OPIC
Office de la propriété intellectuelle du Canada

CIPO
Canadian Intellectual Property Office

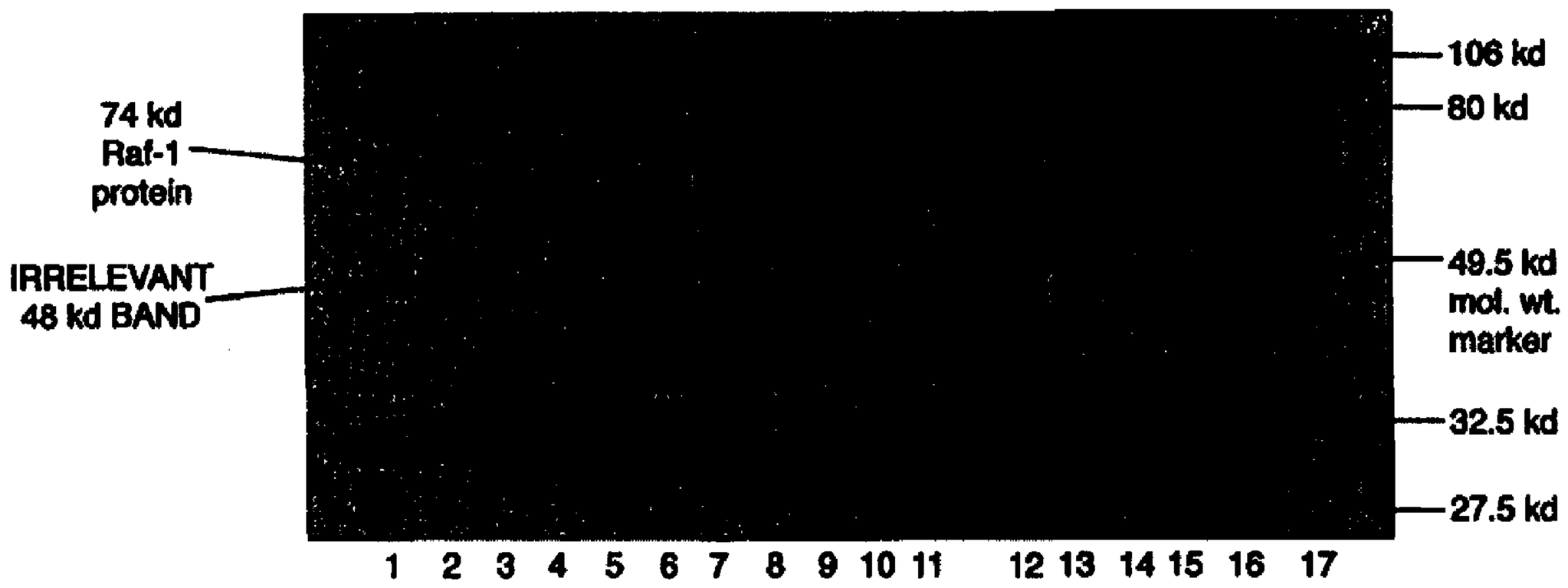
(12) (19) (CA) **Demande-Application**

(21) (A1) **2,321,480**
(86) 1999/02/18
(87) 1999/08/26

(72) WARENIUS, HILMAR MEEK, GB
(72) SEABRA, LAURENCE ANTHONY, GB
(71) THERYTE LIMITED, GB

(51) Int.Cl.[6] G01N 33/574, C07K 16/32
(30) 1998/02/18 (9803446.5) GB
(30) 1998/02/18 (9803447.3) GB
(30) 1998/06/05 (9812151.0) GB
(30) 1998/07/03 (9814545.1) GB
(30) 1999/02/10 (9903035.5) GB

(54) **TRAITEMENT DU CANCER**
(54) **TREATING CANCER**



(57) L'invention concerne un procédé de mesure de la radiosensibilité des cellules, ce procédé consistant à analyser un échantillon comprenant des cellules ou un extrait cellulaire afin de mesurer: (a) le niveau d'expression de p21 ou l'abondance de protéines p21; et (b) le niveau d'expression de Raf-1 ou l'abondance de protéines Raf-1. L'invention concerne également un ensemble permettant de mesurer la radiosensibilité des cellules, cet ensemble comprenant: (i) des moyens de mesure du niveau d'expression de Raf-1 ou de l'abondance de protéines Raf-1; et (ii) de moyens de mesure du niveau d'expression de p21 ou l'abondance de protéines p21.

(57) Provided is a method for measuring the radiosensivity of cells, which method comprises testing a sample comprising cells or an extract therefrom for: (a) the level of expression of p21, or for the abundance of p21 protein; and (b) the level of expression of Raf-1, or for the abundance of Raf-1 protein. The invention also provides a kit for measuring the radiosensitivity of cells, which kit comprises: (i) a means for testing for the level of expression of Raf-1 or for the abundance of Raf-1 protein; and (ii) a means for testing for the level of expression of p21 or for the abundance of p21 protein.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
G01N 33/574, C07K 16/32

A1

(11) International Publication Number: WO 99/42837

(43) International Publication Date: 26 August 1999 (26.08.99)

(21) International Application Number: PCT/GB99/00509

(22) International Filing Date: 18 February 1999 (18.02.99)

(30) Priority Data:

9803446.5	18 February 1998 (18.02.98)	GB
9803447.3	18 February 1998 (18.02.98)	GB
9812151.0	5 June 1998 (05.06.98)	GB
9814545.1	3 July 1998 (03.07.98)	GB
9903035.5	10 February 1999 (10.02.99)	GB

(71) Applicant *(for all designated States except US)*: THERYTE LIMITED [GB/GB]; 5 Castle Street, Liverpool L2 4XE (GB).

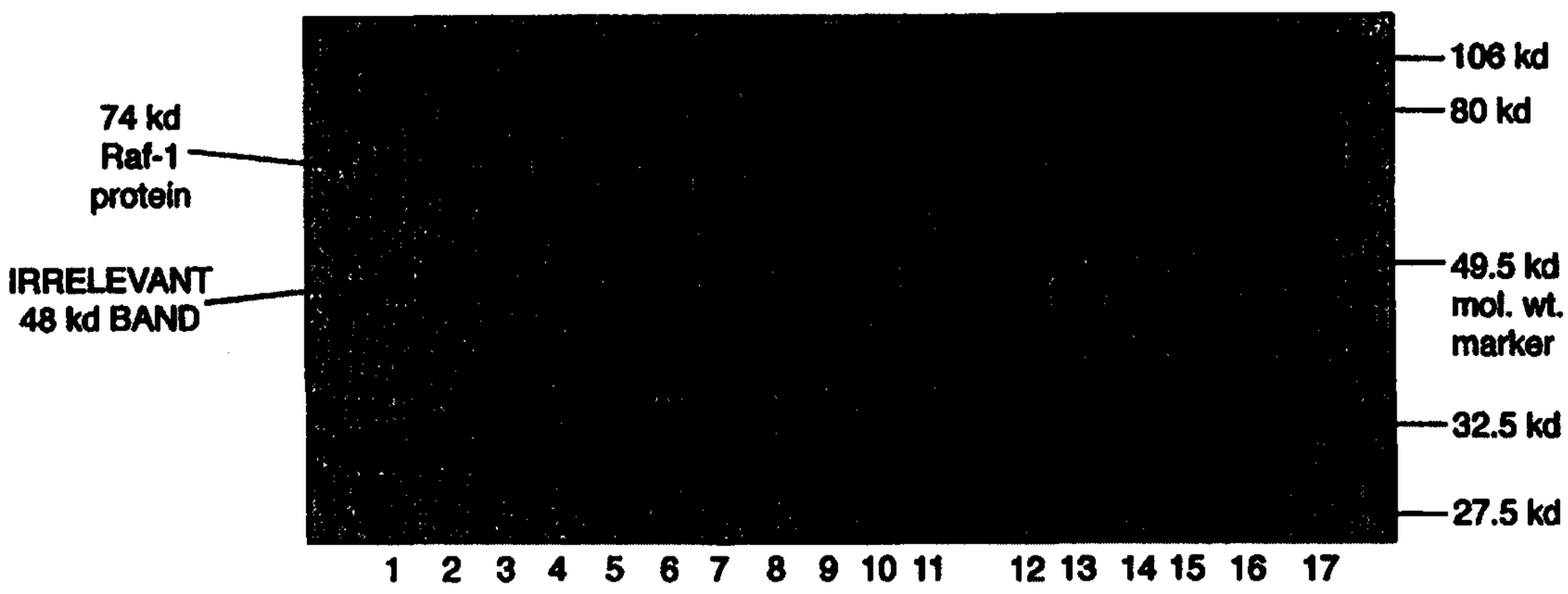
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WARENIUS, Hilmar, Meek [GB/GB]; 14 Delavor Road, Heswall, Wirral L60 4RN (GB). SEABRA, Laurence, Anthony [GB/GB]; 12 Richmond Way, Heswall, Wirral L61 6XH (GB).

(74) Agents: DANIELS, Jeffrey, Nicholas et al.; Page White & Farrer, 54 Doughty Street, London WC1N 2LS (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: TREATING CANCER



(57) Abstract

Provided is a method for measuring the radiosensivity of cells, which method comprises testing a sample comprising cells or an extract therefrom for: (a) the level of expression of p21, or for the abundance of p21 protein; and (b) the level of expression of Raf–1, or for the abundance of Raf–1 protein. The invention also provides a kit for measuring the radiosensitivity of cells, which kit comprises: (i) a means for testing for the level of expression of Raf–1 or for the abundance of Raf–1 protein; and (ii) a means for testing for the level of expression of p21 or for the abundance of p21 protein.

TREATING CANCER

The present invention concerns a method for measuring the radiosensitivity of cancer cells, to predict whether a patient is likely to respond to radiotherapy. The invention also concerns a method for producing an antibody useful in measuring radiosensitivity and a kit for measuring radiosensitivity.

Although radiotherapy has been responsible for curing many people of cancer in the latter half of this century, there still remain a large number of tumours which either show little response to treatment, or respond initially only to recur later. A better understanding of the mechanisms underlying the responsiveness of cancers to radiotherapy could help predict which patients are most likely to benefit from radiotherapy, and also holds the possibility of selectively modulating these mechanisms to improve the treatment of human cancer using radiotherapy.

The molecular basis of intrinsic radiosensitivity has been under investigation for many years. A considerable body of research has focused on the degree of DNA damage and its subsequent repair as reflected in the incidence of double strand breaks (dsbs) in the DNA (Kelland *et al*, 1988; Schwartz *et al*, 1991), the residual damage remaining in the DNA after cellular rejoining of dsbs (Nunez *et al*, 1995; Whitaker *et al*, 1995), and the fidelity of DNA repair (Powell & McMillan, 1994). In addition to DNA damage, however, it has become increasingly apparent that certain oncogenes and tumour suppressor genes may not only be implicated in carcinogenesis, but can also influence the sensitivity of malignant cells to ionising radiation.

As a result of this growing evidence of the role of oncogenes and tumour suppressor genes in the sensitivity of malignant cells to therapeutic agents, attempts have been made to use these and other genes to try and predict the therapeutic response of human cancer to the presently available treatment modalities such as radiotherapy and/or cytotoxic chemotherapy. Research up to the present time, however, has generally attempted to only examine the expression of single tumour related genes as methods of predicting therapeutic response. When investigating the relationship between expression of a chosen

gene and intrinsic radiosensitivity, consideration has not been given as to whether other candidate genes than the one selected for study might also have an affect on the outcome of experiments.

Research into the role of individual genes has focused on a number of cell cycle genes and signal transduction genes. Transfection of normal cell lines with dominant oncogenes such as myc and ras (McKenna *et al*, 1991) has resulted in increased radioresistance even in the absence of detectable changes in the rate of dsb induction (Iliakis *et al*, 1990). Several other dominant oncogenes including c-fms, v-sis, v-erb-B, v-abl, v-src, v-cot (Fitzgerald *et al* 1990, Suzuki *et al*, 1992, Shimm *et al*, 1992) and c-Raf (Kasid *et al*, 1989, Pirollo *et al*, 1989) have also been reported to modulate cellular radiosensitivity in mammalian cells. The potential relevance of these findings to clinical radiotherapy has been emphasised by observations that high levels of Raf-1 (the normal protein product of the c-Raf-1 proto-oncogene) are related to intrinsic radiosensitivity in human *in vitro* cell lines (Warenius *et al*, 1994). However, these results are not sufficient alone to determine the sensitivity of a tumour to radiotherapy in a clinical assay.

An additional body of evidence indicates a positive relationship between mutation in the p53 tumour suppressor gene and increased cellular radioresistance in both rodent and human tumour cells (Fan *et al*, 1994, Radford 1994, Zhen *et al*, 1995, Xia *et al*, 1995, Lee and Bernstein 1993) and in normal cells transfected with mutant p53 (mp53) genes (Pardo *et al*, 1994, Bristow *et al*, 1994, Kawashima *et al*, 1995). Research in the public domain has suggested that mutations in the p53 tumour suppressor gene, which can be found in around 50% of common cancers such as those of the breast, lung and ovary, are associated with resistance to treatment with cytotoxic drugs or radiation. Despite a considerable body of work, however, there are at present no successful clinical tests by which the detection of mutations in the p53 gene alone can be used to predict with an acceptable degree of certainty whether or not a patient's cancer is likely to respond to radiotherapy. A wide disparity of results in clinico-pathological studies comparing tumour response and p53 status leads to the conclusion that at the present time p53 mutation or the over expression of p53 protein are not sufficient alone to predict whether or not a human cancer is likely to respond to radiotherapy.

A number of reports suggest that oncogenes and suppressor genes may modulate intrinsic radiosensitivity by their influence on the progress of irradiated cells through radiation-induced blocks at cell cycle checkpoints. G1/S delay, mediated by p53 following exposure to ionising radiation has been implicated as an important measure of cell cycle perturbation which correlates with relative radiation sensitivity (Kastan *et al*, 1991, McIlwrath *et al*, 1994; Siles *et al*, 1996). Also the expression of dominant oncogenes such as myc and ras (McKenna *et al*, 1991) or SV40 (Su & Little, 1993) has been shown to induce both radioresistance and a concomitant increase in post-radiation delay at the G2/M checkpoint. It has also been shown that the protein product of the normal c-Raf-1 proto-oncogene was related to radiosensitivity in 19 human *in vitro* cell lines (Warenius *et al*, 1994). Recently, it has further been shown that in 6 of the above 19 cell lines, the previously observed Raf-1/radiosensitivity relationship was very strong and related to how rapidly cells exited from a radiation-induced block at the G2/M cell cycle checkpoint. Those radiosensitive human cancer cells with increased expression of the normal Raf-1 protein exhibit more rapid exit from a G2/M block induced by 2Gy of radiation than radioresistant cells with low expression of Raf-1 (Warenius *et al*, 1996). High expression of the Raf-1 protein product of the normal c-Raf proto-oncogene is related to radiosensitivity. The relationship between Raf-1 and radiosensitivity is not, however, strong enough on its own to provide the basis of clinically useful predictive assays. The same is true of other attempts to correlate the effects of single genes to the success of therapies.

Unfortunately, little is known about whether, or how, oncogenes and suppressor genes may interact to influence the radiosensitivity phenotype of human cancer cells. However, transfection experiments using cells from other mammals, such as REF (rat embryo fibroblasts), have demonstrated greater increases in radioresistance in cells expressing dominant plus co-operating oncogenes than expressing the single dominant oncogenes alone (McKenna *et al*, 1990, Su & Little 1992, Pirollo *et al*, 1993). Similarly, radioresistance induced in REF cells by transfection with multiply integrated mutant p53-pro193 alleles was much greater when the mutant p53 gene was co-transfected with H-ras (Bristow *et al*, 1994).

It has been shown more recently (Warenius *et al*, 1994, 1996) that measuring Raf-1 protein in the context of wild-type p53 provides a correlation which could possibly provide the basis of a predictive assay for radiosensitivity. This relationship was demonstrated by measuring Raf-1 protein using quantitative Western blotting. Western blotting is, however, expensive, time consuming and laborious. Furthermore, it requires large numbers of cells. It is thus impractical as a routine clinical test in this particular case. A clinical assay is preferably capable of measuring protein levels in individual cells, rather than in homogenates of a million or more cells as used in Western blotting. It is also important to be able to distinguish Raf protein expression in tumour cells from that in normal cells. This requires the ability to gate out cells on the basis that they are diploid rather than aneuploid in flow cytometry assays, or the ability to measure Raf protein in individual cells that can be observed histologically on tissue sections where morphological criteria enable regions of tumour to be distinguished from connective tissue, blood vessels infiltrating white blood cells, or area of necrosis.

Unfortunately all available antibodies against Raf cross-react with an irrelevant epitope on a 48 kD molecule, when examined on Western blots (see Figure 1). Raf-1 is a 72-74 kD molecule and can thus be distinguished and separately measured on Western blotting. Cellular assays for Raf-1 such as flow cytometry or immunocytochemistry would not, however, be able to distinguish the correct 72-74 kD molecule from the irrelevant 48 kD molecule. The 48 kD protein is unlikely to be a fragment of the 72 kD Raf proto-oncogene because the 48 kD protein is much more abundant than the 72 kD protein on Western blotting.

Thus, on the basis of the above state of the art, at the present time there are no indicators that measuring the mutational status or levels of expression of the protein products of oncogenes, proto-oncogenes or tumour suppressor genes in human cancer cells would be able to provide the basis of a reliable clinical test for whether clinical tumours were likely to respond to drug and/or radiation treatment.

It is an object of this invention to overcome the above-mentioned problems and provide an assay which can be used as a clinical assay to predict whether cancer cells are likely to respond to radiotherapy. It is also an object of this invention to provide an antibody against Raf-1 which is specific for that protein, to facilitate cheaper diagnostic tests. A further object of the invention is to provide a method, using the antibody, for predicting whether cancer cells are likely to respond to radiotherapy by contemporaneously measuring the properties of two or more cancer-related genes.

Accordingly, the present invention provides a method for measuring the radiosensitivity of cells, which method comprises testing a sample comprising cells or an extract therefrom for:

(a) the level of expression of p21, or for the abundance of p21 protein; and

(b) the level of expression of Raf-1, or for the abundance of Raf-1 protein.

The order in which steps (a) and (b) is carried out is not particularly limited. Thus, step (a) may precede step (b), or alternatively step (b) may precede step (a).

The present invention also concerns a method for producing an antibody specific to Raf-1 protein, which antibody does not cross-react with a 48 kD protein co-present in cells containing Raf-1 protein, which method comprises forming a peptide which comprises or forms part of an epitope on the Raf-1 protein that is not present on the 48 kD protein, and preparing an antibody against the peptide. The invention concerns a further method for producing an antibody specific to Raf-1 protein, which antibody does not cross-react with a 48 kD protein co-present in cells containing Raf-1 protein, which further method comprises immunising an animal with Raf-1 protein and an antibody specific to the 48 kD protein so as to mask potential epitopic sites on Raf-1 protein which are also present on the 48 kD protein, and obtaining an antibody against the masked Raf-1 protein.

Furthermore, the present invention provides a kit for measuring the radiosensitivity of cells, which kit comprises:

(i) a means for testing for the level of expression of Raf-1 or for the abundance of Raf-1 protein; and

(ii) a means for testing for the level of expression of p21 or for the abundance of p21 protein.

This application specifically deals with measuring the levels of the Raf-1 protein product of the C-Raf-1 proto-oncogene, in cells whose p21 protein level has been determined (e.g. by Western blotting) to determine the radiosensitivity of the tumour and consequently whether radiotherapy is an appropriate treatment for the patient. In cell lines with elevated p21 protein levels (or elevation of p21 expression), the higher the level of Raf-1 expression the greater the sensitivity of the tumour to ionising radiation.

The level of expression of Raf-1, or the elevation of Raf-1 protein levels can be measured by any appropriate method, as discussed herein, e.g. Western blotting. The point at which it is considered that the protein level, or the expression, is elevated to a sufficient degree above normal indicating useful radiosensitivity, is clear to the skilled person in this field, according to general teaching from the literature regarding usual levels of Raf-1 in human cell lines (see European Journal of Cancer, B Oral Oncology, 1995, Nov., 31B(6), 384-391). This point can be determined according to the judgement of the individual carrying out the present method, depending on the particular cancer cells and patient involved.

The expression of p21, or the level of p21 protein can be measured by any appropriate method. Specifically, p21 is a cyclin dependent kinase inhibitor which can be detected by Western blotting, immunocytochemistry or newer developing techniques, such as determining the relative abundance of p21 mRNA. The point at which it is considered that the p21 expression is effectively elevated or the p21 protein levels are effectively elevated is clear to the skilled person in this field, according to general teaching from the literature regarding usual levels of p21 protein in human cell lines (see Oncogene, 1995, vol. 11, 2021-2028; and Oncogene, 1996, vol.12(6), 1319-1324).

The present invention will be described in further detail by way of example only with reference to the accompanying drawings, in which:

Figure 1 shows a Western blot demonstrating the range of Raf-1 protein levels per total cellular protein, in particular the relative abundance of the 74 kD and 48 kD proteins, in the following 17 human *in vitro* cell lines:

1. KB, oral epidermoid carcinoma
2. HT29, adenocarcinoma, colon
2. MGH-U1, bladder carcinoma
4. HRT18, adenocarcinoma, rectum
5. A431, squamous carcinoma vulva
6. NCTC 2544, skin fibroblasts
7. COR L23, large cell lung carcinoma
8. SK-MEL3, melanoma
9. AT5BIVA, ataxia telangiectasia fibroblast
10. OAW42, ovarian carcinoma
11. I407, embryonic intestinal epithelium
12. 2780, ovarian carcinoma
13. HEP-2, squamous carcinoma
14. HX142, neuroblastoma
15. RT112, bladder carcinoma
16. HeLa, squamous carcinoma
17. NCTC 2544

for a sample protein loading of 100 μg/50μl on a 7.5 % gel, the primary antisera being URP-2653 monoclonal against Raf-1 at a dilution of 1/750;

Figure 2 shows the relationship between radiosensitivity measured as SF2 and Raf-1 abundance in cell lines in which p21 protein levels are not elevated (undetectable); and

Figure 3 shows the relationship between radiosensitivity measured as SF2 and Raf-1 abundance in cell lines in which p21 protein levels are elevated.

Whilst the mechanisms explaining the observed relationships between radiosensitivity, cell cycle progress and the functions of Raf-1 and p21 remain obscure, the strong relationship between Raf-1 and radiosensitivity in human cancer cells in which p21 levels are detectable (preferably elevated), permits the development of a dual parameter Raf-1/p21 test for clinical radiosensitivity.

Figure 3 shows that in cell lines in which p21 protein levels are elevated, the higher the Raf-1 protein, the more radiosensitive the cells are as measured by log surviving fraction at 2 Gy (SF2). On the other hand, Figure 2 shows that in cell lines in which p21 is undetectable there is little or no relationship between Raf-1 levels and radiosensitivity.

The clinical test provided by the present invention requires determination of the level of expression of p21 or the level of p21 protein, in conjunction with measuring the level of Raf-1 expression in biopsy material from tumours in patients.

Determination of the expression level of Raf-1 is effected by measuring the abundance of the Raf-1 protein. Raf-1 protein levels can be measured by immunocytochemistry or flow cytometry (FCM). Previously there was a problem with the latter approach arising out of cross-reactivity of existing antibodies with non-Raf-1 proteins. Until the present, there were no available antibodies to Raf-1 which did not also cross-react with a very abundant but irrelevant 48 kD protein on Western blotting. Techniques such as immunocytochemistry or FCM would only give non-specific results. This necessitated some form of molecular separation, such as by electrophoresis in Western blotting, to separate Raf-1 (a 72-74 kD protein) from the irrelevant 48 kD species. Column chromatography techniques were appropriate, such as gel filtration or ion exchange chromatography. High Performance Liquid Chromatography or Capillary Electrophoresis were also usable as separation techniques. These could all be followed by an immunoassay. Other means of specific recognition may also have been conceivable, including the development of RNA aptamers to the Raf-1 protein. However, all of these

techniques are time consuming and expensive, making them inappropriate for a clinical test. The availability of an antibody specific to Raf-1 allows diagnostic assays to be carried out without the need for separation.

The first method for producing the antibody according to the present invention required isolation and identification of the 48 kD cross-reacting epitope, so that its DNA and protein genetic sequence could be determined. This information was used to compare the 48 kD protein sequence with the full length Raf proto oncogene protein sequence to choose an epitope on the full length proto-oncogene protein that is not shared by the 48 kD protein. This could be achieved using a cell line with high Raf-1 and 48 kD protein levels, such as NCTC 2544 (see Figure 1). Quantities of lysate were produced and the 48 kD protein purified. Purification could be achieved using immunoprecipitation or affinity purification with a monoclonal antibody produced by the inventors, or with a commercial anti-Raf-1 antibody, both of which have been shown on Western blotting to bind strongly to both the 72-74 kD and the 48 kD protein. The cells producing the monoclonal antibody were grown up in high yields for affinity chromatography as ascites in Balb/C mice. The antibody from the ascitic fluid was reacted with cyanogen bromide sephacryl and the antibody-sephacryl used to make an affinity column. The lysates from the NTCT cells were loaded onto the affinity column, non-specific material was washed through and 48 kD and 72-74 kD molecules sharing the same epitope and bound to the antibody on the column were eluted at low pH. The immunoprecipitate or eluate from the affinity chromatography column was then concentrated, a protein estimation carried out and 150 µg per well was run on 10-20 adjacent wells in 10 % SDS poly-acrylamide gel electrophoresis with molecular markers. The 48 kD band was then excised and as long an amino acid sequence as possible was sequenced from the N-terminus (or alternatively from the C-terminus). Using the peptide sequence primers were prepared whose genetic sequences match the protein sequences (allowing for the degeneracy in the coding for certain amino acids) for the N- (or C-) terminus. A series of PCRs was run until a 48 kD length of DNA was obtained. This was then sequenced. A sequence comparison between the 48 kD and the 72-74 kD full length Raf-1 proto-oncogene enabled the rational design of synthetic peptides from potential epitopes on the full length 72-74 kD Raf proto-oncogene protein, which were not present on the 48 kD protein. The unique

synthetic peptides were used to prepare polyclonal and monoclonal antibodies which reacted against the Raf-1 protein, but not the 48 kD protein, these antibodies being valuable in flow or confocal microscopic cytometry and/or immunofluorescence or immunocytochemistry.

The second method for producing the antibody according to the present invention requires immunisation of an animal with Raf protein in addition to an antibody against the 48 kD protein. For example, either the 72-74 kD full length Raf-1 protein (produced from the DNA sequence in an expression vector in bacteria) can be pre-incubated with previously available anti Raf-1 antibodies (which cross-react with the 48 kD protein) and the resulting immune complexes separated by centrifugation and then injected into an animal (such as a mouse), or the anti Raf-1 (anti 48 kD protein) antibodies and the full length 72-74 kD Raf-1 protein can be injected separately into the same animal. Polyclonal and monoclonal antibodies were prepared using the full length 72-74 kD Raf proto-oncogene protein as immunogen. The protein was produced by the recombinant Raf-1 gene in an expression vector. The antibody against the 48 kD protein was a commercially available antibody. Its function was to cover potential epitopic sites on the Raf protein which cross-react with the 48 kD protein. Such a masked Raf protein immunogen more selectively stimulates the production of antibodies which recognise the full length 72-74 kD Raf proto-oncogene protein rather than the 48 kD protein.

Oligonucleotide Arrays

Determination of mRNA levels can be effected in a number of ways. One can readily convert poly-A bearing mRNA to cDNA using reverse transcription - a method is described in the example illustrating this invention. Reverse Transcriptase PCR (RTPCR) methods allow the quantity of single RNAs to be determined, but with a relatively low level of accuracy. Arrays of oligonucleotides are a relatively novel approach to nucleic acid analysis, allowing mutation analysis, sequencing by hybridisation and mRNA expression analysis. Methods of construction of such arrays have been developed, (see for example: A.C. Pease *et al.* Proc. Natl. Acad. Sci. USA. **91**, 5022 - 5026, 1994; U. Maskos and E.M. Southern, Nucleic Acids Research **21**, 2269 - 2270, 1993; E.M. Southern *et al*, Nucleic Acids Research **22**, 1368 - 1373, 1994) and

further methods are envisaged. Arrays that measure expression levels of mRNAs and detect mutations in those RNAs are being developed and these offer an attractive embodiment of the diagnostic test proposed by this invention.

Immunocytochemistry

An alternative embodiment of this invention can measure Raf-1 protein levels by immunocytochemistry using confocal laser fluorescence microscopy. Preferably a scanning system is used such as those described in PCT/US91/09217, PCT/NL/00081 and PCT/US95/01886. Additionally, it is desirable that the microscopy system is also able to analyse multiple fluorescent dyes. In a preferred embodiment, antibodies against p21 are labelled with one dye, an antibody against Raf-1 is labelled with a second dye whilst a third DNA binding dye can be used to select for aneuploid cells. DNA binding dyes such as Hoechst 33258 dye, which binds AT-rich DNA or Chromomycin A_3, which binds GC-rich DNA, are appropriate. A diagnostic test may comprise the steps of:

- Extracting a biopsy of the tumour from a patient.
- Optionally micro-dissecting that material to separate normal tissue from tumour material.
- Preparing the biopsy material for microscopy which includes the steps of:
  - ♦ Labelling the biopsy material with the above fluorescently labelled antibody probes against Raf-1. The biopsy material may also, optionally be labelled with antibody probes against p21 and with a DNA binding dye.
  - ♦ Separating the labelled cells from unbound labelled probes.
- Placing the labelled biopsy material in a scanning confocal microscope to count cells that:
  - ♦ Over-express or show elevated levels of Raf-1, i.e. are labelled with at least a threshold quantity of antibody against Raf-1.
  - ♦ Optionally, express p21, i.e. are labelled with at least the threshold quantity of antibodies against p21. Alternatively, p21 expression might be determined by analysis of the mRNA or genomic DNA as discussed above.
  - ♦ Optionally, have chromosomal amplifications as detected by the intensity of fluorescence from DNA binding fluorescent dyes.

Fluorescence Activated Cell Sorting

A further embodiment of the diagnostic test can exploit Fluorescence Activated Cell Sorting (FACS). A FACS instrument separates cells in a suspension in a manner dependent on the cells being labelled with a fluorescent marker. A typical FACS device operates as follows. Cells in a suspension travelling in single file are passed through a vibrating nozzle which causes the formation of droplets containing a single cell or none at all. The droplets pass through a laser beam. Fluorescence excited from each individual cell in its droplet by the laser is measured. After the detector the stream of cells in suspension pass through an electrostatic collar which gives the droplets a surface charge. The cells carrying droplets are given a positive or negative charge. If the drop contains a cell that fluoresces with an intensity above a particular threshold, the drop gets a charge of one polarity. Unlabelled cells get a charge of the opposite polarity. The charged droplets are then deflected by an electric field and depending on their surface charge are directed into separate containers and are counted Droplets that contain more than one cell scatter light more than individual cells which is readily detected and so these are left uncharged and enter a third disposal container. Multi-channel fluorescent detection devices have been constructed that can separate cells on the basis of labelling with multiple different fluorescent labels. These have multiple lasers which can excite fluorescence at different frequencies and the detector will detect different emission frequencies. A three label system is appropriate for this test. The same labelled probes as those described above for use in a confocal scanning fluorescence microscope would be appropriate. A diagnostic test might comprise the steps of:

- Extracting a biopsy of the tumour from a patient.
- Optionally micro-dissecting that material to separate normal tissue from tumour material.
- Disrupting intracellular adhesion to form a single cell suspension.
- Labelling the suspended cells with the above fluorescently labelled probes against Raf-1. The biopsy material may also, optionally be labelled with antibody probes against p21 and with a DNA binding dye.
- Separating the labelled cells from unbound labelled probes.
- Passing the labelled cell suspension through a FACS device to count cells that:

- Over-express or show elevated levels of Raf-1, i.e. are labelled with the anti-Raf-1 antibody above a threshold for 'normal' expression.
- Optionally, express p21, i.e. are labelled with at least a threshold quantity of antibody against p21.
- Optionally, have chromosomal amplifications as detected by the intensity of fluorescence from DNA binding fluorescent dyes.

Example 1

A number of cell lines were selected and their radiosensitivity was tested in combination with their levels of Raf-1 protein. The p21 and Raf-1 expression were each measured using Western blotting according to substantially standard methods. The results for cell lines in which p21 is undetectable are shown in Figure 2 and the corresponding results for cell lines in which p21 levels are elevated are shown in Figure 3.

Materials and Methods

Cell lines

Growth characteristics clonogenic assay procedures of the human *in vitro* cell lines used in this analysis have already been reported (Warenius *et al* 1994). The cell lines used in this analysis are listed, with their histological classification in Table 1. All are well established; many having been growing *in vitro* for several years. Cell lines were either donations or purchased by our laboratories. On receipt all were grown for 5 passages to provide sufficient cells for batch storage in liquid nitrogen. During this period contamination was excluded by at least one passage in antibiotic free medium and mycoplasma testing was carried out on all lines. For cell survival assays, cells were taken from a designated primary liquid nitrogen batch and grown for 3-6 passages until there were sufficient well-growing cells. Further batches from these cells were frozen in liquid nitrogen. Cells were routinely maintained in DMEM medium except RT112 and H322, which were grown in RPMI1640 and MGHU-1 which were grown in Ham's F12 medium. All lines were supplemented with 10% heat-inactivated foetal calf serum (HIFCS).

Table 1

	Cell Line
I407	Embryonic intest. epith.
HEP 2	Squamous carcin. larynx
MGHU 1	Transit. Carcinoma bladder
HRT 18	Adenocarcinoma rectum
2780	Ovarian carcinoma
OAW 42	Ovarian carcinoma
HT 29/5	Adenocarcinoma colon
COLO 320	Adenocarcinoma colon
H 322	Small cell carcinoma lung
H 417	Small cell carcinoma lung
RPMI 7951	Melanoma
RT 112	Transit. Carcinoma bladder
MOR	Adenocarcinoma lung
MEL 2	Melanoma

The relationship between Raf-1 levels, p21 levels and radiosensitivity was examined for the cell lines. In cells in which p21 protein levels were elevated, a useful correlation was found between Raf-1 protein levels and radiosensitivity.

Thus, in cell lines in which p21 protein levels are elevated, the higher the Raf-1 levels, the more likely it is that the cells are radiosensitive (Figure 3). This correlation is not found in cell lines in which p21 protein is not detectable (Figure 2).

References

Barraclough *et al*, J. Cell Physiolog **131**: 393 - 401, 1987

Bristow *et al.*, Oncogene **9**: 1527 - 1536, 1994.

Bristow *et al.*, Radiotherapy and Oncology **40**: 197 - 223, 1996.

P. W. G. Browning, "Proto-oncogene expression and intrinsic radiosensitivity, PhD Thesis, University of Liverpool, 1997.

Chirgwin *et al*, Biochemistry **18**: 5294 - 5299, 1979.

Deacon *et al*, Radiotherapy and Oncology **2**, 317 - 323, 1984.

Fan *et al*, Cancer Res. **54**: 5824 -5830, 1994.

Fertil & Malaise, Int. J. Radiat. Oncol. Biol. Phys. 7: 621 - 629, 1981.

FitzGerald *et al*, Radiat. Res. **122**: 44 - 52, 1990.

Hollstein *et al*, Science **253**: 49 - 53, 1991.

Iliakis *et al.*, Cancer Res. **50**: 6575 - 6579, 1990.

Kasid *et al.*, Cancer Res. **49**: 3396 - 3400, 1989.

Kastan *et al*, Cancer Res. **51**: 6304 - 6311, 1991.

Kawashima *et al.*, Int. J. Cancer **61**: 76 - 79, 1995.

Kelland *et al*, Radiat. Res. **116**: 526 - 538, 1988

Lee and Bernstein, Proc. Natl. Acad. Sci. USA **90**: 5742 - 5746, 1993.

McIlwrath *et al.*, Cancer Res. **54**: 3718 - 3722, 1994.

McKenna *et al.*, Cancer Res. **50**: 97 - 102, 1990.

McKenna *et al.*, Radiat. Res. **125**: 283 - 287, 1991

Nunez *et al*, Br. J. Cancer **71**: 311 - 316, 1995

Pardo *et al.*, Radiat. Res. **140**: 180 - 185, 1994

Pirollo *et al.*, Int. J. Radiat. Biol. **55**: 783 - 796, 1989

Pirollo *et al.*, Radiat. Res. **135**: 234 - 243, 1993.

Powell & McMillan, Int. J. Rad. Oncol. Biol. Phys., **29**: 1035 - 1040, 1994.

Radford, Int. J. Radiat. Biol. **66**: 557 - 560, 1994.

Sanger *et al*, Proc. Natl. Acad. Sci. USA **74**: 5463 - 5467, 1977.

Schwartz *et al.*, Int. J. Radiat. Biol. **59**: 1314 - 1352, 1991.

Shimm *et al.*, Radiat. Res. **129**: 149 - 156, 1992.

Siles *et al.*, Br. J. Cancer **73**: 581 - 588, 1996.

Su & Little, Int. J. Radiat. Biol. **62**: 461 - 468, 1992.

Su & Little, Radiat. Res. **133**: 73 - 79, 1993.

Suzuki *et al.*, Radiat. Res. **129**: 157 - 162, 1992.

Warenius *et al.*, Eur. J. Cancer **30**, 369 - 375, 1994.

Warenius *et al.*, Rad. Research **146**,485 - 493, 1996.

Whitaker *et al.*, Int. J. Radiat. Biol. **67**: 7 - 18, 1995.

Xia *et al.*, Cancer Res. **55**: 12 - 15, 1995.

Zhen *et al.*, Mut. Res. **346**, 85 - 92, 1995.

CLAIMS:

1. A method for measuring the radiosensitivity of cells, which method comprises testing a sample comprising cells or an extract therefrom for:

(a) the level of expression of p21, or for the abundance of p21 protein; and

(b) the level of expression of Raf-1, or for the abundance of Raf-1 protein.

2. A method according to claim 1, wherein the testing is carried out using a probe for Raf-1 mRNA or using an antibody specific to Raf-1 protein.

3. A method according to claim 2, wherein the antibody does not cross-react with a 48 kD protein co-present with the Raf-1 protein in the sample.

4. A method according to claim 3, wherein the antibody is obtainable by forming a peptide which comprises or forms part of an epitope on the Raf-1 protein, which epitope is not present on the 48 kD protein, and preparing the antibody against the peptide.

5. A method according to claim 3, wherein the antibody is obtainable by immunising an animal with Raf-1 protein and an antibody specific to the 48 kD protein so as to mask an epitopic site on Raf-1 protein that is also present on the 48 kD protein, and obtaining the antibody against the masked Raf-1 protein.

6. A method according to claim 4 or claim 5, wherein the antibody is a monoclonal antibody.

7. A method according to any of claims 2-6, wherein the antibody is a labelled antibody.

8. A method according to claim 7, wherein the label is a fluorescent label.

9. A method according to any preceding claim, further comprising contacting the sample with a DNA binding dye for labelling aneuploid cells.

10. A method according to claim 9, wherein the DNA binding dye is Hoechst 33258, or Chromomycin A_3.

11. A method according to any preceding claim, wherein the sample is a sample of cells.

12. A method according to claim 11, wherein the testing is carried out by performing a cell count.

13. A method according to claim 12, wherein the cell count is performed using multi-parameter flow cytometry.

14. A method according to claim 12, wherein the cell count is performed using scanning confocal microscopy.

15. A method according to claim 12, wherein the cell count is performed using fluorescence activated cell sorting.

16. A method according to any of claims 12-15, wherein the sample of cells is micro-dissected prior to performing the cell count, to separate normal tissue from tumour tissue.

17. A method according to any of claims 12-16, wherein prior to performing the cell count, intracellular adhesion in the sample is disrupted, to form a single cell suspension.

18. A method for producing an antibody specific to Raf-1 protein, which antibody does not cross-react with a 48 kD protein co-present in cells containing Raf-1 protein, which method comprises forming a peptide which comprises or forms part of an epitope on the Raf-1 protein that is not present on the 48 kD protein, and preparing an antibody against the peptide.

19. A method for producing an antibody specific to Raf-1 protein, which antibody does not cross-react with a 48 kD protein co-present in cells containing Raf-1 protein, which method comprises immunising an animal with Raf-1 protein and an antibody specific to the 48 kD protein so as to mask potential epitopic sites on Raf-1 protein which are also present on the 48 kD protein, and obtaining an antibody against the masked Raf-1 protein.

20. An antibody obtainable according to a method as defined in claim 18 or claim 19.

21. An antibody specific to Raf-1 protein, which antibody does not cross-react with a 48 kD protein co-present in cells containing Raf-1 protein.

22. An antibody according to claim 20 or claim 21, which is a monoclonal antibody.

23. A method for selecting an agent for treating cancer, which method comprises:

(a) testing a sample comprising cells in which p21 is expressed or in which p21 protein is detectable, or an extract therefrom, for the level of expression of Raf-1 or for the abundance of Raf-1 protein; and

(b) if Raf-1 is over-expressed, and/or Raf-1 protein is present at elevated levels, selecting ionising radiation for treatment;

(c) if Raf-1 is not over-expressed and/or Raf-1 protein is substantially not present at elevated levels, selecting for treatment an agent other than ionising radiation.

24. A method according to claim 23, wherein the selection for treatment is carried out according to step (c) and the agent other than ionising radiation is an agent comprising a platinating agent, such as CDDP, and/or a taxane, such as taxol.

25. A kit for measuring the radiosensitivity of cells, which kit comprises:

(i) a means for testing for the level of expression of Raf-1 or for the abundance of Raf-1 protein; and

(ii) a means for testing for the level of expression of p21 or for the abundance of p21 protein.

26. A kit according to claim 25, wherein the means for testing for the abundance of Raf-1 protein comprises a probe for Raf-1 mRNA, or an antibody specific to Raf-1 protein.

27. A kit according to claim 26, wherein the antibody is an antibody as defined in any of claims 21-23.

28. A kit according to claim 26 or claim 27, wherein the antibody is a labelled antibody.

29. A kit according to claim 28, wherein the label is a fluorescent label.

30. A kit according to any of claims 25-29, further comprising a DNA binding dye for labelling aneuploid cells.

31. A kit according to claim 30, wherein the DNA binding dye is Hoechst 33258, or Chromomycin A_3.

32. Use of a means for testing for the level of expression of Raf-1 or for the abundance of Raf-1 protein, for measuring the radiosensitivity of cells.

33. Use of a means for testing for the level of expression of p21 or for the abundance of p21 protein, for measuring the radiosensitivity of cells.

#2321480

UNSCANNABLE ITEM

RECEIVED WITH THIS APPLICATION

(ITEM ON THE 10TH FLOOR ZONE 5 IN THE FILE PREPARATION SECTION)

DOCUMENT REÇU AVEC CETTE DEMANDE

NE POUVANT ÊTRE BALAYÉ

(DOCUMENT AU 10 IÈME ÉTAGE AIRE 5 DANS LA SECTION DE LA

PRÉPARATION DES DOSSIERS)

Fig.2.

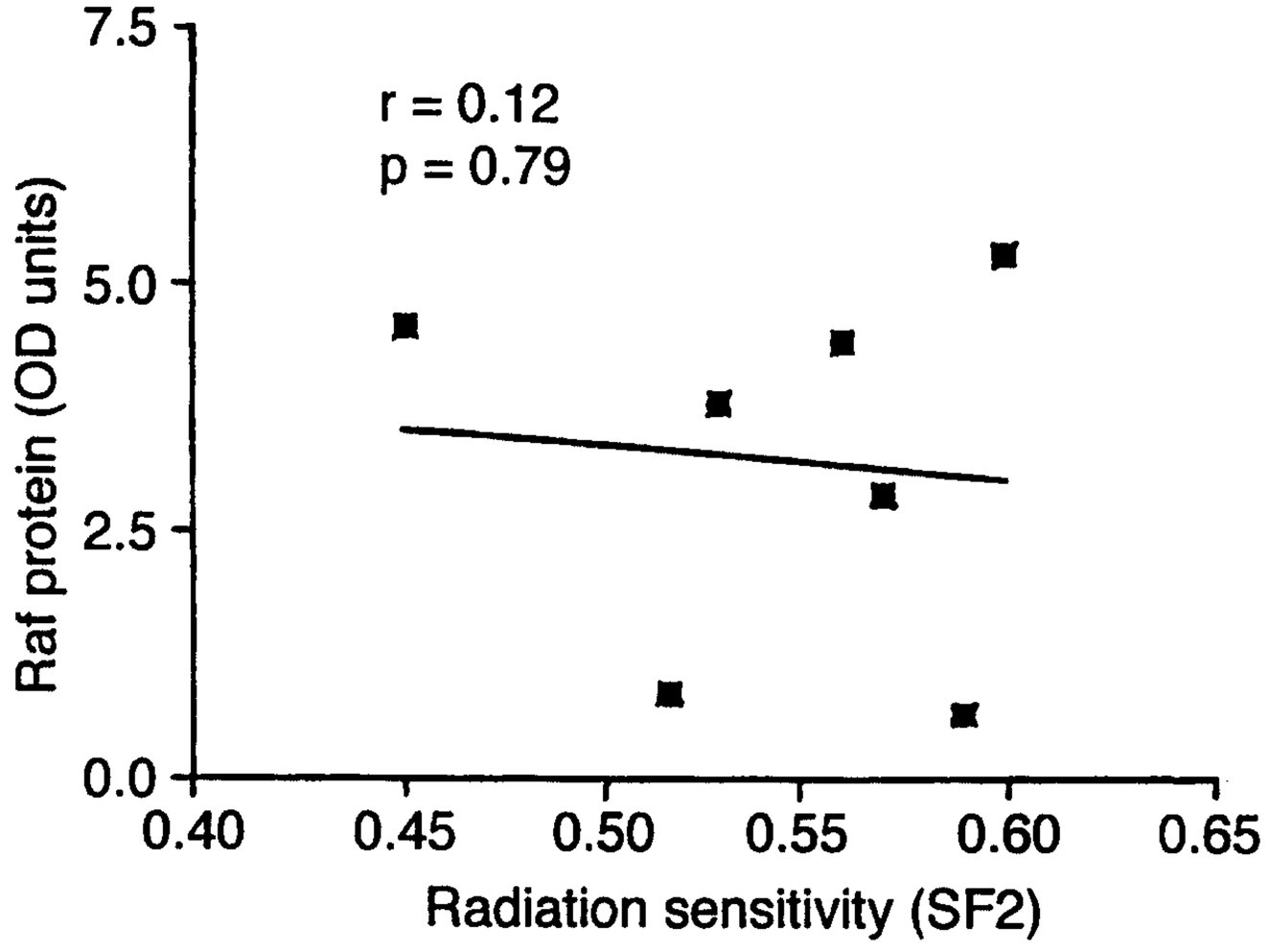
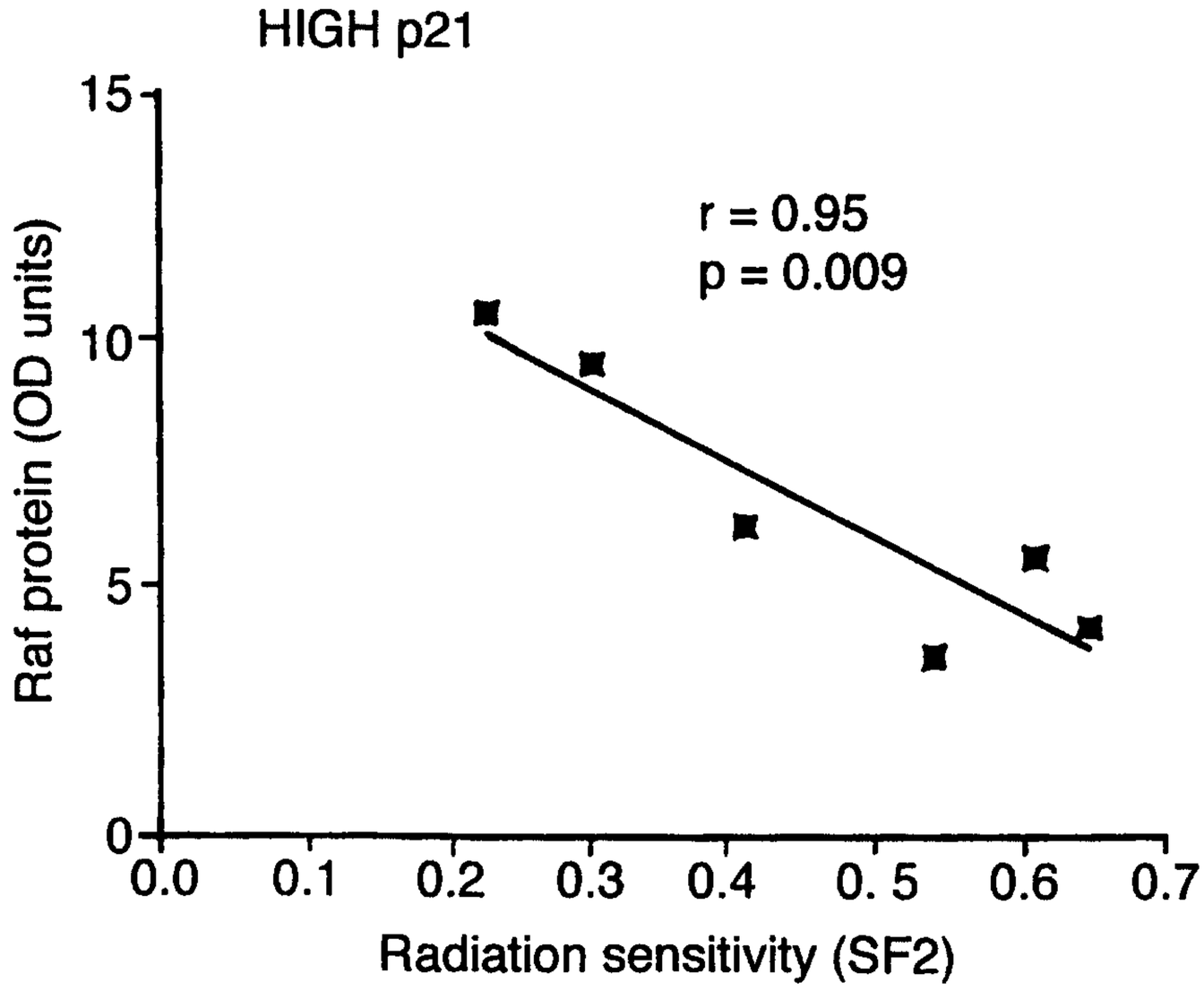
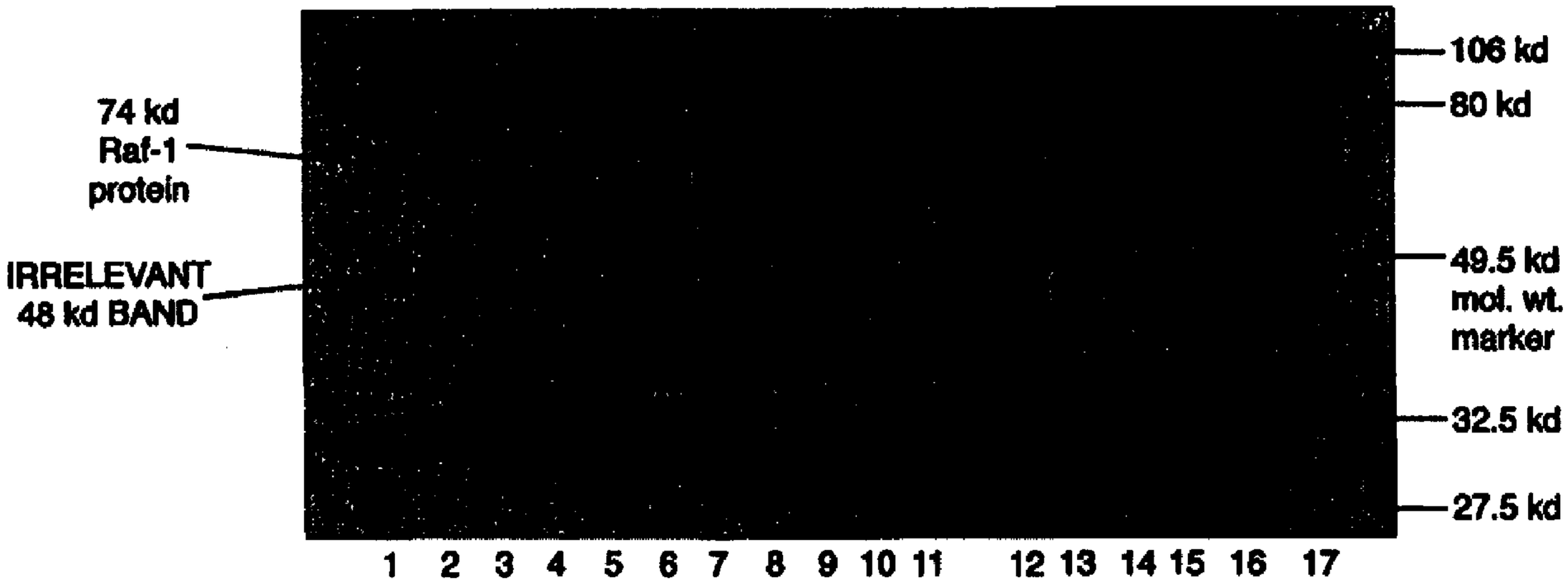

UNDETECTABLE p21
r = 0.12
p = 0.79
Raf protein (OD units)
7.5
5.0
2.5
0.0
0.40
0.45
0.50
0.55
0.60
0.65
Radiation sensitivity (SF2)

Fig.3.


HIGH p21
r = 0.95
p = 0.009
Raf protein (OD units)
15
10
5
0
0.0
0.1
0.2
0.3
0.4
0. 5
0.6
0.7
Radiation sensitivity (SF2)


74 kd
Raf-1
protein
IRRELEVANT
48 kd BAND
106 kd
80 kd
49.5 kd
mol. wt.
marker
32.5 kd
27.5 kd
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17