EXPRESSION OF TRANSFORMING GROWTH FACTOR ALPHA, EPIDERMAL GROWTH FACTOR RECEPTOR, C-*ERB*B-2 AND C-*MET* GENES IN PRIMARY HUMAN COLORECTAL AND LUNG CARCINOMAS

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Dedicated to

my husband, Dayong Gao

and

my son, Frank Liu Gao

ABSTRACT

The mRNA expression of transforming growth factor α (TGF-α), epidermal growth factor receptor (EGFR) and the proto-oncogenes c-*erb*B-2 and c-*met* has been studied in an attempt to elucidate the pathogenesis of primary human colorectal and non-small cell lung carcinomas (NSCLC).

Colorectal carcinomas contain, on average, 4 times more immunoreactive TGF-α molecules than the normal colonic mucosa. This enhancement, in general, is not associated with the over-expression of TGF-α mRNA. Among the three tyrosine kinase receptors studied, only c-*met* is consistently and significantly over-expressed by colorectal carcinomas (mean 6 fold). C-*met* is also over-expressed in colonic adenomas, suggesting that activation of this proto-oncogene may be a significant mechanism contributing to the early stage of human colorectal carcinogenesis.

Again, human NSCLCs contain approximately twice as much immunoreactive TGF-α molecules as compared to the normal lung parenchyma. However, this increase is associated with the over-expression of TGF-α mRNA. When normal lung parechyma is compared with carcinomas, the level of mRNA is over-expressed by a factor of 2 in 60% cases for TGF-α, 55% cases for EGFR, 15% cases for c-*erb*B-2 and 42% cases for c-*met*. While adenocarcinomas tend to express higher levels of TGF-α, c-*erb*B-2 and c-*met*, epidermoid carcinomas often express higher levels of EGFR mRNA. Eight new cell lines have been established from 29 primary NSCLCs. The high levels of TGF-α and c-*met* mRNAs in the primary tumors appear to correlate with their facility in forming propagable cell lines *in vitro*. The high level of c-*myc* mRNA expression in cell lines is correlated with the xenotransplantability of the cell lines into nude mice. When the genotypes and phenotypes of these cell lines are compared to their corresponding primary and xenograft tumors, we find that tumor cells which grow continuously as a cell line often represent a selective subpopulation from a heterogeneous mix of neoplastic cells in the primary tumors.

These results indicate that the c-*met* proto-oncogene is a marker for colonic cancer, and that TGF-α is indicative of marked proliferative activity

associated with neoplastic cells. The expression of these proteins may have therapeutic significance as they can be targeted on the cell surface, albeit in an indirect manner for TGF-α. In addition, the use of cell lines as the only study of human tumors may not be representative of the biological and functional activity of the tumors *in vivo* since it has been shown that these cell lines have been selected from a heterogeneous mix of cells in the primary tumors.

RÉSUMÉ

L'expression par l'ARNm du facteur de croissance transformant α (TGF-α), du récepteur du facteur de croissance épidermique (REGF) et des proto-oncogènes c-*erb*B-2 et c-*met* a été étudiée dans le but de percer le mystère de la pathogenèse du carcinome colorectal primaire humain et du carcinome bronchopulmonaire "non à petites cellules" (CBNPC).

Les carcinomes colorectaux contiennent, en moyenne, quatre fois plus de molécules TGF-α immunoréactives que la muqueuse colique normale. Ce phénomène n'a aucun rapport avec la surexpression de TGF-α par l'ARNm. Parmi les trois récepteurs de la tyrosine kinase, seule c-*met* est surexprimé en permanence et de façon significative dans les cancers colorectaux (six fois plus). C-*met* est également surexprimé dans les adénomes coliques, ce qui donne à penser que l'activation de ce proto-oncogène peut être un mécanisme significatif des premiers symptômes de la carcinogenèse colorectale humaine.

De plus, les CBNPC humains contiennent approximativement deux fois plus de molécules TGF-α immunoréactives que le parenchyme pulmonaire normal. Toutefois, cette hausse est associée à une surexpression de TGF-α par l'ARNm. En comparant le parenchyme pulmonaire normal aux carcinomes, le taux d'ARNm est surexprimé par un facteur de 2 dans 60 % des cas pour les TGF-α, dans 55 % des cas pour le REGF, dans 15 % des cas pour c-*erb*B-2 et dans 42 % des cas pour c-*met*. Même si les adénocarcinomes tendent à exprimer des concentrations supérieures de TGF-α, de c-*erb*B-2 et de c-*met*, les carcinomes épidermoïdes expriment souvent des concentrations supérieures de REGF ARNm. Huit nouvelles lignées cellulaires ont été établies à partir de 29 CBNPC primaires. Les fortes concentrations de TGF-α et de c-*met*

exprimées par l'ARNm dans les tumeurs primaires semblent être liées à leur facilité à former des lignées cellulaires propageables *in vitro*. La forte concentration de c-*myc* exprimée par l'ARNm dans des lignées cellulaires est liée au caractère xénotransplantable des lignées cellulaires chez les souris athymiques. Lorsque les génotypes et phénotypes de ces lignées cellulaires sont comparés à leurs tumeurs de greffe hétérologue primaires correspondantes, on s'aperçoit que les cellules tumorales qui croissent en permanence sous forme de lignées cellulaires représentent très souvent une sous-population sélective d'un mélange hétérogène de cellules néoplasiques dans les tumeurs primaires.

Ces résultats indiquent que le proto-oncogène c-*met* est un marqueur du cancer du côlon et que TGF-α est révélateur de l'activité proliférative marquée liée à l'apparition de cellules néoplasiques. L'expression de ces protéines peut avoir une importance thérapeutique puisqu'elles peuvent être ciblées à la surface cellulaire, encore que de manière indirecte pour le TGF-α. Par ailleurs, l'utilisation des lignées cellulaires pour la seule étude des tumeurs humaines ne peut être représentative de l'activité fonctionnelle et biologique des tumeurs *in vivo* puisqu'il a été démontré que ces lignées cellulaires avaient été choisies dans un milieu hétérogène de cellules de tumeurs primaires.

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ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CAM	Cell Adhesion Molecule
CSF	Colony Stimulating Factor
DNA	Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
FAP	Familial Adenomatous Polyposis
FPC	Familial Polyposis Coli
GAP	GTPase-Activating Protein
GDP	Guanine Diphosphate
GTP	Guanine Triphosphate
HGF	Hepatocyte Growth Factor
HMW	High Molecular Weight
HPLC	High-Pressure Liquid Chromatography
IGF	Insulin-Like Growth Factor
IGF-1R	Insulin-Like Growth Factor-1 Receptor
kbp	Kilo-Base Pair
LMW	Low Molecular Weight
MMTV	Mouse MammaryTumor Virus
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MOPS	3-(N-Morpholino) Propanesulphonic Acid
mRNA	messenger Ribonucleic Acid
MT1	Metallothionein-1
NSCLC	Non-Small Cell Lung Carcinoma

NRK	Normal Rat Kidney
PDGF	Platelet Derived Growth Factor
PDGFR	Platelet Derived Growth Factor Receptor
PKC	Protein Kinase C
PLCr	Phospholipase-C-r
PtdIn 3-Knase	Phosphatidylinositol 3 kinase
RTK	Receptor Tyrosine Kinase
RNA	Ribonucleic Acid
SCLC	Small Cell Lung Carcinomas
SDS	Sodium Dodecyl Sulfate
SGF	Sarcoma Growth Factor
STP	Signal Transfer Particle
TGF	Transforming Growth Factor
TPA	12-O-Tetradecanoyl-Phorbol-13-Acetate
tpr	(Translocated Promoter Gene)

CHAPTER 1

INTRODUCTION AND GENERAL OBJECTIVES

The molecular pathogenesis of cancer involves both activation of cellular proto-oncogenes and inactivation of tumor suppressor genes. Cellular proto-oncogenes are defined as eukaryotic genes which are highly conserved during evolution, and act fundamentally to regulate cell growth and differentiation. The proto-oncogenes can be activated into oncogenes by a variety of mechanisms including gene amplification, point mutation, insertional mutation, deletion and translocation (Weinberg, 1989). Activated oncogenes act in a dominant manner, they are able to transform cells when introduced, either alone or in combination with another gene. The protein products of oncogenes fall into 5 categories according to their functions (Weinberg, 1989): (1) protein kinases, (2) growth factors, (3) growth factor receptors, (4) signal transducers and (5) transcriptional factors. Therefore, alterations in expression of these oncoproteins, either qualitatively or quantitatively, are capable of mediating abnormal growth of cells, and transforming them to malignant cells. The linkage between oncogene products and growth factors / receptors further suggests that abnormal expression of growth factors / receptors also plays an important role in malignant transformation. The autonomous growth of cancer cells involves a variety of mechanisms including: (a) their ability to synthesize more growth factors and / or their receptors, (b) the enhanced responsiveness of cancer cells to the growth factor, (c) the ability of cancer cells to synthesize structurally altered and constitutively activated receptors independent of the ligand stimulation, and (d) the diminished responsiveness of cancer cells to negative growth regulators. After the discovery of nerve growth factor (Levi-Montalcini and Hamburger, 1951), many other growth factors / receptors have

been identified and characterized. In this project, we selectively studied the expression of TGF-α, EGFR, c-*erb*B-2 and c-*met* in primary human colorectal and lung carcinomas, as the previous studies have suggested that: (1) they act primarily to regulate the growth of epithelial cells; and (2) their expressions have been frequently altered in many tumors, and in certain tumors, a high level of these gene expressions has been correlated with a poor prognosis.

1.1 TRANSFORMING GROWTH FACTOR-ALPHA

1.1-1 SGF, TGF-α and -β

Transforming growth factor–alpha (TGF-α) was first identified as one of the components of sarcoma growth factor (SGF) which had been discovered in the serum free conditioned medium of murine sarcoma virus transformed mouse NIH3T3 fibroblasts (Delarco and Todaro, 1978). It has been shown that addition of the conditioned medium causes normal rat kidney fibroblasts (NRK) in monolayer culture to assume the morphology of neoplastic cells, and in soft agar to form progressively growing colonies (anchorage independent growth). This conditioned medium also competes with epidermal growth factor (EGF) for binding to the EGF receptor (EGFR), even though it fails to react with the monoclonal antibody raised against the EGF. Such conditioned medium is thought to contain an EGF homologue which after purification has been termed SGF. Subsequent studies using high-pressure liquid chromatography (HPLC) demonstrate that the SGF is actually composed of two different types of growth factor: TGF-α and -β (Anzano et al, 1983). These two types of TGF are classified based on their interaction with the EGFR and their differential requirement for the presence of EGF to enable NRK cells to form colonies in soft agar. TGF-α is able to compete with EGF for binding to the EGFR and exerts its biological activities through an interaction with the EGFR (Anzano et

al, 1983; Carpenter et al, 1983), while TGF-β demonstrates no structural similarity to EGF and functions through interaction with its own specific high affinity cell surface receptor (Roberts et al, 1981 and 1983; Frolik et al, 1984) TGF-α alone is sufficient to induce NRK cells to grow anchorage independently in soft agar, whereas TGF-β can only do so in the presence of exogenous EGF or TGF-α (Roberts et al, 1983). Genes for TGF-α and TGF-β have been cloned, they reside on chromosome 2 for TGF-α, 19, 1 and 14 for TGF-β1, -β2, and - β3 respectively (Fujii et al, 1986; Barton et al, 1988). TGF-α belongs to a group of growth stimulatory factors, especially for epithelial cells (Smith et al, 1987; Borellini et al, 1989; Tateishi et al, 1990); whereas TGF-β acts bifunctionally. TGF-β stimulates the growth of some fibroblasts, but inhibits the growth of most epithelial cells, especially non-transformed epithelial cells (Roberts et al, 1985). Some tumor cells are resistant to the growth inhibitory effect of TGF-β, and this has been postulated as a mechanism whereby tumor cells may gain a growth advantage over their surrounding normal cells.

1.1-2 STRUCTURE OF TGF-α AND ITS MULTIPLE SPECIES

Mature rat TGF-α is a single chain polypeptide containing 50 amino acids and 3 internal disulfide bonds (Figure 1-1), with an apparent molecular weight of 6 KDa. It shares approximately 33% to 44% sequence homology to mouse and human EGF respectively (Marquardt et al, 1983 and 1984). TGF-α is synthesized from a 4.8 kb mRNA as a 160 (human) or 159 (rat) amino acid glycosylated transmembrane precursor protein (proTGF-α) (Figure 1-1). Secreted TGF-α is released from the extracellular domain of the proTGF-α by an elastase-like proteolysis (Derynck et al, 1984; Lee et al, 1985). Multiple

species of TGF-α ranging in size from 5 to 20 KDa have been reported (Bringman, 1987, Teixido, 1988, Luetteke, 1988). Such a size heterogeneity may be explained by differential proteolytic cleavage in the extracellular domain of the precursor. Larger forms of TGF-α may represent incompletely cleaved intermediates. The extent of proteolytic cleavage may depend on the cellular origin of TGF-α and the type of glycosylation of the precursor molecule. Mature TGF-α, its incompletely cleaved forms, and even the non-processed proTGF-α can bind to and stimulate tyrosine kinase activity of the EGFR (Brachmann et al, 1989; Wong, 1989). It has been suggested that the membrane-bound proTGF-α plays a role in intercellular communications (Wong et al, 1989).

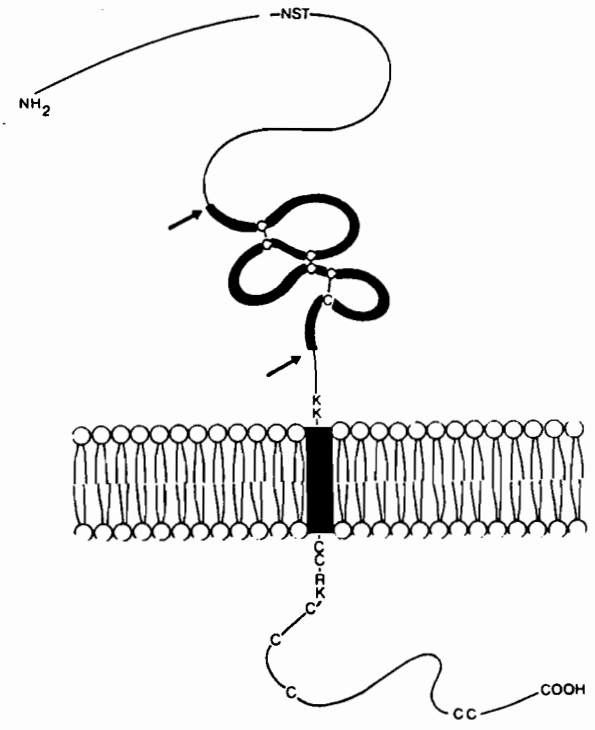


Figure 1-1 Depiction of a hypothetical model of the TGF-α precursor as a transmembrane protein. The 50- amino-acid TGF-α with its three proposed cysteine (C)-disulfide bridges is shown as a heavy line, flanked by the proteolytic cleavage sites (arrows). The transmembrane region is flanked at each side by two basic amino acids (KK and RK). The carboxy-terminal cytoplasmic domain shown below the membrane is rich in cysteines (C). (adapted from Derynck 1986).

1.1-3 CELLULAR SOURCES OF TGF-α

TGF–α expression has been reported in many cells transformed by viral and cellular oncogenes, in cells treated with chemical carcinogens or tumor promoters (Delarco and Todaro, 1978; Salomon et al, 1987; Liu et al, 1988; Raymond et al, 1989), and in many neoplastic cells or tissues derived from spontaneously arising human or experimentally induced animal tumors (Derynck et al, 1987; Tateishi et al, 1990; Imanish et al, 1988). TGF–α is also expressed at high level in both rat and mouse embryonic tissues (Matrisian et al, 1982; Twardzik et al, 1982), and in human placenta (Stromberg et al, 1982). These observations coupled with significant structural homology between TGF-α and EGF led to an initial suggestion that TGF–α might represent an oncofetal counterpart of the EGF (Goustin et al, 1986). However, a growing list has shown that TGF–α is expressed in normal adult tissues / cells, such as skin keratinocytes (Coffey et al, 1987), breast ductal epithelial cells (Zajchowski et al, 1987), activated macrophages (Madtes et al, 1988), gastrointestinal mucosa (Bennett et al, 1989; Cartlidge and Elder, 1989), bovine anterior pituitary cells (Samssonder et al, 1986), kidney (Gomella et al, 1989), and human lung parenchyma and colonic mucosa (Liu et al, 1990). Therefore, it is now realized that TGF–α also plays an important role in regulating physiological functions of cells.

1.1-4 IN VITRO TRANSFORMATION BY TGF–α

Gene transfer experiments have demonstrated that high expression of TGF–α can neoplastically transform immortalized rat-1 fibroblasts (Rosenthal et al, 1986), normal rat kidney (NRK) fibroblasts (Watanabe et al, 1987), and NOG-8 mouse mammary cells (McGeady et al, 1989), as assayed by colony forming ability in soft agar and tumorigenicity in nude mice. Membrane-bound

TGF-α precursor can also transform NRK cells (Blasband et al, 1990). However, TGF-α alone is not sufficient to transform NIH3T3 cells (Di Marco et al, 1989). In the latter cells, malignant transformation can only be achieved when there is a concurrent over-expression of both the TGF-α and EGFR. It is therefore proposed that, at least in NIH3T3 cells, the mitogenic signal transduced by the TGF-α / EGFR ligand-receptor system needs to reach a certain threshold in order to induce malignant transformation.

1.1-5 TGF-α IN TRANSGENIC MICE

Transgenic mouse technology has made it possible to study the function of specific genes and their proteins in the context of the whole animal. TGF-α transgenic mice have been generated, and depending upon the types of transcriptional enhancer / promoter used, its expression can be selectively targeted to various tissues or organs. Matsui et al (1990) have reported that, under the control of mouse mammary tumor virus (MMTV) long terminal repeats, TGF-α expression can be detected in testis, seminal vesicle, salivary gland and lung in the male offspring, but only in mammary gland in the female offspring. While no morphological abnormality is observed in the organs over-expressing TGF-α in male mice, the over-expression of TGF-α in mammary glands of transgenic female mice induced a range of histologic abnormalities including lobular hyperplasia, cystic hyperplasia, adenoma and carcinoma. The expression of TGF-α can be specifically targeted to the pancreas of transgenic mouse by using a rat-elastase enhancer / promoter. This pancreas-restricted TGF-α expression can stimulate the growth of both acinar cells and fibroblasts, and induce multifocal pseudoductular acinar metaplasia (Sandgren et al, 1990). In transgenic mice (MT-TGF-α-hGH) generated by using metallothionein (MT1) as a promoter, expression of TGF-α is detected

in many organs, especially liver, pancreas, kidney and stomach. In these organs, the high level of TGF-α expression results in epithelial cell hyperplasia (Sandgren et al, 1990), whereas in the mammary gland, over-expression of TGF-α induces breast carcinoma. Ihappan et al (1990) have used the same MT1 promoter to generate transgenic mice, they found that over-expression of TGF-α in the liver of these animals produced spontaneous hepatocellular carcinomas. However, no carcinomatous change in the mammary gland was observed. The inconsistence between the results obtained from Sandgren's and Ihappan's groups may be explained by: 1) genetic variations between transgenic mice they have generated; 2) Sandgren's group used rat TGF-α cDNA to construct fusion gene (MT-TGF-α-hHG) for microinjection, whereas Ihappan's group used human TGF-α cDNA. Nevertheless, the findings on the consequence of over-expression of TGF-α in transgenic mice suggest that TGF-α is a potential oncogene *in vivo*.

1.1-6 TGF-α IN HUMAN MALIGNANCIES

The enhanced expression of TGF-α has been reported in many human solid tumors including carcinomas of the kidney (Mydlo et al, 1989; Petrides et al, 1990), colon (Anzano et al, 1989; Liu et al, 1990), lung (Liu et al, 1990; Tateishi et al, 1990), pancreas (Barton et al, 1991), stomach (Bennett et al, 1989), thyroid (Aasland et al, 1990) and brain (Samules et al, 1989). The frequent co-expression of EGFR in these tumors suggests that TGF-α may operate through an autocrine growth stimulatory loop to promote tumor growth (Figure 1-2) (Retrides et al, 1990; Todd et al, 1989; Aasland et al, 1990; Bennett et al, 1990; Tateishi et al, 1990). I*n vitro*, autocrine stimulatory function of TGF-α has been demonstrated clearly in human lung (A549 and PC-9) (Imanishi et al, 1989) and pancreatic carcinoma cell lines (Smith et al, 1987).

These cells express both TGF-α and EGFR. The addition of exogenous TGF-α into their culture media stimulates the proliferation of these cells, and such a stimulation is abolished when monoclonal antibody to TGF-α is concurrently added. Tateishi et al (1990) have studied immunohistochemically the expression of TGF-α in primary human non-small cell lung carcinomas, and demonstrated that, in adenocarcinomas of the lung, the high level of TGF-α correlates significantly with shortened relapse-free and overall survivals. This correlation is not observed in other types of lung carcinomas.

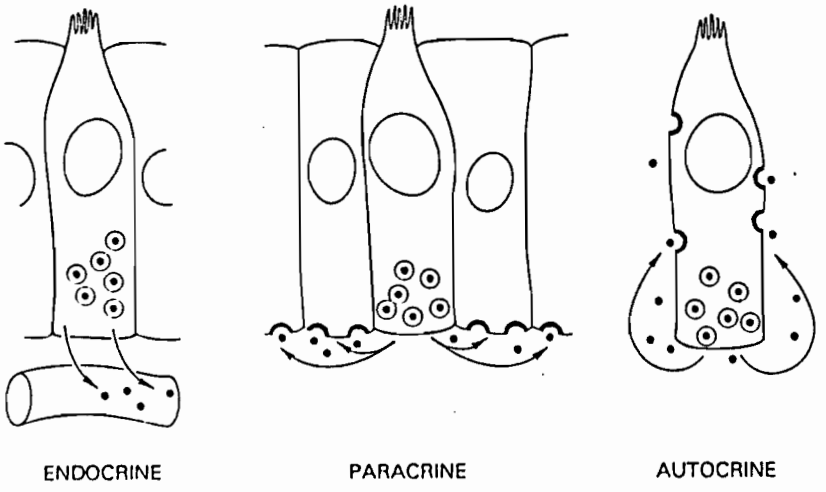
The presently realized functions of TGF-α in the biology of human neoplasia include:

(1) conferring on neoplastic cells a growth advantage over their surrounding normal cells, and mediating an anchorage independent growth of some non-neoplastic cells (Delarco and Todaro, 1978; Derynck, 1986);

(2) promoting neovascularization (Schreiber et al, 1986) and tumor stroma formation (desmoplasia) (Bergh et al, 1988) through a paracrine growth simulatory mechanism (Figure 1-2);

(3) mediating some paraneoplastic syndromes such as hypercalcemia and hypochlorhydria. *In vitro* treatment of cultured bones with TGF-α induces the Ca^{2+} release which may correlate with bone resorption and hypercalcemia in vivo (Stern et al, 1985; Ibbotson et al, 1986). TGF-α has also been shown to inhibit the histamine-stimulated secretion of gastric acid from cultured gastric mucosa obtained from normal guinea pig (Rholes et al, 1986). The observation suggests that over-expression of TGF-α in gastric carcinomas may account for, at least in part, the lack of gastric acid associated with this tumor.

Figure 1-2 Diagrammatic representation of autocrine, paracrine and endocrine secretion. Growth factors are shown in latent form within the cell (before activation). The thickened semicircular regions of the cell membrane represent receptor sites. (Adapted from Sporn et al, 1980)



Endocrine loop: Growth factors (or hormones) are secreted into bloodstream and act extensively on entire body.

Paracrine Loop: Secreted growth factors act on adjacent cells expressing their receptors.

Autocrine Loop: Seceted growth factors act on their producer cells to autostimulate their proliferation.

1.2 TYROSINE KINASE RECEPTOR FAMILY (EGFR, c-*erb*B-2 and c-*met*)

1.2-1 MOLECULAR TOPOLOGY

Many polypeptide growth factors mediate their pleiotropic actions by binding to and activating cell surface receptors with tyrosine kinase activity. The receptors with tyrosine kinase activity, or receptor tyrosine kinases (RTKs) are thought to play an important role in regulating cell growth and differentiation by transducing the extracellular signals, induced by ligand binding, to the intracellular space, leading eventually to a mitogenic response. Such RTKs include the receptors for: EGF, insulin, insulin-like growth factor (IGF), platelet derived growth factor (PDGF), colony stimulating factor (CSF) and the proto-oncogene products of c-*erb*B-2, c-*fms*, c-*kit* and c-*met* (Yarden and Ullrich, 1988).These RTKs share a common molecular topology, they all possess 3 major domains (Figure 1-3):

1. an extracellular ligand binding domain (500-900 amino acids):

This hydrophilic domain contains many glycosylated sites and is characterized by the presence of cysteine-rich repeat sequences (or residues) which are thought to yield rigid, proteinase-resistant conformation. Affinity labelling experiments with ^{125}I-EGF indicates that a region flanked by the two cysteine-rich domains of the EGFR constitutes the major binding sites for EGF (Lax et al, 1989). The low degree of homology in this region among the different RTKs is consistent with the divergent ligand specificity displayed by the different types of receptor.

2. a single transmembrane region (22-26 amino acids):

This hydrophobic domain functions mainly to anchor the receptor to the

plasma membrane. It probably plays a passive role in signal transduction since experiments using chimeric receptors with various combinations of the 3 domains of different types of receptor (EGFR, c-*erb*B-2, PDGF-R, insulin-R and IGF-1R) demonstrate that heterogeneous ligand binding and kinase domains are able to interact regardless of the nature of the transmembrane region (Lammers et al, 1989; Lee et al, 1989, Riedel et al, 1989).

3. cytoplasmic tyrosine kinase domain (400-600 amino acids):

This domain is the most highly conserved among all receptors, it contains a consensus sequence, GlyxGlyxxGlyx(15-20)Lys, which acts as an ATP binding site and is therefore essential for a functional tyrosine kinase activity (Chen et al, 1987; Hanks et al, 1988; Schlessinger, 1988 and Williams et al, 1989). It has been shown that replacement of the consensus lysine residues completely abolishes the kinase activity of the EGFR, PDGF etc. (Chen et al, 1987; Williams et al, 1989).

The carboxy terminal of this domain is the most divergent portion among all the different known RTKs, containing several autophosphorylation tyrosine residues (Downward et al, 1984a). The autophosphorylation sites may modulate the mitogenic responses by competing with other cellular substrates for binding to the substrate binding sites on the tyrosine kinase domain.

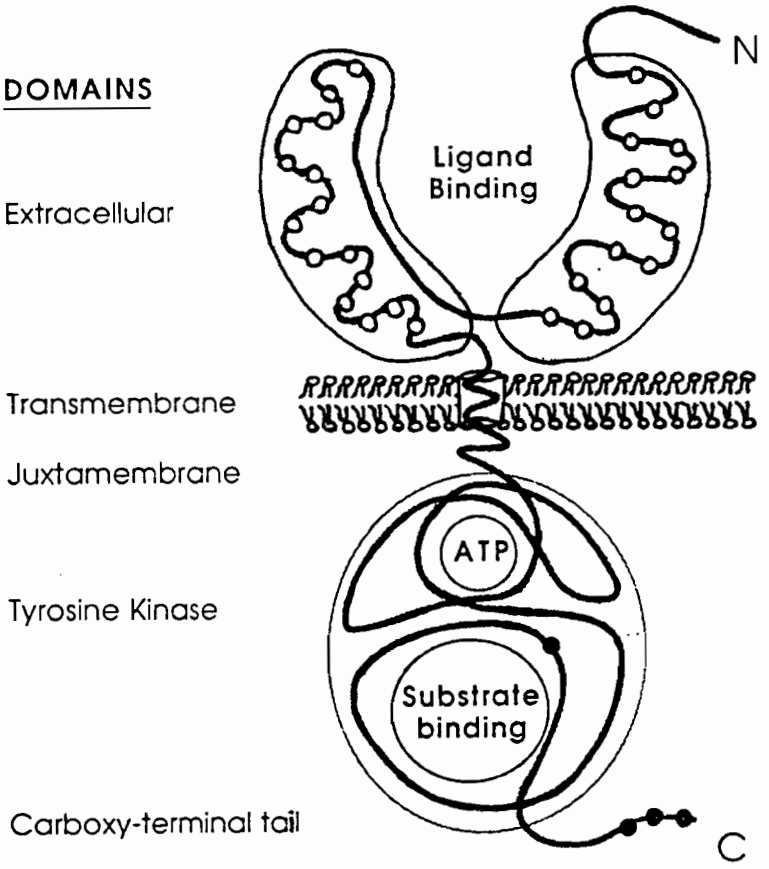


Figure 1.3. Prototypic structure of RTKs. Indicated are amino (N) and carboxy (C) terminal ends of the receptor polypeptide chain, potential binding sites for the ligand, ATP, and endogenous substrates. Cysteine residues (open circles) that may play a role in forming the ligand-binding sites. (adapted from Yarden and Ullrich, 1988).

1.1-2 SUBCLASSES OF RTKS

RTKs can be subclassified into 4 major classes according to the difference in their extracellular cysteine-rich repeat sequence and cytoplasmic tyrosine kinase domain (Yarden and Ullrich, 1988 and Giordano et al, 1989b) (Figure 1-4).

Class I:

This class is defined by monomeric receptors containing two cysteine-rich sequences in their extracellular domains. This class of receptors includes EGFR, c-*erb*B-2 and c-*erb*B-3 (Ullrich et al, 1984; Yamamoto et al, 1986b; Bargmann et al, 1986 and Kraus et al, 1989)

Class II:

The functional structure of this class of RTKs is a disulfide-linked heterotetramer containing $\alpha 2\beta 2$ subunits. The α-subunits are responsible for the extracellular ligand binding. They are linked by disulfide-bonds to the β-subunits which are the transmembrane protein containing the tyrosine kinase domain. Within each α subunit, there is a cysteine-rich domain. This class of receptors includes insulin-R and IGF-1R (Ullrich et al, 1985; 1986).

Class III:

This group of receptors is characterized by the presence of 3 or 5 cysteine residues in their extracellular domain, and a spliced tyrosine kinase domain formed by inserting peptide chains of varying length. The biological significance of this inserting sequence is unclear. It has been shown that this sequence is essential for signal transduction by PDGF through Phosphatidylinositol 3 kinase pathway. These receptors include PDGF-R (Yarden et al, 1986), c-*fms* / CSF-1R (Coussens et al, 1986), c-*kit* (Yarden et al, 1987c) and FGF-R (Ruta et al, 1989).

Class IV:

C-*met* is a prototype of this class of receptor with $\alpha\beta$ heterodimeric structure. The extracellular domain of c-*met* shows a structure in common with other classes of receptors, it contains one cysteine-rich repeat domain and several cysteine residues (Giordano et al, 1989b). No other receptors belonging to this class have been identified yet.

Even though these RTKs share many common structural features, activation of different receptors mediates different biological activities.

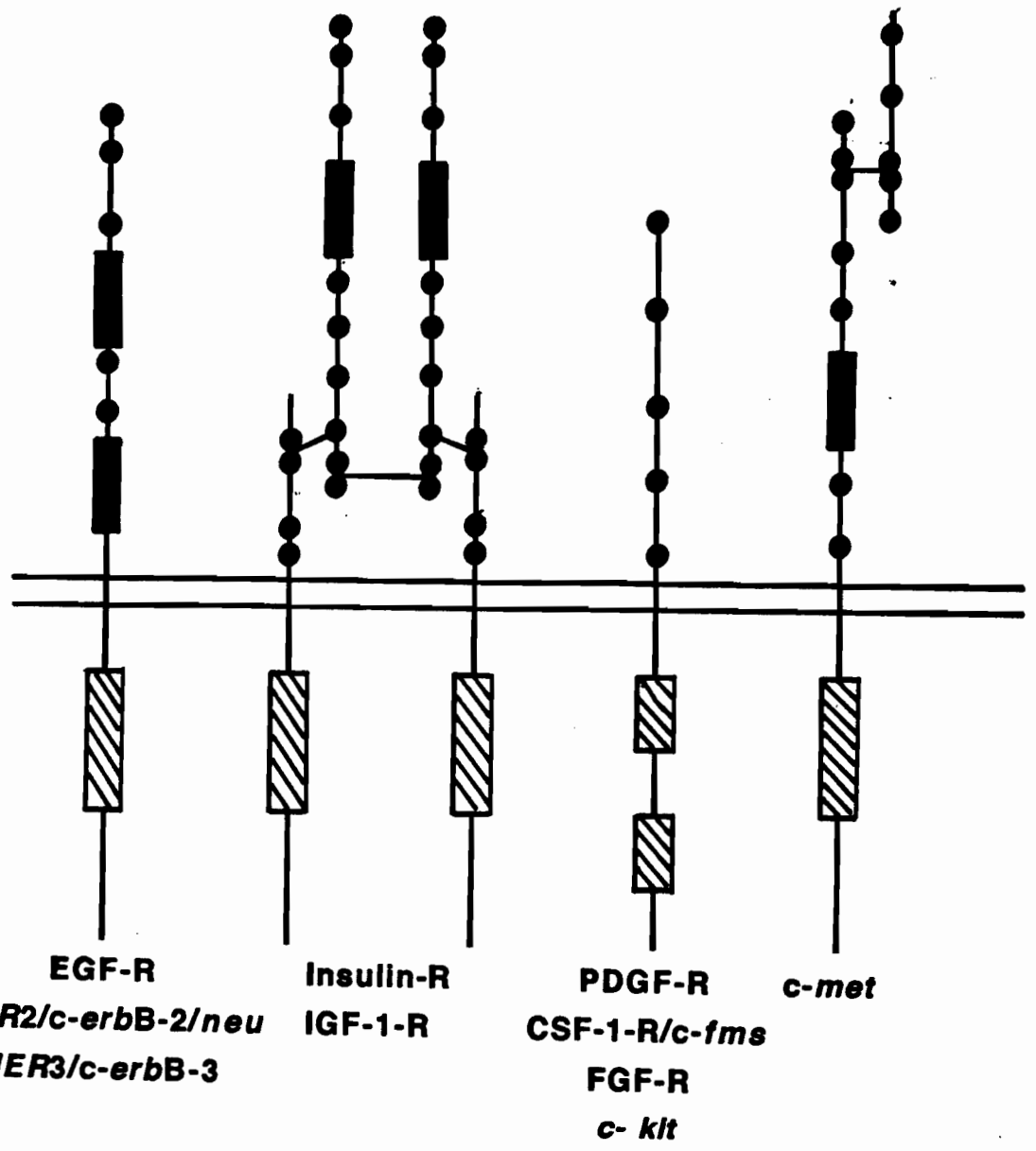


Figure 1-4 Schematic representation of RTK subclasses. ▬: cysteine-rich sequence repeat regions; ●: cysteine-rich residues; ▨: tyrosine kinase domain; EGF: epidermal growth factor, IGF: insulin-like growth factor, PDGF: platelet-derived growth factor, CSF: colony-stimulating factor and FGF: fibroblast growth factor (modified from Yarden and Ullrich, 1988 and Giordano et al, 1989).

1.2-3 LIGAND-STIMULATED RECEPTOR OLIGOMERIZATION

Oligomerization between growth factor receptors is a common phenomenon in response to ligand stimulation, which has been demonstrated in living cells, membrane preparations and purified receptors. Ligand stimulation results in adjacent kinase domains of two monomeric receptors, or within a dimeric receptor, to cross-phosphorylate each other, thereby causing a conformational change which leads eventually to oligomerization (Cochet et al, 1988; Yarden and Schlessinger, 1987a and b). Different classes of receptors mediate dimerization through different pathways (Figure1-5). When class I receptors are activated, they change their extracellular conformation and dimerize with the neighbouring receptors which may be the same type of receptor (EGFR-EGFR), or another type of receptor of the same class (EGFR-NEU). For class II receptors, ligand binding induces only allosteric interaction between the α and β subunits bound by disulfide bridges, resulting in a stabilized complex. Dimerization between class III receptors is mediated by the interaction of two receptors with a dimeric ligand, such as PDGF-AB. Ligand-stimulated c-*met* dimerization has not been demonstrated. It has been shown that dimerized receptors are usually more stable, with elevated protein tyrosine kinase activity and higher ligand binding affinity.

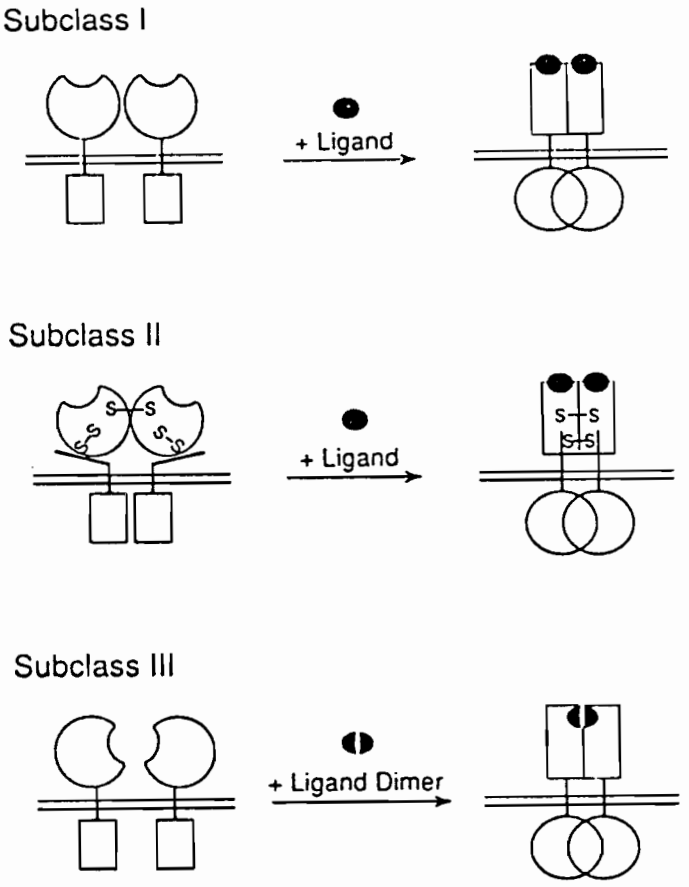


Figure 1-5 Models for ligand-stimulated receptor dimerization. Receptor activation may occur by binding of monomeric ligands resulting in a conformational change of the extracellular domain and dimer formation (subclass I), by interaction of the ligand with a disulfide-stabilized receptor dimer and subsequent intracomplex conformational change (subclass II), or by mediation of dimer formation through a dimeric ligand (subclass III) (adapted from Ullrich and Schlessinger, 1990).

1.2-4 POSSIBLE PATHWAYS INVOLVED IN SIGNAL TRANSDUCTION BY RTKS

Binding between tyrosine kinase receptors and their ligands stimulates tyrosine kinase activity which phosphorylates various cellular substrates and autophosphorylates the receptors themselves, thus inducing a cascade of biochemical responses including inositol-lipid breakdown, ion fluxes, and changes in the expression of growth regulatory genes (Ullrich and Schlessinger, 1990). The targets for tyrosine kinase identified thus far are either components of the second messenger pathways, proto-oncogene products, or factors that regulate the activity of proto-oncogenes (Cantley et al, 1991). SH-2 (*Src* homology 2) domain on the cellular substrates has been shown to play an important role in the substrate utilization by activated RTKs. This domain was first demonstrated in *Src* family gene products, and is not contained in the RTKs, even though many RTKs share structural homology with the cytoplasmic kinase domain of the *Src* gene product ($pp60^{src}$) (Hanks et al, 1988). SH-2 domain is important in the signal transduction by $pp60^{src}$. Mutations at the SH-2 domain affects ability of $pp60^{src}$ to associate with specific cellular substrates and to transform cells (Hirai and Varmus, 1990; Wang and Parsons, 1989). SH-2 domain binds specifically to proteins containing phosphotyrosines (Matsuda et al, 1990). Although RTKs do not contain SH-2 domain, their major cellular substrates usually contain it, such as phospholipase-C-r (PLC-r) (Stahl et al, 1988, Suh et al, 1988), phosphatidylinositol 3 kinase (PtdIns 3-kinase) (Cantley et al, 1991) and *ras* GTPase-activating protein (*ras*-GAP) (McCormick, 1989). It has been presumed that RTKs serve as the critical gathering place, whose autophosphorylation provides important binding sites for the cellular signal-

transducing molecules that contain SH-2 domains (Anderson et al, 1990). Four frequently involved tyrosine kinase targets (cellular substrates) in the signal transduction by RTKs are PLC-r, PtdIns 3-kinase, *ras* GAP and c-*raf* proto-oncogene (Table 1-1). It has been proposed that these target molecules may complex together to form the so called 'signal transfer particle' which preexists in an inactive form. Ligand binding will convert this particle into an active form which is phosphorylated to generate pleiotropic mitogenic responses (Figure 1-6).

The signal transduction pathway of PDGF has been best studied. In response to PDGF stimulation, PDGF-R binds to and phosphorylates all four cellular substrates mentioned above (secondary responses). Many of the cellular responses mediated by activated PDGF-R can be explained as the tertiary responses to the phosphorylated PLC-r, PtdIns 3 kinase, *ras* GAP and c-*raf* (Cantley et al, 1991). For instance, direct phosphorylation of PLC-r results in inositol-lipid breakdown which generates diacylglycerol, the activator for protein kinase C (PKC), and 1.4.5-triphosphate, leading to Ca^{2+} influx, changes in cytoplasmic pH, potassium level and transcription of certain genes. Although *ras* GAP, PtdIns 3 kinase and c-raf are capable of binding to many activated RTKs, their mediated tertiary responses have not been specified.

It seems that different RTKs preferentially phosphorylate different targets, thereby mediating different cellular responses (Table 1-1). This explains why more than one growth factor is usually required in order to sufficiently induce cell growth and transformation.

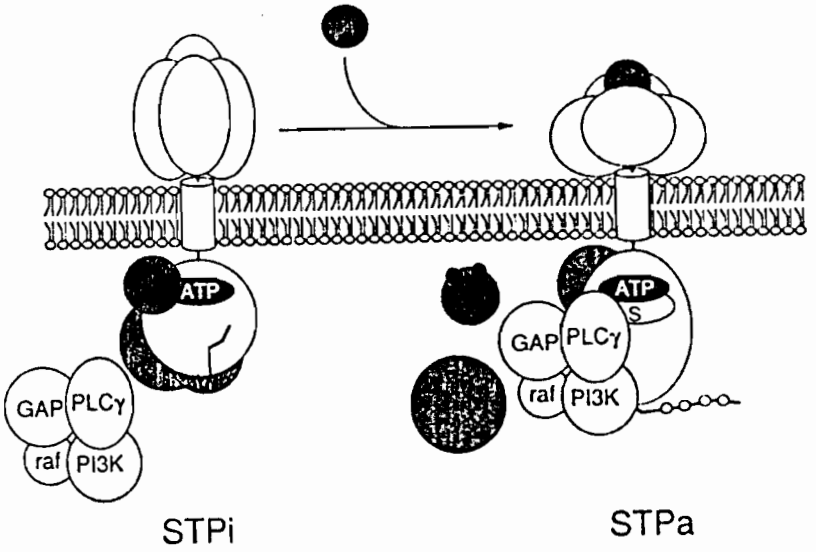


Figure 1-6 Schematic structure of proposed signal transfer particles. Regulatory factors (dark shading) are bound to the inactive tyrosine kinase domain of the receptor, forming the inactive signal transfer particle (STPi). Activation by ligand stimulation may lead to dissociation of regulatory factors that may be phosphorylated on tyrosine residues (black dots), and the newly formed active conformation presents binding sites for substrates. Substrates associated with the activated receptor result in the active signal transfer particle (STPa) (adapted from Ullrich and Schlessinger, 1990)

Table 1-1 Cellular substrates affected by activated RTKs.

	PtdIns 3-kinas	ras GAP	PLC-r	c-raf
PDGF-R	+	+	+	+
EGF-R	+	+	+	?
CSF-R	+	+	-	+
I-R	+	?	-	+
IGF-R	+	?	?	?

PDGF-R: platelet-derived growth factor receptor; EGF-R: epidermal growth factor receptor; CSF-R: colony-stimulating factor receptor; I-R: insulin receptor; IGF-R: insulin-like growth factor receptor (Cantley et al, 1991)

1.2-5 EPIDERMAL GROWTH FACTOR RECEPTOR

EGFR is the prototype of RTKs, which was first purified from the A431 human epidermoid carcinoma cell line by immunoaffinity chromatography (Cohen et al, 1982). EGFR was subsequently detected in various normal and neoplastic cells and tissues of human and rodent origins (Carpenter and Cohen, 1979). However, its expression has not been reported in hematopoietic cells nor in human small cell lung carcinomas (Gamou et al, 1987). The receptor is a 170 KDa transmembrane protein encoded by the gene located on chromosome 7p1.1-1.3 (Conen et al, 1982). The primary structure of EGFR demonstrates 3 typical major domains of class I RTKs (Ullrich et al, 1984) (Figure1-4, Table 1-2). It has been shown that v-*erb*B oncogene of the avian erythroblastosis virus codes for a truncated EGFR which lacks most of the extracellular ligand binding domain and 32 amino acids at the carboxy-terminal tail (Downward et al, 1984b) . The v-*erb*B protein induces transformation by acting as a constitutively activated receptor. This suggests that abnormal expression of the EGFR can lead to malignant transformation.

Binding of EGF or TGF-α activates receptor tyrosine kinase which phosphorylates various cellular proteins and autophosphorylates the receptor itself. Autophosphorylation sites of EGFR are clustered at the carboxy-terminal tail, which act to compete with other substrates for binding to the substrate binding sites at the kinase domain, thereby modulating mitogenic responses (Downward et al, 1984a; Mardolis et al, 1989b).The occupied EGFR clusters in a coated pit which is internalized via a receptor-mediated endocytosis, and is delivered to the lysosomes. Therein, both EGFR and its ligand are degraded by lysosomal enzymes into low molecular weight fragments. The degraded EGFR is inactive and does not recycle back to the plasma membrane (Brown

et al, 1983).

Prior to exposure to the ligand, EGFR exists in a low affinity state. High affinity receptors occur only in response to the ligand stimulation. These findings are consistent with the migration pattern of EGFR in native gel where both the fast-migrating or EGF-induced slow-migrating forms are observed. The fast form (low LMW form) represents the monomeric 170 KDa EGFR, while the slow form (HMW form) represents high affinity, dimerized EGFR induced by ligand stimulation (King et al, 1982). The mitogenic response is mainly mediated through the high affinity receptors (King et al, 1982).

The threonine residue at 654 (Thr654), located at the juxtamembrane of the EGFR, is an important regulatory site whose phosphorylation by protein kinase C provides a negative feed back for receptor activity control (Lin et al, 1986). Activation of receptors for PDGF and bombesin by their ligands abolishes the high affinity EGF binding sites, this effect is thought to be mediated through activation of protein kinase C by these two growth factors (Brown et al, 1983; Wrann et al, 1980). In addition, several other compounds have been shown to down-regulate the high affinity EGFR, and therefore, subsequently affect the binding between EGFR and its ligands. Most of these compounds are protein kinase C activators, such as phorbol ester TPA (12-O-tetradecanoyl-phorbol-13-acetate) (Brown et al, 1979; Shoyab et al, 1979).

The over-expression of EGFR has been a hallmark for squamous cell carcinomas of mouth (Todd et al, 1989), larynx (Scambia et al, 1991), head and neck (Ishotoya et al, 1989) and esophagus (Ozawa et al, 1989). Amplification and / or over-expression of this receptor has also been observed in other types of human solid tumors including malignant gliomas (Wong et al, 1987), carcinomas of the bladder (Berger et al, 1987), breast (Harris et al, 1989), stomach (Yasui et al, 1988; Yonemura et al, 1989), and lung (Veale et

al, 1987). In breast, bladder and gastric carcinomas, its over-expression has been correlated with shortened relapse-free and overall survivals (Harris et al, 1989; Berger et al, 1987; Neal et al, 1990; Yasui et al, 1988).

Table 1-2 Gene location, mRNA size, protein product and ligand for EGFR, c-*met* and c-*erb*B-2.

	EGFR	c-*erb*B-2	c-*met*
Gene Location	7p1.1-1.3	17q21	7q21-31
mRNA	5.8, 10 kb	4.8 kb	9, 7, 6 &5 kb
Protein	175 KDa	185 KDa	190 KDa (NR) 145 KDa (R)
Ligand	EGF, TGF-α	gp30	HGF
Extracellular Domain	621 aa	632 aa	926 aa
Transmembrane region	23 aa	22 aa	23 aa
Cytoplasmic Domain	542 aa	580 aa	435 aa

EGFR: epidermal growth factor receptor; HGF: hepatocyte growth factor; NR: non-reducing condition; R: reducing condition; aa: amino acid

1.2-6 *NEU* /C-*ERB*B-2/*HER*-2 PROTO-ONCOGENE

Neu oncogene was identified in NIH 3T3 cells which had been transfected with ethylnitrosourea-induced rat neuroblastoma gene (Schechter et al, 1984). Molecular cloning of the transforming *neu* gene and cellular *neu* proto-oncogene revealed that activation of this gene is due to a single point mutation from adenine to thymidine (A→T transversion) at 644 in the transmembrane region, resulting in a substitution of glutamine for valine (Bargmann et al, 1986). The human counterpart of the *neu* gene is designated as c-*erb*B-2 (HER-2) by its structural homology to c-*erb*B which encodes EGFR (Semba et al, 1985). The c-*erb*B-2 gene, located on chromosome 17 at q 21, codes for a 185 KDa transmembrane glycoprotein ($p185^{neu}$) (Table 1-2, Figure 1-4) with extensive homology to EGFR, especially in their tyrosine kinase domains (80% homology) (Coussens et al, 1985; Fukushinge et al, 1986; Yamamoto et al, 1986b). $p185^{neu}$ cross-reacts with the polyclonal antibody to EGFR, but it does not bind to the ligands for EGFR i.e. EGF or TGF-α (Stern et al, 1988; King et al, 1988). A putative ligand for c-*erb*B-2 protein has recently been identified as a secreted glycoprotein with an apparent molecular weight of 30 KDa (gp30) from a cultured MDA-MB-231 human breast carcinoma cell line (Lupu et al, 1990). In contrast to EGF or TGF-α which binds only to the EGFR, gp30 binds and phosphorylates both c-*erb*B-2 and EGFR independently, even though its effect is much less efficient in activating EGFR.

As a proto-oncogene, the amplification of c-*erb*B-2 is only capable of the neoplastic transformation of NIH 3T3 cells when acting in combination with either serum, insulin, EGF, or fibroblast growth factor, (Di Fiore et al, 1987). C-*erb*B-2 is widely expressed in many normal and neoplastic cells and tissues.

By using immunocytochemical staining, its expression in human adult tissues has been demonstrated in the membranes of epithelial cells in gastrointestinal mucosa, in the reproductive and urinary tracts, in skin, breast and placenta (Press et al, 1990). The level of c-*erb*B-2 expression in these tissues is generally lower than in their corresponding human fetal tissues (Press et al, 1990). The expression of c-*erb*B-2 is also frequently detected in malignant tumors taking origin from these tissues. Like the expression of EGFR, c-*erb*B-2 is expressed at a very low or undetectable level by human small cell lung carcinomas (Schneider et al, 1989), and its expression has not been reported in hematopoietic cells.

Several lines of evidence have suggested that $p185^{neu}$ and EGFR are not only structurally related, but also are functionally associated. These include:

(1). structurally homology between the EGFR and $p185^{neu}$;

(2). ligand cross-reactivity between gp30 and EGFR as described previously;

(3). EGF-induced, EGFR-dependent $p185^{neu}$ activation: although TGF-α and EGF fail to bind to $p185^{neu}$, their binding to EGFR can subsequently induce phosphorylation of $p185^{neu}$ on threonine, serine and tyrosine residues, as demonstrated in rat-1 embryonic fibroblast cell line (Kokai et al, 1988), in MKN-7 human adenocarcinoma cell line (Akiyama et al, 1988), BT474 human breast carcinoma cell line (Lin et al, 1990) and in the SK-BR-3 mammary carcinoma cell line (King et al, 1988). The EGF-induced $p185^{neu}$ phosphorylation is dependent upon the presence of a functional

EGFR, and is apparently unique, since none of the other growth factors tested, such as PDGF, TGF-β and insulin, can mediate such an effect (Kokai et al, 1988). The detailed molecular mechanism for the EGF-induced p185^{neu} activation is unclear, which may be due to EGF-stimulated heterodimerization between the EGFR and p185^{neu}. This molecular association modulates ligand affinity of the EGFR, since down-regulation of p185^{neu} with monoclonal antibody to the p185^{neu} converts high affinity EGFR to a low affinity state (Wada et al, 1990);

(4). inverse expression of EGFR and p185^{neu} in certain human tissues:

In human epidermis, the more differentiated keratinocytes express more c-*erb*B-2 protein but less EGFR as compared to the less differentiated keratinocytes (Maguire et al, 1989; Nanney et al, 1984; Green et al, 1985). In human colonic mucosa, the left colon expresses a higher level of EGFR but a lower level of c-*erb*B-2 mRNA, while the right colon expresses a lower level of EGFR but a higher level of c-*erb*B-2 mRNA (discussed in Chapter 4). These observations suggest that, under certain physiological conditions, EGFR and c-*erb*B-2 may co-operate to regulate cell growth and differentiation, and a combined activity of the two receptors is important in maintaining normal cell functions.

Gene amplification appears to be the main mechanism underlying c-*erb*B-2 activation. It has been found predominantly in adenocarcinomas, such as human salivary gland adenocarcinomas (Semba et al, 1985), breast and ovarian carcinomas (Parkes et al, 1990; Slamon et al, 1987; Zhang et al,

1989). Occasionally, the amplification of c-*erb*B-2 is also detected in carcinomas of the other histotypes, for example, epidermoid carcinomas of the lung (Schneider et al, 1989). C-*erb*B-2 has been generally accepted as an important prognostic factor for human breast carcinomas. It is amplified in approximately 30% of cases (Salmon et al, 1987). The amplification is associated significantly with both over-expresson of the c-*erb*B-2 mRNA and the over-production of the $p185^{neu}$ (Kern et al, 1990). Both amplification of c-*erb*B-2 gene and over-expression of $p185^{neu}$ have been significantly correlated with a reduction in both relapse-free and overall survivals (Salmon et al, 1987), especially in the lymph node negative patients. It is noted that the $p185^{neu}$ and EGFR are additive in their prognostic value, patients who over-express the two receptors tend to have the poorest prognosis (Harris et al, 1990). Moreover, EGFR and c-*erb*B-2 are more frequently amplified in inflammatory breast carcinomas as compared to non-inflammatory carcinomas (Guerin et al, 1989), and the authors suggest that their amplification is associated with aggressive breast carcinomas. A similar prognostic significance of c-*erb*B-2 has been demonstrated in human lung adenocarcinomas (Kern et al, 1990), and in ovarian and gastric carcinomas (Salmon et al, 1989; Berchuck et al, 1990; Yonemura et al, 1991), but this remains to be further confirmed.

1.2-7 C-*MET* PROTO-ONCOGENE

Met oncogene was identified by NIH 3T3 transfection assay in a human osteogenic sarcoma cell line (HOS) which had been treated with a chemical carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Cooper et al, 1984). In this case, the activation of c-*met* proto-oncogene was due to a DNA

rearrangement which created a hybrid gene containing the 5'-end of tpr (translocated promoter gene) from chromosome 1 and the 3'-end of c-*met* from chromosome 7 (Park et al, 1986). The c-*met* proto-oncogene is expressed predominantly as a 9.0 kb transcript in human fibroblasts and epithelial cells. Other c-*met* RNA species (7.0 kb kb and 6.0 kb) have also been detected depending on the cell type examined (Park et al, 1986). In MNNG-treated HOS cells, a novel 5.0 kb transcript is identified, which represents activated oncogene (tpr-*met* fusion gene) and codes for a 65 KDa protein ($p65^{tpr\text{-}met}$) (Park et al, 1986). Nucleotide sequence analysis of both human (Park et al, 1987) and mouse (Chan et al, 1988) cDNA suggests that c-*met* encodes a cell surface receptor with tyrosine kinase activity (Table 1-2, Figure 1-4). The kinase domain of human c-*met* exhibits approximately 44% and 41% homology to human insulin receptor and v-*abl* respectively (Park et al, 1987). C-*met* protein is synthesized as a 190 KDa disulfide-linked heterodimer consisting of a 50 KDa α chain exposed on the cell surface, and a 140 KDa transmembrane glycoprotein (β-chain). The $p140^{met}$ represents a functional c-*met* receptor which is generated after releasing an α-chain from the heterodimer under a reducing condition (Giordano et al, 1988a; 1989b). Both $p140^{met}$ and $p65^{tpr\text{-}met}$ are phosphorylated on tyrosine residues by an *in vitro* kinase assay, but only the $p65^{tpr\text{-}met}$ is phosphorylated on tyrosine residues *in vivo*, suggesting that, in analogous to the v-*erb*B, the $p65^{tpr\text{-}met}$ may represent a truncated, yet constitutively activated c-*met* receptor (Gonzatti-Haces et al, 1988).

The ligand for c-*met* has been identified, and is known as hepatocyte growth factor (HGF) (Bottaro et al, 1990). HGF was first isolated from the

serum of partially hepatectomized rats , and initially called hepatopoietin-A (Nakamura et al, 1984; Thaler et al, 1985). The growth factor was subsequently purified from rat platelets (Nakamura et al, 1987), rabbit serum (Zarnegar et al, 1989), human serum (Selben et al, 1986), the plasma of a patient with fulminant hepatic failure (Gohda et al, 1989) and human placenta (Wolf et al, 1991). HGF strongly stimulates the DNA synthesis of adult rat hepatocytes in primary culture, and promotes liver regeneration after partial hepatectomy and liver injury (Nakamura et al, 1984). These effects are most likely mediated through a paracrine loop since, in liver, HGF is synthesized by non-parenchymal cells (fibroblasts), but not by parenchymal hepatocytes (Kinoshita et al, 1989). HGF is a disulfide-linked heterodimer composed of a large 65KDa and a small 34 KDa subunits, which is derived from a single-chain precursor protein by proteolytic processing (Miyazawa et al, 1989). HGF differs structurally from other known growth factors, but demonstrates sequence homology to plasminogen (Nakamura et al, 1989; Tashiro et al, 1990).

The expression of c-*met* has been demonstrated in several human cancer cell lines. By the *in vitro* kinase assay, p140^{met} phosphorylation is detected in two colonic (SW-620, DLD-1), three pancreatic (Capan-1, -2, Panc-1), five lung (Calu-1, -6, A549, SK-MES1, SK-lu-1) and two cervical (C-41, Hela) carcinomas cell lines (Gonzatti-Haces et al, 1988). By using the polymerase chain reaction (PCR) technique, the tpr-*met* rearranged gene is detected at very low frequencies in 7 of 14 human tumor cell lines examined, including a pancreatic (Capan-1) and a colonic (DLD-1) adenocarcinoma cell lines, a bladder (ScaBER) carcinoma line, a squamous cell carcinoma line of the tongue (SCC-4), a cervical lymphoma line (Hs602), a retinoblastoma line

(Y-79) and an osteosarcoma line (U-2 OS) (Soman et al, 1990). However, it is not known whether the tpr-*met* gene in these cell lines also expressed rearranged RNA or protein. In a human gastric carcinoma cell line, GTL-16, activation of the c-*met* is associated with amplification and over-expression of the normal (non-rearranged) cellular c-*met* proto-oncogene (Ponzetto et al, 1990). Nevertheless, the expression of c-*met* in primary human tumors has been rarely studied.

1.3 HUMAN COLON CANCER

Colon cancer is one of the most common human malignancies in the world (Figure 1-8). Its incidence varies markedly from country to country, being high in the United States, Saskatchewan in Canada, the United Kingdom, New Zealand, Australia, Denmark and Sweden, and low in Colombia, Japan, India, South Africa, Israel, Finland, Poland and Puerto Rico (Cotran et al, 1989). This geographic variation of the incidence is also associated with the distribution of tumors within the colon. In general, in the low incidence countries, the lesions tend to be located more frequently in caecum and ascending colon, whereas, in the high incidence countries in sigmoid and rectum. Overall, about 70-75% of colorectal carcinomas are located in the rectum, rectosigmoid and sigmoid. Although the right sided carcinomas (Villous and having a broad base) are grossly very different from the left sided carcinomas, their microscopic characteristics are similar. Histologically, about 95% of the colorectal carcinomas are adenocarcinomas, the rest being adenosquamous carcinomas, carcinoid tumors, sarcomas, etc. (Fenoglio-Preiser et al, 1990). The peak incidence is among people at age 70. Younger patients (30 to 40 years old) are usually associated with hereditary intestinal disorders, such as familial polyposis coli.

1.3-1 ETIOLOGY

Several benign and inflammatory colonic lesions are predisposed to a higher risk of developing colorectal carcinomas. They include colonic adenomas, familial polyposis coli, ulcerative colitis, Crohns' colitis and Gardner's syndrome. Among these lesions, colonic adenomas, especially villous adenomas, and familial polyposis coli are of greatest importance. The incidence of carcinomas arising from adenomas is 1% for tubular adenomas smaller than 2 cm, 10% for adenomas larger than 2 cm, 30% for villous adenomas, and almost 100% for familial polyposis coli (FPC) and Gardner's syndrome if the diseased colon is not removed. The FPC is an inherited disease characterized by the presence of innumerable neoplastic polyps which usually involve the entire colon. Histologically, the majority of these polyps are tubular adenomas. Most of the patients diagnosed are at the age of 20 to 30. Once a diagnosis is made, it is recommended that the whole diseased colon be removed. Gardner's syndrome is another inherited disease with a combination of FPC, multiple osteomas, epidermal cysts and fibromatosis. The patients with this syndrome have the same high risk of developing colon cancer as those with FPC.

There is strong evidence which supports the neoplastic progression from adenoma to carcinoma (polyp-cancer sequence), while some evidence is against this concept :

(1) Arguments Cited in Support of the Concept (Morson and Dawson, 1972)

(a) Close association / parallelism in the epidemiology and anatomic distribution of adenomas and carcinomas;

(b) The common occurrence of invasive cancer in large adenomas, and the presence of residual adenomas at the edge of the carcinoma;

(c) Various grades of dysplasia up to carcinoma in-situ can be observed in adenomas;

(d) Average age of patients presenting with adenomas is approximately 5 years younger than the average age of patients with carcinomas;

(e) Adenomas are frequently found in patients with carcinomas, and the removal of adenomas reduces the incidence of colorectal cancer;

(f) Patients with genetically inherited familial adenomatous polyposis syndrome develop numerous adenomatous polyps, many of which with time develop into carcinomas.

2) Arguments Cited Against the Concept (Fenoglio-Preiser et al, 1990)

(a) Different distribution of adenomas and carcinomas in some studies;

(b) Same incidence of carcinomas developing in patients with and without polyps;

(c) Failure to demonstrate areas of adenoma in "small " carcinomas.

In addition, dietary factors including a low content of unabsorbable vegetable fibers, a high content of refined carbohydrates and animal fat, have also been significantly associated with increased risk of developing colon cancer.

1.3-2 STAGING

The prognosis for patients with colorectal carcinomas is dependent on the extent of bowel involvement, lymph node and distant metastasis, histological differentiation of the lesions, and partially to the location of tumor

within the colon. Among these factors, the extent of the tumor and its metastatic potential are of greatest importance. The most commonly used staging system for colon cancer is Dukes' classification (Dukes et al, 1940) which defines:

Dukes' A: tumor invasion confined to the muscularis propria and without lymph node metastasis;

Dukes' B: tumor invasion extending beyond the muscularis propria but without lymph node involvement;

Dukes' C: tumor with regional lymph node metastasis.

1.3-3 GENETIC MODEL FOR COLORECTAL CARCINOGENESIS

Colorectal carcinogenesis is a multi-step process which involves both the activation of proto-oncogenes and inactivation of tumor suppressor genes, with the latter being predominant.

Activation of the Proto-oncogenes:

(1) Mutational Activation of *ras* Gene

Ras family proto-oncogenes were originally identified as the cellular counterparts of transforming genes of the Harvey and Kirsten murine sarcoma viruses (Ellis et al, 1981; Chang et al, 1982). *Ras* family comprises three functional genes: Ha (Harvey)-*ras*, Ki (Kirsten)-*ras* and N (neuroblastoma)-*ras*. Each of these genes codes for a structurally related, membrane bound 21 KDa protein (p21) with GTPase activity (Willingham et al, 1983; Sweet et al, 1984). The *ras* protein acts as a signal transducer of external stimuli, whose function is activated by the binding to guanine triphosphate (GTP). GTPase hydrolyses and converts GTP to GDP, thus allowing p21 to return to a resting

state (Barbacid et al, 1987). Point mutations at codon 12, 13 and 61 result in amino acid changes which abolish the GTPase activity of p21, leading to constitutively activated p21 molecules (Barbacid et al, 1987).

Ras mutations can be detected in approximately 40-50% of colonic adenomas and carcinomas. Most of these mutations take place at codon 12 of c-Ki-*ras*. Mutations are more commonly observed in adenomas of size larger than 1 cm (58%) than adenomas of size smaller than 1 cm (9%), suggesting a possible role of *ras* mutational activation in the progression from adenomas to carcinomas (Vogelstein et al, 1988).

(2) C-*myc* Proto-oncogene Deregulation:

The c-*myc* proto-oncogene was identified as a cellular counterpart of the retroviral oncogene of avian myelocytomatosis virus no.29 (Mladenov et al, 1967). Other *myc* family genes were identified by virtue of their structural homologies to the c-*myc*, they include L-*myc*, N- *myc*, R-myc, P-*myc* and B-*myc* (DePinhor et al, 1987; Ingvarsson et al, 1988). Each of the *myc* family genes codes for a nuclear phosphoprotein that binds to single or double stranded DNA , thus regulating the DNA replication (Ramsay et al, 1984; Persson et al, 1984). C-*myc* activation is mainly mediated by gene amplification and translocation. 5 to 40 times over-expression of the c-*myc* mRNA has been demonstrated in about 60-80% of human colorectal adenocarcinomas, as compared to the normal colonic mucosa (Erisman et al, 1985; Rothberg et al, 1985). The enhanced mRNA expression of c-*myc* mRNA is not associated with amplification and / or rearrangement of this gene (Erisman et al, 1985). The fact that normal cells in culture produce elevated levels of *myc* RNA and protein only when they are stimulated to go from a resting to a dividing state by a mitogenic stimulus (Armelin et al, 1984), and

that there is constitutively over-expression of c-*myc* RNA by colonic carcinomas, suggests that these colonic cancer cells are in a constant state of proliferation.

Inactivation of Tumor Suppressor Genes:

Tumor suppressor genes are defined as eukaryotic genes whose products normally regulate the growth and differentiation of cells in a negative fashion, thus suppressing neoplastic development (Knudson, 1985). Inactivation of tumor suppressor genes by deletions, translocations and mutations has been an important mechanism contributing to malignant transformation.

In colon cancer, allelic deletions have been observed in almost all of the chromosomes, but predominant and significant losses have been located to 1q, 4p, 5q, 6p, 8p, 9q, 17p, 18q and 22q (Fearon and Vogelstein, 1990). Chromosomal deletions are particularly common in 5q, 17p and 18q, presumably because these chromosomes contain the candidate tumor suppressor genes.

(1) Adenomatous Polyposis Coli (APC) Gene on 5q:

This was first identified from a patient with familial polyposis coli syndrome (Bodmer et al, 1987). Allelic deletions of 5q have been detected in 20-50% of colorectal carcinomas, irrespective of whether patients have this syndrome or not. Losses of 5q are observed in 30% of adenomas from patients without FAP syndrome, but are rare in adenomas from patients with such a syndrome (Sasaki et al, 1989). In FAP patients, it is an inherited mutation on chromosome 5q that is responsible for the increased proliferation of the colonic epithelial cells. Additional 5q loss is observed when these

patients develop colorectal carcinomas.

(2) p53 on 17p

The p53 nuclear protein was first identified as a complex with a large T antigen of the DNA tumor virus SV40, and was originally viewed as an oncogene (Lane and Crawford, 1979). It has only recently been recognized that normal p53 is actually a tumor suppressor gene (Finlay et al, 1989). Its ability to co-operate with activated *ras* gene in transforming primary rat embryo fibroblasts is dependent on a number of mutations at p53, these mutations convert p53 into an oncogene (Hinds et al, 1989). p53 is a nuclear protein involved in the regulation of DNA synthesis and cell cycle progression. Inactivation of p53 results from gene deletions and / or mutations. The loss of a large portion of chromosome 17p is detected in approximately 75% of advanced colorectal carcinomas, but is relatively infrequent in adenomas (Vogelstein et al, 1988; Baker et al, 1989).

(3) DCC (Colon Cancer Deleted) Gene on 18q

DCC gene is a newly identified tumor suppressor gene, whose expression is commonly detected in normal colonic mucosa, but is greatly reduced in the majority of colorectal carcinomas (Fearon et al, 1990). The allelic loss of 18q has been detected in about 70% of colorectal carcinomas and 50% of late adenomas (vogelstein et al, 1988; Delattre et al, 1989). DCC gene codes for a protein with significant homology to the N-CAM (cell adhesion molecule) (Fearon et al, 1990). Thus, the inactivation of this gene may play a role in development of colorectal cancer by altering normal cell-cell, or cell-extracellular matrix interactions.

In general, genetic changes take place according to a preferred sequence, with allelic loss of 5q and mutations of *ras* gene occurring earlier and losses of 17p and 18q at a later stage of colorectal carcinogenesis (Figure 1-7) (Fearon and vogelstein, 1990). However, it has been suggested that an accumulation of these changes, rather than their order with respect to one another, is more important. Genetic changes in at least 4 to 5 genes are required for the pathogenesis of a malignant tumor, whereas fewer changes are only sufficient to induce the formation of a benign lesion. Moreover, patients with more than the median number of losses in their tumors have a considerably worse prognosis than the patients with less chromosomal deletions (Fearon and Volgelstien 1990; Kern et al, 1989).

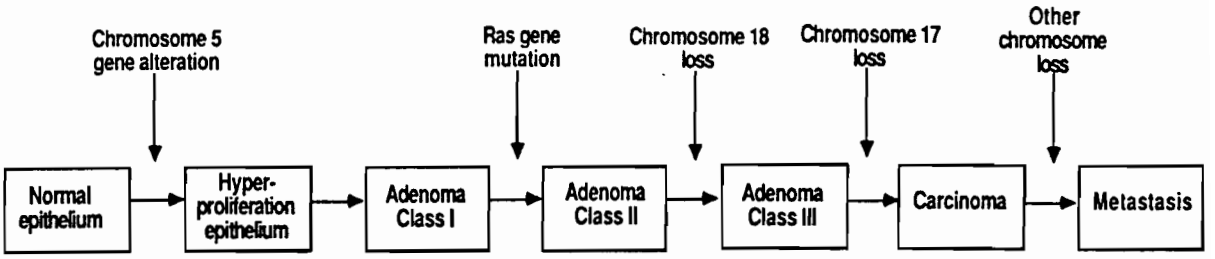


Figure 1-7 A genetic model for colorectal carcinogenesis (adapted from Vogelstein, 1989). Class I adenomas were defined as adenomas of size 10 cm of less. Class II adenomas were greater than 1 cm in size and did not contain foci of carcinoma. Class III adenomas were greater than 1 cm in size and contained foci of carcinoma. Tumorigenesis proceeds through a series of genetic alterations, including *ras* gene activation and loss of putative tumor suppressor genes on chromosomes 5, 17 and 18.

1.4 HUMAN LUNG CANCER

Lung cancer is the most common malignancy in men and the second most common malignancy in women (Figure 1-8) (Silverberg and Lubera, 1988), whose incidence has increased steadily over the past 30 years. Despite many efforts having been made to improve the diagnosis and treatment, 90% of patients still die of this disease (Minna et al, 1989). The highest incidence of lung cancer is among people at ages between 60 to 70. Lung cancer occurs at a relatively higher frequency in men than in women. About 90-95% of human lung cancers are bronchogenic carcinomas, 5% are bronchial carcinoid, and 2-5% are mesenchymal tumors. The major etiologic agents for human lung cancer are tobacco smoking, asbestos and exposure to ionising radiations (Cotran et al, 1989).

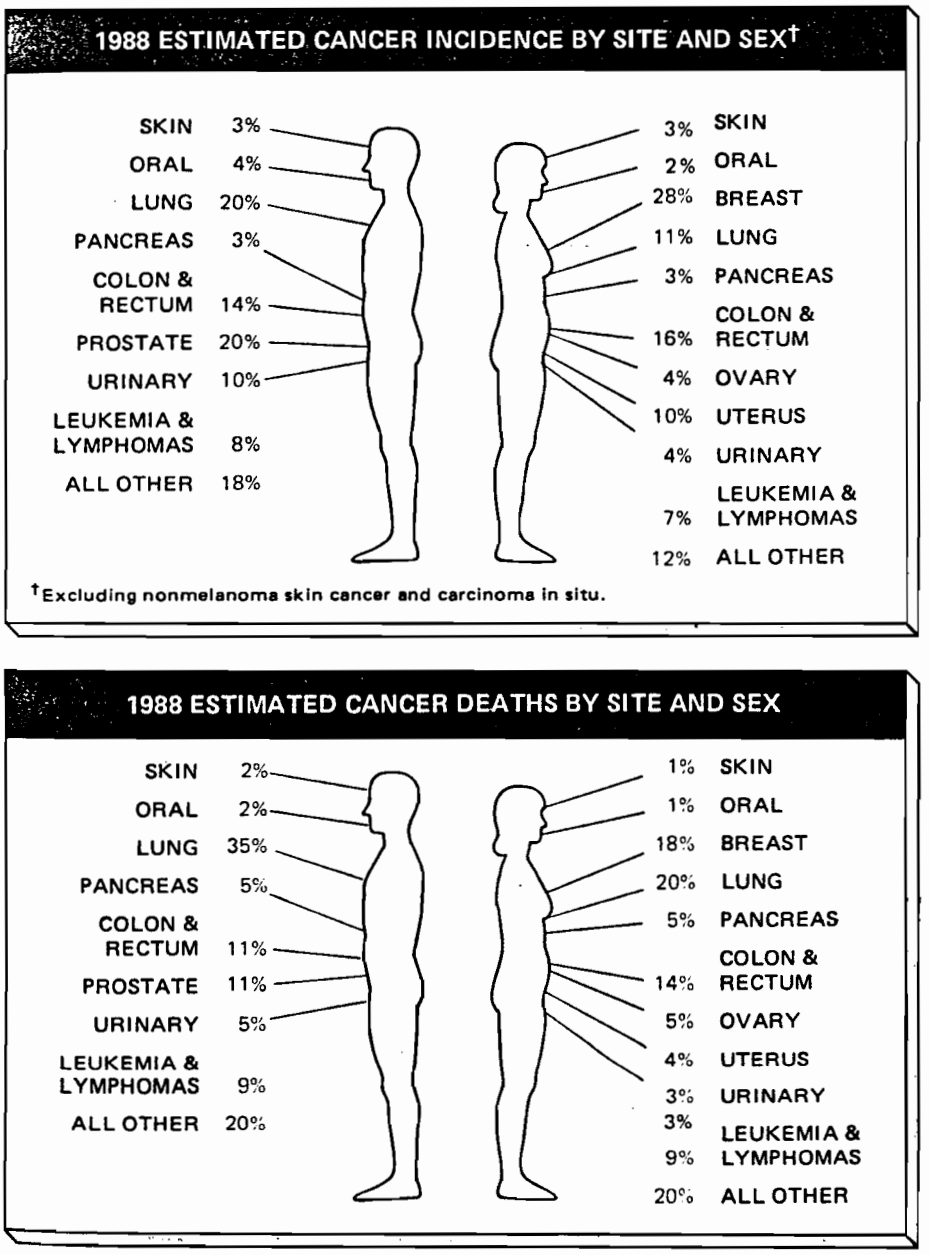


Figure 1-8 Estimated cancer incidence and deaths by site and sex in 1988. (adapted from Sliverberg and Lubera, 1988).

1.4-1 HISTOLOGICAL CLASSIFICATION OF LUNG CANCER

Human lung cancer can be broadly classified into two major groups: small cell lung carcinomas (SCLCs) (25%) and non-small cell lung carcinomas (NSCLCs) (75%). NSCLCs can be subdivided into 3 major histotypes including epidermiod (squamous cell) carcinomas (30%), adenocarcinomas (25%) and large cell carcinomas (15%) (Cotran et al, 1989). SCLCs differ significantly from NSCLCs by their distinct clinical behaviour, biochemical properties and responsiveness to chemotherapeutic agents.

SCLC is a highly malignant disease characterized by rapid growth and pronounced tendency to metastasize. Cancer cells are very sensitive to chemo- and radio-therapy. It has been reported that about 15-25% of patients with limited disease are curable by combined chemo- and radio-therapy. Unfortunately, most patients have already developed distant metastases at the time of diagnosis. The mean survival for this type of lung cancer is only one year after diagnosis (Cotran et al, 1989). Biochemically, SCLC cells are characterized by their expression of neuroendocrine markers such as chromogranins and neuronspecific enolase (NSE). The neuroendocrine secretory granules can be identified by transmission electron microscopy.

In contrast to the SCLCs, NSCLC cells are usually resistant to chemotherapy, therefore, surgical resection has been a major therapeutic modality for this type of cancer. Among the three histotypes of NSCLCs, adeno- and epidermoid carcinomas often present as localized disease allowing treatment by surgery, whereas, large cell carcinomas are usually discovered as an advanced lesion. The 5 year survival rate for male is 10% for adeno- and epidermoid carcinomas and 3% for large cell carcinomas (Cotran

et al, 1989). In addition, neuroendocrine granules are also detected in a subset of large cell undifferentiated carcinomas which have been shown to resemble SCLCs, behaving more aggressively than other histotypes of NSCLC.

1.4-2 STAGING

A uniform TNM system classifies lung carcinomas into 5 major stages according to the anatomic extent of the primary tumors (T), regional lymph node involvement (N) and distant metastasis (M) at the time of diagnosis. (appendix A).

1.4-3 MOLECULAR GENETICS OF LUNG CANCER

It has become clear that the process of bronchial carcinogenesis is associated with multiple genetic abnormalities involving activation of dominant oncogenes and inactivation of tumor suppressor genes.

Activation of Proto-oncogenes

(1) Mutational Activation of *ras* Family Genes

The *ras* family genes can be activated by their over-expression and / or somatic point mutations, with the latter being predominant (Barbard et al, 1986; McGrath et al, 1984). Mutational activation of *ras* gene has been frequently detected in NSCLC cell lines and primary tumors (Birrer and Minna, 1988; Bonfil et al, 1989; Rodenhuis et al, 1988; Santos et al, 1984), whereas, it has been rarely observed in SCLCs. The highest frequency of *ras* mutations is demonstrated in adenocarcinomas of the lung. The most common site affected by mutations is the codon 12 of the c-Ki-*ras* (Bonfil et al, 1989). In

addition, it has been demonstrated that v-Ha-*ras*, a retroviral counterpart of the c-Ha-*ras*, is able to transform normal human bronchial epithelial cells in culture (Yoakum et al, 1985). This finding, together with the development of well differentiated adenocarcinomas of lung in transgenic mice carrying mutated c-Ha-*ras* gene derived from a human bladder carcinoma cell line, suggests a possible participation of an activated *ras* gene in the malignant transformation of normal bronchial epithelial cells. However, no constant correlation has thus far been demonstrated as to the clinico-pathological significance of the *ras* mutations.

(2) Deregulation of *myc* Family Genes

Amplification of *myc* genes can be detected in approximately 10-20% of primary human lung cancer, both of the SCLCs and NSCLCs (Wong et al, 1986; Funa et al, 1987; Johnson et al, 1987; Johnson et al, 1988b; Yokota et al, 1988; Takahashi et al, 1989a). The most common involved myc family genes are c-*myc*, L-*myc* and N-*myc*. Over-expression of *myc* family mRNA is more frequent than the amplification of these genes, which is usually associated with increased level of *myc* proteins (DeGreve et al, 1988; Krystal et al, 1988). The mechanisms contributing to the over-expression of *myc* mRNA include: gene amplification, increased transcriptional initiation and decreased attenuation (Krystal et al, 1988). The over-expression and / or amplification of *myc* family genes in SCLCs have correlated with a more aggressive behaviour and a poor prognosis (Funa et al, 1987; Johnson et al, 1987). The amplification of *myc* family genes in NSCLCs has correlated with their ability to form xenograft tumors in nude mice (Gemma et al, 1988).

Other proto-oncogenes activated in human lung cancer include c-*myb*, c-*raf*-1 and c-*jun* (Schutte et al, 1988). In addition, altered expression of the genes encoding growth factors and / or their receptors have also been frequently detected in lung carcinomas, such as over-expression of TGF-α and c-*erb*B-2 in human NSCLCs, especially lung adenocarcinomas (Kern et al, 1990; Tateishi et al, 1990); amplification and / or over-expression of the EGFR predominantly in epidermoid carcinomas (Veale et al, 1987); and deprived expression of the EGFR in SCLCs (Gamou et al, 1987).

Inactivation of Tumor Suppressor Genes

In lung carcinoma cells, chromosomal deletions can be detected in almost all of the chromosomes, but most consistently and importantly in the chromosome 3p, 13q and 17p. It has been shown that Rb (Retinoblastoma) and p53 genes, two known tumor suppressor genes, reside on chromosomes 13q14 and 17p13 respectively (Lee et al, 1987;). However, the corresponding tumor suppressor gene on 3p has not been identified.

(1) 3p Deletions

By using cytogenetic and restriction fragment length polymorphism (RFLP) analyses, deletions at 3p 14-23 have been detected in almost all of the SCLCs (90-100%), both in cell lines and primary tumors. Its deletion is not as common in the NSCLCs, but still detected in about 50% of cases (Wang-Peng et al, 1982; Falor et al, 1985; Brauch et al, 1987 and Johnson et al, 1988a). The loss of 3p has been frequently detected in human renal cell carcinomas (Zbar et al, 1987). 3p is therefore thought to carry a tumor suppressor gene as yet unidentified. One report has demonstrated that aminoacylase-1 activity in SCLCs is markedly reduced or absent as compared to their normal lung

counterparts (Miller et al, 1986). Since the gene coding for aminoacylase-1 is located on 3p21, within the deleted region, the authors suggested that this gene is a possible candidate for the tumor suppressor gene on the 3p.

(2) Inactivation of Rb Gene

The Rb gene is the first isolated tumor suppressor gene, which is mapped on chromosome 13q14.1 (Friend et al, 1986; Lee et al, 1987). Abnormalities in the structure and expression of this gene have been detected in the vast majority (perhaps all) of the SCLCs, and in about 20-50% of NSCLCs (Harbour et al, 1988; Weston et al, 1989). The abnormalities resulting in the inactivation of the Rb gene include gene deletions, translocations, abnormal size or absent of expression of the mRNA, and absent expression of the protein. In general, Rb gene abnormalities are more frequently detected in epidermoid carcinomas than in adenocarcinomas (Weston et al, 1989).

(3) Inactivation of p53 at 17p13

p53 gene can be inactivated by chromosomal deletions and mutations which commonly involve G to T transversions at the codons 132-283 (Chiba et al, 1990). Mutated p53 gene often encodes mutated p53 protein with increased half life (Iggo et al, 1990; Lane and Benchinol, 1990). p53 mutations can now be detected immunohistochemically by using a monoclonal antibody, PAb240, which specifically recognizes the p53 mutant protein (Gannon et al, 1990). In human lung cancer, deletions and / or mutations of p53 are found in approximately 50% of the cases, both of NSCLCs and SCLCs (Yokota et al, 1987; Takahashi et al, 1989b; Chiba et al, 1990). It is noteworthy that transgenic mice carrying mutated p53 gene

develop lung cancer (Lavigueur et al, 1989). Among the three types of NSCLC, inactivation of the p53 is more common in epidermoid carcinomas than in adenocarcinomas (Iggo et al, 1990).

Other deletions often encountered are located at chromosome 1, 5, 11pter-15.5 (associated with rhabdomyosarcoma, Scrable et al, 1987) and 11p13-q13 (associated with Wilms' tumor, Turleau et al, 1981). Notably, the genetic changes discussed above are not mutually exclusive. In fact, many cell lines and primary tumors demonstrate combined deletions or mutations of several genes, suggesting that multiple genetic changes are required in order to sufficiently transform normal lung bronchial epithelial cells.

1.5 GENERAL OBJECTIVES

Most of the previous studies on the expression of proto-oncogenes have focused on the DNA and protein analyses, and on the cell lines, because of the rapid degradation of RNA in fresh tissues. Therefore, in this project, I have especially studied the mRNA expression of several proto-oncogenes in primary human colorectal and lung carcinomas. The selected genes are those involved in the regulation of the growth and differentiation of epithelial cells, and include TGF-α, EGFR, c-*erb*B-2 and c-*met*. The overall objectives are:

1) To study the frequency and level of their mRNA expression in primary colorectal and lung carcinomas and their corresponding normal tissues;
2) To study the clinicopathological significance of these gene expressions in primary human colon and lung cancers;
3) To study possible roles of these genes in conferring the ability of primary NSCLC cells to form propagable cell lines in culture, and in facilitating the cell lines to form xenograft tumors in nude mice;
4) To determine the phenotypic and genotypic representativeness of the newly established cell lines to their respective primary tumors.

CHAPTER 2

MATERIALS AND METHODS

2.1 Specimens

Tissue from human colorectal and lung carcinomas was obtained as soon as possible and usually within 30 to 60 min after surgical resection. When enough tissue was available, grossly normal colonic mucosa and lung parenchyma were concurrently obtained and used as normal controls. These normal tissues were sampled as far away as possible from the primary tumors, depending on the extent of the resection. Specimens used in this project are summarized as shown in Table 2-1, 2-2 and 2-3.

Table 2-1 Specimens obtained from colon tissues

	TGF-α RIA	mRNA Analysis			
		TGF-α	EGFR	C-*erb*B-2	c-*met*
Normal	25	21	24	24	21
Tumor	29	21	32	32	32
Stage A	2	-	7	7	7
Stage B	18	-	14	14	14
Stage C	9	-	11	11	11
Paired N&T	22	21	23	23	20
Paired N&P	-	-	7	7	7

N: normal, T: tumor, P: polyp. Numbers appearing in the Table represent number of the specimens studied.

Table 2-2 Specimens obtained from lung tissues

	TGF-α R.I.A.	mRNA analysis			
		TGF-α	EGFR	C-*erb*B-2	C-*met*
Normal	18	20	20	20	20
Tumor	16	29	29	29	29
Epidermoid Ca	5	9	9	9	9
Adeno Ca	7	14	14	14	14
Large cell Ca	4	6	6	6	6
Paired N&T	15	20	20	20	19

Ca: carcinoma, N: normal, T: tumor; Numbers appearing in the Table represent number of the specimens studied.

N.B. **DNA analysis** was performed on the same group of samples used for the mRNA analysis.

Table 2-3 Cell lines used in the study

HT-29	Poorly differentiated human colonic adenocarcinoma cell line
A431	human epidermoid carcinoma cell line
MGH-4	large cell undifferentiated human lung carcinoma cell line
MGH-7	poorly differentiated human lung epidermoid carcinoma cell line
MGH-8	poorly differentiated human lung adenocarcinoma cell line
MGH-13	moderately differentiated human lung adenocarcinoma cell line
MGH-14	Human lung large cell neuroendocrine carcinoma cell line
MGH-24	poorly differentiated human lung adenocarcinoma cell line
MGH-26	poorly differentiated human lung adenocarcinoma cell line
MGH-30	poorly differentiated human lung adenosquamous carcinoma cell line

A431 and HT-29 are commercially obtained. MGH-4 to MGH-30 are established in our laboratory.

2.2 Extraction of TGF from Human Tissues

This was performed according to the procedures described by Roberts et al, 1980. Approximately 1 g of tissue was minced to small pieces and suspended in 4 ml of a solution containing 50% (v/v) ethanol, 0.12 N HCl. 0.33 mM phenylmethyl-sulfonyl-fluoride (Sigma) and 0.005 mM of pepstatin (Sigma). Tissue was homogenized with a polytron^{TM} tissue homogenizer (Brinkmann Instruments, Wesbury, NY). After an overnight extraction at 4^{o}C, the homogenate was centrifuged at 1500 g for 10 min. The supernatant was collected and the pellet was re-extracted for 2 hr in 40 ml of a solution containing 73% (v/v) ethanol and 0.18 N HCl. Both supernatants were combined and adjusted to a pH 5.2 with concentrated ammonium hydroxide solution (Sigma). After which 2 volumes of ice-cold anhydrous ethanol and 4 volumes of ice-cold anhydrous ether (BDH Chemicals) were immediately added. The mixture was then kept at -20^{o}C for 30 to 48 hr to ensure an adequate protein precipitation. The resulting precipitate was collected by centrifugation at 1500 g for 10 min, and redissolved in 1M acetic acid solution (3-4 ml / g of tissue) (Anachemia). The acetic acid insoluble residue (which has very little or no TGF–activity as assayed by soft agar colony formation) was removed by ultracentrifugation at 10,000 g for 1 hr. The supernatant was dialysed in distilled water in a Spectrophor membrane (Fisher) which removed proteins smaller than 3500 Da. The concentration of protein was determined by Bio-Rad protein assay reagent. The TGF extracts were eventually lyophilized and reconstituted with 4 mM HCl to a final concentration of 2 mg / ml.

2.3 Radioimmunoassay for TGF-α (RIA)

This was performed using a kit from BioTope (Seattle, Washington) and according to the instructions provided by the manufacturer. The kit contains:

(1) pretreatment solution containing reducing agent for TGF-α;

(2) rabbit anti-rat TGF–α antibody, and normal rabbit serum;

(3) ^{125}I labelled TGF–α tracer;

(4) TGF–α calibrators including varying concentration of TGF–α ranging from 0.312 nM to 10 nM;

(5) prefilled SpinCushoin incubation / separation tubes with 100 μl assay volume. ^{125}I-TGF–α bound to TGF–α antibody can be centrifuged through the cushion and aggregated at the bottom;

(6) FastCounts: reusable radiation shields for counting of bound tracer without removing free tracer.

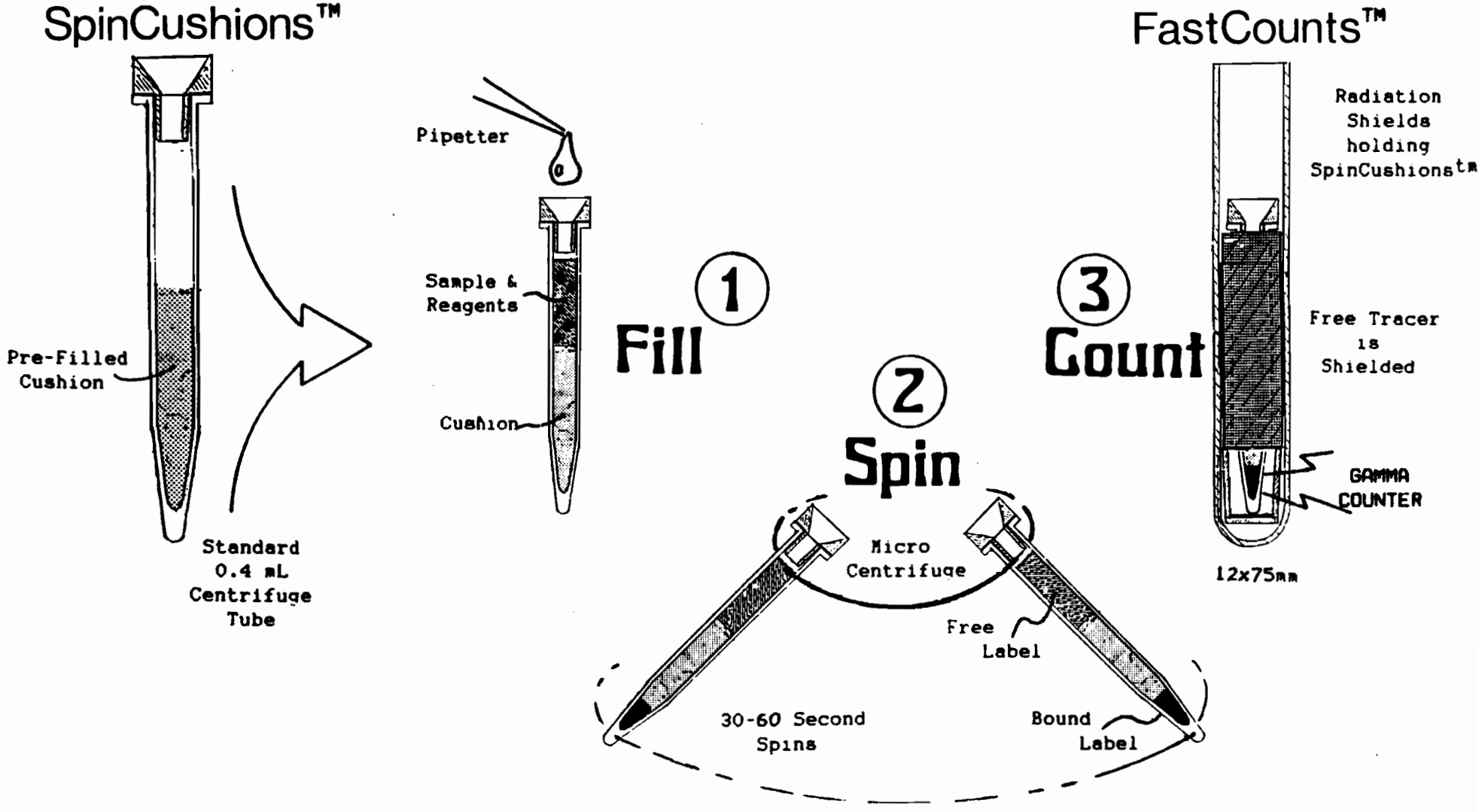
100 μg of protein-extract was lyophilized and reconstituted with 25 μl of 4 mM HCl, and treated with 2.5 μl of reducing agent (pretreatment solution) for 1 min at 95°C. Pretreatment of TGF-α calibrator was also required. The experiment was performed in duplicate.

To start the reaction, 25 μl of pretreated TGF-extract (100 μg of protein), 25 μl of ^{125}I-TGF–α tracer and 50 μl of TGF–α antibody were added sequentially on to the top of the SpinCushion, and mixed carefully by gentle pipetting without the sample penetrating the Cushion. After overnight incubation at 4°C, it was centrifuged for 45 sec in a microcentrifuge to separate the bound-tracer from the free tracer through SpinCushion. The resulting radio-activity was counted in a gamma counter with SpinCushions inserted into FastCounts radiation shields (as illustrated in Figure 2-1).

Non-specific binding was measured for each sample by replacing the TGF-α antibody with pre-immune serum. A standard curve was established using varying concentrations of synthetic mature rat TGF-α (0.312 nM to 10 nM) to compete with a constant amount of ^{125}I-TGF-α tracer for binding to the TGF-α antibody. Therefore, the higher the concentration of TGF-α calibrator , the less radio-activity can be detected because of the less binding of ^{125}I-TGF-α to the antibody.

The degree of inhibition by 100 μg of protein extract was compared to that by standard calibrator, and a relative estimation as to TGF-α concentration in the protein-extract was obtained. The TGF-α content was thus presented as nanogram equivalent of mature TGF-α per 100 μg protein.

Figure 2-1 **TGF-α Radioimmunoassay Procedures**



2.4 Nucleic Acid Probes and Their Preparations

Plasmids carrying cDNAs for human TGF-α, EGFR and c-*erb*B-2 genes were obtained from ATCC (Rockville, MD). TGF-α cDNA probe was a 0.93 kilo-base pair (kbp) *Eco* RI insert of clone phTGF1-10-925. EGFR probe used was a 2.4 kbp *Cla* I EGFR cDNA insert of the pE7 plasmid. C-*erb*B-2 (*neu* /HER-2) cDNA was a 3.4 kbp *Stu* I insert of pCER204. C-*met* probe was a 1.1 kbp *Eco*R I fragment of the human c-*met* cDNA (Park, et al, 1987). C-*myc* cDNA probe was purchased from Oncor (Gaithersburg, MD). Human β–actin cDNA was purchased from Clonotech (Palo Alto, CA).

TGF–α, EGFR and c-*erb*B-2 cDNA inserts were isolated from their plasmid clones by using Large-Scale Alkaline Lysis method (Maniatis et al, 1982). Commercially obtained bacterial clones containing plasmids were rehydrated with LB medium (Luria-Bertani medium, 1% Bacto-tryptone, 0.5% Bacto-yeast extract and 1% NaCl) containing appropriate antibiotics (according to the resistance marker of each bacterial clone). A single bacterial colony, isolated by a streaking technique (Maniatis et al, 1982), was suspended in 20 ml of LB medium containing antibiotics in a 50 ml sterile polypropylene tube (Falcon), and incubated overnight at 37°C with shaking (Orbit Eviron-Shaker, Lab-Line Instruments, Melrose Park, ILL). Next morning, this 20 ml bacterial suspension was transferred into a 2 L flask containing 1L LB medium and antibiotics, and was continuously incubated at 37°C until the optical density (OD) reading at 600 nm reached 0.8. At this point, 5 ml of chloramphenical solution (34 mg / ml) was added to amplify the copy number of the plasmid while stopping bacterial DNA replication. After overnight incubation at 37°C with shaking, the bacterials were collected by

centrifugation at 4000 g and 4°C for 10 min (Beckman, Model J2-21M) using a 500 ml sterile polypropylene bottle (Beckman). The resulting pellet was suspended in 7.5 ml of a solution containing 25 mM Tris-HCl, pH 8.0, 50 mM Na_2-EDTA and 1% glucose (w/v) (Anachemia), and incubated on ice for 10 min. The bacterials were lysed by addition of 2 volumes of 0.2 N NaOH plus 1% SDS, mixing, and incubation on ice for 10 min; and addition of 11.5 ml of cold 5M potassium acetate (Anachemia), pH 4.2, mixing, and another incubation on ice for 5 min. The lysate was filtered through cheese cloth after centrifugation at 12,000 g and 4°C for 30 min. The filtrate was precipitated with 2 volumes of 95% cold ethanol at -20°C for 20 min, and centrifuged at 12,000 g and 4°C for 20 min. The pellet was then suspended in 10 ml of TE buffer (10 mM Tris-HCl, pH 7.4 and 0.1 mM EDTA, pH 8.0). After 20 min incubation with solid ammonium acetate (1.93 g / 10 ml) on ice, and subsequent centrifugation, the supernatant was precipitated again with 2 volumes of 95% ethanol. The pellet was resuspended in 2 ml of TE buffer containing 0.05M NaCl, which was followed by an RNase digestion (100 μg / ml) at 37°C for 30 min. After addition of 0.6 ml of 2 M NaCl, the mixture was extracted with an equal volume of phenol and chloroform (1:1) (Anachemia) until the upper aqueous phase became clear. The mixture was extracted once again with an equal volume of chloroform alone to remove traces of phenol. The plasmid DNA was eventually precipitated with 95% of ice-cold ethanol and resuspended in 100 μl of TE buffer. The DNA concentration was estimated by reading OD at 260 nm. Plasmid DNAs were cut by appropriate enzymes (*Eco* R I for TGF-α and EGFR, Stu I for c-*erb*B-2) to release the cDNA inserts which we used as nucleic acid probes in the subsequent mRNA and DNA analyses.

2.5 Isolation of Total Cellular RNA from Tissues and Cells

The procedures are described in detail in Chapter 3, page 66 (tissues) and Chapter 6, page 134 (cells).

2.6 Northern Blot Analysis.

The procedures are described in detail in Chapter 3, page 66.

2.7 Isolation of DNA from Tissues or Cells

The procedures are described in detail in Chapter 5, page 128.

2.8 Southern Blot Analysis

The procedures are described in detail in Chapter 5, page 129.

2.9 Nucleic Acid Hybridization Technique

The procedures are described in detail in Chapter 3, page 67.

2.10 Establishment of Human Lung Cancer Cell Lines from Primary Non-small Cell Lung Carcinomas (NSCLCs)

The procedures are described in detail in Chapter 5, page 126.

2.11 Establishment of Xenograft Tumors in Nude Mice by NSCLC Cell Lines

The procedures are described in detail in Chapter 5, page 126.

2.12 Determination of DNA Content of the Established Cell Lines by Flowcytometry

The procedures are described in detail in Chapter 5, page 127.

2.13 Growth Rate Determination for the Established Cell Lines

The procedures are described in detail in Chapter 5, page 127.

2.14 Pathological Evaluation

The histopathology of the tumors was reviewed without prior knowledge of the results of molecular analyses. Classification of the tumor grade (differentiation), desmoplastic and inflammatory reactions in the tumors was based on the predominant findings observed. In most cases, 2 to 4 tumor

sections were available for the examination.

2.15 Statistical Analysis

This was performed using the non-parametric Wilcoxon Rank Sum test for independent samples (McClare and Dietrich, 1988). In some cases, Student-t test was also performed.

CHAPTER 3

EXPRESSION OF TRANSFORMING GROWTH FACTOR-ALPHA IN PRIMARY HUMAN COLON AND LUNG CARCINOMAS

(Br J Cancer, 62: 425-429, 1990)

Chi Liu, Anna Woo and Ming-sound Tsao

3.1 ABSTRACT

The expression of TGF-α in human colon and lung carcinoma cell lines have been reported previously, but its expression in primary tumors has not been described in detail. We have used the radio-immunoassay method to measure the specific content of immunoreactive TGF-α in the acid ethanol extracts of normal and cancerous tissues of human colon and lung. The average TGF-α content of colon carcinomas is 4 times that of the normal mucosa, and for non-small cell lung carcinomas it is twice that of the normal parenchyma. Because of variability in the TGF-α expression among individuals and in different segments of colon and lobes of lung, the ratio for TGF-α content of paired tumor and normal tissues was also calculated. On average, the tumor / normal ratio for colon carcinoma is higher than that for lung carcinoma. Although 55% of colon tumors show a ratio 4 times, or greater, only 33% of lung carcinomas demonstrate this ratio. The level of TGF-α in both colon and lung carcinomas does not correlate with histological type, stage, grade nor degree of desmoplasia of these tumors. Northern blot analysis of total cellular RNA confirms the expression of an approximately 4.8 kb TGF-α mRNA in normal colonic mucosa and lung parenchyma. However, in contrast to the results of radio-immunoassay, significant over-expression of TGF-α mRNA is uncommon in primary human colon carcinomas.

3.2 INTRODUCTION

Transforming growth factor-alpha (TGF-α) was first isolated and identified as one of the components of the sarcoma derived growth factor that interacted with the receptor of epidermal growth factor (EGF) (Delarco and Todaro, 1978; Anazano et al, 1983). Subsequent investigations revealed that the TGF-α molecule shared approximately 40% homology to the amino acid sequence of EGF (Marquardt et al, 1983; Marquardt et al, 1984), and it appeared to exert its biological effects exclusively by interacting with the EGF receptors (Carpenter et al, 1983). The initial findings of frequent expression of TGF-α in neoplastic tissues, coupled with the demonstration of its expression in fetal / embryonic tissues in rats led to the suggestion that TGF-α represented an oncofetal counterpart of EGF (Goustin et al, 1986). Recently, the expression of TGF-α has been demonstrated in several human normal adult cells/tissues including skin keratinocytes (Coffey et al, 1987b), breast ductal epithelial cells (Zajchowski et al, 1988), activated macrophages (Madtes et al, 1988; Rappolee et al, 1988), gastrointestinal mucosa (Bennett et al, 1989; Cartildge and Elder, 1989), and kidney (Gomella et al, 1989). TGF-α expression by human colonic and lung carcinoma cell lines has been reported previously (Coffey et al, 1987a; Hanauske et al, 1987; Watkins et al, 1988; Anzano et al, 1989; Derynck et al, 1987), but its expression and possible role in primary human lung and colonic carcinomas has not been studied systematically. We have used both the radioimmunoassay and nucleic acid hybridization techniques to examine the expression of TGF-α in primary human colonic and lung carcinomas.

3.3 MATERIALS AND METHODS

Human colon and lung carcinoma tissues were obtained within 30 to 60 min after surgical resection. When enough tissue was available, grossly normal colonic mucosa at least 10 cm away from the edge of tumor and the posterior basal segment of the lobectomy specimen from each case was concurrently obtained and used as the paired 'normal / contrast' tissue. Some normal mucosa, from colons resected for diverticular disease, was also obtained. The specimens were snap-frozed in liquid nitrogen and then stored at -80^{o}C.

Extraction of TGF From Tissues

The acid ethanol extraction procedure for tissue growth factors was performed exactly according to Roberts et al (1980). The final extracts were dialysed in distilled water, lyophilised and then reconstituted with 4mM HCl.

Radioimmunoassay (RIA)

This was performed using a kit from BioTope (Seattle, Washington) and according to the instructions provided by the manufacturer. The antibody was rabbit anti-rat TGF-α antiserum which recognized both the high and low molecular weight forms of rat and human bio-active TGF-α. We have independently confirmed its non-cross reactivity with EGF. A standard curve was established using varying concentrations of synthetic mature rat TGF-α (BioTope, M.W. 5600 Da) and 100 μg of extract protein from each sample was used for assay. Measurements were conducted in duplicate and non-specific binding was measured for each sample by replacing the antibody with pre-immune serum. All values were represented as nanogram equivalents of

mature TGF–α per 100 μg protein.

RNA Extraction and Electrophoresis

Approximately 1 g of frozen tissue was cut into 2–3 mm cubic fragments and homogenized by a Brinkman's Polytron in 6 ml solution containing 4 M guanidine isothiocyanate, pH 7.0, 25 mM sodium citrate, 0.1 M β–mercaptoethanol and 0.5% sarkosyl. The homogenate was layered onto a 3.3 ml cushion of 5.7 M CsCl solution, pH 5.0, containing 25 mM sodium acetate in a 11 ml polyallomer tube, and centrifuged at 32,000 r.p.m. for 20 hr at room temperature. The clear gelatinous pellet of RNA was dissolved in distilled water pretreated with diethylpyrocarbonate (depc–dH_2O) containing 0.3 M sodium acetate. After one extraction with equal volumes of phenol and chloroform, the aqueous phase containing the RNA was precipitated at –70^{o}C with 3 volumes of 95% ethanol. After a 15 min centrifugation at 12,000 g, the pellet was redissolved in depc–dH_2O and the amount of the total RNA was estimated by measuring the absorbence at 260 nm.

Thirty μg of the total RNA sample was denatured at 65^{o}C for 10 min in a solution containing 20 mM MOPS, 50% formamide and 6% formaldehyde,and was separated electrophoretically in 1% agarose gel containing 0.66 M formaldehyde and in 20 mM MOPS running buffer that contained 5 mM sodium acetate and 1 mM EDTA, pH 7.0. The RNA was blotted on to Hybond–N membrane (Amersham Canada, Oakville, Ont.) with 20 times standard saline citrate (20XSSC; 1XSSC: 0.1 M sodium chloride/0.015 M sodium citrate, pH 7.0). The air–dried membrane was exposed to the ultraviolet light of UV–transilluminator for 1 min to cross link the RNA to the membrane.

Hybridization

This was performed according to a slightly modified procedure of Church & Gilbert (1984). The probe for TGF–α was a 2.3 kb–pairs *Eco*RI insert of the prTGF0.2 plasmid containing the rat TGF–α cDNA (Lee et al., 1985) which shares approximately 90% homologous nucleotides with the coding sequences of the human TGF–α gene (Derynck et al, 1984). Probes were labelled with ^{32}P–dCTP (ICN Canada, Montreal, Que) to high specific activity (approximately 109 c.p.m. μg^{-1}) using the oligolabelling kit of pharmacia (Dorval, Que.). Membranes were prehybridized at 42^{o}C for 1–2 hr in a solution containing 0.5 M NaHPO4, pH 7.2, 5% BSA fraction V, 1 mM EDTA and 5% sodium dodecyl sulphate and 50% deionised formamide. Hybridization was carried out in the same solution containing ^{32}P–labelled probes at 42^{o}C for 48 hr. The membrane was subsequently washed 4 times for 15 min at room temperature in 2XSSC solution containing 0.1% SDS, and twice for 30 min at 55^{o}C in 0.3XSSC solution containing 0.1% SDS After a final rinse in 1XSSC, the membrane was blotted dry and exposed for 5–8 days at –80^{o}C to XAR–5 Kodak X–ray film using an intensifying screen. Densitometric measurements were performed using the Hoefer GS–300 scanning densitometer. All TGF–α values were standardized with the level of β–actin expression as probed by cDNA from the 3'–untranslated region of human β–actin gene (Ponte et al., 1983).

Pathological evaluation

The pathology was reviewed without knowing the results of the biochemical measurements. In most cases, slides from 2 to 4 sections of the

tumors were available for examination by one of us (MST). Classification of the histological type and degree of differentiation (grade) of tumours was based on the predominant finding observed.

Statistical analysis

All statistical analyses were performed using the non–parametric Wilcoxon rank sum test for independent samples (McClare & Dietrich, 1988). In some cases, Student's t–test was also performed.

3.4 RESULTS

Although there was considerable variations among individuals (Figure 3-1), the acid ethanol extracts of both normal colonic mucosa and lung parenchyma contained approximately 0.8 ng immunoreactive TGF–α per 100 μg protein (Table 3-1and 3-2). The mean TGF–α value for the right (caecum, ascending and transverse) colon was 1.19 ± 0.30, and for the left (descending, sigmoid and rectal) colon was 0.55 ± 0.20, but this difference is statistically insignificant. The amount of immuno–reactive TGF–α in extracts of normal mucosa of colons resected for diverticular disease was not significantly different from that of 'normal' mucosa of cancerous colons.

Adenocarcinomas of the colon contained approximately 4 times higher immunoreactive TGF-α than normal colonic mucosa (Table 3-1). There was no difference in the values for tumors of the right versus left colon. One of the left colonic tumors demonstrated an unusually high TGF–α content (39.2 ng per 100 μg protein), and when this specimen is excluded, the mean TGF–α content of the remaining left colonic tumor was 2.08 ± 0.42, which is not significantly different from the mean value for right–sided tumors. The TGF–α

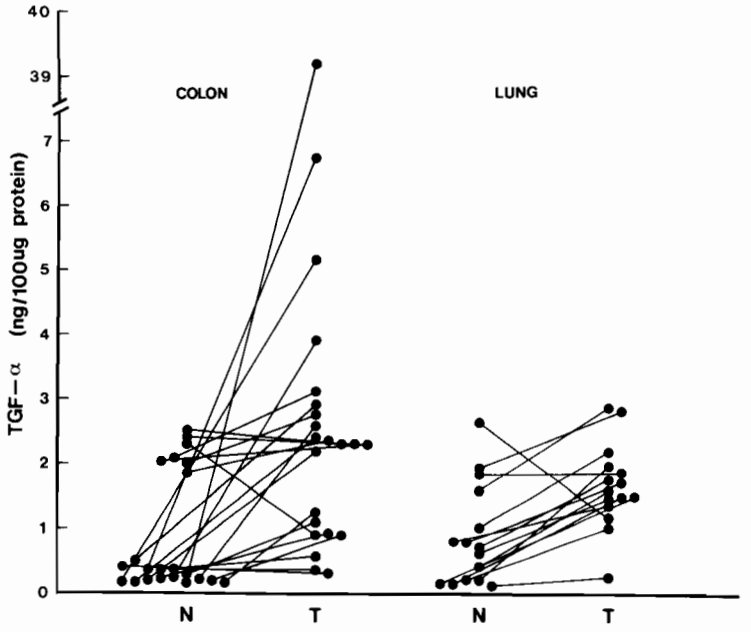


Figure 3-1 The specific content of immunoreactive TGF-α in acid ethanol extracts of paired tumor and normal tissues of patients with colon and lung carcinomas.

Table 3-1 TGF-α levels in acid ethanol extracts of normal and malignant human colonic epithelium

	Normal	Tumor	P value
Means of			
Total	0.83 ± 0.18 (25)	3.40 ± 1.21 (29)	<0.001
Right colon	1.19 ± 0.30 (11)	2.24 ± 0.24 (10)	<0.005
Left colon	0.55 ± 0.20 (14)	4.02 ± 2.00 (19)	<0.001
Duke's staging:			
A	-	2.44 ± 0.13 (2)	NS
B	-	4.05 ± 2.10 (18)	NS
C	-	2.29 ± 0.52 (6)	
Desmoplasia:			
Slight	-	15.12 ± 12.04 (3)	
Moderate	-	1.70 ± 0.24 (19)	NS
Marked	-	3.03 ± 1.01 (6)	
Differentiation:			
Well	-	2.15 ± 0.36 (12)	NS
Moderate	-	4.92 2.68 (14)	
Poor	-	0.55 0.37 (2)	
Inflammatory reaction:			
Sparse	-	2.50 ± 0.20 (15)	NS
Moderate/severe	-	4.47 ± 2.91 (13)	

All values represent means ± s.e. in ng per 100 μg protein; (): the number of specimens; NS: not statistically significant.

Table 3-2 TGF-α levels in acid ethanol extracts of normal and malignant human lung tissues

	Normal	Tumor	p value
Means of			
All specimens	0.84 ± 0.19 (18)	1.79 ± 0.21 (16)	<0.002
Upper lobes	0.52 ± 0.18 (10)		NS
Middle & lower lobes	1.25 ± 0.33 (8)		
Histological types:			
Epidermoid carcinoma	-	1.45 ± 0.37 (5)	NS
Adenocarcinoma	-	1.74 ± 0.37 (7)	NS
Large cell carcinoma	-	2.29 ± 0.53 (4)	
Desmoplasia:			
Slight	-	2.07 ± 0.59 (4)	NS
Moderate/severe	-	1.70 ± 0.21 (12)	
Lymph node metastasis:	-		
Negative	-	1.57 ± 0.22 (10)	NS
Positive	-	1.81 ± 0.30 (5)	

All values represent means ± s.e. in ng per 100 μg protein; (): the number of specimens; NS: not statistically significant.

content of the tumor cannot be correlated to the stage of the disease, to the differentiation (grade) of the tumors, nor to the degree of inflammatory response present in the tumors. Although tumors with marked desmoplasia demonstrated higher TGF–α values than those with moderate desmoplasia, there is no significant difference between these tumors and those with slight desmoplasia. There were not enough specimens to evaluate the significance of a low TGF–α value in poorly–differentiated carcinomas. Interestingly, the specimen with an unusually high TGF–α content shows only slight desmoplastic reaction.

Immunoreactive TGF–α was also consistently measurable in acid ethanol extracts of normal lung parenchyma and the level is approximately twice as high in the lower and middle lobes than in the upper lobes, but the difference is also not statistically significant (Table 3-2). In contrast to colon cancers, lung carcinomas demonstrated only about twice as much TGF–α as the normal lung parenchyma. A significant statistical difference was not found between the different histological types of lung carcinomas, the degree of desmoplasia and the stage of the disease (Table 3-2).

Since TGF–α content among individual patients varied considerably, we also calculated the ratio of TGF–α content of tumor versus normal tissue among specimens with paired samples. The mean of tumor / normal (T/N) ratio for colon carcinomas was 4 times higher than that of lung carcinomas (Table 3-3). The distribution of these T/N ratios is shown in Figure 3-2. Among colon carcinomas, left–sided tumors gave a significantly higher mean ratio than the right–sided tumors; 55% of colonic carcinomas have a T/N ratio higher than 4, whereas only 33% of lung carcinomas demonstrated this high ratio. However, this difference is not statistically significant by Yates corrected x^2 test, or Fisher exact test.

Table 3-3 Relative ratio of TGF-α contents in paired normal and malignant tissues of patients

	Colon	Lung	p value
Mean ratio (T/N)	18.67 ± 11.69 (22)	4.10 ± 1.02 (15)	¶
	or 7.12 ± 1.84 (21)[a]		¶
Right colon	4.31 ± 1.78 (8)		p<0.001
Left colon	28.88 ± 18.21 (14)		
	or 8.84 ± 2.71 (13)[a]		
T/N ≥ 2	55%	67%	NS
T/N ≥ 4	55%	33%	NS

[a]Values represent the means ± s.e. if the specimen with excessively high TGF-α content is excluded. ¶: p<0.001 by Student's t-test, but not significant by Wilcoxon rank sum test. NS: not statistically significant.

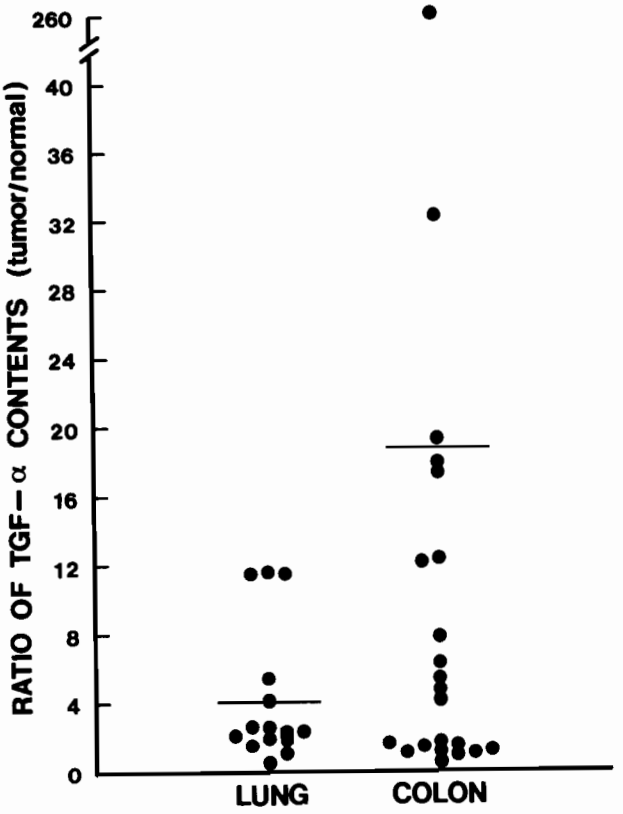


Figure 3-2 The relative increase in the specific content of TGF-α in paired tumor versus corresponding normal tissues of patients with colon and lung carcinomas.

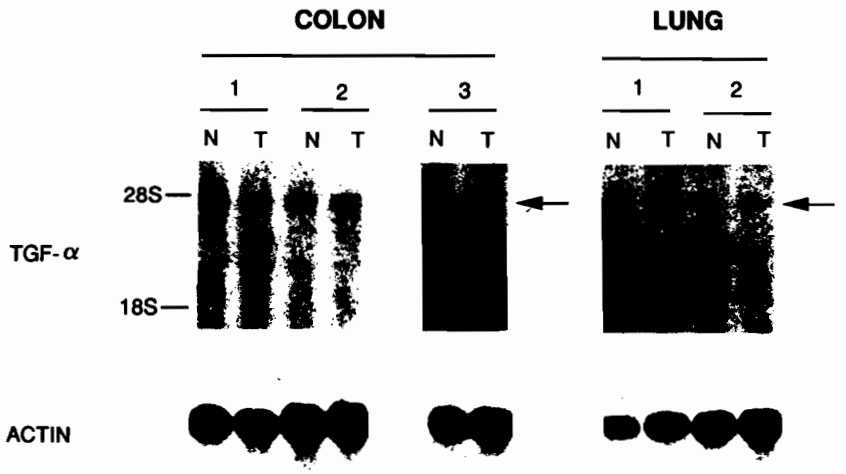


Figure 3-3 Representative Northern blot hybridization showing the expression of an approximately 4.8 kb TGF-α mRNA in normal and cancerous colon and lung tissues.

Northern blot analyses of total RNA extracted from normal colonic mucosa and lung parenchyma consistently demonstrate the presence of an approximately 4.8 kb mature TGF–α mRNA in these tissues (Figure 3-3). However, in contrast to the radio–immunoassay findings, the relative expression of TGF–α mRNA in colon carcinomas is rarely much more than that of the contrasting normal mucosa (Figure 3-4). Fewer than 20% of these tumors expressed 2 to 3 times more TGF–α mRNA than their corresponding normal mucosa. However, in 2 of 5 tumors whose TGF–α mRNA levels were twice as much, or more, than their contrasting normal mucosa, immunoreactive TGF–α content was also measured and found to be very high, indicating good correlation between high mRNA expression level and high level of TGF–α peptide in tumor tissues. A comparative study indicates that the level of TGF–α mRNA expression in these colonic tumors or normal mucosa is approximately one hundredth of that expressed by HT–29 colon carcinoma cell line (data not shown).

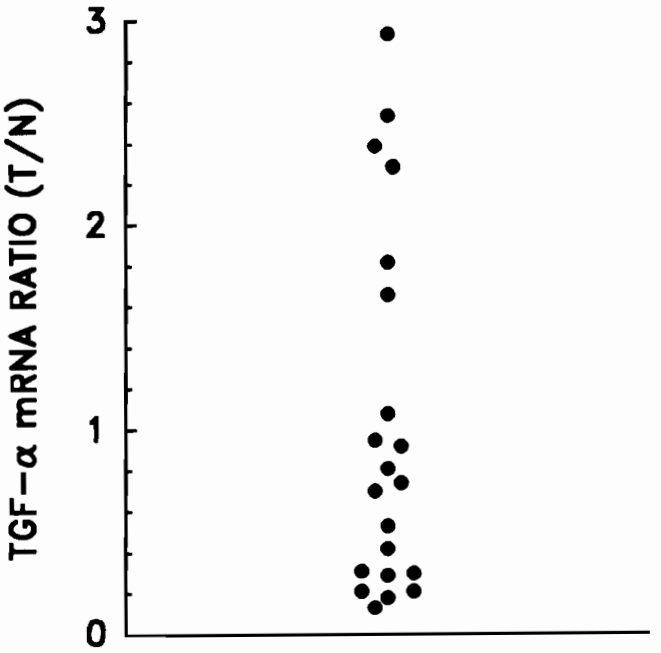


Figure 3-4 The relative expression of TGF-α mRNA in colon cancer tissues compared to their paired adjacent normal mucosa. All values have been normalized to the β-actin expression.

3.5 DISCUSSION

We have confirmed that normal human adult colonic mucosa contains acid–ethanol extractable immunoreactive TGF–α, and the level of TGF–α is higher in the right than left colon. Cartlidge & Elder (1989) have recently reported that the level of immunoreactive TGF–α decreased progressively from the proximal to distal colon, and the levels in ascending and transverse colon are approximately 2 to 3 times higher than those found in the descending and sigmoid colon. Our results also show a two–fold higher TGF–α level in mucosa of the right than left colon. EGF–like activity as detected by the radioreceptor assay method has also been reported in acetic acid extracts of normal colonic mucosa, and the levels also varied among individuals (Rothbauer et al., 1989). Since the level of immunoreactive EGF molecule in acid–ethanol extracts of human normal colon is very low and does not appear to vary between the different segments of the colon (Cartlidge & Elder, 1989), the EGF–binding activity detected by Rothbauer et al. (1989) most likely also represented TGF–α. We have further demonstrated the presence of the full–length (4.6–4.8 Kb) TGF–α mRNA species in the total cellular RNA extracts of normal human colonic mucosa, thus further confirming the expression of this growth factor in normal adult colon.

The expression of TGF–α in normal adult lung tissue has not been previously reported, although Nickell et al (1983) have previously indicated its presence using the classical bioassay method for detecting TGFs. The mean immunoreactive TGF–α activity in normal lung parenchyma is comparable to that found in the colon. In contrast to a previous study which failed to show the presence of TGF–α specific mRNA in normal lungs (Derynck et al., 1987), we have demonstrated its presence in our study. With the continuing additions to the list of normal adult human tissues that express TGF–α, it appears that the

role and importance of TGF–α in the growth and function of normal human adult tissues, especially epithelial cells requires re–evaluation.

Expression / secretion of TGF–α has been reported in most of the human colon carcinoma cell lines studied, but the levels of expression are highly variable (Anzano et al., 1989; Coffey et al., 1987a; Watkins et al., 1988; Hanauske et al., 1987). TGF–α expression in primary human colon carcinoma tissues has not been adequately reported. Rothbauer et al. (1989) used a radio–receptor assay method to study EGF–like activity in extracts of 15 paired normal and carcinomatous colonic tissues and found that carcinomas contained slightly, but significantly, more activity than the normal mucosa (1.98 ± 0.29 vs 1.38 ± 0.19 ng mg^{-1} protein, $P<0.025$). Our results indicate that the mean immunoreactive TGF–α activity in cancerous tissue is 4 times higher than that of the normal mucosa, but when the value for paired normal and tumor tissues are normalized for each patient, the average tumor/normal ratio is even higher. Fifty–five per cent of the colon cancers have a T/N ratio higher than 4, and interestingly, tumors of the left side of the colon appear to express higher levels of TGF–α than the right sided tumors. Unfortunately, when the TGF–α levels are analysed for their clinical and pathological relevance, no significant correlation could be found with the stage of the disease and the grade of the tumor, nor with the degree of desmoplastic and inflammatory reaction in these tumors.

In contrast with the radio–immunoassay findings, significant over–expression of TGF–α mRNA in primary colon carcinoma tissues is rare. The highest over–expression in tumor versus normal mucosa is 2– to 3–fold, and this is only seen in less than 20% of the specimens studied. This is different from renal cell carcinomas where more than 3–fold over–expression was found in 50% of cases (Gomella et al., 1989). The discrepancy between

the levels of protein and mRNA expression could be caused by different post–transcriptional controls that exist in normal and neoplastic cells. It is also possible that in normal colonic mucosa, most of the TGF–α is synthesized by the epithelial cells and secreted into the lumen, hence lower amount is measurable in the tissue, while most of the TGF–α synthesized by invading tumor cells is secreted extracellularly and retained within the tumor tissue, thus yielding a higher specific content.

Carcinomas of the lung appear to express less TGF–α than those from the colon. The mean TGF–α content for lung tumors is only 2 times higher than the normal parenchyma, the mean T/N ratio for paired specimens is 4.1 ± 1.02 (versus at least 7.12 ± 1.84 for colon), and only 33% of lung carcinomas demonstrate a T/N ratio of greater than 4. However, similar to colon cancers, the TGF–α levels cannot be correlated to the histological types of tumor, the stage of the disease, nor the degree of desmoplastic reaction in the tumors. Bergh (1988) has also reported that expression of TGF–α alone in non–small cell lung carcinoma cell lines could not be correlated with the extent of fibrosis in tumors formed by these cells in nude mice.

TGF–α has been considered as one of the prototypes of an autocrine growth factor which may play important roles in carcinogenesis and tumor growth (Sporn & Todaro, 1980; Sporn & Roberts, 1985; Goustin et al., 1986; Derynck, 1988). TGF–α may also play an important role in tumor angiogenesis (Schreiber et al., 1986), and in the pathogenesis of paraneoplastic hypercalcemia (Ibbotson et al., 1986; Tashjian et al., 1985). Nonetheless, the importance of TGF–α in tumor cell biology *in vivo* remains speculative, and our own analyses on colon and lung carcinomas have not provided further insights. Rothbauer et al reported that in 2 of 3 patients with familial polyposis, the TGF–α levels in either the preneoplastic adenomatous

polyps, or carcinoma, were actually lower than those in the 'normal' mucosa, suggesting that TGF-α expression is not related to the mechanism of multistage carcinogenesis. The introduction and expression of hTGF-α gene into mouse keratinocytes from normal skin or papillomas did not result in the formation of carcinoma, suggesting that this growth factor does not influence tumor progression directly (Finzi et al., 1988). However, correlation of TGF-α synthesis or secretion with tumorigenicity and the pathological stage also cannot be demonstrated in human breast cancer cell lines / tissues (Dickson et al., 1986; Perroteau et al., 1986; Zajchowski et al., 1988; Ciardiello et al., 1989). The marked heterogeneity of TGF-α expression, even among cancer of the same organ or histological type, suggests that it is unlikely to play a consistent and direct role in the pathogenesis of all cancers. However, TGF-α may still play an important indirect but synergistic role in the tumor biology of some individual human cancers, especially through its autocrine effects on cell proliferation and tumor growth rate, on pericellular matrix proteolysis involving the plasminogen activator systems (Laiho & Keski-Oja, 1989), and through its paracrine effect on angiogenesis (Schreiber et al., 1986). Further knowledge on the level and pattern of expression of TGF-α in primary human cancers may also contribute understanding to its use as a tumor marker (Yeh et al, 1987; Ciardiello et al, 1989), prognostic indicator (Arteaga et al., 1988) and therapeutic target (Greig et al., 1988).

The most significant finding in Chapter 3 is that, in comparison to the normal tissues, the level of TGF-α in both colonic and non-small lung carcinomas is markedly elevated. The elevation is especially obvious in the colonic carcinomas. Therefore, a further study concerning the expression of its receptor, epidermal growth factor receptor, is conducted in order to assess if the over-expressed TGF-α acts directly on its producer cells to stimulate the proliferation of tumor cells, or if it actually acts on the other types of cells displaying EGFR. In Chapter 4, the expression of 3 tyrosine kinase receptors including EGFR is studied in the primary human colorectal carcinomas and colonic adenomas. The possible clinicopathological significance of these genes is discussed.

The expression and clinicopathological significance of these receptor genes in lung carcinomas are discussed in Chapter 7.

CHAPTER 4

OVER-EXPRESSION OF C-*MET* PROTO-ONCOGENE BUT NOT EPIDERMAL FROWTH FACTOR RECEPTOR OR C-*ERB*B-2 IN PRIMARY HUMAN COLORECTAL CARCINOMAS

(Oncogene, 1991)

Chi Liu, Morag Park and Ming-Sound Tsao

4.1 ABSTRACT

The epidermal growth factor receptor (EGFR) and the protein products of c–*erb*B–2 and c–*met* proto–oncogenes belong to a family of growth factor receptors with tyrosine kinase activity. In human colonic carcinomas, the expression of the EGFR and c–*erb*B–2 have been studied at the protein level only, while c–*met* expression has not been reported. We have examined the mRNA expression of these genes in human normal colorectal mucosa and primary carcinomas. The results demonstrated that the normal mucosa showed highly variable levels of EGFR and c–*erb*B–2 mRNAs, but expressed consistently low amounts of c–*met* mRNA. Colorectal carcinomas did not express significantly higher levels of the EGFR and c–*erb*B–2 mRNAs than the normal mucosa. In contrast, c–*met* was consistently and significantly over–expressed (mean 6 fold) in carcinomas as compared to normal mucosa. 70% of paired normal–tumor specimens showed a tumor to normal c–*met* mRNA ratio of greater than 4. The expression of c–*met* mRNA was also enhanced in the adenomas, suggesting that over–expression of this proto–oncogene may have mechanistic significance in the early stages of human colorectal carcinogenesis.

4.2 INTRODUCTION

Structural and functional homologies between the protein products of viral oncogenes and cellular growth factors or their receptors suggest that the latter play important roles in carcinogenesis (Kahn & Graf, 1986). Over–expression of some growth factor receptors and proto–oncogene protein products with tyrosine kinase activity, such as the epidermal growth factor (EGF) receptor and the protein product of c–*erb*B–2 proto–oncogene, have been shown to exert significant influence on the biology of some human cancers. The EGF receptor (EGFR) gene is frequently amplified and/or over–expressed in glioblastomas (Liebermann et al, 1985), squamous cell carcinomas of the head and neck (Yamamoto et al, 1986a; Ishitoya et al, 1989), carcinomas of the breast (Harris et al, 1989) and stomach (Pfeiffer et al, 1990; Yasui et al, 1988a & b; Yonemura et al, 1989). In human breast cancer, the EGFR level is inversely related to the estrogen receptor level, and over–expression of the EGFR is correlated with a shortened relapse–free survival and a higher risk of recurrence (Harris et al, 1989). In human gastric carcinomas, the concurrent expressions of EGF and EGFR have been correlated with the depth of tumor invasion, metastasis and a poor prognosis (Yasui et al., 1988a). The c–*erb*B–2 proto–oncogene codes for a 185 KDa transmembrane glycoprotein (p185^{neu}) that exhibits approximately 50% homology with the EGFR (Schechter et al, 1984; Coussens et al., 1985; Bargmann et al., 1986; Yamamoto et al., 1986b), but it does not bind to EGF or TGF–α. The putative ligand for p185^{neu}, gp30, has been identified recently (Lupu et al, 1990). The amplification and/or over–expression of c–*erb*B–2 and p185^{neu} in breast cancer patients appear to correlate with a reduction in their relapse–free and overall survival (Slamon et al., 1987; Berger et al, 1988;

Wright et al., 1989). In fact, p185^{neu} and EGFR are additive in their prognostic values. Patients who over–express both proteins tend to have the poorest prognosis (Harris et al., 1989). A similar prognostic value for p185^{neu} in human lung adenocarcinomas was reported recently (Kern et al., 1990).

The expression of EGFR and c–*erb*B–2 in primary human colonic neoplasm has been examined only at the protein level using immunohistochemical, immunoblot and immunoprecipitation techniques (Yasui et al., 1988b; D'Emilia et al., 1989; Cohen et al., 1989; Rothbauer et al., 1989; Koretz et al., 1990). The expression of the c–*met* proto–oncogene which encodes a cell surface tyrosine kinase receptor for the hepatocyte growth factor (Park et al., 1987; Bottaro et al., 1991), has not been reported in primary human colorectal carcinomas. In the present study, we have examined the expression of EGFR, c–*erb*B–2 and c–*met* mRNAs in human normal colonic mucosa and colorectal carcinomas. The results demonstrate that, among the three tyrosine kinase receptors, only c–*met* is consistently over–expressed in these tumors, and enhanced expression of the c–*met* mRNA may be associated with the early stages of human colon carcinogenesis.

4.3 MATERIALS AND METHODS

Tissue was obtained from thirty two cases of colonic carcinoma within 30 to 60 min after surgical resection. In 24 cases, grossly normal colonic mucosa at least 10 cm away from the edge of tumor was concurrently removed and used as the paired "normal" tissue. Seven adenomatous polyps and their normal mucosa were also obtained. Specimens were snap–frozen in liquid nitrogen and stored at –80^{o}C.

HT29 human colonic adenocarcinoma cell line was obtained from ATCC (Rockville, MD) and cultured in RPMI–1640 containing 5% fetal bovine serum (FBS) (Gibco, Grand Island, N.Y.).

Nucleic Acid Probes

EGFR mRNA was probed with a 2.4 kilo base–pairs (kbp) *Cla* I cDNA insert of the pE7 plasmid (Merlino et al, 1984). The c–*erb*B–2 (*neu* / HER–2) mRNA was probed with a 3.4 kbp Stu I insert of pCER204 (Yamamoto et al, 1986b). Both pE7 and pCER204 were obtained from the ATCC. The c–*met* mRNA was probed with 1.1 kbp *Eco*R I fragment of the human c–*met* cDNA which codes the transmembrane and cytoplasmic protein kinase domains (Park et al., 1987). Human β–actin cDNA probe was purchased from Clonotech (Palo Alto, CA).

RNA Isolation

Total cellular RNA was extracted from approximately 1 gm of tissue according to the procedure of Liu et al (1990). RNA from cultured cells was similarly isolated in 4M guanidine isothiocyanate solution, pH 7.0, containing 25 mM sodium citrate, 0.1 M β–mercaptoethanol and 0.5% sarkosyl (Tsao et al, 1989).

Northern Blot and Nucleic Acid Hybridization Analysis

The procedures were described previously (Liu et al, 1990) and membranes were probed sequentially with all four cDNAs. 30 μg of total cellular RNA from pairs of normal and neoplastic tissues were electrophoretically separated in 1% formaldehyde–agarose gel and transferred to Hybond–N membrane. In order to circumvent the difficulty of

comparing signals from different membranes exposed to the X–ray films for varying length of time, 30 μg total cellular RNA of HT29 cells were included in every gel electrophoresis. Hybridized membranes were exposed for several exposure time to obtained the best non–saturated autoradiographic signals. Densitometric measurements of these signals for each tissue sample were normalized first to their respective β–actin mRNA signals, then were calculated as the percentage of mRNA level expressed in HT29 cells.

Pathological Analysis

The histology of the tumors was reviewed without prior knowledge of the results of the molecular analyses. Classification of tumor grade was based on the predominant findings observed. The level of inflammatory infiltrate was comparatively scored as slight or moderate to marked. In most cases, 2 to 4 tumor sections were available for examination. The Dukes' stages for colon carcinoma are as follows: A, tumor invasion confined to the muscularis propria and without lymph node metastasis; B, tumor invasion extending beyond the muscularis propria but without lymph node involvement; C, tumor with regional lymph node metastasis (Dukes et al, 1940).

Statistical Analysis

This was performed using the non–parametric Wilcoxon rank sum test for independent samples (McClare and Dietrich, 1988) and / or Student's *t*–test.

4.4 RESULTS

The expressions of EGFR, c–*erb*B–2 and c–*met* mRNA were studied in 32 colorectal carcinomas and in normal mucosa from 24 colons. Only 21

normal specimens were available for the study of c–*met* expression (Table 4-1). Multiple species of the EGFR and c–*met* mRNA were expressed, but only the full length 9.5 kb EGFR mRNA was measured densitometrically; however, both the 8.0 and 5.0 kb c–*met* mRNA were measured. The c–*erb*B–2 was expressed as a 4.8 kb mRNA (Figure 4-1).

There was a significant variability in the expressions of EGFR and c–*erb*B–2 mRNAs in normal colonic mucosa, but c–*met* mRNA expression was consistently low (Figure 4-2). The mRNA levels of EGFR and c–*erb*B–2 in the left colon (descending and recto–sigmoid colon) were the inverse of their expression levels in the right colon (caecum, ascending and transverse colon) (Figure 4-3). The normal mucosa of left colon showed a higher but statistically insignificant mean level of EGFR mRNA (59.5 ± 22.7% vs 33 ± 6.9%, $p<0.1$ by Student's *t*–test), and a lower level of c–*erb*B–2 mRNA (35.2 ± 6.6 vs 60 ± 11.3, $p<0.05$) than the right colon. However, the levels of c–*met* mRNA were similar in mucosa from both sides of the colon (2.2 ± 0.01% vs 3 ± 0.01%, and Figure 4-3).

The mean level of EGFR mRNA in carcinomas was not significantly different from that in normal mucosa, while the mean c–*erb*B–2 mRNA level was relatively lower in carcinomas as compared to the normal mucosa (Figure 4-3). In contrast, the mean c–*met* mRNA level in carcinomas was 6–fold higher than that in normal mucosa ($p<0.001$), both in the left and right colon (Table 4-1 and Figure 4-3).

Figure 4-2 shows the intra–individual changes in the levels of mRNA expression between normal and tumor tissues in paired samples. Changes in the EGFR and c–*erb*B–2 mRNA levels were highly variable, being increased only in a few pairs and unchanged or decreased in the majority (Table 4-2). On the contrary, in 17 of 20 (85%) carcinomas, c–*met* expression was

Table 4-1. Relative mRNA expression of EGFR, c-*erb*B2 and c-*met* in colorectal normal mucosa and carcinomas.

	Relative mRNA level (% of HT-29)		
	normal mucosa	Carcinoma	p value*
EGFR	46.0 ± 11.4 (24)	39.7 ± 7.8 (32)	0.281
C-*ERB*B-2	47.5 ± 6.9 (24)	31.2 ± 21.9(32)	0.018
C-*MET*	2.6 ±0.7 (21)	14.8 ± 2.2 (32)	<0.001

Values represent means S.E.; ( ) represents the number of specimens.

* Wilcoxon rank sum test.

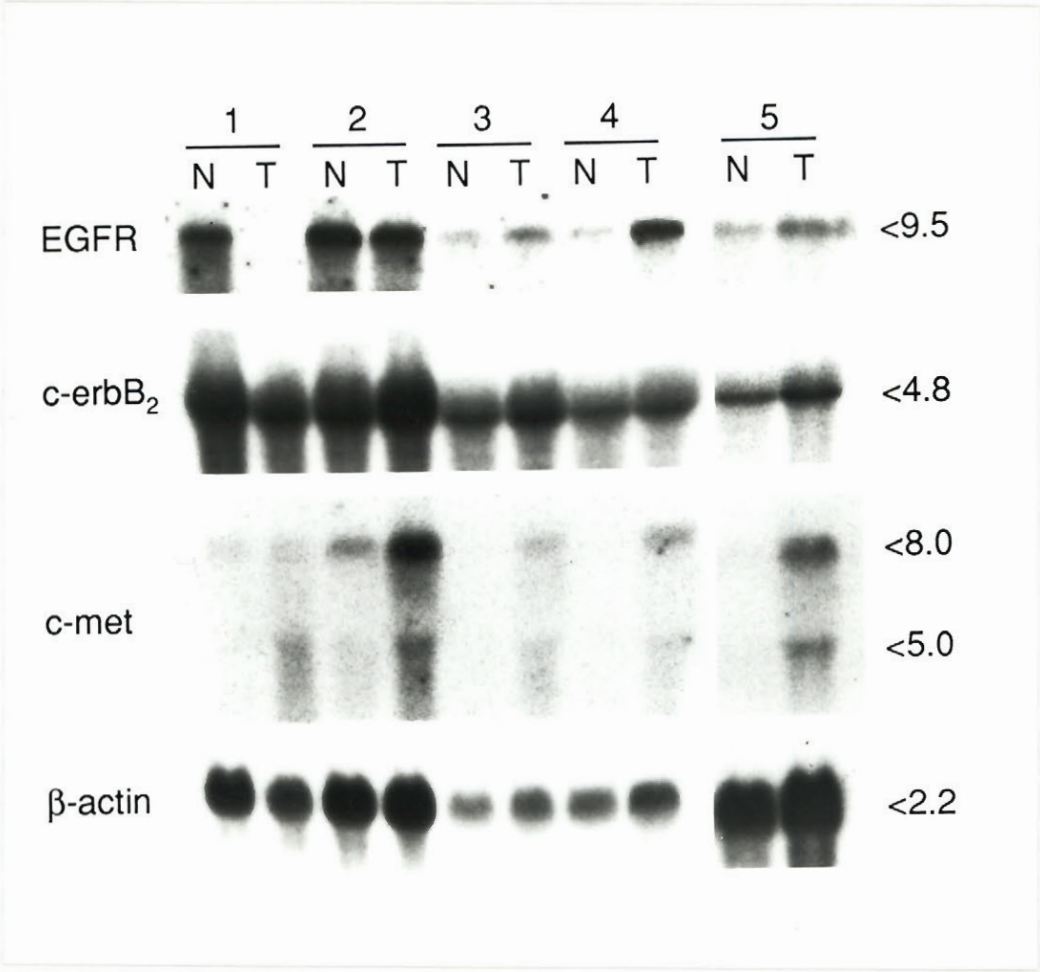


Figure 4-1 Representative northern blot analysis showing the expressions of 9.5 kb EGFR, 8.0 and 5.0 kb c-*met*, and 4.8 kb c-*erb*B-2 mRNAs in normal colonic mucosa (N) and colonic adenocarcinomas (T).

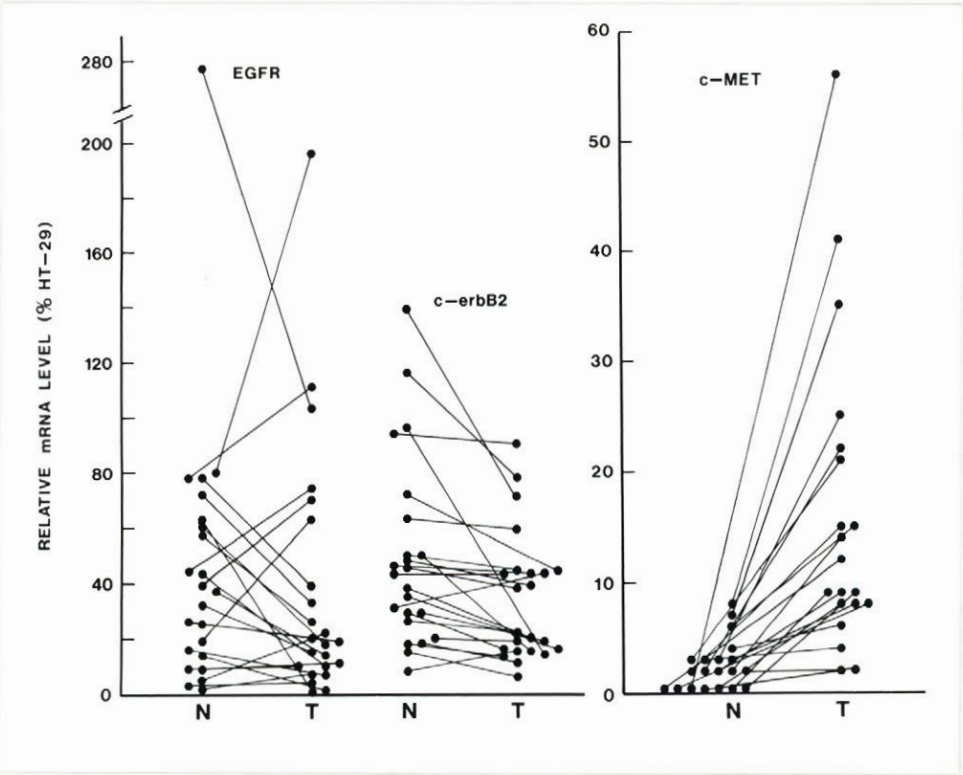


Figure 4-2 The relative mRNA levels of EGFR, c-*erb*B-2 and c-*met* in paired normal colonic mucosa (N) and colonic adenocarcinomas (T). Each pair is linked by a solid line.

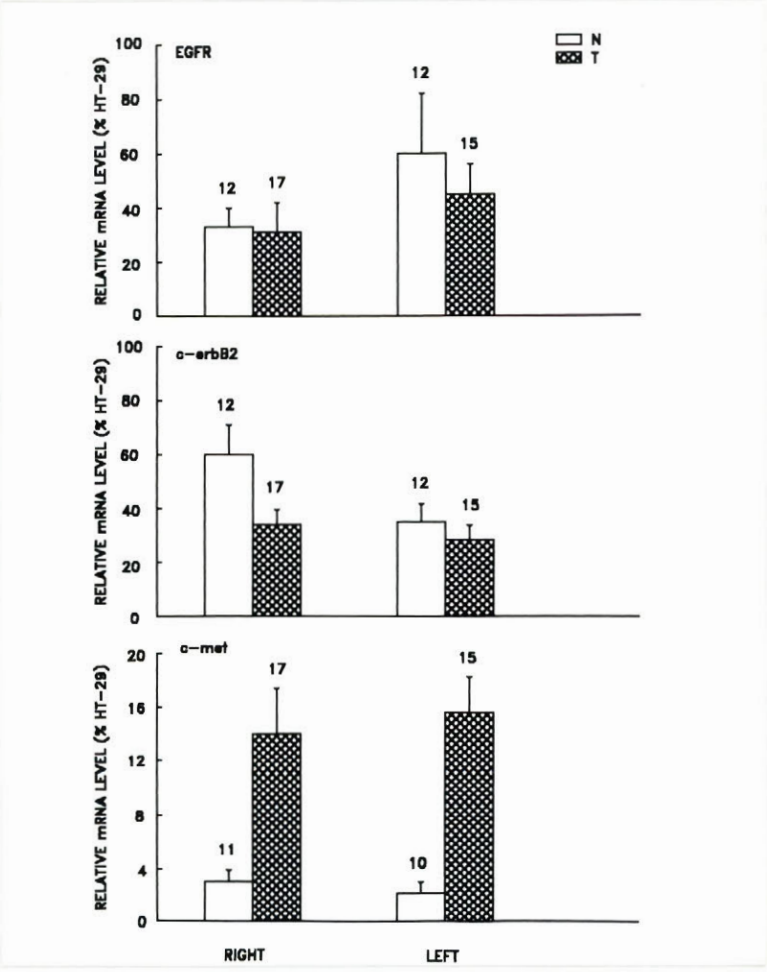


Figure 4-3 The relative mRNA levels of EGFR, c-*erb*B-2 and c-*met* in normal (N) and neoplastic (T) mucosa of the right and left colon.

Table 4-2. The distribution of specimens according to the ratio of mRNA levels in paired tumor and normal tissues.

T/N ratio*	EGFR	C-*ERB*B2	C-*MET*
<2.0	19/23 (83%)	22/2(96%)	3/20 (15%)
≥2.0	4/23 (17%)	1/23 (4%)	17/20 (85%)
<4.0	21/23 (91%)	23/23 (100%)	6/20 (30%)
≥4.0	2/23 (9%)	0/23 (0)	14/20 (70%)

*The ratio between mRNA levels in tumor and normal of paired specimens.

increased 2 fold or greater than their paired normal tissues, and 70% of the cases demonstrated a greater than 4 fold increase in the tumor versus normal (T/N) ratios.

The levels of mRNA expression for EGFR, c–*erb*B–2 and c–*met* could not be correlated with the grade (differentiation) of the tumors. The degree of inflammatory reaction in the tumors also failed to influence the level of expression of these genes, indicating that the non–neoplastic host cells in either the normal or cancerous tissues most likely did not significantly alter the relative mRNA expression of these genes (Table 4-3). We also failed to find a significant correlation between the levels of EGFR and c–*erb*B–2 mRNA and the Dukes' stages of the primary carcinomas. Although c–*met* expression was slightly higher in the stage A and B tumors than in the stage C tumors, this difference was insignificant (Table 4-3). The over–expression of c–*met* was also observed consistently in a tubular and a villous adenoma (Figure 4-4), and in 5 other adenomatous polyps ranging in size from 3 to 10 mm in diameter (data not shown).

Table 4-3 The relationship between the expression levels of EGFR, c-*erb*B2 and c-*met* mRNA with the pathological features of the tumors.

	Relative mRNA level (% of HT-29 expression)					
	EGFR	p*	c-*erb*B-2	p*	c-*met*	p*
Differentiation/grade						
well/moderate	48.1±1.9 (24)		30.7±1.9 (24)		14.9±0.5 (24)	
		ns		ns		ns
poor	34.6±4.9 (8)		32.5±3.5 (8)		14.8±1.5 (8)	
Dukes' stage						
A	36.7±15.1 (7)		35.6±7.4 (7)		15.3±3.9 (7)	
		ns		ns		ns
B	52.1±14.9 (14)		28.2±5.2 (14)		18.6±4.2 (14)	
		ns		ns		0.06
C	25.1±7.6 (11)		30.8±8.1 (11)		9.7±1.7 (11)	
Inflammatory reaction						
slight	45.7±3.1 (12)		32.7±1.9 (12)		14.8±1.0 (12)	
		ns		ns		ns
marked	35.8±2.4 (20)		30.3±1.1 (20)		14.9±0.7 (20)	

Values represent means± s.e., ( ): the number of specimens, *: Wilcoxon rank sum test. ns: non-significant.

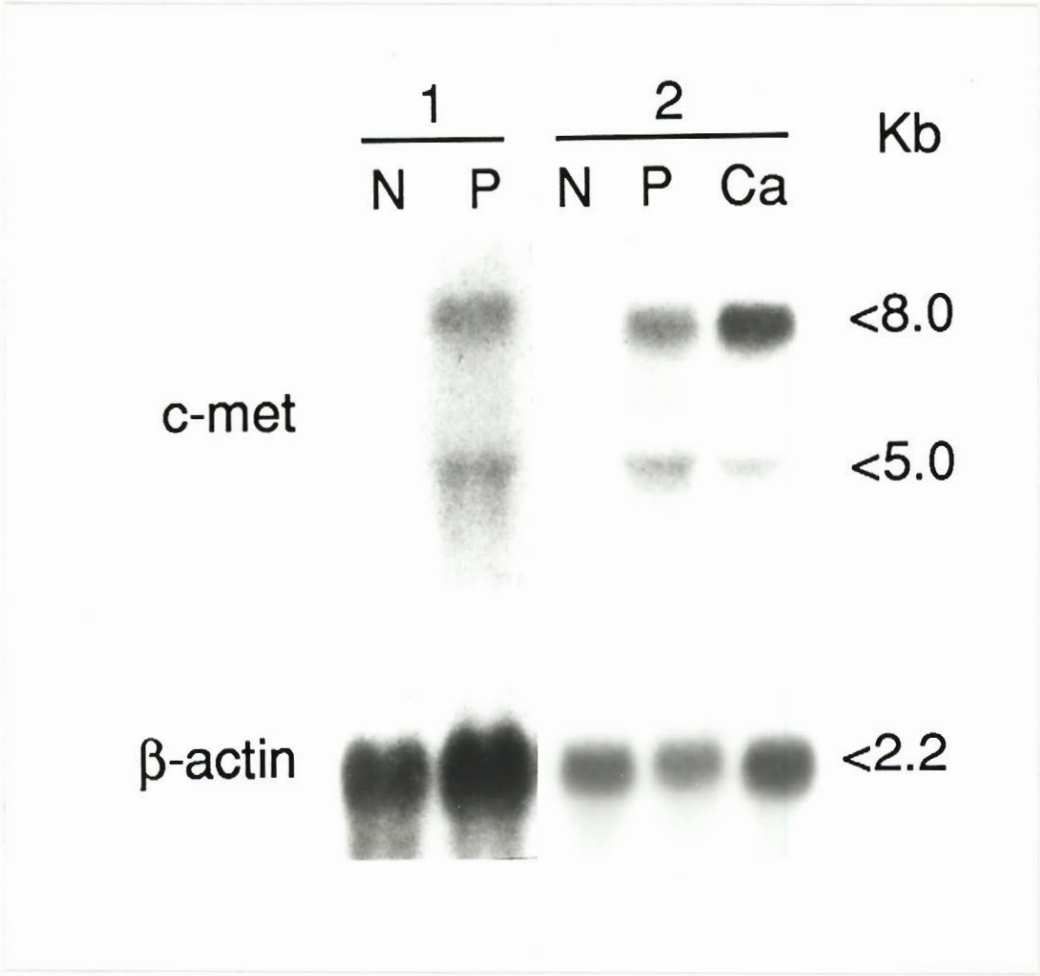


Figure 4-4 Northern analysis showing the expression of c-*met* mRNA in A tubular adenoma (case 1) and a villous adenoma (case 2) where concurrent normal (N), polyp (P) and carcinoma (Ca) were obtained.

4.5 DISCUSSION

We have demonstrated that both the EGFR and c–*erb*B–2 mRNAs are expressed at high level in normal colonic mucosa, and there appears to be an inverse relationship in their levels of expression between the left and right side of the colon. The expression of EGFR is higher in the normal colonic mucosa of the left side than that of the right side. In contrast, c–*erb*B–2 mRNA level is higher in the right colon than in the left colon. Such an inverse relationship between the expression of EGFR and c–*erb*B–2 has been reported in human normal kidney tissue and renal cell carcinomas (Weider et al, 1990), and in normal skin (Nanney et al, 1984; Green et al, 1985; Maguire et al, 1989). In human epidermis, the inverse relationship is associated with the differentiation of epidermal keratinocytes, with the more differentiated keratinocytes expressing less EGFR but more c–*erb*B–2 protein (Maguire et al, 1989). Even though EGF and TGF–α do not bind to p185^{neu} (the protein product of c–*erb*B–2), binding of EGF or TGF–α to EGFR is capable of stimulating the phosphorylation of p185^{neu} (Kokai et al, 1988; King et al, 1988; Akiyama et al, 1988). In addition, gp30, the putative ligand for p185^{neu}, can bind independently to and phosphorylate both the EGFR and p185^{neu} proteins (Lupu et al, 1990). The EGFR and p185^{neu} have recently been shown capable of forming a heterodimer with a ligand–binding affinity higher than the wild type EGFR (Wada et al, 1990). These observations suggest that EGFR and c–*erb*B–2 may co–operate in regulating their physiological effects, and a balance in their combined activity may be important in maintaining their normal functions.

Of the three tyrosine kinase receptors, only c–*met* expression is

consistently enhanced in primary colorectal carcinomas as compared to the normal mucosa. The c–*met* expression has been reported in several human tumor cell lines (Park et al, 1986; Giordano et al, 1988a and b) and its amplification was detected in 2 of 28 primary human gastric and esophageal adenocarcinomas (Houldsworth et al, 1990). We have demonstrated a significantly enhanced expression of the c–*met* mRNA in human primary colon carcinomas, as compared to the normal mucosa. The importance of c–*met* over–expression in these tumor samples is further emphasized by the observation that levels of EGFR and c–*erb*B–2 mRNAs in the same group of carcinomas do not differ from the control normal mucosa. The enhanced expression of c–*met* in adenoma suggests that c–*met* may play important mechanistic roles in the early stages of human colonic carcinogenesis.

Colorectal carcinomas do not express higher levels of EGFR mRNA than the normal mucosa. Only 17% of the tumors expressed greater than 2 fold higher EGFR mRNA than the paired control mucosa. This is consistent with previously reported findings on the EGFR protein levels in human colorectal mucosa and neoplasms. Rothbauer et al (1989) reported that the binding capacities for ^{125}I–EGF were increased only in 3 of 16 (19%) neoplastic tissues as compared to their paired normal mucosa. Koretz et al (1990) have studied immunocytochemically the EGFR expression in paired colonic mucosal and cancerous tissues, and demonstrated that only one third of the carcinomas over–expressed EGFR as compared to their paired normal mucosa. They failed to observe any significant correlation between the level of EGFR expression and the grade and stage of the carcinomas. It is interesting to note that TGF–α is expressed both in the normal colonic mucosa and carcinomas and the mean level in carcinomas is significantly higher than

in the normal tissues (Liu et al, 1990). The co–expressions of TGF–α and EGFR suggest the existence of a functional autocrine mechanism for cell proliferation in these tissues.

The expression of p185^{neu} in colonic carcinomas has been examined previously by immunocytochemistry. Cohen et al (1989) reported that the immunoreactivity of c–*erb*B–2 / *neu* protein was consistently stronger in adenomas than in the adjacent normal mucosa or in areas showing carcinomatous changes. D'Emilia et al (1989) demonstrated that in contrast to 78% (29 of 37 cases) adenomas showing strong reaction with c–*erb*B–2 / *neu* antibodies, only 20% of primary colorectal carcinomas revealed positive staining. Our data on the mRNA expression confirms that over–expression of c–*erb*B–2 is not a common finding in colorectal adenocarcinomas.

The adenoma–carcinoma sequence that occurs in human colonic mucosa is probably the best model of multi–stage carcinogenesis in human tissues. Extensive molecular studies have identified several genetic alterations which occur at various stages of neoplastic progression in this model. These include mutations or deletions in various genes and/or chromosomal segments including the 12p(Ki–ras), 5q (familial polyposis gene), 18q(DCC) and 17p(p53) (Fearon & Vogelstein, 1990). Other reported changes include elevated c–*myc* expression (Finley et al, 1989), enhanced pp60^{src} tyrosine kinase activity (Bolen et al, 1987; Cartwright et al, 1990) and DNA hypomethylation, but their significance in the adenoma–carcinoma progression has not been emphasized. The finding that c–*met* and c–*erb*B–2 are consistently over–expressed in adenomas suggests that the over–expression of the growth factor receptors with tyrosine kinase activity may play important roles in the early stages of human colonic carcinogenesis.

Human lung cancer cell lines have been used extensively to study the genetic, biological and molecular aspects of the lung cancer. The question is whether tumor cells in the cell lines are phenotypically and genotypically representative of tumor cells in the primary tumors. In Chapter 5, we described the establishment of eight new cell lines from primary human non-small cell lung carcinomas, and of five xenograft tumors by injecting the propagable tumor cells into nude mice. DNA and total cellular RNA were isolated from cell lines, primary and xenograft tumors. The system thus allows a phenotypic / genotypic comparison between cell lines, primary tumors and xenograft tumors.

The expression of some growth and differentiation genes (TGF-α, EGFR, c-*erb*B-2 and c-*met*) in the established cell lines, and the relationship between the expressions of these genes is discussed in Chapter 6.

The expression and the possible clinicopathological significance of these genes in the primary human non-small cell lung carcinomas is discussed in Chapter 7.

CHAPTER 5

CHARACTERIZATION OF EIGHT NEWLY ESTABLISHED HUMAN NON-SMALL CELL LUNG CARCINOMA CELL LINES

Chi Liu and Ming-Sound Tsao

5.1 ABSTRACT

We have established eight new cell lines from the primary cultures of 29 unselected human non-small cell lung carcinomas. Seven of these cell lines were established from 14 poorly-differentiated carcinomas, one of which was a large cell neuroendocrine carcinoma. Only one of fifteen well or moderately differentiated carcinomas formed a cell line. The cultured tumor cells exhibited heterogeneous nutritional requirements for their growth *in vitro*. All cell lines were aneuploid. Five of these cell lines formed tumors when injected subcutaneously into nude mice, and their tumorigenicity correlated with the over-expression of the c-*myc* proto-oncogene. The high levels of expression of transforming growth factor-alpha and c-*met* proto-oncogene in the primary tumors appear to correlate with their facility for propagable growth *in vitro*. When the genotypes and phenotypes of the cell lines were compared to their corresponding primary or xenograft tumors, the results suggest that tumor cells which grow continuously as a cell line often represent a selective subpopulation from heterogeneous neoplastic cells in the primary tumors.

5.2 INTRODUCTION

Lung cancer is the leading cause of death from neoplasia in both men and women in the United States, and its incidence continues to increase steadily (Silverberg and Lubera, 1988). Human lung carcinomas are classified morphologically into two major groups, small cell carcinoma (SCLC) and non-small cell carcinoma (NSCLC). They show striking differences in morphology, biochemical and molecular properties, clinical behaviour and their response to therapeutic strategies. The four major types of NSCLC include epidermoid (squamous cell) carcinoma, adenocarcinoma, large cell carcinoma and adenosquamous carcinoma (The world health organization histological typing of lung tumors, 1982). In spite of progress made in the early diagnosis and treatment of lung cancers, the mortality remains very high. A more complete understanding of the molecular pathogenesis and biology of these tumors may contribute further to their accurate classification and therefore to the future design of effective therapies.

Lung carcinoma cell lines have been used extensively in the study of the molecular genetics and biochemistry of human lung cancers, however, the establishment of cell lines from primary human NSCLCs has been unpredictable. Even with the best effort, propagable cell lines have been established successfully from only 30 to 40% of the primary tumors (Gazdar and Oie, 1986a; Brower, et al, 1986; Siegfried and Owens, 1988). The reason for failure of many NSCLCs to grow continuously in monolayer culture is unclear. It is possible that the continuous proliferation of these cells *in vitro* requires the expression of a specific set or sets of growth factor/receptor genes and/or proto-oncogenes. It is generally recognized that polypeptide growth factors, their receptors and the protein products of proto-

oncogenes/oncogenes play important roles in the pathogenesis and biology of human malignancies (Kahn and Draf, 1986). Approximately two thirds of human lung carcinomas demonstrate an increased expression of the transforming growth factor-alpha (TGF-α) (Tateishi, et al, 1990; Liu, et al, 1990) which functions as an autocrine growth factor for the proliferation of some lung carcinoma cell lines (Siegfried, 1987; Imanishi, et al, 1989; Imanishi, et al, 1988). Epidermal growth factor receptor (EGFR) which transduces the effect of TGF-α, is also frequently over-expressed in NSCLCs, especially epidermoid carcinomas (Ozanne and Richards, 1986; Yamamoto, et al, 1986a). Siegfried et al (1987) have reported that the serum-free conditioned medium from cultured A549 , a human bronchioloalveolar carcinoma cell line, contained growth factors which promote strongly the proliferation of human lung cancer cells in primary culture, thus supporting the existence of factors which promote selectively the continuous *in vitro* growth of these tumor cells.

Although cell lines have been used extensively to study the biology of human NSCLC, it is not clear if their phenotypes and genotypes are representative of the neoplastic cells in the primary tumors from which they are derived. Antequera et al (1990) have reported that in long term cultured cells, a great number of their genes become methylated, and the consequent loss of expression of some of these genes may prevent the cells from undergoing terminal differentiation, hence allowing their continuous proliferation *in vitro* (Antequera, et al, 1990). We have established eight new NSCLC cell lines from the primary cultures of 29 unselected surgically removed lung carcinomas. The expression of TGF-α, EGFR, c-*erb*-B2, c-*met* and c-*myc* genes in tumors which succeeded or failed to yield cell lines have been compared.

5.3 EXPERIMENTAL DESIGN

Twenty nine NSCLCs resected at the Montreal General Hospital during a 6 month period in 1989 to 1990 were obtained unselectively as soon as but usually within 30 min after their surgical resection. These tumors included 17 adeno, 3 large cell , 8 epidermoid and one adenosquamous carcinomas. The pathological diagnoses were based on light- and electron-microscopic findings. Approximately 0.5 gm of tumor tissue from each specimen was used for the primary culture. Tumor tissues were also snap frozen in liquid nitrogen and stored at -80^{o}C for subsequent isolation of the RNA and DNA.

For each tumor, establishment of cell lines was attempted in both serum-free and serum-containing media. When pure epithelial tumor cell lines were established, they were injected into the subcutaneous tissue of nude mice to establish tumorigenicity. Tumors which form in the nude mice were examined by electron microscopy. RNA and DNA were isolated from cultured tumor cells and xenograft tumor tissues. Cell lines were also examined for their growth in serum-free and serum-containing media, and for their DNA content by flow cytometry.

5.4 RESULTS AND DISCUSSION

Primary Cultures and Establishment of Cell Lines:

In all cases, tumor cells consistently attached well during the first 3 to 5 days of primary culture. The abundance of tumor cells varied depending on the cellularity, the extent of desmoplasia and necrosis in the primary tumors. In most cases, these neoplastic epithelial cells started to form small colonies during the next 5 to 7 days, but thereafter, they ceased to expand, gradually

degenerated and disappeared (Figure 5-1), or they were overwhelmed by the growth of surrounding fibroblasts.

Only eight of these twenty nine tumors formed propagable cell lines. They included four adenocarcinomas (MGH-8, -13, -24 and -26), two large cell carcinomas (MGH-4 and -14), one epidermoid carcinoma (MGH-7) and one adenosquamous carcinoma (MGH-30) (Figure 5-2). The 27% success rate for establishment of these NSCLC cell lines is comparable to that reported previously by other investigators (Gazdar and Oie, 1986a; Brower, et al, 1986), and is significantly lower than the 75% success rate for SCLC cell lines (Garney and Leij, 1988). The success rate also varied among the different types of NSCLCs. In general, poorly differentiated adenocarcinomas and large cell undifferentiated carcinomas were more capable of growing propagably in vitro (Table 5-1). The difference between well or moderately and poorly differentiated tumors is statistically significant ($p<0.05$, Chi Square Test). The result is consistent with the notion that one of the factors which determines the ability for tumors to form cell lines is their facility to differentiate terminally.

Another parameter which may determine the ability of tumors to form propagable cell lines is their ability to express, synthesize or respond to a specific set or sets of growth factors and/or their receptors. The results of our investigation on the growth capacity of these cell lines in different culture media indicate clearly that tumor cells exhibit variable but specific requirements for their proliferation *in vitro*. MGH-4 and -14 were successful in forming cell lines only in ACL-4 serum-free medium, while MGH-13 and -24 lines established only in RPMI containing 10% fetal bovine serum. The remaining 4 cell lines (MGH-7, -8, -26 and -30) established equally well in both media. When the proliferation of these cells in both the ACL-4 and

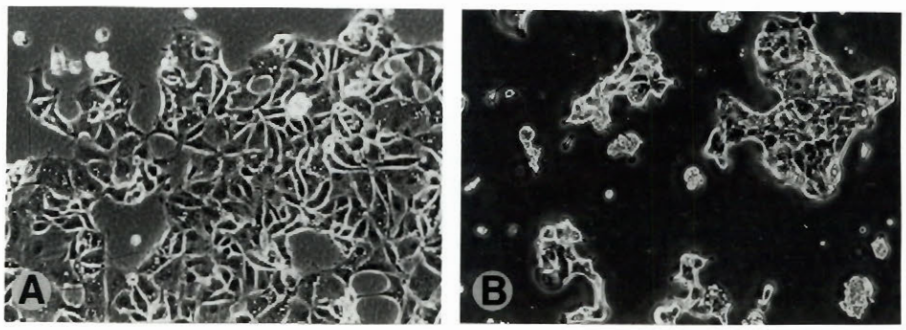


Figure 5-1 A representative primary culture of one of the tumors which failed to form propagable cell line. Colonizing tumor cells at 6 days (A) and 21days (B) after the start of primary culture. (phase contrast, x 200).

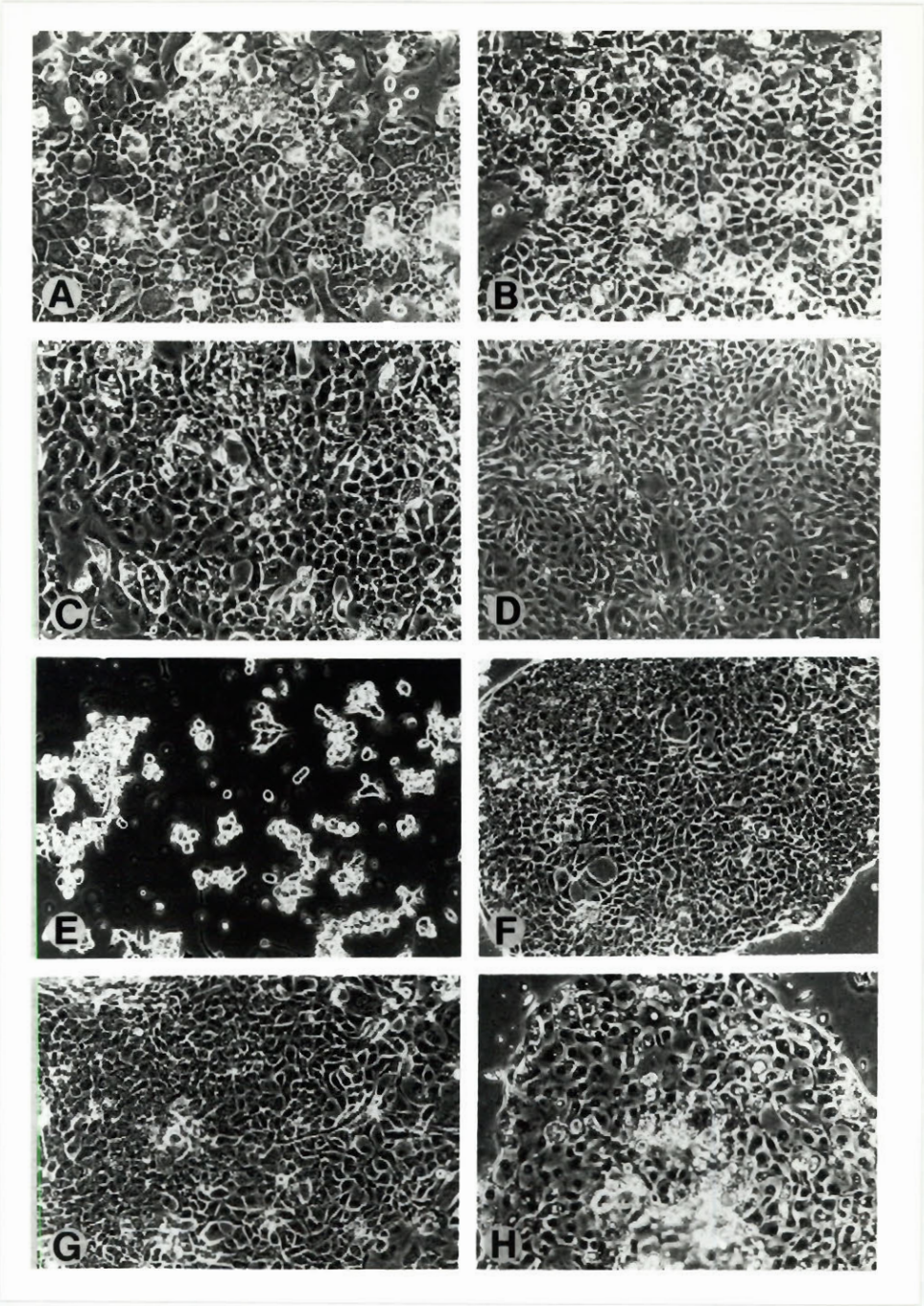


Figure 5-2 The microphotographs of all eight newly established human non-small cell lung carcinoma cell lines (phase contrast, x 200).

A: MGH-4, a large cell undifferentiated carcinoma;
B: MGH-7, a poorly differentiated epidermoid carcinoma;
C, F, G: MGH-8, -24 and -26 respectively, poorly differentiated adenocarcinomas;
D: MGH-13, a moderately differentiated adenocarcinoma;
E: MGH-14, a large cell neuroendocrine carcinoma;
H: MGH-30, a poorly-differentiated adenosquamous carcinoma.

Table 5-1. The success rate of establishing cell lines from the various histological types and grades of primary human non-small cell carcinomas.

	No. of tumor	No. of cell line	success rate
Epidermoid carcinoma	8	1	12.5%
Adenocarcinoma	17	4	23.5%
Large cell carcinoma	3	2	66.7%
Adenosquamous carcinoma	1	1	-
Well or moderately differentiated	15	1	7.0%
Poorly-differentiated*	14	7	50.0%

* Including large cell carcinomas.

serum-containing RPMI-1640 media were tested, it became quite clear that cell lines which were established only in ACL-4, were unable to proliferate in serum containing medium (Figure 5-3). Likewise, cell lines which were established in serum containing medium grew better in this medium than in serum-free medium (Figure 5-3). In contrast, cell lines which were established in both types of media with equal facility demonstrated no preference for growth in either type of medium, regardless of the medium in which they were initially derived (Figure 5-4).

All cell lines demonstrated long population doubling times ranging from 53 to 204 hrs (Table 5-2) and aneuploid DNA composition (Figure 5-5 and Table 5-2). Although these cell lines were not obtained clonally, the results of flow-cytometric analysis demonstrated that each of them are composed of a relatively homogeneous population of cells with a single and relatively narrow aneuploid peak.

Twenty two out of twenty nine primary NSCLCs were available for the study of mRNA expression of TGF-α, EGFR, c-*erb*B-2 and c-*met* proto-oncogenes, and these included 6 cell line-forming carcinomas and 16 carcinomas which failed to grow continuously in culture. The former tend to express comparatively higher levels of TGF-α and c-*met* mRNAs (Table 5-3), especially among the adenocarcinomas. However, the differences are not statistically significant. One of the 16 carcinomas which did not form cell lines expressed unusually high c-*met* mRNA, and when this sample is excluded, the mean c-*met* mRNA level was significantly higher in the cell line-forming tumors than in those which failed to establish cell line ($p=0.05$). There was no correlation between the mRNA level of EGFR or c-*erb*B-2 and the ability of the NSCLCs to form propagable cell line. TGF-α has been reported previously to function as an autocrine growth stimulatory factor in

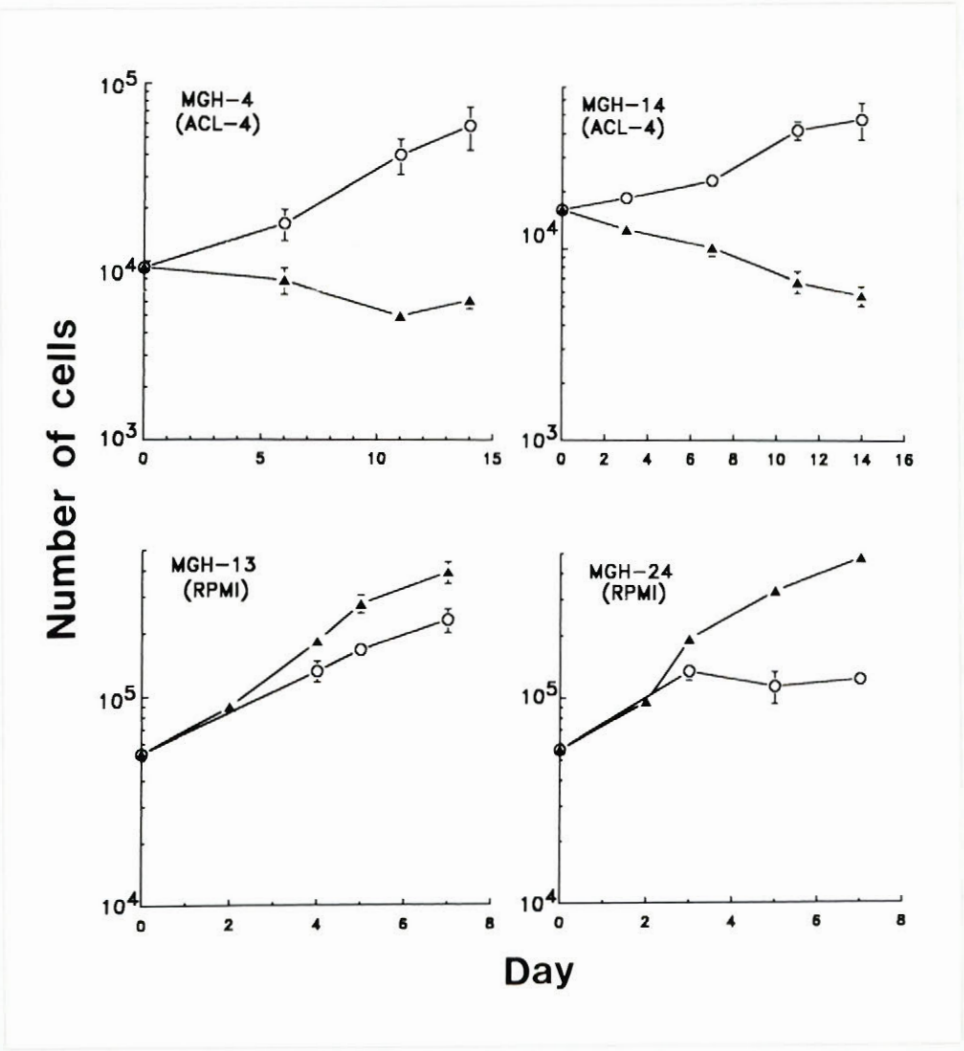


Figure 5-3 The growth curves of MGH-4, -13, -14 and -24 cell lines in ACL-4 serum-free (o) or serum-containing RPMI (▲) media. The brackets indicate the type of medium in which the cell line was initially established.

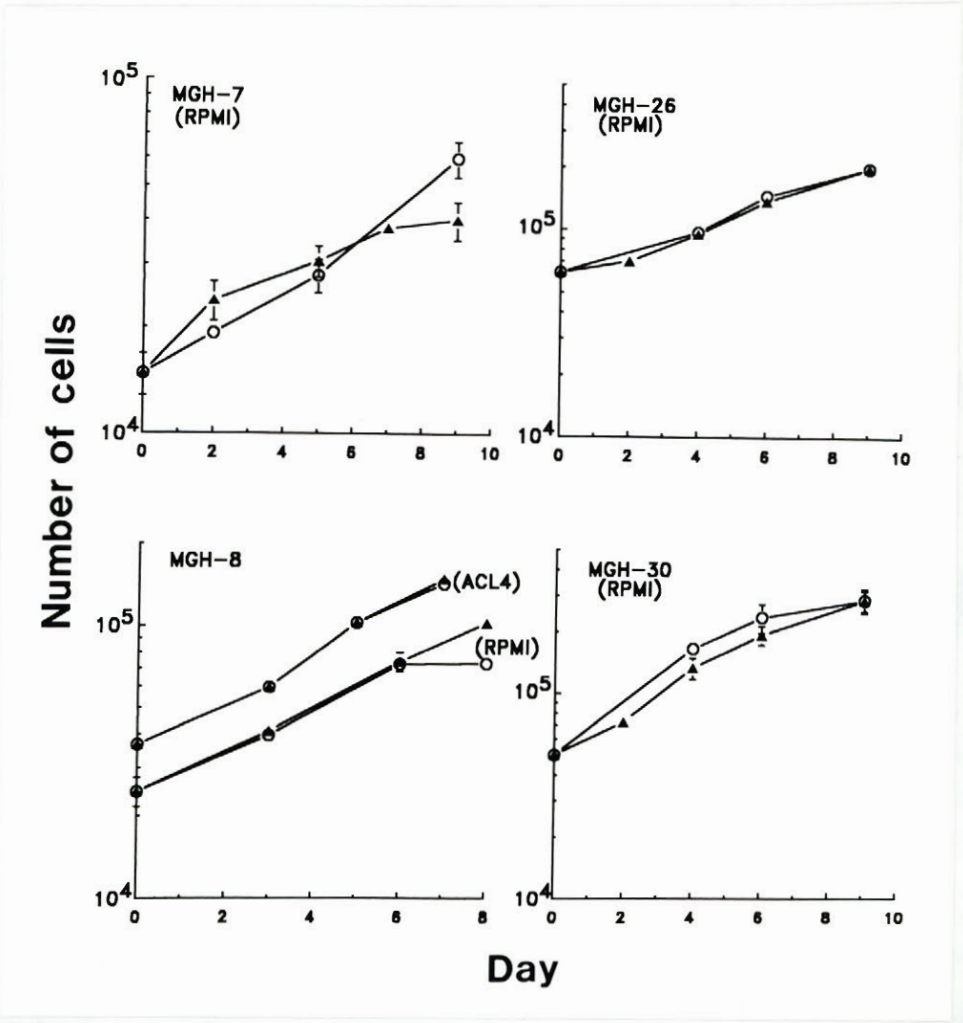


Figure 5-4 The growth curves of MGH-7, -8, -26 and -30 cell lines in ACL-4 serum-free (o) or serum-containing RPMI (▲) media. The brackets indicate the type of medium in which the cell line was initially established.

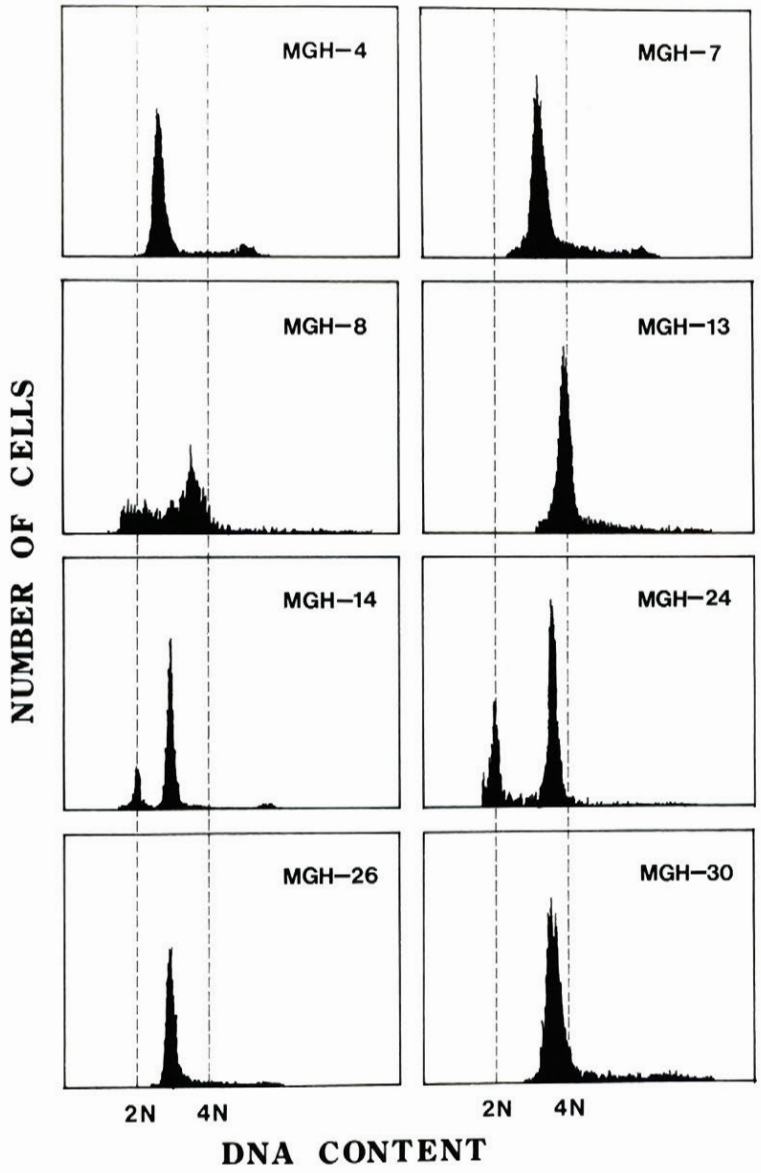


Figure 5-5 The flow cytometric profile of the DNA content of these cell lines. Normal fibroblasts were added to the samples of MGH-14 and -24 to demonstrate position of the diploid (2N) DNA content. 4N represents the normal tetraploid DNA content.

Table 5-2. The population doubling time and ploidy index of eight newly established human lung carcinoma cell lines

Cell Line	Type[a]	Doubling Time (hr)	DNA index
MGH-4	Large cell undifferentiated	125	1.40
MGH-7	Epidermoid (p.d.)	120	1.62
MGH-8	Adeno (p.d.)	80	1.74
MGH-13	Adeno (m.d.)	55	1.94
MGH-14	Large Cell neuroendocrine	204	1.35
MGH-24	Adeno (p.d.)	53	1.77
MGH-26	Adeno (p.d.)	134	1.43
MGH-30	Adenosquamous (p.d.)	79	1.77

[a] p.d.: poorly-differentiated; m.d.: moderately differentiated.

Table 5-3. A comparison of the mRNA expressions between the primary tumors which succeeded or failed to form cell lines [a]

	Cell line forming	Non cell line-forming	p value
TGF-α	2.11 ± 1.32	1.22 ± 1.44	0.06
EGFR	0.09 ± 0.07	0.12 ± 0.12	ns
c-*erb*B-2	0.45 ± 0.41	1.06 ± 1.91	ns
c-*met*	0.78 ± 0.76	0.67 ± 1.56 (16)	0.09
		0.29 ± 0.38 (15)[b]	0.05

[a] All values represent the mean ± S.E.M.; 6 cell line-forming and 16 non cell line-forming tumors were analysed; p values are calculated using the non-parametric Wilcoxon Rank Sum test.

[b] The mean ± S.E.M. after one sample with an unusually very high c-*met* expression was excluded.

A549 and PC-9 lung adenocarcinoma cell lines (Imanishi, et al, 1989). Monoclonal antibody to TGF-α can inhibit the proliferation of these cells in a dose dependent manner, and this inhibition can be reduced by the addition of exogenous TGF-α. The observation that primary tumors which formed cell lines demonstrated higher expressions for TGF-α and c-*met* than tumors which fail to form cell lines suggests that both the TGF-α/EGFR and HGF/c-*met* autocrine growth loops may play critical roles in the ability of human NSCLC cells to grow continuously in culture. Further studies with a larger sample size are necessary to confirm this hypothesis.

Tumorigenicity in Nude Mice

Five of eight cell lines (MGH-4, -7, -14, -24 and -30) injected into nude mice formed tumors within one to three months, and the histological appearance of the xenograft tumors were remarkably similar to their respective primary tumors. The four cell lines (except MGH-14) which formed tumors in nude mice expressed relatively higher levels of c-*myc* mRNA than the cell lines which failed to form xenograft tumors; however, MGH-13 was not tumorigenic despite a comparatively high level of the c-*myc* expression (Figure 5-6). A similar result has been reported by Gemma et al (1988). They demonstrated that, while 11 of 13 primary lung carcinomas with c-*myc* gene amplification were able to form tumors in nude mice, only 5 of 9 tumors without amplification demonstrated tumorigenicity in nude mice. Unfortunately, they did not report the level of c-*myc* mRNA expression in their tumors. Gazzeri et al (1990) reported c-*myc* over-expression in all 5 non-small cell, but only in 2 of 14 small cell xenografted carcinomas established directly from fresh tumor specimens. Thus, the over-expression of c-*myc*

appears to be an important determining factor for the tumorigenicity of human NSCLC cells in nude mice.

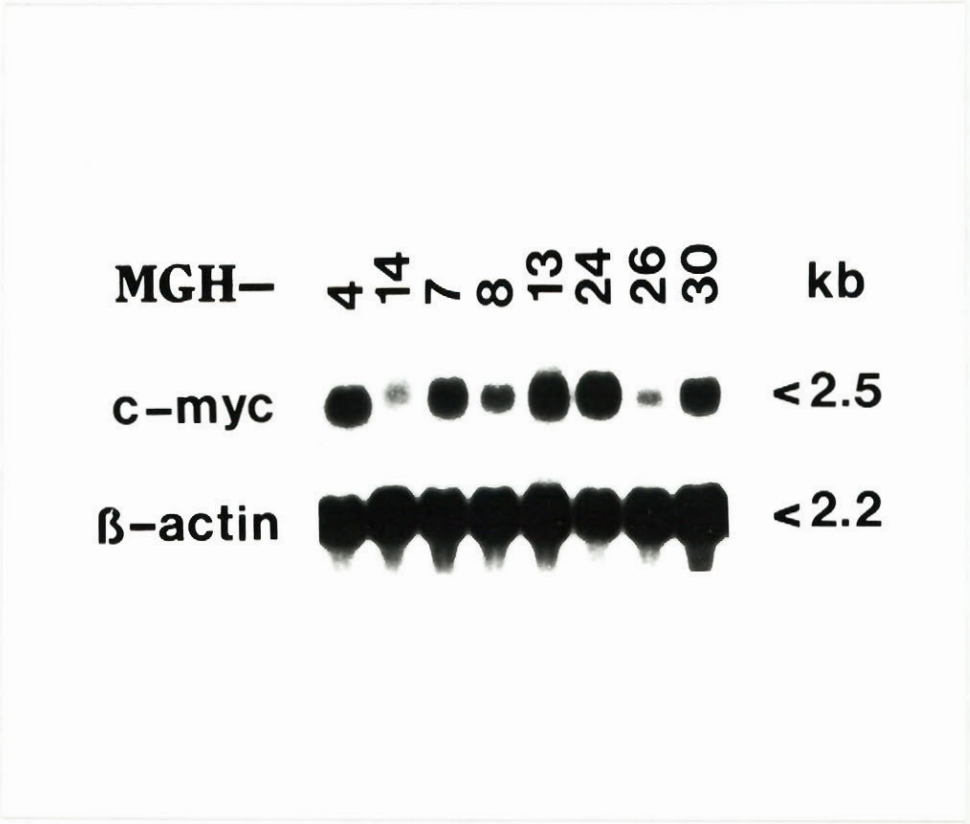


Figure 5-6 The relative levels of c-*myc* and β-actin mRNA expression in confluent cultures of these cell lines.

Comparative Phenotypic Expressions in Primary Tumors, Cell Lines and Xenograft Tumors.

When the mRNA of the TGF-α, EGFR, c-*erb*B-2 and c-*met* in four primary tumors and their corresponding cell lines were compared, expressions were generally higher in cultured cells. It is possible that the cells in culture up-regulate their expression of the various genes, especially the growth factor and/or receptor genes, in order to adapt to their new growth environment. It is also possible that, *in vivo*, tumor cell population is diluted by the presence of non-neoplastic host cells, resulting in relatively lower levels of mRNA as compared to the homogeneous population of the neoplastic cells in culture. However, a comparative study of the gene expression in the primary tumors and the xenograft tumors formed by these cell lines suggest that selection of subpopulations of tumor cells with the capacity to grow continuously in culture may be more common than generally realized (Figure 5-7). In the case of MGH-4, both an amplification and over-expression of the EGFR gene were observed in the primary tumor, but were absent or insignificant in the cultured cells and in the xenograft tumor. In constrast, a marked amplification of the c-*myc* gene was primarily demonstrated in the cultured cells and its xenograft tumor, and at a lower level in the primary tumor (Figure 5-8). In the case of MGH-7, the cell line showed marked over-expression of the EGFR and c-*met* genes, and, although their expressions in the xenograft tumor were down-regulated, they remained significantly higher than in the primary tumor. However, the EGFR gene of the MGH-7 cell line was not amplified. In the case of MGH-14, the xenograft tumor showed a markedly down-regulated c-*met* expression when compared to its expression in the primary tumor. Although it is likely that the culture of MGH-7 and -14 tumors showed selection for tumor cells with specific but randomly altered phenotypic

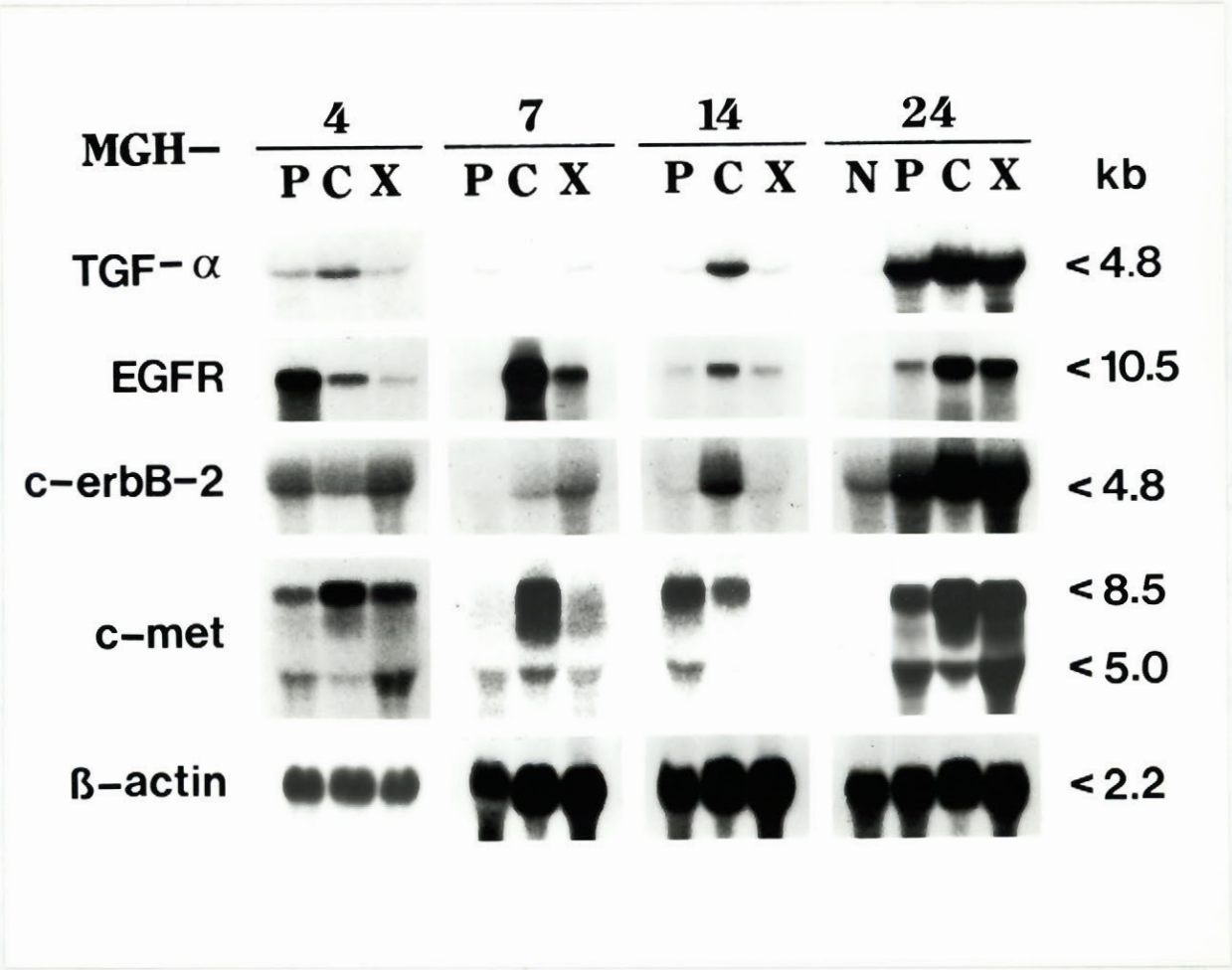


Figure 5-7 The comparative mRNA expression of various growth factor and receptor genes in the primary tumor (P), cell line (C), and xenograft tumors (X) of 4 of the tumorigenic cell lines. N: corresponding normal lung tissue.

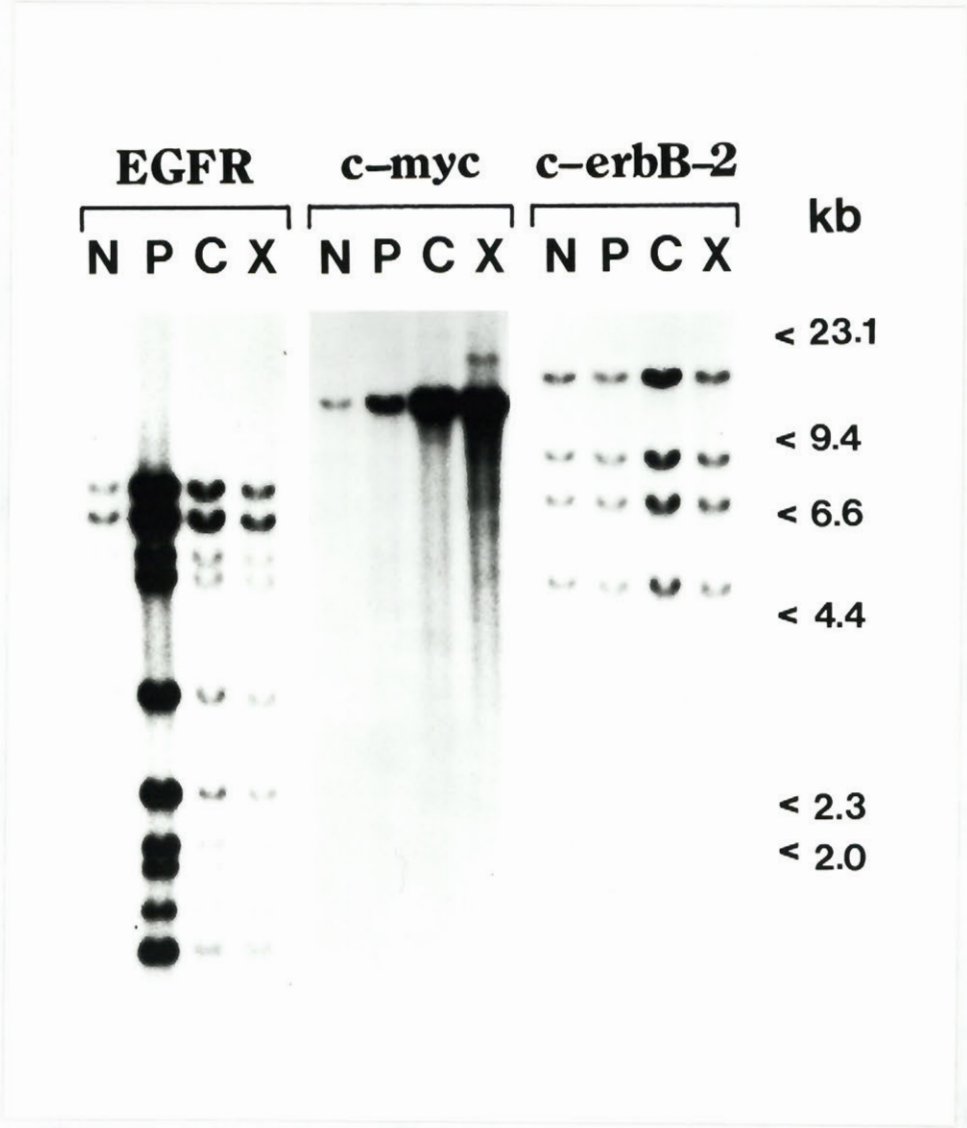


Figure 5-8 The Southern analysis of DNA from the primary tumor (P), cell lin(C), xenograft tumor (X) of MGH-4, showing amplification of EGFR gene in the primary tumor but not in the cell line, and amplification of the c-*myc* gene in the cell line but not in the primary tumor.

expression without the accompanying genotypic changes, it remains possible that the observed phenotypic differences between the primary and xenograft tumors may indicate phenotypic modulations by tumor cells after they have been proliferating in culture. Only the phenotypes and genotypes of MGH-24 cell line and its xenograft tumor were similar to the primary tumor. These results suggest that cell lines may not be representative of the primary tumor cell population from which they are derived, and this should be taken into consideration in the interpretation of data on the phenotypic studies of human lung carcinomas using propagable cell lines, and on the use of cultured tumor cells to evaluate sensitivity to chemotherapeutic agents.

Large Cell Neuroendocrine Carcinoma

All cell lines except MGH-14 grew as an adherent monolayer on tissue culture dishes. MGH-14 was derived from a large cell neuroendocrine carcinoma. The primary tumor was diagnosed morphologically as an undifferentiated large cell carcinoma but transmission electron-microscopic study showed the presence of neuroendocrine granules, and immunohistochemical studies revealed strong positive staining for chromogranin (Figure 5-9) and neuron-specific enolase. These tumor cells grew *in vitro* as loosely attached cell aggregates (Figure 5-2E) thus resembling the growth of SCLC cells in culture. However, in contrast to SCLCs which usually lack the expression of EGFR and c-*erb*B-2 at both the mRNA and protein levels (Schneider, et al, 1989; Gamou, et al, 1987), MGH-14 cell line and its corresponding primary and xenograft tumors expressed EGFR and c-*erb*B-2 mRNAs. In contrast to the SCLC variants, MGH-14 cells show neither amplification nor over-expression of the c-*myc* proto-oncogene (Little, et al, 1983; Johnson, et al, 1986; Keifer, et al, 1987). The large cell

neuroendocrine carcinomas (Hammond and Sause, 1985), or atypical endocrine tumors of the lung (McDowell, et al, 1981; Havens, et al, 1986), have been grouped as a specific type of lung tumor which falls into the "gray area" between the NSCLC and SCLC (Mool, et al, 1986), and their clinical behaviour and response to chemotherapy is controversial. Some investigators reported that they behave like the SCLC, with a rapid clinical course and a good initial responsiveness to chemotherapy (Hammond and Sause, 1985; Mool, et al, 1986). Others have reported that they resemble more closely the NSCLC than the SCLC in their prognosis and poor chemotherapeutic responsiveness (Havens, et al, 1986). In the context of these controversies, we prefer to classify MGH-14 as a large cell neuroendocrine carcinoma, which some investigators may refer to as a "morphological or biochemical" variant of SCLC (Carney, et al, 1985; Gazdar, et al, 1985).

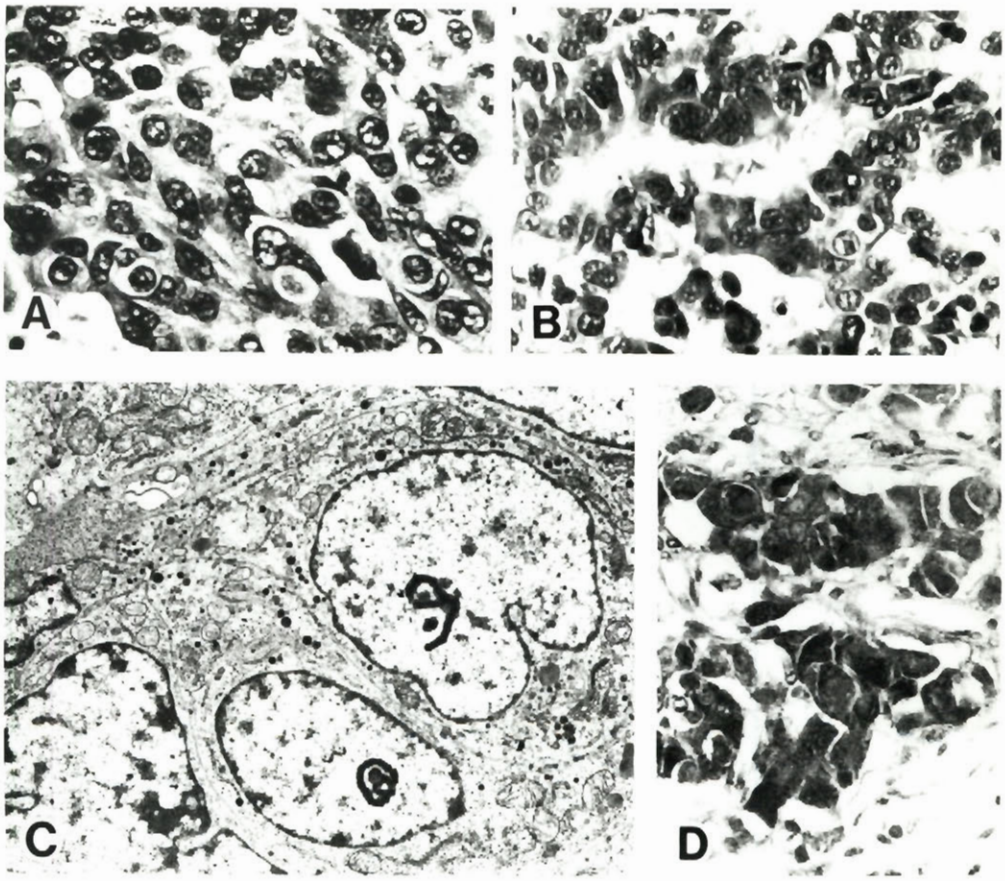


Figure 5-9 The light and electron microscopic appearances of the MGH-14 tumor.

A: primary tumor, B: xenograft tumor in nude mouse, C: transmission electron micrograph of the primary tumor showing numerous dense-core endosecretory granules, and D: strong positive immunohistochemical staining of tumor cells for chromogranin. (A, B and D: x 400, C: x4000).

5.5 METHODS

Establishment of Cell Lines from Primary Tumors

Tumor tissue was gently minced with a scalpel into approximately 1 to 3 mm fragments. The dissociated cells and tissue fragments were washed once with Mg^{2+}- and Ca^{2+}- free Hanks' balanced salt solution (HBSS) (Gibco Laboratories, Grand Island, NY), then were plated into six or eight of 100 mm tissue culture dishes (Falcon) in RPMI-1640 medium plus 10% fetal bovine serum (FBS, Gibco). The cultures were incubated at $37^{o}C$ in a humidified 5% CO_2 atmosphere. Three or four days later, when cell attachment was considered adequate and an initial colony formation had commenced, the medium in half (3 or 4 plates) of the culture dishes was changed with fresh RPMI containing 10% FBS, and the rest with hormonally defined serum-free ACL-4 medium (Gazdar and Oie, 1986b). The media were subsequently changed twice a week. Subculture was performed by dissociating the colonizing cells with HBSS containing 0.05% trypsin and 0.53 mM EDTA (Gibco). Occasionally, the maintenance of primary cultures in confluence with frequent medium changes was used to enrich preferentially the proliferation of neoplastic epithelial cells over their surrounding fibroblasts. Pure epithelial cell cultures were usually obtained after 2 to 4 subcultures, and these were used for further phenotypic and genotypic studies.

Xenografts of Tumor Cells in Nude Mice

CD1 athymic (nu/nu), 6-week old male nude mice were purchased from Charles River (Kingston, NY) and were maintained in a pathogen-free condition. About $1x10^6$ trypsinized tumor cells were suspended in 0.5 ml of

HBSS solution and were injected subcutaneously into the back of these mice (2-4 mice / cell line). They were checked every two weeks for up to 3 months for the formation of tumor. Tumors which had reached 0.5 cm or greater in diameter were removed and processed for routine histology, electron microscopic study and RNA/DNA isolation.

Growth Rate

Trypsin dissociated cells were plated in 6-well tissue culture dishes (Falcon) at a density of 2 or 5 x 10^4 cells per well in RPMI-1640 medium plus 10% FBS. After 3-5 days, the medium was changed with either fresh serum containing RPMI medium, or with ACL-4 medium. The number of cells in each of the triplicate wells was counted every 2 or 3 days, and the population doubling times were determined from the exponential phase of each growth curve.

DNA Content Determination

Approximately 2 x 10^6 trypsin dissociated cells from each cell line were processed according to Lee et al (1989). In order to obtain single cell suspension, cells were trypsinized, washed in a buffer containing 0.1 M NaCl, 11 mM glucose, 5.6 mM Na_2 HPO_4, 5.4 mM KCl, 0.4 mM $CaNO_3$ and 0.4 mM $MgSO_4$, passed 3 times through a 16-gauge syringe and fixed by the addition of cold 95% ethanol to a final concentration of 70%. Cultured normal lung fibroblasts, isolated from one of the NSCLC primary cultures which failed to grow epithelial tumor cells, were used as a reference for diploid DNA content. Cells were stained with propidium iodide and examined by an Epic Profile II (Coulter Electronics, Hialeah, Fl)

flowcytometer. The relative DNA content of these cell lines were expressed as the DNA index, which is the ratio between the model value of the G_1/G_0 aneuploid peak and that of the diploid peak of normal fibroblast.

Nucleic Acid Probes

Plasmid carrying cDNA for human TGF-α, EGFR and c-*erb*B-2 genes were obtained from ATCC (Rockville, MD). TGF-α cDNA probes was a 0.93 Kilo-base pair (kbp) *Eco*R1 insert of clone phTGF1-10-925. EGFR mRNA was probed with a 2.4 kbp *Cla*I EGFR cDNA insert of the pE7 plasmid. C-*erb*B-2 (*neu* / HER-2) mRNA was probed with a 3.4 kbp *Stu* I insert of pCER204. C-*met* mRNA was probed with a 1.1 kbp *Eco*RI fragment of the human c-*met* cDNA (Park, et al, 1987). C-*myc* cDNA probe was purchased from Oncor (Gaithersburg, MD). Human β-actin cDNA was purchased from Clonotech (Palo Alto, Ca).

RNA Isolation and Northern Blot Analysis

Total cellular RNA was extracted from approximately 1 gm of tissue as described previously (Tsao, et al, 1989). RNA from cultured cells was isolated similarly in a 4 M guanidine isothiocyanate solution, pH 7.0, containing 25 mM sodium citrate, 0.1 M β-mercaptoethanol and 0.5% sarkosyl. Northern blot analysis for mRNA expression was performed as described previously (Tsao, et al, 1989).

DNA Isolation

Trypsinized cells were digested overnight at 37^{o}C with 100 μg/ml of proteinase K in an aquaous solution containing 10 mM Tris-HCl, pH 7.4, 0.1 M EDTA, pH 8.0 and 0.5% dodecyl sodium sulfate (SDS) (Anachemia,

Rouses Point, NY). After two extractions with equal volumes of phenol and chloroform, and once with choroform alone, the aquaous phase containing DNA was precipitated overnight at -20^{o}C with 3 volumes of 95% ethanol. The DNA was recovered by spooling and was solubilized in 5 ml of TE buffer containing 10 mM Tris-HCl, pH7.4, and 0.1 mM EDTA, pH 8.0. After digestion with DNase-free RNase (Boehringer Mannheim, Dorval, Quebec), DNA was re-extracted with phenol and chloroform, and precipitated in 95% ethanol. The recovered DNA was dried in a SpeedVac concentrator for 5-10 min and dissolved in TE buffer. The amount of DNA was estimated by measuring the absorbance at 260 nm wavelength.

DNA was isolated similarly from the primary and xenograft tumors. Approximately 0.5 gm of frozen tissue was pulvarized in liquid nitrogen with a mortar and a pestal, and then digested overnight at 37^{o}C with1 mg/ml of proteinase K in a buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 25 mM EDTA, pH 8.0 and 1% SDS. The DNA was extracted as described above.

Endonuclease Digestion and Southern Blot Analysis

Ten microgram of DNA was digested overnight at 37^{o}C with 10-fold excess units of *Eco*RI enzyme. The digested DNA samples were separated in a 0.8% agarose gel in TAE running buffer containing 40 mM Tris-acetate and 0.1 mM EDTA, pH 8.0, and at 35 V for 16 hr. After depurination and denaturation by sequential washes in 0.25 M HCl solution for 15 min, 0.2 M NaOH solution containing 0.6 M NaCl for 30 min, and 0.5 M Tris solution, pH 7.6, containing 1.5 M NaCl for 30 min, the DNA was transfered onto Hybond-N membrane in 20 x SSC solution, and cross-linked by exposure to the

UV-light.

Nucleic Acid Hybridization

The membranes were probed sequentially with each of the six cDNAs as described previously (Tsao, et al, 1989). Membranes was exposed to XAR-5 Kodak X-ray film using an intensifying screen for 3 to 5 days at -80^{o}C.

CHAPTER 6

THE CO-EXPRESSION OF TRANSFORMING GROWTH FACTOR-ALPHA, EPIDERMAL GROWTH FACTOR RECEPTOR, C-*ERB*B-2 AND C-*MET* mRNA IN HUMAN LUNG CARCINOMA CELL LINES

Chi Liu and Ming-Sound Tsao

6-1 ABSTRACT

The mRNA expression of transforming growth factor–alpha (TGF–α), epidermal growth factor receptor (EGFR), c–*erb*B–2 and c–*met* proto–oncogenes in eight newly established human non–small cell lung carcinoma cell lines have been studied. The expressions of TGF–α, c–*erb*B–2 and c–*met* were expressed more consistently at high levels in adenocarcinomas, while EGFR was expressed highest in a squamous cell carcinoma cell line. c–*met* mRNA expression was also highly expressed in adenocarcinoma cell lines. The levels of expression between TGF–α and EGFR or c–*erb*B–2, and between EGFR and c–*erb*B–2 were linearly correlated. The mRNA expression of c–*met* was also correlated with the expression of TGF–α, EGFR and c–*erb*B–2. The results support the functioning of autocrine loops for the proliferation of human non–small cell lung carcinoma cells, especially in adenocarcinomas.

6-2 INTRODUCTION

With the realization that many proto–oncogenes encode for polypeptide growth factors or their receptors (Hunter, 1991; Kahn and Graf, 1986), and that a structural modification of these receptors can induce oncogenic potential, it is becoming clear that the study of expression of growth factors and receptors may provide paramount implication in the understanding of the pathogenesis, and in the diagnosis and treatment of human cancers (Bishop, 1991; Heldin and Westermark, 1989; Weinberg, 1989). Lung cancer is the leading cause of death from malignancies for both men and women in the United States, and its incidence continues to increase steadily (Silverberg and Lubera 1988). Some preliminary reports have suggested that the over–expressions of transforming growth factor–alpha (TGF–α), epidermal growth factor receptor (EGFR) and c–*erb*B–2 proto–oncogene may be correlated with the poor survival rate of patients in subsets of human non–small cell lung carcinomas (Veale et al, 1987; Kern et al, 1990; Tateishi et al, 1990). Important insight into the functional effects of these proteins on biological behaviour of these cancers is best studied using lung carcinoma cell lines. This report describes the mRNA expression of TGF–α, EGFR, c–*erb*B–2 and c–*met* in eight new lung carcinoma cell lines that we established recently.

6-3 MATERIALS AND METHODS:

Cell Line:

The derivation and growth properties of the eight newly established human lung carcinoma cell lines (Table 6-1) have been discussed in Chapter 5. These cell lines were routinely cultured in 100 mm tissue culture dishes (Falcon) in either RPMI–1640 medium containing 10% fetal bovine serum (Gibco–BRL, Grand Island, NY), or in ACL–4 hormonally defined serum free medium (Gazdar and Oie, 1986a). Studies were carried out using pure carcinoma cell lines at passage 4 or 5 after primary culture. A–431 vulvar epidermoid carcinoma cell line were obtained from ATCC (Rockville, Md).

RNA Isolation and Northern Blot Analysis:

Total cellular RNA was extracted from cells in confluent cultures according to a modified method of Chirgwin et al (1977). Cultured cells in 100 mm tissue culture dishes were washed once with ice–cold phosphate–buffered saline and then lysed in 2 ml of 4M guanidine isothiocyanate solution, pH 7.0, containing 25 mM sodium citrate, 0.1 M β–mercaptoethanol and 0.5% sarkosyl. The cell lysate was scraped off with a teflon policeman, and then collected in a conical centrifuge tube. After one minute vortexing, the lysate was layered onto a 1.3 ml cushion of 5.7 M CsCl solution, pH 5.0, containing 25 mM sodium acetate in a 4 ml polyallomer tube. The lysate was centrifuged with a swinging bucket roter in an IEC–B60 ultracentrifuge at 22^{o}C and 32,000 rpm for 20 hr. The overlying solution was decanted by quickly inverting the tube, and the clear gelatinous pellet of RNA was dissolved in 100 μl of distilled H_2O pretreated with diethylpyrocarbonate (depc) and containing 0.3 M sodium acetate. The RNA was extracted once

with phenol–chloroform mixture, once with chloroform, and then precipitated at –70^{o}C with 3 volumes of 95% ethanol. After a 20 min centrifugation at 10,000 x g, the pellet was redissolved in depc–treated H_2O. The amount of RNA was estimated by measuring the absorbence at 260 nm wavelength. Thirty microgram of RNA samples were electrophoretically separated in 1% formaldehyde–agarose gel, and transferred to Hybond–N membrane (Amersham Canada, Oakville, Ontario) as described previously (Liu et al, 1990).

cDNA Probe:

Plasmids carrying cDNA for human TGF–α, EGFR and c–*erb*B–2 were obtained from ATCC (Rockville, MD). TGF–α cDNA probe was a 0.93 kilo-base pairs (kbp) *Eco*R I insert of phTGF1–10–925. EGFR mRNA was probed with a 2.4 kbp *Cla* I cDNA insert of the pE7. C–*erb*B–2 (*neu*/HER–2) mRNA was probed with a 3.4 kbp *Stu* I insert of pCER204. C–*met* mRNA was probed with a 1.1 kbp *Eco*R I fragment of the human cDNA (Park et al, 1987). Human β–actin cDNA probe was purchased from Clonotech (Palo Alto, Ca).

Nucleic Acid Hybridization:

The membranes were probed sequentially with each of the five cDNA probes as described previously (Liu et al, 1990). Membranes was exposed to XAR–5 Kodak film using an intensifying screen for 3 to 5 days at –80^{o}C. Densitometric analyses were performed using the Hoefer GS–300 scanning densitometer. Signal intensity for the growth factor or receptors were normalized against the β–actin signals.

For quantitative comparison of the expression of EGFR and c–*met* mRNA

among the different cell lines, only the full length transcripts of 10.5 kb (EGFR) and 8.5 kb (c–*met*) were measured densitometrically. Both the TGF-α and c–*erb*B–2 only expressed one major species of mRNA transcript.

6-4 RESULTS

All eight cell lines expressed variable amount of the mRNA for TGF–α, EGFR, c–*erb*B–2 and c–*met* (Figure 6-1), and their relative levels of expression, including the expressions in A–431 cells, are illustrated in Table 6-2. Cell lines with glandular differentiation (adeno– and adenosquamous carcinomas) tended to express higher levels of TGF–α, c–*erb*B–2 and c–*met* transcripts. In contrast, the MGH-7 squamous cell carcinoma cell line expressed low levels of these three genes, while it over-expressed EGFR at a higher level than the A431 cell line, which is commonly used in the study of EGFR expression. Both the large cell undifferentiated (MGH–4) and large cell neuroendocrine (MGH–14) carcinomas expressed relatively low levels of these three receptor genes.

There was a significant linear correlation between the levels of mRNA expression for TGF–α and EGFR or c–*erb*B–2, and between EGFR and c–*erb*B–2, especially when the MGH–7 squamous cell carcinoma cell line with markedly over–expressed EGFR is excluded (Figure 6-2). There was also significant linear correlation between the c–*met* and TGF–α, c–*erb*B–2 or EGFR mRNA expressions (Figure 6-3).

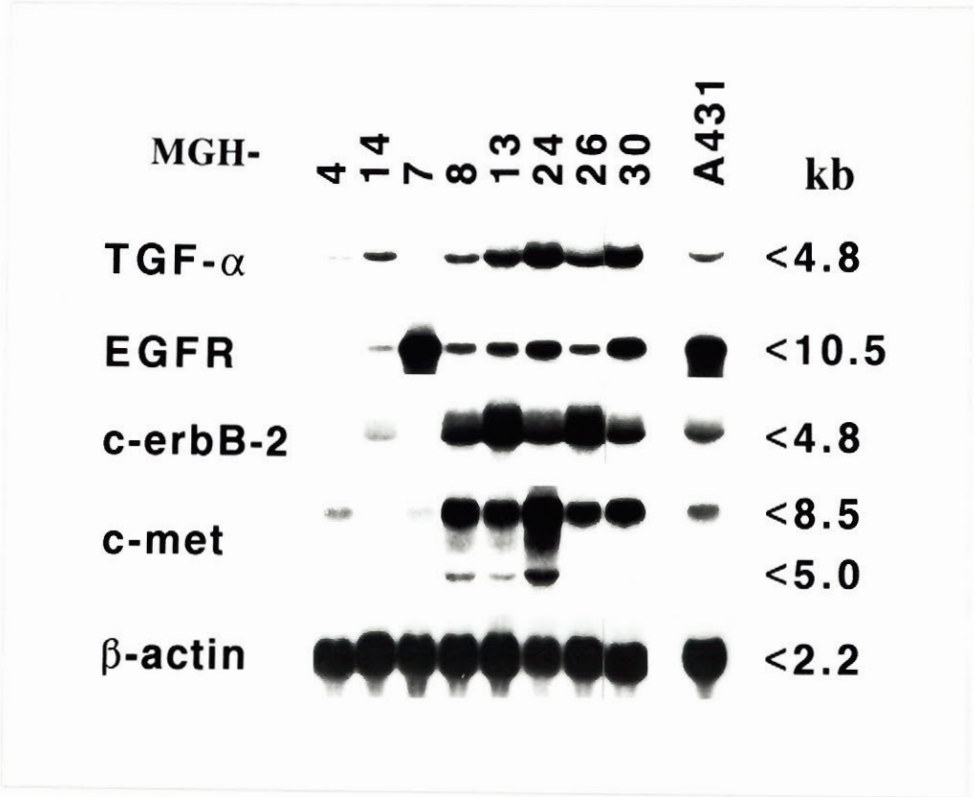


Figure 6-1. Northern blot analyses for the comparative mRNA expression of transforming growth factor (TGF)–α, epidermal growth factor receptor (EGFR), c–*erb*B–2, c–*met* and β–actin genes in eight human lung carcinoma cell lines and A–431 cells.

Table 6-1. The differentiation and media requirements of the eight newly established human lung carcinoma cell lines

Cell line	Differentiation	Growth Medium
MGH–4	Large cell undifferentiated	ACL–4
MGH–7	Squamous cell carcinoma (p.d.)	RPMI & ACL–4
MGH–8	Adenocarcinoma (p.d.)	RPMI & ACL–4
MGH–13	Adenocarcinoma (m.d.)	RPMI
MGH–14	Large cell neuroendocrine	ACL–4
MGH–24	Adenocarcinoma (p.d.)	RPMI
MGH–26	Adenocarcinoma (p.d.)	RPMI & ACL–4
MGH–30	Adenosquamous carcinoma (p.d.)	RPMI & ACL–4

p.d.: poorly differentiated; m.d.: moderately differentiated; RPMI: RPMI–1640 plus 10% fetal bovine serum; ACL–4: serum free hormonally defined medium.

Table 6-2. The relative levels of expression for TGF-α, EGFR, c-*erb*B-2 and c-*met* in human lung carcinoma cell lines.

Cell line	TGF-α	EGFR	c-*erb*B-2	c-*met*
A-431	1.00	1.00	1.00	1.00
MGH-4	1.07	0.06	0.33	1.05
MGH-7	2.35	1.70	0.29	0.79
MGH-8	1.99	0.24	1.94	2.64
MGH-13	4.08	0.38	2.95	2.95
MGH-14	0.24	0.11	0.54	0.21
MGH-24	7.45	0.82	3.04	4.98
MGH-26	2.47	0.21	2.15	2.17
MGH-30	4.20	0.51	1.39	2.17

All RNA samples were electrophoretically separated and hybridized in a single gel/membrane. For each sample, the signals for the various genes were first normalized to their relative β-actin levels, and then calculated relative to the expression in A-431 cells, whose values are arbitrarily designated as 1.00.

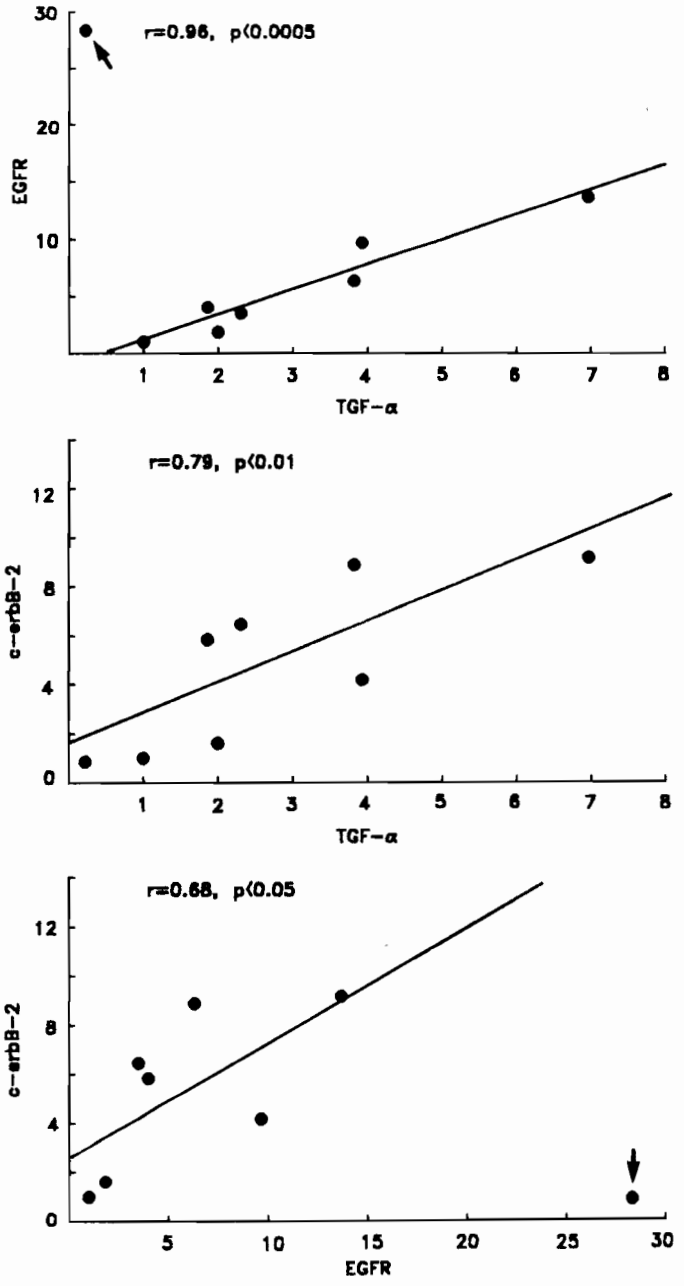


Figure 6-2. Correlations between the mRNA expression levels of TGF–α to EGFR, TGF–α to c–*erb*B–2, and c–*erb*B–2 to EGFR in human lung carcinoma cell lines. The relative mRNA levels of various cell lines are normalized to the values for MGH–4 line, whose expression levels are arbitrarily designated as one. The arrows indicates MGH–7 squamous cell carcinoma cell line which markedly over–expresses the EGFR. Linear correlations between the EGFR and TGF–α or c–*erb*B–2 are noted only when the the MGH–7 line is excluded.

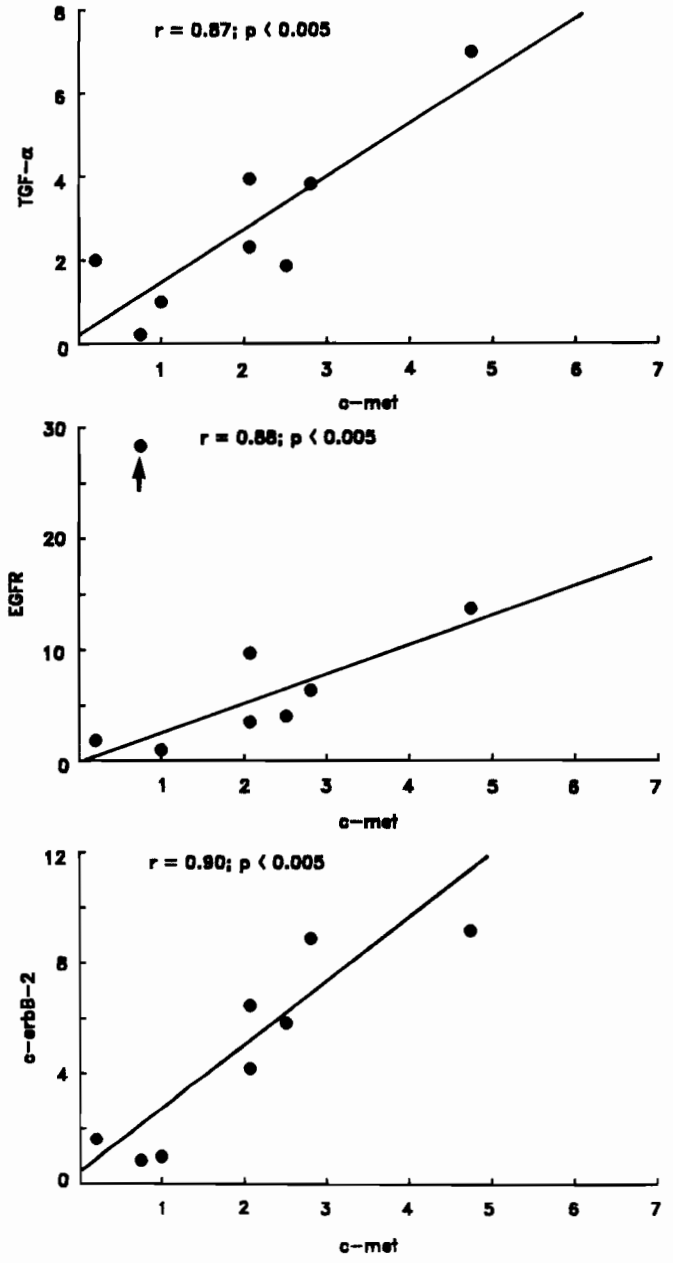


Figure 6-3. Correlations between the mRNA expression levels of c–*met* to TGF–α, EGFR or c–*erb*B–2. The relative mRNA levels of various cell lines are normalized to the values for MGH–4 line, whose expression levels are arbitrarily designated as one. The arrow indicates MGH–7 cell line which markedly over–expresses the EGFR mRNA. Linear correlation between the c–*met* and EGFR is noted only when the MGH–7 cell line is excluded.

6.5 DISCUSSION

We have demonstrated that human lung carcinoma cell lines commonly express the mRNA for TGF–α, EGFR, c–*erb*B–2 and c–*met*. Among the different types of carcinomas, the expressions of TGF–α, c–*erb*B–2 and c–*met* were consistently higher in the adenocarcinoma cell lines. We have further demonstrated that there were linear correlations between the expression of TGF–α and EGFR or c–*erb*B–2, and between c–*met* and TGF–α, EGFR or c–*erb*B–2, among the non–epidermoid carcinomas.

Comparative quantitative studies on the expression of these growth factor and receptor genes in human carcinoma cell lines have not been performed extensively. Sukiyama et al (1986) and Soderdhal et al (1988) reported previously that high level of expression for EGFR was seen in 3 of 4 squamous cell, 6 of 7 adeno and 2 of 2 large cell undifferentiated carcinoma cell lines. Soderdahl et al (1988) also reported that high level of expression for TGF–α was seen in a single case of squamous cell carcinoma cell line, but was low in 2 cases of adenocarcinoma cell lines studied. One of three large cell carcinoma cell lines also demonstrated relatively high level of TGF–α expression.

Two reports described the expression of c–*erb*B–2 proto–oncogene in non–small cell lung carcinomas cell lines at the protein and/or mRNA levels. Kern et al (1990) reported that the $p185^{neu}$ protein level detected using Western blot analysis was correlated to the mRNA expression level measured by the Northern blot technique, and Weiner et al (1990) detected relatively high expression of c–*erb*B–2 in 1 of 2 squamous cell, 5 of 12 adeno and 2 of 3 large cell carcinoma cell lines. Sneider et al (1989) also reported relatively high expression of c–*erb*B–2 mRNA especially in lung adenocarcinoma cell

lines, however, the lack of detailed quantitation of the expression levels relative to a reference cell line, e.g. A–549 or A–431 cells, does not allow a precise correlation for the expression levels of these genes in the various human lung carcinoma cell lines they studied.

Since the binding of TGF–α to EGFR activates both the dimerization and the kinase activities of the EGFR and c–*erb*B–2 proteins (king et al, 1988; Kokai et al, 1988; Akiyama et al, 1988; Wada et al, 1990), their frequent co–expression by lung carcinoma cell lines suggest strongly the operation of an autocrine growth regulation in these cells. It has been previously reported that the addition of neutralizing antibody to TGF–α into the culture medium of PC–9 and A–549 human lung adenocarcinoma cell lines resulted in an inhibition of the proliferation of these cancer cells (Imanishi et al, 1989). The operation of such autocrine growth loop in lung cancer cells may have significant manifestation on the biological behavior of these tumors, and it may account for the apparent adverse effect of over–expression of c–*erb*B–2, or the concurrent over–expression of TGF–α and EGFR on the survival of patients with lung adenocarcinoma (Kern et al, 1990; Tateishi et al, 1990). The transfection of proto–oncogenes which encode various serine/threonine and tyrosine kinases into the NIH 3T3 cells have also been correlated with the induction of increased metastatic potential (Egan et al, 1987).

The consistently high level of expression of the c–*met* proto–oncogene may also indicate the operation of another autocrine or paracrine growth regulatory loop. C–*met* encodes a tyrosine kinase receptor (Park et al, 1987; Giordano et al, 1989a) for the hepatocyte growth factor (Bottaro et al, 1991) which is ubiquitously expressed in many adult cells and tissues (Zarnegar et al, 1990; Tashiro et al, 1990). High levels of p140*met* expression have been reported previously in 5 other non–small cell carcinoma cell lines including

poorly differentiated squamous cell (Calu–1, SK–MES1) and adeno (Calu–6, A549, SK–LU–1) carcinoma cell lines (Gonzatti-Haces et al, 1988). Further studies on the pattern of expression of c–*met* in a larger panel of lung carcinoma cell lines and primary tumors may provide potentially important information on the clinical significance of this proto–oncogene (growth factor receptor) in human lung cancer. The correlation which we observed between the expression levels of c–*met* and TGF–α, EGFR or c–*erb*B–2 needs further confirmation, but it may signify the presence of a common regulatory mechanism in the expression of these genes.

MGH–14 is a large cell neuroendocrine carcinoma cell line, and it may correspond to the variant type of the small cell lung carcinoma (SCLC) cell lines reported by other investigators (Carney et al, 1985; Johnson et al, 1986; Bepler et al, 1987; Hammond and Sause, 1985). The expression of TGF–α and the three tyrosine kinase receptors we studied in the variant type of SCLCs has not been reported, but the expression of EGFR and c–*erb*B–2 in the classical type of SCLC has been reported as absent or extremely very low (Soderdahl et al, 1988; Schneider et al, 1989; Gamou et al, 1987). The MGH–14 cell line clearly expresses, albeit at lower levels, the TGF–α, EGFR, c–*erb*B–2 and c–*met* gene transcripts. It is unclear if a significant expression of these tyrosine kinase receptors may distinguish the large cell neuroendocrine carcinomas from the classical type of SCLC.

CHAPTER 7

EXPRESSION OF TRANSFORMING GROWTH FACTOR-α, EPIDERMAL GROWTH FACTOR RECEPTOR, c-*erb*B-2 AND c-*met* PROTO-ONCOGENES IN PRIMARY HUMAN NON-SMALL CELL LUNG CARCINOMAS

Chi Liu and Ming-Sound Tsao

7.1 ABSTRACT

Northern analyses of the expressions of transforming growth factor-α (TGF-α), epidermal growth factor receptor (EGFR), c-*erb*B-2 and c-*met* proto-oncogenes were performed in 29 primary human non-small cell lung carcinomas (NSCLC), which included 14 adeno, 9 squamous cell and 6 large cell carcinomas. The results demonstrated that TGF-α and EGFR mRNA were expressed at significantly higher levels in carcinomas than in normal lung tissues (2.8 and 1.7 times respectively). In general, although the mRNA level of c-*erb*B-2 in carcinomas did not differ significantly from that in the normal lung tissues, adenocarcinomas expressed significantly higher level of c-*erb*B-2 mRNA than their corresponding normal tissues (2.2 times). Studies of paired normal and tumor samples obtained from individual patients indicated that at least 2 fold over-expression in carcinomas was observed in 60% of the cases for TGF-α, 55% of the cases for EGFR, 15% of the cases for c-*erb*B-2 and 42% of the cases for c-*met*. Among the major histological subsets of NSCLCs, adenocarcinomas appear to express higher levels of TGF-α, c-*erb*B-2 and c-*met* mRNAs. Although the over-expression of these genes is common in human lung cancers, their amplification is a very rare event.

7.2 INTRODUCTION

The pathogenesis of human lung cancer putatively involves both the activation of proto-oncogenes and inactivation of the tumor suppressor genes (Viallet and Minna, 1990). Activated proto-oncogenes may act as growth factors or growth factor receptors which mediate the uncontrolled growth of these neoplastic cells (Kahn and Graf, 1986). Among several growth factor/receptor systems, transforming growth factor-α (TGF-α) and its receptor, the epidermal growth factor receptor (EGFR), have been frequently studied in human solid tumors. The over-expression of TGF-α has been demonstrated in cancers of the kidney (Mydlo, et al, 1989; Petrides, et al, 1990), stomach (Bennett, et al, 1989), colon (Liu, et al, 1990), lung (Liu, et al, 1990; Tateishi, et al, 1990), pancreas (Barton, et al, 1991) and brain (Samules, et al, 1989). TGF-α has been demonstrated to function as an autocrine growth factor for the proliferation of some cultured lung adenocarcinoma cells (Imanishi, et al, 1989), and the over-expression of TGF-α in this type of cancer has been correlated with poorer prognosis (Tateishi, et al, 1990). EGFR is frequently amplified and/or over-expressed in malignant gliomas (Wong, et al 1987), carcinomas of the bladder (Berger, et al, 1987; Neal, et al, 1990), breast (Harris, et al, 1989), stomach (Yasui, er al, 1988; Yonemura, et al, 1989) and lung (Berger, et al, 1987, Veale, et al, 1987), squamous cell carcinomas of the mouth (Todd, et al, 1989), laynx (Scambia, et al, 1991), head and neck (Ishitoya, et al, 1989) and esophagus (Ozawa, et al, 1989; Yano, et al, 1991). Its over-expression in the breast and gastric carcinomas has been correlated with shortened relapse-free and overall survivals (Berger, et al, 1987; Neal, et al, 1989; Harris, et al, 1989; Yasui, et al, 1988a). C-*erb*B-2 proto-oncogene is homologous to the EGFR (Bargmann, et al, 1986; Yamamoto, et al, 1986). The

amplification and/or over-expression of the c-erbB-2 has been correlated significantly with poor prognosis in human breast and lung adenocarcinomas (Slamon, et al, 1987; Wright, et al, 1989; Kern, et al, 1990). C-*met* proto-oncegene encodes the tyrosine kinase receptor of the hepatocyte growth factor receptor (Bottazo, et al, 1991; Naldini, et al, 1991). Its expression has been previously demonstrated in 5 human NSCLC cell lines (Park, et al, 1987), but has been rarely studied in primary human cancers.

7.3 MATERIALS AND METHODS

Specimens

Twenty nine non-small cell lung carcinoma tissues were obtained from surgically resected specimens, these include: 14 adeno, 9 squamous cell and 6 large cell carcinomas. Normal lung tissues from the same lobe of each case were also obtained in 20 of these cases.

Isolation of total cellular RNA and northern blot analysis

Total cellular RNA and DNA were isolated as described previously (Liu, et al, 1990). Thirty microgram of total cellular RNA was used for the mRNA analysis. To obtain a comparative quantitation of the mRNA signals in these tissues, 30 μg total cellular RNA from A431 human epidermoid carcinoma cells was co-electrophoresed in every gel. densitometric measurements of the mRNA signals for each specimen were first normalized to their respective β-actin mRNA levels, and then were calculated as the percentage of mRNA level expressed in A431 cells.

Statistical analysis

This was performed by using Wilconxon Rank Sum test (McClare and Dietrich, 1988).

7.4 RESULTS AND DISCUSSION

The majority of primary human NSCLCs and normal lung parenchyma expressed predominantly the 4.8 kb TGF-α, 10.5 kb EGFR, 4.8 kb c-*erb*B-2, and both the 8.5 and 5.0 kb c-*met* mRNAs (Figure 7-1). The mean mRNA levels of TGF-α and EGFR were significantly higher in the carcinomas than in the normal lung tissues (Table 7-1). The mean c-*met* mRNA level was also higher in the tumors, but the difference is not statistically significant.

Among the various histological subsets of NSCLC, adenocarcinomas expressed higher levels of TGF-α, c-*erb*B-2 and c-*met* mRNA, whereas EGFR expression was significantly higher in squamous cell carcinoma. Large cell carcinoma also showed high level of c-*met* mRNA expression (Table 7-1).

Since there was considerable variation among individuals in the mRNA expression levels of these genes in normal lung parenchyma, intra-individual changes of their expressions were compared in 20 cases in which both the normal and cancerous lung tissues were available. In each case, the ratio of mRNA levels in tumor and normal tissues (T/N) was calculated (Figure 7-2). The mean T/N ratio for TGF-α was 4.67 ± 1.48. Among 12 out of 20 cases in which the tumor expressed at least 2 folds higher TGF-α mRNA than its corresponding normal lung parenchyma, six were adenocarcinomas, four of which demonstrated a T/N ratio of greater than 4. The mean T/N ratio for EGFR was 2.81 ± 0.53, and a greater than 2 fold EGFR over-expression was

observed in 11 of the 20 tumors, including 5 adeno, 3 squamous cell and 3 large cell carcinomas. Six of 20 cases demonstrated a T/N ratio of greater than 2 for c-*erb*B-2, and 4 of these were adenocarcinomas. Out of 8 tumors which showed greater than 2 fold c-*met* mRNA over-expression, 6 were adenocarcinomas (Figure 7-2).

DNA analysis on these samples demonstrated that only one of the 29 tumors showed amplification of the EGFR gene. None of the other genes were amplified (data not shown).

The 29 tumors showed a wide range of differentiation grades or stages (Table 7-2), however, neither of them showed significant correlation with the mRNA expression levels of these four growth factor or receptor genes. However, in four patients who died of distant metastatic carcinoma within 12 months after their lung resection, specific genotypic or phenotypic characteristics were noted (Table 7-3). These included neuroendocrine differentiation, amplification and / or over-expression of the EGFR, c-*myc*, c-*erb*B-2 or c-*met* genes in either the primary tumor or cultured tumor cell line .

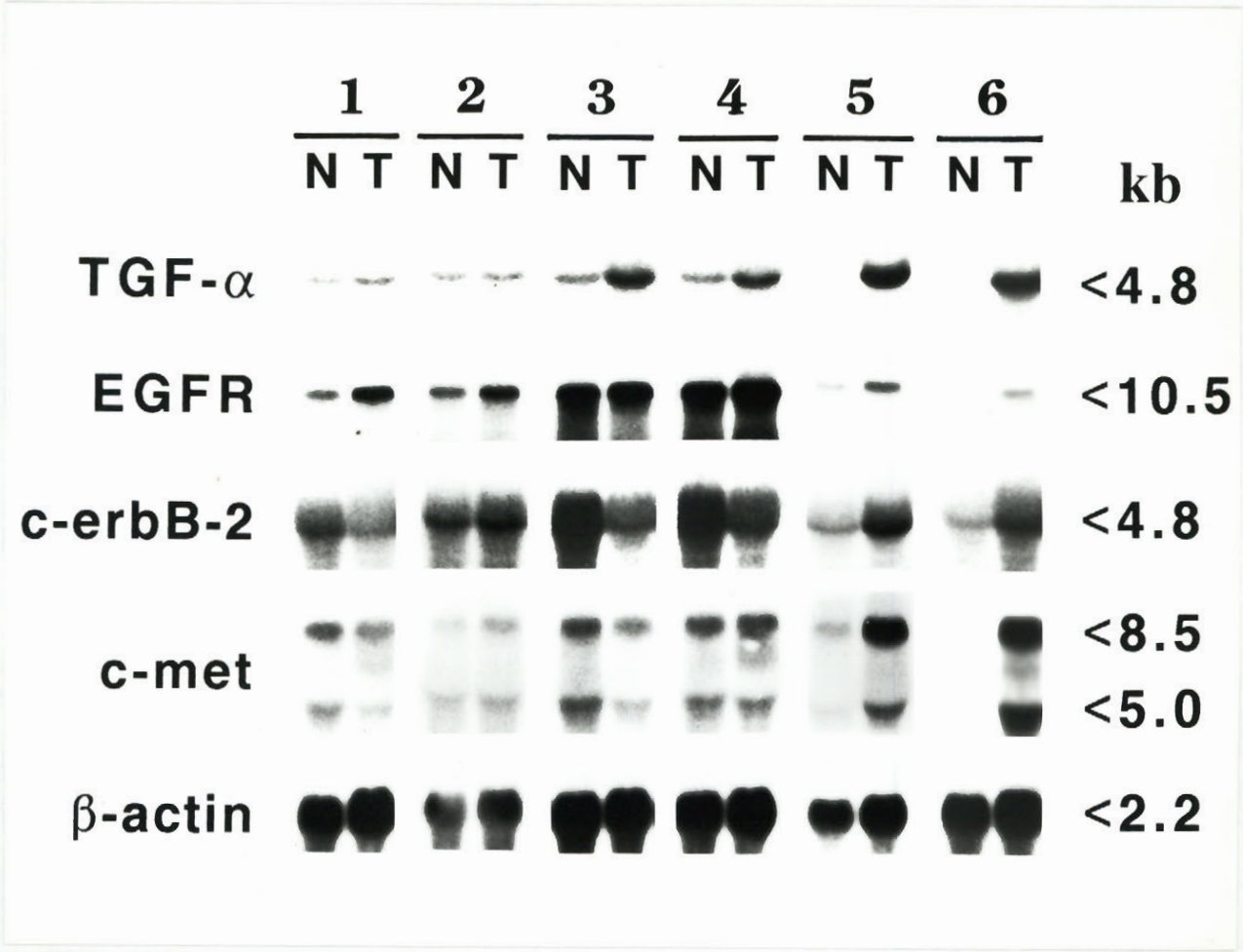


Figure 7-1 Representative Northern analysis showing the mRNA expression of TGF-α, EGFR, c-*erb*B-2 and c-*met* in 6 pairs of normal and neoplastic lung tissues. The cDNA inserts were isolated from phTGF-1-10-925 for TGF-α, pE7 for EGFR, and pCER204 for c-*erb*B-2 (ATCC, Rockville, MD). C-*met* mRNA was probed with a 1.1 kbp *EcoR*1 insert of human cDNA (Park et al, 1987). Human β-actin cDNA probe was obtained from Clonotech (Palo Alto, CA).

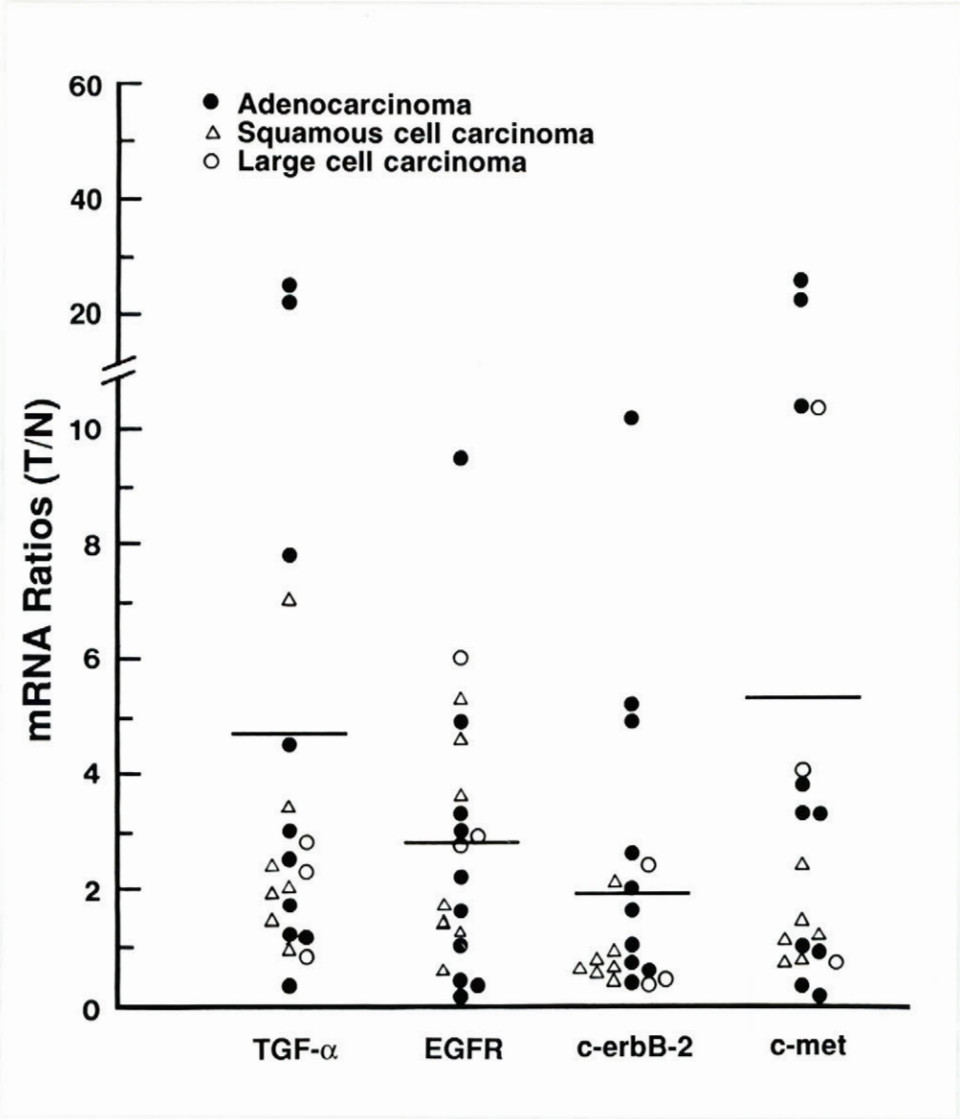


Figure 7-2 Distribution of the ratios for mRNA expression of TGF-α, EGFR, c-*erb*B-2 and c-*met* in carcinomas versus that in normal lung tissues. N: normal, T: tumor.

Table 7-1. Relative mRNA levels of TGF-α, EGFR, c-*erb*B-2 and c-*met* in normal and primary non-small cell carcinomas of the lung

	mRNA level (% of A431)							
	TGF-α	p	EGFR	p	c-*erb*B-2	p	c-*met*	p
Normal	0.44±0.08 (20)	0.007	0.10±0.03 (20)	0.015	0.70±0.12 (20)	ns	0.23±0.04 (20)	ns
Tumor	1.23±0.25 (29)		0.17±0.03 (29)		0.96±0.28 (29)		0.68±0.24 (29)	
ADC	1.66±0.47 (14)	ns	0.12±0.03 (14)	0.01	1.55±0.54 (14)	0.005	0.95±0.45 (14)	ns
SQC	0.97±0.17 (9)		0.26±0.06 (9)		0.44±0.06 (9)		0.18±0.04 (9)	
LCC	0.87±0.27 (6)		0.16±0.06 (6)		0.38±0.12 (6)		0.81±0.46 (6)	

All values represent the mean+s.e.m. (): indicates the number of specimens analysed. ADC: adenocarcinoma, SQC: squamous cell carcinoma, LCC: large cell carcinoma.

p values are calculated using the non-parametric Wilcoxon rank sum test, ns: non-significant.

Table 7-2. The distribution of cases according to the grade and stage of the tumors.

	Number of cases
Grade	
Well differentiated	3
Moderately differentiated	10
Poorly-differentiated[a]	16
Stage[b]	
I	9
II	10
III	10

[a] Includes the large cell carcinomas.

(b) Determined from the preoperative clinical data and pathological diagnosis.

Table 7-3. The clinicopathological findings of the four patients who died with metastatic disease within 12 months after lung resection

Case No.	Stage	Diagnosis	Phenotypic & Genotypic Changes
MGH-4	IIIa (T1N2)	Large cell undifferentiated	Amplified and over-expressed EGFR amplified c-*myc*
MGH-7	II (T2N1)	Epidermoid (p.d.)	Marked over-expression of EGFR in cultured tumor.cells
MGH-14	II (T1N1)	Large cell neuroendocrine	Neuroendocrine differentiation
MGH-23	II (T2N1)	Adeno (p.d.)	Marked over-expression of c-*erb*B-2 (8 folds > A431), and c-*met* (6.4 folds >A431)

p.d.: poorly-differentiated.

Although the expressions of TGF-α, c-*erb*B-2, EGFR and c-*met* have been studied in NSCLC cell lines (Kern, et al, 1990; Park, et al, 1987; 33-36), their mRNA expression in primary tumors have not been reported extensively. We have previously reported that primary human NSCLCs contain approximately two folds higher level of immunoreactive TGF-α molecule as compared to the normal lung tissue (Liu, et al, 1990). The present results indicates that the mean TGF-α mRNA level in NSCLC is also increased 2 to 3 folds as compared to the normal lung tissue, and this enhanced expression is not associated with the amplification of the TGF-α gene. Over-expression of the EGFR gene was commonly observed in squamous cell carcinomas (Veale, et al, 1987). The concurrent over-expression of TGF-α and EGFR in a majority of the NSCLCs supports the operation of an autocrine growth stimulatory mechanism in the proliferation of these tumor cells.

Predominant over-expression of both TGF-α and c-*erb*B-2 among adenocarcinomas of the lung may provide a molecular basis for previous reports that over-expressions of these two molecules correlated with poor prognosis in adenocarcinomas of the lung (Ishitoya, et al, 1989; Kern, et al, 1990). The biological significance of over-expression of the c-*met* in adenocarcinomas of the lung is unclear. A separate study on human NSCLC cell lines established from 8 of the tumors reported here also showed that higher levels of TGF-α, c-*erb*B-2 and c-*met* mRNAs were observed more consistently in adenocarcinoma cell lines (Chapter 5).

In conclusion, our studies demonstrated that over-expression of TGF-α, C-*erb*B-2 and c-*met* is more characteristic among adenocarcinomas, whereas EGFR tends to be expressed at a higher level in squamous cell carcinomas of the lung. Although the number of cases studied is too small to draw any

conclusion, the fact that all 3 patients with non-neuroendocrine tumors, who died of metastatic disease within 12 months after primary resection, over-expressed significantly one or more of these growth factor receptor genes suggests that their usefulness as markers of unfavorable prognosis in NSCLC patients should be further investigated.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

Both colorectal carcinomas and NSCLCs contain significantly higher amounts of immunoreactive TGF-α than do their corresponding normal tissues (4 times and 2 times respectively). The increased protein level of TGF-α in colorectal carcinomas is not associated with its mRNA over-expression, it occurs presumably due to the differential post-transcriptional modification between normal and tumor cells. In contrast, the increased level of TGF-α protein in human NSCLCs appears to take place at the transcriptional level, which is associated with significant over-expression of TGF-α mRNA unrelated to the amplification of the TGF-α gene. As to the role of TGF-α, it is presently not clear whether its over-expression is a primary event which causes neoplastic transformation, or is only secondary to the neoplastic proliferation. It has been suggested that the over-expression of TGF-α is not directly involved in the mechanism of multistep carcinogenesis, because:

(1) TGF-α is widely detected in human normal adult tissues at variable level. Its level varies among different segments of the colon and different lobes of the lung, and varies even among carcinomas of the same organs and histotypes (Coffey et al, 1987b; Zajcholowkis et al, 1988; Madtes et al, 1988; Liu et al, 1990) ;

2) adenomas from patients with familial polyposis coli (high risk of developing colon cancer) actually contain lower levels of TGF-α than that contained in the normal mucosa (Rothbauer et al, 1989);

3) introduction of the TGF-α gene into mouse keratinocytes from normal skin or papillomas does not cause the formation of carcinomas (Finzi et

al, 1988);

4) introduction of the *ras* gene into mouse fibroblasts mediates neoplastic transformation equally well in cells over-expressing EGFR and cells devoid of EGFR. The latter therefore does not respond to TGF-α (Mchay et al, 1986).

However, more recent publications have shown that transgenic mice over-expressing the TGF-α gene develop hepatocellular and breast carcinomas (Matsui et al, 1990; Sandgren et al, 1990; Ihappan et al, 1990). Even though the primary role of TGF-α remains speculative, the fact that this molecule and / or its receptor is frequently over-expressed in many tumor systems suggests that TGF-α does play important roles in the process of tumor progression. Most likely, TGF-α functions via an autocrine loop to confer on tumor cells a growth advantage over their surrounding normal cells. Moreover, TGF-α may also act through a paracrine mechanism to promote neovascularization (Schreiber et al, 1986) and the formation of tumor stroma.

Among the three tyrosine kinase receptors studied, only the c-*met* level is very low in the normal colonic mucosa and consistently increased in the colorectal carcinomas (mean 6 fold). Significant over-expression of c-*met* mRNA is also observed in colonic adenomas, suggesting that the activation of c-*met* proto-oncogene may be one of the important mechanisms contributing to the early stage of colonic carcinogenesis. Over-expression of c-*met* has recently been reported in gastric carcinomas and in some cases of preneoplastic gastritis (Soman et al, 1991), and the . authors suggest that activation of c-*met* is involved in the early stage of gastric carcinogenesis. Their results together with our findings on c-*met* expression in colorectal carcinomas suggest that the activation of c-*met* proto-oncogene may be an important mechanism in the early stage of carcinogenesis involving

gastrointestinal epithelium. C-*met* may also act as a potential tumor marker for carcinomas of gastrointestinal mucosa. In contrast to the consistent over-expression of c-*met* in colorectal carcinomas, 2 times more over-expression of c-*met* mRNA is detected only in 42% of the NSCLCs.

Hepatocyte growth factor (HGF), the ligand for c-*met* , has been purified from serum, placenta, plasma and platelets. The expression of HGF in rat tissues has been demonstrated in lung, kidney, brain, thymus and liver (Tashiro et al, 1990), but has not been reported in the gastrointestinal epithelium. We have performed Northern blot analysis on the HGF expression in the same group of lung and colon samples, but failed to detect any signals. It is not clear whether the expression pattern of HGF in colonic and lung tissues resembles that in liver where HGF produced by the non-parenchymal cells acts on the hepatocytes through a paracrine loop. Another possibility is that HGF may be secreted into the blood stream and exert its action on the whole body through an endocrine pathway. To solve this problem, immunohistochemical staining of HGF on colonic and lung tissues is required to assess its autocrine / paracrine function. In addition, measuring the blood concentration of HGF in both normal individuals and patients will provide an important rationale for assessing an endocrine function of this molecule and its potential clinical applications. Moreover, it is also possible that the c-*met* molecules over-expressed in cancerous tissues represent constitutively activated receptors independent of ligand-stimulation. Receptor tyrosine kinase assays would be useful in solving this issue.

Significant over-expression of both TGF-α and EGFR is demonstrated in human NSCLCs, indicating that this growth factor / receptor system constitutes an autocrine loop commonly involved in the proliferation of NSCLC cells. While adenocarcinomas express higher levels of TGF-α, c-

*erb*B-2 and c-*met*, epidermoid carcinomas tend to express higher levels of EGFR mRNA. This cell-type associated gene expression pattern indicates that different genes function primarily to regulate the growth and differentiation of different types of cells. Both TGF-α and c-*erb*B-2 are predominantly associated with adenocarcinomas of the lung, providing a molecular basis for previous reports that the over-expression of these two molecules correlates with a poorer prognosis in this histotype in comparison with the other histotypes of lung cancer. Unfortunately, we failed to observe any significant correlation between the mRNA levels of the genes tested (TGF-α, EGFR, c-*erb*B-2 and c-*met*) and the grade, and stage of the primary tumors. We also did not find any correlation between the level of their expression and the degree of inflammatory and desmoplastic reactions. The latter suggests that the presence of non-neoplastic cells in the primary tumors does not significantly influence the mRNA levels of these genes. In contrast to frequent mRNA over-expression of the genes tested in primary human colorectal and lung carcinomas, gene amplification is a rare event in these tumors.

Eight new cell lines have been established from 29 non-selective primary NSCLCs. The success rate is 24% for adenocarcinomas, 14% for epidermoid carcinomas, 50% for poorly differentiated carcinomas, and 7% for well or moderately differentiated carcinomas. The factors which determined the ability of the tumor cells in primary NSCLCs to continuously proliferate and form cell lines include:

(1) The ability of NSCLCs to express, synthesize or respond to a specific set or sets of growth factors and / or their receptors. Our results on the growth capacity of the established cell lines in various media indicate clearly that different cells required different external stimuli for their proliferation. Our mRNA analysis on the gene expression of TGF-α, EGFR, c-*erb*B-2

and c-*met* have demonstrated that a high level of TGF-α and c-*met* expression in the primary tumors appear to correlate with their facility in forming propagable cell lines in vitro. As mentioned above, adenocarcinomas tend to express higher levels of TGF-α and c-*met* as compared to other histotypes of the human NSCLC, thus explaining why adenocarcinomas form cell lines more readily in culture than do the epidermoid carcinomas;

(2) The facility of cells to differentiate terminally. We have demonstrated that cell lines are more readily established from poorly differentiated than from well and/ or moderately differentiated carcinomas. This result is consistent with the knowledge that the less differentiated cells are generally more proliferative, and the terminally differentiated cells are less proliferative, as they have achieved stability.

Five of the eight cell lines injected into nude mice formed xenograft tumors. Their tumorigenecity is correlated with a high expression of the c-*myc* mRNA. The xenograft tumors formed by cell lines demonstrate remarkable histological similarities to their corresponding primary tumors. However, when the expression pattern of tested genes in cell lines is compared to that in their corresponding primary and xenograft tumors, we find that tumor cells grown in culture usually undergo a selection which allows only a subpopulation of cells with specific gene expression to proliferate continuously as a cell line. Thus, tumor cells growing as a cell line are not fully representative of the heterogeneous cell population in the primary tumors. Investigators should be aware of this finding, and take it into consideration when they interpret their data on the phenotypic studies of human NSCLCs using propagable cell lines and on the use of cultured tumor cells to evaluate sensitivity to the chemotherapeutic agents.

CONCLUSIONS

(1) Both colorectal and lung carcinomas contain significantly increased amounts of immunoreactive TGF-α as compared to their normal counterparts. This increase in majoriety of the lung carcinomas is associated with the over-expression of TGF-α mRNA, whereas in only a small percentage of colorectal carcinomas it is;

(2) While the mRNA levels of EGFR and c-*erb*B-2 in colorectal carcinomas do not differ significantly from that in the normal colonic mucosa, c-*met* is consistently over-expressed in the neoplastic tissues;

(3) C-*met* is also over-expressed in colonic adenomas, suggesting that the activation of c-*met* proto-oncogene plays a mechanistic role in the early stage of colorectal carcinogenesis;

(4) Both TGF-α and EGFR are significantly over-expressed in NSCLCs as compared to the normal lung tissues. Among various histotypes of NSCLC, adenocarcinomas express higher levels of TGF-α, c-*erb*B-2 and c-*met*, whereas epidermoid carcinomas express higher levels of EGFR mRNA;

(5) High mRNA expression of TGF-α and c-*met* in primary tumors is correlated with their facility to form propagable cell lines in culture;

(6) High expression of c-*myc* mRNA in cell lines is correlated with the xenotransplantability of cell lines into nude mice;

(7) Tumor cells after proliferating for a prolonged time in culture often undergo phenotypic and/or genotypic selection, which results in them being not fully representative of the heterogeneous tumor cell population in the primary tumors.

CHAPTER 9 CLINICAL SIGNIFICANCE

The significant structural homology between the viral oncogene products and the growth factors / receptors suggests that the latter play important roles in the pathogenesis of neoplasms. The identification of specific growth factors / receptors in association with particular tumors will allow us to gain a more complete understanding of the molecular pathogenesis of human cancer. It will also provide a rationale to assess the clinical potential of growth factors / receptors as possible tumor markers, or prognostic factors, and furthermore, to develop more effective therapeutic strategies. As discussed above, the mRNA level of c-*met* is consistently low in normal mucosa, and is significantly elevated in both colonic adenomas and carcinomas. These findings suggest that:

(1) the activation of c-*met* proto-oncogene is an important mechanism involving the early stage of colonic carcinogenesis and subsequent tumor progression;

(2) c-*met* is a possible marker for colon cancer and may be useful in the early diagnosis of colonic carcinomas;

(3) the over-expression of c-*met* may account for, at least partially, the high proliferative activity associated with both colonic adenomas and carcinomas. In this regard, c-*met* is of therapeutic significance. Antibody generated against c-*met* should be able to inhibit c-*met* mediated hyper-proliferation of cells in a cell-type specific manner since c-*met* is a cell surface molecule.

C-*met* may also have a prognostic value which, however, needs to be further explored by using a large sample size and a long period of follow-up on patients.

TGF-α is significantly over-expressed in both colonic and lung carcinomas, and also in many other tumors as previously reported, suggesting that this molecule is indicative of high proliferative activity commonly observed in cancer cells. The potential therapeutic significance of TGF-α and its receptor (EGFR) is currently under investigation. Advances have been made in both immunotherapy and gene therapy. Collectively, high proliferative activity mediated by TGF-α / EGFR may be suppressed by the application of:

(1) monoclonal antibody to EGFR, which has been shown to inhibit the growth of tumor cells both *in vitro* and *in vivo*;

(2) monoclonal antibody to TGF-α, which is capable of inhibiting TGF-α stimulated proliferation of A549 human lung adenocarcinoma cells;

(3) reagents that mimic the structure of TGF-α, and compete with TGF-α for binding to the EGFR;

(4) toxins conjugated with TGF-α, which kill tumor cells upon the binding of growth factors to their receptors, in a cell-type specific manner. In fact, several TGF-α-toxin conjugates have been engineered; they kill tumor cells which express EGFR both *in vitro* and *in vivo* (Berger et al, 1991);

(5) application of antisense TGF-α mRNA, which specifically bind to, and inactivate sense mRNA by preventing its translation into protein. It has been shown that introduction of human TGF-α antisense RNA inhibits the estrogen-stimulated production of TGF– α and also inhibits the growth of MCF-7 human breast cancer cells (Saeki et al, 1991).

Despite the progress has been made in developing and characterizing these new agents, there is no doubt that the final investigation has to be clinical trial. It is essential to determine the toxicity, side effects and optimal method and schedule of the administration of these agents before they can be

used in humans.

Cancer cell lines have been used extensively to study the biological and molecular aspects of human cancer, especially to study the sensitivity of tumor cells to the chemotherapeutic agents. However, a comparative study of gene expression in cell lines, primary tumors from which cell lines are derived, and the xenograft tumors formed by cell lines, indicates that cell lines are not always phenotypically and genotypically representative of the primary tumors. This should be taken into consideration when investigators interpret their data obtained from using cell lines as the only study of human tumors.

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APPENDIX A

The Staging of Cancer

AMERICAN JOINT COMMITTEE ON CANCER

Staging for Lung Carcinoma (3rd edition)

T = Primary Tumor **N** = Regional Lymph Nodes **M** = Distant Metastasis

PRIMARY TUMOR (T)

TX Primary tumor cannot be assessed, or tumor proven by the presence of malignant cells in sputum or bronchial washings but not visualized by imaging or bronchoscopy

T0 No evidence of primary tumor

Tis Carcinoma in situ

T1 Tumor 3 cm or less in greatest dimension, surrounded by lung or visceral pleura, without bronchoscopic evidence of invasion more proximal than the lobar bronchus,* (i.e., not in the main bronchus)

T2 Tumor with any of the following features of size or extent: More than 3 cm in greatest dimension
Involves main bronchus, 2 cm or more distal to the carina
Invades the visceral pleura
Associated with atelectasis or obstructive pneumonitis that extends to the hilar region but does not involve the entire lung

T3 Tumor of any size that directly invades any of the following: chest wall (including superior sulcus tumors), diaphragm, mediastinal pleura, parietal pericardium; tumor in the main bronchus less than 2 cm distal to the carina* but without involvement of the carina; or associated atelectasis or obstructive pneumonitis of the entire lung

T4 Tumor of any size that invades any of the following: mediastinum, heart, great vessels, trachea, esophagus, vertebral body, carina; or tumor with a malignant pleural effusion†

***NOTE:** The uncommon superficial tumor of any size with its invasive component limited to the bronchial wall, which may extend proximal to the main bronchus, is also classified T1.

†**NOTE:** Most pleural effusions associated with lung cancer are due to tumor. However, there are a few patients in whom multiple cytopathologic examinations of pleural fluid are negative for tumor. In these cases, fluid is non-bloody and is not an exudate. When these elements and clinical judgment dictate that the effusion is not related to the tumor, the effusion should be excluded as a staging element and the patient should be staged T1, T2, or T3.

REGIONAL LYMPH NODES (N)

NX Regional lymph nodes cannot be assessed

N0 No regional lymph node metastasis

N1 Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes, including direct extension

N2 Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)

N3 Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)

DISTANT METASTASIS (M)

MX Presence of distant metastasis cannot be assessed

M0 No distant metastasis

M1 Distant metastasis

STAGE GROUPING

Occult carcinoma	TX	N0	M0
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
	T2	N0	M0
Stage II	T1	N1	M0
	T2	N1	M0
Stage IIIA	T1	N2	M0
	T2	N2	M0
	T3	N0, N1, N2	M0
Stage IIIB	Any T	N3	M0
	T4	Any N	M0
Stage IV	Any T	Any N	M1

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