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Involvement of the Met receptor tyrosine kinase in the development of human breast cancer.

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Preface

This thesis has been written according to the guidelines for a manuscript-based thesis issued by the Faculty of Graduate Studies and Research of McGill University. These guidelines read, in part:

"Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

"If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

"The thesis must still conform to all other requirements of the 'Guidelines for Thesis Preparation'. The thesis must include: a Table of Contents, and abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

"Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

"In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers."

This thesis consists of a General Introduction, two published papers, a third manuscript submitted for publication and a General Discussion. A section concerning the Contribution of the Authors is included.

Contributions of Authors

Three first-author publications arose from the work in this thesis:

1) **J.C. Lin**, S.W. Scherer, L. Tougas, G. Traverso, L.-C. Tsui, I.L. Andrulis, S. Jothy and M. Park, "Detailed deletion mapping with a refined physical map of 7q31 localizes a putative tumor suppressor gene for breast cancer in the region of *MET*.",*Oncogene*, 1996, 13(9):2001-2008.

3) **J.C. Lin**, M. Naujokas, H. Zhu, S. Nolet and M. Park, "Intron-exon structure of the *MET* gene and cloning of an alternatively-spliced Met isoform reveals frequent exon-skipping of a single large internal exon.", *Oncogene*, 1998, 16:833-842.

3) **J.C. Lin**, H. Khoury and M. Park, "Exon-skipping of *MET* exon 2 is a novel mechanism for the loss of expression of Met protein in breast cancer cells", *manuscript in preparation*.

The specific contributions of the other authors to these manuscripts is as follows. All other experimental work reported in these papers was performed by myself (see Contributions to Original Research). I wrote all manuscripts, with editorial help from Dr. Morag Park, and made all figures, apart from those specified below.

1) L.T. and S.J. provided tumors from the Royal Victoria Hospital, Montreal. I.L.A. provided tumors from Mount Sinai Hospital, Toronto. S.W.S., G.T. and L.-C.T. provided the clarified order of the polymorphic markers at chromosome 7q31. S.W.S. provided figure 4.

2) M.P. cloned cDNAs pOK8 and pOK14. M.N. sequenced these cDNAs. S.N. and H.Z. characterized these two cDNAs and provided data for figure 2a, 2b, figure 3b and figure 5b, 5c.

3) H.K. performed Western blot experiments for SR proteins in breast cancer cell lines, and provided data for figure 3.

Other publications:

1) L. Tougas, F. Halwani, G. Tremblay, J. Sampalis, **J. Lin**, M. Park and S. Jothy, "Human breast cancer : Genetic alterations in the *MET* gene region.", *Clinical and Investigative Medicine,* 1996, 19(4):222-230.

2) W.R. Zeng, P. Watson, **J. Lin**, S. Jothy, R. Lidereau, M. Park and A. Nepveu, "Refined mapping of the region of loss of heterozygosity on the long arm of chromosome 7 in human breast cancer defines the location of a second tumor suppressor gene at 7q22 in the region of the *CUTL1* gene.", *Oncogene,* 1999, 18(11):2015-2021.

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I dedicate this thesis to my parents, Heng and Ping Lin, whose love and unconditional support have played a huge role in helping me to finish my studies, and for which I am eternally grateful.

Finally, to my husband, Dr. Patrice Tétreault, who has listened to all my presentations, looked at every figure and has even helped to assemble my posters - un énorme merci!

Abstract

Met is a transmembrane receptor tyrosine kinase whose ligand is hepatocyte growth factor/scatter factor (HGF/SF). HGF/SF stimulates epithelial cell mitogenesis, motility and branching tubulogenesis. Met and HGF/SF are essential for embryonic development, and are implicated in oncogenesis. In this thesis, the possible role of Met in the development of human breast cancer is examined. The *MET* gene is located at chromosome 7q31, a region frequently deleted in breast cancer. In this thesis, I demonstrate that *MET* is included in the smallest common region of deletion of chromosome 7q31 in breast cancer, and is therefore a candidate for a breast cancer tumor suppressor gene. To investigate this further, I elucidated the intron-exon structure of the *MET* gene. The *MET* gene is composed of 21 exons, spanning approximately 130 kilobases(kb) of chromosomal DNA. Interestingly, the first coding exon of *MET*, exon 2, is unusually large, at 1214 nucleotides (nt). Exon 2 is subject to alternative splicing in all epithelial cells studied, and is skipped to produce a 7kb mRNA species which does not encode a protein product. Furthermore, in seven of 13 human breast cancer cell lines studied, exon-skipping of *MET* exon 2 occurs more frequently when compared to two immortalized breast epithelial cell lines. These seven breast cancer cell lines have an altered ratio of Met mRNA isoforms, with increased levels of the non-protein-encoding 7kb exon 2-skipped mRNA, and a corresponding decrease in the expression of Met receptor in these cells. Significantly, in all other carcinoma types studied to date, the Met receptor is expressed at high levels, as in normal epithelial cells, indicating that loss

of Met protein is specific to breast carcinoma cells. The alteration in the normal splicing pattern of *MET* is correlated with alterations in the normal pattern of expression of SR splicing proteins, which are responsible for regulating alternative splicing. I propose that loss of Met is an event selected for in breast cancer. This occurs through changes in the alternative splicing of the *MET* gene, which represents a novel mechanism for gene inactivation in tumorigenesis.

Résumé

Met est un récepteur transmembranaire dont l'activité tyrosine kinase est stimulée par le facteur de croissance hépatocytaire HGF/SF. HGF/SF est sécrété par les cellules mésenchymateuses et se lie à son récepteur Met qui est exprimé dans différents tissus épithéliaux dont, entre autres, l'épithelium des glandes mammaires. HGF/SF stimule la mitogenèse, la motilité et la différenciation tubulogénique des cellules épithéliales. Met et son ligand HGF/SF sont requis pour le développement embryonnaire. De plus, cette paire ligand-récepteur jouent un rôle important dans l'oncogenèse. Cette thèse étudie le rôle joué par Met dans le développement du cancer du sein chez l'humain. Le gène *MET* est localisé sur le chromosome 7q31, une région fréquemment alterée chez les patients atteints de cancer du sein. En examinant 73 tumeurs d'origine mammaire, j'ai démontré que *MET* est inclus dans la plus petite région de délétion du chromosome 7q31, et par conséquent, ce gène est un candidat de gène supresseur de tumeur impliqué dans le développement du cancer du sein. Afin de mieux caractériser ceci, j'ai déterminé la structure du gène et son organisation exon-introns. Par la suite j'ai généré des amorces d'ADN pour amplifier chacun des exons, ce qui permet d'analyser les mutations de *MET* dans les tumeurs d'origine mammaire. L'analyse structurale du gène *MET* a permis d'identifier 21 exons répartis sur 130 kilobases d'ADN chromosomique. Le premier exon codant de MET, l'exon 2, est anormalement long, composé de 1214 nucleotides. Cet exon subit un épissage alternatif dans toutes les cellules épithéliales examinées; s'il n'est pas inclus dans l'ARNm, un mRNA de 7kb est produit et celui-ci n'est pas

traduit en protéine. En effet, sept des 13 lignées cellulaires de cancer du sein d'origine humaine expriment l'isoforme de 7kb (qui ne contient pas l'exon 2) à des niveaux plus élevés. Par contre, seulement deux lignées épithéliales mammaires immortalisées produisent cet mRNA. Il en résulte une altération du rapport relatif des différents isoformes de Met, manifestée par une augmentation de l'isoforme de 7kb qui ne produit pas de protéine, et une réduction de l'expression du récepteur Met dans ces cellules. Il est à noter que le récepteur Met est exprimé à des niveaux élevés dans tous les autres types de carcinomes examinés jusqu'a ce jour, de façon similaire aux cellules épithéliales normales. Ceci indique que la perte d'expression de Met est spécifique aux cellules cancéreuses provenant des glandes mammaires. Le changement observé au niveau de l'épissage de *MET* correspond à une modification du profil d'expression de facteurs d'épissage, les protéines SR impliquées dans la régulation de l'épissage alternatif. Je propose que la perte d'expression de Met est essentielle pour le développement du cancer du sein. Ceci est dû aux changements au niveau de l'épissage alternatif du gène, ce qui représente un nouveau mécanisme d'inactivation d'un gène impliqué dans le développement de la tumeur.

Chapter I - Introduction and Literature Review

Breast cancer is the leading cause of death for North American women who are 40 to 55 years of age. Incidence rates for this disease have steadily risen since statistics were first recorded, rising at an average 1.2 percent per year between 1940 and 1982 in Connecticut, which has the oldest cancer registry in continuous operation, and increasing at 4 percent per year in the United States between 1982 and 1986. These increases have occurred in all age groups, although the increase has been greatest in older women. Furthermore, these increases in breast cancer incidence are being observed worldwide, in both industrialized and developing countries. Although the recent increase in incidence may be explained in part by better detection of breast cancer as diagnostic methods improve, the incidence of larger tumours and those with metastases at diagnosis has not decreased, indicating that the long-term increase in the incidence of breast cancer is real (Hulka and Stark, 1995; Pisani et al., 1993).

In contrast to the incidence rates, the age-adjusted mortality rates for breast cancer have remained relatively stable in the United States, perhaps reflecting an increase in reporting of more benign forms of disease or earlier detection, or advances in treatment. However, breast cancer is a problem that is still far from solved, and it is estimated that in the year 2000, it will account for 500 000 deaths annually (Harris et al., 1992; Hulka and Stark, 1995; Pisani et al., 1993).

In an effort to develop better, more effective treatments for breast cancer, it is essential to gain an understanding of how the development of the normal breast occurs and is regulated, since it is the dysregulation of

this process that leads to the development of breast cancer.

I. The Mammary Gland

The function of the mammary gland is to produce milk proteins during lactation and provide nourishment for the newborn. Because of its unique function, the development of the mammary gland takes place in several stages, unlike the development of other mammalian organs. This involves development of the mammary gland *in utero*, during puberty, with the final stage taking place in adulthood, during pregnancy.

A. The development of the mammary gland

The embryological development of the human mammary gland has been studied since the 1800s, and Dabelow in 1957 described this process in the most systematic and detailed way, dividing the process into 10 stages (reviewed in Russo and Russo, 1987).

These stages describe the development of the rudimentary mammary gland from an ectodermal invagination in the first trimester of human fetal development to the formation of branching lobulo-alveolar structures in the final trimester in response to the entry of placental sex hormones into fetal circulation. The lobulo-alveolar units are thought to undergo involution due to the withdrawal of placental hormones after birth, and remain dormant until puberty in the female, and throughout adult life in the male.

In girls, puberty occurs between the ages of 10 to 12 years, during which time ovarian hormones begin to circulate and induce the growth and maturation of the breasts. The overall effect of the estrogens on the

maturing breast is to stimulate the growth and proliferation of the ductal epithelium. Experimentally, estrogen alone will induce ductular growth, while progesterone alone does not. The two hormones together are required for complete ductular and lobulo-alveolar development of the mammary gland. The full differentiation of the mammary gland proceeds throughout puberty into adulthood and may not be completed if full-term pregnancies do not take place (Osborne, 1996).

The adult breast parenchyma is divided into 15 to 20 segments that converge at the nipple in a radial arrangement. Between 5 and 10 major collecting milk ducts open at the nipple. The mature mammary ducts consist of a lining of epithelial cells with microvilli on their cell membranes, responsible for the secretion of milk proteins. Outside this luminal layer of epithelial cells is a layer of starlike myoepithelial cells. These myoepithelial cells contract in response to the hormones prolactin and oxytocin, and serve to expel the milk. Most breast cancers are thought to arise from the layer of epithelial cells lining the ducts and lobules (Osborne, 1996).

B. Regulation of Breast Development by Hormonal and Local Factors

i) Estrogen and Progesterone

As has been discussed, the development of the mammary gland is under the control of systemic ovarian hormones and placental lactogens. Specifically, estrogen and progesterone have been shown to act synergistically to cause proliferation of breast epithelial cells during development, and also during each menstrual cycle (Anderson et al., 1988; Longacre and Bartow, 1986; Masters et al., 1977; Meyer, 1977). The role of

these hormones in breast cancer has also been studied, and certain rodent strains will develop malignant breast tumours following exposure to high doses of estrogens or progestins (Molinolo et al., 1987). Furthermore, the risk of breast cancer in men has been found to increase with the presence of conditions causing an increase in endogenous estrogen levels (Thomas, 1993), and women who undergo bilateral oophorectomy at an early age, and therefore have lowered levels of endogenous estrogen, have a decreased risk of developing breast cancer later in life (Brinton et al., 1988). The ER- and PR-expressing status of breast cancer cells is also important in predicting the outcome of disease (discussed in more detail later).

ii) Peptide Growth Factors

Within the gland, however, it is thought that these systemic hormones exert their function by inducing the production of locally acting peptide growth factors. These, together with other factors such as cellular adhesion, stromal and cell-cell interactions, lead to the formation of the mature mammary gland (Dickson and Lippman, 1995). Although several growth factor families are thought to be involved in mammogenesis, most of what is known about the specific role of such growth factors in mammary gland development is inferred from the study of the action of these growth factors on *in vitro* models of mammary epithelial cell proliferation or in breast cancer. Unfortunately, the precise physiological role played by each growth factor and the way they function together to produce a mature mammary gland has not been determined, due to the absence of a good model with which to study normal mammary gland development *in vitro*, and the difficulty of studying its development *in vivo*.

However, much is known about the general mechanism of action of polypeptide growth factors such as the ones implicated in breast development and neoplasia. These growth factors act primarily by attaching to specific cell-surface receptors, which undergo dimerization or oligomerization and activation of their inherent tyrosine or serine-threonine protein kinases. Activated receptor protein kinases act to recruit a complex of signalling proteins and trigger intracellular signal transduction cascades, eventually leading to altered expression patterns of genes and altered cell properties, resulting in cell division, differentiation, motility or even death (reviewed in Heldin, 1996).

Insulin was one of the first peptide growth factors shown to stimulate cell proliferation in mammary explants and supraphysiological levels are usually necessary to maintain mammary cells in culture (Dickson and Lippman, 1995). However, there is little evidence for a physiological role of insulin in mammary cell proliferation, and it is now believed that in high doses, insulin can mimic the effect of the insulin-like growth factors (IGFs) on mammary epithelium. Receptors for IGF-1 and IGF-2 have been described on the cell membranes of certain human breast cancer cell lines (Furlanetto and DiCarlo, 1984; Myal et al., 1984), and IGF-1 can induce mammary gland differentiation under experimental conditions (Ruan et al., 1992), suggesting that this family of growth factors may play a role in the normal differentation of breast epithelium.

One of the best characterized growth factor families involved in breast development and neoplasia is the epidermal growth factor (EGF) family, which now includes, in addition to EGF, transforming growth factor-α (TGF-α), heparin-binding EGF (HbEGF), and the heregulins,

among others (reviewed in Dickson and Lippman, 1995). Four members of the EGF receptor family have been identified to date: EGFR or c-erbB-1, HER-2/*neu* or c-erbB-2, erbB-3 and erbB-4. These receptors can form homo and heterodimers, and have the ability to bind multiple members of the EGF family of growth factors, resulting in a large number of potential ligand-receptor interactions and downstream cellular effects. The exact role of each member of the EGF and EGFR families in breast development and breast cancer is difficult to ascertain. However, overexpression of TGF-α in transgenic mice can induce hyperproliferation in mammary tissues and delayed ductal differentiation (Jhappan et al., 1990), an observation which had previously been described following local EGF mammary implants (Coleman and Daniel, 1990). Furthermore, the heregulins appear to induce differentiation of breast cancer cells in culture (Peles et al., 1992), and all four members of the EGFR family have been detected in breast cancer, suggesting that this family of growth factors and their receptors may be important in breast development. In breast cancer studies, overexpression of EGFR is correlated with poor prognosis and also poor response to tamoxifen drug therapy (Klijn et al., 1994; Toi et al., 1994), while overexpression of c-erbB-2 has been detected in 20-30% of primary, invasive breast cancers, and is also associated with a poor prognosis (Gusterson et al., 1992; Paik et al., 1990; Ravdin and Chamness, 1995).

The fibroblast growth factor (FGF) family has also been studied in breast cancer, and although some family members have been detected in both mouse and human mammary gland elements, the roles played by this family of growth factors is not well defined. However, it has been suggested that the biologic role played by the FGF family in breast cancer is

its contribution to inflammation and angiogenesis (Dickson and Lippman, 1995).

Other growth factor families, including the transforming growth factor-β (TGF-β) family, the platelet-derived growth factor (PDGF) family and the vascular endothelial growth factor (VEGF) have also been investigated for their possible roles in the development or progression of breast cancer. Again, these roles have not yet been fully determined.

Recently, the hepatocye growth factor/scatter factor (HGF/SF) and its receptor, the Met receptor tyrosine kinase, have been implicated in both breast development and in the development of breast cancer. Both genes have been found expressed in mammary gland elements of normal and malignant tissues, and treatment of mouse mammary gland explants with HGF/SF can stimulate ductal proliferation (Yang et al., 1995). These findings will be discussed in more detail later.

In summary, although the role of peptide growth factors and their receptors have been the subject of intense interest in past years, much remains unknown of the way these factors interact at each stage of breast development or how dysregulation of these signalling cascades can lead to the development and progression of breast cancer. Many conflicting reports exist regarding the exact effect of each growth factor on mammary tissues, and it seems likely that the effect a specific growth factor has on any particular mammary tissue being studied depends also on the local cell-cell interactions, stage of differentiation of the gland, and other local factors such as cell-stromal interactions and state of cellular adhesion, among others.

II. Breast Cancer

Any perturbation of the normal developmental process that leads to uncontrolled growth and immortality in a clone of cells results in the formation of a neoplasm or tumour. Further genetic alterations in the cells of the tumour may cause them to acquire the ability to move, to invade and metastasize to other sites in the body, eventually causing death.

A. Risk Factors

In the United States, breast cancer is one of the most frequent causes of cancer death among women, second only to lung cancer (Boring et al., 1994). Furthermore, it is the most frequently diagnosed cancer in women, at 32% of all cancer types (Boring et al., 1994). Based on data from the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) program for 1988 to 1990, the lifetime risk of being diagnosed with breast cancer is 12.2% or 1 in 8 women, and the lifetime risk of dying of this disease is 3.6% or 1 in 28. By comparison, breast cancer is extremely rare in men, with only 1000 cases reported in the U.S. in 1994 (Brinton and Devesa, 1996). Thus, simply being female is a significant risk factor for developing breast cancer.

Although many risk factors have been identified for breast cancer, not many of these are life style factors suitable for alteration with the ultimate goal of breast cancer prevention. In this respect, breast cancer differs from other highly publicized cancers with clear preventative modifying factors such as skin cancer or lung cancer.

The risk of developing breast cancer increases with age, with

women aged 65-69 years old having an estimated relative risk of 17, versus women aged 30-34. Furthermore, breast cancer is associated with demographic factors such as place of birth and residence (risk higher in North America or Europe vs. Asia), higher socioeconomic status (which may be related to other lifestyle choices, such as having children at later ages), and residency in urban areas (Brinton and Devesa, 1996).

A family history of breast cancer in one first-degree relative is associated with a doubling of risk for breast cancer, and is even higher for women with more than one affected first-degree relative (ie. mother and sister). The effect of family history was found to be even greater if there was early-onset cancer (before age 40), bilateral disease, and if associated with a family history of ovarian cancer. Recently, the gene responsible for this inherited susceptibility to breast and ovarian cancer, located on chromosome 17q21, was identified as BRCA-1 (Miki et al., 1994), and encodes a zinc-finger containing DNA-binding protein with tumour suppressor function and the ability to suppress cell proliferation (Holt et al., 1996; Thompson et al., 1995). Mutations in a second gene located on 13q12-13, called BRCA-2, are also responsible for increasing risk of breast cancer but at a lower frequency (Wooster et al., 1995). However, the impact of the identification of these genes on the prevention of breast cancer in the general population is probably not as great as hoped, since only 5-10% of all breast cancers are thought to result from genetic predisposition (Weber and Garber, 1996). Furthermore, inactivation of these two genes is uncommon in sporadic breast cancer, as evidenced by the low frequency of mutations in BRCA-1 and BRCA-2 in sporadic breast and ovarian cancers (Futreal et al., 1994; Lancaster et al., 1996). Other genes found to be

involved in familial breast cancer include p53, a tumour suppressor gene in which germline mutations are associated with the Li-Fraumeni cancer syndrome (Malkin et al., 1990) and the gene for ataxia-telangiectasia (AT), in which heterozygote carriers of the mutation causing AT may have a higher risk of breast cancer than the general population (Easton et al., 1993).

Other known risk factors for breast cancer relate to the menstruation and reproductive histories of patients. An early age at menarche (<12 years old) is associated with a 50% higher risk of breast cancer than those with a later onset of menarche (>15 years old). Similarly, women with a late age of menopause (>55 years old) have a higher risk of developing breast cancer than those with early-onset menopause (<45 years old), and oophorectomy before age 40 is also associated with a decrease in risk of breast cancer (Brinton et al., 1988). These findings suggest that it is the increased exposure of breast tissue to ovarian hormones that increases the risk of developing breast cancer later in life. This may be due to the cyclic proliferation and apoptosis of breast epithelial tissue that occurs under the regulation of the menstrual cycle (Anderson et al., 1988; Longacre and Bartow, 1986).

A mother's late age at first birth has been shown to correlate with an increased risk for developing breast cancer, with women with a first birth after age 30 having double the risk of women with a first birth before age 18 (MacMahon et al., 1970). Other groups have studied the effect of pregnancy and lactation on the subsequent risk of developing breast cancer, and suggest that the pregnancy must be full-term to be protective (Brinton et al., 1983), and that longer periods of breast feeding may further

reduce the risk of breast cancer (Byers et al., 1985; Newcomb et al., 1994; Yoo et al., 1992). These findings have been interpreted to suggest that it is the early differentiation of the mammary gland, induced by a full-term pregnancy, followed by lactation, that protects the epithelium from further insults and prevents the development of breast cancer (Bernstein et al., 1995; Russo and Russo, 1994).

Another important class of risk factors are medical or biological factors, such as having a personal history of breast, ovarian or endometrial cancer, which increases the risk of developing breast cancer (Bernstein et al., 1992). Also, in recent studies, women in whom 65%-75% or more of the breast was composed of areas of density on mammography were found to have an increased risk (Oza and Boyd, 1993; Saftlas et al., 1991), as did women with biopsy-confirmed atypical hyperplasia of the breast (Dupont and Page, 1985). Obesity, high-dose ionizing radiation to the chest and moderate alcohol consumption have also been associated with an increased risk for developing breast cancer (Hulka and Stark, 1995).

B. Natural History and Cellular Origin of Breast Cancer

An understanding of the natural history of untreated breast cancer allows a better appreciation of the treatment and prognostic difficulties faced with this disease. Clinically, untreated breast cancer is characterized by heterogeneity in patients' outcome, and also by a long course of the disease, which complicates the definition of a breast cancer "cure".

The survival curve of breast cancer patients is biphasic, with one subgroup representing approximately 2/3 of the breast cancer patients studied, surviving past 10 years with a force of mortality rate of 2.5% per

year, while the remaining 1/3 of patients had a more aggressive disease with a force of mortality rate of 25% per year (Fox, 1979). It has been proposed that this data illustrates that there are at least two clinical types of breast cancer, with one type having a much higher mortality rate than the other. Alternatively, breast cancer may be a single disease, but heterogeneous, with a large range of aggressivity in various patients. To date, this question has not been answered in any definitive way, and it is still unknown whether breast cancer represents many diseases, or whether it evolves along one pathway from normal cell to malignant tumour.

Due to the long natural history of the disease, and the fact that breast cancer generally affects women at a relatively late age, establishing whether or not a patient has been "cured" of her disease presents a difficult problem. Most studies have found a persistent increase in mortality rates of treated breast cancer patients even 30 to 40 years after diagnosis (Brinkley and Haybittle, 1984; Hibberd et al., 1983; Rutqvist and Wallgren, 1985). Furthermore, distant metastases from a primary breast carcinoma (most common sites being bone, lung and liver) can occur even 10 years or more after the initial diagnosis. Thus, to speak of being "cured" of breast cancer after being disease-free for a certain amount of time is not as relevant as with other types of cancer.

In an effort to understand how breast cancer evolves, several groups have attempted to identify the cells from which breast cancers arise. Studies which investigated the pattern of expression of intermediate filaments in the epithelial cells of the mammary gland have clearly identified two major cell types (discussed earlier); the luminal epithelial cell and the basal or myoepithelial cell, based on the differential expression

of keratins and vimentin (Taylor-Papadimitriou et al., 1993). In studies of malignant breast tissue, more than 90% of breast tumours were found to express keratins 8, 18 and 19 (Bartek et al., 1985), as are found in the normal luminal epithelial cells of the terminal ductual lobular units, suggesting that most malignancies arise from these cells. The remaining 10-20% of breast cancers show some expression of basal myoepithelial cell markers, such as vimentin, laminin or keratin 14 (Wetzels et al., 1989), which is associated with more aggressive cancers (Domagala et al., 1990). However, it has not been clearly established whether these represent two types of breast tumours, which evolve from two different cell lineages, or whether they represent a continuum of breast cancer evolution.

Similarly, the expression pattern of estrogen receptor (ER) and progesterone receptor (PR) by normal and malignant breast cells has been the subject of study. Estrogen acts through its receptor to induce the expression of the progesterone receptor, and both hormones regulate the expression of other genes. ER and PR have been localized by immunohistochemistry to a population of luminal epithelial cells in the mammary gland (Fuqua, 1996). Approximately 60% of breast cancers are ER-positive, and most of these respond well to antihormonal therapy (Osborne et al., 1980). In general, the hormone unresponsive, ER- and PR-negative breast cancers represent a group of more aggressive tumours, with higher proliferative and invasive rates *in vitro* and *in vivo*. *In vitro* cell culture studies of ER-positive cell lines have shown that prolonged exposure to the chemotherapy drug adriamycin or prolonged withdrawal from estrogen induces these cells to acquire characteristics of ER-negative lines, suggesting that breast cancers arise from a population of luminal,

ER- and PR-positive cells, which then undergo a process termed "dedifferentiation" with the progression of breast cancer to a more invasive, aggressive disease (Dickson and Lippman, 1995; Fuqua, 1996).

C. Disease Staging and Prognostic Factors

A primary breast carcinoma arising from the epithelial cells of the mammary gland can spread by direct infiltration into the surrounding tissue and also through the lymphatic system, which presumably leads to the formation of distant metastases. It is the direct infiltration of the tumour into the stroma, skin, and other structures which leads to the characteristic stellate appearance of malignant breast tumours on mammograms or gross examination. The cancer can also spread directly along mammary ducts, which results in intraductal carcinoma being frequently observed in association with infiltrating carcinoma. Lymphatic spread is most often evidenced by involvement of the axillary lymph nodes, which is the major regional drainage site of the breast. The larger the primary tumour, the more likely it is that the axillary nodes will be involved (Harris and Hellman, 1996).

The clinical staging of breast cancer, designated TNM (Tumour, Nodes, Metastasis), is based on these characteristics and is used to group patients according to the extent of their disease, and for choosing the treatment program and estimating their prognosis. The specific criteria for each breast cancer stage can be found in any clinical oncology text (Harris, 1996), and will not be reiterated here. In general, however, the "T" refers to the size of the primary tumour, the "N" to the number of involved axillary lymph nodes, and "M" to the presence or absence of distant

metastases. Together, the scores from these three criteria are used to determine the clinical stage of the disease, from stage 0 (carcinoma *in situ*) to stage IV (any tumour with distant metastases, representing disseminated and advanced disease). This staging system is then used as a predictor of the patient's prognosis.

Other standard prognostic factors currently used in breast cancer include histologic subtype, nuclear grade, ER and PR status, and proliferation rate of the tumour. However, even with all these prognostic markers in use, some breast cancer patients who seemingly should have a good prognosis continue on to develop recurrent disease. For example, even though the two most powerful prognostic markers that are in common use are tumour size and presence or absence of axillary lymph node involvement, up to 30% of patients with node-negative breast cancers will go on to develop metastatic breast cancer (Page and Johnson, 1995). Thus, investigators continue to search for new prognostic markers, in the hope of eventually being able to identify the small subset of breast cancer patients with a poor prognosis that we are currently unable to distinguish using the markers available today.

It has been suggested that new prognostic markers would be useful in at least three situations (Clark, 1994). One of these is to identify patients whose prognosis is excellent, such that adjuvant therapy after surgery (currently used for all patients) would not be beneficial, and is therefore unnecessary. The second situation involves patients whose prognosis with current treatments is extremely poor. These patients might then be candidates for newer, more aggressive therapies. The third situation is to try to indicate which patients would or would not benefit from specific

therapies.

In an attempt to address these problems with regards to prognosis in breast cancer, many new prognostic markers have been proposed and studied, including the expression of certain growth factors and their receptors, such as the EGFR, the erb-B2 receptor (also called Her-2 or neu), and other genes such as the tumour suppressor gene p53 (Clark, 1996). However, as yet, none of these have been proven to definitively clarify any of the situations described above, and none have been accepted into general use. Thus, the need for new prognostic factors remains.

D. Oncogenes and Tumour Suppressor Genes

It is generally accepted that a cancer develops with the step-wise accumulation of genetic abnormalities within a cell or cells which leads to the dysregulation of growth and differentiation in these cells. Thus, they gain the ability to proliferate, generating a clone of cells which, with additional genetic changes caused by carcinogens and accentuated by faulty DNA repair mechanisms, may then gain the ability to invade the surrounding stroma, and eventually, to travel through the bloodstream or lymphatic system to other areas of the body where metastases may develop.

A cancer can develop when these genetic abnormalities affect two classes of genes; oncogenes, which contribute to oncogenesis if inappropriately activated, and tumour suppressor genes, which contribute to oncogenesis when inactivated (reviewed in Callahan and Salomon, 1993). Thus, oncogenes are typically genes involved in the growth and proliferation of cells, and when activated, confer a growth advantage to

the cancer cell, or which causes the cell to proliferate in a dysregulated manner. Tumour supressor genes, however, are thought to be genes whose function is required to maintain normal organ architecture or to prevent uncontrolled cellular proliferation, and when that function is lost, the cell aquires the potential to become malignant. Full transformation from a normal cell to frank malignancy requires mutations in many such genes, some of which are affected in many types of cancer, and others which are only found affected in specific cancer types.

Two types of genetic abnormalities are commonly observed in human cancer (Callahan et al., 1992). One of these is the amplification of specific chromosomal regions, leading to the activation and/or overexpression of growth-promoting oncogenes in the amplified region. Mutations within the target gene may be associated with these gross genetic abnormalities, but sometimes, gene amplification may occur in the absence of gene mutation. The other type of genetic abnormality frequently observed in breast cancer is the deletion of specific chromosomal regions, commonly referred to as a loss of heterozygosity, or LOH. These areas of LOH are thought to contain tumour suppressor genes.

This multistep model of oncogenesis, in which tumours develop and progress with the accumulation of specific genetic mutations and abnormalities has been elegantly demonstrated for colon cancer. Based on studies of genetic lesions which exist at each clearly defined stage of colon cancer, (Fearon and Vogelstein, 1990) have proposed a genetic model for colorectal tumourigenesis. According to this model, a series of accumulating genetic alterations involving both oncogenes and tumour suppressor genes are necessary for the normal colonic epithelium to

progress from benign proliferation (adenoma) through increasingly malignant stages to a fully metastatic cancer. Although the genetic alterations observed in colon cancer tend to occur at characteristic stages of tumour progression, (Fearon and Vogelstein, 1990) propose that it is the accumulation of several key changes, rather than the order in which they appear, that is most important in the development of colon cancer. However, this understanding of which genetic lesions are associated with each stage of colon cancer may permit clinicians to follow the progression of disease using molecular biology techniques to detect these genetic lesions.

Such a genetic model of oncogenesis would be invaluable in breast cancer, a disease in which clearly defined stages of tumour progression are not established, unlike for colon cancer. If the genetic lesions associated with more advanced or aggressive breast cancer could be identified, these would provide better prognostic and staging information than is presently available. Thus, much effort has gone into studying the genetic lesions present in breast cancer, their prognostic significance, and in attempting to arrange these into a model like the one proposed for colon cancer.

Many of the genes found to be affected by genetic alterations in breast cancer through their amplification, mutation or deletion are normally involved in the growth and development of the breast. By altering the wild type function of these genes, an environment is created in which atypical non-malignant or frankly malignant cells with a growth advantage could develop, and by accumulating more genetic alterations, could eventually evolve into metastatic breast cancer.

Several proto-oncogenes have been found amplified in breast

cancers, including the c-*erb*-B2 gene, c-*myc*, and *int2* (reviewed in Callahan et al., 1993; and Walker and Varley, 1993). Some groups have found amplification of c-erb-B2 to be associated with poorer prognosis, while others have been unable to confirm this finding (reviewed in Walker and Varley, 1993). Although the rat homolog of the c-erb-B2 gene, *neu*, can be activated by point mutation, no such corresponding mutation has been detected in human breast cancer, and it is thought that activation of this gene in humans is by overexpression of the wild-type gene.

However, the most frequent genetic abnormality detected in breast cancer is loss of heterozygosity (LOH). These are areas of chromosomal deletion commonly referred to as a region of LOH, since the technique used to detect such abnormalities depends on using polymorphic markers to demonstrate allelic heterozygosity in the normal tissue, which is reduced to homozygosity in the tumour, indicating that one allele was lost, or deleted. By using multiple polymorphic markers, it is possible to determine the exact chromosomal region covered by the deletion, which is thought to contain tumour suppressor gene(s). The remaining allele of the putative tumour suppressor gene is presumed to be inactivated by a mutation, as postulated by Knudson's two-hit hypothesis for the inactivation of tumour suppressor genes (Knudson, 1971). However, although many regions of LOH have been identified in breast cancer, and several of these correspond to known tumour suppressor genes such as RB-1 (retinoblastoma gene) at chromosome 13q14 and TP53 at chromosome 17p13, LOH of one allele of the tumour suppressor gene is not always found to be associated with a detectable mutation in the remaining copy of the gene (Callahan et al., 1993). Regardless, considerable

effort continues to be invested in determining which chromosomal regions are subject to frequent LOH in breast cancer, as well as the prognostic significance, if any, of these regions of LOH.

LOH of the long arm of chromosome 7 has long been described for cancers of myeloid origin, specifically LOH at chromosome 7q22. However, in 1992, Bièche and his collegues reported frequent LOH of chromosome 7 at q31 in human breast cancer, occuring in 40% of breast cancers studied. This genetic alteration was found to be significantly associated with those patients having a poorer prognosis than patients without this LOH (Bièche et al., 1992). Specifically, Bièche used a polymorphic RFLP marker within the *MET* gene, mapped to chromosome 7q31 to carry out these studies. Thus, *MET* was clearly included in this deletion of chromosome 7q31. However, since only one marker was used, the extent of the deletion was not established, and therefore, the potential tumour suppressor gene identified by the frequent LOH in breast cancer could be any gene in that chromosomal region, not necessarily *MET*. From this study, the candidate gene list could include hundreds of genes, most not yet identified.

After the publication of Bièche's paper, several other papers were published, reporting frequent LOH of the same chromosomal region of 7q31 in other types of cancer, including gastric, colon, ovarian, prostate and head and neck cancers, suggesting the presence of a tumour suppressor gene at chromosome 7q31 which might be important in the development of many types of human cancers (Kuniyasu *et al.*, 1994; Zenklusen *et al.*, 1994b; Latil *et al.*, 1995; Muller *et al.*, 1995; Takahashi *et al.*, 1995; Zenklusen *et al.*, 1995a; Zenklusen *et al.*, 1995b). These findings warranted an investigation into the potential role of *MET*, one of the few known genes

mapped to this region, as the tumour suppressor gene at chromosome 7q31.

III. Met and HGF/SF

A. *TPR-MET*

The *MET* gene encodes a transmembrane receptor tyrosine kinase, whose ligand is hepatocyte growth factor/scatter factor (HGF/SF). *MET* was first isolated from a human osteogenic sarcoma cell line treated with N-methyl-N-nitro-N-nitrosoguanidine (MNNG-HOS) as a rearranged oncogene, *TPR-MET*. *TPR-MET* was formed by the translocation of sequences from the *TPR* gene on chromosome 1 to the *MET* gene on chromosome 7, resulting in the production of a fused oncoprotein in which 142 amino acids derived from the *TPR* sequences were directly fused 5' to the kinase region of the Met protein (Park et al., 1986). The Tpr-Met fusion protein lacks the extracellular and transmembrane domains of the wild-type Met protein, and is therefore an intracellular protein. No mutations exist in the *MET*-derived sequences of *TPR-MET*. However, it is constitutively dimerized through two leucine zipper motifs in the *TPR*-derived portion of the gene, and its *MET*-derived kinase domain is constitutively phosphorylated and activated (Rodrigues and Park, 1993). Tpr-Met is able to transform fibroblasts in vitro. It has been demonstrated that Tpr-Met acts through similar signalling pathways as does the wild-type Met receptor (Fixman et al., 1995). However, *TPR-MET* has not been definitively described as a naturally-occuring oncogene in human cancers.

B. Signal Transduction through the Met receptor

The wild-type *MET* gene product is encoded by a full-length 8kb Met

mRNA, and is synthesized as a single chain precursor of 150kDa, which is glycosylated to generate a 170kDa protein. The glycosylated Met precursor is then cleaved to yield the two Met receptor subunits; a transmembrane β-subunit of 145kDa containing the intracellular tyrosine kinase domain and an α-subunit of 45kDa that remains extracellular (Giordano et al., 1989; Rodrigues et al., 1991). The α- and β- subunits are linked together through disulfide bonds to form the mature wild-type heterodimeric Met receptor of 190kDa (p190Met) which is expressed at the cell surface of many cell types, mainly those of epithelial origin, including breast epithelium (Di Renzo et al., 1991; Gonzatti-Haces et al., 1988; Niranjan et al., 1995; Pepper et al., 1995; Prat et al., 1991; Soriano et al., 1995; Tsarfaty et al., 1992; Yang and Park, 1995; Yang et al., 1995).

The ligand for the Met receptor was originally identified as a potent mitogen for hepatocytes, and was thus named hepatocyte growth factor (HGF) (Bottaro et al., 1991; Naldini et al., 1991). It is a 90kDa disulfide-linked heterodimeric protein of two subunits, post-translationally derived from an inactive single chain precursor, and is structurally similar to the serine protease plasminogen, but lacks any enzymatic activity. Independently, scatter factor (SF) was identified as a protein secreted by fibroblasts and smooth muscle cells that induces the dissociation and movement of epithelial cells (Stoker and Perryman, 1985). HGF and SF were shown to be identical (Naldini et al., 1991), and is now known to be a factor with pleiotropic activity through a single receptor, Met, that is capable of inducing mitosis, motility, and invasion of normal epithelial and carcinoma cells, as well as branching tubulogenesis of epithelial cells of kidney, breast and lung origin when grown in gel matrix culture

conditions (reviewed in Rosen *et al.*, 1994). These observations suggest an important role for the Met-HGF/SF signalling pathway in the growth and differentiation of epithelially-derived organ systems. While the Met receptor tyrosine kinase is expressed on cells of epithelial origin, HGF/SF is expressed by cells of mesenchymal origin, and in the stroma of various tissues.

Binding of the ligand to the Met receptor induces oligomerization and trans-phosphorylation of the Met receptor on tyrosine residues within the kinase domain, thereby activating the tyrosine kinase enzymatic activity. The Met receptors subsequently become phosphorylated on other tyrosine residues in the carboxy-terminal tail. These phosphorylated tyrosine residues can then serve as specific binding sites for proteins containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains, that act to transmit the signal from the Met receptor to downstream signalling pathways. Binding of these substrates is the first step in the activation of downstream signalling pathways, which leads to the biological effects of HGF/SF stimulation of Met-expressing cells, as described earlier.

C. Role in Development

Using immunohistochemical analyses, it has been demonstrated that during mouse embryonal development, HGF/SF is expressed in mesenchymal tissue in close proximity to Met-expressing epithelium (Sonnenberg et al., 1993), suggesting a role for Met-HGF/SF signalling pathways in epithelial-mesenchymal interactions *in vivo*. Transgenic mouse studies, in which null mutant mice of both HGF/SF and MET were generated, demonstrated that the Met-HGF/SF signalling pathway is

essential for full embryonic development (reviewed in Birchmeier and Gherardi, 1998; Schmidt et al., 1997; Uehara et al., 1995). Both the HGF/SF -/- and the MET -/- transgenic mice fail to survive beyond day E16.5 of embryogenesis, and died *in utero*. Heterozygote mice showed normal development and were healthy. More detailed analyses of the *met* and *HGF/SF* null mutant embryos revealed a deficiency in placental and liver development which likely were responsible for the fetal demise. Furthermore, myogenesis in limb buds, the diaphragm, and the tip of the tongue, was arrested, and no myogenic precursor cells were found to have migrated into these areas. This essential role of the Met-HGF/SF signalling pathway in limb myogenesis was also demonstrated by whole-mount *in situ* hybridization studies on mouse embryos, which revealed that Met-expressing muscle precursor cells detach from somites and migrate into the limb buds in response to HGF/SF secreted by the mesoderm (Yang et al., 1996).

Other studies have shown a role for Met and HGF/SF for directing the growth of axonal growth cones (Ebens et al., 1996), and inducing morphogenesis of various epithelial cell lines when grown in 3-D collagen gels, including prostate, pancreas, lung, and mammary cell lines (Brinkmann et al., 1995).

A role for Met-HGF/SF has been suggested in breast development. Immunohistochemical studies have demonstrated that HGF/SF is produced by the fibroblast elements of human and mouse mammary glands (Jin et al., 1997; Tuck et al., 1996), while Met is expressed by both luminal epithelial and myoepithelial cells of the mammary gland (Yant et al., 1998). Expression levels of Met and HGF/SF in the rat mammary gland

are high in the pre-pregnant state, then decrease in pregnancy, becoming virtually undetectable during lactation, and increase again during involution to pre-pregnancy levels (Pepper et al., 1995). Furthermore, *in vitro* studies show that HGF/SF can induce branching morphogenesis of both mouse and human mammary epithelial cell lines when grown in collagen gels (Berdichevsky et al., 1994; Soriano et al., 1995).

Several other observations point to an important role for Met-HGF/SF in the development of the ductal branching system of the mammary gland. HGF/SF is mitogenic for luminal epithelial cells and is morphogeneic for myoepithelial cells, causing the formation of multiple branching tubules when myoepithelial cell cultures in collagen gels are stimulated with HGF/SF (Niranjan et al., 1995). Furthermore, organ cultures of mouse mammary glands revealed that HGF/SF induces growth and expansion of the ductal system, while neuregulin is responsible for lobulo-alveolar budding and the production of milk proteins. These sequential roles in breast development are paralleled by observations *in vivo*, showing HGF/SF production in breast mesenchymal cells during ductal branching in the pre-pregnant mouse, while neuregulin is expressed during lobulo-alveolar development during pregnancy (Yang et al., 1995).

IV. Possible role of Met in breast cancer

As has been discussed, an oncogenic variant of *MET*, *TPR-MET*, can transform fibroblasts in culture. However, *TPR-MET* has not been found to contribute to human oncogenesis *in vivo*. Nevertheless, several groups have studied the status of the Met receptor in human cancers. Met is

overexpressed and/or deregulated in certain human cancers, including gastric, colorectal, thyroid and ovarian carcinomas, leukemia, and sarcomas (Di Renzo et al., 1992; Di Renzo et al., 1994; Jucker et al., 1994; Liu et al., 1992; Rong et al., 1995; Rong et al., 1993; Scotlandi et al., 1996; Yonemura et al., 1996). Deregulation of the Met receptor via an autocrine loop transforms fibroblasts in culture (Rong et al., 1994), and transgenic mice overexpressing HGF/SF exhibit tumorigenesis of various organs (Takayama et al., 1997).

Met is expressed in human breast cancer cell lines, and HGF/SF was shown to stimulate the formation of lumen-like structures in a human breast cancer cell line (Tsarfaty et al., 1992). A study of serum concentrations of HGF/SF in breast cancer patients demonstrated an association between increased levels of circulating HGF/SF and increased malignancy (Taniguchi et al., 1995; Yamashita et al., 1994).

Historically, receptor tyrosine kinases are regarded as protooncogenes in human cancer. In general, tyrosine kinases are involved in cell growth and division, and when deregulated, tend to become constitutively activated, leading to unregulated proliferation and therefore, progression of oncogenesis. However, study of the Ret receptor tyrosine kinase reveals a more complex role for tyrosine kinases in the development of human disease. Ret has been found mutated and constitutively activated in the cancer syndrome MEN2b, and also in sporadic papillary thyroid cancers. However, inactivating mutations have also been described for *RET*, which have been shown to cause the hereditary syndrome Hirshprung's disease (reviewed in Pasini et al., 1996). Thus, the possibility remained that *MET* might be the tumour suppressor

gene on chromosome 7q31, despite its depiction until now as a protooncogene. The role of Met in the differentiation of breast epithelium suggests that the inactivation of *MET* at a crucial moment before the terminal differentiation of the breast epithelium occurs might leave the immature cells susceptible to other oncogenic events, and predispose these cells to oncogenesis.

V. Goals of this thesis

The main goal of this thesis is to further investigate the role that *MET* may play in the development of human breast cancer. Specifically, the first objective was to determine the smallest common region of deletion of chromosome 7q31 in breast cancer, and to establish if *MET* is included in this region. This would provide support for *MET* as a tumour suppressor gene in breast cancer.

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Detailed deletion mapping with a refined physical map of 7q31 localizes a putative tumor suppressor gene for breast cancer in the region of *MET*

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In breast cancer, loss of heterozygosity (LOH) has been described on the long arm of chromosome 7 at band q31, suggesting the presence of a tumor suppressor gene in this region. To define the deleted region, we analysed 73 cases of breast cancer and matched normal DNAs with 17 polymorphic markers. A minimal area of LOH was identified as the chromosomal interval flanked by markers D7S687 and metH, spanning a segment of 2 Mb on chromosome 7q31. Of the 73 breast cancer patients studied, all were informative for at least one marker in this region and nine patients showed LOH at one or more loci (12.3%). To define the physical size of the deletion and to ensure the correct interpretation of the LOH deletion studies, we redefined the physical map of markers within this region of 7q31. We present a new physical order for markers at 7q31. More significantly, we have mapped the minimum deletion of 7q31 in the breast cancers studied to date to a physical distance of 1000 kb, contained on a single YAC clone, which includes the *MET* receptor tyrosine kinase but no other known genes.

Keywords: breast cancer; tumor suppressor gene; chromosome 7q31; MET receptor tyrosine kinase; loss of heterozygosity

Introduction

Breast cancer continues to be a leading cause of death in women. Much effort has been invested into understanding the molecular mechanisms underlying this disease. As has been shown for other cancers, chromosomal deletions thought to identify the location of tumor suppressor gene(s) important in the development of the tumor have been detected in breast cancer (Devilee *et al.*, 1994). Such deletions are commonly identified by the detection of loss of heterozygosity (LOH) at specific chromosomal loci using polymorphic markers. In breast cancer, LOH has been detected at many chromosomal locations, including 1p, 1q, 2p, 3p, 6q, 7q, 8q, 9q, 11p, 11q, 13q, 15q, 16q 17p, 17q, 18p, 18q and 22q (Devilee *et al.*, 1991; Callahan *et al.*, 1992, 1993; Chen *et al.*, 1993; Hampton *et al.*, 1994). Several tumor suppressor genes that are implicated in breast cancer, such as *p53* at chromosome 17p13 (Isobe *et al.*, 1986; McBride *et al.*, 1986; Miller *et al.*, 1986), *BRCA-1* at 17q21 (Miki *et al.*, 1994), and *BRCA-2* at 13q12-13 (Wooster *et al.*, 1995) have been mapped to regions which overlap with some of the previously described regions of LOH. However, for most of the genetic abnormalities, no candidate genes have been identified.

The presence of a tumor suppressor gene on chromosome 7 at band q31 is suggested from two independent studies of primary breast cancer, demonstrating LOH at the *MET* locus (7q31) at frequencies of 40.5% (Bièche *et al.*, 1992) and 27% (Deng *et al.*, 1994). LOH at 7q31 is thought to be an early event in tumor progression (Champème *et al.*, 1995) and is associated with a higher risk of relapse and shorter metastasis-free survival and overall survival (Bièche *et al.*, 1992). Recently, frequent allelic loss on chromosome 7q31 has been described for other cancers, including gastric, colon, ovarian, prostate and head and neck cancers (Kuniyasu *et al.*, 1994; Zenklusen *et al.*, 1994b, 1995a, b; Latil *et al.*, 1995; Muller *et al.*, 1995; Takahashi *et al.*, 1995). This suggests the presence of a tumor suppressor gene at chromosome 7q31, which may be important in many types of human cancer.

To facilitate positional cloning efforts to identify the putative tumor suppressor gene at 7q31 in breast cancers, we have refined the physical map of genetic markers at 7q31 and used 17 polymorphic markers to determine the smallest common region of LOH in 73 patients with breast cancer. Our data indicate that the smallest region of LOH in the tumors studied is located in an interval of 2 Mb, flanked by D7S687 and a marker within the 3' untranslated region of the *MET* gene (metH). Moreover, using the new physical map presented here, we further define the location of a putative breast tumor suppressor gene at chromosome 7q31 to a 1000 kb physical distance, contained on a single YAC clone.

Results

Interstitial deletions involving 7q31

Allele deletion of a polymorphic marker located within the *MET* gene at chromosome 7q31 has been demonstrated by two independent studies (Bièche *et al.*, 1992; Deng *et al.*, 1994). To establish if the *MET* locus is deleted in our cohort of breast cancers, oligonucleotide primers (see Table 1 for primer sequences) were used to amplify the DNA fragments containing the polymorphic restriction enzyme sites recognized by the RFLP probes metH (MspI) and metD (BanI). Both polymorphic restriction enzyme sites are located within 21.5 kb (Kerem *et al.*, 1989) and are localized to the 3' portion of the *MET* gene

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Table 1 Results of loss of heterozygosity analyses by PCR of 73 breast cancer cases using 17 polymorphic markers on chromosome 7

Name of marker[a]	*5' Primer sequences 3'*	*Informative patients*	*Patients with LOH*[b]
D7S506	CCCTTCAAATGCACAGATA GCGTCAGTTACTGGAACTT	37	0 (0)
D7S523	CTGATTCATAGCAGCACTTG AAAACATTTCCATTACCACTG	38	2 (5.3)
D7S471	AGCAGCTATTATGGAATTGC CAAACTATGCAAGGTGCCTA	37	3 (8.1)
D7S461	GGGAAACTCAAAGGCAGAAGA AAACTCAGCATTGTCCTGCC	52	6 (11.5)
AFMa062zd5	ATGGCTTTTTGCATACTAAATGCTT GGTTCTGGCTGCTGA	58	7 (12.1)
D7S687	AAAATATTACACATGCCTGAGTG ACAGTGAAGCGACACCAATC	23	4 (17.4)
D7S486	AAAGCCCAATGGTATATCCC GCCCAGGTGATTGATAGTGC	62	5 (8.1)
D7S522	GCCAAACTGCCACTTCTC ACGTGTTATGCCACTCCC	46	5 (10.9)
D7S2460	CACATCCACTGTGTCTCATTT TATCTGGGACTTNACGCTTC	37	3 (8.1)
metD GH367 GH364	GAGAAATGAGGTTCTTGGATG GCCTCTGGGTAAAATGAGTC	34	5 (14.7)
metH GH220 GH324	CCATGTAGGAGAGCCTTAGTC GTCTAAGGACACACCTTGC	38	4 (10.5)
D7S677	ATCATTCACTATGGGATAGC GAATTACAAGTCACTCTATACAAAA	41	3 (7.3)
D7S655	CAAAATAGTGGGGTATTGGTAAA CCAAGTTAATCTNTGTGAAAGTGTA	28	3 (10.7)
D7S643	CTTGGGGACTGAACCATCTT AGCTACCATAGGGCTGGAGG	42	4 (9.5)
D7S504	TCTGATTGCTGGCTGC GCGCGTGTGTATGTGAG	45	1 (2.2)
D7S500	CCAGAATTGAAAACTCAGCA ATTGATTGAGGAACTGAACTTACCT	49	0 (0)
D7S483	AGTGGTCATTAGCCTTGGCAAAATC AACCAGAGTTGTAAGCCATGAAAGT	44	0 (0)

[a] The order of the DNA markers shown was determined using the published genetic mapping data (Gyapay *et al.*, 1994) and for some of the markers, the order was refined using YAC contig analysis.
[b] The number of informative patients showing LOH with each marker is given, while the percentages are indicated in parentheses

(Figure 1a). Representative results obtained with oligonucleotide pair GH220 and GH324, that amplify the polymorphic MspI (metH) site, and oligonucleotide pair GH367 and GH364, that amplify the polymorphic BanI (metD) site, are shown in Figure 1b and c respectively. In each experiment, control DNA from a patient homozygous for the allele containing the restriction enzyme site was included to ensure that complete digestion by the restriction enzyme had taken place (Figure 1b, patient 2631; Figure 1c, patient 067). All cases scored as LOH were repeated three times, and cases scored as non-LOH were repeated twice. In this study, 38 patients were informative for the MspI (metH) polymorphism and 34 patients were informative for the BanI (metD) polymorphism (Table 1). In total, 54 of 73 cases were informative with respect to at least one of the two polymorphisms studied, and seven of these 54 cases demonstrated LOH at the *MET* locus (13%).

To define the extent of LOH at chromosome 7q31 in our panel of 73 breast tumors we used oligonucleotide pairs to amplify 15 different $(CA)_n$ repeat microsatellite markers (Table 1). The level of informativeness observed for each of the markers in our cohort of breast cancer patients was consistent with the published values (Gyapay *et al.*, 1994). Figure 2 shows representative results obtained with four markers (D7S461, D7S486, D7S522 and D7S643). The data from the $(CA)_n$ repeat analyses as summarized in Table 1 reveals that LOH was observed with all $(CA)_n$ repeat markers studied at 7q31–32 (from D7S523 to D7S504, Table 1). In contrast, no informative patients showed LOH for D7S506 on 7p, D7S500 at 7q35 or D7S483 at 7q36. Of the 73 patients in this study, LOH was scored with at least one marker on chromosome 7 in a total of nine tumors (12.3%), from patients 005, 007, 009, 015, 017, 025, 074, 540 and 2632 (Figure 3).

The superposition of the overlapping regions of loss in these tumors allows the identification of the smallest common region of deletion. The proximal (centromeric) and distal (telomeric) boundaries of this critical region are defined by the flanking markers D7S687 and metH, as demonstrated by tumor samples 017 and 007 respectively (summarized in Figure 3). Figure 2 shows representative results obtained with two markers (D7S486 and D7S522) within the critical region. Of the other 62 tumors, all were informative for at least one marker within the critical region, but none showed LOH.

Physical mapping of chromosome 7q31 markers using YAC contig analysis

To physically map the location of the putative tumor suppressor gene on chromosome 7q31 as defined by

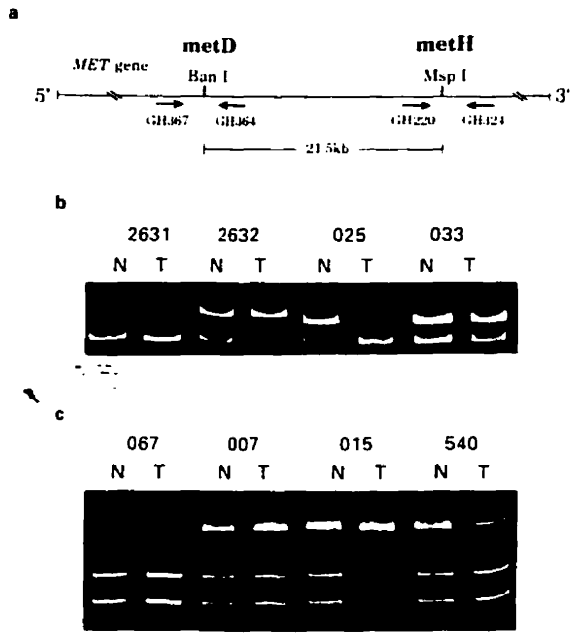

Figure 1 (a) *MET* has been mapped to chromosome 7q31, with the 5' region of the gene proximal to the centromere. The location of the restriction enzyme polymorphisms recognized by the RFLP probes metH (MspI) and metD(BanI) probes are indicated, as are the oligonucleotide primers used to amplify DNA containing the restriction enzyme sites. (b) Tumor DNA (T) and matching normal DNA (N) from the same patient were amplified using metD oligonucleotide primers GH367 and GH364. The PCR product was digested with BanI and electrophoresed on 12% non-denaturing polyacrylamide gel. In the normal DNA lane of an informative patient, the top band corresponds to the uncut allele, while the bottom band corresponds to the cut allele. LOH is scored if one of the alleles is absent or shows diminished intensity. Patient 2631 is uninformative, patient 033 is informative but shows no LOH, while patients 2632 and 025 are informative and show LOH at *MET* in the tumor DNA when compared with the matching normal DNA. (c) Tumor DNA (T) and matching normal DNA (N) from the same patient were amplified using metH primers GH220 and GH324. The PCR product was digested with MspI and electrophoresed on 12% non-denaturing polyacrylamide gel. For an informative patient, three bands, the top band corresponding to the uncut allele and the bottom two bands corresponding to the cut allele, are present in the normal DNA lane. LOH is present if one of the alleles is absent or shows diminished intensity. Patient 067 is uninformative, patient 007 is informative but shows no LOH, while patients 015 and 540 are informative and show LOH at *MET* in the tumor DNA when compared with the normal DNA

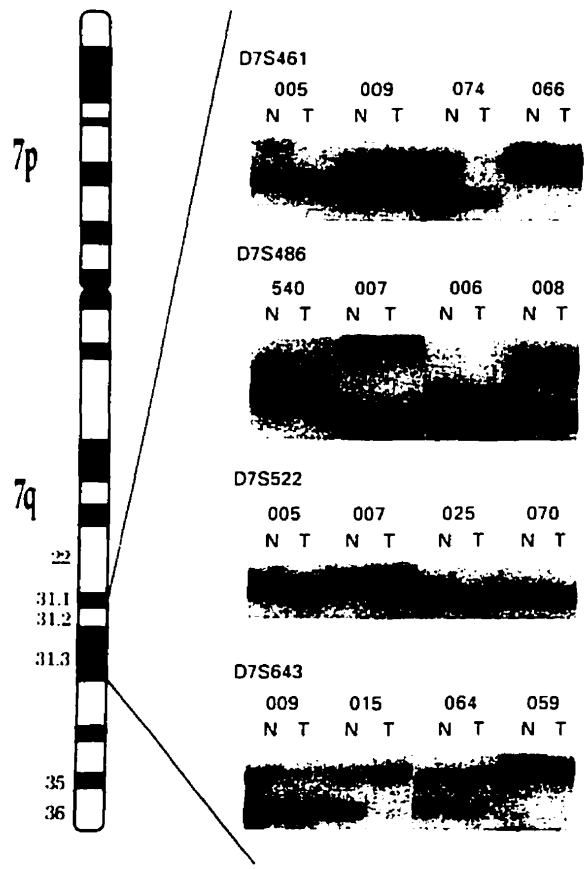

Figure 2 Representative PCR amplifications of (CA_n) microsatellite repeats D7S461, D7S486, D7S522 and D7S643 are shown. Oligonucleotide primers were used to PCR amplify the regions of DNA containing these markers, in the presence of [α-^{32}P]dCTP. Tumor DNA (T) and matching normal DNA (N) were amplified from 73 patients with breast cancer. A patient is considered to be informative if there are two bands (corresponding to two alleles) in the normal DNA lane. A patient shows LOH if, in the tumor DNA lane, one of the alleles is absent or shows diminished intensity. For example, patient 540 is informative for the marker D7S486 and shows LOH at that locus, while patient 008 is informative with this marker, but shows no LOH

our LOH studies, it was necessary to establish the correct order of the genetic DNA markers in this region. To accomplish this, a YAC contig encompassing the *MET* locus was constructed (Figure 4). The contig, which spanned approximately 3.5 Mb of DNA, encompassed five of the genetic markers (D7S486, D7S522, metD, metH, D7S677) used in the LOH study (Figure 4). The position of four cloned genes (*MET*, *CAPZA2*, *WNT2*, and *CFTR*), and seven ESTs (expressed sequence tags) or cDNA fragments within the contig were determined. The chromosomal order and physical distance separating the loci D7S18, *MET*, *WNT2* and *CFTR* as established by our YAC contig analysis is in agreement with previous studies using pulsed-field gel electrophoresis mapping (Poustka *et al.*, 1988; Rommens *et al.*, 1989a, b).

A large YAC contig surrounding D7S687, the proximal endpoint of the region of LOH in our study, has been linked by one clone to the contig shown in Figure 4 (SWS, unpublished data). Based on these studies, the physical distance between D7S687 and metH is 2 Mb (SWS, unpublished data). The combination of genetic analyses and physical mapping data allows us to establish the following order of markers across the critical region of deletion: 7cen-D7S687-D7S486-D7S522-D7S2460-metD-metH-7qter.

Immunohistochemical analyses of Met protein expression in breast cancer

The *MET* gene is the only known gene mapped to the region deleted in our cohort of breast tumors (Figure

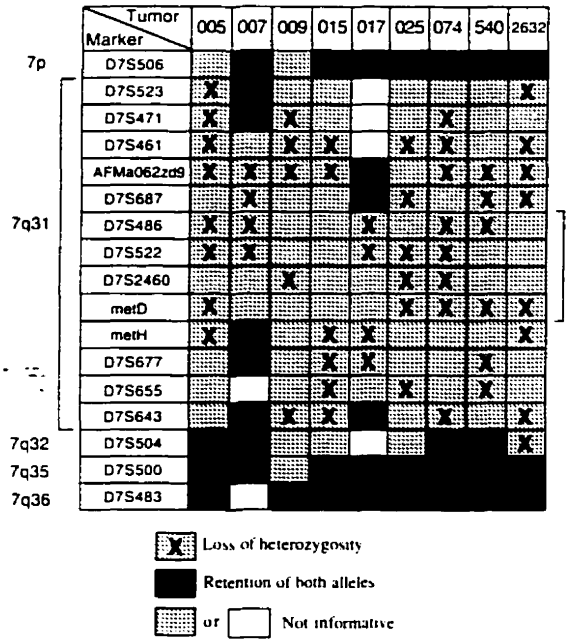

Figure 3 Summary of deletion analyses in nine breast cancer patients. Names of polymorphic markers used are given, along with their chromosome band assignments, in column 1. Nine columns representing the nine patients with a loss of heterozygosity of chromosome 7 are shown. Explanation of the symbols used is given, and the extent of the region of loss in each patient is indicated by the grey shading. The smallest common region of deletion in these nine patients is indicated by the vertical line to the right, and extends from marker D7S687 to metH

4). *MET* encodes a transmembrane growth factor receptor tyrosine kinase (Park *et al.*, 1987), whose ligand is hepatocyte growth factor scatter factor (HGF SF) (Bottaro *et al.*, 1991; Naldini *et al.*, 1991). The Met receptor is expressed in many cell types, but is found at highest levels in epithelial cells (Gonzatti-Haces *et al.*, 1988; Di Renzo *et al.*, 1991; Prat *et al.*, 1991; Yang and Park, 1995), including breast epithelial cells (Tsarfaty *et al.*, 1992; Niranjan *et al.*, 1995; Pepper *et al.*, 1995; Soriano *et al.*, 1995; Yang *et al.*, 1995).

From studies of other tumor suppressor genes (reviewed in Weinberg, 1991; Levine, 1993), if *MET* is a candidate tumor suppressor gene for breast cancer, the deletion of one copy of the *MET* gene may be accompanied by a loss of function mutation in the remaining copy of the gene which may be reflected by an absence of detectable Met protein, or the presence of non-functional protein. To investigate the status of Met protein in breast tumors, we have used an anti-peptide antibody which recognizes the carboxy-terminus of the Met receptor (Rodrigues *et al.*, 1991) to perform immunohistochemical analyses on sections of paraffin-embedded breast reduction tissue. Met immunoreactivity is detected in the epithelial cells of the normal breast ducts and lobules (Figure 5a). Furthermore, this staining can be blocked by competition with the antigenic peptide (Figure 5b). Paraffin blocks were available for six patients, 005, 007, 009, 015, 017 and 025, whose tumors showed LOH at chromosome 7q31. These tumors, and an equivalent number of samples from patients whose tumors showed no apparent LOH, 016, 021, 031, 062, and

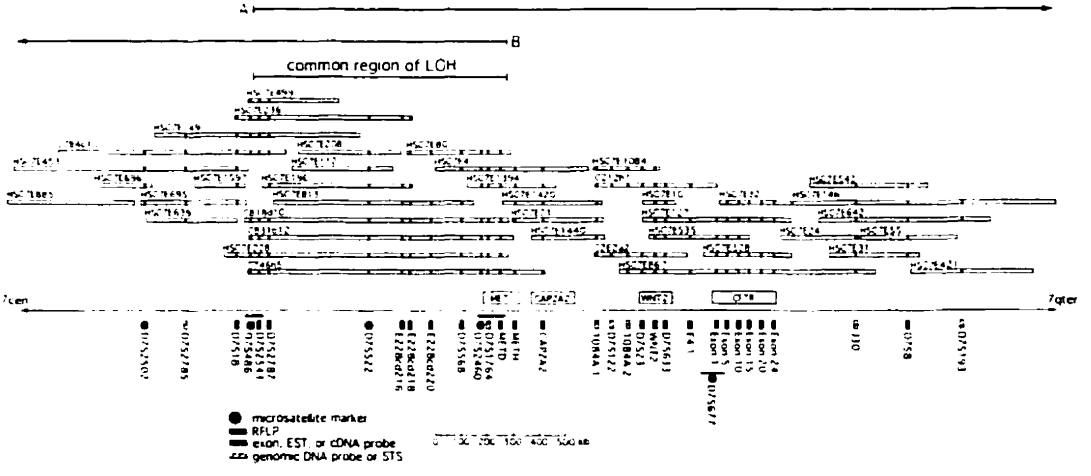

Figure 4 Determining the order of DNA markers and genes around the *MET* locus using YAC contig analysis. The presence of each DNA marker in the YAC clone is shown by a vertical bar. The YACs were aligned based on their DNA marker content, by DNA fingerprinting with repetitive elements, and by Alu-PCR to Alu-PCR hybridization experiments. The black horizontal bars indicate markers that could not be ordered and the minimal region to which they could be mapped. The HSC7E-YACs were from a chromosome 7-specific library, the C-YACs were CEPH clones. Our studies indicate that all of the YAC clones shown contain contiguous segments of genomic DNA as determined by DNA marker and FISH analysis, except for HSC7E208 and C784c10 which are chimeric. The total length of the YAC contig is estimated to be approximately 3.5 Mb. Full-length cDNA probes were used for *CAPZA2*, *WNT2* and *CFTR*. Therefore, these genes should be confined to the region shown. The complete genomic structure of *MET* has not been determined, thus only the positions of metD and metH are shown. The smallest common region of deletion defined in this study (B) and the smallest common region of deletion as determined by Zenklusen *et al.* (1994a) (A), are indicated by the arrows at the top. The common region of LOH as defined by these two studies is shown

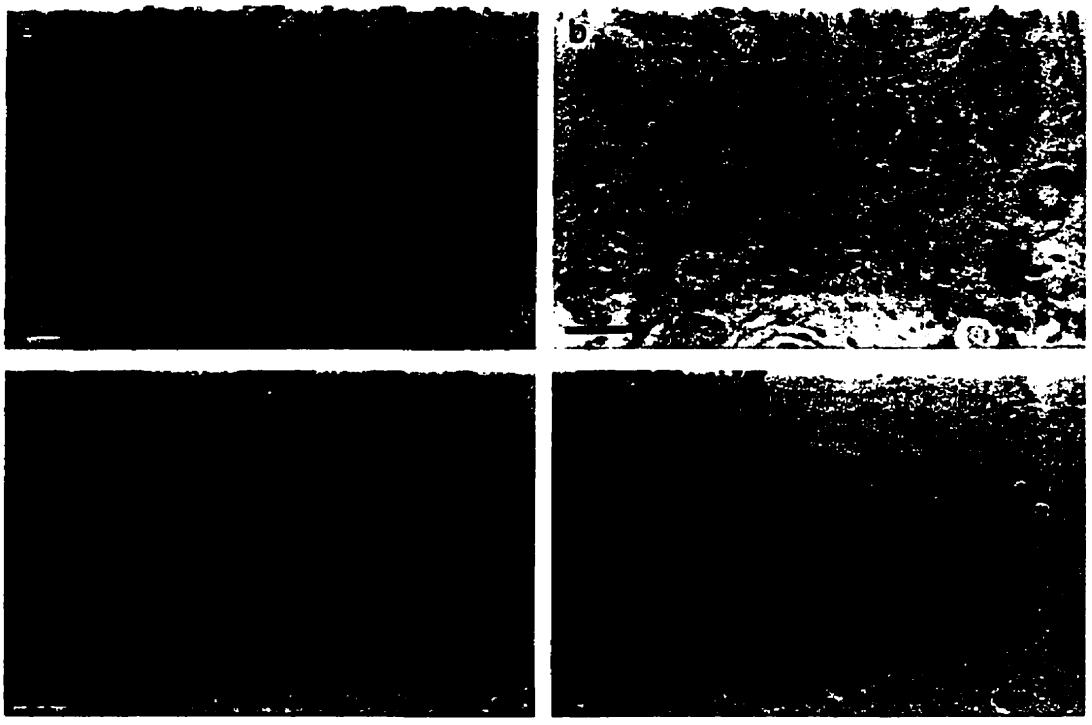

Figure 5 Immunohistochemical analyses of Met in breast cancer. Met immunoreactivity, indicated by the red staining, is present in the epithelial cells of the normal ducts and lobules of tissue obtained from a reduction mammoplasty (**a**). Staining can be blocked by competition with the antigenic peptide (**b**). Met immunoreactivity is detected in the cells of a breast tumor with no apparent LOH of chromosome 7 (**c**) and also in the cells of a breast tumor with LOH of *MET* (**d**). (**a**) and (**b**) Black bar at bottom left corresponds to 50 *μm*. (**c**) and (**d**) Black bar at bottom left corresponds to 125 *μm*.

065, were characterized by immunohistochemistry. In all cases, Met-specific immunoreactivity was detected in the carcinoma cells as well as in adjacent normal ductal epithelial cells. In addition, no significant difference was noted between Met expression in tumors with LOH of chromosome 7q31 (Figure 5d), or tumors without LOH (Figure 5c).

Discussion

With the incidence of breast cancer rising to alarming numbers in North America, great efforts are underway to elucidate the molecular mechanisms of oncogenesis and progression of this malignancy. Observations of loss of heterozygosity at 7q31 in breast cancer have suggested the presence of a tumor suppressor gene (Bièche *et al*., 1992; Deng *et al*., 1994). To define the specific region of chromosome 7 that is deleted in breast cancer, we have used 14 microsatellite repeat markers for 7q31-7q36, a marker on 7p and two polymorphic restriction enzyme sites within the *MET* gene (MspI (metH) and BanI (metD)) to perform LOH analyses on 73 cases of breast cancer. We have identified LOH at 7q31 in nine of these 73 cases, and show that in each case, the LOH reflects a specific loss of a portion of chromosome 7, and is not the result of a random loss of the whole chromosome. More importantly, we define the location of a putative tumor suppressor gene to a 1000 kb region of chromosome 7q31, which is proximal to and includes the *MET* receptor tyrosine kinase, but no other known genes. The 1000 kb critical region is contained on one YAC clone, C746h5 (Figure 4) and thus, this represents an important step towards the eventual isolation of the tumor suppressor gene by positional cloning.

The loss of chromosomal regions harboring tumor suppressor genes has been shown to be an important characteristic of human malignancies such as colon cancer (Fearon and Vogelstein, 1990). In breast cancer, the incidence of LOH of markers at 7q31 has been reported to occur from 0–40% in several independent studies (Sato *et al*., 1990; Devilee *et al*., 1991; Bièche *et al*., 1992; Deng *et al*., 1994; Kerangueven *et al*., 1995; Nagy *et al*., 1995), which in some cases was suggested to reflect a random loss of chromosome 7 (Kerangueven *et al*., 1995; Nagy *et al*., 1995). However, we have found LOH of markers at 7q31-7q32 but not on 7p or at 7q35-7q36 (Table 1), demonstrating the loss of a specific chromosomal region in all tumors showing LOH on chromosome 7, centered on 7q31. Using our refined mapping order for markers at 7q31 (Figure 4) and the superposition of the regions of LOH observed, we have localized the smallest common region of LOH to a chromosomal interval of 2 Mb, flanked proximally by D7S687 and distally by metH (Figure 3). In our cohort of 73 patients, all patients were informative for at least one marker in the critical region and LOH was scored in a total of nine tumors (12.3%) (Figure 3). This frequency of LOH of 7q31 (12.3%) is lower than that previously reported by Bièche *et al*. (1992) and

Deng *et al.* (1994) (40.5% and 27% respectively), yet is consistent with levels reported by others (Kerangueven *et al.*, 1995). However, our observation of loss of a specific chromosomal region in all tumors showing LOH on chromosome 7, centered on 7q31, supports the findings of Bièche *et al.* (1992) and Deng *et al.* (1994) that LOH in this region is a specific event in breast cancer.

The region D7S687-metH is 2 Mb (SWS, unpublished). This partially overlaps with the region of loss mapped by Zenklusen *et al.* (1994a), which was 2.5 cM in genetic distance, flanked proximally by D7S486 and distally by D7S480 (Figure 4). The D7S480 marker lies telomeric to our distal endpoint of metH (Gyapay *et al.*, 1994). Because the loci D7S486 and D7S522 had been previously suggested to be distal to the *MET* locus, it was concluded that *MET* was not included in the region of loss flanked by D7S486 and D7S480 (Zenklusen *et al.*, 1994a). However, from the revised order of the chromosome 7 markers reported here, D7S486 and D7S522 are proximal to *MET* (Figure 4), placing the *MET* gene within the region of loss mapped by us and by Zenklusen *et al.* (1994a). Together, these data allow us to establish a critical region of LOH in the breast tumor samples studied thus far to be between D7S486 and metH, which we show to be a 1000 kb physical distance. Furthermore, we have localized this critical region within a single YAC clone, C746h5 (Figure 4). The *MET* tyrosine kinase gene is included in this critical region, while other genes mapped to chromosome 7q31, such as *CFTR* and *WNT2*, are not included (Figure 4).

Within the critical region, Zenklusen *et al.* (1994a) identified LOH of marker D7S522 at 81.8%, in nine of 11 cases of informative tumors. In our cohort of breast cancer patients, only five of 46 (10.9%) samples informative for D7S522 demonstrated LOH at this locus (Table 1). Such discrepancy in the frequency of LOH of D7S522 may be due to a population variation, since Zenklusen *et al.* (1994a), studied a selected subpopulation of tumors previously characterized by Bièche *et al.*, 1992. The level of LOH of D7S522 observed in our population is in agreement with the level of LOH observed by others for D7S522 (9%) (Kerangueven *et al.*, 1995), and is consistent with the level of LOH of closely linked markers metD and metH in our study (14.7% and 10.5%, respectively) (Table 1).

A tumor suppressor gene for a particular cancer type would be expected to have protein expression patterns and biological functions consistent with the hypothesis that its loss of function would contribute to the development or progression of that cancer. The *MET* receptor tyrosine kinase gene is the only known gene mapped to the critical region containing the tumor suppressor at 7q31, and its role as a potential candidate for the tumor suppressor gene must therefore be evaluated. Met is predominately expressed in epithelial cells, including breast epithelial cells (Gonzatti-Haces *et al.*, 1988; Di Renzo *et al.*, 1991; Prat *et al.*, 1991; Tsarfaty *et al.*, 1992; Niranjan *et al.*, 1995; Pepper *et al.*, 1995; Soriano *et al.*, 1995; Yang and Park, 1995; Yang *et al.*, 1995) (Figure 5a) while its ligand, HGF/SF, a soluble cytokine, is produced primarily by mesenchymal cells, including breast stromal cells (Rahimi *et al.*, 1994; Niranjan *et al.*, 1995). HGF/SF has been postulated to play a role in morphogenetic epithelial-mesenchymal interactions (Stoker *et al.*, 1987; Weidner *et al.*, 1990; Montesano *et al.*, 1991). It stimulates the formation of branching tubules of established mammary gland epithelial cell lines when grown in three-dimensional culture conditions (Berdichevsky *et al.*, 1994; Brinkmann *et al.*, 1995; Soriano *et al.*, 1995), and also stimulates ductal outgrowth from mouse mammary gland explants (Yang *et al.*, 1995). These results suggest that Met – HGF/SF interactions may play a crucial role in breast development.

Although some studies have failed to detect Met expression in normal breast tissue or in breast cancer cells (Di Renzo *et al.*, 1991; Prat *et al.*, 1991), in keeping with the study by Tsarfaty *et al.* (1992), we show that Met is expressed in normal breast epithelium and Met-specific immunoreactivity is also detected in breast carcinoma cells (Figure 5). No difference in Met expression is observed between tumors that do and do not show LOH at the *MET* locus (Figure 5c and d). However, the observation that the Ret receptor tyrosine kinase is inactivated by point mutations in Hirschsprung's Disease (Edery *et al.*, 1994; Romeo *et al.*, 1994), provides a precedent for the inactivation of a receptor tyrosine kinase in human disease by a mechanism other than loss of expression. It is not known if the Met protein expressed in tumors with LOH at *MET* is functional, and until this can be determined, we cannot rule out *MET* as a potential candidate for the putative tumor suppressor gene on chromosome 7q31.

In summary, we have defined the minimum region of chromosome 7 that is deleted in breast cancer to a 1000 kb region of 7q31, flanked by D7S486 and metH. The detection of LOH of *MET* and closely linked loci at 7q31 in many neoplasms in addition to breast cancer, including gastric, colon, ovarian, prostate and head and neck cancers (Kuniyasu *et al.*, 1994; Zenklusen *et al.*, 1994b, 1995a, b; Latil *et al.*, 1995; Muller *et al.*, 1995; Takahashi *et al.*, 1995), indicate the presence of a tumor suppressor gene in the vicinity of *MET* which may be implicated in a variety of tumor types. Our study provides a basis for a positional cloning approach to identify the putative tumor suppressor gene at chromosome 7q31.

Materials and methods

Tissue specimens and genomic DNA extraction

Tumor and matching normal tissue samples were obtained from 43 breast cancer patients at the time of mastectomy or tumorectomy at the Royal Victoria Hospital and from nine patients at St. Mary's Hospital, Montreal, Canada. Forty-six tumors were classified as infiltrating ductal carcinomas, five as infiltrating lobular carcinomas, and one was a mixed ductal and lobular infiltrating carcinoma. An additional 21 tumors were obtained from the Mount Sinai Hospital, Toronto, Canada. Seventeen of these tumors were infiltrating ductal carcinomas and four were infiltrating lobular carcinomas. Tissue samples were snap frozen in liquid nitrogen immediately after surgery and stored at −70°C until DNA extraction. DNA was extracted from tumor and normal tissue and in some cases from blood leukocytes, using a standard proteinase K digestion and phenol-chloroform extraction protocol (Maniatis *et al.*, 1982).

LOH analysis of two markers within the MET gene by PCR

Oligonucleotide primers spanning two polymorphic restriction enzyme sites were used to identify tumors with LOH of *MET*. These sites, a BanI site recognized by the RFLP probe metD and a MspI site recognized by the RFLP probe metH, are located within the 3' region of the *MET* gene (Figure 1). For BanI (metD) analysis, primer pair GH367 and GH364 were used to amplify genomic DNA. For MspI (metH) analysis, primer pair GH220 and GH324 were used (for primer sequences, see Table 1) (Horn *et al.*, 1990). PCR was performed in a volume of 50 μL, containing 50 ng DNA, 1.5 mM $MgCl_2$, 5 μL 10 × standard PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 50 pmol of each primer, 1.25 mM dNTPs and 2.5 units of Taq polymerase (GibcoBRL, Burlington, Ontario). An initial step of 3 min at 85 C was followed by 35 cycles of 1 min of denaturation at 94 C, 2 min of annealing at 55 C and 1 min of extension at 72 C, followed by a final extension step of 7 min at 72 C. Product specificity was confirmed by separation on a 1% agarose gel. One-tenth of the PCR reaction was then digested with an excess of the appropriate restriction enzyme. The digestion products were resolved by 12% non-denaturing polyacrylamide gel electrophoresis (PAGE).

Microsatellite repeat analysis

Fifteen $(CA)_n$ repeat microsatellite markers on chromosome 7: D7S506, D7S523, D7S471, D7S461, AFMa062zd5, D7S687, D7S486, D7S522, D7S2460, D7S677, D7S655, D7S643, D7S504, D7S500 and D7S483 (Gyapay *et al.*, 1994), were used to identify the region of loss of chromosome 7 in breast cancer (for primer sequences, see Table 1). These $(CA)_n$ repeats were amplified by PCR in a final volume of 50 μL, containing 50 ng DNA, 1.5 mM $MgCl_2$, 5 μL standard 10 × PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 50 pmol of each primer, 1.25 mM dNTPs, 1 μCi [α-^{32}P]dCTP and 2.5 units of Taq polymerase (GibcoBRL, Burlington, Ontario). An initial step of 3 min at 85 C was followed by 35 cycles of 40 s of denaturation at 94 C, 30 s of annealing at 55 C, and 40 s of extension at 72 C, followed by a final extension step of 2 min at 72 C. PCR products were separated in a 6% sequencing gel containing formamide (Litt *et al.*, 1993) or a conventional 6% sequencing gel (Maniatis *et al.*, 1992).

Determination of LOH

Allelic loss was scored only on informative patients whose normal DNA samples were polymorphic at a given locus. Patients who were uninformative were not considered. LOH was identified, visually or following phosphoimager densitometric analysis, as a loss in intensity (>50%) or complete loss of one allele in the tumor DNA when compared with the normal DNA from the same patient. All cases of LOH were confirmed by three separate experiments with two different reviewers.

Immunohistochemistry

Three μm sections were cut from paraffin blocks of the breast tumors from patients 005, 007, 009, 015, 016, 017, 021, 025, 031, 062 and 065. Adjacent normal breast tissue as well as paraffin-embedded normal breast tissue obtained from a reduction mammoplasty were also studied. Sections were deparaffinized in xylene and rehydrated, then processed according to the instructions included in the Dako LSAB immunostaining kit (Dimension Laboratories, Mississauga, Ontario). The following changes were made to the standard protocol: endogenous peroxidase was blocked by treatment with 0.3% H_2O_2 for 15 min at room temperature. Following protein blockage, incubation with the primary antibody, Ab144, a rabbit polyclonal antibody recognizing the carboxy-terminus of the Met protein (Rodrigues *et al.*, 1991), or Ab144 in the presence of competing peptide, was carried out at a dilution of 1:500 at room temperature for 30 min. Slides were counterstained with Gill's hematoxylin and mounted with Crystal-mount (Esbe Laboratories, Montreal, Quebec).

YAC clones and contig assembly

The YAC clones were isolated from a chromosome 7-specific YAC library (Scherer *et al.*, 1992) or from the CEPH mega-YAC library (Cohen *et al.*, 1993). The presence or absence of DNA markers was determined by PCR or hybridization analysis of each individual YAC clone. The insert size of the clone was determined by pulsed-field gel electrophoresis followed by comparison to YPH149 size standards (Scherer and Tsui, 1991). Specific information on each of the DNA markers shown in Figure 4 is available in the Genome Database.

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Chapter III - Intron-exon structure of the *MET* gene and cloning of an alternatively-spliced Met isoform reveals frequent exon-skipping of a single large internal exon.

Preface

If *MET* is the tumor suppressor gene important for breast cancer at 7q31, it is predicted that a deletion of one copy of the *MET* gene would be accompanied by a recessive, inactivating mutation in the remaining copy of the gene. However, the intron-exon structure of the *MET* gene had not been determined, and therefore, sequence analysis of the *MET* gene in breast tumors with LOH of 7q31 was not possible. Therefore, in chapter three of this thesis, I used PCR-based techniques to elucidate the complete intron-exon structure of the *MET* gene, and to design primers that could be used for amplification of each individual exon of *MET* for mutation analysis.

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Intron-exon structure of the *MET* gene and cloning of an alternatively-spliced Met isoform reveals frequent exon-skipping of a single large internal exon

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Hepatocyte growth factor/scatter factor (HGF/SF) is a multifunctional factor that stimulates epithelial cell mitogenesis, motility, invasion, and morphogenesis. Its receptor is encoded by the *MET* proto-oncogene, a transmembrane receptor tyrosine kinase. Several studies have suggested a role for *MET* as a dominant oncogene in tumor development and progression. Conversely, *MET* is located at a region on chromosome 7q31 frequently deleted in carcinomas, suggesting that recessive mutations in *MET* may exist in certain cancers. To facilitate a search for mutations in *MET*, we have obtained the intron-exon structure of the human *MET* gene. We present the genomic structure of the first member of the Met receptor family to be characterized. Interestingly, *MET* contains a large second exon of 1214 nucleotides. We show that this exon, containing the AUG for the Met receptor, is frequently skipped in normal human tissues and cell lines, and corresponds to a ubiquitously expressed 7 kb Met transcript. This transcript yields no detectable protein product *in vivo*. Thus, unlike other genes, in which alternative splicing often gives rise to proteins with distinct activities, exon-skipping of *MET* exon 2 is predicted to decrease the abundance of a Met mRNA encoding a functional Met receptor.

Keywords: *MET* receptor tyrosine kinase; genomic structure; alternative splicing; exon-skipping; hepatocyte growth factor receptor

Introduction

The *MET* gene encodes a transmembrane protein receptor tyrosine kinase (Park *et al.*, 1987) whose ligand, Hepatocyte Growth Factor/Scatter Factor (HGF/SF) (Bottaro *et al.*, 1991; Naldini *et al.*, 1991), is a mesenchymally-derived growth factor. Addition of HGF/SF to epithelial cells produces pleiotropic biological effects, including mitogenesis of primary hepatocytes and renal tubule cells, and scatter, invasion and branching tubulogenesis of epithelial cells (reviewed in Rosen *et al.*, 1994). During development, HGF/SF is expressed in mesenchymal tissue in close proximity to Met-expressing epithelium (Sonnenberg *et al.*, 1993), supporting a role for Met-HGF/SF signaling pathways in epithelial-mesenchymal interactions *in vivo*. Genetic studies show a critical role for Met and HGF/SF in normal embryonic development, and have demonstrated a role for Met and HGF/SF in the development of liver and placenta, the development and innervation of skeletal muscle, the ductal growth of mouse mammary explants, and directing the growth of axonal growth cones (Schmidt *et al.*, 1995; Uehara *et al.*, 1995; Yang *et al.*, 1995; Ebens *et al.*, 1996; Maina *et al.*, 1996; Takayama *et al.*, 1996; Yang *et al.*, 1996).

The Met receptor and HGF/SF are also implicated in oncogenesis. Met was first isolated as an oncogenic variant, generated following a genomic rearrangement (Park *et al.*, 1986). Deregulation of the Met receptor via an autocrine loop transforms fibroblasts in culture (Rong *et al.*, 1994), and transgenic mice overexpressing HGF/SF exhibit tumorigenesis of various organs (Takayama *et al.*, 1997). Met is overexpressed and/or deregulated in diverse human tumors including gastric, colorectal, thyroid and ovarian carcinomas, leukemia and sarcomas (Di Renzo *et al.*, 1992, 1994; Liu *et al.*, 1992; Rong *et al.*, 1993, 1995; Jucker *et al.*, 1994; Scotlandi *et al.*, 1996; Yonemura *et al.*, 1996). Although in some cases, constitutive activation of Met in the absence of ligand has been correlated with amplification and overexpression of a wild-type Met protein (Giordano *et al.*, 1989b), it is possible that activating mutations in *MET* may exist in human cancers. In addition, *MET* is located on a 1000 kilobase (kb) region of chromosome 7q31 that is deleted in breast cancer (Lin *et al.*, 1996). Deletions in this region of 7q31 have also been described for prostate and ovarian carcinomas (Zenklusen *et al.*, 1994, 1995; Takahashi *et al.*, 1995). Since loss of chromosomal regions are generally thought to correlate with the presence of recessive mutations, this would suggest that recessive mutations may be found in *MET* in these cancer types. Thus, in a situation analogous to the Ret receptor tyrosine kinase, where both activating and inactivating mutations have been described in human disease (reviewed in Pasini *et al.*, 1996), different mutations affecting the activity of Met may be found in various cancers.

MET is the prototype for a family of receptor tyrosine kinases, including *RON* and the chicken c-*sea* gene (Huff *et al.*, 1993; Ronsin *et al.*, 1993), that have overall sequence and protein structural similarity. The major *MET* product is synthesized as a single chain precursor of 170 kDa that is cleaved post-translationally to yield a transmembrane β-subunit of 145 kDa containing the intracellular tyrosine kinase domain and an α-subunit of 45 kDa that remains extracellular (Giordano *et al.*, 1989a; Rodrigues *et al.*, 1991). The

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α- and β-subunits are linked together through disulfide bonds to form a mature heterodimeric Met receptor of 190 kDa (p190Met) which, upon binding HGF/SF, becomes phosphorylated on tyrosine residues in the β-subunit (Bottaro *et al.*, 1991; Naldini *et al.*, 1991). The p190Met high-affinity HGF/SF receptor is encoded by the most abundant Met mRNA, which is 8 kb in size (Rodrigues *et al.*, 1991). However, another Met transcript of 7 kb is observed by Northern hybridization analysis of RNA from cell lines and tissues (Park *et al.*, 1986; Giordano *et al.*, 1989b). The functional significance of this transcript and its protein product is unknown.

To understand the biochemical significance of alternative splicing in the Met receptor and to facilitate a search for mutations within *MET* in human cancer, we have obtained the intron-exon structure of the human *MET* gene. We have cloned and elucidated the structure of an alternatively spliced Met cDNA that corresponds to the ubiquitously expressed Met mRNA of 7 kb. We show that the 7 kb Met mRNA lacks exon 2 of *MET*, which contains the AUG for the p190Met receptor product. Interestingly, exon 2 is skipped in normal human tissues and cell lines. However, this transcript yields no detectable protein product *in vivo* and only low levels (<5% of wild type receptor) of multiple truncated proteins in transient assays. Thus, the 7 kb Met transcript is unlikely to give rise to a protein product *in vivo* and we suggest that exon-skipping may represent a mechanism that regulates the abundance of the 8 kb Met mRNA encoding a functional Met receptor.

Results

Intron-exon organization of the human MET *gene*

To isolate the *MET* genomic locus, cDNA corresponding to the 8 kb Met transcript, which encodes the p190Met receptor, was used as a probe to screen two libraries prepared from human genomic DNA. The entire *MET* locus was isolated in nine overlapping λ phage clones from a human placental genomic library (Rodrigues *et al.*, 1991) and two cosmid clones from the Lawrence Livermore National Laboratory chromosome 7-specific cosmid library (obtained from Dr SW Scherer). DNA prepared from the λ phage and cosmid genomic clones was used to analyse *MET* genomic structure. Oligonucleotide primers derived from the Met cDNA sequence (Park *et al.*, 1987) were used to obtain sequence information from each clone. The intron-exon boundaries were mapped by sequencing each exon in its entirety, along with portions of the adjacent introns (Table 1). The approximate size of each intron was estimated by PCR amplification of human genomic DNA using oligonucleotide primers flanking each intron (Table 1 and data not shown). Introns 1 and 2 gave variable-sized amplification products. Therefore, the exact sizes of these introns were obtained from sequence information recently deposited at the Human Genome Sequencing Project's World Wide Web site (http://genome.wustl.edu/gsc/gschmpg.html).

A graphical representation of the *MET* gene is shown in Figure 1a. All *MET* exons were found to be flanked by the canonical consensus splice sites: AG at the 3' splice site, and GT at the 5' splice site (Table 1) (Breathnach and Chambon, 1981). The exons range in size, from 81 nucleotides (nt) (exon 16) to 2.5 kb for the last exon (exon 21), which encodes the carboxy-terminus of the Met protein and a long 3'UTR (untranslated region) (Figure 1b and Table 1). The sizes of the introns range between 100 nt and 31 kb. Exon 1 of *MET* is non-coding, and contains most of the 5'UTR. Exon 2 is the largest internal coding exon of *MET* (1214 nt), and contains 14 nt of 5' untranslated sequence followed by the initiating methionine codon. Thus, the 4170 nt open reading frame coding for the p190Met receptor protein is distributed over 20 exons. The correlation of the exon structure of *MET* with the functional domains of the Met protein is shown in Figure 1b. Exon 2 contains the entire coding sequence for the 45 kDa α-chain of the p190Met receptor, whereas the transmembrane 145 kDa β-chain is encoded by exons 2–21.

Cloning and characterization of an alternatively spliced Met mRNA isoform

Northern hybridization analysis of RNA derived from various cell lines reveals that multiple transcripts are expressed from the *MET* locus, the most abundant being estimated to be 8 kb and 7 kb in size (Park *et al.*, 1986; Giordano *et al.*, 1989b). Using a probe derived from the cDNA corresponding to the major 8 kb Met mRNA (probe A; nt +641 to +2055; see Figure 2c), Met transcripts corresponding to the 8 kb and 7 kb Met mRNAs are detected in various human cell lines (Figure 2a). We have previously isolated cDNA, pOK1 (6.8 kb), that corresponds to the most abundant 8 kb Met transcript, and shown that it encodes the p190Met receptor heterodimer (Rodrigues *et al.*, 1991). A second cDNA, pHOS (6.7 kb), corresponds to an 8 kb Met transcript, present at low levels, and results from the use of an alternate splice acceptor for exon 10, located 54 nt upstream of the p190Met splice acceptor site (Park *et al.*, 1987; Rodrigues *et al.*, 1991). The 7 kb Met transcript has not been characterized. We show that a probe derived from the 5' portion of the Met cDNA (probe B; nt −194 to +641; Figure 2c), which contains sequences derived mainly from exon 2, hybridizes to the 8 kb Met transcript, but only weakly to the 7 kb Met isoform (Figure 2b), suggesting that the 7 kb transcript is lacking these sequences.

To preferentially isolate cDNA clones that correspond to the 7 kb Met transcript, we screened a cDNA library prepared from a human cell line that expresses the 7 kb Met mRNA at high levels (Okajima, Figure 2a) with probes A and B (Figure 2c), and chose clones which hybridized strongly with probe A, but not with probe B. Two clones with this profile, pOK14 (3196 nt), and pOK8 (1650 nt), were characterized further and sequenced (Figure 2c). The pOK14 cDNA extended from position −145 to +4265 of *MET*, and contained a deletion of 1214 bp from −14 to 1200 (inclusive) (Figure 2c), whereas the pOK8 cDNA extended from −382 to +2482, and contained the same deletion as pOK14 (Figure 2c). Neither of the pOK14 nor pOK8 cDNA clones contains a poly(A)$^+$ tail, and appear to have been reverse-transcribed following hybridization of the oligo-dT primer to

Table 1 Nucleotide sequence of intron-exon junctions of the human *MET* gene

Exon #	*3′intron*	*Exon sequence*	*5′intron*	*Size of Exon*	*Size of Intron*
1[a]		. . . CAC CGA AAG	gtaaaattgc	398 bp	26 kb
		−14[c]			
2	gttttggcag	ATA AAC CTC . . . TTT AAT AGG	gtaagtcaca	1214	31 kb
		1201			
3	tttattccag	ACA CTT CTG . . . TTC ATG CAG	gtaagtgctt	192	8 kb
		1393			
4	tgctgtttag	GTT GTG GTT . . . GGG AAG AAG	gtaagctgtt	135	770 bp
		1528			
5	ctcttcacag	ATC ACG AAG . . . ATC TAC AAG	gtaggaatct	174	14 kb
		1702			
6	gtccttgtag	GTT TTC CCA . . . GAT GAA TAC	gtaaggatct	161	2 kb
		1863			
7	ttttttccag	ATT GAA ATG . . . TCC TAT GTG	gtaaggaaga	103	100 bp
		1966			
8	tccccttccag	GAT CCT GTA . . . TTT AAA AAG	gtgttgtaaa	137	700 bp
		2103			
9	ttttgttcag	TGT GTC AAA . . . TTT TAT TAG	gtaagtagaa	162	850 bp
		2265			
10[b]alt	tctcttacag	TAC TTG GTG . . .		154	
		2319			
10	gctttgccag	TGG TGG GAG . . . TTT ACA GTG	gtaagtcctt	100	3.6 kb
		2419			
11	ctttctctag	GCA TGT CAA . . . GAA ATT AAG	gtaagaaatg	219	9 kb
		2638			
12	ttcctttcag	GGA AAT GAT . . . AAT ATA GAG	gtgggattcc	147	1.7 kb
		2785			
13	tcatttttag	TGG AAG CAA . . . AAA TTA AAG	gtgcattttt	157	250 bp
		2942			
14[c]	ctgttttaag	ATC TGG GCA . . .TTC CAG AAG	gtatatttca	141	3.3 kb
		3083			
15[d]	catcttacag	ATC AGT TTC . . . TAG GAA GAG	gtaagtattt	231	2.5 kb
		3314			
16	ttttgcacag	GGC ATT TTG . . . CCT TGA ACA	gtaagtggca	81	1.5 kb
		3395			
17	ccttcgaaag	GAA TCA CTG . . . GAG ACT CAT	gtaagttgac	182	3.6 kb
		3577			
18	ttgactgcag	AAT CCA ACT . . . AAA CTG TAT	gtaagtatca	110	1.5 kb
		3687			
19	ctttctgtag	GCT GGA TGA . . . TCA GAT GTG	gtaatgtatt	166	15 kb
		3853			
20	gttttactag	TGG TCC TTT . . . AGA CCC CTT	gtaagtagtc	137	100 bp
		3990			
21	tttggaacag	ATA TGA AGT . . .		2.5 kb	NA

[a]For full sequence of Exon 1, see (Gambarotta *et al.*, 1994). [b]Exon 10 is subject to alternative splicing, in which this alternate splice junction located 54 bp upstream of the normal 5′ splice site is used (Rodrigues *et al.*, 1991). [c]Exon 14 is alternatively spliced in mouse (Lee & Yamada, 1995). [d]The *TPR-MET* breakpoint occurs at exon 15 (Park *et al.*, 1986). [e]Base number is indicated above the first nucleotide of each exon. Bases are numbered from the translation start site (+1) as defined by (Park *et al.*, 1987)

internal A-rich regions. However, the longer clone, pOK14, contains part of the 5′UTR as well as the stop codon at base number +4170 used for the p190Met protein, and lacks only part of the long 3′UTR last exon of *MET* (from +4265 to the poly(A)$^+$ addition site; Figure 2c). Thus, the size of the pOK14 cDNA (3.19 kb) plus the additional 3′UTR (2.2 kb, Figure 2c) and 5′UTR (267 bp, Figure 2c), generates a transcript of 5.66 kb, which is consistent with the size of pOK1 cDNA (6.8 kb) minus exon 2 (1214 nt). Moreover, the structure of the pOK14 and pOK8 clones are consistent with that expected for the 7 kb Met mRNA from Northern analysis, in that they would hybridize with probe A but fail to hybridize or hybridize poorly with probe B (−194 to +641).

The pOK14 and pOK8 cDNAs correspond to a ubiquitously expressed, alternatively spliced Met mRNA lacking exon 2

The pOK14 and pOK8 clones contain no new sequences when compared with the full length Met cDNA pOK1 (Rodrigues *et al.*, 1991) (data not shown). To verify that pOK14 and pOK8 are authentic copies of a Met mRNA, we performed S1 nuclease protection analyses on RNA isolated from various human cell lines. A 2 kb fragment derived from the 5′ end of the pOK8 clone and containing vector sequences (856 bp) was end-labeled (at position +1964 in Met; Figure 3a) and hybridized with RNA derived from cell lines shown to express the 8 kb and 7 kb Met mRNAs by Northern analysis (Figure 2a and data not shown). Following S1 nuclease digestion, two fragments were protected (Figure 3b), one of 1144 nt that corresponds to a Met transcript lacking exon 2 (see predicted fragments; Figure 3a), and one of 764 nt corresponding to a Met transcript containing exon 2 (see Figure 3a). Thus, the S1 nuclease protection assay demonstrated that an alternatively spliced Met transcript lacking exon 2 that corresponds to the cloned cDNAs pOK14 and pOK8 is detected in each cell line tested (764 nt protected fragment; Figure 3b).

To determine if a Met transcript lacking exon 2 is normally expressed in human tissues, we used RT–

PCR (reverse transcriptase-polymerase chain reaction) to identify Met transcripts that either contain or lack exon 2 in a variety of human tissues. Oligonucleotide primer A, located within exon 1 and primer C, located within exon 3, were chosen flanking exon 2 (Figure 4a). These would generate an RT-PCR product of 197 bp if exon 2 is not incorporated, whereas primer B (located within exon 2; see Figure 4a) and primer C generate an RT-PCR product of 225 bp, diagnostic for Met transcripts containing exon 2. All tissues exhibited a 197 bp RT-PCR product with primers A and C, indicating the existence of a Met transcript lacking exon 2 (Figure 4b). Moreover, the same tissues also express Met transcripts containing exon 2, as indicated by the presence of a 225 bp RT-PCR product with primers B and C (Figure 4c) or a 1411 bp RT-PCR product with primers A and C (data not shown). Thus, Met transcripts lacking exon 2 are expressed in all normal human tissues tested.

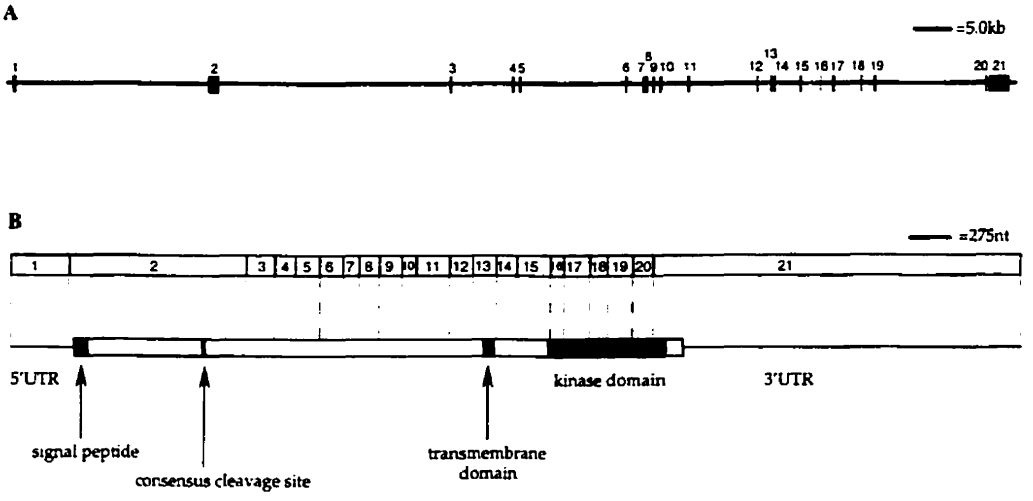

Figure 1 Met genomic structure. (**a**) The *MET* gene locus is drawn schematically and to scale. Exons are indicated by solid boxes and numbered above while introns are indicated by the horizontal line. (**b**) The cDNA that encodes the p190Met receptor product is shown and the protein domains (Park *et al.*, 1987) are correlated with the exon structure of the gene. Numbered boxes indicate the exons of *MET*. UTR = untranslated region; 'consensus cleavage site' represents the predicted site for cleavage of the Met receptor precursor to generate the α-chain (amino-terminal portion) and the β-chain (carboxy-terminal portion)

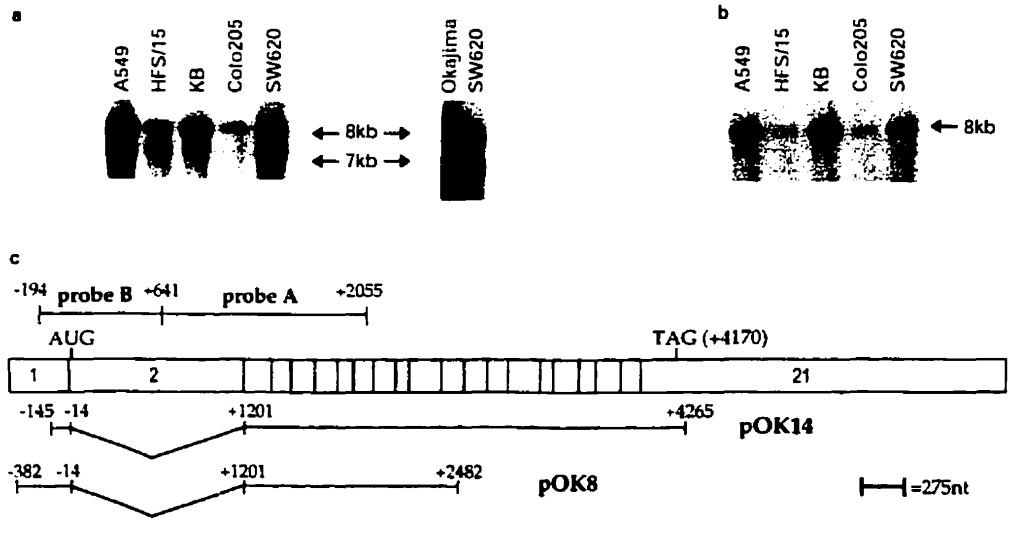

Figure 2 (**a**) Northern hybridization analysis of Met mRNA expression in various cell lines. 10 μg of poly(A)$^+$ selected RNA isolated from each cell line was probed with Met-specific probe A, isotopically labeled with α-^{32}P-dCTP. Two transcripts were detected, one corresponding to the previously characterized 8 kb Met mRNA that encodes the p190Met receptor, and another transcript of 7 kb. (**b**) Met-specific probe B was used to re-probe the Northern blot shown in (**a**). Only the 8 kb band is detected. (**c**) The positions and corresponding base numbers of Met-specific probes A and B are shown. Bases are numbered from the translation start site (+1) as defined by (Park *et al.*, 1987). Exons 1, 2 and 21 are numbered, and the positions of the AUG and stop codons utilized for the p190Met receptor are indicated. The structure of the cDNA clones pOK14 and pOK8 are diagrammatically represented below

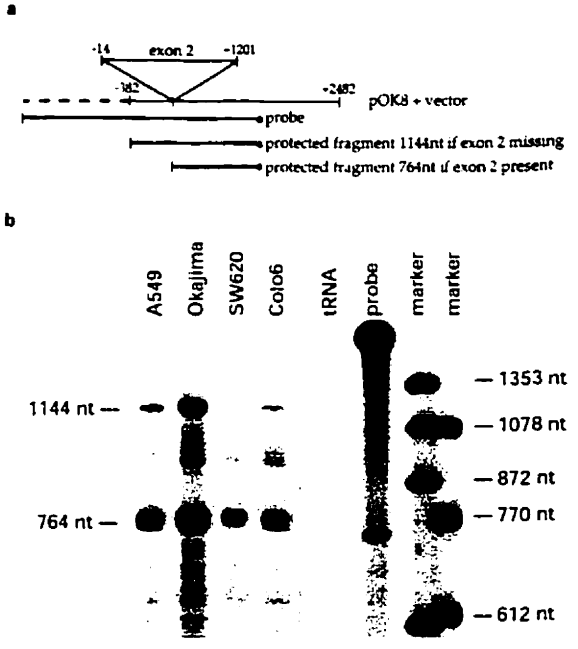

Figure 3 S1 Nuclease Assay. (**a**) A 2 kb ^{32}P-labeled antisense probe was generated from the plasmid containing pOK8 cDNA lacking exon 2 as described in Materials and methods. Vector sequences are indicated by the dashed line. This probe was hybridized to 40 μg of total RNA prepared from A549, Okajima, SW620 and Colo6 cells. (**b**) Following hybridization, samples were treated with S1 nuclease and then analysed on a 4% denaturing gel. ΦX DNA digested with *Hae*III and *Hin*cII serves as molecular weight markers. The sizes of these markers are indicated on the right. Protected fragments of 1144 nt, corresponding to the exon 2-lacking Met mRNA and 764 nt, corresponding to exon 2-containing mRNA are detected

The pOK14 and 8 cDNAs lack the AUG for the p190Met receptor product

The cDNA clones pOK14 and pOK8 lack exon 2, which contains the initiating methionine codon of p190Met. Therefore, although pOK14 cDNA contains the stop codon for the p190Met receptor product (Figure 2c), it would not be predicted to encode a full-length Met protein. To examine the ability of these cDNAs to encode a protein, the pOK8 clone, which contained an additional 237 nt at its 5' end, was fused to pOK14, and the entire cDNA (pOK8+14) was examined for potential open reading frames (ORFs) (data not shown). Only one ORF of any significant length was identified, and it is 2880 nt long (the second largest is 129 nt). This pOK8+14 ORF (2880 nt) is in frame with the ORF of the p190Met protein (Figure 5a). The first methionine codon, which is downstream from an in-frame stop codon (see Figure 5a), is located within exon 3 of *MET*, at position +1291, and would be predicted to encode a protein of 960 amino acids with a size of ~110 kDa. This protein would be identical to the p190Met protein in the carboxy-terminus, but would lack 430 amino acids at the amino-terminus. Such a protein should be recognized by an antibody raised to a peptide in the carboxy-terminus of p190Met (Ab144) (Rodrigues *et al*., 1991). To identify and characterize this putative product, we generated a construct containing pOK8+14 (Figure 5a) in the mammalian expression vector pXMT2. Expression plasmids containing the pOK1 Met cDNA, which encodes the p190Met receptor, or the pOK8+14 Met cDNA were transiently transfected into COS-1 monkey cells and proteins were immunoprecipitated with Ab144. Immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted with Ab144 (Figure 5b). The full-length pOK1 Met cDNA encoded high levels of Met proteins of predicted sizes; the Met receptor precursor (170 kDa) and the cleaved Met β-chain (145 kDa) (Figure 5b, lane 2). However, the pOK8+14 construct expressed low levels (<5% of wild type) of multiple truncated proteins of 100-110 kDa (Figure 5b, lane 3). These data suggest that this construct is either inefficiently translated or that its protein product is highly unstable. In support of these observations, when Met proteins were immunoprecipitated from cell lines which express Met transcripts containing exon 2 (8 kb) and lacking exon 2 (7 kb) (see Figures 2a and 3b) and immunoblotted with Ab144, the 145 kDa Met β-chain and 170 kDa Met precursor proteins can be readily detected, whereas Met proteins of ~110 kDa were not readily detected (Figure 5c). These data suggest that the 7 kb exon 2-skipped Met mRNA, although ubiquitously expressed, fails to give rise to a detectable protein product *in vivo*.

Discussion

To facilitate a search for mutations or alterations that activate or inactivate *MET*, we have obtained the intron-exon structure of the human *MET* gene. We present the genomic structure for the first member of the Met receptor tyrosine kinase family to be characterized. Moreover, we report extensive exon-skipping in human Met mRNA in normal tissue and tumor cell lines, generating a transcript that lacks the large internal coding exon 2.

The human *MET* gene consists of 21 exons distributed over ~130 kb of genomic DNA (Figure 1). The exon nucleotide sequence obtained by the sequencing of genomic DNA matched that of previously sequenced cDNAs corresponding to the 8 kb Met mRNA (Park *et al*., 1987; Rodrigues *et al*., 1991). All *MET* exons were found to be flanked by the canonical consensus splice donor and acceptor sites; AG at the 3' splice site, and GT at the 5' splice site (Table 1) (Breathnach and Chambon, 1981). As a consequence of the intron-exon distribution, the extracellular and transmembrane portion of the p190Met receptor is encoded by exons 2-13 which span ~100 kb of genomic DNA, whereas the cytoplasmic portion containing the catalytic kinase domain is encoded by exons 14-21 that span ~30 kb (Figure 1).

Interestingly, missense mutations, predicted to cause constitutive activation of the Met kinase in the absence of ligand, have recently been identified in *MET* in patients with hereditary and spontaneous papillary renal carcinoma, suggesting that constitutive activation of Met contributes to this disease (Schmidt *et al*., 1997). The multiple independent mutations thought to activate Met in papillary renal carcinomas are localized to the cytoplasmic kinase domain in exons 17, 18 and 19, and include three which are similar to mutations

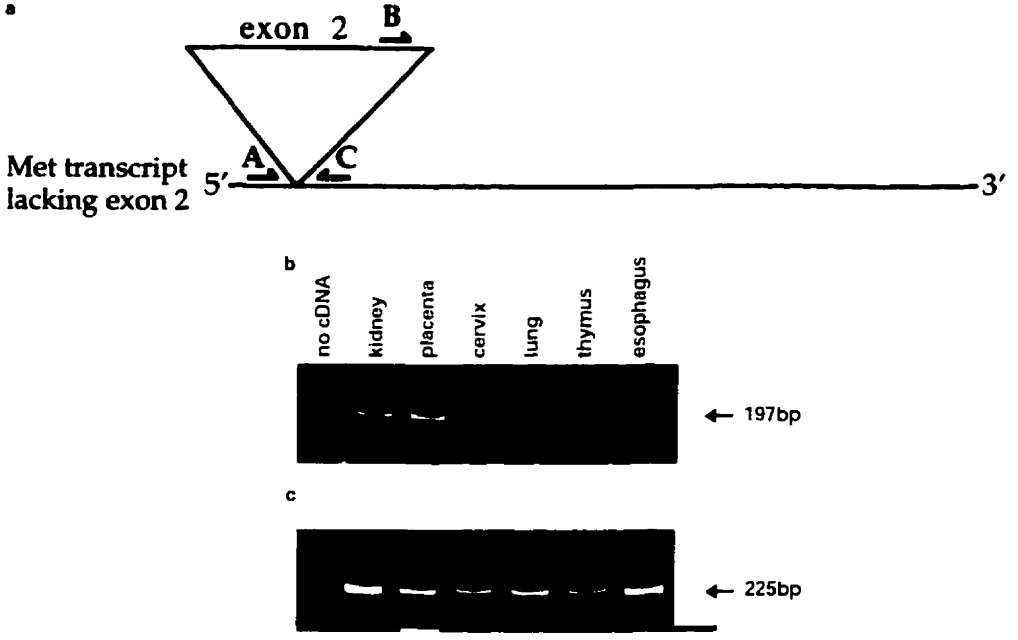

Figure 4 RT-PCR analysis. RNA from various human tissues was used for first-strand cDNA synthesis and subjected to PCR analysis for the presence of exon 2-containing and exon 2-lacking Met messages. (**a**) The location of the oligonucleotide primers A, B and C, are indicated relative to a Met transcript lacking exon 2. (**b**) Primer A, which hybridizes to *MET* −44 to −24 in exon 1, and primer C, which hybridizes to *MET* +1367 to +1345 in exon 3, were used to amplify a 197 bp fragment corresponding to exon 2-lacking Met transcripts. A 197 bp PCR product was detected in all tissues studied. (**c**) Primer B, which hybridizes to *MET* +1142 to +1162 in exon 2, and primer C were used to amplify a 225 bp fragment corresponding to exon 2-containing Met transcripts. A 225 bp PCR product was detected in all tissues studied

that activate the Ret and Kit receptor tyrosine kinases (Schmidt *et al*., 1997). However, mutations that activate receptor tyrosine kinases generally fall into two classes: those localized to the extracellular or transmembrane domain that promote receptor dimerization/oligomerization, and those localized to the kinase domain that alter catalytic activity or substrate specificity (Rodrigues and Park, 1994). Thus, the intron-exon structure of *MET* presented here will now allow a critical evaluation of mutations in the entire Met receptor in other human cancers.

MET is unique among the receptor tyrosine kinases whose genomic structure has been determined to date, due to the presence of a large internal coding exon, exon 2. Exon 2 is absent in Met cDNA clones pOK14 and pOK8 (Figure 2 and data not shown). The structure of these clones is consistent with that of a ubiquitously expressed 7 kb Met mRNA. By Northern hybridization analyses, the 7 kb Met mRNA fails to hybridize with a probe derived mainly from exon 2 (Figure 2b). Moreover, S1 nuclease analyses demonstrate that Met cDNA clones pOK14 and pOK8 correspond to an authentic Met mRNA in human cell lines expressing the 7 kb Met transcript (Figure 3b). All of the Met cDNAs that have been characterized to date (Park *et al*., 1987; Rodrigues *et al*., 1991), including pOK14 and pOK8, share 5'UTR sequences, suggesting that they are transcribed from the same promotor (Gambarotta *et al*., 1994). Therefore, we conclude that the 7 kb and 8 kb Met mRNAs are derived from the same pre-mRNA by alternative splicing of exon 2.

The pOK8 and pOK14 Met cDNA clones lack the initiating methionine codon of p190Met which is present in exon 2. From sequence analysis, the pOK8+14 cDNA contains only one ORF, which would initiate at an AUG in exon 3 (position +1291 in Met), and would correspond to the ORF for the p190Met receptor (Figure 5a). Although by Northern blot analysis the 7 kb Met isoform is expressed in relative abundance in many human cell lines (Figure 2a), and S1 nuclease protection assays demonstrate that a mRNA corresponding to pOK14 or pOK8 is expressed (Figure 3b), a protein product predicted from this message was not readily detected in these cell lines (Figure 5c). Similarly, the expression of pOK8+14 cDNA in COS-1 cells in transient transfection assays yielded extremely low levels of truncated Met proteins of ~100-110 kDa when compared with cDNA encoding the p190Met product (Figure 5b). This suggests that either the pOK8+14 cDNA is inefficiently translated, or alternatively, that a protein product translated from this mRNA is rapidly degraded. Consistent with the former, the first AUG codon of the pOK8+14 transcript (TTATTCAUGG) is in a poor Kozak consensus for initiation of translation and most importantly, lacks an adenine at position −3 (Kozak, 1991). This suggests that the multiple products observed are initiated from downstream in-frame AUGs.

Work from many laboratories has shown that alternative splicing is determined by a balance of several pre-mRNA features, including splice site strength, exon size, presence of splicing enhancers or

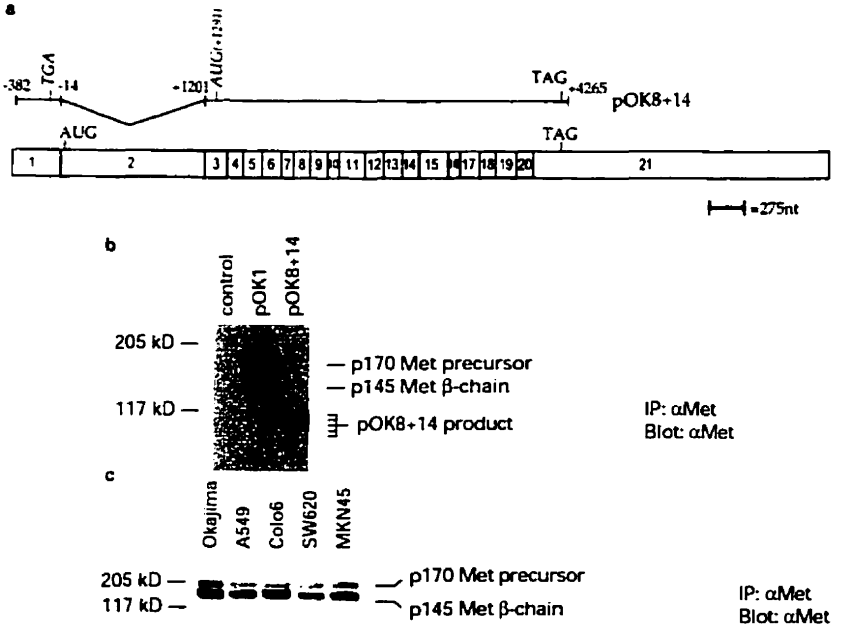

Figure 5 (a) The structure of the pOK8 + 14 cDNa construct is shown, relative to the transcript encoding the p190Met receptor protein. The AUG and stop codons utilized by the p190Met protein are indicated in horizontal letters. The presence of an in-frame stop codon in the pOK8 + 14 5'UTR and the first AUG following this stop codon are indicated by vertical, italic letters. (b) Expression of pOK1 Met cDNA and pOK8 + 14 Met cDNA in COS-1 cells. COS-1 cells were transfected with 4 μg of pXMT2 vector alone (control) or pXMT2 vector containing pOK1 Met cDNA encoding p190Met, or pOK8 + 14 Met cDNA. Met proteins were immunoprecipitated from the COS-1 cell lysates using anti-Met antisera (Ab144). The same antisera was used for blotting. The p170Met precursor, p145Met β-chain and truncated ~p110Met products are indicated by arrows. The positions of molecular weight markers are indicated at the left. (c) Western blot of Met proteins in cell lines. Met proteins were immunoprecipitated from 500 μg of total cell lysate from Okajima, A549, Colo6, SW620 and MKN45 cells using anti-Met antisera (Ab144). The same antisera was used for blotting

repressors and secondary structure (reviewed in Chabot, 1996; Manley and Tacke, 1996). Several receptor tyrosine kinases undergo alternative splicing which generates proteins with similar or distinct structures and activities (Clary and Reichardt, 1994; Piao *et al.*, 1994). For example, alternative splicing in mouse of the conserved *met* exon 14 generates a protein which shows altered substrate specificity (Lee and Yamada, 1995). However, the alternatively spliced 7 kb Met mRNA presented here is unique in that it fails to synthesize a detectable protein product *in vivo*. This was unexpected since the 7 kb Met mRNA is the second most abundant Met mRNA and is ubiquitously expressed.

The inclusion of differentially-spliced exons is a highly regulated event, thought to be dependent on both enhancer sequences localized within either the exon or intron sequences, and the proteins that recognize them (reviewed in Chabot, 1996; Manley and Tacke, 1996). However, since large internal exons are uncommon in vertebrate genes, with less than 1% of all primate internal exons studied being larger than 400 nt (Berget, 1995), the mechanisms that regulate their splicing are poorly understood. In experimental systems, the artificial expansion of internal exons to more than 500 nt results in constitutive exon-skipping when flanked by large introns (>1500 nt) (Sterner *et al.*, 1996). Thus, this would predict that exon 2 of *MET* (1214 nt), which is significantly larger than the average vertebrate exon (134 nt long) and is flanked by two large introns (26 kb and 31 kb; see Table 1), may be inefficiently included in mature Met mRNA. However, although the sequences or factors responsible for the observed alternative splicing of *MET* exon 2 are not understood, the inclusion of this exon in the 8 kb Met mRNA demonstrates that its presence is selected for *in vivo*. Moreover, the abundance of the 7 kb Met mRNA varies in tumor cell lines in culture (Figure 2a and data not shown). Since exon-skipping of *MET* exon 2 is predicted to decrease the amount of mature mRNA encoding a functional Met receptor, in some circumstances, Met pre-mRNA splicing may represent a mechanism of regulating the amount of a functional Met product. Therefore, a further study of the prevalence of exon 2-skipping of *MET* during development and in human tumors is important, and will increase our understanding of the mechanisms of Met regulation *in vivo*.

Materials and methods

Isolation of genomic clones

A human genomic placental DNA library cloned in the bacteriophage vector λDASH 1 (Stratagene) (Rodrigues *et al.*, 1991) was screened with a 4.6 kb cDNA probe (pOK1) containing the complete coding sequence of the *MET* gene (−195 to +4356) (Rodrigues *et al.*, 1991). Positive clones were selected, purified and amplified in LE392 bacteria as described previously (Cooper *et al.*, 1984). The Lawrence

Livermore National Laboratory chromosome 7-specific cosmid library was screened with a *MET* cDNA probe corresponding to *MET* +3081 to +4406 to identify overlapping cosmid clones containing genomic DNA sequences not isolated from the phage library (obtained from Dr SW Scherer). DNA from each phage or cosmid clone was isolated as described in Maniatis *et al.* (1992), and used in cycle sequencing reactions to determine intron-exon boundaries of *MET*.

Cycle sequencing of phage and cosmid DNA

The appropriate purified genomic DNA from phage or cosmid clones (500 ng) was used in cycle sequencing reactions with Met-specific primers to determine the intron-exon junctions and flanking intron sequences. These reactions were carried out using the GibcoBRL dsDNA Cycle Sequencing System (GibcoBRL, Burlington, Ontario) according to the instructions included in the kit. The sequences of the primers used for this purpose are available from the authors.

PCR to estimate intron sizes

To estimate the sizes of the introns of the *MET* gene, appropriate Met-specific oligonucleotide primers were generated and used in PCR reactions to amplify each individual intron. PCR for introns up to 4 kb in size was carried out in a final volume of 50 μL, containing 100 ng genomic DNA prepared from one of two different cell lines (HeLa and Okajima), 1.5 mM $MgCl_2$, 5 μL standard 10 × PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 50 pmol of each primer, 1.25 mM dNTPs and 2.5 units of Taq polymerase (GibcoBRL, Burlington, Ontario). An initial step of 3 min at 85°C was followed by 35 cycles of 40 s denaturation at 94°C, 30 s of annealing at 55°C, and 40 s of extension at 72°C, followed by a final extension step of 2 min at 72°C. Introns between 2 kb and 6 kb were amplified in conditions as described above, with the addition of 2.5 units of Taq extender PCR additive (Stratagene) and substituting the Taq extender buffer for the regular PCR buffer. Introns larger than 6 kb were amplified using the Expand Long Template PCR System (Boehringer Mannheim) according to the instructions included in the kit. PCR products were analysed by electrophoresis of 1 10th of the reaction on a 1% agarose gel. The size of each intron was estimated by comparison of the PCR product with molecular size markers. Each intron was amplified at least twice from two different cell lines. The sizes of all introns except 1 and 2 were estimated in this manner. The sizes of introns 1 and 2 were confirmed from analysis of genomic DNA sequences of the appropriate portion of chromosome 7, available at the Washington University Human Genome Sequencing Project's World Wide Web site (http://genome.wustl.edu/gsc/gschmpg.html). The appropriate contig is H_RG253B13, which covers *MET* genomic DNA from exon 1 to exon 9. The estimated sizes of introns 3 to 8, obtained by PCR as described above, corresponded to the sizes as determined from analysis of this contig.

RNA analysis

Total RNA was isolated from various human cell lines by the method of Chomczynski and Sacchi (1987). Poly(A)$^+$ RNA was twice selected on oligo(dT)-cellulose. For Northern blot analysis, poly(A)$^+$ RNA was denaturated with formamide and separated on a denaturing formamide-formaldehyde agarose gel as described previously (Park *et al.*, 1986). Filters were hybridized for 48 h at 42°C with 2×10^6 c.p.m./ml of probe in hybridization buffer (6 × SSC, 50% formamide, 5 × Denhardt, 1% SDS, 0.2 mg/ml salmon sperm DNA) and washed in 2 × SSC−0.1% SDS at room temperature for 30 min, then three times in 0.2 × SSC−0.1% SDS at 65°C (30 min each wash). Two *MET* derived probes were used. Probe A is derived from an *Eco*RV-*Eco*RI fragment (position +641 to +2055). Probe B consists of an *Eco*RI-*Eco*RV fragment located in position −194 to +641 in the published sequence (Park *et al.*, 1987). Both probes were labeled with α-^{32}P-dCTP by random primer labeling (Feinberg and Vogelstein, 1983).

Isolation of Met cDNA clones

A cDNA library was constructed from twice Poly(A)$^+$ selected RNA isolated from a gastric carcinoma cell line, Okajima (Rodrigues *et al.*, 1991). The cDNA was synthesized following the method of Gubler and Hoffman (1983), semi-*Xho*I synthetic adaptors were added, and the cDNA was cloned into the *Xho*I site of the mammalian expression vector pXMT2. Approximately 10^6 colonies were screened using probes derived from Met cDNA (probe A, probe B as described above). Positive clones were purified, and the longest clones were selected for sequencing using the dideoxynucleotide chain termination method with [α-^{35}S]dATP (ICN Radiochemicals) and Sequenase (USB) as specified by the manufacturers. Clones pOK14 and pOK8 were selected as putative representative cDNAs for the 7 kb Met mRNA, and were sequenced entirely by a combination of ExoIII-S1 nuclease deletion method and synthetic oligonucleotide-directed sequencing of subclones in pBluescript II KS$^-$ (pBS). PC/Gene (IntelliGenetics Inc.) and MacVector (Oxford Molecular Group) software was used for data analysis.

S1 Nuclease protection analysis

A *Bam*HI-*Pvu*II fragment isolated from the 5' end of pOK8 was end-labeled at the *Bam*HI site (position +1964 in *MET*) with T4 DNA polymerase using standard protocols (Maniatis *et al.*, 1992). The labeled *Bam*HI-*Pvu*II 2.0 kb fragment was purified on a low melting agarose gel by extraction with phenol. The probe (25 000 c.p.m.) was hybridized to 50 μg total cellular RNA in 50 μl of 80% formamide, 400 mM NaCl, 40 mM PIPES (pH 6.7) and 1 mM EDTA. Samples were denatured at 85°C for 10 min and incubated at 45°C overnight. 450 μl of S1 digestion buffer (30 mM NaOAc pH 4.6, 50 mM NaCl, 1 mM $ZnSO_4$, 0.5% glycerol and 1000 U/ml of S1 nuclease) were added and the samples were incubated at 25°C for 45 min, then transferred to 37°C for 15 min. After addition of 20 μg yeast t-RNA, samples were ethanol precipitated, washed and resuspended in 80% formamide loading dye, heated for 10 min at 95°C, chilled on ice, and loaded onto a 4% denaturing acrylamide:urea gel.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was performed on single stranded cDNA. Briefly, total RNA was extracted from biopsies as described above. First-strand cDNA was synthesized from 4 μg total RNA using the Expand Reverse Transcriptase system (Boehringer Mannheim) with 50 pmol oligo $(dT)_{15}$ $_{18}$ primer (Pharmacia), according to the instructions provided. Three μL of the RT reaction was used for PCR in a final volume of 50 μL, containing 1.5 mM $MgCl_2$, 5 μL standard 10 × PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 50 pmol of each primer as indicated in Figure 5, 1.25 mM dNTPs, and 2.5 units of Taq polymerase (GibcoBRL, Burlington, Ontario). An initial step of 3 min at 85°C was followed by 35 cycles of 40 s of denaturation at 94°C, 30 s of annealing at 55°C, and 40 s of extension at 72°C, followed by a final extension step of 2 min at 72°C. PCR products were analysed by electrophoresis in a 1%

agarose gel. The primers used were: A = GACTTCTCCACTGGTTCCTGG, which hybridizes to *MET* −44 to −24; B = ACAATGTGAGATGTCTCCAGC, which hybridizes to *MET* +1142 to +1162; and C = CCAAGATTAGCTATGGTGAGGTC, which hybridizes to *MET* +1367 to +1345.

Cell lines and transfections

The Okajima and MKN45 cell lines, derived from poorly differentiated gastric carcinoma (Motoyama *et al.*, 1979), were obtained from Dr GF Vande Woude. The A549 cell line is derived from a lung carcinoma, SW620, Colo6 and Colo205 from colon carcinomas, HFS/15 from human foreskin fibroblasts, and KB from an epidermoid carcinoma. All lines were obtained from American Type Culture Collection. Cell lines were maintained in Dulbecco's modified Eagle serum (DMEM) or RPMI medium, supplemented with 10% fetal bovine serum (Flow laboratories). Transient transfections into COS-1 cells were performed as described previously in (Rodrigues *et al.*, 1991).

Immunoprecipitation

Immunoprecipitations were carried out using a polyclonal anti-Met C-terminal peptide antibody (Ab144) (Rodrigues *et al.*, 1991). The immunoprecipitated proteins were collected on protein A-Sepharose (Pharmacia) and washed three times with RIPA buffer. The proteins were separated on 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with Ab144. Immune complexes were identified with horse radish peroxidase (HRP) conjugated Protein A and revealed by enhanced chemiluminescence (ECL) (Amersham).

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Chapter IV - Exon-skipping of *MET* exon 2 is a novel mechanism for the loss of expression of Met protein in breast cancer cells.

Preface

Many groups have attempted to elucidate the role of the Met receptor in human breast cancer. However, the findings have often been conflicting. Some data suggested that Met may function as a tumor suppressor gene in breast cancer, while others supported a role for Met as an invasion-promoting factor. In the next part of my thesis work, I turned to studying the status of the Met receptor in cell lines derived from human breast cancers, in an attempt to clarify the role(s) that Met might play in the development of breast cancer. While studying cell lines is not ideal to elicit this information, it is a model that can provide valuable insight into the situation *in situ*. Investigating human breast cancer is difficult due to the relative lack of banked specimens and their great demand by many investigators. Thus, in the fourth chapter of this thesis, I examined the status of the Met receptor in 13 breast cancer cell lines, in comparison with two immortalized breast epithelial cell lines.

Abstract

The *MET* gene is a transmembrane receptor tyrosine kinase, whose ligand is hepatocyte growth factor/scatter factor (HGF/SF). HGF/SF, by binding to the Met receptor, stimulates epithelial cell mitogenesis, motility and morphogenesis. Met and HGF/SF have been implicated in human mammary development and tumorigenesis. Several studies have suggested a role for *MET* as a dominant oncogene in breast cancer, while others have described frequent deletions of *MET* in breast cancer, suggesting a tumour suppressor function. In this paper, we describe the status of the Met receptor in thirteen human breast cancer cell lines, which may reflect the role Met plays in the development of this disease *in vivo*. We demonstrate that in seven of these lines, there is decreased or no expression of the Met receptor protein, as compared with levels in two immortalized human breast epithelial cell lines. Furthermore, in these seven lines, there is altered expression of Met mRNA, such that there is relatively increased expression of an alternatively-spliced 7kb Met mRNA which has no protein product. This alteration in Met mRNA expression is not due to mutation within the affected exon of *MET* (exon 2) or in its donor or acceptor splice sites. Rather, we demonstrate that this alteration in the normal splicing pattern of Met is correlated with a change in the expression pattern of a family of proteins responsible for regulating alternative splicing, the SR proteins. We propose that exon-skipping of MET exon 2, common in breast cancer cell lines, may be a mechanism of inactivation of Met in tumours *in situ*.

Introduction

It is generally accepted that cancer develops with the step-wise accumulation of genetic abnormalities within a cell or cells, leading to the dysregulation of growth and differentiation in these cells. Such genetic abnormalities affect two classes of genes; oncogenes, which contribute to oncogenesis if inappropriately activated, and tumour suppressor genes, which contribute to oncogenesis when inactivated.

The *MET* gene has been implicated in oncogenesis. It encodes a transmembrane protein receptor tyrosine kinase (Park et al., 1987) whose ligand is hepatocyte growth factor/scatter factor (HGF/SF) (Bottaro et al., 1991; Naldini et al., 1991). The Met receptor is predominately expressed in epithelial cells, while HGF/SF, a soluble cytokine, is produced primarily by mesenchymal cells. Met-HGF/SF signalling has been postulated to play a role in morphogenetic epithelial-mesenchymal interactions, and HGF/SF can stimulate the growth, movement and tubulogenesis of glandular epithelial cells in culture (reviewed in Rosen et al., 1994). HGF/SF can induce the ductal growth of mouse mammary explants in culture (Yang et al., 1995), and Met and HGF/SF play important roles in embryonic development. Specifically, Met-HGF/SF signalling is essential for the development of placenta, liver, skeletal muscle and for growth of axons (Ebens et al., 1996; Schmidt et al., 1995; Takayama et al., 1996; Uehara et al., 1995; Yang et al., 1996).

Met was first identified as a rearranged oncogene (*TPR-MET*) in which the tyrosine kinase portion is constitutively activated and causes the transformation of fibroblasts *in vitro* (Park et al., 1986). Met is overexpressed and/or deregulated in many types of human tumors

including gastric, colorectal, thyroid and ovarian carcinomas, leukemia, and sarcomas (Di Renzo et al., 1992; Di Renzo et al., 1994; Jucker et al., 1994; Liu et al., 1992; Rong et al., 1995; Rong et al., 1993; Scotlandi et al., 1996; Yonemura et al., 1996). Activating mutations have been described in *MET* in patients with hereditary and spontaneous papillary renal carcinoma, suggesting that constitutive activation of Met contributes to this disease (Schmidt et al., 1997). In other cases, constitutive activation of Met has been correlated with the amplification and overexpression of a wild-type Met protein (Giordano et al., 1989).

In contrast, several studies suggest that *MET* may be a candidate for a tumor suppressor gene located at chromosome 7q31. *MET* is located on a 1000 kilobase (kb) region of chromosome 7q31 that is frequently deleted in breast cancer (Lin et al., 1996). Similar deletions have also been described for prostate, ovarian and renal cell carcinomas (Shridhar et al., 1997; Takahashi et al., 1995; Zenklusen et al., 1994; Zenklusen et al., 1995). Furthermore, while Met protein is expressed in all normal epithelial cells studied, several groups have reported variations in the expression pattern of Met protein in breast carcinoma cells (Beviglia et al., 1997; Byers et al., 1994; Di Renzo et al., 1991; Ghoussoub et al., 1998; Prat et al., 1991). These results suggest that Met may be inactivated in the development of certain breast cancers.

We have studied the status of the Met receptor in a series of thirteen human breast cancer cell lines and two immortalized breast epithelial cell lines. We show that there is decreased Met protein in seven of the thirteen (54%) breast cancer cell lines studied. This is correlated with the loss/decrease in expression of the full length Met mRNA and a

relative increase in amounts of an alternatively-spliced 7kb Met mRNA which encodes no protein product. We propose that increased exon-skipping of *MET* exon 2, and a relative increase in the expression of an alternatively-spliced 7kb Met mRNA with no functional protein product may be a mechanism for the inactivation of Met in breast cancer cells.

Results

Met protein expression is lost in many breast cancer cell lines.

Variable expression levels of Met protein, ranging from no detectable protein to over-expression, have been reported in breast carcinomas and cell lines (Beviglia et al., 1997; Byers et al., 1994; Di Renzo et al., 1991; Ghoussoub et al., 1998; Prat et al., 1991). Notably, high levels of Met protein is associated with more aggressive disease (Camp et al., 1999; Ghoussoub et al., 1998). Since normal human breast epithelial cells express Met protein (Ghoussoub et al., 1998; Lin et al., 1996), this data has been interpreted to suggest that Met expression is lost, then regained in the progression of breast cancer (Ghoussoub et al., 1998). To investigate this further, we used a panel of thirteen breast cancer cell lines and two immortalized breast epithelial cell lines to study the expression pattern of Met receptor protein. Using an antibody directed to the carboxy-terminal portion of Met (Ab144), Met protein was immunoprecipitated from 500μg of total protein from each cell line. Immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted with Ab144 (Figure 1). The Met β-chain of 145kDa is detected at comparable levels in both immortalized breast epithelial cell lines, MCF 10A and MCF 12A. However, the level of p145Met is variable in the breast cancer cell lines, and is decreased or lost compared to the levels of p145Met in immortalized breast epithelial cell lines in seven of these; BT474, T47D, MCF7, MDA468, SKBr3, ZR75-1, and MDA157 (Figure 1). In all Met-expressing cell lines, p145Met became phosphorylated on tyrosine residues upon stimulation with HGF/SF, indicating that the Met receptor expressed in these cell lines was activated by HGF/SF ligand-binding in a wild-type

manner (data not shown).

Loss of Met protein correlates with loss or decrease of the 8kb full-length Met mRNA and relative increase of the exon 2-skipped 7kb Met mRNA.

To determine if the difference in expression levels of p145Met in breast cancer cell lines correlates with changes at the mRNA level, Met mRNAs were examined by Northern hybridization. When full length Met cDNA was used to probe a Northern transfer containing total RNA from the breast cancer cell lines, two isoforms of Met mRNA can be detected. One corresponds to the 8kb full-length Met mRNA and the other a 7kb alternatively-spliced isoform (see representative examples in Figure 2a). We have shown that the 7kb Met mRNA results from exon-skipping of Met exon 2. Exon 2 contains the AUG translation start site for the full-length Met receptor protein and the 7kb Met mRNA fails to give rise to a protein product (Lin et al., 1998).

Cell lines with decreased levels of p145Met, such as BT474, SKBr3, MCF7 and T47D (Figure 1) also demonstrate a decrease in the amount of full-length 8kb Met mRNA (Figure 2a). However, these cell lines express relatively elevated levels of the 7kb Met mRNA isoform, resulting in an alteration in the normal ratio between 8kb and 7kb Met mRNAs. Thus, decreased levels of p145Met receptor in certain breast cancer cell lines is associated with decreased expression of the full-length 8kb Met mRNA and a relative increase in amounts of an alternatively-spliced 7kb message.

To confirm that the 7kb mRNA observed in these lines is identical to the exon 2-skipped Met mRNA isoform previously identified (Lin et al., 1998), RNAse mapping was performed using a riboprobe (see Figure 2c)

spanning the exon 2-exon 3 junction of full-length Met mRNA. A full-length protected fragment of 227nt corresponds with expression of a correctly-spliced 8kb Met mRNA, while an exon 2-skipped 7kb Met mRNA would yield a protected fragment of 166nt (Figure 2c). These studies confirmed the presence of an exon 2-skipped Met mRNA isoform in the cell lines studied (Figure 2b). Furthermore, the cell lines which had decreased or no Met protein expression by Western blot analysis (see Figure 1) had relatively higher levels of the exon-2 skipped Met mRNA than the lines with normal levels of Met protein (Figure 2b), in agreement with the results from the Northern blot analysis.

Alteration of the ratio of alternatively-spliced Met mRNA isoforms is not correlated with any mutation in MET exon 2 or at the exon 2 splice junctions.

Increased exon-skipping in tumorigenesis has been reported for other genes, including tumor suppressor *IRF-1* in human myelodysplasia/leukemia (Harada et al., 1994), *p53* in breast cancer (Voglino et al., 1997), and *E-cadherin* in gastric carcinoma (Becker et al., 1993). In some cases, increased exon-skipping is associated with mutations, either within the exon or at the donor or acceptor splice sites. To determine if increased exon-skipping of *MET* exon 2 is associated with any mutations, exon 2 was PCR-amplified from genomic DNA prepared from each cell line. Sequence of *MET* exon 2 along with additional flanking intronic sequences (20nt of 5' and 3' intron) in all the breast lines studied failed to reveal any mutations (data not shown). Sequencing of *MET* exon 2 in these cell lines also failed to reveal the presence of any

polymorphisms.

Breast carcinoma cell lines demonstrate an altered expression profile of SR splicing proteins when compared with normal breast epithelial cells.

The SR protein family consists of several serine-argenine-rich splicing factors important in the regulation of alternative splicing (reviewed in Chabot, 1996). Alterations in the expression of SR splicing proteins has been reported in the progression of mouse mammary tumorigenesis. This has been correlated with changes in the alternative splicing of CD44, the major receptor for hyaluronic acid (Stickeler et al., 1999). To determine if a similar alteration in the expression profile of SR proteins exists in the human breast cancer cell lines studied, mAb104, which recognizes multiple members of the SR protein family, was used to immunoprecipatate SR proteins from 500μg of total protein of each cell line. Proteins were subjected to SDS-PAGE and immunoblotted with the same antibody (Figure 3). As compared with human mammary epithelial cells (HMEC) and immortalized breast epithelial cell lines, the seven breast cancer cell lines with decreased or no Met protein show variable changes in the expression pattern of SR proteins (Figure 3). Specifically, the cell lines with decreased levels of the 8kb Met mRNA and corresponding increase in levels of the 7kb Met mRNA demonstrate slightly higher levels of SRp20 than normal (HMEC) or immortalized breast epithelial cells, in agreement with (Stickeler et al., 1999). Furthermore, these changes in the expression profile of SR proteins is not present in the cell lines with normal Met expression.

Discussion

Receptor tyrosine kinases (RTKs) play a prominent role in human tumorigenesis. Often, these RTKs function as oncogenes, and are activated in human cancer, either through overexpression or gene mutation (Wilks, 1993). However, in certain cases, RTKs can also contribute to human disease through inactivation. For example, the Ret receptor tyrosine kinase is activated in the cancer syndrome MEN2b, but when inactivated, leads to the development of Hirschsprung's disease (reviewed in Pasini et al., 1996). Similarly, the Met receptor tyrosine kinase has been implicated in diverse roles in human disease. It is overexpressed and constitutively activated in several cancer cell lines/types, and activating mutations in Met have been described in spontaneous and hereditary papillary thyroid cancer (Schmidt et al., 1997). However, other studies have suggested a tumor suppressor role for Met, in that it is frequently deleted in breast cancer (Deng et al., 1994; Lin et al., 1996) and other types of carcinoma (Kuniyasu et al., 1994; Takahashi et al., 1995; Zenklusen et al., 1995). One study found that a deletion in *MET* was associated with a more aggressive breast cancer (Bièche et al., 1992). Furthermore, Met-HGF/SF has been shown to be able to mediate growth arrest and differentation of a breast cancer cell line, T47D, supporting a tumor suppressor role for Met (Ronen et al., 1999).

In this paper, we examine the possibility that Met may be inactivated in certain human breast cancers. We demonstrate that Met protein expression (p145Met) is decreased or absent in seven of 13 human breast cancer cell lines (54%), which is in contrast to the high level of expression of Met in two immortalized breast epithelial cell lines (Figure

1). Met is also highly expressed in normal breast epithelium *in vivo*, as demonstrated by immunohistochemical studies (Lin et al., 1996). Varying levels of expression of Met protein in breast cancer cell lines and tumours has been reported by others (Beviglia et al., 1997; Byers et al., 1994; Di Renzo et al., 1991; Ghoussoub et al., 1998; Prat et al., 1991). Recently, this has been interpreted to suggest that Met expression may be lost early in breast tumorigenesis and regained later, an event which is associated with increased aggressivity of the cancer (Ghoussoub et al., 1998). This hypothesized two-stage involvement of Met in human breast tumorigenesis could explain the conflicting evidence that has been reported involving Met in breast cancer. In this paper, we suggest a mechanism for the loss of the Met receptor, which has been hypothesized to take place in the early stages of breast tumorigenesis.

We studied the expression pattern of Met mRNAs in an attempt to determine whether the alterations in protein expression of Met was correlated with changes in Met mRNA expression. In Northern blot studies, we confirm the presence of two Met mRNA species, one of 8kb, corresponding to the full-length Met mRNA, and the other a 7kb alternatively-spliced isoform lacking exon 2 (Figure 2a). This corresponds with the expression pattern of Met mRNA isoforms in other epithelial cell types (see Figure 2a) (Lin et al., 1998), and with the expression pattern of Met mRNA in breast epithelium as reported by other groups (Beviglia et al., 1997). Furthermore, by ribonuclease protection assays, we demonstrate that the decreased levels of the p145Met protein in certain breast cancer cell lines is associated with decreased levels of the full-length Met mRNA and an increase in the relative amount of the alternatively-spliced 7kb Met

mRNA which fails to express a protein product (Figure 2b).

The arginine-serine-rich (SR) splicing factors are a family of essential splicing proteins which are involved in alternative splicing through their recognition of splice sites and splicing enhancers. They influence changes in alternative splicing when their relative protein concentrations are altered. Specific tissue types have distinct expression patterns of SR proteins, and it has been suggested that alterations in the levels of these proteins may be responsible for alterations in alternative splicing throughout development (reviewed in Manley and Tacke, 1996; Zahler et al., 1993). Recently, alterations in expression patterns of SR proteins has been described in mouse mammary tumorigenesis. These stage-specific alterations was found to be associated with changes in the alternative splicing of CD44, a cell adhesion molecule implicated in metastasis (Stickeler et al., 1999).

Since the observed changes in alternative splicing of *MET* did not correlate with mutations in the gene, we investigated the possibility that the altered expression pattern of the SR proteins reported in mouse mammary tumorigenesis might also exist in the human breast cancer cell lines studied. We observe that the SR splicing protein profile in these breast cancer cell lines is altered from that expressed in normal epithelial cell lines (Figure 3). This is in agreement with the previously described changes in mouse mammary tumorigenesis (Stickeler et al., 1999).

Exon-skipping in tumorigenesis has been reported in other genes, including *IRF-1* in human myelodysplasia and leukemia (Harada et al., 1994), and *E-cadherin* in gastric cancer (Becker et al., 1993). In some cases, this has been shown to result from gene mutations within the exon or at

the splice sites. However, in other cases, no mutation has been associated with the observed increase in exon-skipping. Met inactivation has been proposed to be an important development in human breast tumorigenesis (Ghoussoub et al., 1998). We have confirmed that Met expression is lost or diminished in a significant proportion of breast cancer cell lines (54%). We have also shown that loss of Met is correlated with changes in alternative splicing of the *MET* gene, which results in increased levels of exon 2-skipped 7kb Met mRNA. Furthermore, the increased exon-skipping of Met is not associated with any mutation in the affected exon or at its splice sites. We propose that the observed exon-skipping of *MET* is one mechanism of inactivation of Met in human breast cancer.

Recently, alterations in the expression pattern of SR proteins has been described in mouse mammary tumorigenesis (Stickeler et al., 1999). We propose that alterations in the SR protein expression pattern may result in changes in alternative-splicing of *MET* and therefore affect the expression of the Met receptor in human breast cancer cells. This may contribute to the development of tumorigenesis, without necessarily requiring mutations to the *MET* gene itself, hence providing a novel mechanism for gene inactivation.

Materials and methods

Cell lines.

All lines were obtained from American Type Culture Collection. Cell lines were maintained in the recommended culture medium.

Immunoprecipitation of Met and SR proteins and Western blotting.

Immunoprecipitations were carried out using a polyclonal anti-Met C-terminal peptide antibody (Ab144) (Rodrigues et al., 1991) or mAb104, which recognizes a shared arginine-serine motif of SR protein family members (Stickeler et al., 1999). The immunoprecipitated proteins were collected on protein A-Sepharose (Pharmacia) and washed three times with TBE buffer. The proteins were separated on 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the same antibody used in the immunoprecipitations. Immune complexes were identified with horse radish peroxidase (HRP) conjugated Protein A and revealed by enhanced chemiluminescence (ECL) (Amersham).

RNA Northern blot analysis.

Total RNA was isolated from the cell lines using Trizol reagent (Life Sciences, Inc.). For Northern blot analysis, 40µg total RNA was denaturated with formamide and separated on a 1.2% denaturing formamide-formaldehyde agarose gel as described previously (Park et al., 1986). Filters were hybridized for 48h at 42°C with 2 x 10^6 cpm/ml of probe in hybridization buffer (6X SSC, 50% formamide, 5X Denhardt, 1% SDS, 0.2 mg/ml salmon sperm DNA) and washed in 2X SSC-0.1% SDS at room temperature for 30 min, then three times in 0.2X SSC-0.1% SDS at 65°C

(30min each wash). Full-length Met cDNA probe was labeled with [α-^{32}P]-dCTP by random primer labeling (Feinberg and Vogelstein, 1983).

Ribonuclease protection assays.

The riboprobe (Figure 2c) was prepared by combining 1µg of template DNA; transcription buffer (200mM PIPES, 2M NaCl, 5mM EDTA); 10mM DTT; 40U RNasin RNAse inhibitor; 500mM ATP, CTP and GTP; 12mM UTP, 50µCi α-^{32}P-UTP and 70U T7 RNA polymerase (Pharmacia) and incubating for 1h at 37°C. After 1h, 500mM UTP was added and further incubated for 5min. The riboprobe was then treated with RNase-free DNase at 37°C for 15min and then extracted with chloropane and run through a Sephadex G50 spun column. Forty µg of total RNA was annealed to 8x10^5 cpm of labelled riboprobe at 48°C for 16h in a solution containing 80% formamide; 0.4M NaCl; 0.4M PIPES, pH 6.4; and 1mM EDTA. RNA-RNA hybrids were precipitated with 20µg of tRNA, 295µl of 4M guanidine thiocyanate and 590µl of isopropanol. Pellets were resuspended in formamide dye, denatured and electrophoresed on a 4% acrylamide-8M urea gel. Gels were dried and exposed at -80°C.

Cycle sequencing of cell line DNA.

The appropriate purified genomic DNA from each cell line (500ng) was used in cycle sequencing reactions with Met exon 2-specific primers to determine the exon 2 and flanking intron sequences. These reactions were carried out using the GibcoBRL dsDNA Cycle Sequencing System (GibcoBRL, Burlington, Ontario) according to the instructions included in the kit. The sequences of the primers are available from the authors.

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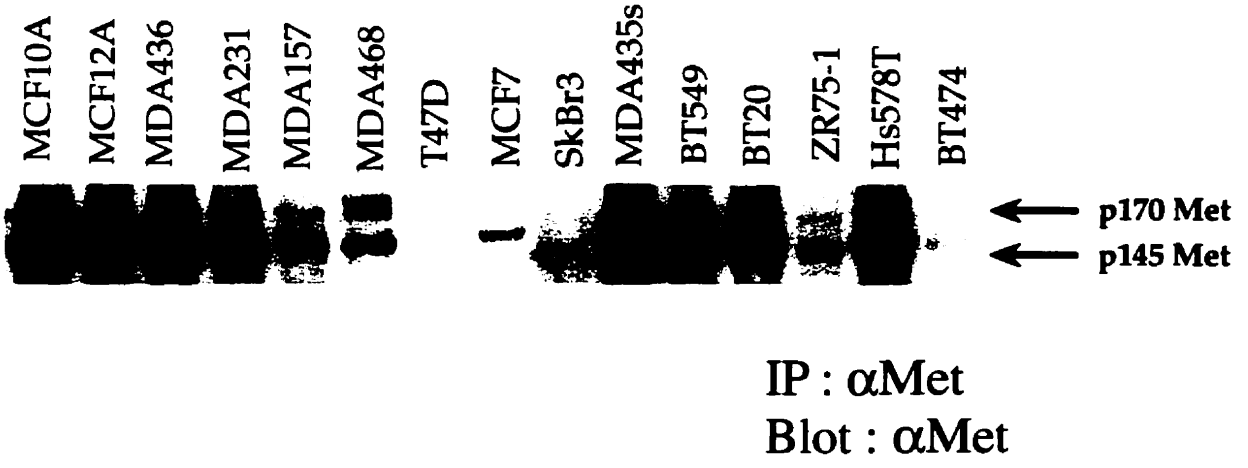

Figure 1: *Expression of Met protein in breast cancer cell lines.*
Met receptor expression was detected by Western blotting using an antibody directed against the carboxy-terminus of Met, Ab144. Varying levels of Met proteins p170 (Met precursor) and p145Met (Met β-chain) are detected in these lines.

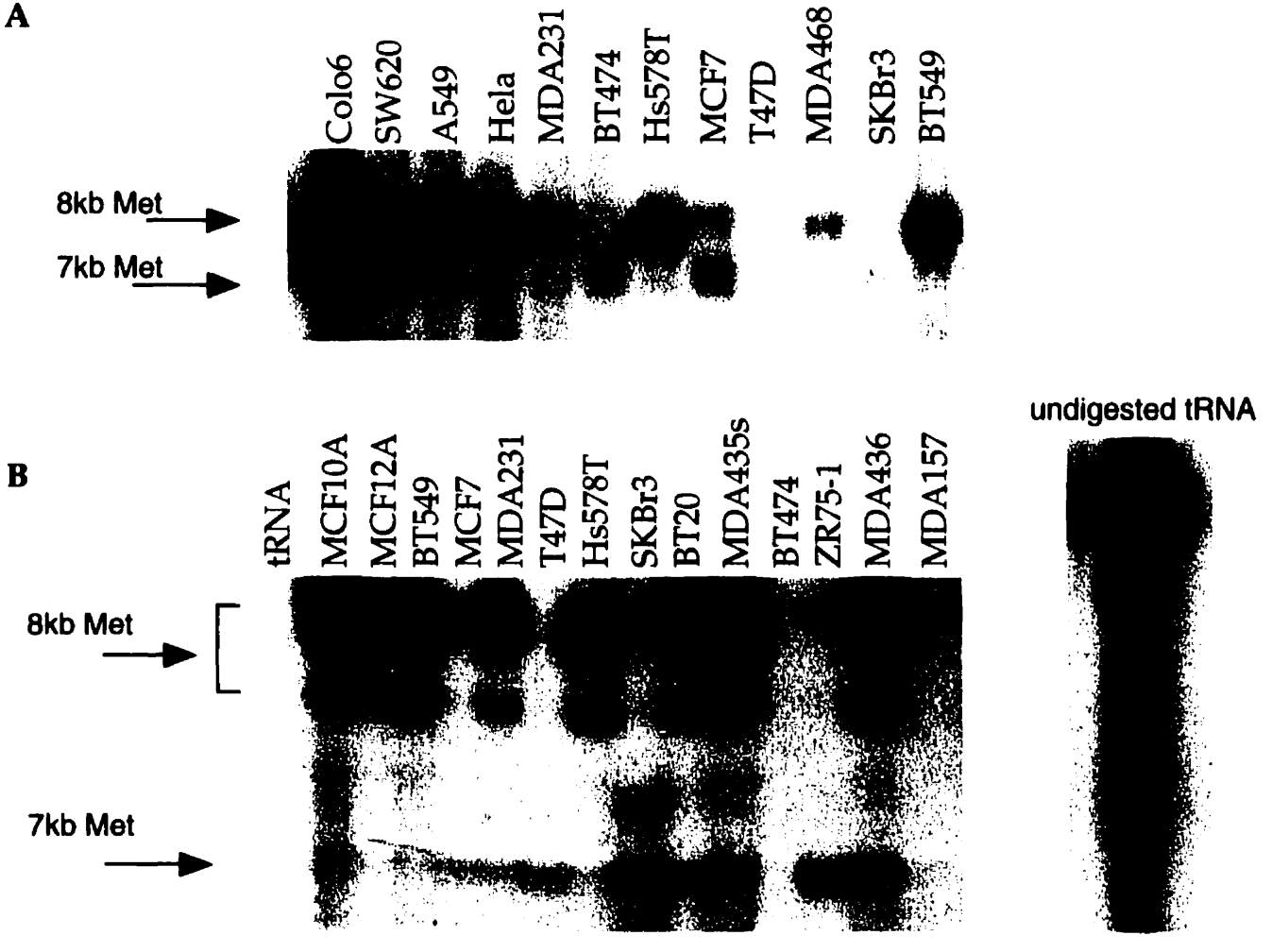

Figure 2 : *Expression of Met mRNA isoforms in human carcinoma cell lines.*

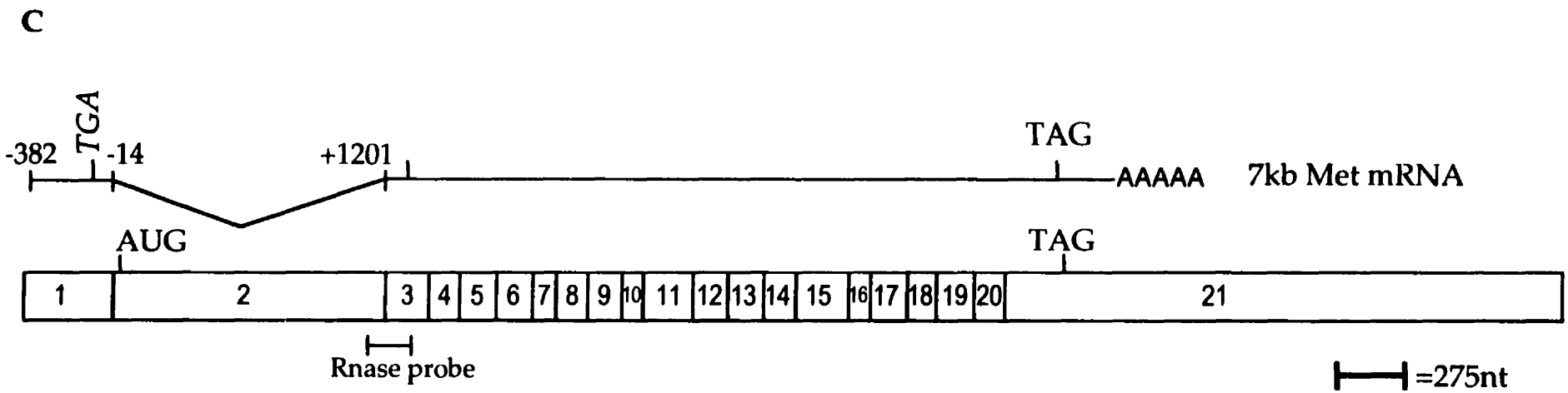

Figure 2 : *Expression of Met mRNA isoforms in human carcinoma cell lines.*
(a) Northern blotting for Met mRNA isoforms in carcinoma cell lines. Two Met mRNA isoforms, 8kb and 7kb, are detected in all carcinoma cells of non-breast origin (Colo6, Sw620, A549 and Hela). Variable levels of these mRNA species are seen in the breast carcinoma cell lines. (b) Ribonuclease protection assays. Protected fragments of 227nt, representing the 8kb Met isoform; and 166nt, representing the 7kb mRNA, are detected at variable levels in the breast carcinoma cell lines. (c) Structure of the 7kb exon 2-skipped Met mRNA isoform. Location of the ribonuclease probe used in (b) is indicated.

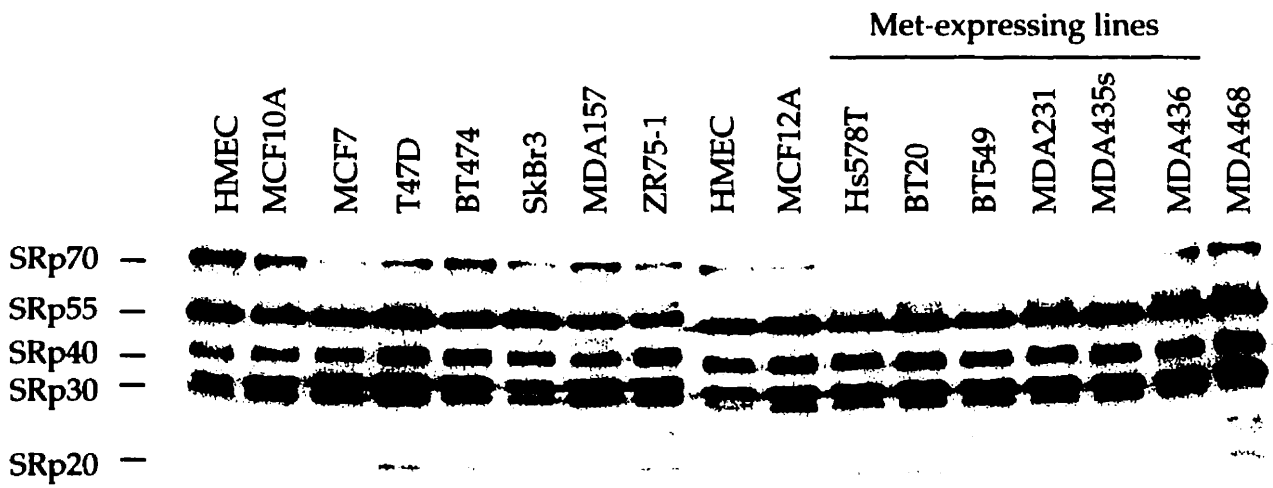

Figure 3: *Expression pattern of SR splicing proteins in breast cancer cell lines.* Western blotting of the SR protein family was carried out using mAB104, which recognizes multiple SR proteins (sizes indicated). Lines with normal to increased Met expression are indicated. Levels of SRp20 are increased in lines with decreased expression of Met, as compared with the HMEC (human mammary epithelial cells) and two immortalized breast epithelial cell lines, MCF10A and MCF12A.

Chapter V - General Discussion

I. Evidence suggesting a role for Met in breast cancer.

Within the past 10 years, several studies have suggested a potential role for the Met receptor and/or HGF/SF in the development or progression of human breast cancer. The evidence amassed has been conflicting, with several reports suggesting that Met may function as a tumor suppressor gene in breast cancer, and others reporting a role for Met as a factor promoting the aggressivity and invasiveness of breast cancer. These conflicting findings have not been fully resolved, and possibly reflect the dual or multiple, complex roles that the Met receptor actually plays in the development of breast cancer.

Bièche and his colleagues were the first to describe loss of heterozygosity (LOH) of *MET* in breast cancer, which was found to be correlated with a poorer prognosis (Bièche et al., 1992). Shortly afterwards, several independent studies reported LOH of loci near the *MET* gene in other carcinoma types, including gastric, colon, ovarian, prostate and head and neck cancers (Kuniyasu et al., 1994; Muller et al., 1995; Odai et al., 1995; Takahashi et al., 1995; Yang et al., 1996; Zenklusen et al., 1995; Zenklusen et al., 1994). These reports sparked an interest in the potential existence of a tumor suppressor gene at chromosome 7q31 which might function in many different carcinoma types. *MET* is a potential candidate for this tumor suppressor gene.

Met is a growth factor receptor tyrosine kinase that is expressed predominantly on epithelial cells, including breast epithelial cells (Di Renzo et al., 1991; Gonzatti-Haces et al., 1988; Lin et al., 1996; Prat et al.,

1991; Yang and Park, 1995) . HGF/SF, the ligand for Met, is a multifunctional soluble cytokine produced by mesenchymal cells, including breast stromal cells (Jin et al., 1997; Niranjan et al., 1995; Rahimi et al., 1994; Tuck et al., 1996). HGF/SF can stimulate mitosis and motility in epithelial cells. In addition, HGF/SF is a potent modulator of epithelial-mesenchymal transition, and induces the inherent morphogenic program of epithelial cells in three-dimensional matrix culture (Brinkmann et al., 1995; Montesano et al., 1991; Stoker et al., 1987; Weidner et al., 1990). Moreover, HGF/SF promotes branching tubulogenesis in several breast epithelial cell lines when grown in three-dimensional culture conditions, demonstrating that HGF/SF can induce differentiation in breast epithelium (Berdichevsky et al., 1994; Soriano et al., 1995). Met and HGF/SF expression varies during the developmental stages of the mammary gland of the rat (Pepper et al., 1995). HGF/SF also stimulates the outgrowth of branching ductules from mouse mammary gland explants (Yang et al., 1995). These results suggest that Met-HGF/SF interactions play a crucial role in breast development. Hence, loss of Met function at a key moment in this developmental process might destabilize epithelial structure, and predispose the affected breast epithelial cells to tumorigenesis.

II. The MET gene is deleted in human breast cancer.

The impetus for the work in this thesis was sparked by the findings of (Bièche et al., 1992), reporting a high frequency (40%) of loss of heterozygosity of the *MET* genomic locus at chromosome 7q31, which suggested that *MET* might be a tumor suppressor gene in breast cancer.

However, the Bièche study used only one polymorphic marker, albeit located within the 3'-untranslated region of the *MET* gene. Thus, the size of the deletion at chromosome 7q31 remained unknown, and the results from this study could not be used to predict the identity of the tumor suppressor gene whose deletion was presumed to be selected for in breast cancer. Furthermore, several groups attempted to repeat the findings of Bièche, but reported only infrequent LOH of chromosome 7q31 (0-4%) (Devilee et al., 1991; Nagy et al., 1995). These results were then suggested to reflect the random loss of chromosome 7, rather than the specific deletion of a tumor suppressor gene important in breast cancer. Thus, although the tissue distribution and role of Met in breast epithelial differentiation made it an attractive candidate for the potential tumor suppressor gene at 7q31, the suggestion that there might be a tumor suppressor gene for breast cancer at chromosome 7q31 remained controversial.

The initial goal of my thesis was two-fold: first, to establish in an independent study, whether a specific deletion of chromosome 7q31 was selected for in human breast cancer, and secondly, to determine if *MET* was included in the smallest common region of deletion, and is therefore a candidate for a putative breast cancer tumor suppressor gene. In the work described in chapter two, 73 breast cancers were obtained from the pathology archives at the Royal Victoria Hospital in Montreal and from Mount Sinai Hospital in Toronto. I used a panel of 17 polymorphic markers on chromosome 7, mostly located at 7q31, but including markers from other regions of chromosome 7q and 7p, to study the extent of the deletion of chromosome 7 in breast cancer. This study demonstrated that chromosome 7 was not randomly lost in breast cancer, since all tumors

with LOH of *MET* (nine in total or 12.3%) had specific deletions of differing sizes of chromosome 7, centered on 7q31 (see Figure 3, Chapter II). By incorporating my data and that from a previous study of a subset of the breast tumors studied by Bièche, (Zenklusen et al., 1994), I was able to map the smallest common region of deletion of chromosome 7 in breast cancer to a 1000kb region of chromosome 7q31, located between the markers D7S486 and metH (Lin et al., 1996; Chapter II). This included the entire coding region of the *MET* gene, but no other known genes.

We also reported a clarified order for polymorphic markers at 7q31 (see Figure 4, Chapter II), which helped to interpret conflicting results reported by (Zenklusen et al., 1994), suggesting that *MET* was not included in the deletion of chromosome 7q31 in breast cancer. The clarified data showed that *MET* was, in fact, deleted in that study.

My LOH data supported the possibility that *MET* might be the tumor suppressor gene at chromosome 7q31 whose deletion was selected for in certain breast cancers. Since Met had been shown to be expressed in normal breast epithelium, it was important to determine whether breast tumors with deletion of one allele of *MET* had lost expression of Met protein, which would further support *MET* as a tumor suppressor gene for breast cancer. Therefore, I performed immunohistochemical studies of Met expression on sections of paraffin-embedded tumors which I had shown to have LOH at 7q31. These were compared with sections from tumors without LOH and also with sections of normal breast tissue obtained from reduction mammoplasties. I showed that Met was expressed in the normal breast epithelium, and that it was also detected in tumors with and without LOH of chromosome 7q31 (Lin et al., 1996;

Chapter II). However, since it was impossible to determine whether or not the Met protein detected in these tumor cells was biologically active or not, it is possible that the Met protein detected in tumors with LOH of 7q31 is inactive. Thus, *MET* remains a potential candidate for the putative tumor suppressor gene at chromosome 7q31.

III. Determination of the gene structure of *MET* and description of an alternatively-spliced mRNA.

If *MET* is the tumor suppressor gene important for breast cancer at 7q31, Knudson's hypothesis (Knudson, 1971) would predict that a deletion of one copy of the *MET* gene would be accompanied by a recessive, inactivating mutation in the remaining copy of the gene. However, the intron-exon structure of the *MET* gene had not been determined, and therefore, sequence analysis of the *MET* gene in breast tumors with LOH of 7q31 was not possible. Therefore, in chapter three of this thesis, I elucidated the complete intron-exon structure of the *MET* gene (Lin et al., 1998; Chapter III). I have also used flanking intron sequences to design primers for the amplification of each individual exon of *MET* for use in mutation analysis of *MET* in human tumors.

Establishing the intron-exon structure of the gene also revealed that *MET* contains an unusually large coding exon, exon 2, which at 1214nt long, was significantly larger than the average vertebrate exon (average size, 137nt (Berget, 1995)). This finding made Met unique among all receptor tyrosine kinases studied to date. *MET* exon 2 also contains the AUG start site for translation of the full-length Met protein. This large exon is flanked by two extremely large introns (25kb and 31kb). Splicing

studies of vertebrate genes suggested that such a large exon, flanked by such large introns, would be inefficiently included in mRNA processing (Berget, 1995). In fact, I demonstrated that a 7kb Met mRNA isoform which had previously been cloned in our lab resulted from skipping of exon 2. This isoform is found expressed in all normal tissues studied, and is the second most abundant Met mRNA species. It contains one large open reading frame, which is predicted to encode a smaller Met protein, lacking the N-terminal region. However, no such protein could be detected *in vivo* using an antibody to the Met carboxy-terminal region, nor was such a protein expressed in a COS cell transient transfection assay. Thus, the exon 2 skipped Met mRNA isoform does not encode protein *in vivo* (Lin et al., 1998; Chapter III). The purpose of this mRNA product is not immediately apparent, but may represent a form of regulation of expression of the Met protein during development, with alterations in the relative amounts of mRNA isoforms determining the expression level of Met protein. The selection for inclusion of *MET* exon 2, despite its large size, suggests the presence of splicing enhancer sequences, not yet identified, which aid in the correct splicing of Met. Such enhancer sequences have been identified in other genes, and have been shown to be important for the regulation of alternative splicing through the binding of splicing proteins, such as the SR protein family (discussed below) (reviewed in Manley and Tacke, 1996).

IV. Study of the Met receptor in breast cancer cell lines.

Investigation of the status of the *MET* gene and protein expression in human breast cancer is difficult due to the relative lack of banked specimens and their great demand by many investigators. The pathology

specimens used for the LOH studies in the second chapter of this thesis were inappropriate for more detailed study of Met mRNA or protein expression due to the lack of fresh frozen tissue. Thus, to further investigate Met in breast cancer, I turned to studying cell lines derived from human breast cancers. Many of these have been previously characterized according to keratin expression, and their invasive/aggressive properties have also been described in the literature. While studying cell lines is not ideal to elicit information about the role Met might play in the development of breast cancer, it is a model which can provide valuable insight into the possible situation *in situ*. Thus, in the fourth chapter of this thesis, I examined the status of the Met receptor in 13 breast cancer cell lines, in comparison with two immortalized breast epithelial cell lines.

I showed that in seven of these 13 breast cancer cell lines, Met protein expression is decreased or absent compared with two immortalized breast epithelial cell lines. Furthermore, the decreased expression of wild-type Met protein is correlated with a relative increase in levels of the non-protein-encoding 7kb exon 2-skipped Met mRNA isoform. However, this is not associated with any mutation in exon 2 or the surrounding splice sites.

Alternative splicing of genes is a tightly regulated process which occurs in a cell-type or developmental-stage specific manner. Coordinated changes of alternative splicing patterns of genes is important in the development of many tissue types, such as the immunoglobulin genes in the development of B-cell lymphocytes. The splicing reaction occurs in the spliceosome, which is composed of five small nuclear ribonucleoproteins

(snRNPs) and numerous other polypeptides. The early stages of spliceosome assembly involves recognition of the 5' splice site by the U1 snRNP, and binding of the two subunits of the U2 snRNP auxiliary factor (U2AF) to the polypyrimidine tract and the 3' splice site (Smith and Valcarcel, 2000). This is followed by the binding of other proteins, such as the members of the SR splicing factor family. These proteins share an arginine-serine-rich motif, and represent a family of splicing factors that are important in the regulation of alternative splicing (reviewed in Chabot, 1996 and Manley and Tacke, 1996). The N-terminal region of these SR proteins contain RNA recognition motifs while their C-terminal arginine-serine rich domains mediate protein -protein interactions with similar domains in the U1 snRNP and U2AF. SR proteins can bind to exon splicing enhancer sequences, when these sequences are not masked by the binding of other polypeptides, and in this way, influence alternative splicing decisions (Smith and Valcarcel, 2000).

Recently, changes in the expression profile of SR splicing proteins have been described in mouse mammary tumorigenesis (Stickeler et al., 1999). To determine whether similar changes in SR proteins are present in human breast cancer, the expression profile of SR proteins was determined in all 13 breast cancer cell lines as well as normal human mammary epithelial cells (HMEC) and two immortalized breast epithelial cell lines (see Figure 3, Chapter IV). Interestingly, levels of the smallest member of the SR protein family, SRp20, was found to be increased in six of the seven cell lines with increased exon 2-skipping of *MET*, as compared with HMECs and two normal breast epithelial cell lines (Lin et al., 2000; Chapter IV). This is in agreement with findings in mouse mammary

tumorigenesis, where an increase in SRp20 is found in tumors and metastases when compared with normal or pre-neoplastic cells (Stickeler et al., 1999). The breast cancer cell lines with normal or increased expression of Met did not demonstrate increased SRp20 expression when compared with HMECs or two immortalized breast epithelial cell lines (see Figure 3, Chapter IV). It is possible that SRp20 plays an important role in the alternative splicing of *MET*, and that the alterations seen in the breast cancer cell lines with increased exon 2-skipping of *MET* are a direct result of the alterations in the levels of this SR protein.

It is not yet known whether other genes are similarly affected by this alteration in the mechanism of alternative splicing. However, changes in alternative splicing of the tumor suppressor gene WT1 and the androgen receptor gene AR have been reported in human breast cancer (Silberstein et al., 1997; Zhu et al., 1997). CD44, a cell surface adhesion molecule and receptor for hyaluronic acid, is also subject to alternative splicing in breast cancer, and has been implicated in metastasis (reviewed in Goodison et al., 1999). Alterations of SR splicing proteins in mouse breast tumors was associated with increased alternative splicing of CD44 (Stickeler et al., 1999). These results suggest that changes in alternative splicing may be a common mechanism for deregulation of genes important for the progression of cancer.

V. The potential role of Met & HGF/SF in breast tumorigenesis.

A link between increased expression of Met or HGF/SF and a poorer prognosis in breast cancer has been reported in the literature (Ghoussoub et al., 1998; Yamashita et al., 1994). Several studies have found increased

expression of both Met and HGF/SF in breast cancer (Jin et al., 1997; Tuck et al., 1996; Yamashita et al., 1994). These findings were proposed to support the fact that Met, as a member of the tyrosine kinase receptor family, may function as a promotor of oncogenesis when deregulated, and not as a tumor suppressor gene. Furthermore, activating mutations in the *MET* gene, which cause the constitutive activation of the kinase activity of Met, have been identified in patients with hereditary and spontaneous papillary renal cell cancer (Jeffers et al., 1997; Schmidt et al., 1997).

In studies of Met expression in breast carcinoma cells to date, there appears to be at least two patterns of Met expression. Immunohistochemical studies show that normal breast epithelium and benign proliferative disease are always positive for Met protein. However, early stage breast cancers and well-differentiated breast cancer cell lines have very little or no Met protein expression (Beviglia et al., 1997; Byers et al., 1994; Ghoussoub et al., 1998). More aggressive breast cancers and poorly differentiated, invasive breast cancer cell lines have been found to have high levels of Met protein (Beviglia et al., 1997; Byers et al., 1994; Ghoussoub et al., 1998). In agreement with this, the seven breast cancer cell lines which demonstrated decreased or no Met expression in chapter four have been classified as well-differentiated breast cancer cell lines, while the Met expressing cell lines are poorly differentiated (Byers et al., 1994). This apparent two-stage involvement of Met protein expression by breast cancer cells has been interpreted to suggest that Met expression may be lost early in tumorigenesis and then regained at a later stage, which is then associated with increased aggressivity of the cancer (Ghoussoub et al., 1998).

The resolution to the conflicting findings regarding the role of Met in breast tumorigenesis may lie in the complex role that Met plays in mammary gland development. Met has been shown to be important for the ductal differentiation of the mammary gland (Neimann et al., 1998; Yang et al., 1995). Furthermore, (Ronen et al., 1999), demonstrated that the creation of an autocrine HGF-Met signalling loop in the breast cancer cell line T47D led to the growth arrest and terminal differentiation of these cells, supporting the role for Met and HGF in breast differentiation. Presumably, well established protective factors for breast cancer, such as early full-term pregnancy and lactation, are related to the early terminal differentiation of the breast epithelium. It is possible that the later this terminal differentiation takes place, the more risk a woman has for developing breast cancer, due to the exposure of pleuri-potent breast epithelial cells to carcinogenic insults. If Met is lost in breast epithelial cells before terminal differentiation takes place, it may prevent those cells from ever achieving complete terminal differentiation, and thus predispose them to developing breast neoplasias later in life.

There may be at least two stages of breast tumorigenesis with regards to Met receptor expression. In the first stage, Met is lost, either through alterations in alternative splicing, as described in chapter four of this thesis, or perhaps through deletion coupled with inactivating mutations (as yet unidentified), as implied by the studies reported in chapter two of this thesis. The loss of Met could be a factor contributing to the initial stages of breast neoplasia, as discussed above. Once the breast tumor develops, regaining Met expression, through mechanisms not yet understood, may allow these cancer cells to gain mitotic and invasive,

motile properties. This could lead to increased aggressivity of the breast cancers expressing Met protein. Experiments where Met is introduced into non-invasive, well-differentiated breast cancer cell lines which normally do not express Met, such as T47D and MCF7, support this theory (Beviglia et al., 1997). In these studies, re-introducing Met resulted in these cells becoming morphologically poorly-differentiated, with invasive properties *in vitro* (Beviglia et al., 1997). This theory is further supported by findings reported by (Ghoussoub et al., 1998) and (Camp et al., 1999), that several breast cancers that initially demonstrated decreased levels or no Met protein expression, became strongly positive in recurrences of the original tumors.

The work in this thesis has identified two mechanisms through which the Met receptor tyrosine kinase may be inactivated in breast cancer, both of which may be important in the development of this disease. One potential mechanism of inactivation of *MET* is through deletion at the DNA level, which may be associated with inactivating mutations in the remaining allele. This was identified by LOH studies, which found a specific deletion of the *MET* gene on chromosome 7q31 in a small percentage of the breast cancers studied (12.3%; 9 of 73) (Lin et al., 1996). Apart from one study showing a high frequency of LOH at chromosome 7q31 (40.5%) (Bièche et al., 1992), this observation is in keeping with other studies of the frequency of LOH of loci at 7q31 (0% to 27%) (Deng et al., 1994; Devilee et al., 1991; Kerangueven et al., 1995; Nagy et al., 1995; Sato et al., 1990), indicating that LOH of the *MET* gene may be found in a small percentage of human breast cancers. Alternatively, a second mechanism for the inactivation of Met was identified in a large percentage of breast

cancer cell lines (53.8%; 7 of 13 lines), in which the alternative splicing of the Met mRNA was found to be altered, resulting in decreased levels of Met protein expression. If this high level of altered Met splicing is reflected in breast cancers (not yet studied), then this mechanism may be more important for the inactivation of Met than through mutation and deletion. Thus, it is important to investigate this further by studying the alternative splicing patterns of Met in breast tumours.

It is possible that both mechanisms for the inactivation of Met occur in breast cancer. Immunofluorescence studies of Met in breast cancer cell lines (data not shown) reveals that the Met protein expressed in cell lines without an altered splicing pattern of Met shows altered intracellular localization when compared to the membrane localization in other epithelial cells. This may represent mutations in the *MET* gene which lead to altered intracellular localization of the protein. Therefore, it is essential to determine if such mutations exist in *MET* in these cell lines, or if any mutation exists in the breast cancers with LOH of *MET*, identified in chapter two.

Traditionally, receptor tyrosine kinases have been thought of as proto-oncogenes, and they are usually believed to contribute to cancer development through their inappropriate activation. However, the work in this thesis addresses the question of whether or not Met could function as a tumor suppressor in breast cancer. I have shown that there are two potential mechanisms for the inactivation of Met in human breast cancer. Alternatively, others have identified activating mutations in Met in hereditary and sporadic papillary renal carcinoma and gastric cancer (Lee et al., 2000; Olivero et al., 1999; Schmidt et al., 1997). I believe that there may

be dual roles for Met in human disease, similar to the Ret tyrosine kinase. Activating mutations in the *RET* gene have been identified in the MEN 2 cancer syndromes and in familial medullary thyroid carcinomas (Jhiang, 2000). However, inactivating mutations in the *RET* gene have also been described in human disease. Loss of function mutations in *RET* are found in Hirschprung's disease, a developmental disorder of the autonomic innervation of the gut (Romeo et al., 1994). Although Hirschprung's disease is not a neoplastic disorder, this finding provides evidence that receptor tyrosine kinases may be involved in human disease through inactivating mutations as well as the more usually described activating mutations. Similarly, inactivating mutations may exist in the *MET* gene, as suggested by the work in this thesis. Furthermore, the expression of receptor tyrosine kinases in breast cancer varies widely. While amplification and overexpression is seen with some receptor tyrosine kinases such as erb-B2, whose amplification is associated with a poorer prognosis(Dickson and Lippman, 1995), the expression of other receptor tyrosine kinases such as FGF-1 and FGF-2 are actually lower in breast cancers than that seen in benign or normal tissue (Bansal et al., 1995; Yiangou et al., 1997). The significance of this has not yet been investigated, but it is reasonable to assume that the decreased expression in breast cancer of tyrosine kinases such as Met, FGF-1 or FGF-2 may be a selected event that is important in the development of this disease, as much so as the overexpression of other tyrosine kinases.

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Chapter VI - Contributions to Original Research

1) In the work reported in chapter two, I used an extensive set of polymorphic markers on chromosome 7 to establish whether or not there is selection for the deletion of a putative tumor suppressor gene at chromosome 7q31 in breast cancer. This allowed me to determine that deletion of the *MET* gene is a selected event in human breast cancer tumorigenesis, and I also identified the smallest common region of deletion of chromsome 7q31, which had not been done before. Using immunohistochemistry, I also demonstrated that Met protein is expressed in normal breast epithelial cells, and also in breast cancers that had a deletion in the *MET* gene.

2) In the work reported in chapter three of this thesis, I identified the complete intron-exon structure of the *MET* gene by PCR amplification and cycle sequencing of Met sequences from genomic DNA. This had not previously been determined, and now provides a valuable tool for studying the regulation of the *MET* gene by alternative splicing, as well as allowing mutation analysis of the *MET* gene in genomic DNA from cancer cells. Furthermore, the elucidation of the *MET* genomic structure led to a new understanding of a cDNA species that had previously been cloned in our lab. My data showing that *MET* had a single large exon, exon 2, which contains the AUG for the full-length Met protein, led to the understanding that Met undergoes alternative splicing of this exon, resulting in a ubiquitously expressed 7kb mRNA species. Although it had been known for some time that this cDNA species did not encode Met

protein, the significance of this data was not understood. However, my data allowed me to re-interpret the old data, and determine that the pOK8/14 cDNA resulted from alternative splicing of the *MET* gene.

3) In the work reported in chapter four, I demonstrated that half of the breast cancer cell lines I studied had decreased levels of Met protein or lacked it altogether. This was significant, since all other cell lines of epithelial cell origin that had been studied had high expression of Met. Furthermore, I used Northern blotting and ribonuclease protection techniques to demonstrate that the loss of Met protein in these breast cancer cell lines is associated with increased levels of the 7kb alternatively-spliced Met mRNA lacking exon 2. Since this mRNA species does not encode any protein, increased amounts of this isoform represents a novel mechanism for the inactivation of Met.