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The Role of *ras* and *myc* Oncogenes in Human Solid Tumours and Their Relevance in Diagnosis and Prognosis (*Review*)

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Abstract. *Advances in the field of oncogenes have produced a tool to investigate the different stages in multistep carcinogenesis. The role of the* ras *and* myc *gene families have been extensively investigated in the progression of carcinogenesis in a range of human solid tumours. This review critically analyses the data available on the role of these oncogenes in the six most common cancers worldwide, (i.e. cancer of the stomach, lung, breast, colon, cervix, and mouth and pharynx). In certain cases the incidence of aberrant gene expression and genetic alterations of the* ras *and* myc *gene families have been shown to be important in the progression of these cancers and may be of use as prognostic indicators.*

Contents

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Key Words: Ras and *myc* oncogenes, human solid tumours, diagnosis, prognosis.

1. Introduction

The transformation of a normal cell into a cancer cell is a complex multistep process resulting in a clone of cells that are no longer under normal regulatory control. Recent advances in the field of oncogenes have provided a tool to investigate the different steps in carcinogenesis, both at an experimental level and also by analysing human tumours. If the control mechanisms of certain genes could be identified at particular steps in tumour development, then it might be possible to develop clinical strategies for intervention, to block further tumour progression.

The evidence that transforming genes were involved in tumourigenesis came initially from work with reteroviruses, which could transform cells in culture and induce tumours in experimental animals (1). The realisation that the acutely transforming reteroviruses had transduced their v-*onc* genes from the host cell led to the discovery of a number of proto-oncogenes in normal cells (2). Subsequently, it was discovered that these normal genes or proto-oncogenes were activated in certain tumours and appeared to be involved in the progression of the disease (3, 4, 5).

Proto-oncogenes may be activated by a number of methods; insertion of a promoter or enhancer element, translocations, point mutations of a structural gene and amplifications. These mechanisms may lead to an increase in the oncogenic product or may produce an altered gene product resulting in malignant transformation of the cell (4,

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5). Other mechanisms as yet unidentified must exist to explain over-expression of certain oncogenes in tumours without obvious genetic alterations. These mechanisms may take the form of other cellular genes influencing the oncogene regulation, or the stability of the oncogene mRNA, of which little is known.

It is now clear that certain tumour suppressor genes or anti-oncogenes may also be involved in tumour progression (for review see ref 6). The existence of these genes has been inferred by both experimental fusion of normal and cancer cells which often suppresses the neoplastic phenotype (7) and by the study of certain forms of hereditary cancer (8, 9, 10). Recently an association between certain chromosomal deletions and cancer of a non-heritable origin has been reported (for review see ref. 11).

The aim of this review is to elucidate the role of the *ras* and *myc* gene families in the six most common solid tumours and also to discuss the relevance of these genes to the diagnosis and prognosis of these carcinomas. Deletions in chromosomes that are pertinent to the role of *ras* and *myc* genes will also be discussed.

2. Tumour development

Carcinogenesis is considered to be a multistep process. Evidence for this comes from epidemiological studies in man, biochemical and histopathological analysis of tumours *in vitro* and *in vivo,* cell tumour formation studies with chemicals, radiation and viruses (for review see refs 12, 13). Carcinogenesis may be divided into three distant stages: initiation, promotion and progression.

Initiation is possibly an absolute requirement involving mutational events which are therefore irreversible. Initiation may occur very rapidly with the use of a single application of a carcinogen. This has been observed in a number of animal model systems where certain chemical carcinogens acting with or without tumour promoters induce the activation of the *ras* genes by point mutation (14-18). It is feasible that the point mutations in the *ras* gene confer a selective growth advantage on these cells. It is not unreasonable to suggest that mutations in other genes probably also occur; some of these mutations will be lethal while others give the cells a competitive advantage and in time undergo phenotype diversification and eventually become a malignant subpopulation. There is also evidence for X-rays inducing point mutations in the *ras* proto-oncogene in lymphomas (19). Viral initiation has also been reported in a number of model systems (20). Evidence from animal model systems has shown that with the use of a particular carcinogen, certain tumour types are formed which carry specific mutations in the *ras* gene family (17-19, 21, 22). A major breaktrough in the understanding of initiation came from the work by Balmain *et all* (14), which demonstrated that a high percentage of mouse skin papillomas had an activated H-*ras* oncogene. The fact that this gene was activated in the premalignant state of skin carcinogenesis supports the hypothesis that this point mutation was intimately linked with initiation. Experimental evidence suggests that over-expression of a single mutated *ras* oncogene is sufficient to transform primary rodent cells into a malignant phenotype (23). Also in early passage rodent cells transformed with recombinant plasmids the mutant T24 H-*ras*1 oncogene was shown to set into motion the malignant conversion, by causing multiple metastasis when intravenously or subcutaneously injected into nude mice (24). The *myc* gene has also been shown to act as an initiator of carcinogenesis when the gene is introduced into cells in a reterovirus or in transgenic mice (25). Even though the *ras* and *myc* genes have been implicated in the initial stages of tumourigenesis, it is most likely that there are more general effects, such as the ability of cells to incorporate and metabolise carcinogens, whether the affected cells are proliferating or non-proliferating, and what selection pressures exist within the tissues.

Tumour promotion is the second stage in the classical model of multistep carcinogenesis. However, the evidence for DNA damage is lacking at this step in carcinogenesis. The major advance in this area has been the identification of protein kinase C as a binding site for the tumour promoter TPA, (26, 27). The role of tumour promoters remains unclear (for a review see refs 28, 29). Hesnnings and Yuspa (29) disagree with the two stage model for phorbol ester promotion. Several test systems have been investigated to ascertain the effects of first and second stage promoters, but as yet our understanding of tumour promoters is incomplete (20, 30, 31).

The progression of the benign tumour to the malignant phenotype is the most important clinical stage. However, at the early stages it is not possible to differentiate experimentally between promotion and progression of the disease, and little is known about the molecular changes involved. Possibly genetic alterations, such as chromosomal rearrangements, deletions, mutations or amplification of specific genes may be important at this stage. Genetic characteristics of malignant cells are aneuploidy, double-minute chromosomes and homogeneously staining regions in chromosomes (32, 33). Also the DNA content per cell is often increased in cancer cells when measured cytochemically (34, 35). Recently Vogelstein *et al* (36) have demonstrated that patients suffering from cancer of the colon with a higher ratio of allelic deletions have a poorer prognosis than those with a lower ratio.

There are now a large number of reports concerning oncogenes in human tumours, some of which have been identified at the different stages of carcinogenesis, on the basis of clinical and pathological evidence. Therefore it is clear that oncogenes can act at all three recognisable stages: initiation, promotion and progression. The question this review addresses is at what stages the *ras* and *myc* oncogenes are involved in the evolution of the common human carcinomas.

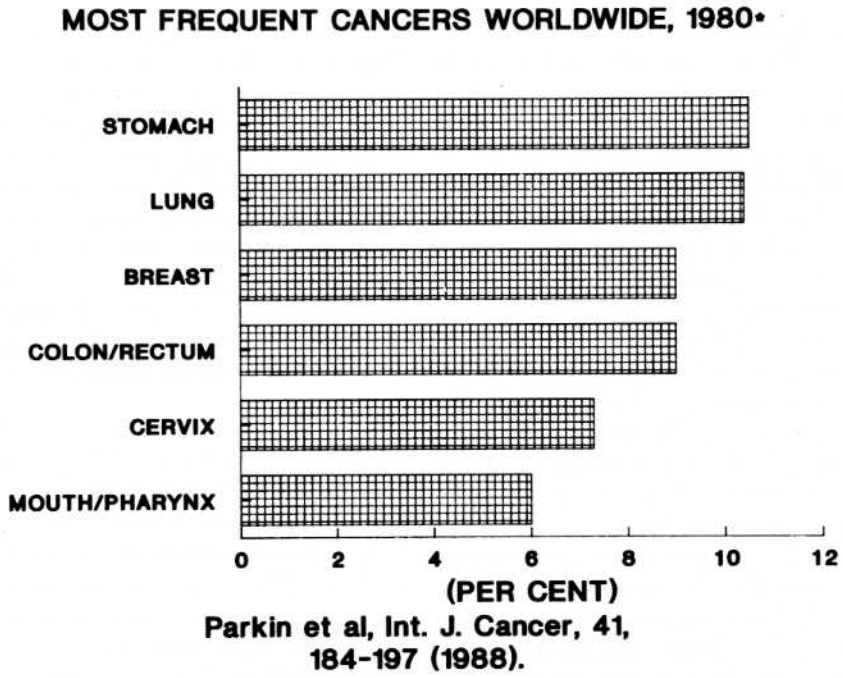

Figure 1. *Most frequent cancers worldwide, 1980. (37)*

3. The role of *ras* and *myc* gene families in carcinogenesis

The role of the *ras* and *myc* gene families in the progression of carcinogenesis in solid tumours has been investigated in a wide range of tissues. The six most common malignancies world wide are cancer of the stomach, lung, breast, colon, cervix and mouth and pharynx (37) (Figure 1). They have all been examined to a greater or lesser extent to ascertain whether the *myc* and *ras* oncogenes are important in their evolution. The incidence of aberrant gene expression or genetic alterations may also be of use as prognostic indicators.

However, even though a large number of studies have been undertaken, few have yielded any real advancement in our knowledge regarding the role these genes play in tumourigenesis. In an effort to dissect out which of these studies further our understanding on the role of oncogenes in carcinogenesis, certain clinical and statistical requirements have to be taken into consideration.

Initially, studies on oncogene expression and alteration of these genes were undertaken in a wide range of human tumours, but with few samples of each tissue type. Even though this provided evidence for the presence of oncogenes in tumours, little could be deduced about their role. In the past six years a large volume of work has been undertaken in this field and much of it falls into pilot or preliminary studies. In many instances there have been insufficient numbers of patients examined for reliable statistical analysis to be undertaken. Also in many cases there have not been complete clinical data with follow up. There are many good reasons for this happening, as publication would be delayd for 3-5 years after analysing fesh tumour tissue to await follow up data and produce survival curves.

In order to separate out the different stages of carcinogenesis it is of particular benefit to study those cancers that have defined clinical stages, as in benign, premalignant, primary tumours and subsequent metastasis. Taking these points into consideration, there are few studies that have all these clinical and statistical requirements.

It is extremely difficult to make any broad generalization about the role of *ras* or *myc* tumourigenesis, as such a range of results have been published; for instance *c-myc* overexpression in carcinoma of the uterine cervix correlates with risk of relapse (38); c-*myc* amplification correlates with a poor prognosis in breast cancer patients (39), elevated c-*myc* oncoprotein correlates with a poor prognosis in head and neck cancer (40) and N-*myc* amplifications are associated with rapid tumour progression in neuroblastomas (41). Whereas in colorectal carcinoma c-*myc* expressions did not correlate with patients survival (42) and N-*myc* and L-*myc* did not correlate with the clinical outcome of patients with small cell lung cancer (SCLC) (43).

Moreover, there is not always an agreement of results between different research groups using the same cancer type. This is very noticeable in breast cancer investigations, where no correlation was found between the 57 per cent incidence of c-*myc* amplification and prognosis (44) and in another study the incidence of c-*myc* amplifications was found to be very low (6 per cent) and again of no prognostic importance (45). Also different results have been found for N-*myc* expression in SCLC and c-*myc* in colon cancer when correlated with survival (53, 77).

The use of oligonucleotides (46) and RNase mismatch cleavage analysis (47) has changed scientific opinion on the incidence of *ras* mutations in human tumours. It is now realised that point mutations in the K-*ras* genes are much more common than originally thought and account for 40 percent in colon tumours. However, no correlation was found between the presence of the mutated *ras* oncogenes and the degree of invasiveness in colon cancer (46, 47), whereas mutation in the H-*ras* gene is significantly associated with poor prognosis in cervical cancer (48, 49). An extremely interesting observation emerged from the colon tumour investigations (46, 47), indicating that mutations in *ras* proto-oncogenes were detected in premalignant colon polyps. *Ras* mutations have also been found in human pre-leukaemia myelodysplastic syndrome (50, 51). Even though amplification of the *ras* genes in tumours is a rare event, over-expression of RNA transcripts of these genes has been reported in the colon (52, 53) and over-expression has been considered important in the clinical course of the disease.

Clinically defined potentially malignant lesions are recognised in both breast and colon cancer, and this provides a very useful tool to investigate the switching on of certain oncogenes in the progression of the disease. It appears from the limited data available in the benign cystic disease of the breast that the c-*myc* gene is amplified in some cases (39, 44) and that c-*myc* expression was elevated (54).

The situation in colon polyps has been more fully investigated and there is a consensus of opinion in the literature that elevated *ras* expression is associated with this premalignant stage of colon cancer (55, 56).

The most reliable data that can be considered to be of prognostic importance are those which has been subjected to thorough statistical analysis such as disease-free survival rates computed by Kaplan-Meier (57), Cox's proportional hazard model (58) or the log rank test (59). These types of analysis have been carried out in breast cancer (39, 60, 61), colon cancer (42), small cell lung cancer patients (43), cancer of the uterine cervix (38), and in head and neck cancer (40). It is only with detailed attention to this type of statistical survival data that we may draw meaningful conclusions from these oncogene studies. As both *ras* and *myc* genes appear to have different roles in many human tumour types, each of the six most common solid human tumours will be discussed separately.

4. Stomach cancer

4i. *C-myc expression in stomach carcinomas.* There is little information on c-*myc* genetic alterations in stomach carcinomas, apart from the report by Yokoto *et al* (62) who looked at nine stomach carcinomas in a survey of 71 epithelial cancers. No amplification of the c-*myc* gene was found in the stomach specimens, compared with a total of eleven per cent in other tumours studied.

The expression of the c-*myc* gene in gastric cancers has been investigated using the monoclonal antibody 1-9E10 (63). Low levels of expression of the protein product p62 c-*myc* were found in normal gastric tissue, compared to those found in inflammatory, metaplastic and displastic specimens (64), suggesting that this oncogene product maybe of use in identifying potentially neoplastic hyperproliferative states. However, the results of Allum *et al* (65) are not in agreement with these findings. Allum *et al* (65) used *myc* 1-6E10 to detect c-*myc* p62 in 93 specimens of gastric cancer; they found that less than 40 per cent of the tumours contained positively staining cells, and also that there was no correlation with the degree of histological differentiation.

4ii. *Ras genetic alteration and expression in stomach cancer.* In the study of 71 epithelial tumours of which 9 were from the stomach, no H-*ras* deletions were reported by Yokota *et al* (62). However, with the use of oligonucleotide probes, Bos *et al* (65a) found a gastric carcinoma with a mutated K-*ras* allele, and also an amplified normal K-*ras* allele. They suggest that these two changes in the K-*ras* genes may indicate two separate steps in the genesis of this particular gastric carcinoma unless the tumour had two seperate clonal origins.

The expression of the *ras* oncogene product p21 has been investigated using a number of monoclonal antibodies, HAS6, HAS5, HAS2, (66) RAP-5 (67, 68), RAS KI-16 (69) and also by a direct binding liquid competition radioimmunassay (RIA) to the p21 protein product (70).

Using the RAP5 monoclonal antibody the p21 *ras* oncogene product was detected in only 1 of 13 cases of normal or benign gastric lesions compared to all of the 20 gastric carcinomas tested (67). A similar result using the same monoclonal antibody, was found in 65 of 96 stomach cancers (68). *Ras* p21 was quantified by De Biasi *et al* in maligant and normal stomach cancer using a direct binding liquid competitions assay (RIA) (70); they reported that the amount of p21 *ras* expressed in maligant stomach cancer was significantly greater than that found in benign tissues ($P<0.005$). Yoshida *et al* (69) reported on the isolation of 16 murine monoclonal antibodies to *ras* p21, and they investigated the expression of p21 *ras* in 101 cases of stomach cancer and in 52 cases of non-stomach cancer. Their results indicate that p21 *ras* is expressed in moderately to well diffentiated stomach cancer, intestinal metaplasia and in atypical hyperplasia. In a recent study, 174 gastric cancers were investigated for the expression of TGFα and H-*ras* p21 immunohistochemically. TGFα immunoreactivity was detected in 7 of 27 early carcinomas and in 110 of 147 advanced cancer ($P<0.01$). Patients with carcinomas showing expression of both TGFα and H-*ras* p21 (59 of 67 cases) had a very poor prognosis compared to those with low levels of expression ($P<0.05$) (71).

The data available on c-*myc* and *ras* gene action in gastric carcinomas indicate that increased *ras* expression is important in the progression of stomach cancer, but the timing of c-*myc* is still uncertain.

5. Lung cancer

There are four major histological types of lung cancer, squamous cell carcinoma, adenocarcinoma, large cell carcinoma and small cell lung carcinoma (SCLC). The former three groups are called the non-small cell lung carcinomas (N-SCLC) and have different clinical features from the SCLC. The SCLC are also treated differently, usually with combination chemotherapy and radiotherapy and have a poor prognosis (72).

There have been a large number of reports on the molecular analysis of lung cancer (reviewed by Minna *et al*, 72, 73) but the majority of the investigations have concentrated on SCLC (43, 74-79). The interpretation of the results in SCLC is complicated by the fact that the majority are from SCLC cell lines or from necropsy specimens post-chemotherapy treatment; however, there are a number of examples taken from fresh primary SCLC prior to any chemotherapy and radiotherapy.

5i. *Myc and ras expression and amplification in SCLC.* A large number of SCLC cell lines have been set up and all members of the *myc* gene family have been investigated. In 31 SCC cell lines, 14 have had a c-*myc* or N-*myc* gene amplified with or without over-expression (75). It is of considerable interest that these authors also found N-*myc* amplification in a tumour cell line prior to chemotherapy and N-*myc* amplification in a tumour metastasis.

A survey of the expression and amplification of 16 proto-oncogenes in 12 SCLC cell lines showed that 7 out of 12 had c-*myc* amplification, 3 out of 12 had N-*myc* amplification and 1 out of 12 had simultaneous amplification of c-*myc* and N-*myc*. All the cell lines had similar levels of expression of N-*ras*, K-*ras*, H-*ras* and c-*raf1* but no amplification of these genes (79). The other oncogenes studied showed no significant expression.

Amplification of the *myc* gene family has also been investigated in 44 SCLC cell lines, of which 19 were established before chemotherapy was initiated. C-*myc* amplification was only seen in cell lines established from treated patients. It was demonstrated that the chemotherapy treated cell lines with c-*myc* amplification survived a significantly shorter time than patients without c-*myc* amplification ($P<0.05$) (78).

In another investigation (43) into 38 different SCLC specimens (34 from necropsy, 4 from surgery prior to treatment), it was found that 4 out of 38 had N-*myc* amplification and 2 out of 38 had L-*myc* amplification but none had c-*myc* gene amplification. All 6 tumour specimens with *myc* amplification were taken from patients who had been treated with combination chemotherapy. However, no difference in the clinical course of the disease was found between patients with and those without N-*myc* or L-*myc* amplification. A particular problem with analysing survival data for SCLC is that these patients have such a poor prognosis anyhow (usually less than 12 months), that one has to be cautions in interpreting the data.

In another study 15 primary biopsies from patients who had SCLC but had no previous treatment, were investigated for N-*myc* expression using *in situ* hybridisation techniques (77). The results indicated that increased N-*myc* expression correlated with poor response to chemotherapy, rapid tumour growth and short survival ($P<0.01$). However, the most likely explanation for the patients without N-*myc* expression having a longer survival was that they had extended treatment periods of cytostatic drugs compared to the patients with high levels of N-*myc* expression. Even so, this study provides very valuable information on fresh primary untreated SCLC specimens, as it indicates that N-*myc* is important in the progression of the disease. The results in fresh tumour specimens indicate that the N-*myc* gene is over-expressed in 6 out of 15 cases of SCLC before chemotherapy (77) and amplified in 4 out of 4 cases after the combined chemotherapy (43). The mechanism for overexpression of N-*myc* in the 6 specimens may not be due to amplification but it is clear that the N-*myc* gene is unregulated in SCLC regardless of whether the tumour has received chemotherapy. This provides further evidence for the involvement of N-*myc* in SCLC and that it is not just an indication of genetic damage due to cytostatic drugs. Recently it has been demonstrated that c-*myc* is overexpressed in a number of biopsy specimens taken from untreated bronchial carcinomas using the *myc* 1-9E10 monoclonal antibody (135). Elevated c-*myc* expression was found in 16 of 37 (43%) squamous carcinomas; 4 of 10 (29%) adenocarcinomas; 3 of 7 (42%) N-SCLC's and 4 of 21 (19%) SCLC's. However no correlation was found between elevated c-*myc* expression and survival of these patients (135).

In contrast to these results in SCLC, there has been a report of K-*ras* and c-*myc* amplification with a point mutation in K-*ras* in a lung giant cell carcinoma (LGCC) (80). This gives weight to the theory of oncogene cooperation in carcinogenesis, but the stage at which these two oncogenes were activated is unknown.

In the context of timing of the activation of oncogenes in lung cancer, the results of Rodenhuis *et al* (81) contribute to this. They examined *ras* gene mutations in N-SCLC patients, the majority of specimens being obtained at thoracotomy, and they found that 9 out of 35 adenocarcinomas of the lung had K-*ras* mutations. On examining the patients' smoking history, they concluded that there was an association between their smoking habits and the incidence of K-*ras* mutations in their lung cancers. It is possible that K-*ras* mutation events may be directly related to carcinogenic substances in tobacco smoke and therefore that these mutations are occurring at the initiation stages of these cancers.

The differential expression of ras p21 in 23 fresh primary lung tumours (82) had been correlated with histological classification; 9 out of 11 tumours with a squamous histology compared to 1 out of 12 non-squamous carcinomas of the

Table I. *Review of* ras *and* myc *expression and genetic alterations in breast cancer.*

This table has been compiled from the published data pertaining to the role of *ras* and *myc* gene families in breast cancer. In order to make comparisons between the data from different papers, the original authors' results have been expressed in a different format in a number of cases. The results have been broken down into: malignant, potentially malignant and benign lesions with the number of patients in each group. Patients with amplification and elevated expression are shown as percentages. Data on rearrangements and mutations are described in the clinical correlation section.

Any clinical correlations pertaining to the review are also included.

Table 1A.

Oncogene	Tumour type	No. of patients studied	Percentage of patients with amplification	Percentage of patients with elevated expression	Clinical correlations	Reference
c-*myc* (RNA)	Carcinoma	23		73	Significant difference in *ras* and *myc* expression between benign and malignant tumour (P>0.01)	105
	Fibrocystic disease	23		17		
	Fibroadenoma	4		0		
c-*myc* (mRNA)	Carcinoma	121	32		c-*myc* amplification correlates with pt age P<0.002 and invasive ductal histology. 6 of the 14 carcinomas with elevated c-*myc* expression had a amplified c-*myc* gene	87
	Fibroadenoma	5	0			
	Carcinoma	14		70		
c-*myc*	Carcinoma	41	17	24	c-*myc* amplification correlated with poor prognosis P<0.02. (Number of pts analysed for c-*myc* expression unclear from original paper)	39
	Lymph node metastasis	10	40			
L-*myc*	Carcinoma	41	24			
N-*myc*	»	41	0			
c-*myc*	Fibrocystic disease	10	10			
c-*myc*	Fibroadenoma	6	0			
c-*myc* (RNA)	Fibrosarcoma	1	0			
c-*myc* (RNA)	Carcinoma	100	6		High levels of c-*myc* expression correlated with lymph mode metastasis (P>0.01)	45
	Carcinoma	98		45		
	Fibroadenoma	6	0	0		
c-*myc* (southern)	Carcinoma	37	57		No clinical correlations	44
	Fibroadenoma	7	29		Rearrangements with amplification seen in 3 malignant tumours	
	Cystosarcoma	2	50			

continued

Table IA continued

	Sclerosant adenosis tumour	1	0		A cystosarcoma specimen was amplified and rearranged	
C-*myc* (myc 1-9E10)	Fibrocystic disease	198			c-*myc* protein found at a higher level in mucous metaplastic cells and multiple papillomas	54
C-*myc* (ELISA)	Carcinoma	24		100	Weak correlation with patients age $P<0.1$ No correlation with prognosis.	61

Table 1B.

Oncogene	Tumour type	No. of pts.	Percentage with elevated expression	Clinical correlations	Reference
H-, K-, N-*ras*	Carcinoma	104		No rearangements or amplification found, one allele lost in 14 of 51 patients	93
K- or N-*ras*	Carcinoma	22	0	heterozygous for H-*ras*[1] correlated with grade III	
H-*ras*	Carcinoma	22	73	tumours.	
Ki-*ras*	Carcinoma	22	0	Breast cancer cells express H-*ras* p21 but not N-*ras* p21 or K-*ras* p21	
N-*ras*			0		
(mRNA) (Southerns)					
H-*ras* (oligonucleotides)	Carcinoma	24		2 G-T mutations (codon 12) 7 amplified 2 loss of H-*ras* allele.	94
H-*ras* (RNA)	Carcinoma	12	100		104
H-*ras* (RNA)	Carcinoma	24	100	Elevated expression correlated with histological grade.	106
N-*ras*	Carcinoma	23	65	Significant difference in c-*myc* and *ras* expression between malignant and benign tumours ($P<0.01$)	105
K-*ras*			48		
H-*ras*			4		
N-*ras*	Fibroadenoma		26		
K-*ras*	Fibrocystic (27)		15		
H-*ras*	disease		4		
(RNA)					
N-*ras* (RNA)	Carcinoma	41		No amplification observed	39
ras p21	Carcinoma	30	63	Correlation between p21 ras and tumour invasion. (positive staining >20% of tumour carcinoma cells scoring positive)	99
RAP-5	fibrocystic	11	0		
(mAb)	fibroadenoma	10	20		
ras p21	Carcinoma	18	83	No clinical correlations found	106
Y13-259	*in situ* CA.	2	100		
(mAb)					

continued

Table IB continued

	Fibrocystic disease	33	42		106
	Fibroadenoma	22	23		
K-*ras* p21 N-*ras* p21	Carcinoma	11	82	Breast cancer cells express a *ras* p21 different from H-*ras*	66
H-*ras* p21 Has 5 Has 21 Has 6 (Western blotting mAb)	Carcinoma	11	0	(elevated expression equals ++ or +++ in this paper)	
ras p21 RAP-5 and Y13-259 (mAb)	Invasive *in situ* CA Fibrocystic disease (46) without hyperplasia with hyperplasia (26) (i) without atypia (ii) with atypia	47 7 20 16 10	77* 71* 0 50‡ 80‡	*ras* p21 expression elevated in invasive CA, compared to hyperplastic lesions. ($P<0.01$) *>50% positive and ‡>20% positive with mAb RAP-5 Patients with hyperplasia, who subsequently developed CA had higher levels of *ras* p21 in 5 of 18 patients at the time of first biopsy	100
ras p21 Y13-259 (mAb)	Carcinoma Fibroadenoma Fibrocystic disease	20 3 5		No significant difference in staining intensity between malignant and benign tumours	107
ras p21	Carcinoma Fibroadenoma	28 12	86 0	Tumour from postmenopausal patients contained more p21 than from pre-menopausal patients	70
Western blotting Y13-259 (mAb)	Hormone response Breast CA pts Hormone Independent Breast cancer pts Fribroadenomas'	7 6 3	100 83 0	Elevated levels of *ras* p21 accompanied by high GTPase activity	111
Western blotting Y13-259 (mAb)	Carcinoma	54	69	High levels of *ras* p21 associated with progression and prognosis of the disease	60

lung demonstrated increased ras p21 expression. Baylin (83) has suggested that there is a developmental relationship between the histological subtypes of the main lung cancers with some tumours showing features of more than one subtype. Kurzrock *et al* (82) argues that this may explain why one adenocarcinoma had evelated ras p21 and that the over-expression of the *ras* gene is involved in the evolution of the squamous cell carcinomas.

Recently another variable has been uncovered in the genesis of lung cancer, with the demonstration of a deletion in the chromosomal region 3p21 in all major types of lung cancer, *i.e.* both N-SCLC and SCLC (84). These authors concluded that loss or inactivation of a gene on 3p21 was involved in the development of all lung cancers. This view is contrary to that reported by Brauch *et al* (85), who found loss of alleles at 3p a consistent feature in SCLC and only occasionally in N-SCLC. Based on the results in Johnson's paper (78), Brauch *et al* (85) calculate that *myc* gene amplification is absent from 89 percent of cell lines from untreated patients. Using these figures they argue that, as *myc* amplification is absent in untreated SCLC patients but as 3p deletions are deleted in cell lines from untreated patients, then in terms of multistep carcinogenesis, the deletion in 3p occurs before the *myc* gene is amplified. It is also of note that

the *erb*AB sequence has been localised to the 3p21-3p25 region which overlaps the deletion in SCLC, and it has been suggested that *erb*AB is possibly the recessive oncogene involved in SCLC (86).

In view of these wide ranging observations, no clear pattern for the genesis of SCLC has emerged. There is obviously a 3p deletion involved in the early stages of SCLC but this may also be seen in other lung cancers, and the *erb*AB sequences may be the recessive oncogene that is involved in the early stages. In addition, the *myc* gene family has a role in the progression of SCLC but the reports vary on which member is important. Clearly the *ras* gene family is also integrated into this process and may correlate with the developmental process of lung cancers in general.

6. Breast cancer

6i. c-myc *genetic alterations in breast cancer.* This is one of the most comprehensively studied human diseases as over 500 breast cancer specimens have been investigated for alterations in their genetic structure or in their levels of expression of the c-*myc* gene (Table Ia). As yet, there is no evidence for amplification or re-arrangement of the N-*myc* gene and only one example of L-*myc* in an infiltrating ductal carcinoma (39). However, amplification, of the c-*myc* gene in malignant breast cancer has been found at varying frequencies by different investigators: six per cent (45), seventeen per cent (39), thirty-two per cent (87) and fifty-seven per cent (44). As the majority of these investigations were all carried out with similar probes using similar techniques, it does appear incongruous that such a wide range of c-*myc* amplifications has been found in breast cancer patients (6-57 per cent). The incidence of c-*myc* re-arrangements is usually very low (0-4 per cent) except in the paper by Bonilla *et all* (44), who found 14 per cent; however, the clinical implications of the results of Bonilla *et al* are unknown, as the authors have not as yet published the survival data on these patients.

Initially, two comprehensive studies undertaken on c-*myc* alterations and breast cancer (39, 87) have come to very different conclusions. In the larger study, with 121 patients, a significant correlation ($P<0.02$) was found between the presence of a genetically altered c-*myc* gene in the tumour tissue and patients' age (87). In this study there were 40 patients who had either c-*myc* amplification or re-arrangements of the gene, and 29 (72.5 per cent) of these patients were over 50 years of age, thereby indicating a correlation between menopausal status and c-*myc* gene alterations. But this study indicated no correlation between c-*myc* gene alteration and oestrogen-progesterone-receptor status, tumour grade, or auxiliary lymph node metastasis. No data are available on the survival of these patients.

The smaller investigations with 41 patients had the benefit of follow up data (39). No association was found between c-*myc* alteration and patient age; however, the authors, have demonstrated a significant correlation between the altered c-*myc* gene and very poor short-term prognosis ($P<0.02$). It is of interest that Varley *et al* (39) also demonstrated that alteratons of the *neu* gene (7 of 41; 17 per cent) correlated well with short-term prognosis in the same group of breast cancer patients ($P<0.0002$). In fact, in this study on 41 patients, none of the patients died or had a tumour recurrence who did not have an altered c-*myc* or *neu* gene. These findings implicate the activation of the c-*myc* gene in the progression of breast cancer, although whether associated with postmenopausal status (87) or with the more aggressive end stage disease (39) has still to be determined. Also the possibility of an interaction of an altered *neu* gene with the c-*myc* gene in the end stage disease has to be considered. One would have hoped that these conflicting results might have been resolved in the two papers published in 1988 (44, 45). However, the interpretation of these results became even more complex. Only six per cent of 100 carcinomas in one study (45) were found to have amplified c-*myc* gene, whilst in the other study Bonilla *et al* (44) found that 21 of their 37 (57 per cent) malignant tumour specimens had c-*myc* amplification. Although a high incidence of c-*myc* amplification was reported by Bonilla *et al,* no clinical correlations were found with the altered c-*myc* gene. Survival data on these patients are not yet available, and so it is not possible to correlate these data with prognosis.

A further development in the study on c-*myc* amplification in breast cancer comes from Guerin *et al* (45), who found that only 6 per cent of the patients had c-*myc* amplification but 20 per cent of them had c-*erb*B-2/*neu* amplification. Significant correlations were found between c-*erb*B-2/*neu* amplification and the number of positive lymph nodes and oestrogen, progesterone receptor status. Lymph node involvement in breast cancers is considered to be the most important clinical factor in predicting recurrence (88). The correlation between *erb*B-2/*neu* oncogene and prognosis confirms earlier results of Slamon *et al* (89) and Varley *et al* (39).

C-*myc* alteration has not been found in many benign cancers: 0 of 5 (87), 1 of 17 (39), 0 of 6 (45), and 3 of 11 (44). Low levels of c-*myc* amplification were found in the one benign fibrocystic specimen that also showed histologically marked hyperplasia with foci of atypia, which is a pathology associated with increased risk of breast cancer (39). It is also of interest that in one specimen of cystosarcoma phylloides the c-*myc* gene was amplified and rearranged (44). These findings are based on very small numbers of benign breast tumours, but there are considerable clinical implications if one can identify premalignant breast lesions from alterations in the c-*myc* gene.

There are no clear cut conclusions that may be drawn concerning c-*myc* gene alterations in breast cancer. It appears that in certain studies (87) it stands out as an important gene in the progression of the disease and is intimately linked with the prognosis of the patients. In other studies (39) there appears to be an interaction between the c-*myc* and the *neu* oncogene in as much as all the patients who died had a

genetic alteration in one of these genes. Moreover a further complication of this story is that c-erbB-2/*neu* oncogene appears to have a much greater role in the course of the disease than the c-*myc* gene (45). Unless we consider that breast cancer patients treated in the different regions covered by these studies have very different diseases, which is highly unlikely, it is more probable that the role of c-*myc* gene alterations in breast cancer is very complex. Moreover, as yet the implication of c-*myc* amplification in benign breast tumours is unclear.

6ii. *Elevated c-myc expression in breast cancers.* It was previously considered that in all cases where there was c-*myc* amplification one would see a concomitant elevation of c-*myc* expression at the RNA level (90). However, this does not appear to be borne out in DNA and RNA studies on breast cancer. The numbers of malignant breast cancer patients reported with an over-expressed c-*myc* gene and also an amplified c-*myc* gene varies: 6 of 14 (87), 7 of 7 (39), 4 of 6 (45). The reason for these findings is unclear.

However, it appears that elevated c-*myc* expression observed at the RNA level does correlate with the prognosis of the patients with breast cancer. In a study of 41 breast cancer patients 10 were found to have an elevated c-*myc* RNA transcript of whom 5 have had a recurrence or have died; the remainder have only been followed up for 8-26 months (39). The results of the follow up of these patients will be of interest, as the remaining 5 patients may also be found to have a poor prognosis.

One of the most informative studies on c-*myc* expression in breast cancer has been by Guerin *et al* (45), who found that 45 out of 98 tumours had high levels of c-*myc* RNA and that this correlated with lymph node involvement ($P<0.01$). Moreover only eleven per cent of these tumours had c-*myc* amplification. They also reported that c-*myc* expression was elevated in lymph node metastases in 3 patients, as well as in the corresponding primary tumours. Therefore c-*myc* overexpression in breast cancer cannot be explained solely by amplification of the gene previously considered. In fact, two cases which had high levels of c-*myc* amplification (10 and >50 fold) had low levels of c-*myc* expression (45).

Using monoclonal antibodies to the c-*myc* protein, it was found that there were high levels of staining intensity in all malignant tumours, and also in the majority of the benign breast lesions analysed, whereas normal breast tissue exhibited very low levels of c-*myc* protein (91). The expression of c-*myc* was specifically studied in 198 specimens of fibrocystic disease compared to normal tissue using an improved immunohistochemical technique to detect c-*myc* by pretreating with neuraminidase (54). The results indicated that c-*myc* was not expressed in normal and epithelial cells in either ducts or lobes. However, high levels of staining were found in mucous metaplastic cells of epitheleosis and multiple papillomas, and the authors suggested that the elevated expression of c-*myc* in these cells may be involved in an early stage of malignant cell transformation.

A sensitive and quantitative ELISA has been developed for the c-*myc* oncoproteins (92) and it has been used to assess the level of c-*myc* in 24 breast cancer patients compared to normal tissue (61). It was demonstrated that all of the tumour specimens had considerably higher levels of c-*myc* oncoprotein than found in normal breast tissue from a patient with no evidence of breast cancer. A particularly intriguing piece of evidence was found in this investigation: a correlation was found between the extent of the tumour (T_1 - T_4) and c-*myc* expression in the tumour tissue ($P<0.02$), and also the level of c-*myc* expression in normal tissue adjacent to the breast cancer tumours correlated with the extent of the tumour. These authors proposed that these high levels of c-*myc* expression found in normal tissue were perhaps due to 'growth factors' being released from nearby large tumours, and that the histologically normal tissues were not in fact molecularly normal. No correlation was found between the survival of these patients and elevated c-*myc* expression in the tumour tissue, and this maybe due to the fact that the majority of the patients had advanced disease.

6iii. *Ras genetic alterations in breast cancer.* In a large study of 104 breast cancer patients it was found that there was no evidence of rearrangements or amplification of the *ras* gene (93). However, recently in 24 breast tumours there was evidence of amplification in 6 tumours and 2 had an activating G-T mutation in codon 12 of H-*ras* (94). No amplification of the N-*ras* gene was found in 41 patients (39) (Table Ib).

The deletion of a normal cellular sequence is thought to unmask recessive mutations, and the analysis of lost genes on particular chromosomes using restriction fragment length polymorphisms (RFLP) has had rewarding results in a number of paediatric hereditary disorders and certain adult malignancies (11, 95, 96). The H-*ras* gene has a number of Bam H1 RFLP and loss of one of the H-*ras* 1 alleles on chromosome 11p was detected in 14 out of 51 (27 per cent) breast cancer patients who were constitutionally heterozygous for this locus (93). Even though loss of this allele did not change the level of p21 *ras* expression, it was found that the loss correlated with histological differentiation, lack of oestrogen and progesterone receptors and distal metastasis. Spandidos (94) also demonstrated that 2 out of 24 (8 per cent) of the breast tumour specimens had lost the H-*ras* 1 allele. The loss of H-*ras* 1 alleles in these tumour tissue specimens may indicate the existence of a regulatory sequence that is important in the initiation of breast cancer. Alternatively, the normal H-*ras* gene or another gene located near to it may act as a onco-suppressor (97) (Table Ib).

6iv. *Expression of the ras gene family in breast cancer.* The expression of the *ras* gene family in breast cancer has been studied with immunohistochemical techniques using the

monoclonal antibodies Y13-259 (98) and RAP-5 (99), by Western Blotting, and by RNA hybridisation experiments (Table Ib). The specificity of the two main monoclonals for p21 *ras* has been debated and is still considered to be contentious (55, 100, 101, 102).

Spandidos and Agnantis (103) found that 12 out of 12 breast tumours over-expressed the H-*ras* mRNA compared to normal tissue using RNA dot hybridisation analysis, and in their follow up paper Agnantis *et al* (104) reported that elevated H-*ras* mRNA expression in 24 patients was associated with advanced histological types. In a Northern Blot analysis of mRNA from malignant breast tumours, 73 per cent were found to have elevated levels of H-*ras*, but K-*ras* and N-*ras* were at base line levels (93).

In another study a completely opposite finding was reported by Whittaker *et al* (105) regarding *ras* gene expression. They assessed the levels of c-*myc*, H, K and N-*ras* mRNA expression in 27 benign and 23 malignant breast cancers, and found that H-*ras* was only over-expressed in one specimen of a benign and in a malignant tumour compared to elevated expression in N-*ras* (65 per cent in carcinomas and 26 per cent in benign tumours) and K-*ras* (48 per cent in carcinoma and 15 per cent in benign tumours). These authors concluded from the analysis of these four oncogenes that there was a significant difference in oncogene expression between the benign and carcinoma specimens ($P<0.01$).

Expression of *ras* p21 was examined by RAP-5 monoclonal antibody by Horan-Hand *et al* (99), and it was found that 63 per cent of the malignant mammary tumours had increased levels of staining compared to 10 per cent of the benign tumours. The Y13-259 monoclonal was used by Agnantis *et al* (106) to ascertain the level of *ras* p21 expression in malignant and benign breast disease, and they reported that 83 per cent of the carcinomas and 42 per cent of cystic disease and 23 per cent of fibro-adenomas had moderate or above staining intensity but no clinical correlation was found in this study. In contrast to these results, a very different result was published by Tanaka *et al* (66). These authors used monoclonal antibodies which had been raised against peptides, two of them had homology with K-*ras* and N-*ras*, and the other with H-*ras*. In 10 breast cancer specimes none of them reacted with the H-*ras* monoclonal but 80 per cent reacted with the K-*ras* and N-*ras* monoclonals.

A comprehensive study using both RAP-5 and Y13-259 monoclonal antibodies to *ras* p21 was performed by Ohuchi *et al* (100) to determine the levels of expression in a range of benign and malignant breast tumours. Invasive carcinomas demonstrated enhanced levels of *ras* p21 expression; 77 per cent had >50% positive cells with RAP-5. The 46 fibrocystic specimens were subdivided into those with and without hyperplasia and further subdivided into those with and without atypia. The subgroup hyperplasia with atypia had higher levels of p21 expression than found in the other benign tumours; however, the levels were much lower than those found in the carcinomas. Analysis of the patients with a 15 year follow up indicated that there was a higher level of *ras* p21 expression in hyperplasia specimens from patients who subsequently developed breast cancer.

In contrast to the results, Candlish *et al* (107) found no significant differential staining in *ras* p21 expression between malignant and benign breast tumours, and normal breast tissue adjacent to the resection margin using the monoclonal antibody Y13-259.

Recently it has been demonstrated with the use of a direct binding liquid competition radioimmuno assay that 24 out of 28 breast tumours had higher levels of *ras* p21 than the average value found in fibroadenomas (70). These authors demonstrated that the higher levels of *ras* p21 were associated with post-menopausal patients.

The level of *ras* p21 protein in malignant, benign and normal breast tissue has been determined by Western Blotting analysis and was found to be elevated in all 7 hormone responsive specimes analysed and in 5 out of 6 hormone independent specimens examined; however, low levels of p21 were found in the benign and normal tissue (108). Another study using Western Blotting analysis demonstrated that 37 out of 54 carcinomas (69 per cent) had elevated levels of *ras* p21 (60) and there was an association between increase in p21 in 60 per cent of the T_3 and T_4 tumours. A significant difference was also found between high *ras* p21 levels and a short disease-free interval, ($P<0.05$).

The results of these investigations indicate that the H-*ras* oncogene is important in the progression of malignant breast cancer, and that K-*ras* and N-*ras* may also play a major role. It is also clear that neither amplification nor rearrangement of the Ha-*ras* 1 gene is important. However, certain reports suggest that loss of one H-*ras* allele was significantly linked to parameters of tumour aggressiveness, (93), while in another study loss of one H-*ras* allele (14 of 65 informative patients) was correlated with paucity of the oestrogen receptor (109). However, Sheng *et al* (110) reported that the presence of variant alleles of H-*ras* 1 locus are not informative markers in breast cancer. The information on *ras* p21 expression in malignant breast tumours points to this gene having a role in the progression of the disease. The higher levels of p21 were identified in invasive tumours, in those that have metastasis and also in those that have a poor prognosis.

7. Colorectal Carcinomas

Cancer of the colon provides an excellent opportunity to study the progression of neoplasia, because most carcinomas appear to be derived from adenomas (111) and therefore specimes from different stages of the disease may be investigated. Both the *ras* and *myc* gene families have been analysed in these tumours, and results are now available from reasonably large scale studies with long-term follow up (36, 42), (Table IIa, b).

7i. *C-myc expression in colorectal carcinomas.* Genetic

Table IIA. *Review of ras and myc expression and genetic alterations in colon cancer.*

Oncogene	Tumour type	No. of patients	Percentage with amplification	Percentage with elevated expression	Clinical correlations	Reference
c-myc (RNA)	Carcinoma	5		100	No clinical correlation reported	138
c-*myc* (RNA)	Carcinoma	6	0	100	Increased expression in these 6 tumours is accompanied by a parallel increase in expression of two G1 specific genes and S phase specific gene	139
c-*myc* (RNA) and (Southern)	Carcinoma	29	0	72	No c-*myc* amplification or rearrangements observed. C-*myc* more abundant in central portions of tumour than at periphery	113
c-*myc* (Southern)	Carcinoma metastasis Adenoma	41 4 15	7 0		No clinical correlation found	112
c-*myc* (RNA)	Carcinoma	38		68	No correlation between c-*myc* expression and recurrence or patient survival	42
c-*myc* (mRNA) and (Southern)	Polyps Carcinoma metastasi	6 14 2	0 0 1(26x)	17 29 50	Pts with increased *fos*, *myc* and H-*ras* expression had poorer prognosis	53
C-*myc*	Polyps (24) adenomatous villous	 11 13		staining intensity ++ ++++	Staining intensity greater in well differentiated tumours	140
Mycl-6E10 (mAb)	Carcinoma moderate to well differentiated poorly differentiated	42		 +++++ ++		
C-*myc* *Mycl*-6E10 (mAb) and immunoblotting. Southerns.	Carcinoma	15	0	80	No c-*myc* amplification or rearrangements found in these tumours.c-*myc* p62 detected by immunoblotting and immunohistology gave similar results. Correlation between c-*myc* p62 and histological grades.	115

continued

Table IIA continued

C-*myc* *Mycl*-6E10 (mAb)	Carcinoma	100		Staining intensity 40% moderate 29% strongly 29% weakly	c-*myc* expression in most colon CA. No correlations with differentiation, staging or survival	116

Table IIB.

Oncogene	Tumour type	No. of patients	Percentage elevated expression	Percentage with RAS mutations	Clinical correlations	Reference
K-*ras* N-*ras* H-*ras* (oligo-nucleotides)	Carcinoma	27		37	In 5 of the 6 patients with K-*ras* mutations same mutation in benign and malignant sections of the tumour. No amplifications of any of the *ras* genes.	46
K-*ras* (RNase) mismatch cleavage analysis)	Carcinoma	66	0	39	In 7 of 8 tumours originating in adenomas has mutant K-*ras* genes	47
K-*ras* N-*ras* H-*ras*	Carcinoma	92		41 5 0	*Ras* gene mutations correlate in colon carcinogenesis, but not necessarily the first event as only 13% of class I adenomas have a mutation (only 1 adenoma had a N-*ras* mutation)	117
	(Adenoma 80)					
K-*ras* N-*ras*	class I	40		13		
	class II	19		50		
	class III	21				
H-*ras* (oligonucleo-tides)	Adenomas (80)			0		
K-*ras*2 K-*ras*2 (oligo-nucleo-tides)	Carcinoma Adenoma	40 12		65 75	K-*ras* mutations occur early in colon carcinogenesis before change to aneuploidy	119
K-*ras* H-*ras* K-*ras* H-*ras* (RNA)	Carcinoma » Adenoma »	12 12 4 4	100 50 100 100		All the carcinomas had elevated K-*ras* expression over that found in normal tissue and six had H-*ras* elevated expression. All adenomas had elevated K-*ras* and H-*ras* gene transcripts	52
K-*ras*	Polyps Carcinoma metastasis	6 14 2	17 36 50		No amplifications or rearrangement of H-*ras* or K-*ras* reported	53
Ha-*ras* (mRNA)	Polyps Carcinoma metastasis	6 14 2	0 14 0		High levels of *fos*, *myc* and K-*ras*, H-*ras* expression correlated with poor prognosis	

continued

Table IIB continued

ras p21 Y13-259 (mAb)	Polyps Carcinomas metastasis	4 12 3		Presence of *ras* p21 found in all stages of colon carcinogenesis	121
ras p21 Y13-259 (mAb) and immuno-blotting	Carcinoma metastasis	17 9	53 0	8 of the 9 positively staining carcinomas Dukes stage B or C only 1 stage D. None of the metastasis had significant staining conclued *ras* p21 expressed in early stage of colon CA	122
ras p21 Y13-259 (mAb)	Carcinoma Adenoma	21 6	29 100	(using their paper's staining intensity + or ++ equals elevated expression) Adenomas showed significantly greater staining intensity compared to carcinomas ($P<0.01$)	56
ras p21 RAP-5 and Y13-259 (MAb's)	Carcinoma Adenoma	6 6		Adenomas showed greater staining intensity than carcinomas using Y13-259, whereas RAP-5 bound to many types of cells, normal and neoplastic	55

alterations in the form of c-*myc* amplifications and rearrangements are rare events in colon carcinoma, with only a few reported cases (53) in 1 out of 2 colon metastases and (112), in 3 out of 45 colon carcinomas. Moreover, in a study of 29 cases of primary adenocarcinomas there was no evidence of c-*myc* amplification or rearrangements (113).

Elevated expression of the c-*myc* gene has been reported in about 70 per cent of all primary adenocarcinomas of the colon by Erisman's group (42, 113, 114). They demonstrated that the c-*myc* gene transcript was elevated about 5 fold greater than that found in normal mucosa. Similar results on c-*myc* RNA transcripts have been reported in smaller numbers of patients by Yokota *et al* (62) and Monnat *et al* (53).

However, the 40 month clinical follow up paper on the 38 patients with colon adenocarcinoma (42) provides evidence for no statistical correlation between patients with elevated levels of c-*myc* RNA and tumour recurrence or survival. These authors suggest that there is no obvious clinical value in measuring c-*myc* expression with respect to these patients' progress and that the expression of the c-*myc* gene is not an important factor in the late stages of tumorigenesis of the colon.

The myc-1-9E10 monoclonal antibody has also been used to analyse c-*myc* expression in colon tumours (64, 115, 116, 140). The reports in the literature concerning the c-*myc* nuclear cellular localisation in colon tumours using *myc* monoclonal antibody are uncertain. However, Jones *et al* (116) critically reviewed the use of this monoclonal and concluded that the staining pattern observed may be indicative of its real distribution at a tissue level (*i.e* in both the cytoplasm and the nucleus) and that this oncoprotein may have a more widespread distribution and may also have other functions not previously considered. Nevertheless, the conclusion drawn from the c-*myc* monoclonal data (116) indicates that the expression of this oncogene is increased in the majority of colorectal carcinomas but is unrelated to clinical behaviour and this is in agreement with c-*myc* mRNA data (42).

7ii. *Ras genetic alterations in colon cancer.* There is now considerable evidence for the involvement of *ras* gene mutations in the progression of colon carcinoma (46, 47, 117), whereas there is no evidence for amplification or rearrangement of the *ras* genes in these tumours (46, 53, 112).

The development of sensitive oligomer hybridization assays in conjunction with methods for selective amplification

of specific sequences has provided a method of reliably quantifying *ras* gene mutations in tumour specimens. Using these techniques approximately 40 per cent of colon tumours have been shown to have an activated *ras* gene (46, 117) and these results are in agreement with those reported using a RNase mismatch cleavage analysis (47). The majority of the activated *ras* genes had a mutation at codon 12 in the K-*ras* gene.

Moreover, these results indicate that the activated *ras* gene in colon carcinomas occurs relatively early in the development of these tumours. A strikingly high incidence of mutant K-*ras* genes were found in colon tumours originating in adenomas or polyps, 7 out of 8 (47) and 5 out of 6 (46). Although mutations in codon 12 of the K-*ras* gene occurred in certain incidences, it was found that N-*ras* activation also occurred in a villous adenoma and in one carcinoma (118), thereby indicating that one of these mutations was probably not associated with the initial or early event in the development of these tumours. This has important implications in the timing of genetic changes during the progression of colon cancer.

Burmer and Loab (119) have also investigated the timing of *ras* mutations in colon cancer, using techniques of histological enrichment, cell sorting, DNA amplification and PCR followed by DNA sequencing. They found that in 40 carcinomas, 27 were aneuploid and 26 contained mutations in codon 12 of the K-*ras* 2 gene. Moreover 4 of the 12 adenomas were aneuploid and 9 had the same K-*ras* 2 mutations. These authors thereby suggested that the mutation in K-*ras* 2 pre-empted the change in ploidy status.

The possibility that constitutional differences in oncogene structure or expression may increase the chance of malignant transformation was investigated by Wyllie *et al* (120). They postulated that the chance of malignant transformation may be determined by additional genetic events apart from *ras* gene mutations to drive the cell into tumourigenesis. However, analysis of H-*ras* restriction fragments length polymorphisms (RFLPs) in patients with colorectal carcinomas showed that the frequency of rare alleles was not statistically different in these patients, compared to control groups.

An association between *ras* gene mutations and allelic deletions of chromosomes 5, 17 and 18 was investigated in adenomas and carcinomas from 172 specimens (117). The adenomas were divided into three classes representing different stages of the disease (increasing from Class I to III). The Class II and III adenoms contained *ras* mutations at the same frequency as carcinomas (50%), whereas in Class I only 13 per cent had *ras* mutations ($P<0.001$), mainly in the K-*ras* gene. Also none of the Class I adenomas had an allelic deletion on chromosome 5, whereas Class II and III had 29 per cent. These results indicate that *ras* mutations and allelic deletions in chromosome 5 occurred at earlier stages of the disease than deletion in 18q, which also precedes deletions in 17p. This is one of the first papers that provides evidence for the progressive accumulation of genetic alterations in carcinogenesis.

Recently a comprehensive survey of allelic losses has been undertaken with DNA markers from every nonacrocentric autosomal arm in 56 colon tumours and paired normal specimens (36). They found that the patients with greater than the median percentage of allelic deletions were more likely to develop a tumour rcurrence ($P<0.01$) and were also more likely to die from their cancer ($P<0.01$). However, the indicence of *ras* gene mutations in this group of patients (*i.e.* greater than the median percentage of allelic deletions) was similar to that in the group of patients with less than the median value. The significance of multiple allelic losses (*e.g.* 15 in tumour S141-A) is unclear, but the identification of this feature must make one more cautious in the interpretation of oncogene activation and allelic losses.

7iii. *Ras oncogene expression in colon carcinoma.* Elevated expression of H-*ras* and K-*ras* RNA transcripts was initially reported by Spandidos and Kerr (52). They demonstrated that elevated expression of one or both of these oncogenes was elevated in all of the 4 colorectal polyps and in most of the thirteen adenocarcinomas of the colon analysed, compared to normal tissue, whereas elevated K-*ras* expression was seen in only 4 out of 14 adenocarcinomas and 1 out of 6 polyps; and elevated H-*ras* expression in 2 out of 14 carcinomas and in none of the polyps analysed by Monnat *et al* (53). Clearly these investigations are on small samples and need to be confirmed with prospective studies.

The p21 *ras* protein has been investigated in colon tumours in a number of studies using the monoclonal antibodies Y13 259 (55, 56, 121, 122) and with RAP-5 (67, 99). There has been some debate in the literature concerning the specificity of RAP-5 (55). However, Czerniak *et al* (67) did show a distinct difference in the negatively staining pattern of RAP-5 in benign colonic lesions and the postively staining carcinomas.

The results with Y13 259 monoclonal appear to contradict this as it was demonstrated that adenomas showed a consistently higher level of *ras* p21 expression by staining intensity than that found in carcinomas ($P<0.01$) and in normal tissue ($P<0.002$) (56) and these results are in agreement with Robinson *et al* (55). In contrast to this, Kerr *et al* (121) using Y13 259, reported that *ras* p21 was found in all tissues and was not restricted to any specific stage of colon carcinogenesis.

A direct binding liquid competition radioimmunoassay (RIA) has been used to quantify *ras* p21 in colon tumours (70) and 4 of the 5 tumours tested showed increased *ras* p21 in these tissues compared to normal. In two of these samples high levels of p21 were found in the adjacent normal tissue and it is postulated that the carcinoma cells provide a 'factor' that influences the expression of p21 with normal cells and that this may be important in the early transformation of the histologically normal cells. It is of interest to note that elevated expression of the c-*myc* gene was found in normal tissue adjacent to breast cancers using a c-*myc* ELISA (61). In both of these investigations it is possible that the histolo-

gical normal tissue is not molecularly normal and may be undergoing the initial stages of carcinogenesis.

The role of the *ras* and *myc* gene families in colonic carcinoma is still very uncertain. The c-*myc* gene may be overexpressed in certain carcinomas but as yet appears to have no particular association with the Staging of the disease. Little is known about this oncogene in the benign and premalignant stages of colon cancer. Nevertheless there may be a role for c-*myc* when its action is considered in conjunction with the *fos* and *ras* genes (53). In this study all the five deaths that occurred had elevated expression of *fos*, *myc* and H- or K-*ras* ($P<0.01$).

The *ras* gene family appears to be involved in the early stages of colon carcinoma, but it cannot as yet be correlated just with the initiating event, especially as the Class 1 adenomas in the paper of Vogelstein *et al* (114) had a significantly lower number of *ras* mutations than Class II or III adenomas. All the same, the evidence from immunohistochemical analysis, even though controversial, does point to an association between elevated *ras* p21 expression and benign colon tumours.

8. Cervix cancer

Cancer of the uterine cervix is the most prevalent cancer in under-developed countries (37) and it mainly affects women in lower socioeconomic groups.

A number of factors have been linked to the progression of this disease. Certain human papilloma virus DNA sequences have been found to be integrated into the cells of uterine cervix cancer cells and are transcribed (48) and the presence of these viruses in pre-neoplastic cervical lesions indicates a poorer prognosis (123). Recently certain oncogenes have also been implicated in the progression of the disease (38, 48, 49). In 154 patients with cervical cancer, 6 per cent had an amplified c-*myc* in stages I and II, compared to 49 per cent in stages III and IV. This indicates that c-*myc* amplification is associated with advanced disease ($P<10^{-5}$) Also elevated levels of c-*myc* expression are significantly more frequent in stages III and IV ($P=10^{-4}$). It was recognised by Riou and co-workers (38) that over-expression of the c-*myc* genes was found in some patients with stage I or II cervical cancer who subsequently had a relapse. They then analysed c-*myc* expression in 72 previously untreated patients with cervical cancer (Stage I or II) and found 25 (35 per cent) had high levels of expression. In a multi-variate analysis only c-*myc* over-expression and nodal status correlated with a risk of relaps, and in fact c-*myc* over-expression was the major prognostic factor ($P=0.001$).

This association between c-*myc* over-expression in the early stages of cervical cancer and a poor prognosis points very clearly to the involvement of this gene in the progression of the disease. In a smaller study Ocadiz *et al* (124) reported that 17 out of 35 (49 per cent) cases of cervical carcinoma had an amplified c-*myc* gene, and that 43% presented with both amplification and rearrangement of the gene. Riou and co-workers have also investigated the role of mutations in codon 12 in H-*ras* and also loss of H-*ras* heterozygosity in cervical cancer. No correlation was found between loss of H-*ras* heterozygosity and advanced stages of the disease but mutations in H-*ras* codon 12 correlated with cancers of a poor prognosis ($P<0.01$), (49). It is of interest that these authors also found that 4 out of 10 carcinoms which had a mutated H-*ras* gene also had a deletion of the H-*ras* gene on the other allele. This observation opens up a possibility that perhaps the loss of the H-*ras* gene on one allele contributed to the activation of the mutated H-*ras* gene on the other. Elevated expression of the ras p21 gene has been demonstrated in cervical carcinoma using the Y13-295 monoclonal antibody (125), and demostrated a higher staining intensity in malignant cells than in the benign or premalignant lesions.

The combined results of H-*ras* and c-*myc* data in Riou's and co-workers investigations patients yielded a further interesting association, in as much as all the tumours with a mutated H-*ras* gene also had an amplified c-*myc* gene (49). This may indicate oncogene co-operation in the progression of this cancer which was similarly demonstrated by Land *et al* (126, 127) in an experimental model system where both *ras* and *myc* co-operated to transform the cell line.

9. Head and neck cancer

The incidence of head and neck cancer varies considerably worldwide. Oral cancer accounts for about 2 per cent of all malignancies in the western world where it accounts up to 40 per cent of all malignancies in India and South East Asia. When worldwide figures are computed for mouth and pharynx cancer, this group accounts for 6 per cent of all solid tumour malignancies (37). However, the prognosis for many of these patients is poor and even in the cases that have undergone successful surgery, many suffer severe degrees of dysfunction. The molecular mechanisms operating in neoplasia of the head and neck have not been investigated to the same extent as those in breast, colon or lung, but recent work has indicated that the *ras* and *myc* gene families and possibly *erb*B-1 and EGF may be involved (13, 40, 128-135).

9i. *Myc expression and amplification in head and neck cancers.* We have found evidence for elevated *myc* oncogene expression in 14 head and neck squamous cell carcinoma (SCC), from the tongue, floor of mouth, buccal mucosa, hypopharynx and larynx regions (13, 128, 129, 130). The increase in c-*myc* expression correlated with the stage of the disease, there was a significant difference between c-*myc* oncogene expression in TNM stages I and II and stage III ($P<0.05$), and also combined stages III and IV ($P<0.05$). In the combined stages III and IV it was found that there was a ten fold increase in c-*myc* expression over normal oral tissue (129). Amplification of c-*myc* and N-*myc* oncogenes has been reported in 3 out of 23 and 8 out of 23 oral carcinomas

Table III. *Cenetic alterations and elevated levels of expression of* ras *and* myc *gene families in human solid tumours, correlated with the patients' clinical outcome using specific statistical analysis**.

Tumour site	Oncogene	Correlations with follow-up data	P	Reference
Breast	c-*myc*	Amplification and rearrangements correlated with poor prognosis	<0.02	39
	c-*myc*	Elevated oncoprotein expression does not correlate with prognosis	NSD	61
	ras p21	Increased amount of H-*ras* p21 protein product associated with disease recurrence	<0.05	60
	ras p21	In 15 year follow up - higher levels of *ras* p21 in hyperplasia (benign), in patients who subsquently developed cancer of the breast	<0.01	100**
Cervix	c-*myc*	c-*myc* over expression significantly associated with risk of relapse	<0.001	38
Colon	c-*myc* *fos* H, K-*ras*	High levels of expression of these oncogenes correlated with poor prognosis	<0.01	53
	c-*myc*	No correlation between c-*myc* expression and recurrence or survival	NSD	42
Lung (SCLC)	N-*myc*	Elevated expression correlated with short survival	<0.01	77
	L-*myc* N-*myc*	No correlation between *myc* amplification and survival	NSD	43
Head and neck	c-*myc*	Elevated c-*myc* oncoprotein expression correlates with poor prognosis	<0.02	40
Stomach	TGFα and *ras* p21	Carcinomas with synchronous expression of TGF and H-*ras* correlated with poor prognosis	<0.05	71

* log rank test (59), Cox's proportinal hazard model (58) and survival data compared by the Kaplan Meier method (57).
** Fisher's exact method.

respectively (134). These authors indicate that amplification of the *myc* gene is associated with advanced stages of the disease.

Recently we have analysed c-*myc* oncoprotein in SCC of the head and neck using an ELISA technique (92). In 44 specimens of SCC, the median level of c-*myc* oncoprotein expression in normal tissue adjacent to the resection site was 0.37 (range 0.16-1.01) pg c-*myc*/ug total protein (tp), while the median level of c-*myc* expression in tumour specimens was 0.77 (range 0.12-13.25) pg c-*myc*/ug tp. A significant difference was found between the survival of patients with low levels of c-*myc* expression and those with high levels of c-*myc* expression (P>0.02) (40). We have also demostrated a correlation between elevated c-*myc* expression and prognosis using the *myc* 1-9E10 monoclonal antibody (135).

9ii. *Ras genetic alterations in head and neck cancer.* The expression of H-*ras* and K-*ras* are both elevated in SCC of the head and neck (128, 129) but no correlation was found between the expression of these genes and the progression of the disease.

However, using the monoclonal antibody Y13-259 to *ras* p21, Azuma *et al* (131) have demonstrated a relationship between increased expression of *ras* p21 and poor prognosis in 121 SCC of the head and neck (P<0.001). Moreover, correlations were reported between elevated *ras* p21 expression and histological grading and also with clinical staging. The TNM system of clinical staging in head and neck cancer is considered to be best prognostic indicator of the head and neck region (136).

In this study of *ras* p21 expression, Azuma *et al* (131) also included 44 specimens of oral leukoplakia which are considered to be potentially malignant lesions in approximately 5 per cent of cases, and therefore may represent a potentially

malignant clinical stage in oral cancer. none of these lesions when compared to normal tissue showed an increase in *ras* p21 expression.

There is little information on *ras* gene activation in the head and neck region. In a study on 20 oral SCC, point mutations were rare in H-, K- or N-*ras* genes in head and neck patients in Western Europe. (Chang, unpublished; Chang and Field, unpublished). Recently it has been reported in 2 oral cancer cell lines that there are mutations at codon 12 and 13 of H-*ras* and also concurrent amplification of c-*erb*B-1 and c-*myc* genes (132). The cell lines with these genetic alterations were established from a metastatic lymph node in the neck, the primary site being in the palate. This may be interpreted as probably the most advanced stage in multistep carcinogenesis and that multiple genetic defects have occurred during the development of this cancer. In the context of timing of these genetic alterations in oral cancer, clearly c-*erb*B-1 may correspond to an early event in tumourigenesis as demonstrated by Wong *et al* (137) in the hamster cheek pouch model system. They chemically induced oral carcinomas in this model system using DMBA and they demonstrated that the c-*erb*B-1 gene was amplified. A possible explanation for these results is that *erb*B-1 is involved in initiation events, *ras* genes are overexpressed throughout at the promotion and progression stages, and c-*myc* at the very late stages of tumour progression.

10. The significance of *ras* and *myc* gene families in the progresion of human carcinomas

In this review it has been our intention to summarize the relevant work on the *ras* and *myc* oncogenes in the most common human tumours. Great progress has been made in this decade in determining that both *ras* and *myc* families are important in the progression of human neoplasia, but as yet we do not clearly understand the role of these genes in multistep carcinogenesis. As clearly stated earlier in this review, the number of investigations that contribute to our understanding of the timing of specific genetic effects is quite small. Only breast and colon tumours have clearly defined clinical stages of malignancy that have been studied to any great extent.

In a number of benign breast cancer patients with fibrocystic disease, the c-*myc* gene has been found to be overexpressed when measured by immunohistochemical techiques (54, 91) and by RNA hybridization analysis (105). However, the c-*myc* gene is not normally amplified or rearranged in benign breast tumours (45, 87), apart from one or two cases found by Varley *et al* (39 and Bonilla *et al* (44).

The *ras* p21 gene product also appears to be elevated in a large number of benign breast cancers as shown by Horan Hand *et al* (99), Aganantis *et al* (106), Ohuchi *et al* (100), and Whittaker *et al* (105), but these results were not corroborated by DeBortoli *et al* (108) and DeBiasi *et al* (70). One paper on p21 *ras* expression in benign breast tumours is very informative on the role of *ras* genes in early neoplasia (100). This study has followed up patients with fibrocystic disease for 15 years and found that the patients who subsequently developed breast carcinomas had high levels of p21 *ras* at the initial biopsy. It has been argued that the *ras* and *myc* genes co-operate in neoplasia, (126-127); it is therefore of interest that a significant difference in c-*myc*/*ras* gene expression was observed between benign and malignant breast tumours (105). It may thus be argued that there is evidence for the over-expression of *ras* and *myc* gene families at the initial stages of breast cancer.

In a number of adenomatous and villous polyps of the colon, elevated c-*myc* expression has been observed (25, 53), however, there is no evidence for amplification or rearrangement of this gene (25, 115).

There appears to be a very strong association between over-expression of the *ras* genes and benign colon cancers. Spandidos and Kerr (52) demonstrated that all four adenomas analysed had elevated K- and H-*ras* gene transcripts, and that the *ras* expression in these adenomas was elevated over that found in the colon carcinomas. This result is supported by the finding of Williams *et al* (56), who found that adenomas showed a significantly higher *ras* p21 staining intensity than carcinomas using the Y13-259 monoclonal antibody. A different result was found by Kerr *et al* (121), who reported *ras* p21 expression at all stages of colon cancers. The most striking evidence for the involvement of *ras* genes in colon tumour development comes from the work on *ras* mutations in adenomas (46, 47, 117). It is of specific note that the same K-*ras* mutations were seen in benign and malignant sections of colon tumours, but by subclassifying adenomas into classes I to III, it was found that the earliest stages (*i.e.* class I) had significantly fewer *ras* mutations than class II or III. These reports indicate that the *ras* genes are important in the initiation phase of colon cancer but it is possible that an unknown primary genetic event takes place beforehand.

A number of clinicopathological parameters associated with human cancers have been used to predict the prognosis of these patients. These may be grouped into patient factors, tumour factors and treatment factors; however, all are interlinked and it is extremely difficult to dissect out any specific factors as being wholly responsible for the patients' prognosis. Tumour factors are those that have been used most widely in clinical correlations with oncogene studies, but the most useful parameter is the fate of the patient. A correlation between an oncogene's expression or genetic alteration with survival is one of the most valuable pieces of clinical information available, when risk of relapse is evaluated by Cox's proportional hazard model (58) or the rank log test (59), and survival rates computed by the Kaplan-Meier method (57). This information is not only valuable from a potentially prognostic view point, but it also provides information on the roles of a specific gene at the most aggressive stage of the disease.

Unfortunately, this type of data is not really available for

many investigations, but what published information is available is shown in Table III. C-*myc* genetic alterations are associated with poor prognosis in breast cancer (39), and overexpression of the c-*myc* gene is correlated with a risk of relapse in cervical carcinoma (38) and in SCC of head and neck (40). No correlation was found for c-*myc* over-expression in colon cancer (42). In SCLC opposite results pertaining to correlations between the N-*myc* expression and survival have been reported (43, 77).

There are few reports on *ras* p21 in human solid tumours that have correlated changes in expression with survival data using good statistical analysis. In breast cancer it has been shown by Clair *et al* (60) that elevated p21 *ras* expression correlates with disease recurrence, and Monnat *et al* (53) have demonstrated that high levels of c-*myc*, *fos* and H, K-*ras* expression correlate with poor prognosis in colon neoplasms. However, recently Vogelstein *et al* (36) have studied the allelotype, (polymorphic DNA markers in every nonacrocentric autosomal arm) in 56 colorectal carcinomas. They found that patients with above the median number of allelic losses had a worse prognosis. However, no correlation was found for the prevalence of *ras* gene mutations in the group of patients with a value over the median number of allelic deletions ($P<0.001$ Fisher exact test). In stomach carcinomas Yamamoto and Hattori (71) demonstrated that the synchronous expression of TGFα and Ha-*ras* p21 expression correlated with prognosis.

Reports in the early literature gave an erroneous impression that it might be possible to attribute a particular oncogene or anti-oncogene to certain clinical stages of neoplasia. However, the data reviewed in this paper highlight the difficulties involved in assigning either the *ras* or *myc* gene families to any specific stage in multistep carcinogenesis. The type of investigation now being undertaken requires an accurate sampling of the pathological specimes (*i.e.* to ensure that actual tumour tissue is being analysed (46) and the use of scientifically evaluated probe banks for both oncogene and deletion mapping studies. There must also be good clinical follow-up over a meaningful length of time. Valid statistical analysis (Cox's proportional hazard model or log rank test and survival rates computed by Kaplan-meier's method) of these results can only be undertaken if a sufficiently large number of tumour specimens are available for study.

Despite the reservations outlined in this review, the advances in our understanding of oncogenes in the past decade have been considerable. In future it may be possible to envisage a "molecular index" of human carcinomas and in this way the full clinical potential of oncogene research could be realised.

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