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No Correlation between *ras*, c-*myc* and c-*jun* Proto-oncogene Expression and Prognosis in Advanced Carcinoma of Cervix

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55 patients suffering from stage III or IV carcinoma of cervix were treated with two pulses of neo-adjuvant chemotherapy prior to radical radiotherapy. 51% (26/51) had a partial response. The initial response to chemotherapy is associated with significantly better long-term survival. The 3-year survival of chemotherapy responders is 62% against 21% for non-responders ($P = 0.009$ log-rank test). To detect possible differences in oncogene expression in biopsy specimens taken from responding and non-responding patients, paraffin-fixed material was immunocytochemically stained for the expression of the protein products of *ras*, c-*myc* and c-*jun* proto-oncogenes. The frequency of oncogene expression was *ras* 80.4%, c-*myc* 45.1% and c-*jun* 39.2%. There was no statistically significant association between oncogene expression, time to local recurrence or development of metastases or survival.

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INTRODUCTION

WE HAVE SHOWN that long-term disease-free survival in patients with advanced carcinoma of cervix treated with neo-adjuvant chemotherapy is strongly associated with an objective response to the chemotherapy [1]. There may be a fundamental biological difference between chemoresponsive and non-responsive tumours. Evidence strongly suggests that oncogene expression is an important factor in the initiation and development of a range of malignant tumours [2]. The degree of oncogene expression may be an important prognostic factor for the individual patient with carcinoma of cervix. The c-*myc* oncogene, which is closely associated with cellular proliferation has received the most attention in this disease. Early stage patients (stage I and II) whose tumours showed c-*myc* overexpression have an 8-fold increase in the incidence of early relapse compared with other patients [3]. C-*myc* gene amplification and overexpression is more frequent in advanced tumours (Stage III and IV) than earlier stages [4].

Mutated forms of other oncogenes have been seen more frequently in advanced tumours. Mutations at codon 12 of the c-Ha-*ras* gene were detected in 2% of Stage I and Stage II tumours and 24% of Stage III and IV lesions [5]. Loss of one c-Ha-*ras* allele is relatively common in cervical cancer (36%) and is not associated with increasing tumour stage.

However, tumours with loss of one c-Ha-*ras* allele often contain an activated c-*myc* gene. Both c-Ha-*ras* mutations and allele loss are associated with c-*myc* gene amplification and/or overexpression which was found in 100% and 70% of tumours containing mutation and deletion, respectively [5]. This suggests that the two proto-oncogenes cooperate in the progression of cervical cancer. *In vivo* these two proto-oncogenes have been shown to cooperate in the malignant transformation of fibroblasts [6].

In contrast to *myc* and *ras*, the role of c-*jun* in carcinoma of cervix has not been explored. c-*jun* interacts with c-*fos* to produce the transcription factor AP-1 which controls the transcription of several genes and the resultant synthesis of cellular proteins. Genes activated by AP-1 include those coding for metallothionien$_{IIa}$, collagenase and stromelysin. Interestingly in cultured cells metallothioniens bind to cisplatin and overproduction of metallothionien confers relative resistance to cisplatin [7].

It is possible that the pattern of oncogene expression could predict for clinical behaviour therefore we have retrospectively examined paraffin-fixed biopsy material immunocytochemically stained for the protein products of the *ras*, *myc* and *jun* proto-oncogenes.

MATERIALS AND METHODS

55 patients were treated between January 1984 and June 1987. The median age was 49 (range 29–69). 40 patients were assigned to stage III and 15 to stage IVa.

Two pulses of chemotherapy consisting of cisplatin 50 mg/m^2, bleomycin 30 mg and vincristine 2 mg were given 14 days apart before radical radiotherapy. Further details including side-effects are published elsewhere [1].

Paraffin-fixed histological material removed prior to treatment was immunohistochemically stained to try to correlate prognosis with expression of *ras*, c-*myc* and *jun* oncoproteins. The following antibodies were used: for *ras* p21, the rat monoclonal antibody (Mab) Y13 259 [8]; for c-*myc* p62, the mouse Mab mycl-9E10 [9] and c-*jun* AP-1, the rabbit antipeptide polyclonal antibody 890. This antibody was made against the peptide GSLKPHLRAKNSD which corresponds with aminoacids 47-59 of the human c-*jun* sequence [10]. Y13 259 is specific for

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both natural and mutant p21 protein from all members of the *ras* family (n-*ras*, K-*ras* and H-*ras*). Mycl-9E10 is specific for p62 protein from c-*myc* only. Both Y13 259 and mycl-9E10 antibodies were prepared from hybridomas as previously described [11, 12]. Immunohistochemistry was carried out using the streptavidin-peroxidase DAB system [11, 12]. The ability of Mab Y13 259 to detect enhanced levels of *ras* p21 was confirmed using transfected Chinese hamster lung (FHO 6T1-1) cells which carry and express at high levels the exogenous human T24 H-*ras* 1 gene and the recipient Chinese hamster lung (CHL) cells as negative controls. The positive control for *myc* p62 was the human HL60 promyelocytic cell line which expresses the *myc* gene and the negative control was human embryo fibroblasts.

The negative control for the polyclonal antibody AP-1 was 208r rat fibroblasts. The positive controls were the same cell line transfected with the plasmid pAGT1 which contains T24 H *ras* 1 oncogene. The resultant cell line RFAGT1 overexpresses the *jun* AP-1 protein. An additional control included specimens treated by the staining protocol but without addition of an antibody.

The antibody staining was scored as positive, negative or equivocal. All of the sections were examined by two experienced pathologists who at the time of the examination were unaware of the clinical outcome. Positive staining was confined to tumour cells and not surrounding normal tissue.

RESULTS

51% (26/51) of patients had a partial response to chemotherapy according to WHO criteria. 25 patients had no response to chemotherapy and in 4 cases response was not assessed. All patients who demonstrated a partial response to chemotherapy had a complete response to radiotherapy when assessed 3 months after the end of treatment. The actuarial 5-year survival of all patients is 43% (95% C.I. 29–56%). However an initial response to chemotherapy is associated with significantly better long-term survival. The 3-year survival of chemotherapy responders is 62% against 21% for non-responders ($P = 0.009$ log-rank test). Response to chemotherapy and subsequently good survival is not explained by differences in patients age, tumour pathological grade or stage [1] or expression of oncogene protein products.

The frequency of expression of oncogene protein products is high in these advanced tumours. The percentage of biopsy specimens showing positive immunocytochemical staining was 80.4% for *ras*, 45.1% for c-*myc* and 39.2% for c-*jun*. In the 49 specimens in which an assay for all three oncoproteins was performed only 16.3% (8 cases) had no evidence of expression of at least one oncogene and 28.6% (14 cases) exhibited all three oncogenic protein products.

There is no statistically significant association with positive staining for oncogene products and response to chemotherapy (Table 1), survival, time to distant metastases or local recurrence (Table 2). Similarly pathological grade shows no statistically significant association with individual oncogene expression or the number of oncogenes detected in each biopsy specimen.

DISCUSSION

It has been suggested that cisplatin resistance in human carcinomas may be associated with oncogene amplification. The oncogene c-*fos* (closely associated with c-*jun*) and c-Ha-*ras* have been shown to be amplified in patients failing treatment with cisplatin and 5-fluorouracil [13]. Cultured cell lines of cisplatin resistant breast and ovarian tumours have shown a 3–5-fold increase in c-*myc* messenger RNA [14].

Table 1. Association of oncoprotein expression and response to chemotherapy

Oncogene	Partial response (%)		No response (%)		*P*-value
ras					
Present	80.0	(20)	77.3	(17)	1.00*
Absent	20.0	(5)	27.7	(5)	
	100	(25)	100	(22)	
c-*myc*					
Present	50.2	(13)	33.3	(7)	0.251
Absent	50.0	(13)	66.7	(14)	
	100	(26)	100	(21)	
c-*jun*					
Present	40.0	(10)	31.8	(7)	0.260
Absent	60.0	(15)	68.2	(15)	
	100	(25)	100	(22)	

*This *P*-value from Fisher's exact test.

We can find no statistically significant association between *ras* and *myc* expression and response to chemotherapy. In particular *ras* p21 protein was found in 80.0% of chemoresponsive tumours and 77.3% of non-responding lesions, a virtually even split. c-*fos* forms a heterodimer with c-*jun* to produce AP-1 protein. One possible biochemical mechanism of resistance is control of AP-1 transcription factor. However we could not find any statistical association between AP-1 detection and response to chemotherapy.

Similarly local control development of distant metastases or survival showed no strong evidence of being related to oncogene expression as detected by immunochemical methods in our patients. One reason for the lack of prognostic significance in the gene products is the high level of detection coupled with the size of the sample. Activation of c-*myc* has been shown to be common in advanced cervical carcinomas. Gene amplification was present in 49% (26/53) and overexpression (increased c-*myc* RNA) was present in 75% (30/40) in biopsies taken from stage III and IV patients [4]. Our frequency of detection (45%) is consistent with these findings. The frequency of detection of the *ras* protein p21 (80.4%) is high but perhaps not surprisingly in these aggressive advanced tumours.

Table 2. Log-rank P-values for the association of oncoprotein expression with patient outcome

	Survival	Time to develop distant metastases	Time to local recurrence
ras			
Present vs. absent	0.295	0.061	0.974
c-*myc*			
Present vs. absent	0.183	0.164	0.601
c-*jun*			
Present vs. absent	0.923	0.521	0.995
Number of oncoproteins present in each biopsy			
0v1v2v3	0.709, 0.476*	0.208, 0.404	0.607, 0.636

*First *P*-value compares 0 vs.1 vs.2 vs.3 assuming no ordering for patients for whom three oncoproteins were assayed. The second *P*-value tests for linear trend across these categories. The results in this row are only for patients who had three oncoproteins assayed.

Our data suggest that the detection of the protein oncogene products of c-*myc*, *ras* or c-*jun* by immunochemistry does not give useful prognostic information in advanced carcinoma of cervix and does not predict response to chemotherapy or ultimate outcome.

1. Symonds RP, Burnett RA, Habeshaw T, Kaye SB, Snee MP, Watson ER. The prognostic value of a response to chemotherapy given before radiotherapy in advanced cancer of cervix. *Br J Cancer* 1989, **59**, 473–475.
2. Slamon DJ. Proto-oncogenes and human cancers. *N Engl J Med* 1987, **317**, 955–957.
3. Riou G, Le MG, Le Doussal V, Barvois M, Martine G, Haire C. C-*myc* proto-oncogene expression and prognosis in early carcinoma of the uterine cervix. *Lancet* 1987, **ii**, 761–763.
4. Riou GF. Proto-oncogenes and prognosis in early carcinoma of the uterine cervix. *Cancer Surveys* 1988, **7**, 441–455.
5. Riou G, Barvois M, Sheng Z, Duvillard P, Lhomme C. Somatic deletions and mutations of c-Has *ras* gene in human cervical cancers. *Oncogene* 1988, **3**, 329–333.
6. Land H, Pavada LF, Weinberg RA. The tumourgenic conversion of primary embryo fibroblasts requires at least two co-operating oncogenes. *Nature* 1983, **304**, 596–602.
7. Andrews PA, Howell SB. Cellular pharmacology of cis-platin. Perspectives on mechanisms of acquired resistance. *Cancer Cells* 1990, **2**, 35–43.
8. Furth ME, Davis LJ, Fleurdelys B, Sconlick EM. Monoclonal antibodies to the p21 products of the transforming gene of Harvey murine sarcoma virus of the cellular ras gene family. *J Virol* 1982, **43**, 294–304.
9. Evan GI, Lewis GK, Ramsay G, Bishop JM. Isolation of monoclonal antibodies specific for human c-*myc* proto-oncogene product. *Mol Cell Biol* 1985, **5**, 3610–3616.
10. Hattori K, Angel P, Le Beau M, Karin MM. Structure and chromosomal localisation of the functional intronless human JUN proto-oncogene. *Proc Natl Acad Sci USA* 1988, **85**, 9148–9152.
11. Spandidos DA, Pintzas A, Kakkanas A. Elevated expression the *myc* gene in human benign and malignant breast lesions compared to normal tissue. *Anticancer Res* 1987, **7**, 1299–1304.
12. Papadimitriou K, Yiagnisis M, Tolis G, Spandidos DA. Immunohistochemical analysis of the ras oncogene protein product in human thyroid neoplasms. *Anticancer Res* 1988, **8**, 1223–1228.
13. Scanlon KJ, Lu Y, Kashani-Sabet M, MA J, Newman E. Mechanisms for cis-platin-FUra synergism and cis-platin resistance in human ovarian carcinoma cells both *in vitro* and *in vivo*. In: Rustum Y and McGuire J eds. *The Expanding Role of Folates and Fluoropyrimides in Cancer Chemotherapy*. New York, Plenum Press, 1988, 131–139.
14. Scanlon KJ, Kashani-Sabet M, Miyachi H, Sowers LC, Rossi J. Molecular basis of cisplatin resistance in human carcinomas: model systems and patients. *Anticancer Res* 1989, **9**, 1301–1312.

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Screening of New Anticancer Agents *in vitro* Using Panels of Human Cell Lines Derived from Non-seminomatous Germ Cell Tumours and Transitional Cell Carcinomas of the Bladder

John R.W. Masters, Wendy E.A. Jenkins and Robert H. Shoemaker

Metastatic testis tumours are cured in over 80% of patients using combination chemotherapy, and this hypersensitivity is retained by the cells *in vitro*. To determine whether differential toxicity to testis tumour cells is useful in the screening of novel anticancer agents, we compared the toxicities of 12 compounds against panels of human bladder and testis tumour cell lines using a clonogenic assay. The compounds had screened negative against P388 *in vivo*, and had been retested using the human tumour colony forming assay (HTCFA) and in selected cases against human tumour xenografts. NSC 339004, chloroquinoxaline sulphonamide, was 7-fold more toxic to testis tumour than bladder cancer cells, comparing the mean of the concentrations reducing colony-forming ability by 70%. This was the only one of the compounds selected by the HTCFA shown to have clinical activity. Compound R was selectively toxic to the bladder cancer cells, and might be of value as an intravesical agent. These data indicate that panels of testis and bladder cancer cell lines might be a useful addition to the disease-oriented screening programme.
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INTRODUCTION

ANTICANCER DRUG screening in experimental animals has been conducted by the US National Cancer Institute (NCI) on a large scale for over 30 years. While this programme has discovered or played a key role in the development of nearly all the drugs currently used in cancer treatment [1], the limited activity of these compounds against the common adult solid tumours has led to the development of alternative screening strategies.

The concept of "*in vitro* phase II screening" derives from the testing of new drugs on the colony-forming ability in agar of fresh human tumours of each histological type [2]. Because preliminary data were encouraging, the NCI organised a multicentre analysis of the assay. Approximately 1000 compounds already tested *in vivo* against transplantable P388 leukaemia in mice were entered in the human tumour colony forming assay (HTCFA) screen. Compounds negative against P388 *in vivo*,