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C-*raf*-1 Proto-oncogene Expression Relates to Radiosensitivity Rather Than Radioresistance

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The transfection of several oncogenes, particularly c-*raf*-1, into mammalian *in vitro* cell lines has been reported to be associated with increased radioresistance. We have thus investigated (by scanning photodensitometry of western blots) the phenotypic expression of the c-*raf*-1, c-*myc* and c-*ras* protein products in 19 human *in vitro* cell lines, whose intrinsic cellular sensitivity to 4 MeV photon irradiation has also been determined. High levels of c-*raf*-1 proto-oncogene product expression did not correlate with increased cellular radioresistance, but rather showed a significant correlation with intrinsic cellular radiosensitivity to photon irradiation for α ($r = 0.664$, $P = 0.002$), and SF_2 ($r = -0.655$, $P = 0.002$). There was no significant correlation for the *ras* family, c-*myc* or actin. These results conflict with those of previous studies in which transfection of the activated forms of the c-*raf*-1 oncogene were associated with increased radioresistance, and suggest the possibility that the full length proto-oncogene may influence cellular radiosensitivity in a different manner from that of the activated oncogene.
Eur J Cancer, Vol. 30A, No. 3, pp. 369–375, 1994

INTRODUCTION

SEVERAL STUDIES have indicated that cellular and viral oncogenes, in particular c-*myc* [1, 2], c-Ha-*ras* [3, 4], N-*ras* [5] and c-*raf*-1 [6–8], known to be involved in the malignant transformation of mammalian cells, may also increase the resistance of these cells to ionising radiation. Some reports have found no general correlation between cellular radiosensitivity and oncogene product levels [9–11], and others have described increased radioresistance in cells which have only been transfected with a neomycin-resistance marker [12]. The previous studies demonstrating a positive relationship between oncogene expression and relative radioresistance were mainly conducted on NIH3T3 or embryonic cells transfected with viral or activated cellular oncogenes. It has been noted that the use of NIH3T3 cells for the study of radioresistance may be problematic [4], since they are highly aneuploid, have a very high rate of spontaneous transformation, and there is known to be a wide variation among laboratories in their properties which makes comparisons difficult [13,14]. In addition, transfected murine fibroblastic or embryonic cell lines are not representative of the

Table 1. Cellular radiosensitivity parameters and gene expression in 19 human cell lines

Cell line	$\alpha(Gy^{-1})$	$\beta(Gy^{-2})$	SF_2	c-*raf*-1	c-*myc*	pan-*ras*	Actin
HX142	0.97 (0.15)	0.041 (0.03)	0.128	9.58 (0.88)	0.41 (0.15)	4.75 (0.62)	6.44 (1.10)
OAW42	0.705 (0.07)	0.020 (0.01)	0.230	10.39 (1.16)	3.63 (0.54)	4.56 (0.23)	5.29 (1.15)
2780	0.525 (0.05)	0.036 (0.01)	0.308	9.25 (1.16)	7.25 (0.29)	6.56 (0.34)	7.89 (0.51)
HELA	0.54 (0.02)	0.005 (0.003)	0.337	3.09 (0.62)	7.97 (0.41)	4.13 (0.05)	6.54 (0.81)
HRT18	0.411 (0.04)	0.014 (0.004)	0.421	5.94 (0.38)	10.05 (0.76)	6.07 (0.35)	3.90 (0.11)
HX34	0.33 (0.08)	0.018 (0.01)	0.486	4.16 (0.65)	6.19 (0.89)	4.92 (0.10)	5.90 (0.07)
A431	0.30 (0.04)	0.016 (0.01)	0.519	0.70 (0.20)	5.69 (1.09)	4.65 (0.27)	5.46 (0.60)
SK-MEL3	0.28 (0.03)	0.017 (0.01)	0.521	6.81 (0.71)	4.58 (0.37)	4.35 (0.47)	6.13 (0.67)
H322	0.28 (0.03)	0.014 (0.01)	0.545	3.58 (0.17)	4.00 (0.25)	2.17 (0.33)	5.50 (0.06)
G361	0.25 (0.039)	0.022 (0.003)	0.560	5.16 (0.57)	8.30 (0.65)	5.20 (0.57)	6.13 (0.67)
I407	0.25 (0.03)	0.034 (0.01)	0.556	3.33 (1.31)	4.79 (0.21)	3.40 (0.31)	5.27 (0.07)
KB	0.22 (0.03)	0.040 (0.01)	0.554	4.66 (0.80)	3.71 (0.41)	4.79 (0.57)	5.61 (0.15)
A549	0.199 (0.04)	0.037 (0.01)	0.585	5.23 (0.42)	1.11 (0.42)	5.17 (0.30)	5.09 (0.56)
HT29	0.189 (0.04)	0.033 (0.01)	0.606	0.65 (0.18)	4.32 (0.60)	6.33 (0.60)	1.96 (0.16)
RT112	0.21 (0.21)	0.019 (0.001)	0.614	5.04 (0.91)	7.11 (0.44)	5.20 (0.19)	4.97 (0.18)
MGH-U1	0.20 (0.06)	0.020 (0.01)	0.623	5.27 (0.67)	4.08 (0.76)	7.49 (0.64)	4.48 (0.56)
HEP2	0.113 (0.03)	0.048 (0.01)	0.664	3.82 (0.65)	5.29 (0.75)	4.72 (0.77)	6.05 (0.25)
COR L23	0.476 (0.03)	0.045 (0.01)	0.328	4.71 (0.33)	8.07 (1.18)	1.49 (0.24)	1.59 (0.13)
MOR	0.391 (0.04)	0.005 (0.003)	0.453	4.50 (0.38)	3.13 (1.05)	5.43 (0.67)	4.16 (0.36)

Figures in parenthesis represent 1 S.E.M.

range of different histological types of tumour encountered in the clinical situation, where marked differences in radiation responsiveness may be observed.

In 1981, Fertil and Malaise reviewed published cell survival data for several human *in vitro* tumour cell lines of different histological origin, irradiated under oxic conditions fitted by the linear quadratic equation [15]. Values for SF_2 (the surviving fraction at 2 Gy) and α (the initial slope of the cell survival curve) thus obtained correlated closely with clinical response, as assessed from published estimates of the prescribed 95% tumour control dose for the particular type of tumour in the clinic. Subsequently, Deacon *et al.* [16] observed similar correlations in an analysis of cell survival data from 51 human *in vitro* cell lines. Human *in vitro* tumour cell lines thus appear to provide a potentially more clinically relevant model system in which to study the relationship between cellular radiosensitivity and oncogene expression. We have, therefore, examined the putative relationship between expression of the protein product of three oncogenes and intrinsic cellular radiosensitivity, as measured by α and SF_2 values in 19 human *in vitro* cell lines, originally derived from explantation of spontaneous clinical cancers.

We chose to investigate the protein expression of c-*myc*, pan-*ras* and c-*raf*-1 which have been reported to be associated with increased radioresistance when they have been used as dominant oncogenes in transfection studies. The extent to which these oncogenes are actively involved in producing the malignant phenotype of each of the 19 human *in vitro* cell lines examined in this study is unknown. For this reason, we have measured proto-oncogene protein expression by photodensitometric scanning of western blots prepared from lysates of each of the 19 cell lines, and compared these to the α and SF_2 values obtained from clonogenic cell survival assays of each cell line.

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MATERIALS AND METHODS

Clonogenic cell survival curves

The 19 cell lines, whose radiation cell survival parameters are shown in Table 1, were maintained as exponentially growing adherent monolayer cultures in DMEM medium, with the exception of 2780, RT112 and H322, which were grown in RPMI 1640 medium, and HX34, HX142 and MGH-U1, which were maintained in Ham's F12 medium. All lines were grown in the presence of 10% heat inactivated fetal calf serum (FCS) (Biological Industries, Glasgow, U.K.), and were passaged every 2–3 days to ensure exponential growth. To assay intrinsic cellular radiosensitivity, freshly trypsinised adherent cells were washed in fresh medium, resuspended at 10^5 cells/ml in Ham's F12 medium, supplemented with 10% heat-inactivated FCS and 10 mmol/l HEPES, and maintained at 37°C (5% CO_2) for 20 min prior to being irradiated by 4 MeV X-rays from a clinical linear accelerator at a dose rate of 2.0 Gy/min. The cells were then seeded at densities between 10^2 and 5×10^4 cells per 60 mm petri dish, and incubated in humidified air with 5% CO_2 at 37°C until distinct colonies of greater than 100 cells had formed (10–13 days depending on the cell line). For each cell line, the depicted survival curve was obtained by experiments consisting of at least 12 dose points over the range 0–6 Gy, and thereafter at 1 Gy intervals to 10 Gy. Each dose point was assayed by at least three separate experiments, each assay consisting of three replicate plates. The experimental data were analysed using the linear-quadratic equation:

$$S = e^{-(\alpha D + \beta D^2)}$$

where S is the fraction of cells surviving a dose D and α and β are constants. The data were fitted to the linear quadratic function using the non-linear regression program of the Inplot 4

package. The derived parameter SF_2 was obtained from the fitted curves.

Expression of gene products

Exponentially growing cells were trypsinised, washed twice in ice-cold phosphate buffered saline (PBS) and pelleted by centrifugation at 4°C. Cell pellets were mixed with 100 μl of ice-cold lysis buffer [50 mmol/l Tris–HCl at pH 6.8, 1.1% SDS, 11% glycerol, with 0.11 μmol/l leupeptin and 0.01 μmol/l phenylmethylsulphonyl flouride (PMSF)] for every 3×10^6 cells. After sonication and mixing, lysates were stored as aliquots at −80°C.

Conditions were optimised for each protein analysed. SDS–PAGE electrophoresis was performed in 7.5% (C-*raf*, C-*myc* and actin) and 15% (pan-*ras*) linear gradient acrylamide slab gels. Electrophoretic transfer of peptide bands to nitrocellulose was carried out at constant current of 500 mA for 3–6 h. Nitrocellulose blots were washed in Tris-buffered saline with 0.5% FCS for 1 h. The blots were washed and exposed to Tris-saline with Tween and 0.5% FCS containing the following antisera: C-*raf*-1, URP 26S3 at 1/750, C-*myc* 9E10 at 1/1000 (Dr G. Evan, ICRF, London, U.K.), pan-*ras*, OP22 at 1/200 (Oncogene Science, New York, U.S.A.) and actin at 1/750 000 (ICN Biomedicals, High Wycombe, U.K.). Detection of bound antisera was performed using a second alkaline phosphatase-linked anti-species antibody at 1/1000 dilution and an NBT/BCIP substrate solution. Stained peptide bands were quantified by photodensitometric scanning at 577 nm on a Shimadzu CS-9000. Linearity and precision were assessed for each protein during the optimisation of conditions. Linear regression values for diluted standards/controls ranged from 0.95 to 0.99. Intra-blot coefficients of variation (CV) ranged from 6.8 to 14.1%, and inter-blot CV from 3.5 to 24.5%. The following standards and controls were used to define optimal conditions for each gene product: (a) C-*raf*-1, NIH3T3 ECO (transfected with wild type raf, U Rapp) and NCTC 2544 human 74 kD c-*raf*-1 high expressor. (b) C-*myc*, Colo 320 high expressor human cell line. (c) pan-*ras* 21 kD recombinant protein standard (Oncogene Science). (d) Actin-recombinant protein standard (ICN Biomedicals).

For protein comparisons within the cell line panel, each sample was loaded in duplicate with total protein concentrations of 50 μg (actin) or 150 μg (*raf*, *myc* and *ras*). The mean value of each blot was assigned an arbitrary value of 5.0, and the values of each sample ranged accordingly. Each blot was repeated at least three times.

Cell kinetic parameters

Bromodeoxyuridine (BrdU) labelling. Exponentially growing cells were pulse-labelled for 30 min in medium containing 10 μmol/l BrdU (Sigma Chemical Co, Poole, U.K.). After 30 min, the cells were trypsinised and washed in fresh medium. Half the cells were fixed immediately in 70% ethanol, the other half were replated and incubated for a further 3–5 h before fixation and storage at −20°C. Labelled cells were stained according to the method of van Erp *et al.*[17], using a monoclonal anti-BrdU antibody and a fluorescein isothiocyanate (FITC)-labelled goat anti-mouse second antibody (Dako, High Wycombe, U.K.). Stained cells were resuspended in 20 μg/ml propidium iodide (PI) (Sigma).

Cells were analysed on a Becton Dickinson (Oxford, U.K.) FACS 420 flow cytometer, using an argon ion laser for excitation, a 580-nm dichroic mirror, a high-pass filter RG630 nm for red and a band pass filter 515/530 nm for green. List mode data was stored and analysed on a Hewlett Packard 9133 computer.

Data analysis

S-phase time and potential doubling time were measured using the procedure of Begg *et al.* [18], based on the movement of the labelled S-phase fraction between the 30-min and 4-h samples. Bivariate histograms of FITC (BrdU) and PI (DNA content) were used to measure the distribution of cells within the G1, S and G2/M phases of the cell cycle.

RESULTS

Expression of the protein products of the c-*raf*-1, c-*myc* and pan-*ras* proto-oncogenes were measured by photodensitometric scanning of western blots prepared from lysates of each cell line. Gene product levels were expressed as optical density (o.d.) units per μg of total cellular protein. The actin protein was also measured by the same method. There was considerable variation in gene product expression between individual cell lines (Fig. 1), whether calculated relative to total cell number or total cellular protein. Levels of c-*raf*-1 and c-*myc* protein varied 15- and 24-fold, respectively, whilst pan-*ras* and actin levels showed a 4-fold variation.

The intrinsic cellular radiosensitivities of the 19 human *in vitro* cell lines, covering several different histological types (Fig. 2), were determined by *in vitro* clonogenic cell survival assays, following exposure to a series of single doses of X-irradiation from a 4 MeV clinical linear accelerator at a dose rate of 2 Gy/min. For each cell line the data were fitted by the linear quadratic equation using an inplot 4 programme to give values for α (the initial slope of the cell survival curve) and β. SF_2 was derived by interpolation of the cell survival curves. α values had an 8.3-fold range (Fig. 2a) and SF_2 values a 5.57-fold range (Fig. 2b).

The relationship between expression of proto-oncogenes and intrinsic cellular radiosensitivity is shown in Fig. 3. A significant correlation between the C-*raf*-1 74 kD proto-oncogene product and the intrinsic cellular radiosensitivity parameter α was apparent ($r = 0.664$, $P = 0.002$). There was a similar significant correlation between C-*raf*-1 proto-oncogene product and SF_2 ($r = -0.655$, $P = 0.002$, data not shown). In contrast to results obtained by transfection studies, high levels of C-*raf*-1 proto-oncogene expression correlated with high sensitivity to ionising radiation. The C-*myc* and *ras* family proto-oncogenes and the actin gene products did not, independently or together, show any relationship with either α or SF_2 values.

Because cellular proto-oncogenes might potentially influence intrinsic cellular radiosensitivity as a result of an effect on proliferation, the cell kinetic parameters of the 19 human cell lines were measured by flow cytometry following BrdU incorporation in exponentially growing cultures. These parameters are shown in Table 2. The labelling indices (LI) of individual cell lines varied from 0.3 to 0.57 with a mean of 0.42. The potential doubling times (T_{pots}) ranged from 9.69 to 42.2 h with a mean of 24.34 h.

There was no correlation between the C-*raf*-1 proto-oncogene product and either T_{pot} (Fig. 4a), LI (Fig. 4c) or the duration of S phase, Ts (data not shown). Furthermore, when each T_{pot} or LI value was adjusted (by dividing by the mean for the set of 19 values), and the factor obtained for each cell line (adjusted LI or adjusted T_{pot}, Table 2) was used as the denominator to adjust the C-*raf*-1 value of that line, the significance of the correlation between the proto-oncogene protein product and alpha was not removed (Fig. 4, panels b and d).

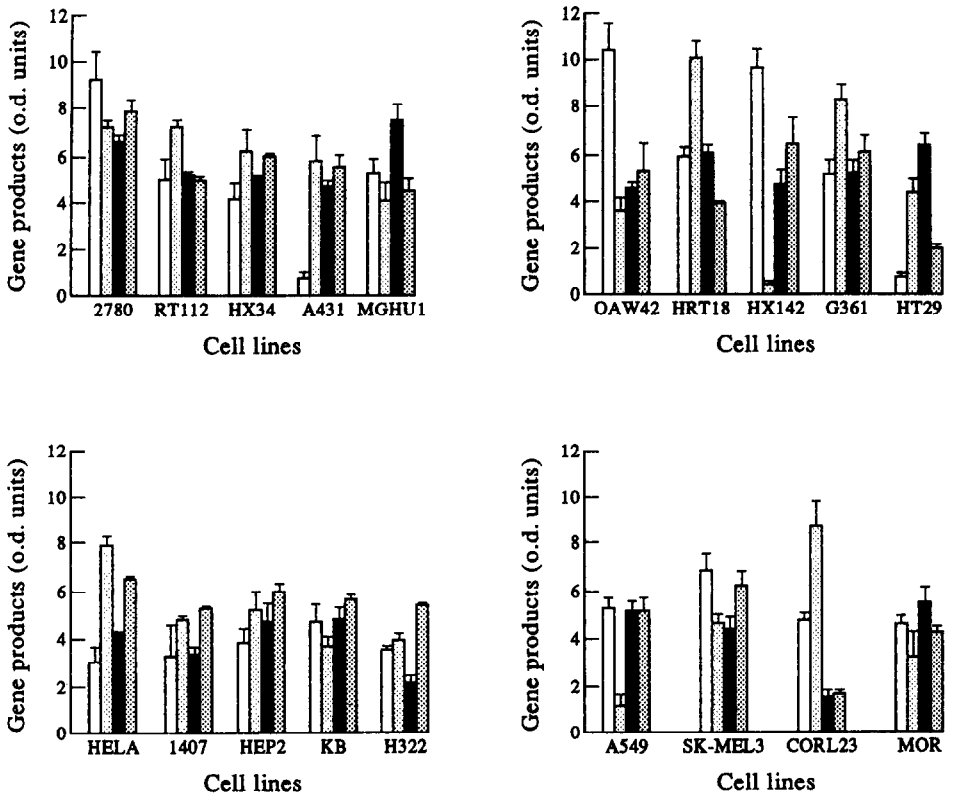
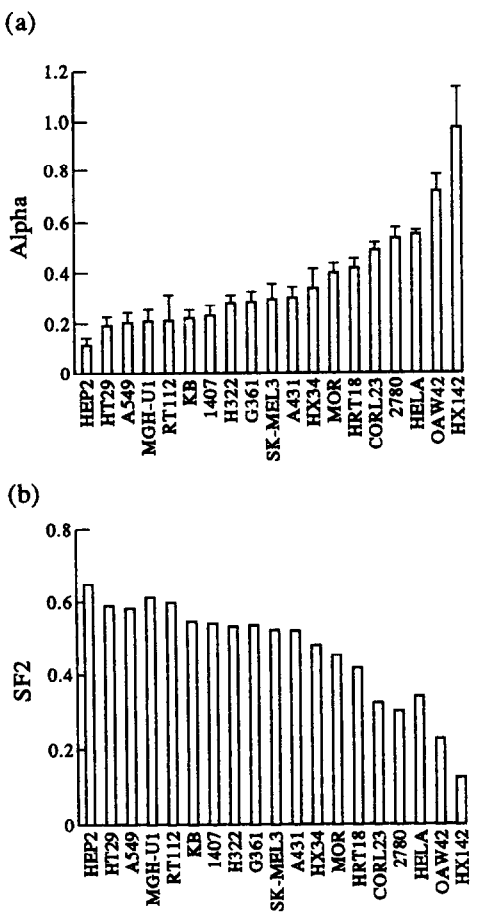


Fig. 1. Expression of gene product in 19 human *in vitro* cell lines ± 1 S.E.M. Histological classification of cell lines is as given in Fig. 2. The four gene products are shown for each cell line. c-*raf*-1 □, c-*myc* □, pan-*ras* ■, actin □.



Cell line	Histology
HEP2	Squamous carcinoma larynx
HT29	Adenocarcinoma colon
A549	Adenocarcinoma lung
MGH-U1	Transitional cell carcinoma bladder
RT112	Transitional cell carcinoma bladder
KB	Oral epidermoid carcinoma
1407	Embryonic intestinal epithelium
H322	Small cell carcinoma lung
G361	Melanoma
SK-MEL3	Melanoma
A431	Squamous carcinoma vulva
HX34	Skin melanoma
MOR	Adenocarcinoma lung
HRT18	Adenocarcinoma rectum
CORL23	Large cell lung carcinoma
2780	Ovarian carcinoma
HELA	Squamous carcinoma
OAW42	Ovarian carcinoma
HX142	Neuroblastoma

Fig. 2. Alpha ± 1 S.E.M. (panel a) and SF_2 (panel b) values of 19 human *in vitro* cell lines and their histogenic origin.

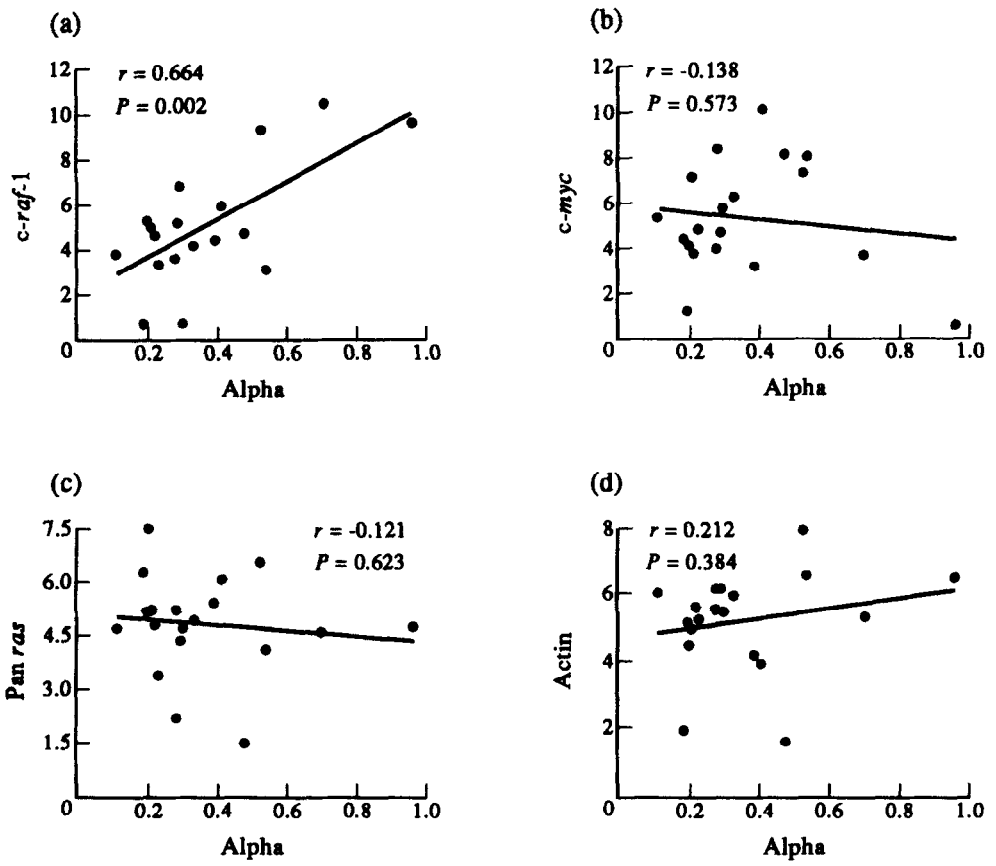


Fig. 3. Linear regression analyses of the relationship between intrinsic cellular radiosensitivity as measured by α (the initial slope of the cell survival curve) and gene product expression of c-*raf*-1, c-*myc*, pan-*ras* and actin.

Table 2. Cell cycle kinetic parameters of 19 *human* in vitro *cell lines*

	LI			TS		T_{POT}		
Cell line	Mean	S.E.M.	Adjusted LI	Mean	S.E.M.	Mean	S.E.M.	Adjusted T_{pot}
HX142	0.42	0.02	0.99	9.03	0.41	21.78	1.63	0.89
OAW42	0.49	0.02	1.16	10.19	1.36	19.33	5.21	0.79
2780	0.57	0.05	1.23	5.49	0.54	9.69	0.29	0.40
HELA	0.42	0.03	0.99	9.87	1.17	23.87	4.09	0.98
HRT18	0.41	0.05	0.97	7.86	0.67	19.66	3.02	0.81
HX34	0.43	0.04	1.02	7.10	1.35	16.03	1.21	0.66
A431	0.41	0.05	0.97	7.85	1.72	19.05	2.77	0.78
SK-MEL3	0.33	0.01	0.78	13.63	1.78	42.20	7.34	1.73
H322	0.42	0.02	0.99	13.92	2.97	32.01	5.83	1.31
G361	0.45	0.04	1.07	9.90	0.36	22.22	2.46	0.91
I407	0.30	0.03	0.71	7.57	0.69	25.14	0.91	1.07
KB	0.30	0.04	0.71	9.08	0.24	31.28	4.96	1.28
A549	0.35	0.06	0.83	12.98	3.38	40.13	14.28	1.65
HT29	0.44	0.04	1.04	10.59	1.19	23.60	4.48	0.97
RT112	0.52	0.03	1.23	8.19	0.37	15.80	1.66	0.65
MGH-U1	0.46	0.03	1.09	8.59	0.75	19.01	2.10	0.78
HEP2	0.34	0.01	0.81	10.24	1.69	29.94	3.87	1.23
COR L23	0.51	0.04	1.21	10.67	0.98	21.10	2.21	0.87
MOR	0.44	0.05	1.04	13.31	1.53	30.68	1.87	1.26

LI, labelling index; TS, S phase duration; T_{POT}, potential doubling time.

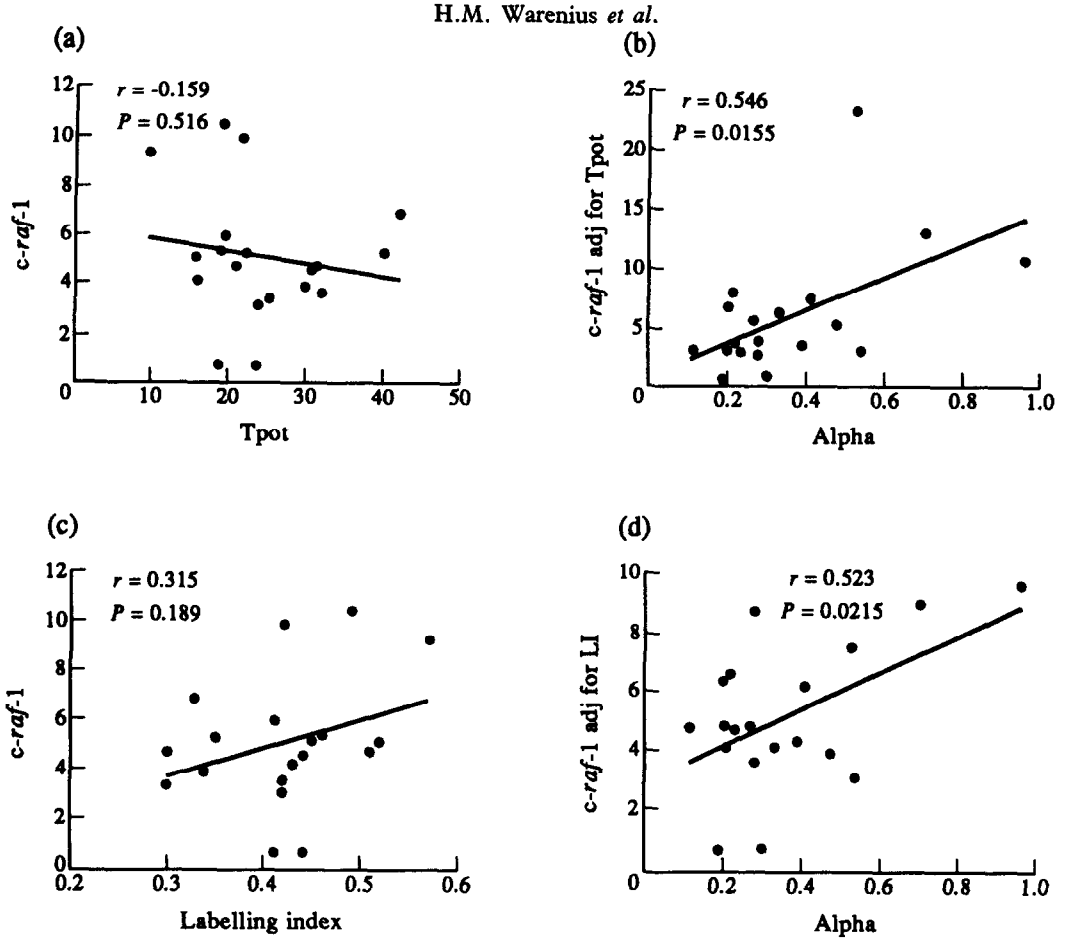


Fig. 4. Linear regression analyses of the relationship between c-*raf*-1 gene expression and the cell kinetic parameters T_{pot} (panel a) and LI (panel c) and between α and c-*raf*-1 levels adjusted for T_{pot} (panel b) and LI (panel d).

DISCUSSION

Possible relationships between oncogene expression and cellular radiosensitivity are of considerable interest because of their potential clinical relevance. Thus, the measurement of appropriate oncogenes in human cancers might be useful in predicting their clinical response. In addition, experiments indicating that antisense mRNA to C-*raf*-1 in transformed mammalian cells may reverse radioresistance [7] suggest the possibility of using, in the future, antisense oligonucleotides (designed to penetrate intact cells [19]) against relevant oncogenes in less radioresponsive tumours. However, such approaches require an understanding of the role of cellular oncogene expression as a factor influencing the radiation response of those human cancers commonly encountered in the clinical situation.

In order to improve this understanding, we have investigated proto-oncogene protein expression in 19 human *in vitro* cell lines of different histological types, which have been established by explantation of viable tissue from spontaneous human cancers occurring in the clinic. These cell lines exhibit a wide range of radiation sensitivities with values of α, the initial slope of the cell survival curve varying by 8.3-fold and SF_2 values varying by 5.6-fold. The potential relevance of such cell lines as models of clinical radioresponsiveness has been established previously [16, 20].

We observed considerable variation between cell lines in protein product expression, both of the three proto-oncogenes we chose to study and the control actin gene product. With this degree of variation in gene product expression and intrinsic cellular radiosensitivity from cell to cell, it was surprising that a significant correlation between c-*raf*-1 proto-oncogene product levels and both α and SF_2 photon values could be observed. Moreover, we have only measured the basal levels of the c-*raf*-1 protein in each cell line. At present, we have no information about the degree of variation in kinase activity that may occur between individual c-*raf*-1 proto-oncogene products, and which itself could possibly affect any relationship between c-*raf*-1 protein and cellular radiosensitivity. The correlation we observed was in the opposite direction to that predicted from previous transfection experiments. However, no other significant correlations were observed between c-*myc*, pan-*ras* and actin gene product levels and radiation sensitivity.

The approach of searching for differences in proto-oncogene activity in tumours, which were already known to have different radiation sensitivities, has been described previously by Rygaard *et al.* [21], who measured c-*raf*-1 and c-*myc* mRNA levels by northern blotting in two sets of small cell lung cancer xenografts. The two tumours in the resistant set showed similar levels of mRNA for c-*raf*-1 and actin as the three tumours in the sensitive set. The authors concluded that there was no relationship between c-*raf*-1 proto-oncogene expression and response to radiation. Given the degree of variation in proto-oncogene expression which we have observed within and between different *in vitro* cell lines, it is unlikely that correlations between proto-oncogene product levels and intrinsic cellular radiosensitivity

are detectable unless a sufficiently large number of cell lines are examined.

In addition, many factors are already understood to contribute to the radiation-resistant phenotype, including those influencing initial DNA damage by radiation [22], the types of lesions produced [23, 24] and the repair or misrepair [25, 26] of, and cellular recovery from, such damage. Within this context, it is surprising that the effect of one gene product is visible. The lack of correlation between c-*raf*-1 proto-oncogene product levels and cell cycle kinetic parameters makes it unlikely that its effect on cellular radiosensitivity is mediated via cellular proliferation. C-*raf*-1 proto-oncogene expression may influence initial damage, its repair or subsequent events in cellular recovery.

A number of mechanisms have been described for activation of c-*raf*-1, including the truncation of exons 2–9 usually during transfection [27–29], gene fusion extending the N-terminal region with activation of protein kinase activity [30], and site mutation in the conserved region 2 (CR2) binding region [30]. The difference between the correlation of the 74 kD c-*raf*-1 proto-oncogene expression to intrinsic cellular radiosensitivity described here, and that observed previously for the activated oncogene in transfection studies, suggest that the full length proto-oncogene may have a different mode of action on the radiation-resistant phenotype than the activated forms of the oncogene. The introduction of exogenous c-*raf*-1 constructs in conditional expression vectors into our human cell lines will be required to further elucidate these actions.

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