) was not different from other studies [9, 27].

Contrary to other studies [11, 29], we did not find any correlation between p53 protein mutation and survival or disease-free survival. Interestingly, in Cordon-Cardos et al. [11] study, the presence or absence in tumour cells of p53 protein in high levels as measured by immunohistochemistry was a very good indicator of patient survival. Treatment modalities, however, were not specified and patients had apparently less locally advanced sarcomas. It should be noted that our results were obtained in a selected group of patients with very extensive disease. All had a locally advanced tumour considered as unresectable and were candidates for amputation. Nine patients of our series had already distant metastasis. The majority of the tumours were high grade (84%) and with a high number of recurrences before ILP [53 patients (48%) had at least one recurrence and 14 patients (13%) had >2]. In this rather restricted group of patients with very bad prognoses, survival rates are difficult to analyse if compared with other studies on sarcoma with all types of patients (low grade, absence of recurrence etc.).

We did not study *p53* gene status mutation in our patients' tumours but we searched for p53 protein mutation by immunohistochemistry. In the case of missense gene mutation, mutant *p53* gene product is characterised by a conformational change of the protein with subsequent prolonged half-life and stability. The accumulated mutant protein is therefore detectable by immunohistochemistry, and immunostaining of tissue sections may be an important surrogate for *p53* genetic analysis [30–32]. In nonsense *p53* gene mutations, products however, are truncated proteins and are undectable by immunohistochemistry. In our experience with carcinomas of the head and neck with *p53* gene sequencing, a good correlation was found between tumours harbouring missense mutations and p53 DO7 mAb detection [33]. Moreover, use of DO7 mAb with antigen retrieval was found to be the most sensitive and specific procedure for assessing p53 protein mutations [34]. In our study, 28 of 110 patients were p53 DO7 accumulated (25.4%). In Cordon-Cardo et al. [11] study, 56 of 211 sarcomas had an overexpression of p53 (26.5%). In his study, in the last analysis with DNA sequencing carried out on 73 tumours, the overexpression of p53 protein correlated with missense mutations characterised by a conformational change of the protein. The majority of *p53* gene mutations are missense (78% in germ line mutations and 74% in somatic mutations) [10] (IARC TP53 mutation database, R11 release, October 2006) and among sarcoma, 69% of the *p53* mutations are missense, 13.4% are silent and 6.06% nonsense [10] (IARC TP53 mutation database, R11 release, October 2006). We, however, could not even do the RT–PCR technique procedure because we did not have any frozen material. In fact, in the postoperative specimens, it was not possible to do the sections for freezing because it was not possible to distinguish the tumour areas.

Moreover, the presence of p53 protein accumulation may be the result of a nonmutational stabilisation of this protein which binds to viral (adenovirus) or cellular (mdm2) oncoproteins [11]. In our study, considering to discriminate p53 protein accumulation with and without any *p53* gene mutation, we used the PAb1620 antibody which recognises

the 1620 epitope present only in the wild-type p53 protein. Out of 39 specimens expressing p53 DO7, 24 did not express 1620 epitope corresponding to 20 patients (18.2%). Prevalence of *p53* gene mutation in soft tissue tumour is 17.2% [10].

The data of the present studies also emphasise that among the 60 patients with both pre- and postoperative specimens available, in nine patients, p53 protein status was different. In five cases, we found p53 protein to be mutated in postoperative but not in the preoperative specimens. This discordance may be explained by the fact that the biopsy was carried out in some cases at the beginning of the disease and the tumour may have modified its *p53* gene status after systemic chemotherapies performance [35]. Moreover, cytotoxic treatment leads to a significant reduction in the number of tumour cells that are sensitive to the therapy. Tumours that survive the treatment are enriched in resistant clones and are likely to display the p53 status associated with chemoresistance. In four other cases, p53 protein was mutated in the pre- but not postoperative specimens. This may be due to the fact that a different tumour clone was selected in the pre- and postoperative specimens.

In addition, as it is known that therapy-caused events in the context of ILP occur timely close to the treatment event [36], the p53 status detected 2 months following ILP may represent the late outcome, resulting not only from the direct effect of the therapy but also from several events such as inflammation and hypoxia. It is, however, not possible for ethical reasons to obtain samples from these patients before the residual tumour excision carried out 2 months after ILP.

p53 status may influence histological response to ILP treatment. If this result is confirmed in a largest study, it may play a role in TNF-α response in clinical use: it could help us to decide on postoperative treatments after ILP. Patients with mutated p53 protein could benefit from a more aggressive treatment including postoperative radiotherapy and further CT. Radiotherapy post-ILP improves local tumour control in limb-saving treatment of STS even after radical resection [37]. The decision of its use in this combined modality treatment might be difficult to take since adjuvant irradiation may result in acute and late morbidity such as worse limb strength, oedema and delayed pathologic fractures with pseudarthrosis which are difficult to treat. In patients for whom the decision is difficult to take, we suggest analysing p53 status because a mutated p53 may tend to have a high risk of histological NR. Moreover, in the future, manipulation of *p53* status may improve effectiveness of ILP treatment and the clinical use of compounds [38] able to restore p53 function or *p53* gene transfer [39] could be a novel strategy for optimising the efficiency of TNF-α in human cancer treatment.

funding

references

1. Pisters PW, Patel SR, Varma DG et al. Preoperative chemotherapy for stage IIIB extremity soft tissue sarcoma: long-term results from a single institution. J Clin Oncol 1997; 15: 3481–3487.

2. Meric F, Hess KR, Varma DG et al. Radiographic response to neoadjuvant chemotherapy is a predictor of local control and survival in soft tissue sarcomas. Cancer 2002; 95: 1120–1126.

3. Creech O, Krementz ET, Ryan RF, Winblad JN. Chemotherapy of cancer: regional perfusion utilizing an extracorporeal circuit. Ann Surg 1958; 148: 616–632.

4. Lienard D, Ewalenko P, Delmotte JJ et al. High-dose recombinant tumor necrosis factor alpha in combination with interferon gamma and melphalan in isolation perfusion of the limbs for melanoma and sarcoma. J Clin Oncol 1992; 10: 52–60.

5. Eggermont AM, Schraffordt KH, Klausner JM et al. Isolated limb perfusion with tumor necrosis factor and melphalan for limb salvage in 186 patients with locally advanced soft tissue extremity sarcomas. The cumulative multicenter European experience. Ann Surg 1996; 224: 756–764.

6. Bonvalot S, Laplanche A, Lejeune F et al. Limb salvage with isolated perfusion for soft tissue sarcoma: could less TNF-alpha be better? Ann Oncol 2005; 16: 1061–1068.

7. Renard N, Nooijen PT, Schalkwijk L et al. VWF release and platelet aggregation in human melanoma after perfusion with TNF alpha. J Pathol 1995; 176: 279–287.

8. Rossi CR, Mocellin S, Pilati P et al. TNF alpha-based isolated perfusion for limb-threatening soft tissue sarcomas: state of the art and future trends. J Immunother 2003; 26: 291–300.

9. Lejeune FJ, Lienard D, Matter M, Ruegg C. Efficiency of recombinant human TNF in human cancer therapy. Cancer Immun 2006; 6: 6.

10. Olivier M, Eeles R, Hollstein M et al. The IARC TP53 database: new online mutation analysis and recommendations to users. Hum Mutat 2002; 19: 607–614.

11. Cordon-Cardo C, Latres E, Drobnjak M et al. Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. Cancer Res 1994; 54: 794–799.

12. Cai Z, Capoulade C, Moyret-Lalle C et al. Resistance of MCF7 human breast carcinoma cells to TNF-induced cell death is associated with loss of p53 function. Oncogene 1997; 15: 2817–2826.

13. Thiery J, Echchakir H, Dorothee G et al. Role of p53 in the sensitization of tumor cells to apoptotic cell death. Mol Immunol 2002; 38: 977–980.

14. Thiery J, Dorothee G, Haddada H et al. Potentiation of a tumor cell susceptibility to autologous CTL killing by restoration of wild-type p53 function. J Immunol 2003; 170: 5919–5926.

15. Ameyar-Zazoua M, Larochette N, Dorothee G et al. Wild-type p53 induced sensitization of mutant p53 TNF-resistant cells: role of caspase-8 and mitochondria. Cancer Gene Ther 2002; 9: 219–227.

16. Ameyar M, Shatrov V, Bouquet C et al. Adenovirus-mediated transfer of wild-type p53 gene sensitizes TNF resistant MCF7 derivatives to the cytotoxic effect of this cytokine: relationship with c-myc and Rb. Oncogene 1999; 18: 5464–5472.

17. Gould KA, Nixon C, Tilby MJ. p53 elevation in relation to levels and cytotoxicity of mono- and bifunctional melphalan-DNA adducts. Mol Pharmacol 2004; 66: 1301–1309.

18. Nelson WG, Kastan MB. DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. Mol Cell Biol 1994; 14: 1815–1823.

19. Anwar K, Nakakuki K, Imai H et al. Over-expression of p53 protein in human laryngeal carcinoma. Int J Cancer 1993; 53: 952–956.

20. Xirodimas DP, Lane DP. Molecular evolution of the thermosensitive PAb1620 epitope of human p53 by DNA shuffling. J Biol Chem 1999; 274: 28042–28049.

21. Wang YC, Lin RK, Tan YH et al. Wild-type p53 overexpression and its correlation with MDM2 and p14ARF alterations: an alternative pathway to non-small-cell lung cancer. J Clin Oncol 2005; 23: 154–164.

22. Kaplan EL, Meier P. Non parametric estimation from incomplete observations. J Am Statisc Assoc 1958; 53: 457–481.

23. Rothman KJ. Estimation of confidence limits for the cumulative probability of survival in life table analysis. J Chronic Dis 1978; 31: 557–560.

24. Peto R, Pike MC, Armitage P et al. Design and analysis of randomized clinical trials requiring prolonged observation of each patient. II. analysis and examples. Br J Cancer 1977; 35: 1–39.

25. Van Der Veen AH, de Wilt JH, Eggermont AM et al. TNF-alpha augments intratumoural concentrations of doxorubicin in TNF-alpha-based isolated limb perfusion in rat sarcoma models and enhances anti-tumour effects. Br J Cancer 2000; 82: 973–980.

26. de Wilt JH, ten Hagen TL, de Boeck G et al. Tumour necrosis factor alpha increases melphalan concentration in tumour tissue after isolated limb perfusion. Br J Cancer 2000; 82: 1000–1003.

27. Grunhagen DJ, de Wilt JH, Graveland WJ et al. Outcome and prognostic factor analysis of 217 consecutive isolated limb perfusions with tumor necrosis factor-alpha and melphalan for limb-threatening soft tissue sarcoma. Cancer 2006; 106: 1776–1784.

28. Bedard V, Vataire A, Desouche C et al. A prospective database of 100 patients with locally soft tissue sarcoma (STS) treated by isolated limb perfusion with melphalan and TNFa 1 mg. ASCO Annual Meeting Proceedings Part I. J Clin Oncol 2007; (18S): (Abstr 10010).

29. Onel K, Cordon-Cardo C. MDM2 and prognosis. Mol Cancer Res 2004; 2: 1–8.

30. Schneider-Stock R, Radig K, Oda Y et al. p53 gene mutations in soft-tissue sarcomas–correlations with p53 immunohistochemistry and DNA ploidy. J Cancer Res Clin Oncol 1997; 123: 211–218.

31. Rieske P, Bartkowiak JK, Szadowska AM et al. A comparative study of P53/MDM2 genes alterations and P53/MDM2 proteins immunoreactivity in soft-tissue sarcomas. J Exp Clin Cancer Res 1999; 18: 403–416.

32. Mousses S, McAuley L, Bell RS et al. Molecular and immunohistochemical identification of p53 alterations in bone and soft tissue sarcomas. Mod Pathol 1996; 9: 1–6.

33. Temam S, Flahault A, Perie S et al. p53 gene status as a predictor of tumor response to induction chemotherapy of patients with locoregionally advanced squamous cell carcinomas of the head and neck. J Clin Oncol 2000; 18: 385–394.

34. Baas IO, Mulder JW, Offerhaus GJ et al. An evaluation of six antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms. J Pathol 1994; 172: 5–12.

35. Righetti SC, Perego P, Corna E et al. Emergence of p53 mutant cisplatin-resistant ovarian carcinoma cells following drug exposure: spontaneously mutant selection. Cell Growth Differ 1999; 10: 473–478.

36. Lassau N, Lamuraglia M, Vanel D et al. Doppler US with perfusion software and contrast medium injection in the early evaluation of isolated limb perfusion of limb sarcomas: prospective study of 49 cases. Ann Oncol 2005; 16: 1054–1060.

37. Thijssens KM, van Ginkel RJ, Pras E et al. Isolated limb perfusion with tumor necrosis factor alpha and melphalan for locally advanced soft tissue sarcoma: the value of adjuvant radiotherapy. Ann Surg Oncol 2006; 13: 518–524.

38. Bykov VJ, Selivanova G, Wiman KG. Small molecules that reactivate mutant p53. Eur J Cancer 2003; 39: 1828–1834.

39. Cristofanilli M, Krishnamurthy S, Guerra L et al. A nonreplicating adenoviral vector that contains the wild-type p53 transgene combined with chemotherapy for primary breast cancer: safety, efficacy, and biologic activity of a novel gene-therapy approach. Cancer 2006; 107: 935–944.