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LOSS OF HETEROZYGOSITY ANALYSIS OF C-*MET* AND AN ADJACENT LOCUS, D7S95, IN HUMAN NON-SMALL CELL LUNG CARCINOMA

Darryl Jordan Gebien

Department of Pathology

McGill University, Montreal

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In Memory of My Uncle,

Tom Shub

Abstract

The c-*met* proto-oncogene encodes a tyrosine kinase receptor for hepatocyte growth factor/scatter factor. Losses of c-*met* alleles have been documented in human carcinomas. In breast carcinoma c-*met* has been suggested to be a potential inactivated tumour suppressor gene (TSG). In non-small cell lung carcinoma (NSCLC) a general reduction of c-*met* expression in squamous cell carcinomas (SQCCs), as well as overexpression in adenocarcinomas (ADCs) have been revealed. Furthermore, many SQCCs and ADCs had undetectable c-*met* mRNA and protein. These results prompted us to explore c-*met* allelic alterations in NSCLC. Our main goal was to address the possible involvement of inactivated c-*met* alleles in the development and progression of NSCLC. In this LOH analysis two polymorphic-sensitive probes corresponding to the c-*met* gene, p-metH and p-metD, were used to analyze normal and tumour DNA samples from patients with primary NSCLC. Of 110 cases examined with p-metH, 56 (50.9%) were informative and 4 (7.1%) exhibited LOH. Among 109 patients examined with p-metD, 28 (25.7%) were informative and 1 (3.6%) was LOH-positive. The combined LOH incidence for c-*met* was 7.4% (5/68 informative cases). Lastly, since a locus adjacent (distally) to c-*met* on chromosome 7, D7S95, was shown to exhibit a significant LOH frequency in gastric carcinoma (43.3%), we also sought to determine if loss of this locus was common in NSCLC. Of 104 patients examined at D7S95, 45 (43.3%) were informative and 1 (2.2%) exhibited LOH. These findings suggest, firstly, that c-*met* does not have a possible role as an inactivated TSG in the tumourigenesis of NSCLC nor is it closely linked to a putative TSG, and secondly, D7S95 does not contain a detectable inactivated TSG or a closely-mapping TSG in NSCLC.

Résumé

Le c-*met* proto-oncogène encode un récepteur tyrosine kinase pour le facteur croissance/facteur dispersion de l'hépatocyte. Des pertes d'allèles c-*met* ont été documentées dans des carcinomes humains. Dans le carcinome du sein c-*met* a été suggéré comme étant un gène supprimeur de tumeur (TSG) potential. Dans le carcinome des non-petites cellules du poumon (NSCLC) une réduction générale dans l'expression du c-*met* a été révélée dans les carcinomes à cellules 'squameuses' (SQCCs) et surexprimé dans les adenocarcinomes (ADCs). Par ailleurs, plusieurs SQCCs et ADCs avaient du mARN et de la protéine c-*met* non détectable. A partir de ces résultats, nous avons décidé d'explorer les altérations alléliques dans le NSCLC. Notre principal but était d'adresser la possibilité d'une participation d'allèles c-*met* inactivées dans le développement et la progression du NSCLC. Dans cet analyse LOH (perte d'hétérozygocité), deux sondes à sensibilité polymorphique correspondant au gene c-*met*, p-metH et p-metD, ont été utilisé pour analyser des échantillons d'ADN normaux et tumoraux de patients atteints de NSCLC primaire. Des 110 cas examinés avec p-metH, 56 (50.9%) étaient informatifs et 4 (7.1%) présentait une LOH. Parmi les 109 patients examinés avec p-metD, 28 (25.7%) étaient informatifs et 1 (3.6%) présentait une LOH. L'incidence combiné du LOH pour c-*met* était 7.4% (5/68 cas informatifs). Finalement, puisqu'il fut démontré qu'un locus adjacent à c-*met* sur le chromosome 7, D7S95, présentait une fréquence LOH significative dans le carcinome gastrique (43.3%), nous avons vérifié si la perte de cet du locus était commun dans le NSCLC. Des 104 patients examinés à D7S95, 45 (43.3%) étaient informatifs et 1 (2.2%) présentait une LOH. Ces résultats suggèrent, premièrement, que c-*met* n'a pas de rôle possible en tant que TSG dans la tumorigénèse du NSCLC et qu'il n'est pas étroitement lié à un TSG putatif, et en second lieu, D7S95 ne contient pas un TSG inactivé détectable ou un TSG 'closely-mapping' dans le NSCLC.

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Lastly, this thesis is dedicated to my uncle, who passed away from cancer in 1982. His profound love and influence helped shape who I have become.

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1. INTRODUCTION

1.1 Lung Cancer Incidence and Mortality

In the Western world, cancer is presently the second leading cause of death next to heart disease. In Canada in 1995, it is estimated that 125,000 new cases were diagnosed and 61,500 deaths resulted from cancer (1). Lung cancer is the most common lethal cancer in the Western world, as it accounts for a third of cancer deaths in men and one-fifth in women. It occurs most frequently between the ages of 40 and 70 and has a peak incidence in the sixth and seventh decades. The incidence of lung cancer is rising in both men and women . Although it was regarded as a rare tumour as late as 1945, carcinoma of the lung now occurs in epidemic proportions. In the United States, the annual number of deaths from lung cancer increased from 18,000 in 1950 to an estimated 153,000 in 1994 (2). Its overall 5-year survival rate is a low 3-15%, depending on the particular subtype of lung neoplasm (49). As a result of its increased incidence and mortality, as well as its poor prognosis, efforts to elucidate the cause and pathogenesis of lung carcinoma were initiated as early as the 1960's. Although the main causes of lung cancer have since been identified (e.g., tobacco smoke, air pollution, certain industrial hazards), and diagnostic, prognostic and therapeutic measures have been refined, the overall outlook for lung cancer patients has not measurably improved over the last 30 years. At present then, the only hope for controlling the disease lies in learning more about its biology, particularly at the molecular level. This may

translate into improved methods of prevention, early detection and treatment to increase the survival of lung cancer patients.

1.2 Pathology of Lung Cancer

The majority of lung cancers arise centrally from the major bronchi of the lung and in general, the upper lobes are more commonly involved than the lower, and the right lung more than the left (61). Most of these originate from the bronchial or bronchiolar epithelium, and are therefore termed 'bronchogenic carcinomas'. Since these constitute 95% of all lung cancers (49) and are the number one cause of cancer-related death in industrialized nations, most studies have focused on improving knowledge of these malignancies, including this thesis.

Bronchogenic carcinomas begin as an area of in situ cytologic atypia that gives rise to a small area of thickening or elevation of the bronchial mucosa. Atypia may include squamous metaplasia, basal cell hyperplasia and dysplasia of the bronchial epithelium (61). The mucosal lesions, which are located in and about the hilus of the lung, may then follow one of several patterns of growth. They may form intraluminal masses; they may penetrate the bronchial mucosa to infiltrate along the peribronchial tissue; or they may form large bulky masses that push into adjacent lung parenchyma. These tumours may then extend to the pleura, followed by spread into the pleural cavity and chest wall, and possibly to adjacent intrathoracic structures. As the neoplasm develops, tumour cells may disseminate (or *metastasize*) via the lymphatics or blood, frequently reaching the regional lymph nodes as well as the liver, brain, bones and

adrenals. In fact, almost every organ of the body can be affected. Due to the propensity of lung cancer cells to disseminate early during tumour development, this carries a very bad prognosis for the patient.

Once a lung tumour has been detected and diagnosed, the main choices available for treatment include chemotherapy, radiotherapy and surgical resection. For most advanced lung carcinoma cases however, palliative care is the only option. Therapeutic strategies, which are designed using a combination of the above treatments, are generally determined by certain features of the tumour in question. These include histological subtype, extent of invasion and spread to regional lymph nodes, as well as the presence/absence of distant metastases. General physical conditions of the patient are also important in making therapeutic decisions.

1.3 Diagnosis and Classification of Lung Cancer

Although clinical symptoms and radiography may suggest the presence of a lung tumour, a firm diagnosis is presently based on microscopic examination of biopsy materials or cell/tissue samples. Specimens for cytologic study are obtained from either sputum, pleural effusions, fine-needle aspiration biopsy or biopsy using a bronchoscope. These are examined for cellular characteristics such as nuclear-cytoplasmic ratio and nuclear pleomorphism. From these and other features, normal cells may be distinguished from neoplastic, and ideally, benign conditions from malignancy (61). During the post-war years it was recognized lung cancers could be subdivided into several different entities of various cellular origin. In 1967, a lung cancer classification scheme was published by the

World Health Organization (WHO) and has since been updated in 1982 (3). It provided histological criteria for the categorization of lung tumour specimens and has served as a baseline for diagnosis. The main advantage of subtyping lung cancers was that since tumours sharing the same histological subtype were known to exhibit similar behaviours, choice of the most appropriate therapeutic strategy for a given tumour could be *inferred* (in part) from its histological characteristics. This would include comparing its features with a WHO-standardized database containing the features, therapeutic measures and clinical outcomes of patients with similar tumours.

The WHO classification scheme, which includes more than 40 categories of lung neoplasms, divides bronchogenic carcinoma into seven major histological subtypes (see Section I. C. in Table 1). Those important to this study include epidermoid or squamous cell carcinoma (SQCC), adenocarcinoma (ADC), large cell carcinoma (LCC), adenosquamous carcinoma (ADSQC) and carcinoid tumours. These subtypes (or *histotypes*) are categorized broadly as non-small cell lung carcinoma (NSCLC). NSCLC is distinguished from small cell lung carcinoma (SCLC) for specific reasons— tumours of these two categories show striking differences in morphology, biochemical and molecular properties, as well as clinical behaviour, response to therapeutic modalities and prognosis (61). NSCLC comprises approximately 70-75% of bronchogenic carcinomas whereas SCLC comprise 20-25% (49).

Table 1- Revised WHO histological classification of lung tumours (1982).

I. Epithelial tumours

A. Benign

1. Papillomas
   a. Squamous cell papilloma
   b. 'Transitional' papilloma
2. Adenomas
   a. Pleomorphic adenoma ('mixed' tumour)
   b. Monomorphic adenoma
   c. Others

B. Dysplasia
Carcinoma *in situ*

C. Malignant (i.e., bronchogenic carcinoma)

1. Squamous cell carcinoma (epidermoid carcinoma)
   Variant:
   a. Spindle-cell (squamous) carcinoma
2. Small cell carcinoma
   a. Oat-cell carcinoma
   b. Intermediate cell type
   c. Combined oat-cell carcinoma
3. Adenocarcinoma
   a. Acinar adenocarcinoma
   b. Papillary adenocarcinoma
   c. Bronchiolo-alveolar carcinoma
   d. Solid carcinoma with mucus formation
4. Large cell carcinoma
   Variants:
   a. Giant-cell carcinoma
   b. Clear-cell carcinoma
5. Adenosquamous carcinoma
6. Carcinoid tumour
7. Bronchial gland carcinomas
   a. Adenoid cystic carcinoma
   b. Mucoepidermoid carcinoma
   c. Others
8. Others

II. Soft-tissue tumours

III. Mesothelial tumours

A. Benign mesothelioma
B. Malignant mesothelioma
   1. Epithelial
   2. Fibrous (spindle-cell)
   3. Biphasic

IV. Miscellaneous tumours

A. Benign
B. Malignant
   1. Carcinosarcoma
   2. Pulmonary blastoma
   3. Malignant melanoma
   4. Malignant lymphomas
   5. Others

V. Secondary tumours

VI. Unclassified tumours

VII. Tumour-like lesions

A. Hamartoma
B. Lymphoproliferative lesions
C. Tumourlet
D. Eosinophilic granuloma
E. 'Sclerosing haemangioma'
F. Inflammatory pseudotumour
G. Others

-adapted from The World Health Organization Histological Typing of Lung Tumours, 2nd edition, (3).

1.3.1 Non-Small Cell Lung Carcinoma (NSCLC)

Squamous cell carcinoma, the most common lung cancer histotype in industrialized nations, is found more commonly in men than women and is tightly correlated with a smoking history (18). This tumour arises in the larger, more central bronchi, tends to spread locally and metastasizes later than the other subtypes of bronchogenic carcinoma. In contrast to these however, its growth rate is usually more rapid. SQCCs exhibit various degrees of differentiation, ranging from well-differentiated squamous cell neoplasms to poorly differentiated with residual squamous cell features, and if untreated, their median survival is less than a year. However, since they tend to develop into large, bulky, centrally obstructing symptomatic masses before metastasis, they are usually surgically resectable (in 60% of cases) and therefore have a better prognosis than the other subtypes (61).

Adenocarcinoma is the most common type of lung cancer in women and non-smokers (18). This lesion refers either to a tumour that forms gland like structures and/or to one that contains mucin-producing cells, as demonstrated by Alcian blue/PAS staining. ADCs are usually located in the lung periphery, tend to be smaller and vary histologically from well-differentiated tumours with glandular features, through papillary lesions, to solid masses with occasional gland structures. ADCs grow slower than other bronchogenic carcinoma subtypes but tend to metastasize widely at an early stage. Only 40% of cases can be resected (61).

Large cell carcinoma constitute a group of malignant epithelial tumours that lack cytologic differentiation and most likely represent squamous cell or glandular neoplasms that are too poorly differentiated to permit categorization (3). LCCs are either composed of anaplastic large cells, clear cells, spindle-shaped cells or sometimes, giant cells. Some show neuroendocrine differentiation. LCCs tend to be large and bulky with substantial necrosis and hemorrhage, and are generally located peripherally. They are characteristically aggressive because of their tendency to metastasize at an early stage, and therefore exhibit poor prognosis.

Bronchial carcinoid tumours, representing 1 to 5% of all lung tumours, were initially classified as 'bronchial adenomas', (18). Unlike benign tumours however, these neoplasms are now known to be often locally invasive and occasionally capable of metastasis. Histologically, they show neuroendocrine differentiation. Although neuropeptides are readily demonstrated in the tumour cells, the large majority are endocrinologically silent (85). Carcinoids frequently arise in young adults, appear to have no association with cigarette smoking and have equal incidences for both sexes.

1.3.2 Small Cell Lung Carcinoma (SCLC)

Small cell lung carcinoma is more common in men than women and has a strong relationship with cigarette smoking (49). This highly malignant tumour, which originates near the hilum or central portions of the lung, is characterized by sheets of small, round, malignant cells with scant cytoplasm. SCLCs are rapidly growing lesions that tend to infiltrate widely and disseminate early in their course. Surgery is therefore not an option, however, a combination of radiotherapy and chemotherapy is used for treatment.

1.3.3 Combined Patterns

A small proportion of bronchogenic carcinomas display more than one line of cellular differentiation. The most frequent combined patterns are mixed squamous cell with adenocarcinoma (so-called *adenosquamous carcinoma*) and mixed squamous with small cell carcinoma. In such cases, many pathologists base their diagnosis on the predominant tumour type present, arguing this is what determines the prognosis of the patient. Mixed tumours are most commonly seen in multiple sections of resected specimens, or, at post-mortem.

1.4 Lung Cancer Staging

Aside from the histologically based WHO classification system, lung cancers are also categorized, for clinical reasons, with respect to degree of tumour dissemination (i.e., staging). This includes tumour size, extent of spread to regional lymph nodes and presence/absence of distant metastases. The most widely used staging method for lung cancer is the TNM system (T: primary tumour size; N: regional lymph node involvement; M: metastases), adopted by the American Joint Committee on Cancer and the Union Internationale Contre le Cancer. TNM information is based on clinical and radiographic examination, and in some cases surgical exploration. Staging provides a basis for treatment choice and also serves as a prognostic factor.

1.5 Poor Prognosis of Lung Cancer: Demand for Molecular Markers

In general, NSCLC tumours tend to remain localized longer and have a slightly better prognosis than SCLC tumours, which are usually advanced lesions by the time they are discovered. Early detection of NSCLC may facilitate cure by lobectomy or pneumonectomy. In contrast, except for cases with very localized disease, surgical resection of SCLC is so ineffective that the diagnosis essentially precludes surgery. If untreated, the survival time for patients with SCLC is 6 to 17 weeks, however, since this cancer is sensitive to chemotherapy, potential cure rates have been reported in some centers to range from 15 to 25% for limited disease cases (18).

Nonetheless, the overall 5-year survival rate for patients with bronchogenic carcinoma is low, (with the exception of carcinoid tumours). In males it is approximately 10% for ADC and SQCC, and a grim 3% for LCC and SCLC cases (18).

Ideally, the best way to reduce the overall mortality from lung cancer is through screening for pre-malignant cells/tissues. However, this is presently not possible nor cost-effective. An alternative approach is to identify factors which may provide additional diagnostic and prognostic value, independent of the conventional histopathological diagnostic/prognostic parameters. These may enable better assessment of tumour behaviour, thereby enhancing the effectiveness of therapeutic strategies.

Certain recent lung cancer studies are attempting to identify *biochemical* markers exhibited by tumour cells. In particular, it is hoped markers of cell proliferation and different aspects of differentiation will provide novel prognostic and diagnostic factors. They may supply additional tumour information to that obtained by the histopathological parameters. Together, this knowledge may facilitate improved understanding of lung tumour behaviours.

Other lung cancer studies are focusing on consistent genetic changes that accompany tumourigenesis (i.e., molecular biology/ pathology). It is anticipated such DNA alterations will serve as *molecular* markers, providing additional criteria for the subtyping and staging of lung tumours as they progress towards malignancy. They may serve as prognostic factors as well. In the long term, other molecular markers may be used to screen the human population for individuals genetically-predisposed to lung cancer. Moreover, if the genetic abnormalities reflected by the markers are

verified to be involved in development of this disease, it is possible novel therapies could be designed based on these genetic markers.

From the voluminous data collected recently concerning altered genes in human cancer and the potential practical implications of molecular markers, it seems likely this system will be at the forefront of future cancer research as well as oncological medicine. Before discussing the genetic changes involved in the pathogenesis of lung cancer however, the molecular basis of cancer must be described. This next section will also discuss how certain of the cancer-implicated genes are *identified* in human neoplasms and will further explore their *practical uses* in clinical medicine.

1.6 The Molecular Basis of Cancer

> *The unlimited tendency to rapid proliferation in malignant tumour cells [could result] from a permanent predominance of the chromosomes that promote division....Another possibility [to explain cancer] is the presence of definite chromosomes which inhibit division....Cells of tumours with unlimited growth would arise if those 'inhibiting chromosomes' were eliminated....[Since] each kind of chromosome is represented twice in the normal cell, the depression of only one of these two might pass unnoticed.....*
>
> —Boveri, 1914; translation published in 1929

Based on over 200 years of deduction and scientific investigation, it is overwhelmingly evident cancer is a cellular disease arising from an accumulation of changes in the normal DNA sequence of a cell's chromosomes. Some of these changes, or *mutations*, may be inherited through the germline of an individual (germinal mutations), while most are acquired by the action of carcinogenic agents, including viruses, a wide

variety of chemicals and both ionizing and ultraviolet radiation, (somatic mutations) (98). All carcinogens share an important biological property—they either damage or alter DNA. This includes point mutations, insertions, deletions, translocations and gene amplifications.

Evidence from numerous cancer studies strongly suggest cellular transformation, an early stage in tumourigenesis, involves alterations that affect genes normally responsible for regulating cell growth (or differentiation) (7,12). Upon mutation of the growth-controlling genes, the expression or biochemical function of their encoded proteins may become distorted or lost completely. This gives rise to loss of critical regulatory mechanisms and deregulated cell growth may then ensue (98). It is thought that over time, further mutations occur in the developing population of transforming cells. Some may be lethal or place the cell at a disadvantage with respect to its neighbours (causing the cell to disappear from the tumour population), while others may convey greater autonomy and a selective growth advantage. These more malignant cells will tend to become dominant in the population, leading to tumour progression. Although it is currently a controversial matter whether an alteration of a specific gene is responsible for a particular step of tumour progression, one thing is for certain— accumulation of genetic aberrations is at the crux of oncogenesis (7,11,12,47,59,108).

Two classes of regulatory genes are the principal targets of genetic damage in cancer. These include growth-promoting proto-oncogenes and growth-inhibiting tumour suppressor genes (TSGs), (also known as anti-oncogenes). Mutant alleles of proto-oncogenes (or *oncogenes*) are considered dominant because they transform cells despite the presence of their normal counterpart. In contrast, both normal alleles of TSGs must be

lost or damaged for transformation to occur, thus they are of a recessive nature. Activation of oncogenes and inactivation of TSGs are considered necessary for tumour development (8). To note, tumour formation also requires disorders in the interactions between neoplastic cells and their surroundings, resulting in invasion and metastasis (59). Changes at several different genes, including both proto-oncogenes and TSGs, have been shown to commonly occur within individual tumours. In addition, these alterations appear to be strongly conserved in most types of cancer (117).

1.6.1 Oncogenes

With an activating alteration, the conversion of a cellular proto-oncogene to an oncogene is accompanied by either: 1) increased gene expression, resulting in the over-synthesis of a structurally normal growth-promoting oncoprotein; or 2) creation of a growth-promoting oncoprotein product with abnormal structure and enhanced function, (i.e., constitutive activation). Overexpression is usually due to an amplification or translocation of a proto-oncogene, and constitutively activated oncoproteins are generally produced by a translocation or by a point mutation within the open reading frame of the proto-oncogene (8). In both cases, genetic aberrations of proto-oncogenes— genes normally responsible for growth promotion— give rise to abnormal or abnormally expressed proteins that are involved in processes leading to transformation of normal cells to a malignant state. Examples of overexpression resulting from gene amplification include the N-*myc* gene in neuroblastoma (7) and the c-*neu* gene in breast cancer (49), while an example of overexpression resulting from *translocation* includes the increased *myc* protein levels in Burkitt's

lymphoma (98). It is caused by a rearrangement of the c-*myc* gene on chromosome 8 to a chromosome 14 immunoglobulin gene that exhibits very high transcriptional activity. An example of a constitutively activated oncoprotein created by *translocation* is the product of the chimerical c-*abl-bcr* Philadelphia chromosome in chronic myelogenous leukemia (CML) (7). It has abnormally high tyrosine kinase activity and is produced by reciprocal fusion of a portion of the c-*abl* proto-oncogene on chromosome 9 to chromosome 22 and the *bcr* locus on chromosome 22 to chromosome 9. An example of an oncoprotein produced by *point mutation* is the abnormally active *ras* protein produced by substitution of a single base pair in the H-*ras* gene (49).

The potential of oncogenes to participate in tumourigenesis arises from the fact that oncoproteins, similar to their normal counterparts (i.e., proto-oncogene-encoded proteins), are relays in the elaborate biochemical signalling networks that govern cell phenotype, including regulation of cell proliferation. The identities of proto-oncogene products and oncoproteins range from extracellular growth factors and cell surface growth factor receptors, through cytoplasmic protein kinases and GTP-binding proteins, to nuclear transcription factors (7). However, in contrast to their normal counterparts, which are tightly regulated in their expression and function, oncoproteins are not. Thus, they may potentially wreak havoc in the otherwise well-controlled, homeostatic signalling network. For instance, many oncoproteins constitutively activate the signalling pathways used by normal cells for stimulating proliferation. As a result, these proteins induce a cellular state similar to that experienced when a normal cell is exposed to growth factors. Consequently, the oncogene-bearing cell acquires growth autonomy as it no longer is dependent on external mitogenic stimuli, and

deregulated clonal cell proliferation may then occur (i.e., immortalization). This may then be followed by malignant transformation, depending on further mutations. It should be noted that evidence from experiments with primary rodent cells *in vitro* indicated the expression of at least two oncogenes is required to produce a malignant phenotype (39). It is believed their encoded oncoproteins are, in fact, cooperating— one causes immortalization and the other elicits malignant transformation (39,98).

Proto-oncogenes, oncogenes and their protein products are classified according to their function in the signal transduction pathway and to date, several have been identified for each step of the signalling network. An examination of human tumours by a variety of methods has revealed that within individual tumours, multiple activated oncogenes are present (8). Moreover, oncogenes have been detected in a wide variety of human cancers and some have been implicated with poor prognosis, including K-*ras* in both lung carcinoma (64) and acute myelogenous leukemia (98). Others are involved more particularly in metastatic disease, including the c-*neu* oncogene in breast cancer (98).

1.6.2 Tumour Suppressor Genes

The discovery of the existence of TSGs converged from several distinct lines of work. Two of these investigations are discussed here, namely a cell fusion study and a genetic investigation of a particular human cancer. In the former, somatic cell hybridization was performed with normal and tumour cells (59). Non-tumourigenic hybrids resulted from the fusion of these two cell types. Moreover, when chromosomes from the normal parent cell were lost due to the unstable nature of the hybrid

karyotypes, the hybrid cells frequently reverted back to a tumourigenic state. These results indicated: 1) cancer cells often lose critical growth-regulating genetic information of a recessive nature during their progression toward full malignancy, and 2) the normal chromosomes in the fusion study delivered such regulating genes to the cancer cells

Independently, a model was proposed by Knudson (1971) for the rare human eye tumour, retinoblastoma (47,59). It was based primarily on a genetic analysis of two forms of the disease— familial and sporadic. The model suggested both forms are triggered by *two* successive, independent lesions at a particular locus of the retinal cell genome, termed the retinoblastoma susceptibility gene (Rb1 gene). This 'two-hit hypothesis' was verified and connected with TSGs several years later, only after the nature of the lesions became elucidated by cytogenetic and molecular analyses. In particular, genetic alterations served to inactivate *both* functional copies of the Rb1 gene situated on the long arm of the chromosome 13 homologues (at 13q14). This created a loss of the normal Rb1 protein product, a nuclear phosphoprotein presently known to be involved in *suppression* of cell proliferation. According to the model, in the familial form the affected individual inherits a mutant, loss-of-function allele of the Rb1 TSG from the afflicted parent. A somatic event, such as a chromosomal deletion, later eliminates the remaining wild-type Rb1 allele inherited from the other parent. It follows that before the second lesion, single mutant Rb1 alleles are present within every cell of the body as a result of the germline mutation. These alleles act in a recessive manner*,

*At the cellular level the Rb1 TSG alleles function as recessive genes, whereas at the level of the whole organism, they are inherited in a dominant Mendelian fashion (105).

since heterozygous children for Rb1 essentially undergo normal development (but are genetically predisposed to the disease), and only the rare cell that loses its wild-type allele shows evidence of growth deregulation (108). In the sporadic form, the Knudson model explains two independent somatic alterations at the Rb1 TSG locus are required for tumourigenesis, the second of which must occur in descendants of the cell that received the first Rb1 mutation.

In both the cell fusion and retinoblastoma studies, critical normal genetic information was shown to be lost (inactivated) from the tumour cell genomes. This presented the first evidence of tumour-suppressing genes. It is now known that loss of genetic information is a frequent mechanism in the predisposition to, and development of, various human cancers (11). More specifically, genetic lesions leading to inactivation of TSGs is thought to liberate cells from the growth constraints normally imposed by these genes, yielding uncontrolled cell proliferation and tumour formation.

Presently, relatively few TSGs have been cloned when compared to the list of oncogenes, but with recent improvements in both gene cloning techniques and methods of identifying genetic deletions, new ones are being rapidly discovered and characterized. Examples of well-characterized TSGs include the gene for p53, which is mutated in a wide variety of human cancers (97), and the Rb1 gene, inactivated not only in retinoblastoma, but also in certain other human cancers (82). Similar to the genetic lesions involved in sporadic retinoblastoma, elimination of growth-inhibitory control by these genes is basically achieved either by mutations that give rise to mutant, loss-of-function proteins, and/or by the simple loss of critical TSG sequences from the cell genome. In general, large interstitial deletions, chromosomal translocations and certain other chromosomal

abnormalities (see below) are responsible for TSG loss, whereas focal deletions, point mutations, insertions and some translocations give rise to the mutant proteins.

It is now becoming clear that the protein products of TSGs, similar to those for oncogenes, are components of the signalling pathways that enable cells to respond to extracellular stimuli. Evidence shows they range from cell surface receptors, through intracellular signal transducers, to nuclear factors that regulate transcription and DNA replication (105). In contrast to oncoproteins however, TSG proteins are thought to be involved with growth-inhibitory signals rather than growth-promoting ones. It is envisaged they enable a cell to receive and process such inhibitory signals from its surroundings. If the TSGs responsible for critical components of this signalling network are inactivated as a result of genetic alterations, the cell loses responsiveness to growth-inhibitory signals even though these signals may still be present in its environment. Deregulated neoplastic growth may then ensue, and may be followed by malignant transformation.

Karyotype data is widely used as a preliminary measure for the general localization of TSGs. In general, frequent non-random chromosomal deletions among a particular class of neoplasm is indicative of an underlying inactivated TSG (11). The pitfall with these techniques however, is they do not detect the entire spectrum of genetic alterations in tumours, including microdeletions, point mutations and homologous recombination with a defective chromatid (117). In addition, due to their inherent sensitivity limitations, cytogenetic procedures are not adequate to reveal the precise chromosomal location of a putative TSG. Molecular techniques though, because of their higher sensitivity provide a more advantageous method of detecting TSGs— they can determine the smallest

chromosomal region involved in genetic alterations. In many cases, their results correlate well with cytogenetic results, and in fact, extend information given by them (108).

Presently, the molecular method of choice is a *loss of heterozygosity* (LOH) analysis. The goal of this method is not only to identify novel tumour suppressor gene regions for subsequent characterization of their pathogenic (and normal biological) roles, but also, to provide oncologists and pathologists with molecular markers of neoplasia.

1.6.3 Loss of Heterozygosity and Detection of TSGs

In retinoblastoma, the sporadic and familial forms of the disease are viewed to result from the same two-step process that eliminates both copies of the Rb1 TSG in retinal cells— the difference being the inheritance or somatic occurrence of the first mutation (M1). Molecular as well as cytogenetic studies of the Rb1 locus suggest the second mutation (M2) in the oncogenesis of the two forms involves somatic alteration of the remaining wild-type Rb1 allele in a way that causes its loss, thus unmasking the mutant Rb1 allele (Figure 1). Cavenee *et al.* (1989) (11) suggest one possible M2 mechanism could be replication of the initially inactivated allele into the cognate chromosome. This could be achieved either by mitotic non-disjunction with duplication of the mutant chromosome, or by a simple mitotic recombination. Alternatively, loss of the normal allele may occur by a deletion of the entire wild-type chromosome, leaving an apparent monosomic chromosome that contains the mutant allele. An additional proposed mechanism is that for cases with no molecular or cytogenetic evidence for changes at the wild-type Rb1 locus, an independent point

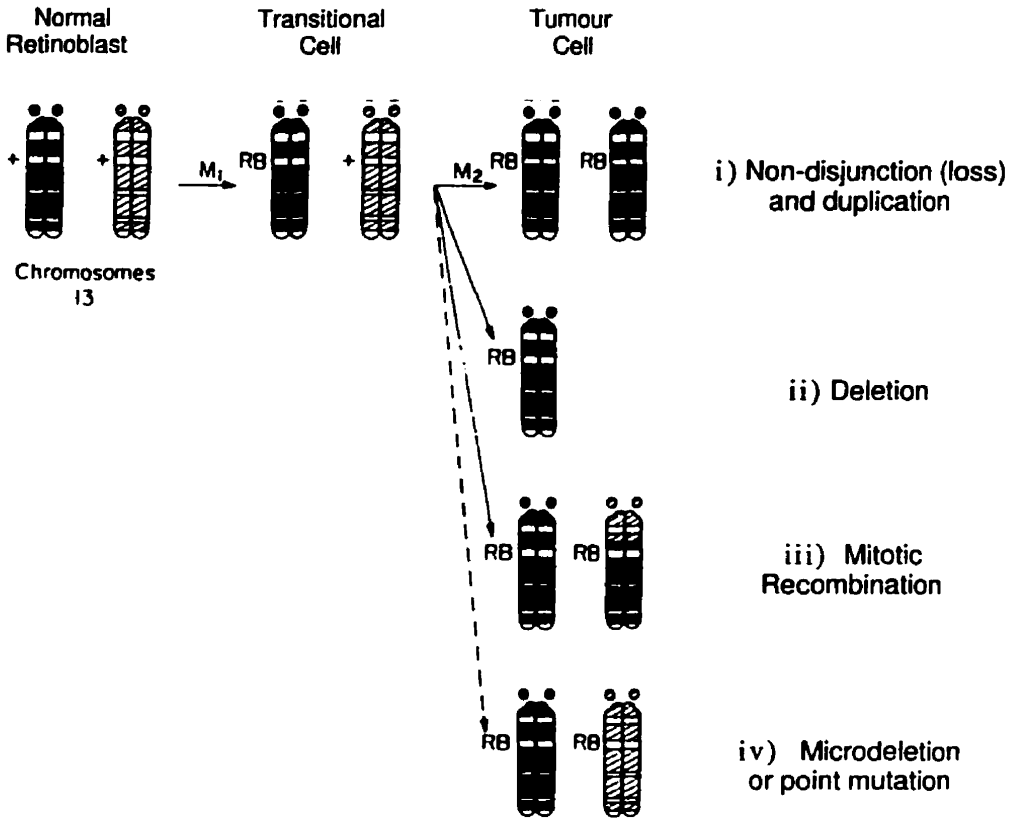

Fig. 1- To explain the origin of familial or sporadic retinoblastoma tumours, two independent mutations, M1 and M2, are thought to inactivate both normal alleles at the Rb1 (RB) locus on chromosome 13. The first mutation, M1 (inherited or non-inherited), is generally localized to the Rb1 locus. Four different mechanisms are proposed to generate the second mutation, M2, in the remaining wild-type allele– i) The chromosome carrying the normal allele is lost entirely and the mutant chromosome is duplicated. ii) The normal chromosome is lost and the mutant chromosome remains as a monosomic chromosome. iii) A mitotic recombination occurs such that the proximal part of chromosome 13 retains the maternal and paternal regions, but the distal portions of chromosome 13 contain loci from the chromosome carrying the original retinoblastoma mutation. iv) In some cases it is presumed that an independent mutation (microdeletion or point mutation) occurs at the wild-type Rb1 locus, resulting in its inactivation. Adapted from Tannock and Hill, *The Basic Science of Oncology, 2nd Ed.* 1992.

mutation or microdeletion may have occurred, resulting in its inactivation (98).

In the familial form, predisposition to the disease arises as a germinal mutation of an Rb1 allele inherited by an individual. This person is therefore an obligate *heterozygote* at the Rb1 locus in each of their somatic and germ cells. A subsequent somatic event in any retinal cell (by the M2 mechanisms above) that ultimately results in loss of the remaining normal allele and concomitant reduction to *homozygosity* for the mutant allele (i.e., mutant at the Rb1 locus now on both chromosome homologues or on a monosomal chromosome), will result in a tumour clone. This generation of homozygosity is alternatively termed *loss of heterozygosity* (LOH), (108). In contrast, sporadic retinoblastoma could also arise through the appearance of homozygosity at the Rb1 locus, but the difference being two somatic events in this case as compared to one germinal and one somatic event for familal retinoblastoma.

Since other human cancers also exhibit loss of recessively acting alleles, the Knudson two-hit hypothesis may also apply to these, serving as a paradigm of allele loss in neoplastic cells. It relates to all types of cancers, including ones with both hereditary and non-hereditary forms, a common mechanism for allele inactivation. Consequently, the LOH principle may be applied to these cancers. And any technique that detects consistent LOH for a particular locus among a given tumour class will indicate its potential role as a inactivated TSG (below). One technique currently used for LOH determination is a restriction fragment length polymorphism analysis.

Certain LOH analyses take advantage of variations in restriction endonuclease sites that are naturally present in human DNA. These create markers that can be followed to trace the presence or absence of specific

chromosomal regions (Figure 2). The variations in recognition sites for restriction enzymes are due to differences in the base sequences between maternal and paternal chromosome homologues. These differences are generated either by silent base mutations that give rise to, or eliminate restriction sites, or, by the presence of a variable number of short stretches of repetitive sequences called tandem repeats* (105). Enzymatic digestion of DNA containing the base sequence variations will give rise to restriction fragment length polymorphisms (RFLPs)— 'polymorphic', because of a difference in the size of DNA fragments produced. Upon a Southern blot analysis with a chromosome-specific, single-copy segment of interest from the human genome which has been isolated in recombinant DNA form (i.e., a cDNA probe), the RFLPs will be *revealed* at the corresponding chromosomal segment in question. This involves autoradiographic visualization of the chromosomal segment RFLPs as *two* distinct signals (if there is a difference of a single restriction site)— one is from the paternally-derived and one is from the maternally-derived chromosomal homologue (Figure 2a). To note, the RFLP chromosomal segment is also termed a *polymorphic marker.*

Because the DNA of this individual contains a single base variation in the chromosomal marker of interest (i.e., heterozygous$^{\aleph}$ at this region) and will therefore give rise to two hybridization signals, this case is considered to be *informative* at this locus. Now suppose this person develops a tumour. Upon a RFLP comparison of the status of the locus in tumour and normal tissues, the autoradiograph will show *two* signals for the

*Both are sufficiently minor as to not alter proteins encoded by these regions.

$^{\aleph}$ Do not confuse this RFLP-heterozygosity with the heterozygosity discussed above, which was used to describe individuals having one mutated copy of a recessive allele.

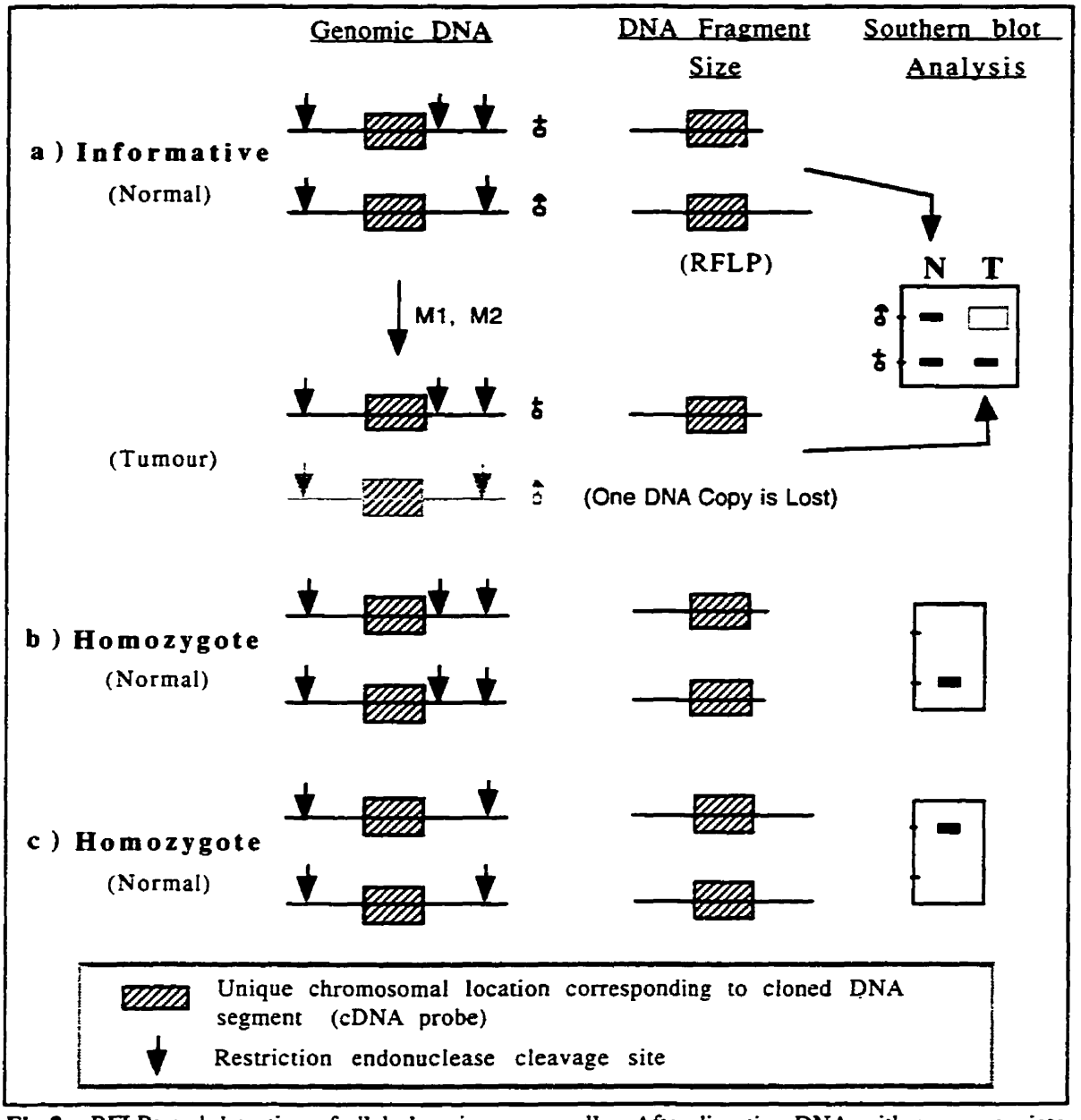

Fig.2- RFLPs and detection of allele loss in cancer cells. After digesting DNA with an appropriate restriction enzyme, the generated fragments are separated according to size by agarose gel electrophoresis, blotted to a nylon filter, and hybridized with the cloned, radioactive cDNA probe. **a)** If a DNA sample contains variations in cleavage sites in the genomic region of interest between maternal and paternal chromosomal homologues, RFLPs will be revealed by the probe as two hybridization signals of different sizes. This case is considered to be informative. If one of the alleles has been deleted, as in the tumour DNA, one of the signals will be missing, allowing molecular confirmation of the deletion event. To explain this result, the Knudson model suggests that one mutation has inactivated the maternal allele in the tumour cells (M1) and a deletion (M2) (or some other secondary event) has removed the paternal allele, resulting in loss of its autoradiograph signal. **b)** Case with 3 restriction sites in region of interest on both chromosomal homologues gives rise to 2 *small* restriction fragments of identical size (homozygote). **c)** Case with 2 restriction sites on both homologues gives rise to 2 *large* restriction fragments of identical size (homozygote). RFLPs are not revealed for either homozygous case because of identical restriction sites in the maternal and paternal copies of the genomic region of interest— only one autoradiograph signal will be present. Allelic loss for homozygotes therefore cannot be distinguished with accuracy and these cases are deemed 'uninformative'.

normal tissue, and possibly, only *one* signal for the tumour tissue (Figure 2a). In light of Knudson's two-hit hypothesis, this signal is representing the inactivated mutant allele caused by the first mutation (M1), whereas the absent band is indicating loss of the wild-type allele caused by a second mutation (M2). At the molecular level, this is deemed 'reduction to homozygosity' or LOH. Figure 3 illustrates autoradiographic visualization of the various M2 mechanisms for achieving loss of the wild-type allele. In the molecular context then, LOH is defined as loss of an informative RFLP hybridization signal (52). In reality however, because tumour specimens are slightly contaminated with non-tumour cells and some tumours exhibit heterogeneity for the allelic loss (68), complete signal absence is not usually detected. Therefore, LOH is operationally defined, in most studies, as a reduction of the signal intensity of more than 50% compared to its partner band, in an informative case.

For a normal case in which the restriction sites are identical (homozygous) for the maternal and paternal alleles of interest, the RFLPs will not be revealed (Figure 2b). A Southern analysis will yield only one hybridization signal, identifying *both* the maternally- and paternally-derived alleles. These cases are deemed *uninformatives* because any loss of an allele cannot, at present, be quantified with accuracy. To note, for a given polymorphic marker some uninformative cases may show the larger hybridization signal, while other uninformatives exhibit the smaller signal.

In general, LOH for a polymorphic marker indicates loss of this particular chromosomal region of interest, as revealed by the corresponding cDNA probe. It follows then, that when screening DNA extracted from a number of tumour specimens, its *frequent* LOH will serve as a sign of non-random mutation events that gave rise to its removal. Once this possibility

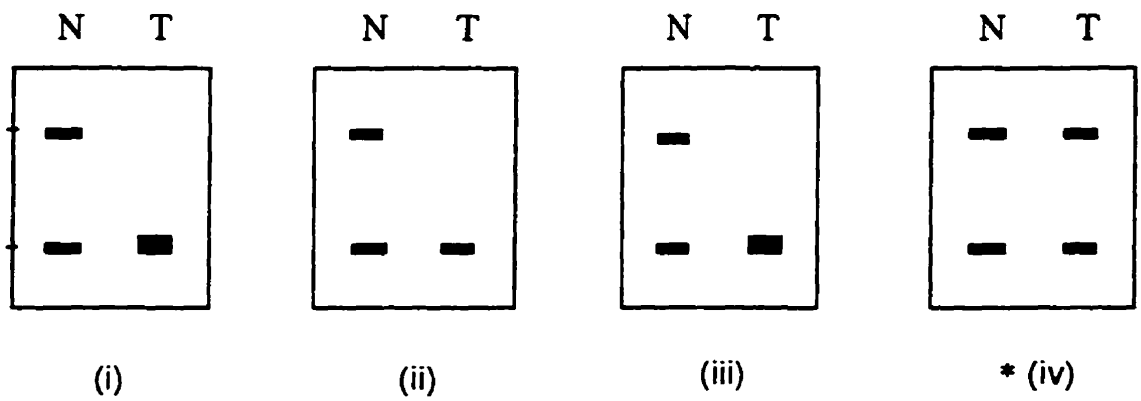

Fig. 3- Southern blot analysis of a polymorphic marker in tumour samples may reveal reduction to homozygosity (or LOH) for the chromosomal region of interest. Loss of the wild-type allele (top band in this example) and subsequent homozygosity for the mutant allele (bottom band), as revealed on the autoradiograph, is believed to occur by a variety of mechanisms that generate M2 (see Fig.1): i) chromosomal non-disjunction and duplication of the chromosome containing the mutant allele; ii) deletion of the chromosome (or chromosomal region) containing the normal allele; iii) chromosomal recombination (resulting in two identical copies of the mutant allele); *iv) Although in some cases there is no molecular evidence for changes in the markers on either the maternal or paternal chromosome, it is possible that a microdeletion or point mutation has inactivated the remaining normal copy of the region of interest. Tumour band sizes in i) and iii) are exaggerated to illustrate the identical mutant alleles.

was recognized, human tumours showing cytogenetic deletions were investigated for allelic loss using RFLP analysis with various polymorphic markers. Several tumours showed significant LOH incidences for these markers. Moreover, it was concluded that frequent LOH for a polymorphic marker among a specific tumour type could be reflecting the inactivation of an underlying TSG (11,12,49,59,108).

To note, a frequent LOH incidence may also be reflecting inactivation of a TSG that is closely mapped to the polymorphic marker of interest. Most cDNA probes used in LOH analyses are anonymous segments of DNA that have been mapped to specific chromosomal areas. Because the event causing removal of the wild-type allele does not respect the gene's specific boundaries and rather involves relatively large areas of DNA, it is highly possible the polymorphic marker is linked to the removal event by virtue of its *proximity* to the gene of interest, rather than being a part of the TSG itself (105). Nonetheless, the closer the linked marker is to a putative TSG locus, the higher will be the frequency with which its LOH is scored in tumours (59).

After the location of a candidate gene has been determined by LOH analysis, functional proof that it is truly a TSG is required. At present, this rests either on reintroducing the normal gene into neoplastic cells to see whether the neoplastic phenotype is abolished, or, in targeting disruption of the gene to observe if this plays a role in transformation (117). The former method is the most widely accepted one. To accomplish this, microcell-mediated chromosome transfer is utilized. This involves introduction of the chromosome suspected of carrying the TSG into a tumour cell line that has apparently lost this gene. This is followed by characterization of any resulting suppression of the neoplastic phenotype. If tumourigenicity is

reduced or abolished, and if it is proven to be the result of a specific gene on the introduced chromosome, this gene is therefore verified to be an inactivated TSG involved in tumour pathogenesis. TSGs verified by this technique include Rb1, the p53 gene and the *D*eleted in *C*olon *C*arcinoma gene (DCC) (8,59).

LOH for various polymorphic markers has been detected in all types of human tumours (117), indicating the wide prevalence of genetic losses during tumourigenesis. Some examples include markers on chromosome 3p in lung carcinoma (73), on 5q in colon carcinoma (52), on 7q in breast carcinoma (5), on 13q in retinoblastoma (Rb1 gene) (59) and on 18q in colorectal carcinoma (DCC gene) (40). LOH analyses have also identified absence of the *same* marker with almost equal frequency among different types of tumours, suggesting that a single locus may be involved in the oncogenesis of various tumours irrespective of the cell or tissue type in which it is expressed (5,59,108). An example is the well-characterized gene for p53. Its locus, 17p13, shows frequent LOH in several cancers, such as astrocytomas, breast cancer, small cell lung cancer, and colon cancer. Furthermore, multiple losses of loci have been detected within individual tumours (39), providing evidence in support of the 'multiple lesion theory' of tumourigenesis.

1.6.4 Implications of LOH Studies

Following repeated experimental observation of LOH for a particular polymorphic marker among human tumours of a given class, this segment of the genome may be used to identify individuals whom are predisposed, heritably or sporadically, to the cancer. For example, LOH analysis of the

Rb1 gene is already being used for screening in retinoblastoma families (108). LOH for a chromosomal segment may also serve as a diagnostic/prognostic *molecular marker* of neoplasia. An example is loss of the APC locus on chromosome 5 and the p53 locus on chromosome 17. Together, these are used to identify an early adenoma stage in gastric cancer (52). Furthermore, molecular markers identified by LOH analyses may be combined with those for oncogenes to delineate the histopathological stages leading to malignancy of certain human neoplastic tissues, (i.e., tumour progression). Ideally, both types of molecular markers will be useful in improving the classification and prognostication of human neoplasms.

For example, in colorectal cancer, acquisition of the malignant phenotype is achieved through a series of morphologically- and histologically identifiable stages: colon epithelial hyperplasia followed by formation of adenomas that progressively enlarge and ultimately undergo malignant transformation to become aggressive carcinomas (49). It is believed that during this progression there is a succession of genetic alterations corresponding to the series of disease states. The following molecular events are observed in carcinogenesis: beginning with adenoma, alterations include deletion of the APC (putative tumour suppressor) gene on chromosome 5 (formerly the *F*amilial *A*denomatous *P*olyposis gene, FAP), followed by activation of the *ras* proto-oncogene and ultimately, loss of the DCC and p53 TSGs by the carcinoma stage (39). To note, the order of mutations is considered less important than their total accumulation during carcinogenesis. Analysis of the status of these markers in a tumour biopsy is currently used to determine if- and where a colorectal specimen lies in the adenoma-carcinoma sequence (49).

Since other human tumours also show multistage progression leading towards malignancy at the morphological, histological and molecular levels, the colorectal cancer model of multistep carcinogenesis may provide a paradigm for the molecular progression of other cancers. This includes lung cancer. However, there is presently only fragmentary evidence linking specific genetic lesions to the steps of progression in certain human cancers. Therefore, it is necessary to gain more knowledge regarding genetic alterations in the various tumour stages and subtypes. The impetus then, is to discover more molecular markers and to determine if they are linked to certain stages of a tumour, as well as if they are of prognostic significance.

Lastly, the ultimate benefit of identifying TSGs is that they may someday be applied to the management of cancer. By understanding the biological function of a TSG, it may be possible to reinstate normal growth control in tumour cells by inserting wild-type suppressor genes into cells that lack them. Early successes have been reported on this front, including the use of retrovirus transducing vectors to insert normal Rb1 gene copies into cells that are missing them (108). These genetically reconstituted cells grew more slowly in culture and lost tumourigenicity when implanted into immunodefficient mice. At present however, this prospect is not imminent for treatment of human cancers, primarily because of the technical difficulties of delivering genes specifically to tumour cells. A more encouraging approach, despite its relatively scarce investigation so far, is based on the protein products of TSGs. Given sufficient information about the structure and function of these proteins, pharmaceutical chemists may be able to provide cancer cells with substitutes or bypasses for damaged TSGs (8). Thus, to apply our knowledge of lost genetic information in cancer to the treatment of cancer, it is necessary to elucidate the biology,

biochemistry and pathological involvement of candidate TSGs and their proteins. This explains why the search for lost information in tumour cells occupies a central place in current cancer research.

1.7 The Molecular Biology of Lung Cancer

Genetic analyses of human lung cancer have been possible mainly because of the establishment of a large panel of human lung cancer cell lines as materials for cytogenetic and molecular genetic studies. Resected human lung tumour specimens and their normal lung tissue counterparts have also provided materials for such analyses. Cell lines and tissues belonging to the various histological subtypes and stages of NSCLC and SCLC have been studied for genetic changes accompanying the progression towards malignancy. This has revealed deregulation of multiple steps in the mechanisms of growth control, involving both activated oncogenes and inactivated TSGs (6,105).

One of the proto-oncogenes known to be implicated in lung cancer is the *myc* gene family— c-*myc*, N-*myc* and L-*myc* (42). These genes, located on separate chromosomes, encode nuclear phosphoproteins important in regulating the cell cycle. Each member of this gene family has been found amplified in certain SCLC and NSCLC tumours or cell lines (105). Of all reported studies to date, 36 of 200 (18%) tumours and 38 of 122 (31%) tumour cell lines derived from patients with SCLC had DNA amplification of one member of the *myc* family. In addition, 25 of 320 (8%) tumours and 3 of 15 (20%) tumour cell lines from patients with NSCLC also had *myc* family DNA amplification. Since the *myc* amplification

frequencies in SCLCs and NSCLCs are relatively low, it seems unlikely amplification of this gene family is an early event in the pathogenesis of lung cancer. Rather, these genes may render a growth advantage to an already transformed cell, which can subsequently become the dominant population in the developing tumour. Supporting, yet indirect evidence of this is that c-*myc* amplification has been found associated with shortened survival in SCLC patients.

The *ras* family of proto-oncogenes, H-*ras*, K-*ras* and N-*ras*, are also implicated in lung cancer (105). They encode GTPase proteins involved in the transduction of intracellular signals. Transforming potential of these proteins is acquired when a critical amino acid residue is replaced as a result of a point mutation in the DNA, giving rise to a loss of GTPase activity. In lung tumour studies of *ras* status, approximately 20% of NSCLC and 30% of NSCLC cell lines exhibited mutation of at least one member of this gene family (63,64). In contrast, none of 55 SCLC tumours and tumour cell lines had *ras* mutations. Among tumours of the NSCLC group, *ras* mutations were most commonly found in ADCs (24%), whereas SQCCs exhibited the lowest mutation incidence. Of the *ras* gene family, K-*ras* had the highest incidence of mutation. From the above results it has been proposed that molecular screening of *ras* gene mutations in clinical lung specimens could be of diagnostic, prognostic and even therapeutic importance. Moreover, since K-*ras* mutations in NSCLC tumours and tumour cell lines have been associated with shortened survival (64), the status of this gene may be used as a prognostic factor. Similar to the situation for the *myc* family, *ras* family mutations seem to participate in the more advanced stages of lung carcinoma, (but in this case, for NSCLC alone).

A number of other proto-oncogenes have been implicated in the pathogenesis of lung cancer, despite lack of evidence for their activating mechanisms. Dysregulated expression of the unmutated human c-*jun* gene, which encodes a transcription factor that was shown to cooperate with *ras* in transforming primary rat embryo cells, has been revealed in SCLC cell lines (105). The gene that encodes epidermal growth factor receptor (EGF-R), *erb*B-1, was found expressed at high levels in the majority of tumours and tumour cell lines from patients with NSCLC (111). Also expressed highly in NSCLC tumours and cell lines, particularly in the ADC subtype, was the c-*erb*B-2 proto-oncogene, (also known as HER2/*neu*) (57). It encodes a homologue of the EGF-R. Although little is known about the *src* tyrosine kinase in the biology of lung cancer, both it and the *lck* gene product (a *src*-related protein tyrosine kinase) have been shown to be abnormally expressed in some SCLC and NSCLC cell lines (105).

Cytogenetic reports of established SCLC and NSCLC cell lines have indicated these cells are grossly aneuploid and exhibit several striking chromosomal abnormalities, including frequent interstitial deletions (105,117). LOH analyses, lagging well behind the cytogenetic studies, have confirmed the presence of only some of the regions exhibiting allelic loss. To date, sites of consistent LOH in lung cancer include chromosomal regions 3p (10,42,73), 5q (19,110), 11p (92,109), 13q (54,82) and 17p (62,97). Some of these regions also show allele loss in other human cancers and may contain a TSG (or putative TSG). This includes the APC gene in colorectal cancer (5q), the putative Wilms' tumour locus, WT1 (11p13), the Rb1 TSG (13q14), and the p53 TSG (17p13). A candidate TSG for the 3p region has yet to be isolated.

As previously described, the Rb1 gene product is a nuclear phosphoprotein normally involved in the suppression of cell proliferation. Analysis of Rb1 expression in lung cancer showed that 60% of SCLC and 10% of NSCLC tumour samples had undetectable Rb1 mRNA transcripts, whereas normal lung tissue samples expressed the transcript (54,82). Furthermore, an abnormal Rb1 protein was frequently noted (>75%) in SCLC cell lines (105). This high frequency suggested a key role for inactivation of the Rb1 gene in either the initiation or development of SCLC.

The assignment of the p53 TSG to 17p13, revealed by RFLP analyses to be commonly deleted in a wide variety of human cancers, has led to analysis of its structure and expression in primary bronchogenic carcinomas (62,95,97). Mutations of this gene have been found in 100% of 25 cell lines derived from patients with SCLC and in 27 of 35 (77%) SCLC tumours. In NSCLC tumour cell lines it was abnormal in 57 of 77 (74%) while in NSCLC tumours, mutations were present in 27 of 55 (49%) cases. Recently, 43% of 120 NSCLC tumours were observed to have p53 DNA mutations (65). From these results it was concluded mutations of the gene for p53 are very common in lung carcinomas, particularly among SCLCs. Furthermore, since the presence of p53 aberrations were found associated with shortened overall survival, this indicated p53 status may be of prognostic significance in patients with advanced stage tumours.

Evidence of 11p abnormalities have been documented in RFLP analyses of human lung carcinomas. Among patients with NSCLC tumours, one study detected 11p LOH in 14 of 22 (64%) informative SQCCs, in 10 of 22 (45%) informative ADCs and in 3 of 7 (43%) informative LCCs (109). In a later report, 9 of 40 (23%) informative NSCLC patients showed 11p

LOH, (15% of SQCCs; 31% of ADCs) (92). These results are particularly interesting since Wilms' tumour, a kidney cancer found in children, has been associated with a deletion of chromosomal region 11p13 (108). One locus at this region, termed WT1, is believed to be a putative TSG. It encodes a transcription factor that is normally present in certain tissues of the human embryonic kidney (59). Moreover, introduction of a normal human chromosome 11 was found to reverse the tumourigenicity of Wilms' tumour cell lines, providing compelling evidence for the existence of a TSG at 11p13 (105).

Two polymorphic sites on the long arm of chromosome 5, MCC and APC, have been demonstrated to be involved in the pathogenesis of colorectal cancer (110). Using cDNA probes corresponding to these 5q21 loci in a RFLP analysis of human primary lung carcinomas, D'Amico *et al.* (1992) (19) showed 17 of 21 (80%) informative SCLC patients and 2 of 5 (40%) informative NSCLC cases had LOH in the MCC/APC region. The high incidence in SCLC suggested inactivation of a 5q TSG is involved in the pathogenesis of this lung cancer subtype. Interestingly, the APC protein has been shown to associate with β-catenin, a cytoplasmic anchor protein of the cadherin cell-cell adhesion molecules (110). It has been proposed that since loss or disturbance of this adhesion property correlates with dedifferentiation and metastasis of lung SQCCs, loss of APC and cell-cell adhesion may play a role in the later stages of tumourigenesis.

Essentially all SCLCs and approximately half of NSCLCs exhibit deletions of the short arm of chromosome 3, as detected by cytogenetic and LOH analyses (10,42,73,109,113). It is therefore highly probable a TSG may reside in this region. However, because of the large size of the deletion, the gene has not yet been identified. Candidate TSGs on this

region include the β-retinoic acid receptor gene, certain transcription factor genes, the *raf*-1 proto-oncogene and the protein tyrosine phosphatase-γ gene (PTP-γ) (26).

From the above reports it is evident that multiple genetic lesions involving both activated oncogenes and inactivated TSGs are implicated in the pathogenesis of human bronchogenic carcinomas. Some of these alterations are not mutually exclusive, as many cell lines combine deletions of 3p, Rb1 and/or p53 (105). Similar to the molecular pathology of colorectal carcinogenesis, a road map for genetic mutations in lung cancer is emerging. With respect to its potentially inactivated TSGs however, due to the numerous lesions detected by cytogenetics there is presently a high demand for new LOH studies to better define the nature and incidence of these abnormalities.

One such cytogenetic abnormality currently under investigation is a deletion of the long arm of chromosome 7. Not only has this been commonly observed among primary bronchogenic carcinomas (117), but also in various other human malignancies (4,58,75). For breast carcinoma, attempts have been made to identify an inactivated TSG situated in this frequently deleted region. In particular, a recent molecular study of a large panel of breast carcinomas has revealed frequent LOH for a certain gene situated on the long arm of chromosome 7 (5). The loss of this gene, named c-*met*, which is known to have important biological functions, was also found to be a strong predictor of poor patient survival. These results suggest c-*met* may be an inactivated TSG in breast cancer. Since bronchogenic carcinomas also show deletions of the chromosomal region containing c-*met*, it is of interest to examine c-*met* LOH in these neoplasms. This is the focus of our investigation. But before exploring loss of this gene

in lung carcinoma, it is worthwhile to review its *normal biology*, as well as its *expression patterns* in various cancers and tumour cell lines.

1.8 The c-*met* Gene and its Encoded Protein

1.8.1 Detection of c-*met*; Characterization, Structure and Biosynthesis of its Protein

The c-*met* gene was originally identified as an activated transforming gene in a chemically treated human cell line, MNNG-HOS (15). These cells, which were morphologically transformed and formed tumours in mice, were derived by treating the human osteosarcoma (HOS) cell line with the potent clastogenic carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). In transfection experiments, DNA from MNNG-HOS cells induced efficient transformation of NIH3T3 mouse fibroblasts whereas no transformation was observed for HOS cell DNA. This indicated treatment of HOS cells with MNNG led to the generation of an activated oncogene. This gene was named *met*. Activation of *met* involved a chromosomal rearrangement that generates a chimeric gene (20,21,77). It contains sequences derived from chromosome 1 (designated *tpr- translocated promoter region*) fused to sequences encoding the kinase domain of the c-*met* proto-oncogene on chromosome 7 (in humans). To note, mouse c-*met* is located on chromosome 6 (13), whereas in humans it is situated at 7q31.1 (20,96).

Sequencing of human c-*met* proto-oncogene cDNA clones (prepared from a normal c-*met* RNA transcript of the HOS cell line) revealed an open

reading frame of 4224 nucleotides that codes for a protein (MET) of 1408 amino acids (78). In comparison, the DNA sequence of the mouse c-*met* proto-oncogene encodes a predicted 1380 amino acid protein exhibiting high (89%) homology with human MET (13). Moreover, the primary amino acid sequence of both mouse and human MET suggested they had features in common with the protein tyrosine kinase family of growth factor receptors (RTKs) (13,20,78). Park *et al.* (1987) (78) noted the following features of the MET sequence (Figure 4): The first 18 N-terminal amino acids were hydrophobic, indicating this domain was a potential signal peptide for insertion into membranes of the endoplasmic reticulum and plasma membrane. A second hydrophobic region, a stretch of 23 amino acids (residues 950-973), shared characteristics with the transmembrane domains of RTKs, suggesting it could serve as the membrane-spanning region of MET. Therefore, it was deduced that the putative extracellular domain (N-terminal) consists of residues 24-950, while the cytoplasmic domain (C-terminal) is comprised of residues 973-1408. Many cysteine residues, including a small cysteine-rich region, and several consensus sequences for asparagine-linked *N*-glycosylation were noted in the extracellular domain. Notably, in other RTKs, cysteine-rich clusters are thought to form a structural backbone for the ligand-binding region of the N-terminal. Most importantly, a putative ATP binding domain, including a consensus ATP-binding site (Lys-1127), and a kinase domain (residues 1101-1351) were observed in the sequence of the cytoplasmic domain. The kinase domain was found to exhibit high homology with human insulin receptor (44%) and epidermal growth factor receptor (38%). Lastly, an unusual feature of the predicted c-*met* protein was the presence of a long

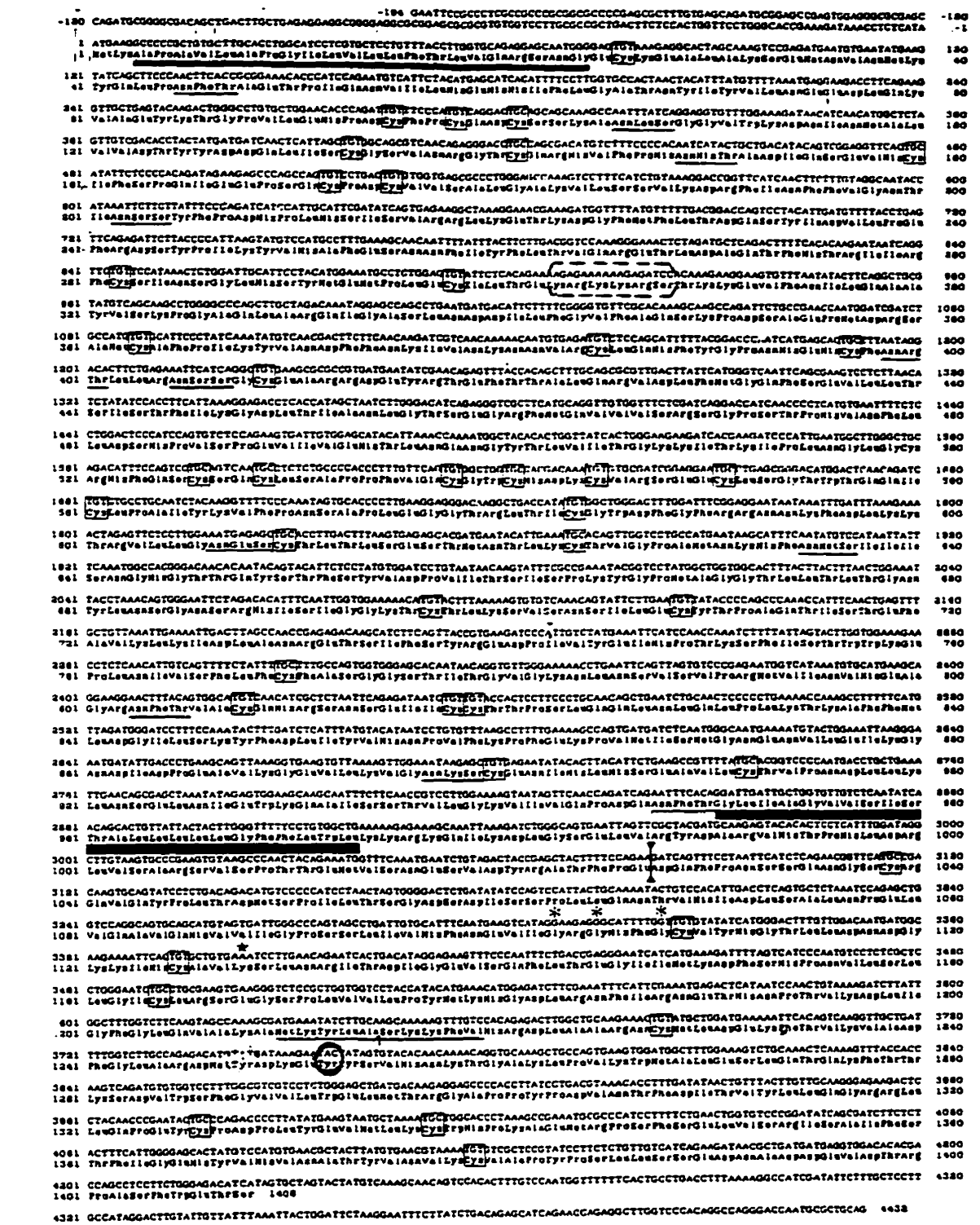

Fig. 4- The c-met proto-oncogene nucleotide sequence and deduced amino acid sequence. Nucleotides are numbered above and amino acids are numbered below the line starting with the first methionine of the open reading frame. The putative signal sequence and transmembrane domain are underlined with a solid bar. The consensus sequences for N-linked glycosylation are underlined and cysteine residues are boxed. Putative ATP binding domain: * (Lys); *** (Gly-Xaa-Gly-Xaa-Xaa-Gly). Broken box indicates characteristic stretch of basic amino acids that is cleaved in forming the mature protein structure. The major autophosphorylation site, Tyr-1253 is circled. Adapted from Park *et al.* (1987), *Proc. Natl. Acad. Sci. USA*, Vol. 84.

stretch of amino acids between the putative transmembrane and kinase domains (Figure 5).

Further examination of the predicted amino acid sequence of MET revealed a Lys-Arg-Lys-Lys-Arg-Ser sequence located 303 residues from the N-terminal (78). Chan *et al.* (1988) (13) as well as Tempest *et al.* (1988) (102) independently noted this basic sequence was similar to sequences used to cleave receptor precursors, including those of the insulin receptor (I-R) and insulin-like growth factor-1 receptor (IGF-1-R), into α and β subunits that are joined by disulfide bonds in the mature receptors. By analogy, these investigators proposed that cleavage of MET at this site and removal of the signal peptide would generate an N-terminal peptide (the α-subunit) that becomes associated with a remaining membrane-bound portion of MET (the β-subunit). Electrophoretic studies of c-*met* proteins under reducing and non-reducing conditions indeed verified the mature protein existed as a two-chain structure (34,102). Furthermore, these studies demonstrated it is a 190 kDa heterodimer ($p190^{MET}$) composed of a 50 kDa α-chain, bound by disulfide bridges to a 145 kDa β-subunit (Figure 5). Similar to the receptors for insulin and IGF-1, the α-subunit is exposed at the cell surface while the β-subunit spans the plasma membrane into the cytoplasm.

To examine the biosynthesis and post-translational processing of MET, Giordano *et al.* (1989a,b) (35,36) took advantage of a human gastric carcinoma cell line, GTL-16, which exhibits an abnormally high level expression of the c-*met* gene. By immunoprecipitation with anti-MET antibodies and using pulse-chase experiments with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, the first translational product appearing after a short metabolic labelling was

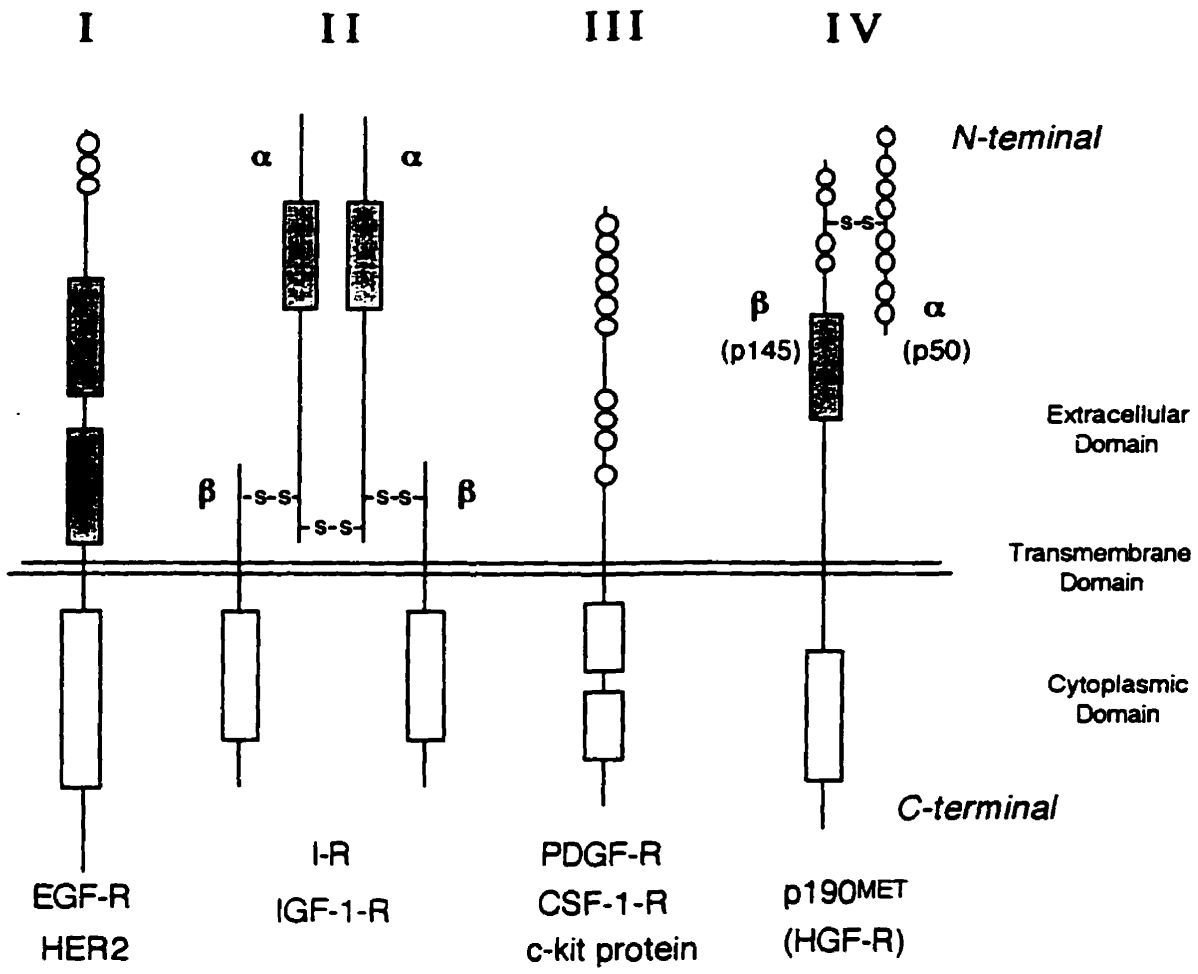

Fig. 5- Comparison of the structures of the known classes of tyrosine kinase receptors (I-III) with the newer class of receptor, the mature product of the c-*met* proto-oncogene (IV),. The abbreviations are: EGF-R, epidermal growth factor receptor; HER2, c-*erb*-B-2 gene product; I-R, insulin receptor; IGF-1-R, insulin-like growth factor-1 receptor; PDGF-R, platelet-derived growth factor receptor; and CSF-1-R, colony stimulating factor-1 receptor. Tyrosine kinase domains (▭), cysteine residues (O) and cysteine-rich regions (▩) are illustrated. Molecular masses of the p190^{MET} subunits are shown.

a single-chain 170 kDa protein. This already-glycosylated precursor was determined not to be present at the cell surface since it could not be labelled by cell-surface iodination procedures. When electrophoresis was run under non-reducing conditions, a shift in its apparent molecular weight to 180 kDa indicated p170 undergoes a conformational change— probably involving modification(s) of intrachain disulfide bonds. After a 1 hour total period of chase the mature 50 kDa α- and 145 kDa β-subunit became detectable (due to proteolytic cleavage), and when under non-reducing conditions, migrated as the 190 kDa complex. Furthermore, when the cells were metabolically labelled by [^{125}I] under non-permeating conditions p190^{MET} was detectable, indicating its exposure at the cell surface. Thus, it was concluded mature p190^{MET} is a cell surface heterodimer derived from a glycosylated c-*met*-encoded 170 kDa precursor protein that undergoes proteolytic cleavage to form both a 50 kDa subunit and a 145 kDa subunit joined by disulfide bonds. To note, the same biosynthetic and processing pathway was observed in a number of epithelial cells other than GTL-16 (14).

Verification of the tyrosine kinase activity of MET began with the work of Comoglio *et al.* (1984) (14) who, using anti-phosphotyrosine antibodies with the GTL-16 cell line, identified a 145 kDa transmembrane glycoprotein having abnormally elevated protein tyrosine kinase activity. Giordano *et al.* (1988) (34) then showed this kinase was the product of a 190 kDa disulfide-linked heterodimeric complex using SDS-PAGE. Electrophoresis of the 190 kDa protein, as well as of an immunoprecipitated c-*met*-encoded protein (both obtained from GTL-16 cells) under reducing and non-reducing conditions subsequently revealed the two proteins were indistinguishable (35). Together, these results demonstrated the product of

the c-*met* proto-oncogene was a 190 kDa kinase consisting of a 145 kDa subunit having tyrosine kinase activity (35).

Concurrent with these investigations, the research group who originally identified c-*met* (15) and who, from the c-*met* sequence, postulated it was a member of the tyrosine kinase family of growth factor receptors (20), also sought direct evidence of its tyrosine kinase activity. Tempest and colleagues (1986) (101) used anti-MET antibodies to immunoprecipitate MET-related products from two kinds of cells— ones containing *normal* c-*met* transcripts (HOS cells) and ones with *tpr-met* transcripts (MNNG-HOS cells and NIH3T3 cells transformed with *tpr-met*). After incubation in [γ-^{32}P]ATP and analysis by SDS-PAGE, phosphorylation of a 140 and 165 kDa protein was detected in the former cells, while phosphorylation of a 60 and 65 kDa protein was identified in the latter cell types*. This indicated the c-*met* proto-oncogene encodes a p145/p165 protein kinase, and in an activated form, codes for a p65 protein kinase. Furthermore, when the cells containing these proteins were metabolically-labelled with [^{32}P]orthophosphate and subjected to a phosphoamino acid analysis, the c-*met* proteins (both normal and oncogenic) were found to catalyze *autophosphorylation* on tyrosine residues.

Now that two groups had independently and definitively showed the c-*met* product had tyrosine kinase activity, support for its role as a *conserved* kinase was needed. Using an immunocomplex kinase assay with

*1) Due to non-conformity of this laboratory's techniques with those of the preceding research group, p140 may be presumed to be identical to the above 145 kDa protein and p165 identical to the above 170 kDa protein. 2) This research group suggested the p60 and p65 proteins were of the same identity, with one being derived from the other due to degradation or post-translational modification. For the sake of simplicity these will be referred to as p65.

anti-MET and anti-phosphotyrosine antibodies, Giordano *et al.* (1989a,b) (35,36) found the $p190^{MET}$ kinase active in a variety of human cell lines, including those derived from lung, gastric, colic, glial and soft mesenchymal tumours. Independently, Gonzatti-Haces *et al.* (1988) (37) detected $p145^{MET}$ kinase activity in human tumour epithelial cell lines of the colon, pancreas, lung and cervix. Since then, $p190^{MET}$ has been detected in a diverse array of cell types, mostly of epithelial origin. Because $p190^{MET}$ could be labelled by cell-surface iodination procedures (37) as well as with [^{3}H]glucosamine (34), this confirmed MET is a glycoprotein situated at the plasma membrane. It was therefore concluded that c-*met* encodes a conserved 190 kDa cell-surface heterodimeric glycoprotein with inherent tyrosine kinase activity— in short, a tyrosine kinase receptor (RTK). In subsequent studies of the RTK, tyrosine residues 1234 and 1235 were identified to be involved in phosphorylation (28,120). These are located within the kinase domain predicted by the Park research group. Other studies revealed the following: 1)the kinase is positively regulated by autophosphorylation of Tyr-1235; and 2) it is negatively regulated by phosphorylation of a critical serine residue, (controlled by both protein kinase-C activation and rises in intracellular Ca^{2+} concentrations). This residue is located in the juxtamembrane domain of the β-subunit (32,33).

1.8.2 The Ligand for $p190^{MET}$ is Hepatocyte Growth Factor/Scatter Factor (HGF/SF); Biological Functions of HGF/SF-MET

Around the same time when c-*met* was being investigated for its tyrosine kinase activity, two apparently unrelated studies were being conducted— one on a polypeptide growth factor that stimulated growth of

cultured hepatocytes; and the other on a fibroblast-secreted factor causing dispersal of cultured epithelial cells. After liver damage in the rat, the first factor was found to have rapidly elevated mRNA and protein levels in platelets, serum and non-parenchymal cells of the liver (but not hepatocytes), and was followed by elevated hepatocyte DNA synthesis and cell growth (60). It appeared to act as a paracrine growth factor for hepatocytes. This liver-regenerating cytokine was therefore termed 'hepatocyte growth factor' (HGF), also known as Hepatopoeitin-A. Yet, it was later shown to be mitogenic for a variety of other cell types including endothelium, melanocytes, keratinocytes and several types of epithelium (14). HGF has since been purified from serum of human patients with hepatic failure and rabbit serum, in addition to rat platelets. Furthermore, it has been detected in a wide variety of human tissues including those of the lung, brain, kidney, pancreas, placenta, prostate, ovaries, small and large intestine, spleen, skin, stomach, thyroid and thymus (114). Examples of human cells producing HGF mRNA and protein include Kupffer cells, sinusoidal endothelial cells and Ito cells (all of which are non-parenchymal liver cells), endothelial cells of the kidney, fibroblasts, as well as alveolar macrophages and endothelial cells of the lung (66). Each of these cells, of mesenchymal origin, are known to target HGF to epithelial cells and hepatocytes. Thus, HGF is thought to be a mesenchymal-derived factor influencing epithelial cell growth through an epithelial-mesenchymal paracrine interaction. Interestingly, after partial hepatectomy or unilateral nephrectomy in the rat, HGF mRNA levels in the endothelial cells of the *lung* are dramatically increased (60). This suggests the lung acts as an endocrine organ with respect to HGF production and concomitant organ regeneration. In addition, recent studies have raised the possibility of

autocrine production of HGF. Using reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analyses, Tsao *et al.* (1993) (104) reported HGF and its receptor are co-expressed in normal bronchial epithelial cells, as well as in certain lung carcinoma cell lines. Autocrine production of HGF has also been shown in the human epidermal keratinocyte cell line, ndk (46), and in human osteosarcoma cell lines (29).

Study of the second factor, originally isolated from mouse fibroblasts, determined it stimulated the matrix invasion, separation and motility, but not necessarily the growth of normally cohesive epithelial and endothelial cell types (71). It was therefore named 'scatter factor' (SF). SF was later found to be secreted by human embryo and adult fibroblasts, as well as by smooth muscle cells (30). It was also shown to promote invasion of carcinoma cells into collagen matrices (107). Due to the type of responses it evoked SF was speculated to be involved in embryogenesis, as well as in the progression of carcinoma cells to a more malignant, invasive phenotype.

While the biological activities of HGF and SF seemed unrelated, purification of the molecules revealed a large degree of amino acid sequence and structural similarity (Figure 6). Both were disulfide-linked heterodimers with a heavy α-subunit of 55-65 kDa containing a unique hairpin structure and four characteristic kringle structures, (thought to be involved in receptor recognition). In addition, both had a light β-subunit of 32-36 kDa* (14). Subsequently, a biological activity comparison analysis and immunochemical studies (30), as well as comparative molecular cloning (14) revealed that HGF and SF were identical. Both factors were interchangeable and equally effective in promoting hepatocyte growth and

*The actual subunit sizes are 69 and 34 kDa, respectively (66).

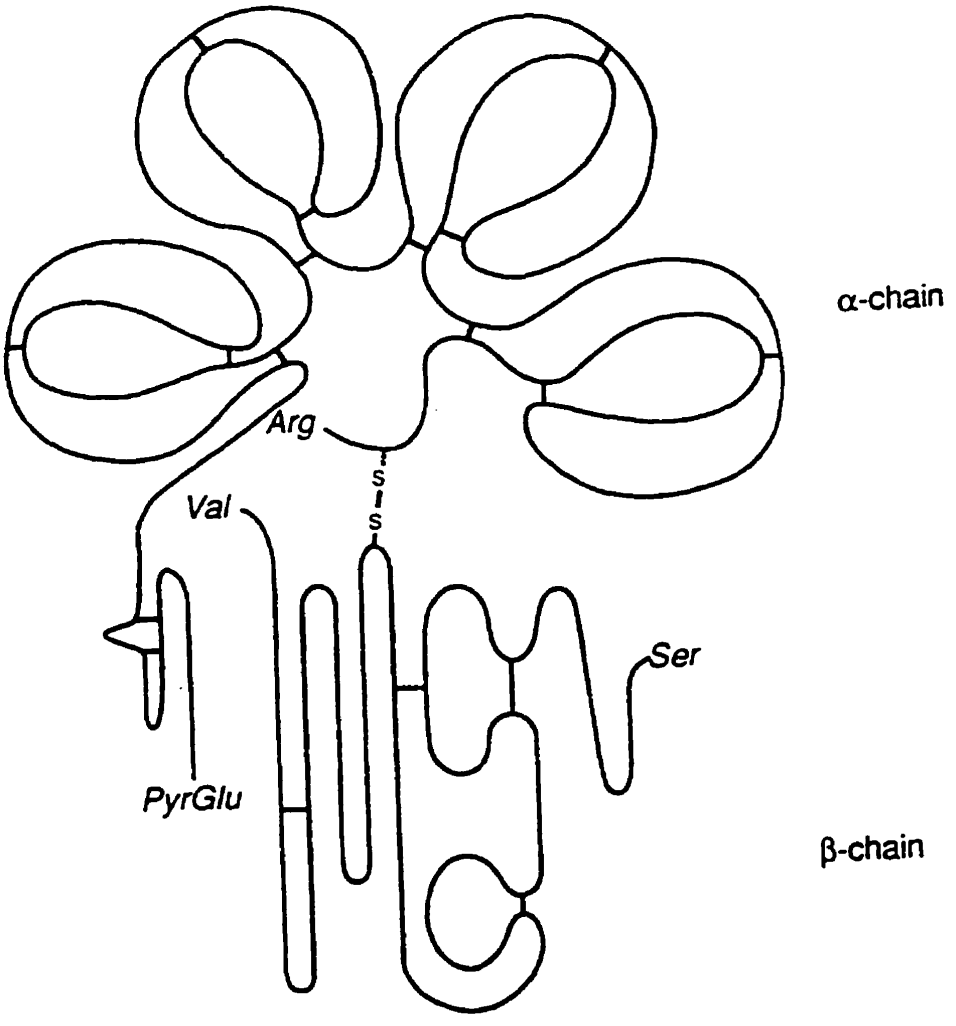

Fig. 6- Schematic diagram of hepatocyte growth factor/scatter factor (HGF/SF). The disulfide bridge connecting the α and β subunits is shown. The larger α-chain contains 4 kringle domains as well as an N-terminal hairpin loop structure, in which all are required for the proper biological activities of HGF/SF. The first two kringle domains and the hairpin structure are required for receptor recognition. Adapted from Mizuno and Nakamura, in *Hepatocyte Growth Factor/Scatter Factor (HGF-SF) and the C-Met Receptor*, ed by Goldberg and Rosen, 1993.

epithelial cell dissociation and matrix invasion. Most notably, they bound with equal affinities to the same, yet unidentified, sites on target cells.

By this time c-*met* was known to encode a RTK, but the identity of its ligand remained unknown. The receptor for HGF/SF was unknown as well. The connection between the two came when, using a human mammary epithelial cell line (B5/589) treated with pure HGF/SF, Bottaro and colleagues (1991) (9) detected specific phosphorylation of the 145 kDa β-subunit of MET. It was identified by immunoprecipitation with anti-phosphotyrosine antibodies, followed by immunoblotting with anti-MET antibodies. Covalent cross-linking of radiolabelled ligand to $p190^{MET}$ then directly established the c-*met* product as the cell-surface receptor for HGF/SF. Chemical cross-linking of HGF/SF to $p190^{MET}$ was also demonstrated in an independent study, but in the GTL-16 cell line (71).

In addition to its mitogenic role in hepatocytes and several other cell types, and its ability to induce motility (motogenesis) and invasion of certain endothelial and epithelial cell types (including Madin-Darby canine kidney (MDCK) cells), other biological responses are exhibited for the HGF/SF-MET ligand-receptor system. This includes promotion of tubule formation in MDCK cells (morphogenesis), chemotaxis for vascular endothelial cells (angiogenesis), and growth inhibition in certain tumour cell lines (27,89). Using a receptor chimera containing the extracellular domain of colony stimulating factor-1 (CSF-1) fused to the transmembrane and cytoplasmic domain of the MET RTK, Zhu *et al.* (1994) (119) demonstrated that stimulation of the MET kinase is sufficient and essential to mediate the motogenic, invasive and morphogenic responses of MDCK cells to HGF/SF. Chimeras with an inactive kinase were incapable of transducing such responses. Moreover, several MET receptor isoforms

(generated by alternative RNA splicing) have recently been revealed (53,80,84). It is possible these could mediate the pleiotropic responses to HGF/SF. Or perhaps each response is regulated by unique components of the MET-mediated signal transduction pathway. Nonetheless, the exact mechanism(s) responsible for this diversity awaits to be determined.

In summary, HGF/SF is a multifunctional cytokine that is most often expressed by mesenchymal and stromal cells such as fibroblasts, macrophages and a wide variety of endothelial cells. It exerts its effects primarily on epithelial cells, and serves as a paracrine effector of mesenchymal-epithelial interactions; an endocrine effector of organ regeneration; and as an autocrine factor in certain normal and neoplastic cells. When HGF/SF activates the kinase of its cell surface receptor, $p190^{MET}$, this ligand-receptor system may exert a mitogenic, motogenic, morphogenic, invasive, angiogenic or growth-inhibiting effect.

1.8.3 The RTK Family and MET-Mediated Signal Transduction

Before elucidation of the $p190^{MET}$ RTK structure, the tyrosine kinase family of receptors consisted of three classes (Figure 5) (76). Receptors belonging to Class I (e.g., epidermal growth factor receptor (EGF-R); the *neu*/c-*erb*B-2 gene product HER2) are monomeric, contain two cysteine-rich regions in the extracellular domain and have an extended C-terminus that exhibits regulatory activity. Class II receptors (e.g., I-R; IGF-1-R) have a heterotetrameric (α_2-β_2) structure that is formed by disulfide-bound subunits. The α and β chains are cleavage products of a receptor precursor that is the product of a single gene (112). Class III receptors (e.g., platelet-derived growth factor receptors α and β (PDGF-R); colony stimulating

factor-1-receptor (CSF-1-R); stem cell factor receptor (c-*kit*)) are monomeric and are characterized by both the presence of a split kinase domain in the intracellular segment and the absence of cysteine-rich regions. By virtue of having a heterodimeric ($\alpha\beta$) subunit structure, $p190^{MET}$ became the prototype of a fourth class of RTK (36). In the evolutionary tree of the tyrosine kinases c-*met* most resembles the gene for insulin receptor (14).

The distinguishing feature of all RTKs is the possession of an extracellular ligand-binding domain that is linked by a transmembrane region to a cytoplasmically-located protein tyrosine kinase domain. The catalytic domain is the most conserved domain of these receptors (76). The role of a RTK is to translate ligand binding into a biological signal and to transmit this via intracellular target proteins to the cell nucleus, leading to a biological response. Tyrosine kinase activation of the receptor is critical for this response (87). In general, the predominant biological activity of certain RTKs is to stimulate cell growth and proliferation, while others promote differentiation and cell motility, or act to arrest growth. In some situations a single RTK can mediate several biological responses. This most likely depends on properties of the RTK, as well as the particular cellular environment in which it is expressed.

For the $p190^{MET}$ RTK, ligand binding is believed to induce receptor dimerization (38). This process promotes interactions between kinase domains and stimulates their intrinsic tyrosine kinase activities (76). This results in trans-autophosphorylation of their tyrosine residues. Autophosphorylation of the MET RTK occurs on Tyr-1234 and -1235 located within the kinase domain (28,120). This gives rise to an increase in tyrosine kinase activity (kinase activation) and phosphorylation of Tyr-1356.

It has been proposed that kinase activation may result in recruitment to the receptor of cytosolic enzymes responsible for the production of second messengers (14). According to this model, the tyrosine kinase activity of the receptor is primarily directed to carry out self-phosphorylation at defined tyrosine residues. These phosphotyrosines then serve as high affinity docking sites for signal transduction molecules containing specific domains responsible for receptor binding, (known as Src-homology 2 (SH2) domains). Numerous SH2-containing proteins have been identified and those that bind with- and are phosphorylated by activated $p190^{MET}$ include phospholipase C-γ (PLC-γ), phosphatidylinositol 3-kinase (PI3-K) and the GTPase-activating protein of *ras* (GAP) (44). Such proteins are thought able to exert their distinct enzymatic activities and transmit a biological signal (frequently involving other signal transduction molecules) upon their tyrosine phosphorylation (87). Ultimately, this leads to a cellular response.

1.9 Expression of c-*met* in Normal Tissues

In contrast to its ligand, Northern, Western and immunocytochemical analyses have determined c-*met* is expressed, and is present, in hepatocytes. Moreover, these analyses have determined its major transcript*, an 8 Kb mRNA species encoding $p190^{MET}$, is selectively expressed in various epithelial tissues (22,81). Using a cDNA probe encompassing the entire c-*met* coding sequence, DiRenzo and colleagues (1991) (22) detected high

*In some fresh tissues transcripts of 7 and 5.2 Kb are also detectable. These correspond to the isoforms of MET.

levels of c-*met* mRNA in epithelial tissues isolated from the human liver, gastrointestinal tract, thyroid, kidney and brain. Lower levels were found in epithelium of the prostate, seminal vessicles and breast. In the lung, uterus, ovary, skin and skeletal muscle epithelia c-*met* mRNA was very low, while in the adrenal gland, bone marrow and spleen it was undetectable. In the adult mouse, levels of c-*met* expression were generally consistent with those in human tissues (41). Exceptions included high levels in the lung, uterus and skin, and no expression in the liver and intestine. In both organisms, the tissue distribution of $p190^{MET}$ reflected the pattern of expression of HGF/SF, thus providing evidence for the paracrine relationship between adjacent tissues of a particular organ.

In addition to the above human c-*met* mRNA studies, a Western blot analysis (22) and immunofluorescence microscopy (81) showed the levels of $p190^{MET}$ in different tissues generally corresponds to the amount of detectable mRNA. However, in the thyroid, $p190^{MET}$ was almost undetectable even though its mRNA level was high, suggesting that either the mRNA was not efficiently translated or the protein was unstable. This indicated that expression of c-*met* may be specifically regulated at the translational or post-translational levels in different tissues/cells (22). The variation in c-*met* mRNA levels among different tissues also suggest there must be some form of regulation at the transcriptional level. Indeed, Gambarotta *et al.* (1994) (31) have provided evidence of this since they have identified and assayed, by cloning and transfection techniques, a 300 base-pair promoter region upstream from the MET transcription start site. Initial analysis has indicated this region contains *cis*-acting elements responsible for the tissue-restricted expression of c-*met*. Despite this

advancement however, the understanding of regulation of c-*met* gene expression is still at a preliminary stage.

Due to the wide distribution of MET among various epithelial cell types; its organ/tissue-specific expression; its high expression levels in certain mouse and human epithelia; and its pluripotent functions, the HGF receptor is thought to play a role in epithelial cell physiology. Since MET has also been detected in melanocytes and endothelial cells (14), it seems to have a physiological role in certain non-epithelial cells as well. Combined with the ubiquitous presence of HGF/SF in embryonic and adult tissues, it is probable this ligand-receptor system plays an important role in normal growth, development and tissue repair. Moreover, since cell proliferation, motility and differentiation are normally tightly regulated, it is possible that abnormal expression and secretion of HGF/SF or the MET RTK might contribute to neoplastic growth and/or tumour metastasis. In fact, co-expression of MET and HGF/SF in NIH3T3 fibroblasts has been reported to induce tumourigenicity (104). This autocrine loop has also been reported in human lung carcinoma and osteosarcoma cell lines (29,104). With respect to the c-*met* gene, its potential involvement in cancer is described in the following section.

1.10 Expression and Activation of c-*met* in Tumour Cells/ Tissues

In an earlier discussion regarding activation of oncogenes (Section 1.6.1), it was explained transforming potentials are generally accompanied by alterations that either give rise to their increased expression, or, by ones

that result in mutant, growth-promoting oncoproteins. Current literature pertaining to involvement of the c-*met* proto-oncogene in tumours, tumour cell lines or experimentally transformed cell lines, suggest this gene may become activated by *both* types of mechanisms depending on the particular cell/tissue type of scrutiny (below). Genetic alterations leading to activation of this oncogene are believed to potentially lead to a subversion of the normal signalling pathways controlled by the RTK (17,27). Moreover, other studies suggest c-*met* also behaves as an inactivated tumour suppressor gene. Despite this apparent complexity, the goal to unravel the possible involvement of c-*met* in tumourigenesis begins with examination of its DNA, gene expression and protein RTK among neoplastic tissues. Subsequent elucidation of the molecular mechanisms governing its dysregulation will also be required. The following subsections give a historical account of c-*met* activation and expression in neoplasia, beginning with the original study that led to detection of this RTK gene.

1.10.1 Activation of c-*met* by Rearrangement

Previously, c-*met* was described to be originally identified in the chemically-transformed human cell line MNNG-HOS. A re-arrangement event (i.e., *tpr-met*) was responsible for activation of the gene (21,77,103). This results in a hybrid transcript that is translated into a 65 kDa oncoprotein lacking the first 1026 amino acids of MET and is endowed with constitutive tyrosine kinase activity (20,37,101). This resembles the Philadelphia chromosome fusion protein in CML. By virtue of this activity, the *tpr-met* fusion protein was believed to be responsible for transformation of the NIH3T3 mouse fibroblast cells. Furthermore, it was suggested this

fusion protein might participate in human tumourigenesis. In fact, its mRNA was later detected at very low levels in certain cell lines derived from human gastric carcinomas (93). Despite this instance however, there are no other reports concerning the presence of *tpr-met*, nor any other examples of rearranged c-*met* DNA in human tumours.

1.10.2 Activation of c-*met* by Defective Post-translational Processing

As explained earlier (Subsection 1.8.1), the mature $p190^{MET}$ RTK is composed of a 50 kDa α-subunit and a 145 kDa β-subunit. These arise from proteolytic cleavage of a 170 kDa single-chain precursor. Mondino *et al.* (1991) (67) have found a human colon carcinoma cell line (LoVo) expressing an abnormal single-chain, 190 kDa MET polypeptide present at the cell surface ($p190^{NC}$). Pulse-chase experiments showed the 170 kDa precursor is normally produced and glycosylated but is not cleaved. Since the proteolytic cleavage site was conserved, it was deduced that a defective post-translational mechanism was responsible for the lack of cleavage. Using immunoprecipitation and a kinase assay, $p190^{NC}$ was determined to be a functional tyrosine kinase *in vitro*. Unlike $p190^{MET}$ however (which is not phosphorylated on tyrosine *in vivo* under physiological conditions), a Western blot analysis of LoVo cells using antiphosphotyrosine antibodies revealed $p190^{NC}$ exhibited constitutive autophosphorylation on tyrosine *in vivo*. This was indicative of activation of the kinase and it was proposed in the LoVo cell line to be a result of $p190^{NC}$ mimicking the activated conformation reached by $p190^{MET}$ upon binding to HGF/SF. Thus, activation of the c-*met* gene in this example is believed to be due to a faulty post-translational mechanism that does not cleave the MET precursor and

results in an oncoprotein endowed with deregulated tyrosine kinase activity. The mechanism responsible for normal cleavage of the precursor remains to be elucidated.

1.10.3 Overexpression of c-*met*

Previously, it has been shown that experimentally induced overexpression of several RTKs, such as EGF-R or CSF-1-R, is sufficient to induce malignant transformation in NIH3T3 mouse fibroblasts (14). Although it has not been shown for human c-*met*, overexpression of mouse c-*met* in transfection experiments with NIH3T3 cells has been reported to elicit the malignant phenotype (41). The first evidence of 'naturally occurring' overexpression of c-*met* came from a study of *spontaneously* transformed NIH3T3 cells (16). Southern analyses with probes corresponding to regions of activated human c-*met* (from MNNG-HOS cells) determined that unlike normal NIH3T3 cells, a high proportion of these transformed ones had a 4- to 8-fold amplification of the mouse c-*met* gene. Northern analyses with the same probes showed these cells had at least a 20-fold overexpression of the major c-*met* mRNA transcript, whereas in untransformed NIH3T3 cells the transcript was undetectable. These observations suggested amplification and concomitant overexpression of c-*met* in the fibroblasts could be involved with their malignant transformation. This fact combined with the results of Tempest *et al.* (1988) (102)— that considerable variation in the kinase activity of MET existed among human tumour cell lines— led to studies of c-*met* expression in human tumours and tumour cell lines.

The first *human* example of c-*met* amplification and overexpression was in the GTL-16 gastric carcinoma cell line examined by Giordano *et al.* (1989) (35). Using Southern and Northern blot analyses with probes corresponding to specific regions of the c-*met* gene (metH and metG respectively), combined with a Western blot analysis with anti-MET antibodies, a 10-fold amplification of c-*met*; high levels of c-*met* transcripts; and over-abundance of MET were revealed in this cell line. As well, the MET kinase was found constitutively activated. Since the primary structure of MET was indistinguishable from that found in normal cells, and there was no evidence for a rearrangement of the amplified c-*met* allele (79), overexpression alone was suggested to be sufficient for activation of the MET kinase. Furthermore, because it was known at this time that mouse c-*met* had transforming activity (41), and that its coding sequence was highly homologous with human c-*met* (13), human c-*met* therefore was thought to have transforming potential. More specifically, the HGF/SF-MET system could have contributed to the development of malignancy in GTL-16 cells, conferring to them a growth advantage by virtue of overexpression and concomitant constitutive activation of the RTK.

Liu *et al.* (1992) (55) studied the mRNA expression of c-*met* and two other growth factor RTK genes in 21 pairs of normal human colorectal mucosa/primary colorectal carcinoma specimens. Northern analysis of normal mucosa revealed highly variable mRNA levels of EGF-R and c-*erb*B-2, and consistently low amounts of c-*met* mRNA. However, in the corresponding tumour tissues only c-*met* showed an alteration in its transcript levels. It frequently had significantly higher amounts of mRNA (6-fold mean). In addition, c-*met* mRNA levels were also enhanced in colorectal *adenomas*. This suggested overexpression of this gene might

play an important role in the early stages of human colorectal carcinogenesis*. In an independent study of seven colorectal carcinoma cell lines, Southern analysis of c-*met* did not reveal any gene amplifications (50). A similar result was obtained in a different Southern analysis of colorectal carcinoma specimens— increased c-*met* expression was not accompanied by gene amplification (22). Hence, the mechanism(s) leading to c-*met* overexpression in these neoplasms is unknown.

In human gastric carcinomas however, c-*met* amplification has been found. It is particularly frequent in those of the scirrhous type, as revealed by Southern analysis with the p-metH and p-metD probes (50). Furthermore, gastric carcinoma patients having c-*met* amplification showed significantly advanced tumour stage and poorer prognosis than those without the amplification. This research team later compared expression of the gene in non-neoplastic gastric mucosa and gastric tumour tissues. Forty-eight percent of gastric carcinomas exhibited c-*met* overexpression (51). These results suggested amplification and overexpression of c-*met* may participate in the development and progression of stomach carcinomas, particularly in the scirrhous type.

As stated above, the MET RTK is barely detectable in normal human thyroids. In contrast, it was reported to have a 100-fold over-abundance in 22 of 41 human carcinoma specimens derived from the thyroid follicular epithelium (23). Western analyses using a rabbit polyclonal antiserum raised against a portion of the β-chain of MET also revealed no over-abundance in other classes of thyroid neoplasms, namely adenomas, medullary carcinomas and anaplastic carcinomas. Moreover, among certain

*See 'Adenoma-carcinoma sequence' in Subsection 1.6.4 (p.25)

of the follicular carcinomas that were sub-classified according to their histological and clinical features, all overexpressing ones showed evidence of an aggressive phenotype, both histologically and clinically. It was therefore proposed that c-*met* might participate in the progression of specific histotypes of human thyroid cancers towards malignancy. Western analysis with monoclonal antibodies directed against various domains of the over-abundant c-*met* proteins revealed no major structural alterations. As well, investigation by Southern analysis of the c-*met* gene in tumours overexpressing the HGF/SF receptor revealed no gene rearrangement or amplification. The mechanism responsible for the elevated MET levels in these tumours thus remains to be identified.

This research group also studied c-*met* DNA and expression levels in non-neoplastic human ovary, benign ovarian tumours, and epithelial ovarian carcinomas using Southern and Western analyses (24). Although MET was detectable in the surface epithelium of normal ovary, and its level was unchanged in benign ovarian tumours, 20% of the carcinomas showed a 3- to 10-fold increase in its expression while an additional five cases had a 50-fold elevation. Histologically, overexpressing tumours generally showed an early stage, well-differentiated phenotype. This suggested overexpression of MET might act early in the progression of ovarian carcinomas, conferring a growth advantage to neoplastic cells derived from the ovarian epithelium. Using Southern analysis with a cDNA encompassing the entire c-*met* coding sequence, no evidence of gene amplification or rearrangement was found.

In the following two studies, c-*met* expression was assessed not in epithelial derived neoplasms (carcinomas), but rather in tumours of mesenchymal and hematopoietic origin, (tissues known not to express c-

met). In the first, Western and Northern analyses showed MET to be highly expressed in 60% of human osteosarcomas examined and in 100% (12/12) of osteosarcoma cell lines (29). The HGF/SF receptor was not detected in any other varieties of bone sarcomas analyzed. C-*met* DNA analysis in the overexpressing tumours by Southern blot hybridization did not reveal evidence for amplification or rearrangement. Interestingly, some osteosarcoma specimens and cell lines co-expressed MET and HGF/SF, as revealed by Northern analysis. In these cell lines MET was constitutively phosphorylated in the absence of exogenously added ligand, indicative of deregulated receptor activation. Together the results suggested c-*met* may possibly contribute to osteosarcoma transformation due to its overexpression. In addition, an autocrine loop between it and its ligand might add a selective advantage for the malignant progression of osteosarcoma cells, making them independent from external support.

In the second report, analysis of 50 primary specimens of human leukemia and lymphoma and 23 tumour cell lines of hematopoietic origin revealed expression of c-*met* mRNA and protein in 6 out of the 73 tumour samples (43). This was considered abnormal since MET is not detectable in lymphocytes, granulocytes or monocytes— the cells from which these tumours are derived. Southern experiments did not show amplification or rearrangement of the c-*met* gene. However, cytogenetic data suggested the abnormal expression of c-*met* might have been due to chromosomal rearrangements. It may be concluded from the above two reports that dysregulated c-*met* expression is not limited to tumours of epithelial origin.

Prat and colleagues (1991) (81) first demonstrated abnormal c-*met* expression in human bronchogenic carcinoma. Using immunohistochemistry with anti-MET antibodies they detected

homogenous staining in carcinoma of the lung, while in normal lung tissues staining was barely detectable. Later, Liu and Tsao (1993b) (57) examined the mRNA expression of c-*met* in 29 primary tumours and 8 cell lines of human NSCLC. The mean c-*met* mRNA level was higher in tumours than normal (although the difference was not statistically significant), and among certain of the NSCLC histotypes, ADCs, ADSQCs and their cell lines expressed relatively high amounts of c-*met* mRNA whereas the level was unchanged or lower in SQCCs. Southern analysis indicated only 1 of the 29 tumours had a c-*met* amplification. These results not only demonstrated a differential expression of c-*met* among histotypes of NSCLC, but also raised suspicions of a potential clinical significance of this gene in human lung cancer.

In a larger panel of NSCLC with their corresponding normal lung tissues (130 pairs), Zhu *et al.* (1993) (118) found variable levels of c-*met* mRNA among ADCs and LCCs, whereas SQCCs generally had decreased levels compared to normal lung parenchyma. In addition, certain SQCC and ADC cases had undetectable c-*met* mRNA and protein. This was unusual since normal bronchial epithelial cells normally express c-*met*. This result could be indicating that loss (or reduction) of c-*met* expression may give rise to loss of a *growth suppressive* effect of MET, thus contributing to the tumourigenesis of certain NSCLCs. Notably, this suggestion contradicts the above observations in other human cancers— that elevated MET levels are associated with *growth promotion*. Yet, reports of tumour cytotoxic activity of HGF/SF-MET in hepatocellular carcinoma cells (89) provide further support for this 'growth-suppressive' possibility.

In summary, the above reports serve to illustrate that c-*met* has transforming activity in both the mouse and MNNG-HOS cells, and in many

human cancers it is overexpressed at various stages of tumour progression (summarized in Table 2). Also demonstrated was the apparent contradiction of the role for c-*met* in cancer, (i.e. growth promotion versus loss of a normal growth-suppressive role). Whether c-*met* overexpression has pathogenic implications in human cancer could be argued since the possibility exists that this abnormality could be a bystander effect of some para-phenomenon, rather than a factor contributing to neoplastic development and malignant progression. However, several lines of evidence strongly suggest c-*met* is directly involved in the tumourigenesis of certain cancers having an oncogenic role, including: 1) its overexpression is common both among and within certain types of human neoplasms; 2) HGF/SF-MET participate in multiple biological functions that are crucial to normal homeostasis and therefore have innate oncogenic potential; and 3) the onset of c-*met* overexpression in certain human neoplasms is associated with an aggressive phenotype. Less clear is the molecular mechanism(s) responsible for MET overexpression. Contrasting with its oncogenic role, c-*met* has been identified in hepatocellular carcinoma cell lines (along with HGF/SF), and suggested from a study of NSCLC tumours, to also have a growth suppressive role. Could it be that the same receptor-ligand system might have opposing biological effects in different tissues? Of particular interest to the 'suppressive function' is the region containing c-*met* on the long arm of chromosome 7 is often deleted in a variety of human neoplasms (117). This is the focus of the next and final section to this introduction.

Table 2- Summary of c-*met* proto-oncogene anomalies in various mouse and human tumour cell lines and tissues.

Cell Line/Tumour Type	Activating Mutation	C-*met* Abnormality	Reference
Transformed MNNG-HOS Cells	rearrangement	tpr-met fusion protein	Cooper *et al.*, 1984 (15)
Human Gastric CA Cell Lines	rearrangement	tpr-met fusion protein	Soman *et al.*, 1991 (93)
Human Colon CA Cell Line (LoVo)	defective post-translational processing	single-chain p190 RTK	Mondino *et al.*, 1991 (67)
Spontaneously Transformed Mouse Fibroblasts (NIH 3T3)	amplification (4-8 fold)	overexpression (20-fold)	Cooper *et al.*, 1986 (16)
Human Gastric CA Cell Line (GTL-16)	amplification (10-fold)	overexpression	Giordano *et al.*, 1989 (35)
Primary Human Colorectal CA	undetermined	overexpression (6-fold)	Liu *et al.*, 1992 (55)
Human Gastric CA	amplification	overexpression	Kuniyasu *et al.*, 1992/93 (50,51)
Human Thyroid CA (derived from follicular epithelium)	unknown	over-abundance of c-*met* protein	DiRenzo *et al.*, 1992 (23)
Human Ovarian CA	unknown	overexpression (3-10 fold, 20%; 50-fold,8%)	DiRenzo *et al.*, 1994a (24)
Human Osteosarcoma	unknown	overexpression (combined with autocrine/paracrine HGF production)	Ferracini *et al.*, 1995 (29)
Human Leukemia and Lymphoma	unknown	8.2% of cases expressed c-*met* abnormally	Jucker *et al.*, 1994 (43)
Primary Human NSCLC	unknown	>2-fold overexpression in 60% of ADC and 14% of SQCC	Liu *et al.*, 1993b (57)

CA- carcinoma; NSCLC- non-small cell lung carcinoma; ADC- adenocarcinoma of the lung; SQCC- squamous cell carcinoma of the lung

1.11 Loss of Heterozygosity of C-*met* and an Adjacent Locus, D7S95, in Cancer

As stated above, cytogenetic techniques are helpful preliminary measures for identifying the locations of putative TSGs. Consistent deletions or inversions of part of a chromosome among a specific tumour type are indicative of inactivation of an underlying TSG during neoplastic development and/or progression. Recently, karyotype reports have demonstrated the long arm of chromosome 7 (7q) is frequently deleted in various types of human cancer. This includes breast carcinoma (103), prostate carcinoma (58), SQCC of the head and neck (75), ADCs of the colon, ovary and rectum (4), bladder carcinoma (4), and seminoma of the testis (4). A higher degree of resolution has been indicated in other cytogenetic reports, including deletions of 7q22-q31.1 in uterine leiomyoma, 7q31.1-32 in malignant andrological neoplasms and 7q31-32 in breast and ovarian carcinomas (117). Thus, these data provide evidence for a potential TSG(s) on the long arm of human chromosome 7. However, due to the sensitivity limitations of cytogenetic techniques, the precise chromosomal location of the TSG(s) is unknown.

Supporting evidence for the existence of a TSG(s) on human chromosome 7 is given in a report describing an unknown gene(s) situated on this chromosome that is responsible for the cessation of cell division in a particular human cell line (74). Insertion by microcell-mediated chromosome transfer of an intact human chromosome 7 to immortalized human fibroblast cell lines having LOH at 7q31-32 led to restoration of their senescent properties. This implies a TSG(s) may be located on this

band and that it (they) restored senescent properties to the cells by replacing the lost function. Interestingly, since HGF/SF-MET has a growth-inhibiting effect in certain tumour cell lines and because c-*met* is situated on 7q, the possibility arises that the unknown TSG could be c-*met*. Yet, before this was even recognized (since the growth-suppressive role of c-*met* had not yet been elucidated), researchers in France were already seeking molecular evidence for c-*met* allele loss on chromosome 7. This was simply based on the facts that deletions of 7q loci were common among various human neoplasms; c-*met* was situated in this region; and that the expression patterns of c-*met* were drastically modified in human tumour tissues and cancer cell lines.

In particular, Bièche and colleagues (1992) (5) attempted to discern if the c-*met* locus exhibits LOH among patients with primary breast cancer— a cancer showing frequent 7q deletions. Using the p-metH polymorphic-sensitive probe in a Southern analysis of normal and tumour samples from 245 patients, a significant proportion (41%) of 121 informative cases indeed showed allelic loss at this locus. No amplifications or rearrangements of the gene were detected. Furthermore, patients with LOH had significantly shorter survival times than those without this anomaly. These observations suggested— on the contrary to the previous studies indicating c-*met* behaves as a proto-oncogene— a possible role for c-*met* as a breast cancer TSG. Notably, this resembles the 'bifunctional' nature of the gene for p53. Although this gene is a tumour suppressor, it exhibits properties of a proto-oncogene, (i.e., at times expressing a mutant, growth-promoting oncoprotein) (59,108). It may be possible that c-*met* is also a bifunctional gene, dependent on the particular type of mutation causing its alteration.

Nevertheless, the results of this study provide evidence that c-*met* could be a TSG residing on human chromosome 7.

In primary human lung cancer, there are presently no molecular reports of 7q allelic losses. Karyotype analyses, however, have revealed a consistent deletion breakpoint at 7q22 in ADCs, as well as frequent trisomy 7 among bronchogenic carcinomas in general (117). In addition, many of the trisomic cases are associated with interstitial deletions on 7q. Combined with the facts that c-*met* is situated distal to 7q22; its expression is dysregulated among NSCLCs; its mRNA transcripts are undetectable in certain SQCCs and ADCs; as well as its significant LOH incidence in the Bièche study, these data were sufficient evidence to explore c-*met* LOH in NSCLC. Perhaps c-*met* is a lung cancer TSG that is inactivated in particular subtypes of NSCLC.

Soon after this project was initiated we learned of a new molecular study that presented evidence of frequent LOH for certain 7q regions among patients with gastric carcinoma. Employing five probes (including p-metH) that covered the full length of 7q in a RFLP analysis of 98 patients, Kuniyasu *et al.* (1994) (52) revealed a significant proportion of informatives (20%) had LOH at c-*met*. Moreover, a higher LOH incidence (43%) was determined at a broad locus distal to this gene, called D7S95 (7q31-q35). Recalling that the higher the LOH frequency is scored for a particular marker, the closer it is situated to a putative TSG locus, it is therefore evident that in gastric carcinoma c-*met* is not a putative TSG. Rather, it is contained somewhere in 7q31-q35, distal to c-*met*. Other important findings were the following: 8 of 13 cases with LOH at D7S95 belonged to the most advanced gastric carcinoma stage; and 6 of 8 cases with abdominal dissemination exhibited LOH at D7S95. These implied cases with D7S95

LOH have worse prognosis than those without this allelic loss. Combined with the high LOH frequency at D7S95, these data led us to investigate allelic loss of D7S95 in NSCLC.

2. SPECIFIC AIMS

1) To study the possible involvement of inactivated c-*met* alleles in the development and progression of NSCLC.

The potential role for c-*met* as an inactivated NSCLC TSG is investigated here. This can be facilitated by RFLP analysis of the c-*met* gene in a large panel of NSCLCs. If its LOH incidence is high in NSCLC, this would suggest a possible growth-suppressive role for this gene, or alternatively, for a closely linked 7q region. Moreover, if the c-*met* LOH frequency is statistically significant among NSCLCs, and supposing a strong correlation exists between LOH cases and worse prognosis, a clinical role for c-*met* as prognostic factor could eventually be possible. Furthermore, if its LOH is common among a particular subtype of NSCLC, loss of this locus could also have a potential use as a diagnostic factor.

2) To determine LOH incidence for the D7S95 locus in NSCLC.

Since a locus adjacent to c-*met* on chromosome 7 (D7S95) was found to exhibit a significant LOH frequency in gastric carcinoma, we wish to determine if loss of this locus is common in NSCLC. This may be accomplished by RFLP analysis. Perhaps the D7S95 locus in NSCLC patients could ultimately serve as a prognostic/diagnostic factor.

3) To investigate whether c-*met* allelic loss is associated with reduced c-*met* transcript levels.

Since it is logical to assume that losses at the DNA level should be accompanied by losses (or reductions) at the RNA level, we chose to

investigate c-*met* expression levels for tumours exhibiting LOH at this locus. This involves a comparison of LOH-positive and LOH-negative cases with their corresponding c-*met* mRNA levels. To note, these mRNA levels were previously measured by our laboratory in a semi-quantitative analysis.

3. MATERIALS AND METHODS

3.1 Normal Lung and Lung Tumour Specimens

Normal lung tissue and lung tumour specimens were obtained from patients undergoing curative resection for primary NSCLC at Montreal General Hospital between 1989 and 1995. Normal and tumour tissues were obtained soon after surgery and were snap frozen in liquid nitrogen. These were stored at -80°C. The tumours to be evaluated in this study were classified using standard histopathological criteria (3). These included 59 adenocarcinomas, 33 squamous cell carcinomas, 11 large cell carcinomas, 2 adenosquamous carcinomas and 4 carcinoid tumours. Details of tumour sizes and pathological stages were obtained from surgical pathology reports and patient medical records. The tumours were staged according to the TNM system.

3.2 DNA Extraction and Isolation

To prepare the normal and tumour samples for DNA isolation, approximately 0.5-1.0g of tissue was immersed in liquid nitrogen. This was immediately pulverized with a pestle and treated with 5ml RSB buffer (Tris/NaCl/EDTA), 1ml 10% sodium dodecyl sulfate (SDS) and 0.5ml proteinase K (10mg/ml). Following overnight digestion at 50°C with gentle agitation, each sample was subjected to a series of equal volume phenol-chloroform extractions. In the first step a 10:10:1

phenol:chloroform:isoamyl alcohol mixture was utilized. This was added to the sample in a 15ml-centrifuge tube and mixed by gentle inversion. After 10min of 4°C centrifugation at 9500rpm, two layers appeared. The aqueous layer, which contained the unpure DNA, was removed for further extraction while the bottom layer was discarded. In the second step the procedure was repeated using another 10:10:1 mixture of phenol, chloroform and isoamyl alcohol. In subsequent extractions only chloroform was used. The extraction procedure was considered complete when the interface between the upper and lower layers was fully transparent. DNA was precipitated using 2.5 volumes of cold 99% ethanol and placed at room temperature for 10min in an open 1.5ml-eppendorf tube, allowing the sample to dry. Depending on the relative amount of DNA isolated, various volumes of TE buffer, pH 7.6 were added, ranging from 200 to 500µl. After remaining overnight to dissolve, DNA samples were gently pipetted to permit further dissolution. To measure the concentration and relative purity, absorbencies were measured at 260 and 280nm using an ultraviolet spectrophotometer, (relative purity= 260nm reading/280nm reading). The paired samples were stored at -20°C prior to use.

3.3 Isolation of DNA Probes for Southern Hybridization

Three polymorphic-sensitive, single-stranded human genomic DNA probes— p-metH, p-metD and CRI-pS148— were used to examine LOH on human chromosome 7 (Table 3). The metH locus is located at the extreme 3' end of c-*met*, while metD is situated further upstream. Both c-*met* probes were used in order to increase the number of informative cases. The CRI-

pS148 probe recognizes the D7S95 locus of chromosome 7, which is adjacent and downstream from c-*met* at 7q31-35 (52). P-metH and p-metD were kindly provided by Dr. Morag Park's laboratory (Royal Victoria Hospital, Montreal, Quebec) and CRI-pS148 was purchased from Collaborative Research Inc. (Waltham, MA).

Table 3- DNA probes used in the present LOH study.

Locus	Probe	Insert Size (Kb)	Location	Plasmid Vector	Restriction Enzyme
c-*met*	p-metH	1.6	7q31.1	pBR322	MspI or TaqI
	p-metD	1.1	7q31.1	pBR322	TaqI
D7S95	CRI-pS148	-	7q31-q35	pUC8	MspI

All probes received were incorporated in plasmid vectors. pBR322 plasmids were used for cloning both p-metH and p-metD, while the pUC8 plasmid was used for CRI-pS148. To culture the plasmid vectors, the metH- and metD-containing plasmids were incorporated in separate DH5 strains of E. coli and grown overnight (12-16 hours) with rigorous shaking at 37°C in 3ml LB-ampicillin (Luria Bertani broth, pH 7) starter cultures. Following this, 2.5ml of each culture was added to a 250ml LB-ampicillin growth culture and grown overnight in the same conditions as above. Since the CRI-pS148-containing vectors were received already incorporated in E. coli, a bacterial stab was inoculated into a 3ml starter culture and grown overnight at 37°C. Two and a half milliliters of this culture was placed the next day in a 250ml growth culture and grown overnight. All plasmid

DNAs were harvested and purified using the QIAGEN Plasmid Maxi-Prep kit (QIAGEN Inc., Chatsworth, CA). These were dissolved in suitable volumes of TE buffer, pH 7.6 and measured for concentration and relative purity using the UV spectrophotometer.

For the pBR322 DNAs it was necessary to isolate the metH and metD inserts to improve the quality of hybridization, (i.e., to reduce autoradiographic background). This was accomplished by digesting the plasmid DNAs with the appropriate restriction enzyme (Table 3), and subjecting them to 1.0% agarose gel electrophoresis (stained with ethidium bromide) in 1X TAE buffer. A 1.6 Kb metH band and a 1.1 Kb metD band were excised from the gel and isolated using the QIAEX protocol (QIAGEN Inc., Chatsworth, CA). These were dissolved in appropriate volumes of TE buffer and measured for concentration and relative purity using the UV spectrophotometer. The protocol for the CRI-pS148-containing plasmid indicated the entire plasmid could be used for Southern blot without any loss of hybridization quality. Further procedures to isolate the DNA insert were therefore unnecessary.

3.4 Digestion of Normal/Tumour DNA Pairs with Restriction Enzymes

To prepare the normal and tumour DNA samples for Southern blot analysis, enzymatic digestion was performed. Samples were treated with either Msp1 or Taq1 restriction endonuclease (New England Biolabs, Beverly, MA), depending on the particular DNA probe to be used (Table 3). This created the polymorphic fragments required to assess loss of heterozygosity. In a 1.5ml centrifuge tube, 50 units of enzyme was added to 10μg sample DNA along with 1/10 final volume of appropriate 10X buffer (New England Biolabs, Beverly, MA). For Taq1-digests, reaction mixtures were supplemented with 1/10 final volume of 100X bovine serum albumin (BSA). Reaction mixture volumes were adjusted with autoclaved distilled water and depending on the concentration of each DNA sample, final volumes ranged from 30 to 50μl. Msp1-digests were incubated at 37°C whereas Taq1-digests were placed in 65°C. To avoid condensation, the latter were treated with approximately 50μl of mineral oil. This was removed from the reaction mixture once digestion was accomplished.

After remaining overnight, approximately 2μl of each sample was subjected to mini gel electrophoresis to ensure digestion was complete. If incomplete, an additional 20 units of appropriate restriction enzyme was added, followed by an additional mini gel test. If complete, the sample was either prepared immediately for large gel electrophoresis or stored at -20°C in 1/6 volume of 6X DNA loading dye.

3.5 Electrophoresis of Digested Normal/Tumour DNA Pairs

Large gel electrophoresis was performed to fractionate the digested DNA samples. Two microliters of the molecular weight DNA marker, λHindIII (0.5μg/μl), combined with 1μl DNA loading dye was placed in the first lane of each 1.0% ethidium-bromide-stained agarose gel. DNA samples containing 1/6 volume of 6X loading dye were placed into the other lanes as normal/tumour pairs. Gels were then ran at 32V for 18 hours in 1X TAE buffer. To facilitate accurate inspection of the normal and tumour autoradiographic signals, and to ascertain if allelic losses (or amplifications) occurred, it was necessary to ensure equal loading of normal and tumour DNAs.

3.6 Southern Transfer

After electrophoresis, each gel was trimmed and treated with various buffer solutions to prepare for DNA transfer to a nylon membrane. The first step involved placing the gel in 250ml of 0.25M HCl for 30min with gentle agitation. Following rinsing with distilled water, it was soaked in a denaturation buffer (1.5M NaCl, 0.5M NaOH) for another 30min with gentle shaking. After rinsing again with distilled water, a neutralization buffer was employed (1.5M NaCl, 0.5M Tris-HCl, pH 7.2, 0.001M EDTA) for 30min with gentle agitation.

The agarose gel, Whatman 3MM filter paper wicks, a stack of absorbent paper towels and a Hybond-N nylon membrane and were used to construct a capillary blot. Transfer was performed overnight in 10X SSC

buffer and was followed by rinsing (2X SSC) and air-drying of the membrane. To cross link the DNA to the membrane, it was placed face-down for 2min on a short-wave ultraviolet light transilluminator (Fotodyne Inc., New Berlin, WI). Membranes were then either stored at 4°C in cellophane wrapping or prepared immediately for hybridization.

3.7 Southern Blot Hybridization and Detection of LOH

Prehybridization was performed using glass hybridization tubes in a hybridization oven. Fifteen milliliters of prehybridization solution, consisting of 0.75g skim milk powder, 7.5ml 40X deionized formamide, 3ml 20X SSPE, 1.5ml 10% SDS, 2.25ml distilled water and 0.75ml denatured salmon sperm DNA (Pharmacia-Biotech, Baie D'Urfe, Quebec) was added to each membrane. Salmon sperm DNA was denatured by placing it in a 95°C dry bath for 8min, followed by chilling on ice for 2min. Tubes were placed in the oven for 1.5-2 hours at 42°C

The metH, metD and CRI-pS148 probes were denatured and radiolabelled with [^{32}P]-dCTP (ICN Radiochemicals, Irvine, CA) to a specific activity of approximately 1-2x10^{9} dpm/μg, using the Oligolabelling Kit protocol of Pharmacia Biotech. Each was then purified by centrifugation in a syringe column containing 1ml Sephadex G-50. Once the prehybridization solution was discarded, a hybridization solution consisting of 0.75g skim milk powder, 20X SSPE, 40% dextransulfate (Pharmacia-Biotech) 40X deionized formamide and 10% SDS was placed in the tube. After denaturing the purified labelled probe it was added and mixed with this solution, and the tube placed in 42°C overnight. To

minimize background for the CRI-pS148 entire-plasmid probe, 625μg of sonicated human placenta DNA was added to it prior to denaturation.

After overnight hybridization, each radioactive membrane was rinsed twice in a pre-warmed (42°C) 2X SSC, 0.1% SDS wash solution. It was sequentially washed for 15min at 42°C in a fresh 2X SSC, 0.1% SDS solution; 15min in 0.5X SSC, 0.1% SDS ; and 15min in 0.1X SSC, 0.1% SDS. A final washing step was performed, if necessary, with another 0.1X SSC, 0.1% SDS solution, but at 52°C. Washing was considered complete only when analysis of the membrane edges with a Geiger counter showed readings below 200cpm. Membranes were blotted dry and exposed for 1-5 days at -80°C to Kodak-X-OMAT AR X-ray film using intensifying screens.

Visual inspection was the first method of choice for identifying LOH cases. Densitometric measurements were planned for cases with apparent (borderline) LOH. A Hoefer GS-300 scanning densitometer was available for this. Our definition (in accordance with most other LOH studies) considered an informative case to be LOH-positive if, in the *tumour* DNA, one RFLP signal was completely absent or reduced by greater than 50% in intensity compared to its partner signal. Verification of cases showing LOH was accomplished by entirely repeating enzymatic digestion and Southern blot analysis.

4. RESULTS

We investigated LOH at the c-*met* gene (chromosome 7q31.1) in NSCLC by RFLP analysis with two polymorphic-sensitive human DNA probes— p-metH and p-metD (see Section 3.3 in *Materials and Methods*). Both probes, corresponding to different regions of c-*met*, were utilized to increase the number of informative cases at this gene. LOH was also explored at the D7S95 locus (CRI-pS148 probe). It is situated downstream from c-*met* at 7q31-35.

A representative set of results obtained for the metH locus is shown in Figure 7. Using the λHindIII molecular weight marker, two distinct RFLP signals of 2.3 and 1.8 Kb in size were exhibited for informative cases at this locus. These sizes were consistent with those obtained by Kuniyasu *et al.* (1994) (52). Each panel shows certain informative cases (e.g., #118, 121, 7, 99, 68, 56); ones exhibiting LOH (#118, 7, 99 and 56); and certain homozygous normal/tumour pairs (e.g., #120, 89, 67). Although each LOH case shows incomplete absence of a tumour signal, its intensity is reduced by more than 50% compared to its partner signal. Incomplete absence is most likely due to contamination of tumour cells with normal cells initially present in the resected tumour samples. Despite persistent attempts to ensure equal loading of normal/tumour DNA, this was not the case for certain DNA pairs, (e.g., #118, 119, 81, 56, 73). No amplifications of the metH locus were observed for tumour samples.

A representative set of results obtained for the metD locus is shown in Figure 8. Two distinct RFLP signals of approximately 6.6 and 4.8 Kb in size were exhibited for informative cases at this locus. The λHindIII marker

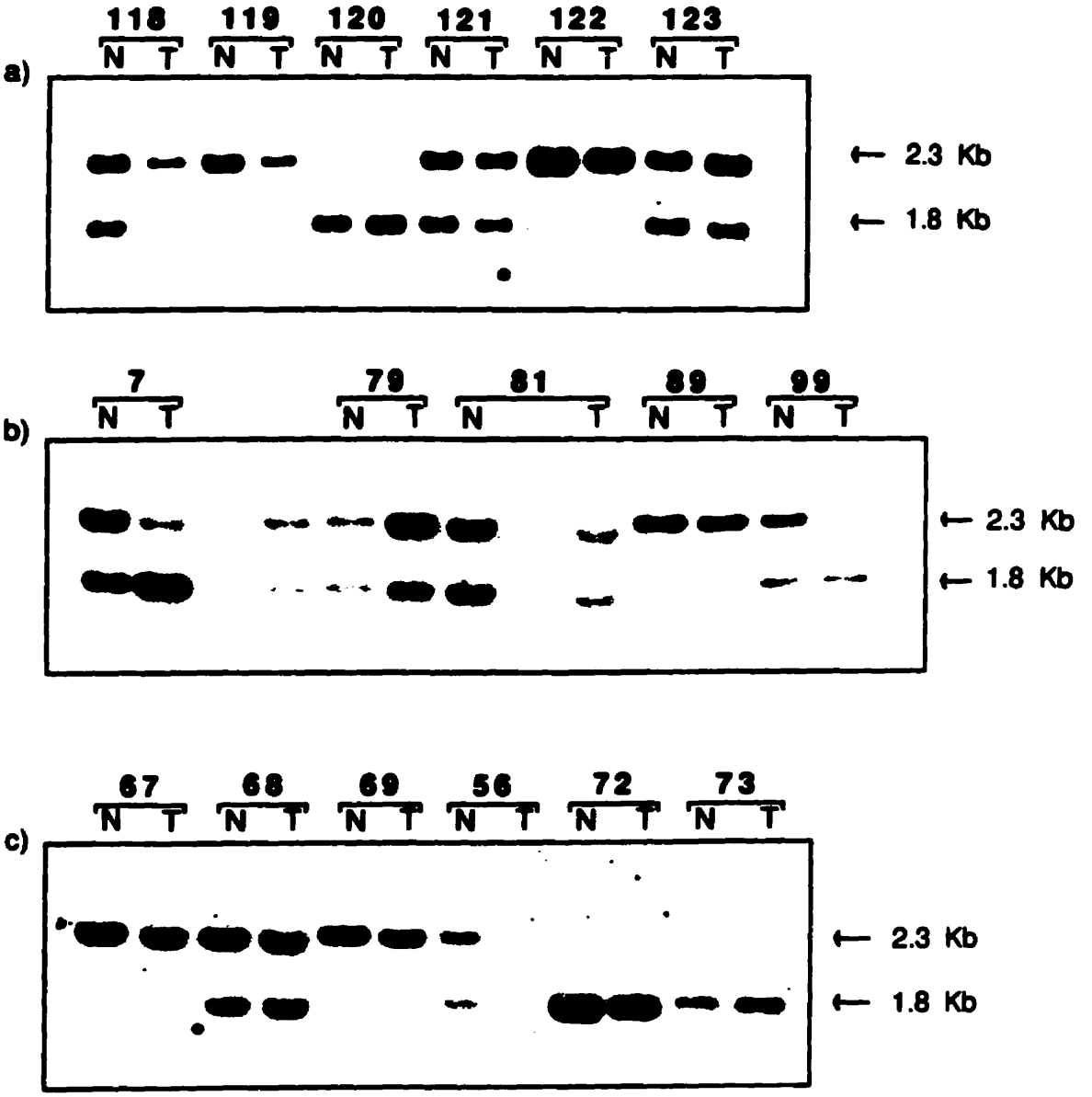

Fig. 7- Representative lung tissue pairs and allele loss at MetH. T: tumour; N: corresponding non-neoplastic lung epithelial tissue. All samples were digested with Msp1 and hybridized with p-metH. Sizes of hybridized fragments are shown at right of each panel. DNA loading is not equal in certain cases. a) Case 118 is informative and shows LOH. Cases 121 and 123 are informatives but have retained both alleles. Cases 119, 120 and 122 are uninformatives (homozygotes). b) Cases 7 and 99 are informatives and are LOH+. Informative cases without LOH include #79 and #81. Case 89 is uninformative. c) Case 56 is informative and LOH+ despite faint 2.3 Kb signal. Case 68 is informative and LOH-. Cases 67, 69, 72 and 73 are uninformatives.

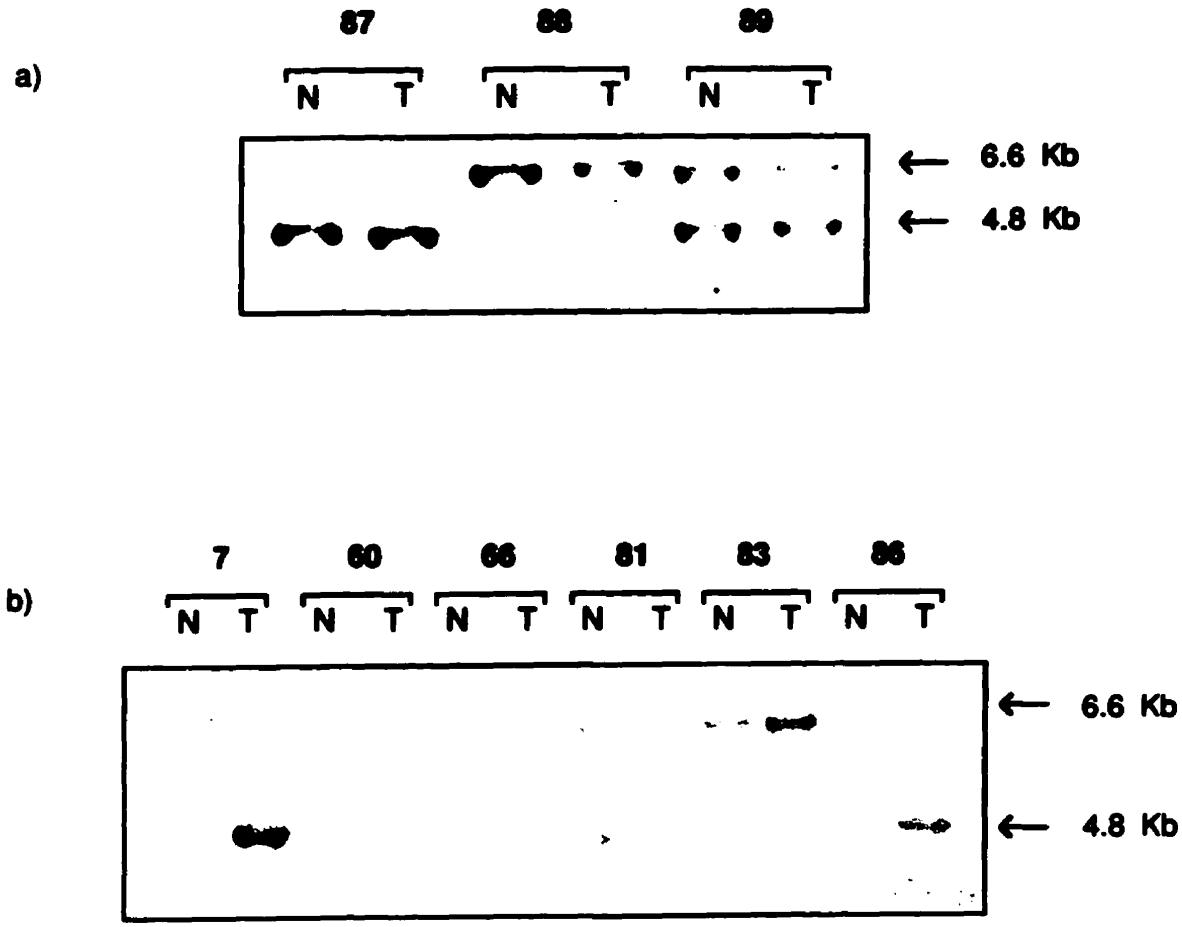

Fig.8- Representative lung tissue pairs and allele loss at MetD. T: tumour; N: corresponding non-neoplastic lung epithelial tissue. All samples were digested with Taq1 and hybridized with p-MetD. DNA loading is not equal in some cases. Approximate sizes of hybridized fragments are shown at right of each panel. a) Cases 87 and 88 are uninformatives. Case 89 is informative and LOH- even though the 6.6 Kb band is reduced in intensity. b) Case 7 is informative and shows LOH with incomplete absence of top signal. This is due most likely to contamination of tumour tissue with normal cells. Cases 60 66, 81 and 86 are informative and do not show LOH. Case 83 is uninformative.

was used to estimate their sizes. The top panel shows two uninformative cases having the different RFLP sizes (#87, 88), and an informative case (#89). Although the upper tumour signal of case #89 is reduced in intensity compared to the lower signal, this reduction is not greater than 50%. Thus, this informative case is LOH-negative. In the bottom panel, case #7 is LOH-positive despite incomplete absence of the upper signal. Again, this is probably due to contamination of tumour cells with normal cells initially present in the resected tumour sample. In addition, DNA loading was not equal for this pair. The quantity of tumour DNA was much higher than that for the normal DNA (electrophoresis photograph not shown), resulting in stronger tumour signals. This panel also shows several informative LOH-negative cases (#60, 66, 81 and 86), as well as an uninformative case (#83). DNA loading was also not equal for cases #81 and 83. Amplifications of the metD locus were not observed.

A representative set of results obtained for the D7S95 locus is shown in Figure 9. Two distinct RFLP signals of 3.3 and 2.2 Kb in size were exhibited for informative cases at this locus, consistent with those obtained by Kuniyasu *et al.* (1994) (52). The top panel shows several uninformative cases (#47, 82, 83 and 86), and an informative LOH-negative case (#87). DNA loading was not equal for cases #47 and 86. The lower panel shows an informative LOH-positive case (#144), as well as an uninformative case (#145). DNA loading was not equal for these two normal/tumour pairs. Amplifications of the D7S95 locus were not observed.

In our LOH analysis, a panel of 110 patients with NSCLC were examined for allelic loss at metH, metD and D7S95. The frequency of informativity and incidence of LOH for the three loci are given in Table 4. Of 110 cases examined at metH, 56 (50.9%) were informative and 4 (7.1%)

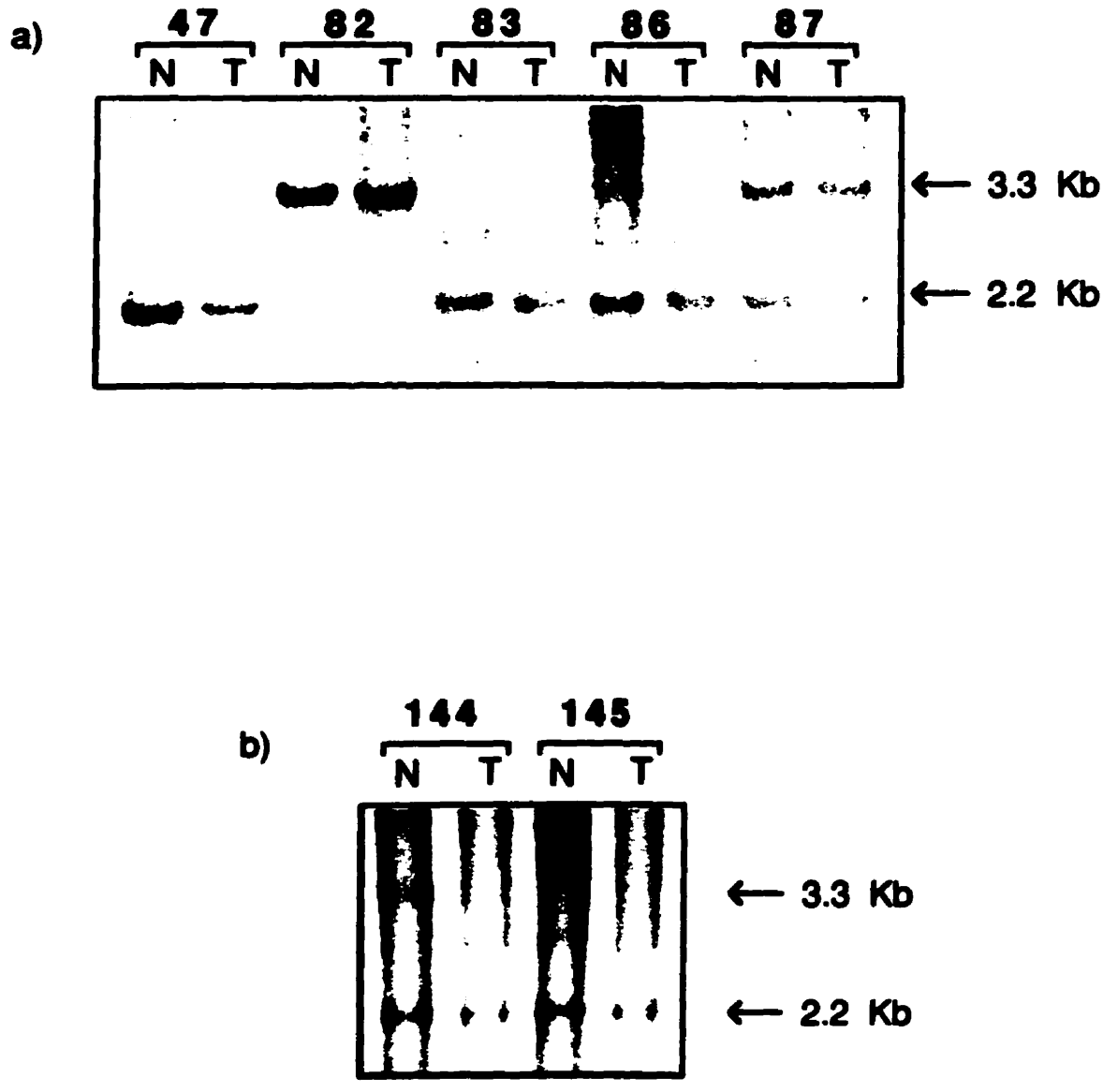

Fig. 9- Representative lung tissue pairs and allele loss at D7S95. T: tumour; N: corresponding non-neoplastic lung epithelial tissue. All samples were digested with Msp1 and hybridized with CRI-pS148. DNA loading is not equal in some cases. Sizes of hybridized fragments are shown at right of each panel. a) Case 87 is an informative. Cases 47, 82, 83 and 86 are uninformatives. b) Case 144 is informative and exhibits LOH. Case 145 is uninformative.

exhibited LOH. These LOH cases are shown in Figure 7. The frequency of informativity at this locus is consistent with those obtained by Bièche *et al.* (1992) (5) (49.4%), Nagy *et al.* (1995) (69) (46.8%) and Tougas (1995) (103) (52.9%). Of 109 patients examined at metD, 28 (25.7%) were informative and 1 (3.6%) was LOH-positive. Notably, this case (#7) was one of those exhibiting LOH at metH. Its LOH is displayed in Figures 7b and 8b. The frequency of informativity at metD could not be compared with that of other LOH studies because this data was unavailable, (i.e., in published c-*met* LOH reports, DNA probes corresponding to either metH or the entire c-*met* gene were utilized and not metD). For the D7S95 locus, 45 of 104 cases (43.3%) were informative and 1 (2.2%) showed LOH. This case is shown in Figure 9b. The frequency of informativity at this locus was consistent with that obtained by Kuniyasu *et al.* (1994) (52) (43.5%).

The number of patients who were uninformative at metH but were informative at metD was twelve. Thus, for the entire c-*met* gene, the total number of informative cases was 68 (56+12). Five of these showed LOH, giving rise to a total c-*met* LOH incidence of 7.4%. With the exception of case 7, all tumours exhibiting LOH at one c-*met* locus were uninformative at the other c-*met* locus (data not shown). Hence, for these tumours it could not be determined if both loci exhibited LOH.

Table 4- Loss of heterozygosity for the three loci on 7q.

	metH	metD	D7S95
cases	110	109	104
informatives	56 (50.9%)	28 (25.7%)	45 (43.3%)
LOH	4 (7.1%)	1 (3.6%)	1 (2.2%)

Cases with LOH at c-*met* included a SQCC and three ADCs. The single D7S95 LOH case was a carcinoid tumour. When comparing the few c-*met* LOH cases with their corresponding relative c-*met* mRNA levels previously measured in our laboratory using Northern analysis (personal communication), all had either very low or undetectable transcript levels. In comparison, ones without this abnormality showed variable c-*met* mRNA levels, ranging from very high to undetectable levels*.

*See c-*met* mRNA levels in NSCLC (p. 54 in Section 1.10).

5. DISCUSSION

Extensive cytogenetic observations of common chromosome 7q alterations among a variety of human neoplasms, as well as microcell-mediated chromosome transfer experiments involving introduction of chromosome 7 to immortalized human cell lines (74) have indicated the presence of at least one TSG on the long arm of human chromosome 7. The precise location, identity and characteristics of this (these) gene(s) have not been elucidated. One technique that could enable identification and localization of the TSG(s) is a loss of heterozygosity analysis. Bièche *et al.* (1992) (5) reported a high LOH incidence (41%) of c-*met* (7q31.1) among primary breast carcinoma patients, indicating it could be an inactivated 7q breast cancer TSG. In contrast, the results of Kuniyasu *et al.* (1994) (52), who investigated 7q allele loss among gastric carcinoma patients, suggested the D7S95 locus (distal to c-*met* at 7q31-q35) contained a gastric cancer TSG. A higher LOH frequency was scored at this locus (43%) compared to that for c-*met* (20%), (Table 5).

In the present study, LOH for the c-*met* gene was detected in 5 of 68 informative samples of primary human NSCLC tumour DNA. At D7S95, only 1 of 45 informative cases showed LOH. Although it is possible c-*met* and D7S95 allele losses may have been underestimated due to cellular heterogeneity of tumour biopsy specimens, it is nonetheless apparent LOH of these loci is very infrequent in NSCLC. Thus, these results are inconsistent with the high incidences of c-*met* and D7S95 allelic loss revealed in the breast and gastric cancer studies respectively (5,52). They indicate firstly, that c-*met* does not have a possible role as an inactivated

Table 5- Molecular evidence for a tumour suppressor gene on chromosome 7q in various human carcinomas.

Tumour Type	Marker (Band)	LOH Frequency (#LOH/#informatives)	Sample Size	Reference
Breast Carcinoma	p-metH (7q31.1)	41% (49/121)	245	Bièche *et al.*, 1992 (5)
Breast Carcinoma	p-metH	4% (2/52)	111	Nagy *et al.*, 1995 (69)
Breast Carcinoma	p-metH	22% (8/36)	68	Tougas, 1995 (103)
Breast Carcinoma	D7S522 (7q31.1-q31.2)	82% (-/-)	—	Zenklusen *et al.*, 1994b (116)
Gastric Carcinoma	p-metH	20% (7/35)	98	Kuniyasu *et al.*, 1994 (52)
	D7S95 (7q31-q35)	43% (13/30)	98	
Head and Neck Carcinoma	p-metH	23% (7/30)	87	Muller *et al.*, 1995 (68)
Head and Neck SQCC	D7S522	53% (-/-)	—	Zenklusen *et al.*, 1995 (117)
Prostate Carcinoma	D7S522	83% (5/6)	16	Zenklusen *et al.*, 1994b (116)
Prostate Carcinoma	D7S486 (7q31.1)	26% (11/43)	54	Takahashi *et al.*, 1995 (96)
	D7S523 (7q31.1)	24% (10/42)	54	
	D7S522	23% (7/31)	54	
Colon Carcinoma	D7S522	80% (-/-)	—	Zenklusen *et al.*, 1995 (117)

TSG in the tumourigenesis of NSCLC nor is it closely linked to a putative TSG, and secondly, D7S95 does not contain a detectable inactivated TSG or a closely-mapping TSG in NSCLC. The possibility exists, however, that small interstitial deletions on other loci nearby the c-*met* and D7S95 regions may not have been detected in the present study.

In addition, it is interesting that despite the few c-*met* LOH cases detected, all exhibited very low or undetectable c-*met* mRNA levels. Presumably, the lost genetic information in these cases served to reduce the c-*met* mRNA levels. This would be in accordance with the common belief that loss at the DNA level is accompanied by loss (or down-regulation) at both the RNA and protein levels (7,8). Unfortunately however, because of the small number of LOH cases detected, we can only hypothesize such a relationship exists for inactivated c-*met*.

Lastly, for the case exhibiting LOH at both metH and metD (case #7) it is plausible to assume a single inactivation event rather than two independent events precipitated losses at these loci. This could have occurred simply by the removal of a large portion of the c-*met* gene, or by removal of a larger 7q segment containing c-*met*. Perhaps the same event happened for the other c-*met* LOH cases but because these were uninformative at one of the two loci, this could not be determined.

Recently, a new RFLP study of c-*met* allele loss among 111 primary breast cancer patients reported a very low (4%) LOH incidence (69), (Table 5). This is inconsistent with the results of the Bièche study (5), as well as with a 22% c-*met* LOH frequency reported by Tougas (1995) (103) among primary breast cancer patients. It is not clear why such a discrepancy exists. Perhaps it is due to differences in technique or in heterogeneous cell populations of tumour samples. Nonetheless, this result raises doubt

concerning the putative role for c-*met* as a breast cancer TSG. Moreover, with the exception of the Bièche study, the low to moderate c-*met* LOH frequencies detected in various cancers (Table 5) might be reflective of inactivation of a *nearby* 7q TSG locus. Thus, the question arises as to the existence, identity and location of this putative closely mapping TSG.

Substantial support for a candidate TSG region situated in close proximity to c-*met* has come from several recent LOH studies investigating loss of various 7q loci among different types of human carcinoma. To date, in breast (116), head and neck (117), prostate (68,116) and colon carcinomas (117) an uncharacterized polymorphic 7q marker termed D7S522 has been shown to exhibit, in general, the highest LOH frequency among markers on 7q. D7S522 corresponds to band 7q31.1-q31.2, which is distal to c-*met* and proximal to the D7S95 locus. The incidences of LOH for this marker are shown in Table 5. In certain carcinomas its LOH frequency is over 80%, strongly suggesting a TSG is situated in the 7q31.1-q31.2 region. Interestingly, in primary prostate cancer, loss of this segment is correlated with higher tumour grade and lymph node metastasis (96). This led to the speculation that genetic alterations of 7q31.1-q31.2 might participate in prostate tumour progression and metastasis. The same might be true for other cancers showing consistent LOH at D7S522 but this remains to be determined, including in lung carcinoma. Nevertheless, it is becoming clear that D7S522 (rather than c-*met*) may contain an inactivated 7q TSG in certain epithelial-derived human cancers.

Additional evidence for a closely mapping candidate TSG region to c-*met* has come from a chromosome transfer experiment. Utilizing microcell fusion procedures to introduce human chromosome 7 to cells of a murine SQCC-derived cell line (CH72 cell clones) and injecting these

clones into nude mice, Zenklusen *et al.* (1994a) (115) found five of seven clones had a 2 to 3-fold increase in tumour latency time* compared to the parental CH72 controls. Furthermore, when certain of these clones expelled the human chromosome, tumourigenicity was regained. These two observations indicated human chromosome 7 contained a gene(s) responsible for the suppression of tumourigenicity. A PCR analysis of the integrity of the inserted chromosome in one of the two unsuppressed clones (MF6) revealed an interstitial deletion at 7q31.1-q31.3. This suggested a TSG might have been located in this segment. In an attempt to reveal the identity of this putative TSG, a Southern analysis of the MF6 clones was performed using certain cDNA probes corresponding to various characterized genes on 7q, (including c-*met*) (115). This analysis did not reveal inactivation of these genes, thus it excluded them (as well as c-*met*) as candidates for the TSG. Unfortunately, the D7S522 chromosomal marker was not investigated in the Southern analysis to determine if it was specifically deleted. Hence, it could not be elucidated whether or not it harboured the TSG. Interestingly though, the researchers noted among nude mice tumours generated by the various clones, the MF6-induced ones (i.e. tumour cells that did not contain the putative TSG in the 7q31.1-q31.3 segment) showed extensive vascularization, whereas the suppressed tumours had reduced vascularity. This may have accounted for the differences in latency between the two groups of tumours. More importantly, this observation provided preliminary insight concerning the mechanism of action of the putative 7q31.1-q31.3 TSG.

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*_Latency_ is defined as the number of days elapsed after injection and before any sign of tumour is detected. Increased latency is equivalent to delayed tumourigenicity.

A potential candidate for a TSG on the long arm of chromosome 7 is the *DRA* gene. To date, it has been mapped to 7q22-31.1 and is known to encode a transcription factor or a transcription factor-related protein. This gene was recently shown to be down-regulated in adenomas and adenocarcinomas of the human colon (117). However, *DRA* exhibits tissue-specific expression patterns, being expressed at substantial levels only in colonic mucosa. It also does not map to the D7S522 region. Furthermore, in contrast to the observed effects of the 7q TSG in the two previously described chromosome transfer studies (i.e., in immortalized human fibroblast cell lines and murine SQCC-derived cell lines), *DRA* does not seem to be involved in the regulation of cell proliferation or angiogenesis. Thus, it is unlikely this gene is the putative 7q TSG. Nonetheless, it may participate along with the 7q tumour suppressor in repressing the tumourigenicity of normal colon mucosal cells.

In conclusion, our investigation revealed c-*met* and D7S95 do not have significant LOH frequencies in human NSCLC. Although cytogenetic and molecular observations have indicated at least one 7q TSG site in various human epithelial-derived tumours, and chromosome transfer experiments have revealed a 7q gene(s) is responsible for both maintaining senescence in human fibroblast cells and suppressing tumourigenicity in induced nude mice tumours, our results suggest that in NSCLC tumours c-*met* nor D7S95 harbour a potential TSG. Since the number of cases exhibiting LOH at these loci was statistically insignificant in this study, it was not feasible to investigate any relationships between LOH cases and prognostic features, (i.e., tumour size, histological subtype, patient survival, etc.). Thus, there is presently no potential clinical value for using c-*met* or D7S95 as diagnostic/prognostic molecular markers in NSCLC.

6. FUTURE STUDIES

Although the c-*met* and D7S95 loci were shown not to contain an inactivated 7q TSG in NSCLC, this does not preclude the possibility of at least one inactivated TSG residing in the 7q segment. This is due to the common cytogenetic finding of 7q abnormalities among lung tumour specimens (117). It may be possible that small interstitial deletions near the c-*met* and D7S95 regions could have been present in the DNA of the tumour samples. The D7S522 polymorphic marker, because of its map location, may have been such a deleted segment since it has been shown to have significant LOH incidences in various human epithelial-derived tumours. Its potential then as a TSG-containing site in NSCLC relies on a future LOH analysis. If it were to exhibit frequent LOH, and if this is associated with poor patient survival (similar to the situation for prostate carcinoma), the D7S522 marker may then serve as a prognostic factor aiding in the assessment and treatment of patients suffering with NSCLC. However, if it is not identified to harbour the 7q TSG in NSCLC, further LOH studies will be needed to determine the location of this suppressor gene. Thus, at present, detailed molecular studies of lung tumours in which cytogenetic alterations have been observed are needed to determine the identity, location, function(s) and characteristics of the tumour suppressor residing on 7q.

In regards to the c-*met* gene and human cancer, although the results of the Bièche study indicated its possible role as an inactivated TSG in breast carcinoma, these observations have yet to be confirmed. Based on the numerous reports describing both dysregulated c-*met* expression and

abnormally enhanced activity of its unmutated tyrosine kinase protein in different types of human cancers, it seems this gene may function solely as an activated oncogene— its overexpression and concomitant constitutive RTK activation potentially contributing to tumourigenesis. Yet, many important questions concerning its normal and cancer biology remain unanswered. This includes the molecular mechanism(s) leading to its overexpression, as well as the exact intracellular signalling pathways implicated in MET-mediated signal transduction. Further studies are also needed to elucidate the particular biological roles for the MET protein isoforms.

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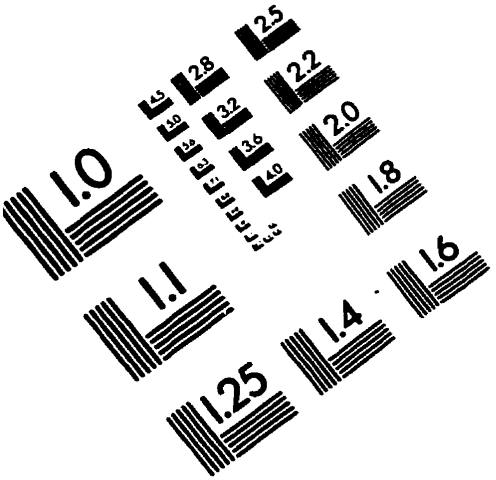
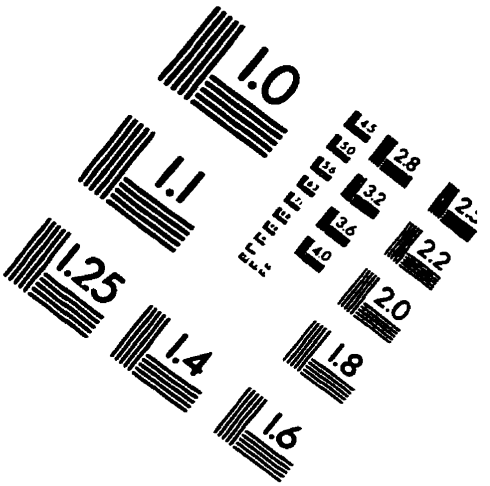
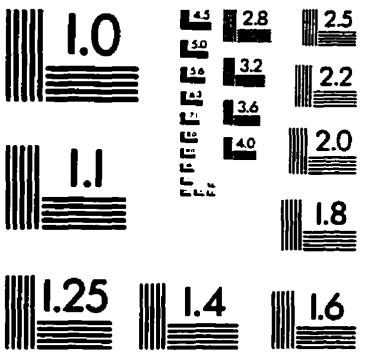
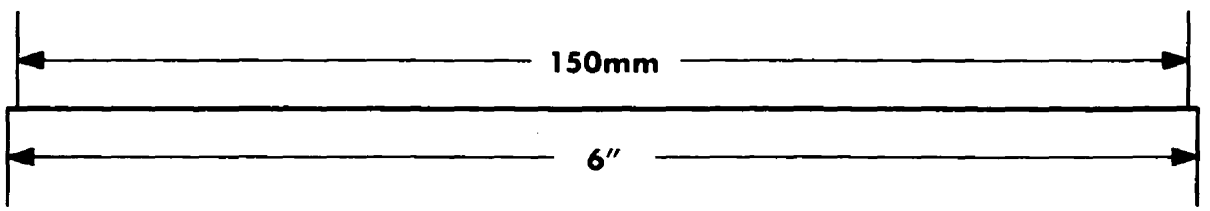
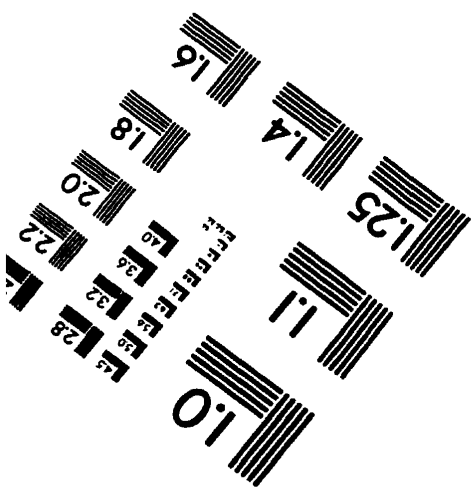
IMAGE EVALUATION TEST TARGET (QA-3)

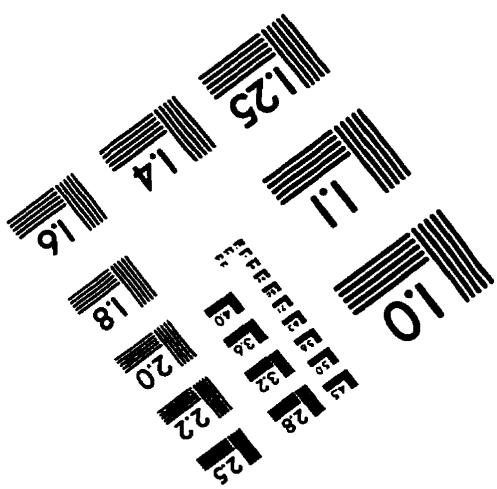












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Fax: 716/288-5989