are unlikely to demonstrate benefit with any significant degree of confidence. This dilemma has been recognised in recent years, and has resulted in the setting up of large multicentre trials. The UK AXIS (Adjuvant X-ray and Infusion Study) is an example of one such trial designed to determine the efficacy of intraportal chemotherapy (with or without radiotherapy for rectal cancer). The data monitoring committee for this trial has recently reviewed data from the first 2200 patients and recommends continuation.

Undoubtedly, the recent trials of systemic chemotherapy using 5-fluorouracil (5FU) and levamisole or 5FU and leucovorin are providing exciting data. These regimes require 6–12 months of treatment and are commenced approximately 6 weeks postoperatively. Important questions remain relating to the optimum dose, the best combination and the minimum length of treatment likely to provide survival benefit. In order to answer them, there is a need for even more prospective randomised trials.

There is also evidence to suggest that adjuvant chemotherapy should be commenced in the early postoperative period (even intra-operatively) to achieve optimum responses. The patient may indeed be at greatest risk of developing micro-metastases during the peri-operative period and high-dose chemotherapy at this stage may be of relevance. The protocol described by Wils and colleagues, and organised in collaboration with the EORTC is designed to address two questions. The first relates to the role of early locally directed peri-operative treatment and the second to the optimum regime of subsequent systemic chemotherapy. Early regional chemotherapy can be delivered either by portal vein infusion or by intra-peritoneal chemotherapy. It is feasible that this study could be combined with the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) AXIS trial with appropriate patients undergoing a second randomisation to determine which systemic chemotherapy regimen (either 5FU and leucovorin or 5FU and levamisole) should be given. Collaboration of this type would undoubtedly increase patient accrual and enable important questions to be answered quickly.

The physical barrier between the U.K. and continental Europe has now been breached, and surely the time has arrived for clinical researchers to collaborate on a pan-European scale. If this could be achieved, then one of the main inhibiting factors in randomised trials, the inordinate length of time required to accrue large numbers of patients, could be overcome. It is opportune for the EORTC and the UKCCCR to join forces in trials relating to colorectal cancer.

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Papers

Serum p53 Auto-antibodies: Incidence in Familial Breast Cancer

J.A. Green, B. Mudenda, J. Jenkins, S.J. Leinster, M. Tarunina, B. Green and L. Robertson

Inactivation of the p53 gene, which codes for a tumour suppressor protein, is known to occur in the majority of human malignancies. An ELISA technique has been developed which has detected auto-antibodies to p53 in the serum of 25.6% of 176 women with breast cancer, considerably higher than previously reported with an immunoblotting technique. The incidence of auto-antibodies in those cases with a family history of breast cancer was 9.1%, compared to 29.4% in those with no family history ($P=0.029$). In women without clinical breast cancer, 4 out of 36 (11.1%) of those with a positive family history were seropositive, compared to 1 out of 73 control women. Auto-antibodies were more frequently seen in the serum of breast cancer patients whose biopsies demonstrated overexpression of p53 protein. We conclude that auto-antibodies to p53 may have a role in the molecular characterisation of familial breast cancer.
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INTRODUCTION

THE PRODUCT of the p53 gene is a nuclear protein which has an important role in regulation of the growth of both normal and malignant cells, and may act as a stop or checkpoint in the late G_1 phase of the cell cycle [1]. Transfection of mutant p53 into immortalised primary human bronchial epithelial cells results in increased tumorigenicity [2], while the insertion of the non-mutated p53 gene into human cancer cell lines has been shown to cause both suppression of growth and reversal of the malignant phenotype [3]. Mutation in the p53 gene is one of a number of related genetic changes in the development of malignancy in a wide range of human tumour types, and may result in the

accumulation of high levels of protein in the transformed cells, allowing detection by immunohistochemistry [1, 4]. The most widely studied tumours have been colorectal carcinomas, but mutations have been described in human cancers as diverse as lung, osteosarcoma, ovary and melanoma [5]. In other situations, complex formation between non-mutated p53 and viral proteins or products of other oncogenes may give rise to immunocytochemically detectable p53 protein [6, 7]. In breast cancer expression of p53 protein is detectable in up to 50% of cases [8, 9].

Serum auto-antibodies to p53 were demonstrated in 14/122 (11.5%) breast cancer patients by Crawford and colleagues [10], using a Western blotting method, at a time when the function of p53 as a tumour suppressor had not been considered, nor was its role in human malignancies established. An ELISA method for the detection of p53 antibodies in human sera has been developed in our laboratory, which provides a more sensitive and rapid assay for these antibodies. Sera from breast cancer patients and volunteer controls were tested for p53 antibodies in a total of 285 patients and controls. The study was carried out to assess the role of these auto-antibodies in screening for familial and sporadic cases of breast cancer, and to determine their prognostic significance.

PATIENTS AND METHODS

176 consecutive newly diagnosed patients attending the breast clinic at the Royal Liverpool University Hospital from December 1990 to August 1991 were entered into the study. Blood was collected and the serum frozen at −20°C within 2 h. Control sera were obtained from women volunteers who attended the breast clinic for minor symptoms in whom no active breast disease was found. Thus patients with radiological, clinical or cytological evidence of benign breast conditions were excluded from the study. A separate group of high-risk patients with a family history of breast cancer was obtained, comprising the first or second degree relatives of breast cancer patients who often had no breast symptoms themselves, and on both clinical and radiological examination were free of tumour.

The definitive study of the serum of 285 patients and controls were carried out by an ELISA technique, in which purified soluble p53 protein was applied to 96 well plates. This was prepared from a fragment of the p53 gene with deletion of 396 base pairs from the *N*-terminus produced in the Marie Curie Institute by the polymerase chain reaction and cloning in the pDS/RSB bacterial expression plasmid [11]. Identity of the polymerase chain reaction product to wild type human p53 was confirmed by sequencing. The fragment of p53 was expressed in *E. coli* and, after centrifugation and lysis, purified on an Ni-NTA agarose column (Quiagen). The purified protein was then renatured by serial dialysis in 25 mM Tris HCl buffer pH8.0 with decreasing concentrations of guanidine HCl (Sigma). After incubation, unbound p53 was removed by washing and 50 µl from each serum sample was then added in pairs to each well for 1 h at 37°C, after a blocking step with 1% bovine serum albumin. Standard controls comprised the monoclonal antibody to p53 PAb 122 (Boehringer Mannheim), pooled positive sera and pooled negative sera from an initial series of samples assayed by immunoblotting. Peroxidase conjugated rabbit anti-human secondary antibody was then added to each well for a further hour at 37°C, and the optical density was determined at 490 nm after reaction with OPD (Dako). As additional proof of the specificity of the method for antibodies to p53, preincubation of the positive control sera with the purified p53 protein caused a downward shift in the curve of optical density plotted against serum dilution in the range 1:100 to 1:1000 for three of the positive controls sera. In all cases, preincubation with purified p53 protein brought the optical density of the positive control sera to a level below that of the negative control (Figure 1).

In order to identify positive sera with p53 antibodies, sera from a separate series of 26 patients with breast cancer were assayed for these antibodies by a western blotting method,

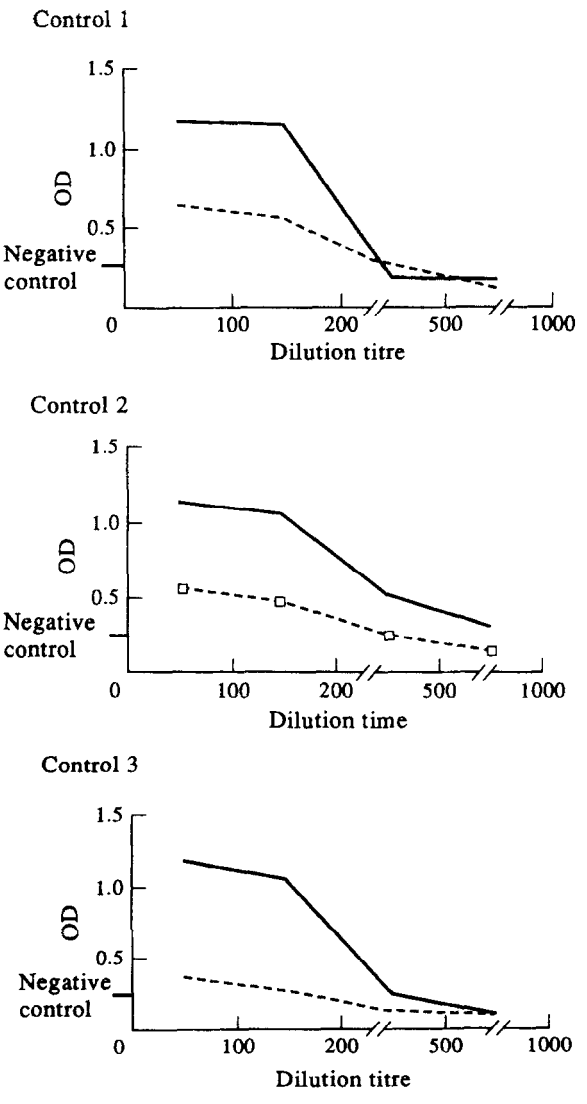

Figure 1. Plots of optical density observed in the ELISA assay of three sera known to be positive for antibodies in the western immunoblot assay. Each serum was preabsorbed with cyanogen bromide sepharose beads conjugated with p53 protein at the dilution titres of the serum shown. All samples, including the negative control serum shown, are the means of readings. —— Positive control serum; ---positive control serum preincubated with p53.

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similar in outline to that employed by Winter and colleagues [12]. Full details of the technique are available from the authors. Briefly, monkey Cos-1 cells are transfected comprising 50% Harlow (conserved box 5) mutant and 50% rational (wild type), the immunoprecipated protein is run on an SDS PAGE gel, and subsequently transferred to nitrocellulose filters before being exposed to the diluted patient's serum. Detection is based on further incubation with ^{125}I-labelled protein A, and a band noted at a molecular weight of 53 kD in the case of positive sera (Figure 2).

Tissue sections from paraffin-embedded material on the corresponding biopsy were analysed by the avidin-biotin immunoperoxidase method using CM1 polyclonal antibody to p53 protein supplied by Dr D. Lane, University of Dundee, U.K., and a biotinylated swine anti-rabbit secondary antibody (Dako), according to the method of Hsu and colleagues [13]. Nuclear staining was classified as positive or negative with reference to p53 transfected Cos-1 cells, which were formalin fixed and embedded in paraffin.

RESULTS

Of the series of 26 breast cancer sera analysed by immunoblotting, four (15.4%) were found to give strong bands corresponding to the molecular weight of 53 kD. The ELISA method was then validated by comparison of the optical density plot of this series compared to the negative control, and the cut-off point was defined as 2.5 times the negative control. 2 of the 22 patients negative by the western technique were found to be positive by the ELISA method. The dilution titre of the six positives gave a linear curve within the dilution range 1:10 up to 1:5000, and for the analysis of the test sera, serum diluted 1:200 was used, a 4-fold greater dilution than employed in the immunoblotting method. Maximum detection of the p53 antibodies by the PAb 122 monoclonal was obtained at a p53 concentration of 3 μg/ml, 50 μl of which was applied to each well.

A separate series of 176 patients with breast cancer presenting to one unit over a period of 12 months was then analysed by the ELISA method with 45 (25.5%) found to be positive. Out of 76 control women, only 1 (1.3%) was positive. A further group of 36 clinically negative women in whom there was a family history of breast cancer contained four positive sera (11.1%). The incidence of auto-antibodies in healthy individuals with a breast cancer family history is significantly greater than that in healthy control patients (Fisher's exact test $P = 0.036$). Conversely, Table 1 shows that in women with breast cancer the incidence of auto-antibodies is lower in those with a family history compared to those without a family history (Yates χ^2 $P = 0.029$). While the numbers are small, there was no apparent effect of closeness to affected relative, and of the 7 patients who had more than 1 affected relative, none exhibited auto-antibodies to p53. There was no correlation seen with the bulk of tumour, the histological subtype of tumour, or the age of the patients (Table 1).

When the presence of the auto-antibodies in the sera by the ELISA technique was compared with immunocytochemical localisation of p53 protein in the corresponding tumours, a high proportion of seropositive patients contained p53 in their tumours (Table 2). Sixty per cent of patients with a family history of breast cancer had p53 protein detectable in their tumours, whereas only 9.1% of 33 cases in this category demonstrated serum auto-antibodies to p53.

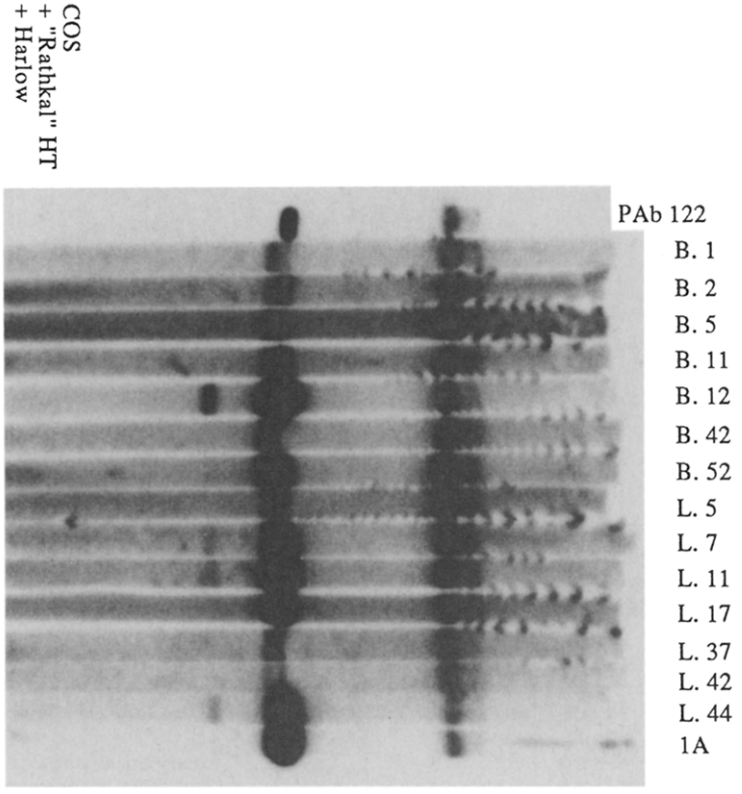

Figure 2. Western immunoblot of p53 protein (50% Harlow mutant: 50% rational) run on an SDS gel and detected after incubation with test serum, by radiolabelled protein A. In lane 1 the mouse monoclonal PAb 122 was applied, while in lanes 2–10, sera from advanced breast cancer patients are shown, and a dense band is demonstrated in lanes 3, 5, 6, 8 and 10 at a molecular weight corresponding to 53 kD. The remaining lanes contain sera from patients with advanced non-small cell lung cancer.

Table 1. Clinical correlations of p53 autoantibodies in breast cancer patients assessed by ELISA

	n	Positive	Per cent
Stage			
DCIS	23	8	34.8%
Stage I and II	98	21	21.4%
Stage III and IV	55	16	29.1%
Presentation			
Symptomatic	118	29	24.6%
Breast screening clinics	58	16	27.6%
Age (years)			
<50	53	14	26.4%
50–64	89	21	23.6%
>64	34	10	29.4%
Family history			
None	143	42	29.4%
Positive	33	3	9.1%
Histological type			
Ductal	130	27	20.8%
Lobular	17	8	47.1%
Other invasive	6	2	33.3%
DCIS	23	8	34.8%

Table 2. Serum p53 autoantibody status (ELISA) and tumour p53 protein expression by immunoperoxidase localisation with CM1 antibody in breast cancer patients

Serum status (ELISA)	p53 expression	*n*
Positive	12* (70.6%)	17
Negative	9* (24.3%)	37
Without family history	3 (11.1%)	27
With family history	6 (60%)	10
	*$\chi^2 = 8.6$ ($P = 0.003$)	

DISCUSSION

This is the first demonstration by ELISA of a high incidence (26%) of serum auto-antibodies to the putative tumour suppressor protein p53, a figure which approximates to the incidence of p53 mutations in breast cancer [14]. One possibility is that the auto-antibodies are raised against the mutant p53 protein, which in syngeneic tumour systems has been identified as auto-antigenic [15]. However, a more recent study of serum autoantibodies in breast cancer has suggested they react with the amino terminal part of the p53 protein, at sites distance from the mutational hot spots in the five evolutionarily conserved sites [16]. The incidence is considerably higher than the 11.5% reported by Crawford and colleagues [10] and the 15% in our own series of 26 using a western blotting technique. In a study of 199 cases of childhood cancer, Caron de Fromentel and colleagues [17] found an overall incidence of 12%, with the highest incidence (21%) in non-Hodgkin's lymphoma patients. The incidence of auto-antibodies would be expected to be higher if the deleted N-terminal peptide were included in the ELISA assay.

The correlation between serum auto-antibody detection and localisation of mutant p53 in the corresponding tumours is not absolute, and is consistent with the observation in lung cancer patients that they are mutation-related [12]. A mutation may conceivably give rise to a protein which is immunogenic, yet not detectable by immunochemistry, and the relationship between individual mutations, protein localisation and subsequent prognosis has yet to be defined for each tumour type. Preliminary data in breast cancer would suggest that nuclear p53 accumulation is a poor prognostic factor [8, 18], based on correlation with other prognostic factors in addition to survival data.

The most significant observation in this study is the demonstration of a lower incidence of autoantibodies to p53 protein in those with breast cancer and a family history of the disease than in patients with breast cancer but an absence of family history. One early theory was that the majority of familial breast cancer could be associated with a germ line p53 mutation, but it is now clear that with the exception of some, but not all, cases of the Li-Fraumeni syndrome [19, 20], the majority of breast cancer patients with a family history carry no such genetic alteration [21, 22]. In their epitope mapping study, Schlichtholz and colleagues [16] concluded that the most likely explanation for the development of the serum auto-antibodies was a loss of tolerance induced by accumulation of p53 protein at sites either in the nucleus or in the process of translocation to the cell membrane. In the present study, one explanation for the differing incidence of auto-antibodies seen in relation to family history is related to tolerance induced to the protein product of a germ line mutation, resulting in a lower incidence of auto-antibodies in a subset of the familial cases. In the sporadic cases, tolerance could be lost to the wild type protein as a result of excessive protein accumulation or conformational changes associated with complex formation with heat shock protein, for example [23].

These preliminary findings suggest a role for estimation of serum auto-antibodies in addition to immunocytochemistry and sequencing of mutations in the characterisation of familial and sporadic breast cancers. These serological changes may also prove useful as a non-invasive method of assessment of prognosis of both early and late breast cancer.

1. Levine AJ. Cancer Surveys 12. Tumour suppressor genes, the cell cycle and cancer. 1992, 59–79.
2. Gerwin BI, Spillare E, Forrester K, *et al.* Mutant p53 can induce tumorigenic conversion of human bronchial epithelial cells and reduce their responsiveness to a negative growth factor, transforming growth factor β. *Proc Natl Acad Sci USA* 1989, **2**, 2759–2763.
3. Baker SJ, Markowtiz S, Fearon ER, Wilson JKV, Vogelstein B. Suppression of human colorectal carcinoma cell growth by wild type p53. *Science* 1990, **24**, 912–915.
4. Varley JM, Bramma WJ, Lane DP, Swallow JE, Dolan C, Walker RA. Loss of chromosome 7p13 sepuences and mutation of p53 in Luman breast carcinomas. *Oncogene 1991*, **6**, 413–421.
5. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. *Science* 1991, **25**, 49–53.
6. Barnes DM, Hanby AM, Gillett CE, *et al.* Abnormal expression of a wild type p53 protein in normal cells of a cancer family patient. *Lancet* 1992, **340**, 259–262.
7. Crook T, Wrede D, Tidy JA, Mason WP, Evans DJ, Vousden KH. Clonal p53 mutation in primary cervical cancer: association with human papillomavirus negative tumours. *Lancet* 1992, **339**, 1070–1073.
8. Cattoretti G, Rilke F, Andreola S, D'Amato L, Delia D. p53 expression in breast cancer. *Int J Cancer* 1988, **41**, 178–183.
9. Davidoff AM, Humphrey PA, Iglehart JK, Marks JR. Genetic basis

for p53 overexpression in human breast cancer. *Proc Natl Acad Sci USA* 1991, **88**, 5006–5010.
10. Crawford LV, Pim DC, Lamb P. The cellular protein p53 in human tumours. *Mol Biol Med* 1984, **2**, 261–272.
11. Genzt R, Chen GH, Rosen CA. Bioassay for trans-activation using purified human immuno deficiency virus rat-encoded protein: trans-activation requires mRNA synthesis. *Proc Natl Acad Sci USA* 1989, **86**, 821–824.
12. Winter SF, Minna JD, Johnson BE, Takahashi T, Gazdar AF, Carbone DP. Development of antibodies against p53 in lung cancer patients appears to be dependent on the type of p53 mutation. *Cancer Res* 1992, **52**, 4168–4174.
13. Hsu SH, Raine L, Frazer H. Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase technique: a comparison between ABC and unlabelled antibody (PAP) techniques. *J Histochem Cytochem* 1988, **19**, 577–579.
14. Coles C, Condie A, Chetty U, Steel CM, Evans HJ, Prosser J. p53 mutations in breast cancer. *Cancer Res* 1992, **52**, 5291–5298.
15. De Leo AB, Jay G, Appella E, Dubois GC, Law LW, Old LJ. Detection of a transformation related antigen in chemically induced sarcomas and other transformed cells of the mouse. *Proc Natl Acad Sci USA* 1979, **76**, 2420–2424.
16. Schlichtholz B, Legros Y, Gillet D, *et al.* The immune response to p53 in breast cancer patients is directed against immunodominant epitopes unrelated to the mutational hot spot. *Cancer Res* 1992, **52**, 6380–6384.
17. Caron de Fromentel C, May-Levin F, Mouriesse H, *et al.* Presence of circulating antibodies against cellular protein p53 in a notable proportion of children with B-cell lymphoma. *Int J Cancer* 1987, **39**, 185–189.
18. Thor AD, Moore II DH, Edgerton SM, *et al.* Accumulation of p53 tumor suppressor gene protein: an independent marker of prognosis in breast cancers. *J Natl Cancer Inst* 1992, **84**, 845–855.
19. Malkin D, Li FP, Strong LC, *et al.* Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas and other neoplasms. *Science* 1990, **250**, 1233–1238.
20. Srivastava S, Zou Z, Pirollo K, Blattner W, Chang EH. Germ line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. *Nature* 1990, **348**, 747–749.
21. Warren W, Eeles RA, Ponder BA, *et al.* No evidence for germline mutations in exons 5–9 of the p53 gene in 25 breast cancer families. *Oncogene* 1992, **7**, 1043–1046.
22. Sidransky D, Takashi T, Helzlsouer K, *et al.* Inherited p53 gene mutations in breast cancer. *Cancer Res* 1992, **52**, 2984–2986.
23. Davidoff AM, Iglehart JD, Marks JR. Immune response to p53 is dependent upon p53/HSP70 complexes in breast cancers. *Proc Natl Acad Sci USA* 1992, **89**, 3439–3442.

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Efficacy and Tolerance of Oral Dipyrone Versus Oral Morphine for Cancer Pain

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In a double-blind, randomised and parallel clinical trial, two oral doses of dipyrone (1 and 2 g) administered every 8 h were compared with 10 mg of oral morphine given every 4 h for the relief of chronic cancer pain. A total of 121 patients with cancer pain without gastric involvement participated in a 7-day treatment course and were allocated to receive either dipyrone 1 g (n = 41), dipyrone 2 g (n = 38) or morphine (n = 42). Drug efficacy was analysed according to the degree of pain relief using a 100-mm visual analogue scale, and the number of patients who decided to increase the dose of the analgesic drug on day 4. The analgesic effect of dipyrone, 2 g every 8 h, was similar to that of morphine. The efficacy of both schedules was significantly greater than that of dipyrone, 1 g every 8 h. Dipyrone at either 1 or 2 g doses tended to be better tolerated than morphine, although the differences were not statistically significant.

Key words: dipyrone, morphine, analgesia, efficacy, tolerance, cancer pain
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INTRODUCTION

SEVERE CANCER pain is usually mitigated by opiates, particularly meperidine and morphine [1, 2]. Dipyrone has a potent analgesic action and has been extensively used in the treatment of acute painful conditions. The use of high doses of orally administered dipyrone to relieve severe pain including chronic cancer pain is a common practice in Spain. However, the optimal dose of dipyrone still has to be determined, and no clinical trials have been conducted to elucidate this question.

The aim of the present study was to compare the efficacy and tolerance of two different oral doses of dipyrone (1 and 2 g), administered every 8 h, with 10 mg of oral morphine given every 4 h for the relief of cancer pain. Morphine was selected as