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Original Paper

Response to Radiotherapy of Human Uterine Cervix Carcinoma is not Correlated with Rearrangements of the Ha-*ras*-1 and/or c-*myc* Genes

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An association between the presence of the activated form of Ha-*ras*-1 and c-*myc* genes and increased cellular radioresistance has been shown in several cell lines. The aim of this study was to determine whether such an association could be observed in clinical tumour biopsies. We examined 70 tumour specimens and 51 samples of peripheral blood obtained from untreated patients with carcinoma of the uterine cervix mainly stage II and III. In addition to initial clinical tumour response to radiotherapy, radiosensitivity was also analysed by the subrenal capsule assay (SRCA). Mutations in exons 1 and 2 of the Ha-*ras*-1 gene were examined using PCR single-strand conformation polymorphism (PCR-SSCP) and direct sequencing; and restriction fragment length polymorphism of the Ha-*ras*-1 gene was analysed using Southern hybridisation. Eight (11%) out of 70 tumours contained mutations in exons 1 and 2 of the Ha-*ras*-1 gene. Eleven (22%) out of the 51 tumours displayed rearrangements of the Ha-*ras*-1 gene (six tumours (12%) showed alterations of allele length, one amplification and four (8%) loss of one Ha-*ras*-1 allele). In addition, 12 (17%) out of 70 patients demonstrated the presence of rare alleles. Only one of the 70 tumours contained an amplified c-*myc* gene. There was no significant correlation between either rearrangements of the structure of the Ha-*ras*-1 and/or c-*myc* genes or presence of rare alleles in tumours and tumour response to radiotherapy. © **1997 Elsevier Science Ltd.**

Key words: radiosensitivity, Ha-*ras*-1, c-*myc* genes, cervical carcinomas

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INTRODUCTION

RADIOTHERAPY IS important in the treatment of cancer of the uterine cervix. Achievements in tumour radiotherapy may be dependent on sensitivity of malignant cells to ionising radiation. Several investigations have demonstrated a connection between expression of oncogenes and cellular radioresistance in rodent as well as human cell lines. In a rodent model, an association between the presence of activated N-*ras*, Ha-*ras*-1, K-*ras*, c-*raf*-1 at high and low dose rates [1–3] and of v-*abl*, v-*fms*, v-*fos* only at a low dose rates of radiation [4, 5] and a radioresistant phenotype of the NIH3T3 transfectants has been shown. The Ha-*ras*-1, v-*abl*, c-*fms*, v-*myc*, v-*erb*-B, v-*src*, v-*mos* and c-*cot* oncogenes are also capable of affecting the radiosensitivity of transfectants of other rodent cells [4–7]. Different results were obtained with the *myc* oncogene, which alone has been shown to alter the sensitivity of transfected rodent cells to radiation [5], although others showed no effect with c-*myc* [6, 8]. However, c-*myc* together with *ras* had an apparent synergistic effect on the radiosensitivity of rat embryo cells (REC) [6].

Some investigations have used human cell lines, but results of these studies are less clear. Activated c-*raf*-1 and

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amplified c-*myc* proto-oncogenes have been found in radioresistant human fibroblast cell lines [9]. It has also been shown that transfection of anti-sense *raf*-1 cDNA into a radioresistant human squamous cell carcinoma line results in a loss of the radioresistant phenotype [10]. No apparent correlation between *ras* expression and radioresistance was found in an immortalised human keratinocyte cell line transfected with an activated Ha-*ras*-1 gene [11]. Grant and associates [12] did not find a consistent association between increased radioresistance and *ras* expression in human embryo retinal cell lines (HERs) transfected with activated N-*ras* and Ha-*ras*-1. However, two out of three lines with the highest D_0 values showed the highest levels of N-*ras* and Ha-*ras*-1 expression. Human fetal fibroblast cell lines transfected by activated Ha-*ras*-1 together with SV40 T-antigen were significantly more radioresistant than their parental cells [13], while clones of transformants obtained by transfection of human epithelial cells with the same genes did not demonstrate increased radioresistance [14]. Such a heterogeneity in the radiosensitivity of recipient cells may be explained by different genetic backgrounds into which oncogenes are transfected and expressed. Furthermore, since the experiments mentioned above were performed using cell lines as well as the transfection assay as the model system, different methodologies and experimental factors may affect radiation response and complicate interpretation of the results.

Only a few investigations have been carried out with human cell lines in which basic levels of oncogene expression have been measured and compared to the cell's radiosensitivity. Amplification of the c-*myc* gene and increased levels of c-*myc* RNA have been observed in a radioresistant variant of a small cell lung cancer line (SCLC-V) [15] and in radioresistant fibroblasts from patients with Li–Fraumeni syndrome, respectively [9]. No association between levels of *myc*, c-*raf*, K-*ras* RNA and radiosensitivity of five subclones of the SCLC-V was found in another study [16]. In 19 human cell lines of different histological types, no correlation between c-myc and ras protein levels and radiosensitivity was observed. A high level of the c-raf-1 protein was, however, associated with increased radiosensitivity, an effect which is opposite to that observed in transfection experiments [17].

The purpose of the present study was to determine whether a correlation between the alterations of the structure of Ha-*ras*-1 and/or c-*myc* genes and radioresistance could be observed in clinical biopsies from human carcinomas of the uterine cervix. Previous studies of Ha-*ras*-1 have demonstrated a restriction fragment length polymorphism (RFLP) for *Bam*H1 and *Msp*I restriction enzymes [18]. RFLP is determined by the presence at the 3′ end of the gene of the variable tandem repetition (VTR) structure unit, which is a 28 bp long palindrome sequence (Figure 1). Four variants of the amplification of this sequence form four common alleles: a1, a2, a3 and a4. In addition, there are several (at least 26) rare alleles of this gene in the human genome. Their sizes differ from those of the common alleles by 0.1–0.3 kb. It has been shown [19] that the loss of one Ha-*ras*-1 allele is relatively common in cervical cancer. Carcinomas that have a Ha-*ras*-1 allele deletion also often have a mutated Ha-*ras*-1 gene. It has been suggested that the loss of the Ha-*ras*-1 gene on one allele might contribute to the activation of a mutated Ha-*ras*-1 gene on the other allele. In the same study, amplification of the c-*myc* gene in all tumours with mutant Ha-*ras*-1 has been found. Thus, based on data cited above, we investigated whether a correlation exists between structural changes in Ha-*ras*-1 and/or c-*myc* and radiosensitivity of human cervix carcinomas. Radiosensitivity was estimated by a clinical tumour response after the end of radiotherapy, as well as by the response of heterotransplanted tumour samples to irradiation (subrenal capsule assay).

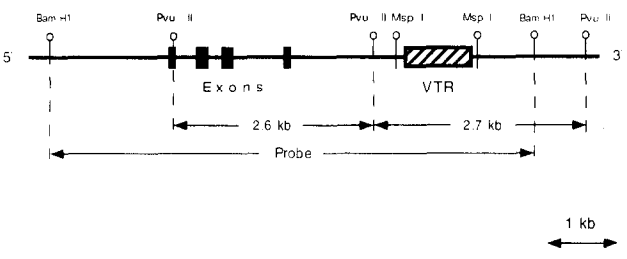

Figure 1. Structure of the human Ha-*ras*-1 gene. The diagram shows a 2.6 kb *Pvu*II fragment containing a sense part of the Ha-*ras*-1 gene and location of the VTR (hatched box). The *Bam*H1 fragment (6.7 kb) was used for Southern blot analysis.

MATERIALS AND METHODS

Cell material

Biopsies of carcinomas of the uterine cervix from 70 untreated patients were obtained from the Department of Gynaecology at the Central Research Institute of Roentgenology and Radiology (CRIRR, St. Petersburg, Russia). 30 patients had stage II, 34 stage III and 6 stage IV disease using the FIGO system. For conformation of histological type, biopsies were examined at the Department of Pathology of the CRIRR. The histopathological diagnosis of the cases analysed was squamous cell carcinoma. Peripheral blood was collected prior to treatment from 51 patients.

Radiation therapy

Patients were subjected to intracavitary and external beam therapy. The intracavitary radiotherapy was performed on "Agat-V" and "Agat-VU" using ^{60}Co sources with afterloading technique. A single dose was 7 Gy/fraction with one fraction per week. The number of fractions was 6–7. External radiotherapy was carried out with γ-rays from two opposing fields with a midline block (single dose was 2 Gy, with 4 fractions/week). The total absorbed dose was 40–42 Gy. Patients with a tumour volume of more than 120 cm^3 were treated with open fields using γ-beams. The absorbed dose at the anterior part of the patient was 18 Gy. Tumour response to radiation therapy was assessed 3 months after the end of therapy. Patients were assigned to two groups, those obtaining a complete remission and those that did not.

The subrenal capsule assay (SRCA)

The SRCA, known as a method for predicting chemosensitivity of human tumours, was used to test radiosensitivity of the cervical cancers. Tumour specimens, which had been stored in liquid nitrogen, were thawed by gradually increasing the temperature and cut into 1 mm^3 pieces. To create temporary immunosuppression, normal immunocompetent mice were subjected to total irradiation (4.5 Gy ^{60}Co) 18–24 h before transplantation. It has been shown [20, 21] that

total irradiation of mice at this dose leads to relatively high temporary immune suppression in animals. Mice were anaesthetised, the left kidney was exteriorised and a piece of the tumour tissue was inserted under the renal capsule. The exact size of the fragment was determined by measuring two perpendicular dimensions under the microscope. The kidney was then replaced into the body cavity, and the wound was closed (day 0). On day 1, irradiation (one fraction with 6 Gy) of the mice was carried out. Each treated and control (without postoperative irradiation) group consisted of 6–8 mice. On day 6, the animals were killed and final tumour sizes were measured. The ratio of final to initial tumour size (ΔTS = final TS/initial TS) was calculated. The variation of ΔTS within each of the groups (control and experimental) expressed as standard deviation divided by the mean was 2–8%. The difference in average relative $\Delta\overline{TS}$ (calculated from 6–8 mice) under subrenal capsule between the control and the treated samples was expressed as a fraction (%) of the control as follows:

$$\frac{\Delta\overline{TS}\ \text{control} - \Delta\overline{TS}\ \text{experiment}}{\Delta\overline{TS}\ \text{control}} \times 100\%$$

Four effects of irradiation on transplant growth were observed: stimulation — $\Delta\overline{TS} > 1$; no effect — $\Delta\overline{TS} = 1$; inhibition — $0 > \Delta\overline{TS} > 1$; regression — $\Delta\overline{TS} > 0$. We considered a tumour to be radiosensitive if $\Delta\overline{TS} < 0$.

DNA extraction

High molecular weight DNA was extracted from biopsies and lymphocytes (lymphocytes were fractionated on a Pharmacia Ficoll-Hypaque gradient) by incubation at 65°C for 15 min in lysis buffer (300 mM sodium acetate, 50 mM EDTA, 0.5% N-lauroylsarcosine, pH 7.5) mixed with an equal volume of buffer-saturated phenol (0.2% 2-mercaptoethanol, 0.1 mM Tris-HCl, pH 8.0). Samples were extracted twice with an equal volume of phenol:chloroform (1:1) and twice with chloroform:isoamyl alcohol (24:1). The proteins were hydrolysed with 100 μg/ml proteinase K (Boehringer Mannheim, Germany). DNA was precipitated with 2 volumes of ethanol and redissolved in 10 mM Tris-HCl, 1 mM EDTA pH 8.0 buffer. RNA was hydrolysed with pancreatic RNase A (100 μg/ml, Sigma, U.S.A.) at 37°C for 1 h.

Southern hybridisation

Two micrograms of cellular DNA was digested with *Pvu*II (5 units/μg DNA, 24 h at 37°C) or *Msp*I (12 units/μg of DNA, 36 h at 37°C) and *Hpa*II (4 units/μg of DNA, 24 h at 37°C) or *Eco*RI (5 units/μg DNA, 24 h at 37°C). The restriction enzymes were from Amersham, U.K. The digested DNA was then separated in 0.8% and 1.2% agarose, respectively, and transferred to Zeta-probe membrane (BioRad, U.S.A.). A 6.6 kb Ha-*ras*-1 *Bam*H1 [22] and 1.2 kb c-*myc* cDNA [23] probes were labelled with [α-^{32}P]dCTP(Amersham) using a multiprimer DNA labelling system (Amersham) to a specific activity 5×10^8 cpm/μg. Prehybridisation, hybridisation and washing of the filters were carried out according to the conditions recommended in the instruction manual for Zeta-Probe membranes. Filters were exposed to Kodak XAR-5 film for 24–48 h at −70°C.

PCR-SSCP analysis

DNA from tumour samples or lymphocytes were amplified at specific sequences around codons 12, 13 and 61 of the Ha-*ras*-1 gene. The primers used in the analyses had the following sequences: for 12 and 13 codons, 5′ primer, 5′-GACGGAATATAAGCTGGTGG; 3′ primer, 5′-TGGATGGTCAGCGCACTCTT; for 61 codon, 5′ primer, 5′-AGACGTGCCTGTTGGACATC; 3′ primer, 5′-CGCATGTACTGGTCCCGCAT. Each amplification reaction was carried out in a 10 μl reaction volume containing 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 2 mM $MgCl_2$; 2 pmol of each primer; deoxynucleotide triphosphates dATP, dTTP, dGTP, at 70 μM each; dCTP at 10 μM; 1 μCi [α-^{32}P]dCTP (3000 Ci/mmol); 100 ng genomic template DNA; 1 unit of *Taq* polymerase (Perkin-Elmer, U.S.A.). Reaction mixtures were subjected to 35 cycles of amplification in a DNA thermal cycler (Perkin-Elmer). Each cycle consisted of denaturation at 95°C for 40 sec, annealing at 65°C (codons 12 and 13) or 60°C (codon 61) for 1 min, followed by polymerisation at 72°C for 1.5 min. Following the last cycle, tubes were incubated for a further 7 min at 72°C. Samples without DNA and with plasmid DNA containing a fragment Ha-*ras*-1 oncogene with a G → T substitution at the second position of the codon 12 were used as negative and positive controls, respectively. Following amplification, 30 μl formamide dye (95% formamide, 10 mM EDTA, 0.05% xylene cyanol) were added to each reaction. Three microliter aliquots were denatured at 80°C for 5 min and electrophoresed in an 8% acrylamide gel containing 5% glycerol (12 and 13 codons) or in a 6% acrylamide gel without glycerol at 17°C (35 W). Dried gels were exposed to Fuji Medical X-ray film for 16–18 h.

Direct sequencing

PCR reactions were performed with one biotinylated (5′-TAAACTGCAGGAGACCCTGTAGGAGG) and one non-biotinylated (5′-GCAGCTTAAGGCTCACCTCTATAGTG) primer. Single-stranded DNA was isolated using Dynabeads M-280 Streptavidin (Dynal, Norway). Sequencing was carried out with one of the internal primers (5′ primer: 5′-CAGGCCCCTGAGGAGCGAT; 3′ primer: 5′-CGTCCACAAAATGGTTCT) using a Sequenase version 2.0 DNA sequencing kit (Amersham).

Statistical analysis

Statistical analysis for correlations was performed by Fisher's exact test. The multivariate analysis of the influence of alterations of Ha-*ras* on response to radiotherapy was carried out by stratifying for tumour stage. The analysis was performed using the program package StatXact (Statistical Software for Exact Nonparametric Inference) (CYTEL Software Corporation, Cambridge, Massachusetts, U.S.A.). First the hypothesis of homogenous odds ratio (OR) over the different strata was tested and then followed by testing the hypothesis that the common OR is equal to one (corresponding to the absence of prognostic value). Exact estimates of the common OR together with exact 95% confidence intervals (CI) are presented.

RESULTS

*Characterisation of rearrangements of the Ha-*ras*-1 and c-*myc *genes*

In our study, rearrangements of the Ha-*ras* gene in 70 tumour biopsies obtained from patients with squamous cell carcinomas of the uterine cervix were analysed and compared to clinical radiotherapy response. DNA from the

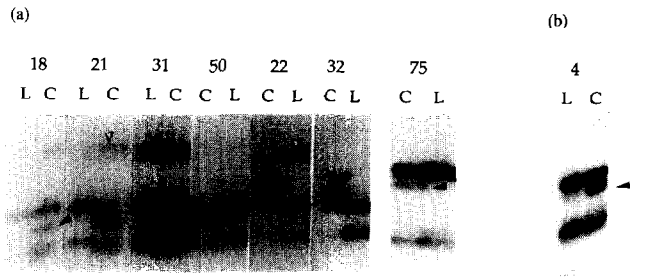

Figure 2. SSCP analysis of the Ha-*ras*-1 exons 1 (a) and 2 (b) mutations in patients with carcinomas of the uterine cervix. Solid arrowheads — band with altered mobility. Open arrowheads — non-denatured product. The patient numbers above each lane correspond to those in Table 1. C, L — DNA from carcinomas and lymphocytes, respectively.

lymphocytes of 51 patients was used as reference for the constitutional genotype.

HA-ras-1

(a) Point mutations. DNA from 70 tumours and 51 samples of peripheral blood lymphocytes were subjected to SSCP analysis (mutant allele that is present in >10% cells can be detected) to identify point mutations within exon 1 and 2 of the Ha-*ras*-1 gene.

The primer pair for codons 12 and 13 amplifies a 63 bp fragment of exon 1 of Ha-*ras*-1. In preliminary experiments, various conditions of electrophoresis were assessed. Optimum differential migration of mutant product from T24 (positive control) and normal PCR products were obtained using 8% acrylamide gel with 5% glycerol run at 17°C. Mutated and wild-type Ha-*ras*-1 gene were distinguished by a mobility shift of the separated strands of the amplified product (Figure 2a, patient nos. 18, 21, 31, 50, 22, 32 and 75). In six cases, strands with mobility similar to those of the amplified products from the normal gene were present in addition to those with altered mobility, indicating the likely presence of one normal and one mutated Ha-*ras*-1 allele in these samples. In one biopsy (Figure 2a, patient no. 21), no normal product was detected, indicating probable mutations in both alleles. The primer pair for codon 61 amplifies a 73 bp fragment of exon 2 of Ha-*ras*-1. For resolution of mutations in this codon, 6% acrylamide gels without glycerol were run at 17°C. Amplified DNA from one tumour showed altered mobility (Figure 2b, patient no. 4). In this case both normal and mutant strands were present. Lymphocyte DNA was available from 7 of 8 patients. SSCP analysis of exons 1 and 2 showed no evidence of constitutional mutations in those 7 patients for whom tumour mutations had been identified.

Direct sequencing revealed nucleotide substitutions in 7 samples in which point mutations in exon 1 were detected by SSCP (Figure 3 and Table 1). Four DNA samples that showed similar single-strand mobility shifts (nos. 18, 21, 31 and 50) had identical nucleotide substitutions in the second position of the codon 12 (GGC → GTC). One tumour (no. 75) showed base substitution in the first position of codon 12 (GGC → AGC). In two tumours (nos. 22 and 32), base substitution in the third position of codon 11 (GCC → GCT) was detected. One (no. 22) showed a very faint band that corresponds to a mutant base despite the fact that mutant allele was present in the tumour in a higher proportion of cells than normal. Sequencing of the codon

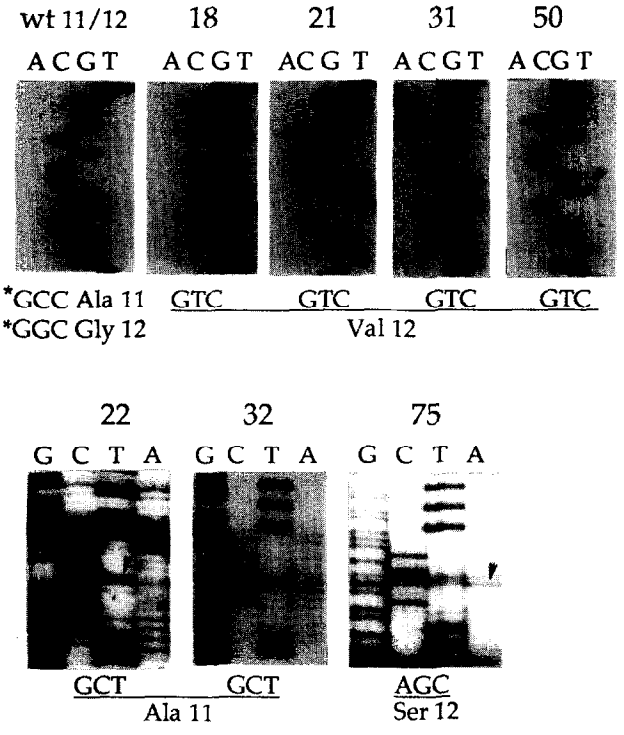

Figure 3. Direct sequencing of PCR products from carcinomas of the uterine cervix. Wild type sequences (wt) for codons 11 and 12 are indicated by asterisks. Arrowheads — mutant bands. Patient nos. 18, 31, 50, 22, 32 and 75 have heterozygous, no. 21 homozygous mutant Ha-*ras*-1.

61 mutation detected by SSCP was not carried out. No mutations were found in lymphocyte DNA obtained from the same patients.

With the exception of one case, mutations were detected in advanced stage tumours (6 patients with stage III and 1 with IV), although the correlation was non-significant ($P = 0.059$).

(b) Structural alterations. In 11 out of 51 biopsies, rearrangements of the Ha-*ras*-1 gene were found (Figures 4 and 5). In four tumours (patient nos. 6, 17, 31 and 41), alteration of the length of one of the Ha-*ras*-1 alleles was found (Table 1). Two other tumours were found to contain alterations of both Ha-*ras*-1 alleles (patient nos. 8 and 40). Four of the six tumours with alterations in the length of one of the Ha-*ras*-1 alleles were found in stage II patients ($P = 0.07$). Loss of heterozygosity was observed in four of the 42 informative cases (patients heterozygous for the Ha-*ras*-1 locus) of carcinoma (patient nos. 7, 19, 36 and 61). The faint hybridisation to the deleted bands could reflect contamination of tumours by constitutional cells. We found that loss of one Ha-*ras*-1 allele is a relatively rare event (8%) in carcinomas of the uterine cervix. Only one case of amplification of the a3 allele of the Ha-*ras*-1 gene was observed (patient no. 27, Figure 4b).

(c) Rare alleles. Rare alleles of the Ha-*ras*-1 gene were detected in 12 (17%) cervical cancers. In 6 cases, we compared constitutive and tumour genotypes (Figures 4 and 5, patient nos. 5, 31, 40, 42, 62 and 70). Only in two tumours was the appearance of the rare alleles in tumour tissue caused by alteration of the length of one of the Ha-*ras*-1 allele (nos. 31 and 40). Other patients had rare alleles in tumour cells as well as in lymphocytes.

Table 1. Rearrangements of the Ha-ras-1 gene and tumour response to radiotherapy

Patient no.	Clinical stage	Alteration of the length of allele	Allele loss	Amplification	Mutation (codon)	Tumour radiosensitivity Clinical	Subrenal capsule assay
27	II B			a3		RS	RS
6	II A	a3 → a1				RS	RR
8	II A	a1 → a4				RS	NA
		a1 → a3					
17	II B	a1 → a3				RS	RR
41	II B	a1 → a3				RR	RR
31	III B	a1 → r.a1				RR	RS
40	III B	a3 → r.a1				RR	ND
		a3 → a2					
						4RS:3RR	2RS:3RR
7	II A		a4*			RS	RR
36	II A		a1			RS	RS
61	II B		a3			RS	RR
19	IV A		a2			NT	RS
						3RS	2RS:2RR
4	II B				61	RS	RR
18	III B				12	RR	RS
21	III B				12	RS	RS
31	III B				12	RR	RS
32	III B				11	RR	ND
50	III B				12	RS	RS
75	III B				12	RS	ND
22	IV A				11	RR	RS
						4RS:4RR	5RS:1RR

Arrows (→) show direction in which alteration occurred; r.al, rare allele.
*Allele that was absent in tumour compared with lymphocytes from the same patient.
RS, radiosensitive; RR, radioresistant; NA, no adherence; ND, not determined; NT, no treatment.

c-myc

No structural alteration of the c-*myc* oncogene was found. Only one case with amplification of this gene was observed (patient no. 61, Figure 4c).

Relationship between clinical tumour radiosensitivity and rearrangements of the Ha-ras-1 gene

We found 11 tumours with structural alterations of the Ha-*ras*-1 gene. Seven were obtained from patients who demonstrated a complete response to radiotherapy when assessed 3 months after the end of treatment (Table 1). One patient was not treated and 3 had non-radioresponsive tumours. The occurrence of Ha-*ras* alterations and response to radiotherapy did not correlate since the frequencies of Ha-*ras* alterations in radioresponsive (7/33) and non-radioresponsive groups of tumours (3/18) were not significantly different ($P = 0.27$).

Of eight tumours with the mutant Ha-*ras* allele, four were radioresponsive and four non-radioresponsive (Table 1). The frequencies of Ha-*ras* mutations in radioresponsive and non-radioresponsive tumours were 8% (4/50) and 20% (4/20), respectively ($P = 0.12$).

Relationship between clinical tumour radiosensitivity and presence of rare alleles of the Ha-ras-1 gene

Five of the tumours with rare alleles were radioresponsive and five were non-radioresponsive (Table 2). The association between the presence of rare alleles and clinical tumour response to radiotherapy was not statistically significant ($P = 0.13$, frequencies of rare alleles 10% — 5/50 and 25% — 5/20 in radioresponsive and non-radioresponsive tumours, respectively).

The result of multivariate analysis showed that Ha-*ras* rearrangement was not a significant prognostic factor for response to radiotherapy (OR = 1.9 (95% CI 0.2–14.3) for mutations and 4.4 (95% CI 0.3–244) for other alterations). The relative prognostic value of tumour stage after stratification of Ha-*ras* status was significant (OR = 10; 95% CI 2–100).

Relationship between radioresponsiveness in the SRCA, clinical response of tumours to radiotherapy and rearrangements of the Ha-ras-1 gene

The radioresponsiveness of the 56 tumours was also analysed using the subrenal capsule assay (SRCA). However, a total of 10 cases had to be excluded: 6 of the tumour tissue specimens did not adhere under the renal capsule after the operation; and 4 patients were not treated with radiotherapy. For the remaining 46 samples, a comparison of the SRCA with the clinical radioresponse showed no clear pattern; only 13 of the 46 tumours with complete remission showed corresponding sensitivity in the SRCA. For the clinical failures, 6 tumour specimens exhibited radioresistance as measured by SRCA. Thus only 41% [6 + 13/46] of the SRCA samples showed a correlation with the clinical response. As seen in Table 1, among 9 tumours showing structural alterations of the Ha-*ras*-1 gene and in which radioresponsiveness was defined by SRCA, 5 tumours were radioresistant and 4 were radiosensitive. Although 5 of the 6 tested tumours with a mutation of the Ha-*ras*-1 gene were

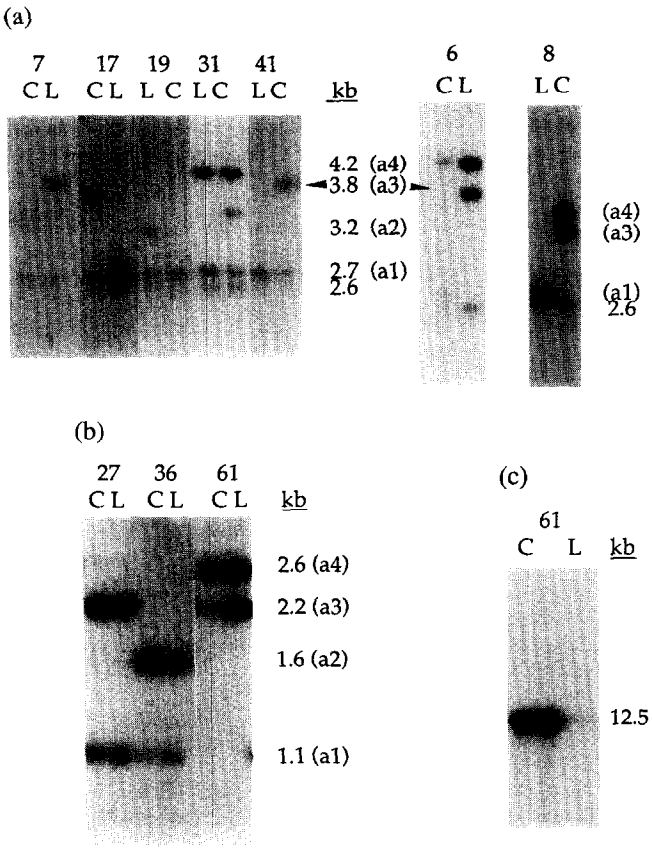

Figure 4. Structural alterations of the Ha-*ras*-1 (a, b) and c-*myc* (c) genes in patients with carcinomas of the uterine cervix (Southern hybridisation). The kb lengths of the *PvuII* (a) and *MspI/HpaII* (b) restriction fragments, corresponding to four common alleles (a1, a2, a3 and a4), the sense region (2.6 kb) of the Ha-*ras*-1 and the *Eco*RI fragment of the c-*myc* genes are shown.

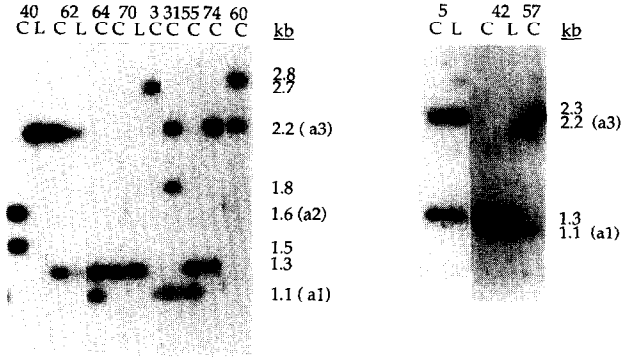

Figure 5. Rare alleles of the Ha-*ras*-1 gene in patients with squamous cell carcinoma of the uterine cervix (Southern hybridisation). The lengths of *MspI/HpaII* restriction fragments, corresponding to common (a1, a2, a3 and a4) and rare (1.3, 1.5, 1.8, 2.3, 2.7 and 2.8) alleles, are indicated.

radiosensitive in the SRCA, the number of cases is too small to make any conclusions about the association between the presence of mutations of the Ha-*ras*-1 gene and radioresponsivcness in SRCA. In addition, there was no statistically significant correlation between the presence of rare alleles of the Ha-*ras*-1 gene and tumour response to irradiation (as measured by SRCA) — 6 tumours were radiosensitive and 2 were radioresistant (Table 2).

The results of measuring radiosensitivity obtained by the subrenal capsule assay should be interpreted with caution.

DISCUSSION

The results demonstrate the absence of a correlation between any type of rearrangement of the Ha-*ras*-1 gene and the response of human uterine cervix carcinomas to radiotherapy. The result of the multivariate analysis indicates that the status of the Ha-*ras* gene may have an additional prognostic value for patients in whom alterations of this gene were found, but the low prevalence of patients with an altered Ha-*ras* gene in the study group makes it very doubtful if such a marker would be useful when considering cost-effectiveness.

In our study, the percentage of tumours with loss of Ha-*ras*-1 alleles was low (8%) compared to other studies [19]. One of the 4 patients with loss of one of the alleles did not receive radiotherapy, while the other 3 had radioresponsive tumours. However, due to the very few cases that showed loss of heterozygosity, it cannot be considered a reliable marker for the predisposition of tumour to radiotherapy. It also seems unlikely that the loss of one of the alleles may contribute to activation of another, as was suggested previously [24], since none of the patients with mutant Ha-*ras*-1 showed allele loss. Finally, we did not find an association between presence of any of the rare alleles and tumour radioresponse.

Table 2. Association of the presence of the rare alleles of the Ha-ras-1 *gene and tumour response to radiotherapy*

Patient no.	Clinical stage	Rare allele size (kb)	Tumour radiosensitivity	
			Clinical	Subrenal capsule assay
3	II B	2.7	NT	RR
60	II B	2.8	RS	RR
42	II A	1.3	RS	RR
55	II B	1.3	RS	RR
64	II B	1.3	RR	ND
5	III B	1.3	RR	RR
31	III B	1.8	RR	RS
40	III B	1.5	RR	ND
62	III B	1.3	RS	RR
70	III B	1.3	RS	ND
74	III B	1.3	RR	ND
57	IV A	2.3	NT	RS

RS, radiosensitive; RR, radioresistant; ND, not determined; NT, no treatment.

It has previously been shown in transfection experiments that the c-*myc* gene alone is incapable of affecting radiation sensitivity, but in combination with the Ha-*ras*-1 gene it can confer radioresistance on certain types of cells [6]. We did not find any structural alterations of the c-*myc* gene. The fact that we detected only one case with amplified c-*myc* indicates that the number of gene copies of this gene cannot be a factor that has a role in the determination of tumour radiosensitivity.

The absence of a correlation between radiosensitivity and structural alterations of Ha-*ras*-1 and c-*myc* genes does not exclude the possibility that overexpressed Ha-*ras* and c-*myc* genes may show such a correlation. For modulation of radiosensitivity, cooperation between other genes, including those involved in apoptosis, is probably also essential. This is of special interest since it has recently been shown that the level of spontaneous apoptosis in cervical carcinomas predicts their response to radiotherapy [25].

It is also possible that a cascade of events which resulted in certain radioresistant phenotype is initiated by exposure of cells to radiation. Ionising radiation generates different DNA damage and especially double strand breaks (DSB), the repair of which has been suggested to be linked to cellular radiosensitivity [26, 27]. Activated products of some oncogenes may interact at certain points of the signal transduction pathway with, for example, components of the DNA DSB repair system. Recently, a DNA-dependent protein kinase (DNA-PK) complex, components of which can complement the radiosensitive phenotype of mutants exhibiting defective DNA DSB repair and inability to perform V(D)J recombination, has been described [28–30]. DNA-PK is activated by binding to damaged DNA and phosphorylates some transcriptional factors including c-*fos*, c-*jun* and c-*myc* [28], which are also nuclear targets for an oncogene-controlled signal transduction pathway. DNA-PK is also able to stabilise the tumour suppressor protein p53 [31] which, in turn, has been shown to modulate the effect of the *ras* oncogene on cytotoxic agents, including X-rays [32], and whose apoptotic and growth arrest functions can be blocked by cooperation between c-*myc* and *bcl-2* genes [33]. The effect of oncogenes on radioresistance may thus be dependent on cell type in terms of the existence of a distinct balance between different regulatory pathways of radiation response. The fact that DNA-PK is much more abundant in primate cells than in rodents [28] can, perhaps, partly explain discordant results obtained in transfection experiments with activated Ha-*ras*-1 that have been observed in human and rodent cell lines. Thus, additional screening of molecular and biochemical alterations in tumour cells is necessary for understanding the mechanisms of resistance of tumours to radiation therapy.

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