MOLECULAR GENETIC STUDY OF HUMAN ESOPHAGEAL CARCINOMA

1991

ZOU

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
F. EDWARD HÉBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

APPROVAL SHEET

GRADUATE EDUCATION

TEACHING HOSPITALS
WALTER REED ARMY MEDICAL CENTER
NAVAL HOSPITAL BETHESDA
MALCOLM GROW AIR FORCE MEDICAL CENTER
WILFORD HALL AIR FORCE MEDICAL CENTER

Title of Dissertation: "Molecular Genetic Study of Human Esophageal Carcinoma"

Name of Candidate: Zhi-qiang Zou's
Doctor of Philosophy Degree
July 16, 1991

Dissertation and Abstract Approved:

Committee Chairperson	8-22-91 Date
Committee Member	8-21-91 Date
Committee Member	8-21-91 Date
Committee Member	8/21/91 Date
Committee Member	8/23/91 Date

The author hereby certifies that the use of any copyrighted material in the dissertation manuscript entitled:

"Molecular Genetic Study of Human Esophageal Carcinoma"

beyond brief excerpts is with the permission of the copyright owner, and will save and hold harmless the Uniformed Services University of the Health Sciences from any damage which may arise from such copyright violations.

Zhiqiang Zou
Department of Pathology
Uniformed Services University
of the Health Sciences

ABSTRACT

Title of Dissertation:

Molecular Genetic Study of Human Esophageal Carcinoma

ZhiQiang Zou, Doctor of Philosophy, 1991

Dissertation Directed by:

Esther Chang Ph.D.

Professor

Department of Pathology

Esophageal carcinoma is one of the most common human malignancies in certain areas of the world. Various etiological factors have been implicated in this disease, but little is known about the molecular genetic changes which accompany tumorigenesis. Two classes of genes, oncogene and tumor suppressor gene, have demonstrated susceptibility to mutations in different types of neoplasia. In the present investigation, two approaches were undertaken in the search for such genes which might be mutated during the development of esophageal carcinoma. In the first, the human HER2 oncogene, encoding a tyrosine kinase type receptor, was identified in a transformed NIH3T3 cell line induced by one esophageal tumor DNA sample. Increased expression of multiple copies of this gene together with rearrangement at the flanking sequences was acquired during the transfection process. Analysis of the complete cDNA sequence of this HER2 revealed a single nucleotide difference in codon 655 within

the transmembrane region compared with a published normal cDNA sequence. This difference was demonstrated to be a polymorphism by allele-specific oligonucleotide hybridization on 47 DNA samples. In addition, human sequences other than HER2 were detected in the same transformant with multiple copies and rearrangement obtained in the transfection. These results suggest that the transformation may have been achieved from overproduction of HER2 due to amplification and/or rearrangement at the flanking sequence during the experimental manipulation. The activation of HER2 gene in this assay may thus be a fortuitous event. In the second approach, the tumor suppressor gene p53 was analyzed in 18 esophageal tumor samples from Linxian, China. p53 gene spanning codon 126 through 330 was amplified by polymerase chain reaction and was sequenced directly. Seven cases (39%) bore a point mutation in the coding region of the p53 gene. These results show that p53 mutations occur frequently in human esophageal carcinoma. Diverse mutagenic carcinogens may contribute to the gene mutation. The resultant impaired function of p53, as one molecular event, may play a role in the development of esophageal carcinoma.

MOLECULAR GENETIC STUDY OF HUMAN ESOPHAGEAL CARCINOMA

by

ZHIQIANG ZOU

Dissertation submitted to the Faculty of the Department of Pathology
Graduate Program of the Uniformed Services University
of the Health Sciences in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy, 1991

Acknowledgment

Special thanks to Dr. Esther Chang for her full support of this study during the five plus years.

Thanks to all my committee members: Dr. Carl Dieffenbach, Dr. Philip Grimley, Dr. Frank Jenkins, Dr. Dvorit Samid and Dr. Robert Silverman.

Thanks to Dr. Kai Mai, Dr. Songliang Qiu, Henan Medical University, Henan Institute of Medical Sciences.

Thanks to Linxian Hospital, AnYang Hospital for providing the tumor samples.

Thanks to Dr. David A. Cooney for helping me in preparation of this dissertation.

Thanks to all my friends and colleagues in Dr. Esther Chang's laboratory for their great help in conducting this study.

Thanks to my parents and my wife.

TABLE OF CONTENTS

Approval Sheet..... i

Copyright Statement. ii

Abstract.....iii

Title Page..... v

Acknowledgment.....vi

Table of Contents..... vii

List of Tables..... xi

List of Figures..... xii

INTRODUCTION.....1

Oncogenes and Tumor Suppressor Genes..... 1

Identification by DNA Transfection Assay..... 3

The Use of Polymerase Chain Reaction (PCR)..... 6

HER2 Gene Encodes a Receptor with

Tyrosine Kinase Activity..... 7

Rat neu Gene..... 8

HER2/c-erbB2.....9

Amplification of HER2 in Human Tumors..... 13

Overexpression of HER2 in Human

Breast Carcinoma..... 14

HER2 Promoter Region..... 15

HER2 Rearrangement in Human

Gastrointestinal Tumor..... 18

Study of the Transforming Function

of HER2 *in vitro*..... 18
p53 Gene Encodes a Protein with
Tumor Suppressor Effects..... 20
History.....20
Structure of p53 Gene and Product..... 20
Biological Function of the p53 Gene Product..... 22
p53 Gene Acts as a Tumor Suppressor Gene..... 24
p53 Gene Mutation in Human Malignancy..... 26
Risk Factors Implicated in the Development
of Human Esophagal Carcinoma..... 31
Pathogenesis of Human Esophageal Carcinoma..... 37
Current Molecular and Genetic Studies of
Esophageal Carcinoma..... 37

MATERIALS AND METHODS..... 41
Tumor Samples and High Molecular
Weight (HMW) DNA Preparation..... 41
DNA Transfection and Tumorigenesis Assay..... 42
Genomic DNA Cloning and cDNA Cloning..... 43
Polymerase Chain Reaction..... 45
Transformation.....45
Nucleotide Sequencing..... 47
Nucleic Acid Hybridization..... 49

RESULTS.....51

Identification of HER2 Oncogene in a
Transformed NIH3T3 Cell Line by DNA
from One Esophageal Carcinoma..... 51
Identification of Human DNA Sequences
in a Secondary Transformant DNA..... 51
Partial Genomic Cloning of the
Human DNA Sequences..... 59
Identification of HER2 Oncogene
in the Transformant..... 62
No Equivalent Mutation in the Transmembrane
Region to the Active neu..... 68
The HER2 Flanking Sequences Were Altered in Mouse
Transformant DNA, but the Gene Was Structurally
Normal in the Original Tumor..... 69
Multiple Copies of the HER2 and Elevated
Expression Were Detected in the Mouse
Transformant, Amplification Was Not
Detected in the Original Human Tumor..... 77
Analysis of HER2 Promoter Region..... 81
Molecular Cloning of HER2 cDNA from Secondary
Transformant Cell Line..... 84
Preliminary Test of Tumorigenic
Activity of the HER2 cDNA..... 85
HER2 Alleles in Amino Acid Codon 655 within
Transmembrane Region..... 93
Analysis of the Second 6.6 kb EcoRI

Genomic Fragment (S1)..... 98
Detection of p53 Mutation in Human
Esophageal Carcinoma from a High
Incidence Area Linxian, China..... 105
Development of a Strategy for PCR and Direct
Sequencing of p53 Gene from Tumor DNA..... 105
Frequent Mutations of p53 Gene in Esophageal
Carcinoma from the High Incidence Area..... 111
Development of a Rapid PCR Method to Clone
Human Transforming Sequences
from a Mouse Transformant..... 120

DISCUSSION..... 128

CONCLUSION..... 141

BIBLIOGRAPHY..... 144

List of Tables

Table 1. Frequency of p53 Gene Mutation in Human Tumors... 27

Table 2. Oligonucleotides Used in This Study..... 46

Table 3. Test of Tumorigenesis of DNA from Human Esophageal Carcinomas..... 52

Table 4. Test of Tumorigenesis of Alu-Positive Mouse Tumor DNA..... 56

Table 5. Tumorigenesis Study of HER2 Expression Plasmids..... 92

Table 6. HER2 Alleles at Codon 655 within Transmembrane Region..... 97

Table 7. p53 Mutation in Human Esophageal Carcinomas..... 112

List of Figures

Figure 1. A Schematic Diagram of HER2 cDNA and the Predicted Protein..... 12
Figure 2. Schematic Illustration of HER2 Promoter Region..... 17
Figure 3. A Schematic Diagram of the "Hot Spots" of the p53 Gene..... 30
Figure 4. Southern Blot Analysis of High Molecular Weight DNA from Mouse Tumors..... 54
Figure 5. Detection of Human EGF Receptor Related Sequences in DNA of the Mouse Transformant.... 58
Figure 6. Restriction Maps of Two Human Alu Positive 6.6 kb EcoRI Fragments..... 61
Figure 7. Analysis of the Human DNA Sequences for Their Evolutionary Conservation..... 64
Figure 8. Northern Blot Analysis..... 67
Figure 9. Codon 655 within the HER2 Transmembrane Region..... 71
Figure 10. Southern Analysis of HER2 Gene in the Mouse Transformant 74
Figure 11. Loss of the 5' Flanking Sequence of HER2 Gene in the Mouse Transformant..... 76
Figure 12. Analysis of the Human DNA Sequences (S3 Fragment) in the Transformant DNA..... 79
Figure 13. DNA Sequence of HER2 Promoter Region..... 83

Figure 14. Complete Nucleotide and Amino Acid Sequence of HER2..... 87
Figure 15. Construction of the Expression Plasmids..... 90
Figure 16. Allele Specific Hybridization of HER2 Transmembrane Region..... 96
Figure 17. Analysis of the Human DNA Sequence (S1 fragment) in the Transformant..... 101
figure 18. Comparison of the Banding Pattern of SphI Fragment with that of HER2 cDNA by Southern Analysis..... 103
Figure 19. Strategy for Amplifying and Sequencing the "Hot Spots" of p53 Gene..... 108
Figure 20. DNA Sequence of Exons 5 and 6 of the p53 Gene..... 110
Figure 21. DNA Sequence of Exon 7 of the p53 Gene..... 114
Figure 22. Single Base Deletion in the p53 Gene..... 117
Figure 23. Missense Point Mutation in the p53 Gene..... 119
Figure 24. Schematic Illustration of Amplification of Inter-Alu Sequences..... 122
Figure 25. Amplification of Human Genomic Sequences in DNA of the Mouse Transformants..... 124
Figure 26. Southern Analysis of Amplified Human Sequences from DNA of the Mouse Transformants..... 126

INTRODUCTION

The incidence of esophageal carcinoma is remarkably high in certain geographical areas, such as the borders between Henan, Hebei, and Shanxi provinces in northern China; the Caspian Littoral in Iran; and South Africa. Over 90% of cancers of the esophagus are squamous cell carcinomas. The remaining cases are predominantly adenocarcinomas of the esophagus, which is strongly associated with Barrett's esophagus, a condition in which the squamous epithelium is replaced by columnar epithelium arising, in most cases, from reflux esophagitis. The aim of this dissertation is to explore the genetic alterations occurring in esophageal carcinoma from one of the high incidence areas, Linxian, China.

Oncogenes and Tumor Suppressor Genes

Tumor oncogenes were originally derived from normal cellular genes called proto-oncogenes which undergo activating processes including point mutations, deletions, gene fusions caused by chromosomal translocation, and gene amplifications which can lead to overexpression of a given gene (Bishop, 1987; Varmus, 1989). These changes have been found in a variety of human malignancies (Bishop, 1991). Proto-oncogenes play a critical role in cell growth and differentiation, with their functions ranging from the regulation of DNA

replication, and the control of transcription, to the action of growth factors, receptors, altered cytoplasmic protein kinase and GTP binding proteins (Hunter, 1991). Abnormal functioning of these cellular genes accompanies neoplastic growth and contributes to the tumor progression.

Tumor suppressor genes were discovered as a consequence of several cardinal observations (Levine and Monard, 1990; Sager, 1989; Marshall, 1991; Hollingsworth and Lee, 1991). The phenotype of the tumor cell was lost in the hybrids of tumor cells and their normal counterparts, suggesting that the genome of the normal cell was responsible for the suppression of the transforming phenotype (Stanbridge, 1988). The transfer of normal tumor suppressor genes into tumor cells (in which the corresponding endogenous gene was inactive) was shown to suppress tumor cell growth (Baker, *et al.* 1990; Bookstein, *et al.* 1990). Furthermore, many types of tumors lost heterozygosity at certain specific loci (Ponder, 1988; Hollingsworth and Lee, 1991). The chromosomal regions lost in tumor cells may harbor the suppressor genes for cell growth. Based on statistical analysis of retinoblastoma incidence, Knudson proposed the "two hit" hypothesis, that is, two alleles of a tumor suppressor gene were sequentially inactivated by a somatic event and an inherited mutation (Knudson, 1971). This hypothesis was strongly supported by the identification of retinoblastoma susceptibility gene (RB1) on chromosome 13q (Friend, *et al.* 1986; Lee, *et al.* 1987).

The biochemical functions of the tumor suppressor gene

products are not clearly elucidated. Nuclear protein products of these genes may be involved in DNA replication, or in the regulation of gene expression for cell proliferation or differentiation as a transcription factor (Marshall, 1991). A cell surface molecule involved in cell adhesion might regulate cell-to-cell interactions (Fearon, *et al*. 1990). In contrast to the dominant oncogenes, two genetic lesions are required for the manifestation of tumor suppressor gene, one each to inactivate the maternal and paternal allele. Inactivation of tumor suppressor genes is oncogenic.

It is generally accepted that cancer results from multiple genetic mutations. Oncogenes and tumor suppressor genes are frequently involved in the pathogenesis of human cancers. The transformation of a normal cell into a tumorigenic cell involves the activation and concerted expression of several proto-oncogenes, as well as, the inactivation of tumor suppressor genes. Using a variety of molecular techniques, these changes can be identified in human tumors, to provide valuable information regarding the tumorigenesis, and its diagnosis, prognosis, and subsequent treatment of cancer.

Identification of Oncogene by DNA Transfection Assay

Gene transfer is one of the approaches used to identify activated oncogenes. A number of oncogenes implicated in human cancers have been identified by DNA

transfection studies. The transfection study was performed mostly with NIH3T3 cells, a mouse fibroblast line which was maintained as a non-tumorigenic cell line *in vitro*, and in the nude mouse (Varmus, 1989; Pimentel, 1989). Exogenous DNA, (transfected commonly by calcium-phosphate precipitation method (Wigler, *et al.* 1978), conferred the transformed phenotype on a recipient cell if it contained the genetic injuries responsible for the transforming phenotype. DNA from the transformed cells and tumors could be analyzed for the presence of the human Alu sequences (highly repetitive sequence and somewhat species specific) and for further identification of the putative transforming gene (Shih, *et al.* 1981; Cooper and Neiman, 1980). The actual mechanism of gene transfer in this system was not clearly understood except for the fact that foreign DNA was randomly integrated into the mouse cell genome. Multiple copies of the exogenous DNA were usually obtained either by formation of concatameric structure through a process of intermolecular ligation (Perucho, *et al.* 1980), or by amplification of the integrated gene (Glanville, 1985).

The transforming activity of cellular DNA can be detected in the original tumor DNA or acquired during the experimental manipulation. The ras gene family constituted the most frequently identified transforming genes in human tumors and in the transfection assay. Activation occurred as a consequence of a single amino acid substitutions in codons 12 or 61 of the gene. The ras gene encoded a GTP binding

protein involved in the cellular signal transduction (Barbacid, 1987; Bos, 1988). Similarly, the rat neu gene was also activated by a point mutation within the transmembrane region of the gene leading an elevated tyrosine kinase activity (Bargmann, *et al*. 1988). Next to the ras family, the hst1 gene was the second most frequent gene to be encountered in the DNA transfection assay. Not only has hst1 been isolated from cell lines transformed by gastric carcinoma DNA, but also from cell lines transfected and transformed by normal gastric mucosal and leukocyte DNA (Sakamoto, *et al*. 1986; 1988) as well as from hepatoma and Kaposi's sarcoma (Yuasa and Sudo, 1987; Delli Bovi, *et al*. 1987). The mechanism of such activation was not clear yet, DNA rearrangement or replacement of the 5' regulatory sequences has been observed in some of the above cases resulting from the transfection procedure.

Actually, many oncogenes identified in the NIH3T3 transfection assay were artificially activated by recombination, rearrangement, point mutations, or deletions occurring during or after the integration of the foreign DNA, while the original tumor DNA lacked these genetic changes (Pimentel, 1989). Thus there were clearly limitations to this assay. The NIH3T3 cell was considered to be a preneoplastic cell which has undergone some genetic alterations; it was unstable and highly susceptible to transfection by exogenous DNA; and the resultant transformation might not reflect the changes in the original tumor DNA. On the other hand, the mesenchymally-derived NIH3T3 cells might not be susceptible to

transformation by oncogenes from other cell types (Bos, 1988). However, it has been a useful and sensitive system to isolate oncogenes and to study a single event in the carcinogenesis.

The Use of Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) was recently developed for the rapid amplification of a chosen DNA sequence *in vitro* (Saiki, *et al.* 1985; Mullis, *et al.* 1987). The targeted nucleic acid sequences were amplified enzymatically by repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers (Gibbs, 1990). The availability of Taq DNA polymerase from the thermophilic bacterium *Thermus aquaticus* has greatly simplified the automation of the reaction (Saiki, *et al.* 1988). Exponential amplification of the target sequence made it possible to detect and analyze very small quantities of nucleic acids. Since its discovery, a variety of procedures derived from the basic principle have been developed and applied to individual purposes (Gibbs, 1990). For example, single-stranded products could be produced by an asymmetric PCR reaction in which an imbalanced ratio of the two primers lead to unidirectional extension when one of the primer was exhausted. The single-stranded template would be much more suitable for direct sequencing than the double-stranded products (Gyllensten and Erllich, 1988). Nowadays, this powerful technology has been widely applied to

biomedical research, as well as to the clinical diagnosis of disease. Changes occurring in genetic diseases and malignancies were rapidly identified by PCR. These included chromosomal translocation in hematological malignancies, point mutations of ras oncogene family in a variety of tumors and mutations of tumor suppressor gene, p53 in human common neoplasia (Kawasaki, *et al.* 1988; Bos, *et al.* 1987; Baker, *et al.* 1989).

HER2 Gene Encodes a Receptor With Tyrosine Kinase Activity

To date, three members of the epidermal growth factor receptor (EGFR) family have been identified: the first, the EGF receptor gene, shared homology with the v-erbB oncogene of the avian erythroblastosis virus in its carboxyl half, and was designated erbB-1 or HER-1 (Downward, *et al.* 1984); the second member, c-erbB2 or HER2 exhibited an overall structural and functional similarity to the EGF receptor (Coussens, *et al.* 1985; Yamamoto, *et al.* 1986); c-erbB3 or HER3 was the third member of this family (Kraus, *et al.* 1989) whose predicted protein shared structural similarities to other members of this family. These genes play an important part in the regulation of normal cell growth and differentiation by encoding a growth factor receptor possessing intrinsic tyrosine kinase activity. Overexpression of HER1 and HER2 genes has been causally implicated in the neoplastic process of human malignancies (Slamon, *et al.* 1987; 1989; Yoshida, *et*

al. 1990).

Rat neu gene. The neu gene was the rat version of the human HER2. It was first identified in rat neuroblastomas that had been induced by ethyl nitrosourea. DNA extracted from these rat tumors or tumor cell lines was capable of transforming NIH3T3 cells and the transformants induced rapidly-growing fibrosarcomas in NFS mice (Shih, *et al.* 1981). A unique antigen was expressed on the surface of the transformed NIH3T3 cell. It was a 185 kd phosphoprotein sharing a strong homology with the EGF receptor gene product (Schechter, *et al.* 1984). The complete copy of a cDNA isolated from the transformed cell lines possessed transforming ability (Bargmann, *et al.* 1986). An identical point mutation was consistently found in independently induced tumors. The transversion of A to T resulted in the replacement of valine (a hydrophobic amino acid) by glutamic acid (a charged hydrophilic amino acid) at position codon 664; this alteration was, in turn, responsible for the activation of the neu gene.

The biochemical consequence of this mutation was an increased tyrosine kinase activity which appeared to be essential for transformation (Bargmann and Weinberg, 1988). In addition, a construct containing only the intracellular portion of the neu gene product demonstrated transforming activity (Flanagan and Leder, 1988), and the activated neu gene induced mammary adenocarcinoma in transgenic mice as well (Muller, *et al.* 1988). The mutant neu gene product was shown

to have a shorter halflife and a greater tyrosine specific phosphorylation than the normal protein in the absence of ligand stimulation (Huang, *et al.* 1990). Furthermore, when the normal neu was expressed at high level, it induced the cellular transformation (Marco, *et al.* 1990). Monoclonal antibody against the epitope of the external domain of the neu product inhibited tumor growth effectively both *in vitro* and *in vivo* (Drebin, *et al.* 1985; 1986; 1988). This result raised the possibility of immunotherapy for certain human tumors expressing increased levels of HER2.

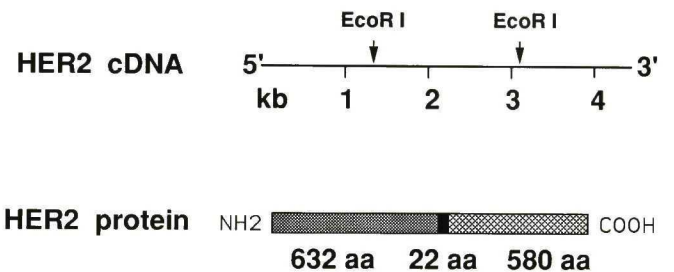
HER2/c-erbB2. Human HER2 was initially identified by screening a human cDNA library with a v-erbB probe (Coussens, *et al.* 1985; Yamamoto, *et al.* 1986). The rationale for this strategy of cloning was that the viral oncogene and several receptors for polypeptide growth factors shared extensive homology in the tyrosine kinase domain, and novel genes possessing tyrosine kinase activity could be identified under hybridization condition of low stringency. Using this approach, in addition to the EGF receptor gene, several, as yet, unidentified restriction fragments were revealed with the v-erbB probe. These fragments were amplified in a salivary gland adenocarcinoma cell line (Semba, *et al.* 1985) and in the MKN gastric carcinoma cell line (Fukushige, *et al.* 1986). Subsequently, the complete cDNA sequence was obtained from placenta and the MKN cell line (Coussens, *et al.* 1985; Yamamoto, *et al.* 1986). Analysis of the sequence indicated that the gene was the human counterpart of the neu gene and

that it was similar to, but distinct from the human EGF receptor gene. It was designated as HER2/c-erbB2. The polypeptide encoded by a 4.5 Kb messenger was 1225 amino acids in length and 185 kd in molecular weight. The putative HER2 protein product was typical of many transmembrane proteins in that it was composed of a cytoplasmic domain, a transmembrane domain, and an extracellular domain (Figure 1). The highest homology to the EGF receptor was found in the cytoplasmic domain, the region exhibiting the tyrosine kinase activity. The HER2 gene was located on the long arm of chromosome 17 q21-q22 which was in close proximity to the protooncogene c-erbA, the receptor for thyroid hormone (Coussens, *et al.* 1985; Fukushige, *et al.* 1986). HER2 gene was widely expressed on the cell membrane of normal adult epithelial cells and normal fetal tissue. A higher expression was seen in the fetal tissue. In normal tissue the level of expression was similar to that found in non-amplified non-overexpressing breast cancers and breast cancer cell lines, suggesting that HER2 was constitutively expressed in a variety of epithelial cell types (Press, *et al.* 1990)

The activation mechanism of human HER2 seemed to differ from the mechanism of the rat neu gene. To date, a natural point mutation in HER2 transmembrane region has not been reported despite the fact that the engineered mutant HER2, which is similar to the mutant neu, could transform the fibroblast. Moreover, in contrast to the neu gene, HER2 has not been identified by the NIH3T3 DNA transfection assay. The

Figure 1

A schematic diagram of HER2 cDNA and the predicted protein. The diagram was drawn according to the published sequences (Coussens, *et al.* 1985; Yamamoto, *et al.* 1986). aa, amino acid. The predicted protein consists of an extracellular domain, a transmembrane domain, and a cytoplasmic domain.


EcoR I
EcoR I
HER2 cDNA
5'
3'
kb
1
2
3
4
HER2 protein
NH2
COOH
632 aa
22 aa
580 aa
extracellular domain
transmembrane domain
cytoplasmic domain

entire coding sequence of the gene obtained from either placenta or tumor was identical except for a single nucleotide change at codon 655 (within the transmembrane domain); this was apparently not homologous to the position of the mutant neu gene (Coussens, *et al.* 1985; Yamamoto, *et al.* 1986; Slamon, *et al.* 1989; Di Fiore, *et al.* 1987). The resulting replacement of an isoleucine for a valine was not considered significant due to the similar hydrophobicity of these two amino acids. A study of 100 cases of breast carcinoma did not reveal any point mutations in the transmembrane region at codon 659 corresponding to the rat mutant codon (Lemoine, *et al.* 1990). Thus, a genetic alteration of HER2 other than a point mutation was implicated in the activation of the human HER2 gene.

Amplification of HER2 in human tumors. Extensive investigations have been undertaken to elucidate the relationship between the HER2 gene and human malignancy. Interestingly, this gene was often found amplified and highly expressed in adenocarcinomas, such as gastric, salivary ovarian and breast carcinomas (Semba, *et al.* 1985; Yokota, *et al.* 1987; Slamon, *et al.* 1989). Significantly, amplification and overexpression of HER2 were frequently observed in breast carcinoma. A survey of 189 cases of breast carcinoma indicated that 30% of the cases showed a various degree of amplification of the HER2 gene; the amplification correlated with poor overall survival, short time to relapse, more number of lymph nodes involved, large size of the primary tumor and

estrogen receptor negativity (Slamon, *et al.* 1987). Further confirmation of the contention that HER2 amplification was predictive of a poor prognosis in patients with breast carcinoma has been published by a number of subsequent investigators (Zhou, *et al.* 1987; Cline, *et al.* 1987; Guerin, *et al.* 1988; Slamon, *et al.* 1989). A recent retrospective case-controlled study showed that 18% of node-negative patients with recurrent cancer harbored additional copies of the HER2 gene in their malignant tissue, as compared to only 5% of the patients in remission. This result suggested that amplification of HER2 might contribute to the pathogenesis of some forms of node-negative breast cancer and serve as a prognostic marker to identify high risk patients (Paterson, *et al.* 1991). However, controversy still existed regarding the prognostic value of HER2 amplification in breast carcinoma (Ali, *et al.* 1988; Zhou, *et al.* 1989).

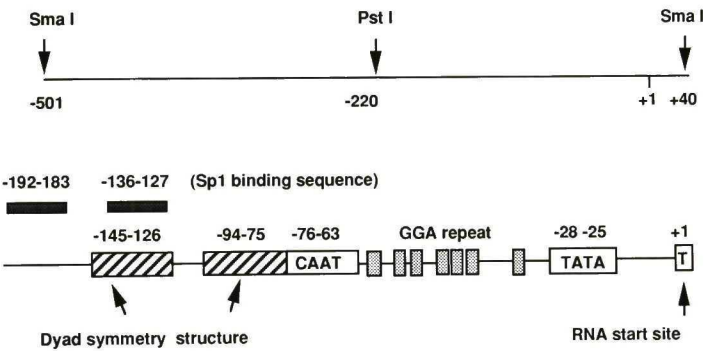
Overexpression of HER2 in human breast carcinoma. The presence of the HER2 gene product in breast carcinoma cell lines and fresh tumor tissue can be evaluated by Northern blot analysis, Western blot analysis, as well as by immunohistochemical staining. A high level of expression has been observed in tumors which harbor the amplified gene (King, *et al.* 1988; Venter, *et al.* 1987; Gusterson, *et al.* 1988). One study also found that the gene product of overexpressed HER2 was phosphorylated on its tyrosine residues, suggesting that the activated HER2 might act as a tyrosine kinase (Wildenhain, *et al.* 1990). As with amplification of HER2 gene, expression

of the gene product correlated with poor prognosis in some studies (van der Vijver, *et al* 1988; Venter, *et al*. 1987).

HER2 promoter region. HER2 gene amplification was, in general, associated with overexpression and increased tyrosine kinase activity. However, increases of HER2 mRNA were detected both in the presence or absence of gene amplification and translocation, suggesting that different molecular mechanisms might be involved in the regulation of HER2 expression (Kraus, *et al*. 1987). This possibility prompted a consideration of the HER2 promoter region. The HER2 promoter region was cloned and analyzed. Full promoter activity was localized to a 260 bp stretch of DNA from the start site of transcription. S1 nuclease mapping revealed several transcription start-sites. The nucleotide sequence of the HER2 promoter region contained a typical TATA box, and a CAAT box separated by 40 bp; seven GGC repeats between the TATA box and the CAAT box which presumably interacted with nuclear proteins; two Sp1 binding sequences; and two structures of dyad symmetry which were believed to participate in regulation of the gene (Figure 2) (Tal, *et al*. 1987; Ishii, *et al*. 1987). Transcription of HER2 appeared to be regulated by multiple factors and elements. The HER2 promoter could be induced by EGF, TPA (12-0-tetradecanoylphorbol-13-acetate), dibutyryl-cAMP, and retinoic acid, suggesting that HER2 expression would vary in response to changes in developmental and environmental conditions, allowing cells to signal growth and other physiological response in an appropriate way (Hudson, *et al*.

Figure 2

Schematic illustration of HER2 promoter region. The figure was constructed using published data (Tal, *et al.* 1987; Ishii, *et al.* 1987). The top panel represents the SmaI fragment containing the full activity of HER2 promoter. Number +1 denotes the most downstream transcription start site. Minus numbers denote the nucleotides upstream of the transcription start site; plus numbers denote nucleotides downstream of the transcription start site. Each element of the promoter region is indicated in the figure.


Sma I
Pst I
Sma I
-501
-220
+1
+40
-192-183
-136-127
(Sp1 binding sequence)
-145-126
-94-75
-76-63
GGA repeat
-28 -25
+1
CAAT
TATA
T
Dyad symmetry structure
RNA start site

1990).

HER2 rearrangement in human gastrointestinal tumor.

Rearrangements of HER2 have also been observed in certain gastrointestinal tumors and in a fetal tissue. In the gastric carcinoma MKN cell line, translocation was found to be responsible for generating a fusion fragment which might result in a 2.3 kb species messenger. Internal deletions have been observed in a primary gastric carcinoma with elevated expression of HER2 (Park, *et al.* 1989). In one colon carcinoma, HER2 rearrangement in the carboxyl-terminal coding sequence was associated with expression of a slightly larger-than-normal HER2 protein species (Tal, *et al.* 1988). Furthermore, another study of a gastric adenocarcinoma reported a structurally altered HER2 gene retaining coding sequences for the cytoplasmic domain (Yokota, *et al.* 1987). Rearrangement was also found in a normal fetal small intestine (Press, *et al.* 1990).

Study of the transforming function of HER2 *in vitro*.

In vitro studies have shown that when HER2 cDNA was overexpressed, transformation of NIH3T3 cells occurred (Di Fiore, *et al.* 1987; Hudziak, *et al.* 1987). Whether the HER2 cDNA was put under the control of the LTR of Moloney murine leukemia virus or whether multiple copies of the HER2 cDNA were obtained by methotrexate-selection of the cells cotransfected with dihydrofolate reductase cDNA, the increased generation of the HER2 product led to transformation. This data further supported the contention that amplification of

HER2 was the mechanism of activation in human malignancies.

When HER2 cDNA was engineered to have a mutation equivalent to the mutant neu or to have a deletion in the NH2-terminal extracellular domain, cells bearing such mutant HER2 genes exhibited increased *in vitro* tyrosine activity. This increase has been attributed to conformational changes and could transform cells at a 10 to 100 folds greater efficiency than the wild type gene (Segatto, *et al.* 1988). The intracellular portion of the gene product was thought to be responsible for transducing the mitogenic signal by phosphorylating specific intracellular substrates. Genetic manipulations which destroy the intrinsic tyrosine kinase activity of this molecule could abolish biological effects. On the other hand, the carboxyl-terminal domain of HER2 could exert an enhancing effect on HER2 kinase and transforming activity, which was partly responsible for the constitutive level of the kinase activity in the absence of ligand (Di Fiore, *et al.* 1990).

Finally, activated HER2 might potentiate tumorigenesis by inducing tumor cell resistance to host defense mechanisms. Increasing levels of expression of transfected HER2 in NIH3T3 cells led to induction of resistance to the cytotoxicity elicited by tumor necrosis factor (TNF) or macrophage (Hudziak, *et al.* 1988). Similar resistance was also found in cultured human cell lines derived from breast and ovarian carcinoma overexpressing the HER2 gene product (Lichtenstein, *et al.* 1990). It appeared that the resistance was due to the

altered binding activity of TNF receptor to its ligand (Hudziak, *et al.* 1988).

p53 Gene Encodes a Protein with Tumor Suppressor Effects

History. The p53 gene is currently categorized as a tumor suppressor gene. It was first detected in association with the SV40 T antigen in SV40 transformed cells (Lane and Crawford 1979; Linzer and Levine, 1979). The expression of p53 in chemically transformed cells and embryonal carcinoma cells devoid of the SV40 genome suggested that it might be a tumor antigen capable of interacting with the SV40 T antigen (Linzer and levine, 1979). Another line of evidence for this possibility was that the anti-p53 activity was found in the sera from cancer patients (Crawford, *et al.* 1982). However, when the genomic DNA and cDNA of p53 were cloned and their function analyzed, it was found that p53 could cooperate with the ras oncogene in the transformation of primary rat embryo fibroblast cells (Parada, *et al.* 1984; Eliyahu, *et al.* 1984). Thus, p53 was accorded a second attribute, that of tumor oncogene. However, the p53 genes used in these studies were shown to be the mutant form later on. Now, substantial evidence suggests that p53 itself behaves as a suppressor of the growth of tumor cells, and that only mutant p53 enhances the process of cellular transformation.

Structure of p53 gene and product. p53 genes from several species have been identified (Soussi, *et al.* 1987).

The gene was mapped to the short arm of human chromosome 17 in humans, and to mouse chromosome 11 (Czosnek, *et al* 1984; McBride, *et al*. 1986). The human p53 gene spanned 20 kb DNA, and produced a 2.8 kb messenger RNA which encodes a product with 393 amino acids whose molecular weight was 53 kilodaltons (Harlow, *et al*. 1985).

The p53 gene appeared to have been conserved during the course of evolution. Thus, similar genomic structures have been observed in three species, human, mouse and frog (Soussi, *et al*, 1987). The gene contained 11 exons interrupted by introns of variable size. The protein produced from different species also exhibited similar structure domain and shared extensive sequence homology (Soussi, *et al*. 1990). Five most conserved regions have been identified. These were located between amino acid residue 13-19, 111-136, 165-175, 230-252, 264-280 at domain I, II, III, IV, and V corresponding to exon 2, 3 and 4, 7, and 8 of the gene respectively. These domains, containing few charged residues, were found in the hydrophobic central part of the molecule; mutations in these domains might cause a conformational change and, thus, rendered a abnormal function to p53.

The expression of p53 in normal adult cells and tissues was extremely low. Increased expression has been found in various tumor cell lines and tumor tissues, usually in mutant form. The gene was also well expressed in undifferentiated stem cells, during mouse organogenesis (Rogel, *et al*. 1985). The p53 protein was mainly localized to

nucleus and was capable of binding to DNA (Gannon and Lane, 1987). It was, moreover, phosphorylated on multiple serine and threonine residues (Meek and Eckhart, 1988).

Biological function of the p53 gene product. p53 may participate in DNA replication and play a role in cell cycle progression. The p53 protein product was largely absent from cells that have just completed division but accumulated in the G1 phase of the cell cycle (Diller, *et al.* 1990). Expression of p53 in proliferating cells inhibited progression from Go/G_1 to S phase (Mercer, *et al.* 1990). Inhibition of cell cycle progression was, in turn, associated with a marked decrease in histone H3 mRNA expression, an S-phase marker gene whose function was tightly linked to DNA replication. These effects might be mediated partly through down-regulation of proliferating cell nuclear antigen, which has been identified as a cofactor of DNA polymerase delta, an important component of replicative DNA synthesis (Mercer, *et al.* 1991). It has also been noted that p53 inhibited SV40 DNA replication by repressing the binding of SV40 large T antigen to polymerase alpha (Wang, *et al.* 1989). This result suggested that wild type p53 might interfere with the binding of cellular initiation proteins to polymerase alpha.

p53 may participate in the regulation of gene transcription as a transacting protein. The fusion protein between portions of p53 and the binding domain of yeast GAL4 exhibited transcriptional activity on the reporter genes both in yeast and mammalian cells (Fields and Jang, 1990).

Analogous fusion proteins, formed instead from a mutant p53, lacked the ability to induce the activity of a reporter gene (Raycroft, *et al.* 1990). These data suggested that wild-type p53 protein might coordinately activate the transcription of a set of genes responsible for negatively regulating key events in cell proliferation.

Some DNA tumor virus have been shown to be the causative agent for human cancer, as human papilloma-virus (HPV) was associated with genital tumors. Gene products, such as SV40 tumor antigen, E1a and E1b in adenovirus, E6 and E7 protein of human papilloma virus type 16 and type 18, encoded by the viral genome, could lead to cellular immortalization and transformation, Interestingly, the p53 gene could form complexes with certain of these viral protein, such as adenovirus E1b, SV40 T antigen, and HPV E6 protein (Werness, *et al.* 1990). It has been shown that murine p53 protein could bind to SV40 large T antigen and thus inhibit its ability to initiate and replicate SV40 DNA (Braithwaite, *et al.* 1987; Sturzbecher, *et al.* 1988). However, it is not known whether the p53 protein has this effect on viral tumorigenesis in humans, particularly, the genital cancers in which infection by HPV16 and HPV18 is prevalent. This laboratory has observed that p53 mutations occurred frequently in cervical carcinoma cell lines, two out of the five cell lines examined showed mutations in this gene. Three of these cell lines contained HPV16 or HPV18, which showed non-detectable level of p53 protein but higher levels of p53 mRNA than in those devoid of

HPV genome (unpublished data). A recent study reported that the E6 oncoprotein encoded by HPV16 and HPV18 could selectively stimulate the degradation of p53 (Scheffner, *et al.* 1990). This result might explain the low levels of p53 protein detected in many cervical carcinoma cell lines and HPV16, HPV18 transformed cell lines. The deprivation of cellular proteins, like p53, by the viral oncoprotein may result in the deregulation of cellular proliferation and differentiation process.

p53 gene acts as a tumor suppressor gene. The cumulative data gathered to date suggest that p53 functions as a cell growth suppressor gene, whose defective or lack of function contributes to the development of malignancy. It has been found that rearrangement and functional inactivation of the p53 gene occurred at a high frequency in mouse spleen tumors induced by the friend erythroleukemia virus (Mowat, *et al.* 1985; Chow, *et al.* 1987). Similarly, mutation of p53 has been observed in a broad range of human malignancies. The recent findings of germline mutation of the p53 in cancer prone families with the Li-Fraumeni syndrome further supported the two "hit hypothesis" proposed by Knudson (Srivastava, *et al.* 1990, Malkin, *et al.* 1990). Members of such families tend to develop different types of tumors, such as, breast carcinomas, soft tissue sarcomas, brain tumors, osteosarcomas, and leukemia, all at an early age. Fibroblast cells derived from normal skin biopsies from those cancer patients in the same family were found to harbor an identical point mutations

in one allele (the other allele remained normally) at the most conserved region, but the tumor cells were found to have only the mutant allele. This result suggested that mutation of p53 might be one of the primary genetic defects in these cancer-prone families.

Direct experimental evidence showing that p53 was an antioncogene came from the finding that wild type p53 inhibited oncogene-mediated cellular transformation and tumor cell growth (Finlay, *et al.* 1989; Eliyahu, 1989). Wild type p53 was shown to be capable of inhibiting focus formation in rat embryo fibroblast cell transfected with a combination of oncogenes, such as E1a plus ras, myc plus ras, as well as mutant p53 plus ras; but the mutant p53 failed to exhibit such inhibitory activity. When the wild type p53 was introduced into human colon cancer cell lines which contained only one mutant allele of the p53 gene, clonal growth of these cell lines was inhibited (Baker, *et al.* 1990).

While wild type p53 possessed antiproliferative effect, certain mutant p53 genes could enhance the oncogene mediated cellular transformation. Mutant p53 could immortalize and enhance the transforming ability of the ras gene in early passage rodent cell (Eliyahu, *et al.* 1984). Moreover, different p53 mutants might exhibit distinct biological activities, such as, transforming function; or they might form oligomers with the wild type p53 and neutralize its suppressor function, the so called negative dominant effect (Halevy, *et al.* 1990; Lavigueur, *et al.*1989). All of the

mutant p53 proteins that were active during transformation had rather extended half-lives, ranging from about 10 (6 - 20) minutes to 6 (2 - 12) hours and were found in correspondingly higher concentrations in transformed cell lines and tumor tissue. Because of its very short half life, wild type p53 protein was almost undetectable in conventional immunochemical and histochemical assays. Thus, the ready detection of this protein could be taken as synonymous with mutation. Most transforming variants of p53 exhibited a specific interaction with the major heat-shock protein, hsc70 which might be an important step in endowing these variants with oncogenic properties (Hinds, *et al.* 1987; Finlay, *et al.* 1988). Conversely, the mutant protein failed to bind SV40 large-T antigen as efficiently as the wild type, and also was incapable of inhibiting SV40 replication *in vivo* and *in vitro* (Braithwaite, *et al.* 1987; Sturzbecher, *et al.* 1988).

p53 gene mutation in human malignancy. Using the polymerase chain reaction and direct nucleotide sequencing, a number of studies have shown that p53 mutations were prevalent in a wide variety of human malignancies, ranging from pediatric tumors to the most common adult solid tumors (Table 1). Since the conserved regions were essential for the function of p53 gene product, these regions were commonly amplified and analyzed. As a matter of fact, most of the mutations found were clustered in these regions. The four most frequent target regions for mutation of p53 gene clustered around codons 132-145; 171-179; 239-248; and 272-286

Table 1. Frequency of p53 Gene Mutation in Human Tumors

Tumor	Case Number	Mutation Rate	Reference
Colon	58	50%	Baker, (1990)
Lung	30	57%	Takahashi, (1989)
Esophagus	18	39%	Hollstein, (1990)
Liver	16	50%	Hsu, (1991)
Diverse tissue	21	71%	Baker, (1990)

(Figure 3) (Nigro, *et al.* 1989). Cytogenetic studies and restriction fragment length polymorphism (RFLP) analysis of tumors have shown that a loss of heterozygosity of chromosome 17p at loci flanking the p53 gene occurred in tumors derived from colon, breast, lung, ovaries, cervix, adrenal cortex, bone, bladder, skeletal muscles and brain tumor (Baker, *et al.* 1990; Mulligan, *et al.* 1990). The remaining p53 allele usually was mutated as revealed by sequencing. Frequent mutations were also observed in tumors without allelic deletions (Baker, *et al.* 1990). Although the mutations in diverse tumors scattered over four of the five evolutionarily conserved domains, a specific point mutation of codon 249 appeared to be associated to the hepatocellular carcinoma, which was possibly caused by infection with hepatitis B virus or exposure to the hepatocarcinogen, aflatoxin B1 in high incidence areas (Hsu, *et al.* 1991). The mechanistic basis of such a site specific mutation was not known. DNA adducts formed by aflatoxin were able to induce G to T transitions in animal mutation assays (McMahon, *et al.* 1990).

Besides point mutations, p53 rearrangements and homozygous deletions were also prevalent in certain types of tumors. One study has shown that 14 out of 31 rhabdomyosarcomas and 12 out of 29 osteosarcomas exhibited the structural alterations of the p53 gene, or altered expression of its product (Mulligan, *et al.* 1990). In chronic myelogenous leukemia, rearrangements of the p53 gene (30%) appeared to be associated with progression to the "blast

Figure 3

A schematic diagram of the "hot spots" of the p53 gene. The figure was constructed according to published data (Baker, *et al*. 1990). Numbers denote the amino acid codon. Four exons, V to VIII are indicated by open boxes. Black boxes denote the frequent mutation sites.

codon #
100
200
300
400
exons
V
VI
VII
VIII
hot spots
132-145
171-179
239-248
272-286

crisis" phase (Kelman, *et al.* 1989). The wide involvement of the p53 in human malignancies is indicative of the central function this gene plays in regulating cell growth. Further study will contribute to a better understanding of the molecular mechanisms underlying such tumorigenesis.

Risk Factors Implicated in the Development of Human Esophageal Carcinoma

Many studies have been undertaken to elucidate the causative factors of esophageal carcinoma. The risk factors appear to vary with geographical region and cultural differences of the populations. A high intake of alcohol and cigarette smoking seemed to be associated with the development of esophageal cancer in western countries and South Africa (McGlashan, *et al.* 1982), whereas high-level exposure to chemical carcinogens or mutagens, nutritional deficiency, and genetic predisposition might play important roles in northern China (Yang, 1980). However, it would appear that none of these factors alone could explain the high incidence in this area of China.

The convincing evidence of genetic susceptibility to esophageal carcinoma was found in a rare cancer-prone syndrome, tylosis, which was an autosomal dominant disease characterized by thickening of the skin on the palms and soles. Cosegregation of the esophageal carcinoma with the disease was observed in the tylosis families (Harper, *et al.*

1970). However, there were only few such families worldwide and accounted for negligible cases of the esophageal cancer. More evidence emerged from the epidemiological surveys in the high incidence population (Wu, *et al*. 1989). The esophageal carcinoma showed a strong tendency for familial aggregation. A study carried out in Yangcheng, China, a high incidence area, revealed that 54% of the patients with esophageal cancer had a positive family history, 65% of cancer families had patients with esophageal carcinoma in 2 successive generations, and 6% of cancer families had patients in 3 successive generations. The youngest patient was only 15 years old (Li and He, 1985). Another study has shown that the standardized mortality rate, relative risk, and indirect age-adjusted mortality rate in blood relatives of patients with esophageal cancer were significantly higher than those of non-blood relatives and matched controls living in the same environment (Ding and Wu, 1983). A recent case-controlled study of 1244 patients and 1314 control individuals found that the risk increased 70% for patients whose parents had esophageal or stomach cancer, but only slightly increased for those whose spouses had such cancers. No other single strong environmental or dietary risk factor could be defined (Li, *et al*. 1989). In addition, a high incidence of esophageal carcinomas was observed in populations who migrated from a high incidence area to a low incidence area (Wu, *et al*, 1989).

In laboratory investigations, a decreased level of unscheduled DNA synthesis of peripheral lymphocytes after UV

irradiation was found in subjects with a positive family history, and an increased rate of sister chromatid exchange was detected in short-term cultivated lymphocytes after induction with nitroso-methylbenzylamine (Wu, *et al.* 1986; Wu, *et al.* 1989). Studies on the chromosomes of peripheral lymphocytes from 103 members of 5 high-risk families and from 30 members of 4 low-risk families showed that the frequency of structural and numerical aberrations were much higher in members from high-risk families than in those from low-risk families (Wu and Ran, 1979). These results provided supporting evidence for decreased DNA repair capacity and increased genetic instability in the cancer prone families. Taken together, these results suggested that genetic predisposition or exposure to environmental factors early in life might provide an explanation for the high susceptibility seen in certain families.

Esophageal cancer was prevalent mainly in poor populations. In high incidence areas, the population subsisted mainly on corn, wheat or millet, and generally consumed small amounts of fresh vegetables, fruits and animal products. As a result, dietary intake of vitamins A, C, B_2, trace elements, such as, zinc and molybdenum was low among these populations (Yang, 1980). The mechanism by which nutritional deficiency promoted esophageal cancer was not understood clearly. In a pilot clinical trial conducted in China, the incidence of precancerous lesion was lower in subjects whose serum levels of retinol, riboflavin, and zinc

were elevated medically than in those who served as controls (Wahrendorf, *et al.* 1988). Experimentally, a zinc deficient diet led to induction of the esophageal tumors with higher frequency and shorter lag time in rats treated with Methylbenzylnitrosamine (MBNA) (Fong, *et al.* 1978). Riboflavin was important for the maintenance of the integrity of the esophageal squamous epithelium. It also participated in the biotransformation of certain precarcinogens in the liver (Wang, *et al.* 1985). Vitamin A played a role in the normal cellular differentiation of the epithelium and to inhibit tumor promotion (Sporn and Roberts, 1983). Nutritional deficiency may lead to a poor defense against the assaults of chronic irritation and/or inflammation. The vulnerable mucosa and submucosa exhibit defective repair capacity and are easily succumbed by the attack of mutagenic and carcinogenic agents, a process culminating in the formation of invasive cancer.

Frequent contamination of foodstuffs with fungi has been observed in high incidence areas. Pickled vegetables, consumed frequently in Linxian, have long been suspected to be an important source of such contamination (Yang, 1980; Li, *et al.* 1986). During the fermentation process, the contamination with many species of fungi could occur. Among the molds, Fusarium moniliforme has been shown to be a particularly common contaminant; indeed, it has been documented that prolonged feeding of fungus-inoculated cornbread alone was capable of inducing the development of papillomas and

carcinomas in the forestomach of rats (Li and Cheng, 1984). Fungal contamination might also increase the level of secondary amines in food and promote the synthesis of nitrosoamines (Li, *et al.* 1986; Singer, *et al.* 1986). Moreover, extracts of pickled vegetables have been shown to be mutagenic and tumorigenic in animal models (Lu, *et al.* 1981). On the other hand, a recent case-controlled study conducted in Linxian failed to establish an association with adult intake of pickled vegetables and esophageal neoplasia (Li, *et al.* 1989).

It has been suggested that any condition associated with chronic injury to the esophageal mucosa predisposed this tissue to the development of squamous cell carcinoma (Correa, 1982; Crespi, *et al.* 1984; Shu, 1985). Therefore, attention has been focused on the prevalence of non-specific chronic esophagitis in high incidence areas. Based on the results from mass endoscopic survey in high incidence populations, chronic esophagitis was now considered to be a precancerous condition (Crespi, *et al.* 1979). The condition was prevalent in high incidence area, but uncommon in low risk area (Crespi, *et al.* 1984; Oettle, *et al.* 1986). It was frequently accompanied by atrophy and dysplasia (Crespi, *et al.* 1979; Munoz, *et al.* 1982). The location of the mucosal lesions was similar to that of cancer, both involving the middle and lower thirds of the esophagus. Progression from mild to severe lesion was observed in a limited follow up study (Munoz, *et al.* 1985). The incidence of chronic esophagitis was

significantly higher in high risk population among young individuals aged 15 to 26 years old, and in those with a family history of esophageal cancer (Wahrendorf, *et al.* 1989; Chang-Claude, *et al.* 1990). These findings suggest that chronic injury to the esophageal mucosa during early life and or any esophagitis of long standing may predispose to the ultimate development of esophageal carcinoma.

Nitrosamines were the principal compounds known to induce esophageal tumors in animal models. High levels of nitrosamines, such as dimethylnitrosamine, diethyl-nitrosamine and methyphenylnitrosamine were found in the foodstuffs and water in the high incidence areas of China (Lu, *et al.* 1986). Moreover, higher level of nitroso-compounds were excreted in the urine of subjects from these areas (Lu, *et al.* 1986). These data indicated that subjects living in high-risk areas were exposed to greater levels of N-nitroso compounds than those in low-risk areas. Alkylation of DNA was one of the most likely mechanisms for the initiation of mutagenic and carcinogenic processes (Doerfler, 1983). Direct evidence has shown that the DNA alkylation product, O^6-methyl deoxyguanosine was higher in the DNA samples from Linxian than in those from control areas (Lu, *et al.* 1986). This DNA adduct was known to induce G to T transition during DNA replication (Mitra, *et al.* 1989), a substitution that may activate oncogenes, or alternatively, inactivate tumor suppressor genes, ultimately resulting in tumor progression.

Pathogenesis of Human Esophageal Carcinoma

Carcinogenesis of the esophageal epithelium appeared to unfold in multiple stages. The insult of chronic inflammation to an esophageal mucosa in individuals with certain genetic predispositions might last for a considerable proportion of the life span of the susceptible individual (Chang-Claude, *et al.* 1990). Extensive histo-cytological surveys disclosed that esophageal dysplasia characterized by a disruption of the normal architecture of the esophageal epithelium accompanied by proliferation of atypical cells was the advanced precancerous lesions seen (Shu, 1985). These studies also revealed that the incidence of esophageal dysplasia coincided with that of esophageal cancer in the high-risk population. Severe dysplasia preceded invasive cancer by seven to eight years (Crespi, *et al.* 1984; Qiu and Yang, 1988). Accordingly, the following sequential events in the genesis of esophageal cancer were: normal epithelium -> chronic esophagitis with hyperplasia or atrophy -> dysplasia-> intraepithelial carcinoma -> invasive carcinoma -> metastasis (Correa, 1982; Crespi, *et al.* 1979; 1984; Munoz, *et al.* 1982).

Current Molecular and Genetic Studies of Esophageal Carcinoma

The molecular genetic study of neoplasia has progressed rapidly since the discovery of oncogenes over a

decade ago. A better understanding of the mechanism of tumorigenesis now exists as result of the discovery of the tumor suppressor genes in hereditary and sporadic malignancies in recent years. However, the esophageal cancer has drawn less attention than the more common cancers arising in the colon, lung and breast. Nevertheless, a limited numbers of studies have suggested the involvement of oncogenes and antioncogenes in esophageal carcinoma. Most notable was the finding that coamplification of the hst1 and int2 genes, members of the fibroblast growth factor (FGF) family, was found in about 50% of the esophageal cancers studied (Tsuda, *et al.* 1989), though products of these genes have not been detected in the tumor tissues. The FGF family has been implicated in many aspects of cell-growth, angiogenesis, organogenesis, and tumor development (Burgess and Maciag, 1989). A recent report indicated that coamplification of these two genes correlated with a poorer clinical outcome and increased incidence of distant organ metastasis (Kitagawa, *et al*, 1991).

EGF-receptor amplification and/or overexpression were found in many squamous cell carcinomas (Yamamoto, *et al.* 1986). Studies have shown this gene to be amplified and its expression to be elevated in human esophageal squamous cell carcinoma (Ozawa, *et al.* 1989; Yoshida, *et al.* 1990). Similarly, frequent amplification of the gene for EGF receptor and myc oncogene was detected in DNA samples from the Linxian area (Lu, *et al.* 1988). Recently it has been shown that the

elevated level of EGF receptor predicted a poor prognosis for patients with esophageal cancer (Ozawa, *et al.* 1989).

The ras gene family was most frequently activated in human tumors (Bos, 1988). In one study, the DNA sample of an esophageal carcinoma in a patient from Linxian exhibited the transforming activity due to an activated H-ras gene (Jiang, 1988). Rat esophageal papillomas induced by MBNA also showed an activated H-ras gene (Wang, *et al.* 1990). However, other reports, using the polymerase chain reaction failed to detect mutated ras genes in large numbers of samples of this type of tumor (Hollstein, *et al.* 1988).

Cytogenetic studies of the esophageal carcinoma cell lines revealed several nonrandom chromosomal structural abnormalities, most of which were chromosomal deletions (Whang-Peng, *et al.* 1990). The chromosome deletions may indicate the loss of certain genes whose functions are the negative regulator of cell growth. Loss of function of tumor suppressor genes, due to deletion or point mutation, was implicated in a variety of tumors. Of the suppressor genes, the p53 gene, which encodes a nuclear phosphoprotein, has been demonstrated in a wide spectrum of malignancies. Recently, p53 mutations were also found to occur frequently in esophageal carcinoma cell lines and in tumor tissues from patients in the United States (Hollstein, *et al.* 1990). Consistent with this finding, a frequent loss of heterozygosity on 17p (45%) has been reported in Japanese patients with esophageal squamous cell carcinoma (Wagata, *et*

al. 1991).

Since both oncogenes and tumor suppressor genes are frequent targets for the genetic changes induced by a variety risk factors, a search for the active oncogene or defective tumor suppressor should provide insight into the mechanisms underlying the causation and progression of esophageal carcinoma. Because there are fewer molecular genetic studies on esophageal carcinoma as compared to other common tumors, we have used the DNA mediated gene transfer assay and the polymerase chain reaction to identify the dominantly transforming genes and suspected mutant tumor suppressor genes in human esophageal carcinoma from a high incidence area in China.

MATERIALS AND METHODS

Tumor Samples and High Molecular Weight (HMW) DNA Preparation

Eighteen esophageal carcinoma samples were kindly provided by the Henan Institute of Medical Sciences; these samples were obtained at Linxian County Hospital and other regional hospitals. The tumor samples were quickly frozen and stored at -70°C freezer prior to the isolation of HMW DNA. The tumor tissue was minced, and incubated with buffer A (0.01 M Tris pH 8, 0.01 M NaCl, 0.01 M EDTA and 0.5% SDS) in the presence of Proteinase K (100 μg/ml) at 37°C overnight to 2 days. The isolation of HMW DNA was carried out with the method described previously (Gross-Bellard, *et al*. 1973). The DNA was gently extracted with half volume of water-saturated phenol and half volume of chloroform / isoamyl alcohol (24:1) three times, equal volume of chloroform / isoamyl alcohol one time. The DNA was precipitated with two volumes of absolute alcohol and 1/15 volume of 3 M NaOac (pH 5.5) at -20°C overnight. The DNA pellet was dissolved in Buffer D (0.01 M Tris pH 8.0, 0.01 M NaCl 0.01 M EDTA) and dialyzed against a large excess of the same buffer at 4°C for 3 days. Five cervical carcinoma DNA samples were isolated from cervical carcinoma cell lines HTB31, HTB32, HTB33, HTB34, HTB35 (ATCC). Six breast carcinoma DNA samples with their peripheral

lymphocyte DNA samples were provided kindly by Dr. J. Taylor-Papadimitriou, Imperial Cancer Research Funds. The normal control DNA samples were collected in this laboratory obtained either from human placenta or fibroblast cell lines.

DNA Transfection and Tumorigenesis Assay

NIH3T3 cells were cultured in Dulbecco's modified essential medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS). Using the calcium phosphate precipitation method (Graham and Van Der Eb, 1973; Wigler, 1978), 2X 10^5 cells in a 75 cm^2 flask were cotransfected with 25 μg HMW DNA and 600 ng pSV_2 neo plasmid DNA. The cells were cultured in the presence of 0.4 mg/ml of G418 (Geneticin, GIBCO) for two weeks and in the absence of G418 for one additional week. Surviving cells were harvested (without selection of morphological transformed colony) and injected subcutaneously into nude mice approximately 200 to 250 colonies from one flask per mouse. The mouse was examined for tumor development twice a week. Any induced tumors were excised when they reached a size of 1 cm x 1 cm. From these tumors, the transformed mouse cell lines were established *in vitro* as follows: the tumors were minced and treated with 2 ml trypsin (0.25 mg/ml) for 2 minutes; 10 ml DMEM was added to the trypsinized tissue; the supernatant was seeded into two 75 cm^2 flasks and fed with DMEM supplemented with 15% FBS and 3X PSN (1X PSN, 50 μg/ml each of penicillin, streptomycin and

neomycin). The medium was replaced the next day with regular DMEM medium.

The DNA from the induced mouse tumors was analyzed for the presence of human Alu sequences by Southern hybridization. The Alu positive primary tumor DNA was then used as donor for the second round of transfection. The transfection of the expressional plasmids DNA was performed under the same conditions as above except for the use of 1 μg of plasmid DNA with 15 μg of sheared and phenol extracted calf thymus DNA as carrier.

Genomic DNA Cloning and cDNA Cloning

Preparation of DNA fragment. HMW DNA was isolated from the secondary mouse tumor 128-15:19T #5 (m693). The DNA was digested with EcoRI under the conditions recommended by the supplier. Digested DNA was electrophoresed in 0.6% agarose gels. DNA fragment around the marker for 6.6 kb was excised. The DNA was purified by a rapid method, in which the excised piece of agarose gel was frozen at -70°C for one hour, and then squeezed tightly to obtain the DNA containing buffer. The DNA was precipitated with two volumes of absolute ethanol plus 1/15 volumes of 3M NaOAC (pH, 5.5).

***In vitro* packaging.** 0.1 μg of DNA fragment was ligated to 0.9 μg of λgtwes EcoRI arms (BRL) by 2 U T4 DNA ligase in 8 μl buffer (as described in Transformation section). The ligated materials were added to two tubes of

Freeze/Thaw (Gigapack packaging extracts, Stratagene) on ice. 15 μl of Sonic Extract was then added and mixed. The reaction was carried out at room temperature for two hours. The reaction was then diluted with 0.5 ml of SM buffer (Per Liter: 5.8 g NaCl, 0.2 g $MgSO_4.7H_2O$, 50 ml 1 M Tris.HCl, pH 7.5, 5 ml 2% gelatin) with addition of 20 μl chloroform.

Screening of DNA library. The bacteria host (LE 392 for λgtwes, Y1088 for λgt11) was cultured in LB (Per liter: 10 g Bacto-tryptone 10 g NaCl, 5 g Yeast extract) medium supplemented with 10 mM $MgSO_4$ and 0.2% maltose overnight. The bacteria was collected by centrifugation and resuspended in 0.4 volume of the original culture with 10 mM $MgSO_4$. The optical density was adjusted to 0.5. For plating on a 130 mm plate, 0.1 ml phage and 0.6 ml bacteria were mixed and incubated at 37°C for 15 minutes. 7 ml of LB top agar (0.7% agarose in LB medium) was added to the mixture and poured on to a LB bottom agar plate (1.2% bacto agar in LB medium). The plates were incubated at 37°C for 7-9 hours.

The partial genomic library constructed was screened by a human Alu probe. A cDNA library (λgt11) was established from the poly A^+ enriched mRNA of the secondary transformant (128-15-19T #5 m693) (constructed by America Biotec, Inc.). The cDNA library was screened by a 1.8 kb BamHI genomic fragment containing portion of the HER2 gene encoding the cytoplasmic domain. This 1.8 kb fragment was isolated from the 6.6 kb genomic fragment originally from the secondary transformant mouse DNA 128-15:19T #5 (m693).

Polymerase Chain Reaction

PCR was performed in 100 μl of reaction volume containing 20 μM dNTP, 1 μg sense, and 1 μg antisense primer, 1-2 μg HMW DNA, and 2.5 units Taq polymerase (BRL) in a buffer mixture (10 mM Tris.HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin). Asymmetric PCR reactions were carried out for generating single stranded product by using diluted primary regular PCR product with a pair of primers consisting of 1 or 2 nested primers in a molar ratio of 1 to 100. All PCR reactions were performed on Perkin Elmer Cetus DNA Thermal Cycler. The conditions for all PCR reactions were determined by the primer's Tm value and the length of the amplified fragment, and were described in each figure legend. The primers used in all PCR reaction are listed in Table 2.

Transformation

Ligation. The plasmid vector and DNA fragment were ligated (molar ratio 1:1) by 1 unit T4 DNA ligase (BRL) in 10 μl of ligation buffer (66 mM Tris.HCl, pH 7.6, 6.6 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, 10 mM Spermidine) at room temperature for 1 hour, and at 12°C for overnight with addition of 1 u T4 DNA ligase.

Preparation of competent cell. DH5α (BRL) was used as the bacteria host cell. The cells from 100 ml of exponential growing culture ($O.D._{600}$ 0.4) were collected by centrifugation,

Table 2. Oligonucleotides Used in This Study

1. HER2 transmembrane region:

   1). 5′-TCCGCCCCCAGCCCTCTGACG-3′ (SENSE)
   2). 5′-CCGTTTCCTGCAGCAGTCTCCGC-3′ (ANTISENSE)
   3). 5′-CCGGATCTTCTGCTGCCGTCGC-3′ (ANTISENSE)
   4). 5′-GTCCATCGTCTCTGCG-3′ (oligonucleotide A)
   5). 5′-GTCCATCATCTCTGCG-3′ (oligonucleotide B)

2. HER2 promoter region primers:

   1). 5′-TTTTACTAGAGGATGTGGTGGG-3′ (SENSE)
   2). 5′-AGGCCTGCGCGAAGAGAGGG-3′ (SENSE)
   3). 5′-GCTGAGTTCCCCTCCATTGG-3′ (ANTISENSE)
   4). 5′-CCCTTTACTGCGCCGCGCGCC-3′ (ANTISENSE)

3. p53 primers:

   1). 5′-GTAGGAATTCGTCCCAAGCAATGGATGAT-3′ (SENSE)
   2). 5′-CATCGAATTCTGGAAACTTTCCACTTGAT-3′ (ANTISENSE)
   3). 5′-TTCCTCTTCCTGCAGTACTCC-3 (SENSE)
   4). 5′-CGGAATTCAGGCGGCTCATAGGGC-3′ (ANTISENSE)
   5). 5′-CAGGTCTCCCCAAGGCGCACTGGCC-3′ (SENSE)
   6). 5′-AAGGGTGAAATATTCTCC-3′ (ANTISENSE)
   7). 5′-CTTCCAGTGTGATGATGGTGAGG-3′ (ANTISENCE)

4. Alu primers:

   1). 5′-GGTTGCAGTGAGCCGAGATC-3′ (SENSE)
   2). 5′-GTTTCACCATGTTGGTCAGGC-3′ (ANTISENSE)

resuspended in 20 ml ice-cold buffer I (50 mM Mops pH 7.0, 10 mM RbCl), and repelleted. The pellets were then resuspended in 20 ml ice-cold buffer II (100 mM Mops pH 6.5, 10 mM RbCl, 70 mM $CaCl_2$); incubated on ice for 30 minutes, repelleted and resuspended in 2 ml buffer II.

Transformation. 250 μl of competent bacteria, 1 μl (5-10 ng) DNA and 4 μl of DSMO were mixed in a polypropylene tube (Fisher) and incubated on ice for 30 minutes. The tube was then put at 42°C for 60-80 seconds and returned on ice for 30 minutes. 3 ml LB medium was added to the tube and mixed. The tube was placed in a shaking incubator at 42°C for 90 minutes. An aliquot of the culture was plated on a 1.2% agar LB plate with 50 μg/ml of ampicillin, incubated at 37°C overnight.

Nucleotide Sequencing

Double strand plasmid DNA sequencing. The genomic DNA or cDNA fragment was subcloned in vector PGEM blue 4 (Promega) containing the SP6 and T7 promoters flanking the multiple cloning site. The plasmid DNAs were prepared by following the protocol provided by the DNA sequencing kit, K/RT™ (Promega) with modification. The bacterial pellet from a 1.5 ml overnight culture was suspended in 100 μl ice-cold solution containing 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl for 5 minutes at room temperature. 200 μl of newly-made solution containing 0.2 N NaOH, 1% SDS was added, mixed and incubated

on ice for 5 minutes. Then, 150 μl ice-cold KOAC was added, mixed and put on ice for 5 minutes. The mixture was centrifuged on a microcentrifuge for 15 minutes. The supernatant was extracted with phenol/chloroform three times and chloroform two times. The DNA was precipitated with absolute ethanol at -70°C for 20 minutes. The DNA pellet was washed with 70% ethanol two times and resuspended in TE buffer. The DNA sequencing reactions were carried out by dideoxy termination method using a Sequenase kit (USB) or a K/RT™ kit)(Promega).

PCR product sequencing. The single stranded templates were generated by asymmetric PCR. Reaction products were purified by filtration though Centricon 30 (Amicon). The sequencing reactions were performed by a modified procedure of the manufacturer's protocol (Sequenase kit, USB). It is described here as follows: A mixture containing 2 μl of primer (40 ng), 2 μl of reaction buffer and 6 μl (one fourth of product from a single PCR reaction) was boiled for 5 minutes, quenched on ice for 2 minutes, and then incubated at 50°C for 15 minutes. Samples were put on ice again prior to the labeling and termination reactions which were performed according to the protocol supplied with the kit. The sequencing reaction was run on 6% polyacrylamide gel. The gel was processed, dried and exposed to Kodak XAR 5 film for overnight.

Nucleic Acid Hybridization

Southern and Northern hybridization. HMW DNA was digested by restriction enzymes according to the conditions recommended by the enzyme manufacturers. The samples were analyzed on 0.6% agarose gels, and transferred to nylon membrane (ICN) by standard Southern method (Southern, 1975). Cytoplasmic total RNA was isolated by the Triton-phenol-chloroform method (Bastos, *et al.* 1977). The RNA was fractionated on 1% agarose gel in the presence of 1.96 M formaldehyde and transferred to nylon membrane as described (Fasano, *et al.* 1984). The membranes were hybridized with ^{32}P-labeled DNA fragment at 40°C in a solution of 40% formamide, 5X Denhardt's solution (50X Denhardt's, 1% of each Ficoll, polyvinylpyrrolidone, BSA), 5X SSC (20X SSC, 3 M NaCl, 0.3 M NaCitrate), 0.01 M Tris (pH7.5), salmon sperm DNA (0.25 mg/ml), and 10% dextran sulfate for 16-18 hours. The blots were washed with 2X SSC and 0.1% SDS 3 times at room temperature followed by 2 washes at 50°C in 0.1% SSC and 0.1% SDS. The membrane was exposed to Kodak film. The complete HER2 cDNA probe was isolated from the library constructed from mRNA of 128:15-19T #5 m693. The human EGF receptor probe was a 798 bp cDNA fragment and a 1838 bp cDNA fragment. N-ras probe was a 500 bp cDNA fragment. The c-raf probe was a 3 kb cDNA fragment.

Filter hybridization. The bacterial colonies or bacteriophage plaques were transferred onto nitrocellulose

filters (MSI) and were lysed with a solution containing 0.9 M, NaOH and 1.5 M NaCl for 1-2 minutes, neutralized with a solution of 1 M Tris.HCl and 1.5 M NaCl for 3 minutes, three times. The filters were baked at 80°C for two hours and were ready to be used. The filters were incubated with ^{32}P-labeled DNA probe in Solution 3 (4XSSC, 1.5% BSA, 1.5% Ficoll, 1.5% PVP, 0.1% SDS) at 65°C for 12-16 hours, and then washed with 0.1X SSC and 0.1% SDS 2 to 3 times at the same temperature.

Oligonucleotide probe hybridization. The PCR products were electrophoresed on 2% agarose gel and were transferred onto nylon membrane by standard Southern method. The nylon membranes were hybridized with ^{32}P labeled oligonucleotides in a solution containing 5X SSPE (20X SSPE, 3.6 M NaCl, 0.2 M NaH2PO4, 20 mM EDTA), 5X Denhardt's solution, 0.1% SDS, at 50°C for 3 hours, and washed in 2X SSPE at RT, and then with 5X SSPE, 0.1% SDS at 54°C. The washed membranes were exposed to Kodak XRP 1 film for 2-4 hours. The end-labeling reaction was carried out in 20 μl solution (70 mM Tris.HCl, pH 7.6, 0.1 M KCl, 10 mM $MgCl_2$, 5 mM DTT, 0.5 mg/ml BSA) consisting of ^{32}P-ATP 40 μC, 1 μg oligonucleotides, 10 u T4 kinase. The oligonucletides of HER2 transmembrane region are listed in Table 2.

RESULTS

Identification of HER2 Oncogene in a Transformed NIH3T3 Cell Line by DNA from One Esophageal Carcinoma

Identification of Human DNA Sequences in a Secondary Transformant DNA

A number of oncogenes implicated in human cancer have been identified by the DNA transfection assay. In an attempt to identify the gene involved in human esophageal squamous cell carcinoma, tumor samples were obtained from a high incidence area, Linxian, China and used in a DNA transfection assay. NIH3T3 cells, cotransfected with tumor DNA and a selective marker pSV2neo, were pooled and inoculated into nude mice subcutaneously. Of the 12 tumor DNA samples examined by transfection, 44 out of 55 injected mice developed tumors. All of these tumors appeared at about 4 weeks after inoculation and were excised when they reached a size of 1 cm x 1 cm. The mice injected with the parental NIH3T3 cells did not develop tumors within a 2 month period of time. Seventeen of the 44 tumors were found to contain human Alu sequences (Table 3) as detected by hybridization with Alu probe. Figure 4 shows the 5 examples of the DNA from these tumors containing the human sequences. Five of tumor DNA samples (97-40:44T #1 to #5) induced by original tumor DNA of m693 were strongly hybridized to the human Alu probe (Figure 4 B). Because of the large

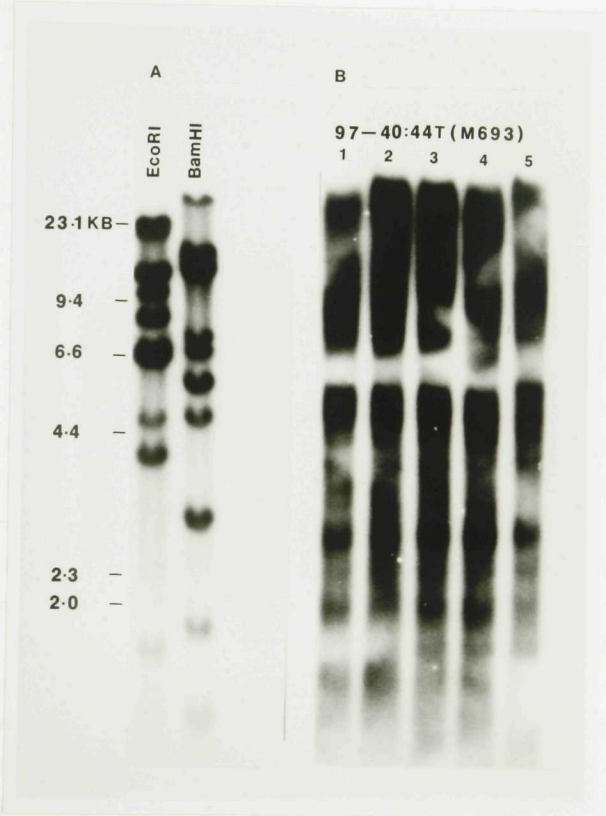
Table 3. Test of Tumorigenesis of DNA from Human Esophageal Carcinomas.

DNA Sample[1]	# Mice injected[2]	# Tumors Formed	Alu+ Tumors[3]
m685	3	3	2
m686	5	0	0
m687	5	4	0
m688	4	4	4
m689	5	5	0
m690	5	5	5
m691	3	3	1
m693	5	5	5
m694	5	5	0
m695	5	0	0
m697	5	5	0
m700	5	5	0
Total	55	44	17

1. DNA samples from the human esophageal carcinomas.
2. Numbers of mice injected with G418 resistant colonies. Each mouse received 200-300 colonies from a single T75 flask.
3. Numbers of human Alu positive tumors detected.

Figure 4

Southern blot analysis of high molecular weight DNA from mouse tumors. Panel B shows five primary transformant tumors 97-40:44T #1 to #5, panel A shows the secondary mouse tumor (128:15:19T #5) induced by DNA of 97-40:44T #2 with molecular weight marker shown. 10 μg of each DNA sample was digested with EcoRI or BamHI, electrophoresed, transferred and hybridized to a ^{32}P-labeled human Alu probe as described in Materials and Methods.


A
B
EcoRI
BamHI
97—40:44T (M693)
1
2
3
4
5
23·1 KB
9·4
6·6
4·4
2·3
2·0

amount of human DNA in the mouse genome, no specific band could be visualized on the radiography.

Five Alu+ tumor DNA samples induced by 3 original esophageal tumors respectively were used in the second round transfection (m690, #4 and #5; m691; m693, #2 and #3). Twenty out of 24 mice injected developed tumors as shown in Table 4. Southern analysis of these mouse tumors revealed that six tumors induced by one of the primary mouse tumors (97-40:44T #2, m693) were Alu positive with a similar band pattern (data not shown). These mouse tumors were designated as 128-15:19T #1 to 128-15:19T #6. In Figure 4 A, one of these tumor DNA was digested by EcoRI (lane 1) and BamHI (lane 2), nine Alu positive bands appeared in the EcoRI digested DNA (lane 1), and 10 bands appeared in BamHI-digested DNA. The intensity of a 6.6 kb EcoRI band was much stronger than the intensity of all other bands suggesting the presence of more abundant repetitive sequences in this band. To test whether these human transforming sequences represented any known oncogene, Southern analysis was performed with oncogene probes. Among the four oncogene probes (c-raf, mos, v-erbA, human EGF receptor) examined, it was found that the cDNA probe of human EGF receptor gene hybridized to several bands of the same size as the bands detected by Alu probe in the transformant tumor DNA (data not shown). Figure 5 showed the comparison of the band patterns of EGF receptor gene among the human DNA, mouse NIH3T3 DNA and the transformant DNA. The band pattern in the transformant DNA (lane 1, first round transformant; lane 2,

Table 4. Test of Tumorigenesis of Alu-Positive Mouse Tumor DNA

Alu+ DNA[1]	# Mice Injected[2]	# Tumors Formed	Alu+ tumors[3]
m690(#4)	5	5	0
m690(#5)	3	0	0
m691(#2)	4	3	0
m693(#2)	6	6	6
m693(#3)	6	6	0
Total	24	20	6

1. DNA from the first round transformants containing the human sequences.
2. Numbers of mice injected with the G418 resistant colonies. Each mouse received 200-300 colonies from a single T75 flask.
3. Numbers of human Alu positive tumors detected.

Figure 5

Detection of human EGF receptor related sequences in DNA of the mouse transformant. 10 μg of genomic DNA were digested with EcoRI, electrophoresed on a 0.8% agarose gel and transferred to nylon membrane. The membrane was hybridized with a human EGF receptor cDNA (789 bp plus 1838 bp EcoRI fragments) under conditions described in the Materials and Methods section. Size of bands is shown. Lane 1, first round tumor; lane 2, second round tumor; lane 3, original esophageal tumor; lane 4, original tumor adjacent tissue; lane 5, NIH3T3; lane 6, human placenta.

1
2
3
4
5
6
23.1 KB
9·4
6·6
4·4
2·3
2·0

second round transformant) was different from the one in human DNA (lane 3, original esophageal tumor, lane 4, original tumor adjacent tissue, lane 6, placenta) and in mouse DNA (lane 5, NIH3T3). This result indicated that the induced mouse tumor contained human DNA sequences, and these DNA sequences probably shared homology with human EGF receptor gene.

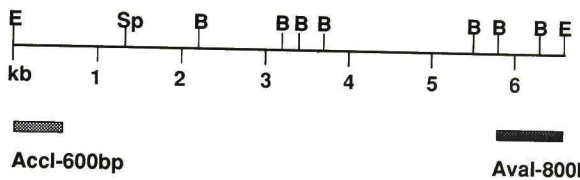
Partial Genomic Cloning of the Human DNA Sequences

To identify the human transforming sequences in these secondary transformants, a partially enriched genomic library was established from the secondary mouse tumor DNA (128-15:19T #5). It was assumed the 6.6 kb EcoRI fragment contained some coding sequence of the gene because it was hybridized to EGF receptor cDNA. For this reason, the EcoRI fragments ranging approximately from 5.5 to 7.5 kb were fractionated on agarose gel and used to construct a λgtwes library. About twenty million clones were screened with Alu probe. Of these, four clones, designated S1, S2, S3 and S4, were Alu positive. Restriction mapping analysis indicated that all four clones were about 6.6 kb in size. Two of theses clones, S3 and S4, were identical, and were ultimately identified as HER2 oncogene fragments. The other two clones, S1 and S2, were identical, but were not a fragment of the HER2 gene. Figure 6 shows a restriction map of these two genomic fragments. The top panel represents the S3 EcoRI 6.6 kb fragment and the

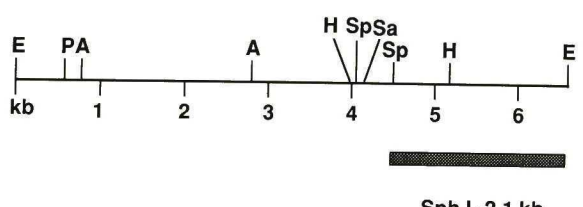
Figure 6

Restriction maps of two human Alu positive 6.6 kb EcoRI fragments. Alu positive clones were subcloned into PGEM blue 4 plasmid. Maps were constructed according to the standard procedure for restriction digestion analysis of plasmid DNA. The top panel represents clones S3 and S4. The bottom panel represents clones S1 and S2. A. AvaI, B. BamHI, E. EcoRI, H. HindIII, P. PstI, Sa. SacI, Sp. SphI. The box below each map indicates the fragments which have been used as DNA probes and have been partially sequenced.

S3 6.6kb EcoR I fragment



S1 6.6 kb EcoR I fragment



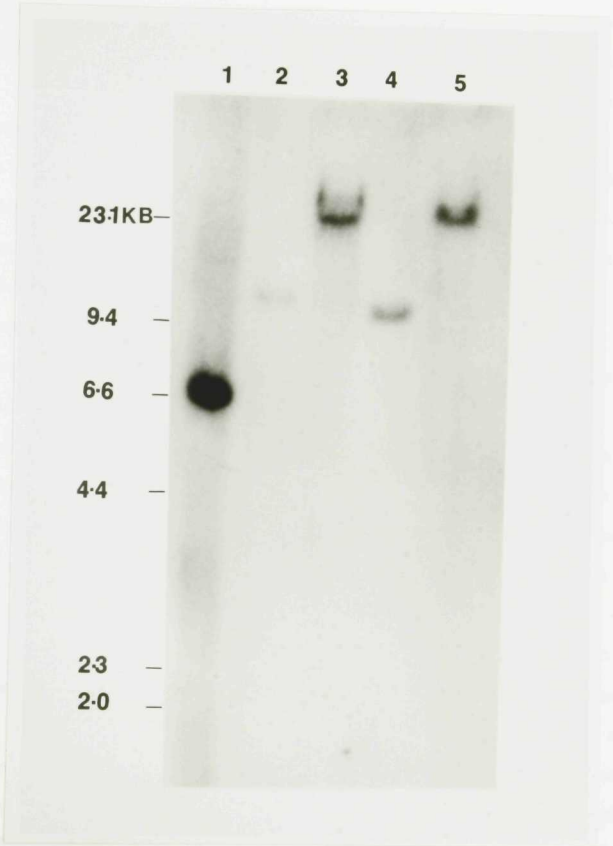
bottom panel represents the S1 EcoRI fragment.

Identification of the HER2 Oncogene in the Transformant

To further characterize the cloned genomic fragments, the S3 DNA fragment was first digested with a variety of restriction enzymes. Southern hybridization was carried out with an Alu probe. Fragments not hybridizing with Alu were selected for further analysis to evaluate the degree of evolutionary conservation. An 800 bp-AvaI fragment and a 600 bp-AccI fragment were found to contain no Alu sequences (see Figure 6). These were subcloned into a PGEM blue 4 vector for generating DNA probes and sequencing. A Southern blot containing DNA samples from a variety of species was hybridized with the 600 bp-AccI fragment. The results showed that the AccI fragment detected different size band on the EcoRI digested DNA samples. As shown in Figure 7, the human DNA sample had a 6.6 kb band as expected (lane 1), consistent with the size in the transformant DNA, the mouse sample showed a 24 kb band (lane 5), the mink DNA sample had a 10 kb band (lane 4), the rat had also a 24 kb (lane 3), and the syrian hamster DNA sample showed a 11 kb fragment (lane 2). This result suggested that the AccI fragment represented a human sequence evolutionarily conserved across many species. Because the exonic sequences was generally well conserved, the AccI fragment might contain the protein coding sequence.

Figure 7

Analysis of the human DNA sequences for their evolutionary conservation. 15 μg of genomic DNA were restricted with EcoRI, electrophoresed on a 0.8% agarose gel and transferred to nylon membrane. The membrane was hybridized with a 600 bp AccI fragment of the 6.6 kb EcoRI fragment under conditions described in Material and Methods. The sizes of molecular weight marker are shown. Lane 1, human; lane 2, syrian hamster; lane 3, rat; lane 4, mink; lane 5, NIH3T3.

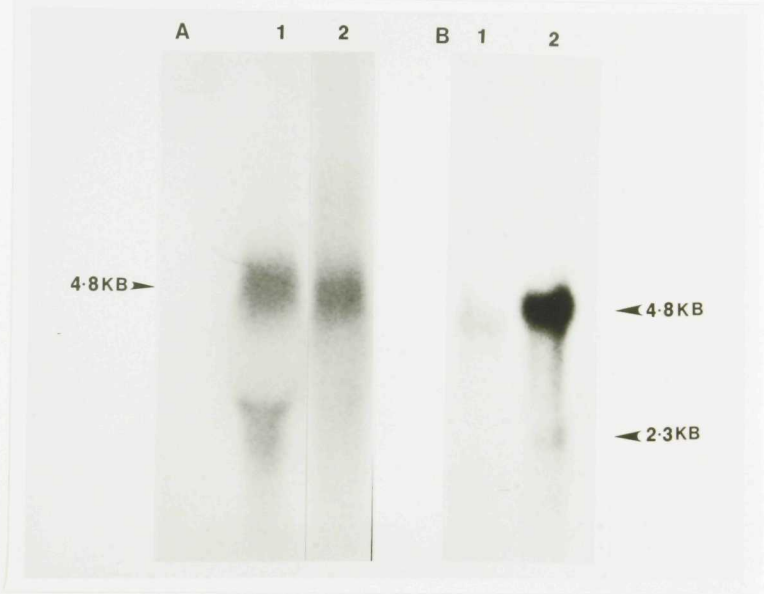

1
2
3
4
5
23·1KB
9·4
6·6
4·4
2·3
2·0

Northern hybridization with the AccI fragment indeed detected a 4.8 kb band in both primary and secondary transformant cellular RNA (Figure 8A, lane 1, primary tumor, lane 2, secondary tumor). No hybridization could be detected in NIH3T3 cells under the condition used. This result indicated that both primary tumor cells and secondary tumor cells expressed the introduced human gene.

Partial DNA sequences of the 800 bp-AvaI fragment and the 600 bp-AccI fragments were obtained at both ends by using a sequencing primer complementary to the flanking region of the cloning site of the vector. A comparison of the AvaI sequences with those of known genes in the GenBank DNA Sequence Data Bank showed identical sequences to HER2 gene, encoding a human epidermal growth factor receptor like protein, over a stretch of 90 nucleotides in the cytoplasmic region of this putative transmembrane protein (Coussens, *et al.* 1985; Yamamoto, *et al.* 1986). However, the sequence obtained at both ends of the 600 bp-AccI fragment did not exhibit homology with any of the sequences in the Genbank data bank, possibly because the exon was small and located in the middle portion of this fragment. To verify such sequence data, other BamHI fragments from the 6.6 kb fragment were sequenced partially at the end of the fragment and compared with the sequence of the HER2 gene. A 1.8 kb BamHI fragment (see Figure 6, top panel) was identical to the published sequence which contained four exons spanning residue 740 to 910 of the cytoplasmic domain of the HER2 protein (Coussens,

Figure 8

Northern blot analysis. 30 μg of total cytoplasmic RNA was fractionated on 1% agarose gel in the presence of 1.96 M formaldehyde and transferred to nylon membrane. A. The membrane was hybridized with a ^{32}P-labeled 600 bp AccI fragment (of the 6.6 kb EcoRI fragment S3) under conditions described in Materials and Methods. Lane 1, secondary tumor (128-15:19T #5 (m693)), lane 2, primary tumor (97-40:44T #2). B. The membrane was hybridized with the full size HER2 cDNA probe (1.3 kb, 1.4 kb, 1.6 kb EcoRI fragments). Lane 1, NIH3T3, lane 2, secondary tumor 128-15:19T #5 (m693). Size of the mRNA is shown.


A
1
2
B
1
2
4·8KB
4·8KB
2·3KB

et al. 1985).

In summary, a human DNA sequence hybridizing with the human EGF receptor gene has been identified. The cloned DNA fragment showed evolutionary conservation. A 4.8 kb messenger was present in the transformant cell line. Partial sequence analysis demonstrated complete homology with regions of human HER2 gene. It was concluded that a human HER2 gene has been identified in this transfection assay. Of particular interest was the fact that this HER2 gene was isolated from a esophageal squamous cell carcinoma, a type of malignancy in which HER2 was not commonly involved.

No Equivalent Mutation in the Transmembrane Region to the Active neu

The activation of neu was known to result from the substitution of glutamic acid for valine at codon 664 in the transmembrane region (Bargmann, *et al.* 1986). At first, it was suspected that this transforming HER2 might harbor the same mutation. When several genomic BamHI fragments were subcloned from the 6.6 kb fragment and sequenced, it was found that the transmembrane region resided in a 1 kb BamHI fragment (see Figure 6, top panel). The sequence showed a normal valine residue at the position equivalent to the mutant codon of neu (at codon 659 of HER2). However, a notable change was found in the second codon of the transmembrane region next to the extracellular domain (codon 655); a single nucleotide

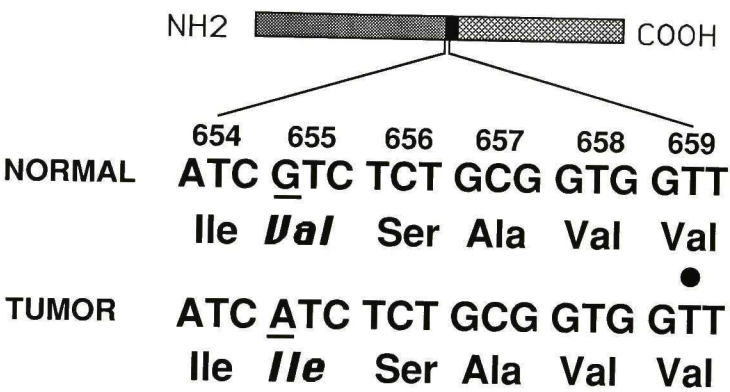
transition from G to A resulted in the substitution of an isoleucine for a valine (Figure 9). The HER2 of esophageal cancer origin had an isoleucine residue at codon 655, as did the gastric carcinoma-derived cDNA sequence (Yamamoto, *et al.* 1986). Placenta cDNA, however, showed a valine residue at this position (Coussens, *et al.* 1985). Together, these results suggested that the activation of this HER2 was not due to the point mutation that occurred in the neu gene, and that the change of valine to isoleucine at codon 655 presumably did not play a major role in this case because of the similar hydrophobicity of these two amino acids.

The HER2 Flanking Sequences Were Altered in Mouse Transformant DNA, but the Gene Was Structurally Normal in the Original Tumor

The transfer of HER2 to NIH3T3 cells in two consecutive cycles implies that gene alteration occurred either in the original human tumor DNA from which the transformants were derived, or during the transfection procedure. It was reasoned that there might be other changes, such as gene rearrangement, causing HER2 activation since the point mutation has been excluded as the mechanism of activation. The original tumor DNA of m693 and the transformant DNA derived from it, therefore, were analyzed by Southern analysis using various restriction enzymes to compare with a panel of DNA samples from normal placenta and other

Figure 9

Codon 655 within the HER2 transmembrane region. The DNA sequence of the transmembrane region of HER2 gene was obtained from a 1 kb BamHI fragment of the 6.6 kb EcoRI fragment isolated from the secondary transformant cell line 128-15:19T #5. The normal sequence was taken from a published data (Coussens, *et al*. 1985). Numbers above the sequence denotes the amino acid codon number. The different nucleotide is underlined. The black dot denotes the equivalent position to the mutant neu gene.


NH2
COOH
654 655 656 657 658 659
NORMAL
ATC GTC TCT GCG GTG GTT
Ile Val Ser Ala Val Val
TUMOR
ATC ATC TCT GCG GTG GTT
Ile Ile Ser Ala Val Val

esophageal tumors. The results showed that the original tumor DNA after EcoRI digestion shared the same HER2 band-pattern with all other samples. The full size HER2 probe detected a 15 kb band which was the carboxyl terminal fragment, an 8 kb band, a 6.6 kb band, a 4.6 kb band and a light 1.7 kb band (Figure 10, lane 3, m693.). The sequence further upstream to the 5' end of the gene was also analyzed with a HER2 promoter region probe and a 5'extreme end probe of HER2 cDNA. Both the 24 kb EcoRI and 17 kb SacI bands appeared in the tumor of m693 and placental DNA (Figure 11, lane 1, 4, placenta; lane 2, 5, m693). Furthermore, HER2 rearrangement of m693 was not observed when DNA was digested with BamHI, HindIII, (data not shown). This result indicated that HER2 rearrangement did not occur in the original tumor DNA.

On the other hand, the mouse transformant tumor DNA contained a HER2 positive band of different size compared with the original tumor DNA. Using 5'end HER2 cDNA probe, or promoter region probe, an EcoRI 23 kb band and a 8 kb band (not detected by promoter region probe), a SacI 17 kb band, and a 15 kb band (not detected by promoter region probe) were detected in both normal placental DNA and original tumor m693 DNA (Figure 11, lane 1, 2, 4, 5,). However, the transformant DNA showed alteration, an EcoRI 21 kb and a SacI 8 Kb appeared instead of the EcoRI 23 kb and the SacI 17 kb, respectively (lane 3,6), but an HindIII 2.5 kb (further upstream to the 8 kb band)(lane 9, transformant, lane 7, placenta, lane 8, tumor m693) retained normally. These results suggest that the

Figure 10

Southern analysis of HER2 gene in the mouse transformant. 10 μg of (2 μg in lane 4) genomic DNA were digested with EcoRI, electrophoresed on a 0.8% agarose gel and transferred to nylon membrane. The membrane was hybridized with full size HER2 cDNA (1.3 kb, 1.4 kb, 1.6 kb EcoRI fragments) under conditions as described in Materials and Methods. Sizes of molecular weight marker are shown. Lane 1 and 2: human normal tissue; lane 3: original tumor (m693); lane 4: secondary tumor (128-15:19T #5); lane 5: original tumor adjacent tissue (contains less DNA); lane 6 and 7: esophageal tumors; lane 8: NIH3T3; lane 9: NIH3T3 DNA containing HER2 cDNA.

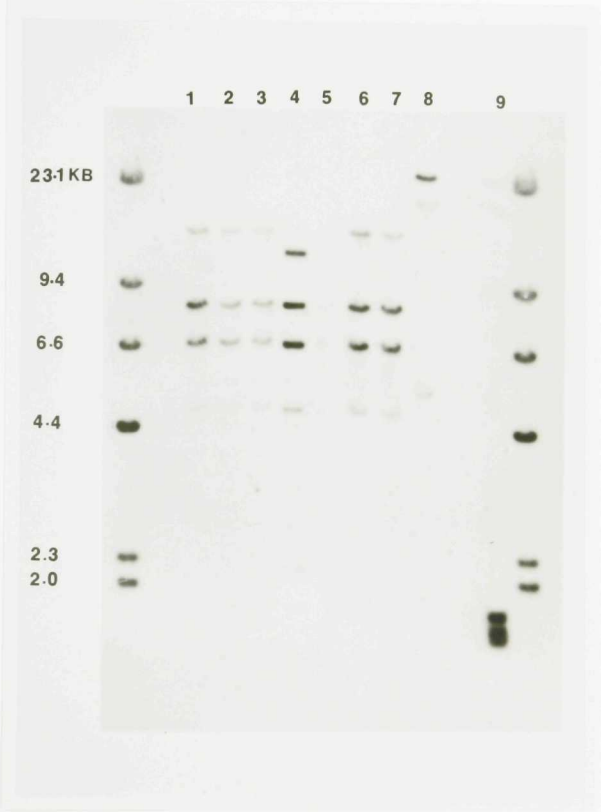

1 2 3 4 5 6 7 8 9
23·1 KB
9·4
6·6
4·4
2·3
2·0

Figure 11

Loss of the 5' flanking sequence of HER2 gene in the mouse transformant. 10 μg of genomic DNA were digested with EcoRI, SacI or HindIII and electrophoresed on a 0.8% agarose gel and transferred to nylon membrane. The membrane was hybridized with a 400 bp EcoRI-PstI fragment of HER2 5' end under conditions described in Materials and Methods. Sizes of molecular weight marker are shown. Lane 1, 4 and 7, human placenta; lane 2, 5 and 8, tumor m693; lane 3, 6 and 9, secondary tumor 128-15:19T #5 (m693).

EcoR I
SacI
Hind III
1 2 3 4 5 6 7 8 9
23.1KB
9.4
6.6
4.4
2.3
2.0

alteration at upstream flanking to HER2 occurred during the transfection, and that less than an 8 kb sequence upstream to the HER2 gene remained during the integration to the mouse genome.

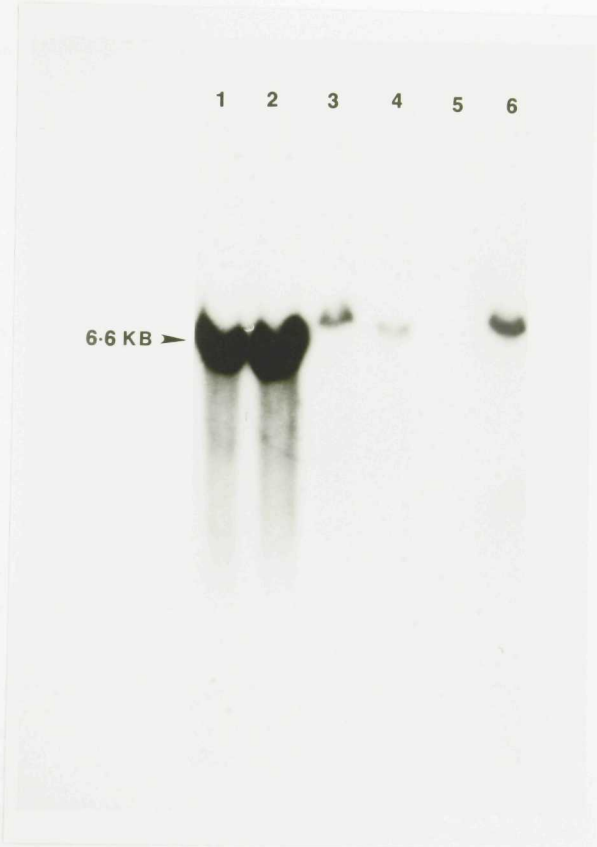
By using a 3'-end cDNA probe, a 13 kb EcoRI band appeared in the transformant DNA instead of the normal 15 kb band, indicating that flanking sequence downstream from the 3'-end of HER2 was also altered during the transfection (Figure 10, lane 4). The BamHI 14 kb band, the SacI 7 kb band and the HindIII 14 Kb band detected by 3'-extreme-end probe remained unchanged in the transformant DNA (data not shown). The results suggested that the alteration of 3' end did not affect the integrity of the HER2 gene product, though the length of altered sequence could not be determined exactly. All together, the sequences flanking the HER2 gene at both its 5'-end and 3'-end were altered in the transformed cell.

Multiple Copies of the HER2 and Elevated Expression Were Detected in the Mouse Transformant, Amplification Was Not Detected in the Original Human Tumor

Genomic fragment of AccI-600 bp was first used in a Southern blot analysis. As shown in Figure 12, it detected a 15-20 folds increase of signal intensity of the 6.6 kb EcoRI band in both primary and secondary transformants as compared with the intensity seen in the human placental and original tumor DNA (Figure 12, lane 1, secondary transformant, lane 2,

Figure 12

Analysis of the human DNA sequences (S3 fragment) in the transformant DNA. 10 μg of genomic DNA were digested with EcoRI, electrophoresed on a 0.8% agarose gel and transferred to nylon membrane. The membrane was hybridized with a 600 bp AccI fragment of the 6.6 kb EcoRI fragment under conditions described in Materials and Methods. Size of band is shown. Lane 1, primary tumor; lane 2, secondary tumor; lane 3, original esophageal tumor; lane 4, original tumor adjacent tissue; lane 5, NIH3T3; lane 6, human placenta.


1
2
3
4
5
6
6·6 KB

primary transformant, lane 3, original tumor, lane 4, adjacent tissue, lane 5, NIH3T3 cells, lane 6, placenta). When the full size HER2 cDNA probe was then used on another Southern blot, it detected a 3 folds, even increase of intensity in all bands in the transformant DNA when one-sixth of the amount of DNA of that control was loaded on the gel indicating that the entire HER2 gene was amplified (Figure 10, lane 4, secondary tumor, lane 1, placenta). This results suggested that multiple copies of HER2 were present in the transfected mouse genome.

To reevaluate the expression level of HER2 in transformant cell lines, full size HER2 cDNA was hybridized to the transformant total cellular RNA. As expected, the 4.8 kb mRNA was detected, and, in addition, a minor 2.3 kb mRNA was also detected in the transformant cell line (Figure 8B, lane 2). The expression level of HER2 in the transformant was greatly increased as compared with the basal level in NIH3T3 cells (lane 1). Of particular interest was the presence of the 2.3 kb species mRNA in the transformant cell line (lane 2). A similar 2.3 kb mRNA has been detected in the MKN-7 gastric carcinoma cell line from which HER2 has been isolated in another study (Yamamoto, *et al.* 1986). This 2.3 kb mRNA species was presumably the result of gene rearrangement in this cell line. The product of this 2.3 kb mRNA corresponded to the HER2 ligand-binding. In the present study, the 2.3 kb mRNA might also result from rearrangement and or alternative processing of the gene, and they might provide a growth

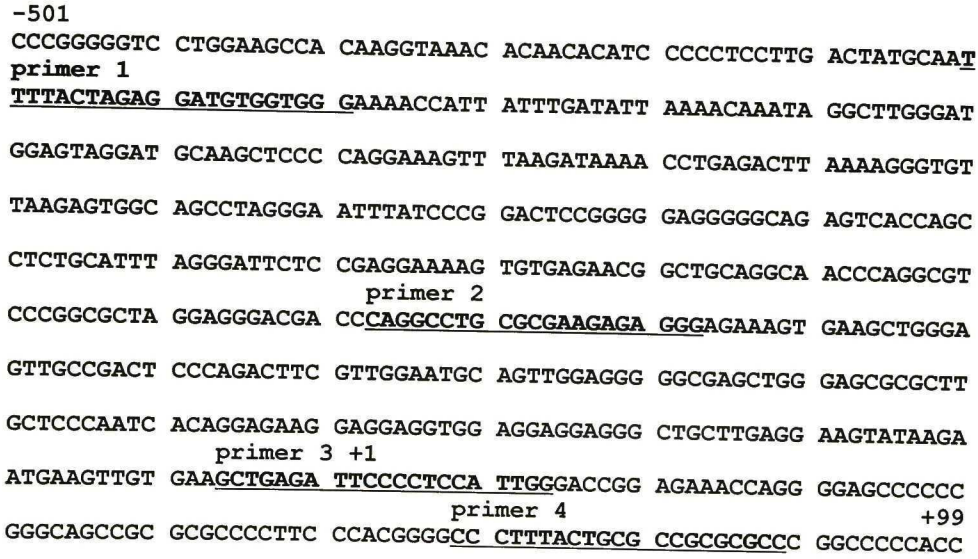
advantage to the cell in culture. The overly expressed 4.8 kb gene products might also be responsible for the transformation of the mouse NIH3T3 cell.

Analysis of HER2 Promoter Region

Overexpression of the growth factor receptor gene may affect a cell's phenotype and growth pattern and result in its transformation. In some breast carcinomas, it was found that overexpression of the HER2 gene occurred without accompanying gene amplification (Tal, *et al.* 1987). Characterization of the HER2 promoter region suggested multiple mechanisms involved in the regulation of HER2 expression (Hudson, *et al.* 1990). Thus, analysis of the HER2 promoter region in the original tumor tissue and in the transformants could help to elucidate the mechanism of activation in the current study. Several studies showed that the promoting activity of the HER2 promoter was confined to a short DNA stretch upstream of the transcription start site (Tal, *et al.* 1987; Ishii, *et al.* 1987). In order to examine the nucleotide sequence of HER2 promoter region, oligonucleotide primers were synthesized according to the published sequence and were used to amplify and sequence the promoter region. As indicated in Figure 13, the HER2 promoter region, spanning nucleotide -618 to -88 was amplified with the primers highlighted and underlined in the sequence. The DNA was first amplified by primer 1 (sense primer) and 4 (antisense primer), the products were diluted

Figure 13

DNA sequence of HER2 promoter region. Four PCR primers are highlighted and underlined in the sequence. Numbers above the sequence denotes the position upstream (minus) or downstream (plus) to the mRNA start site. Primer 1 and 2 are sense primers, primers 3 and 4 are antisense primers.


-501
CCCGGGGGTC CTGGAAGCCA CAAGGTAAAC ACAACACATC CCCCTCCTTG ACTATGCAAT
primer 1
TTTACTAGAG GATGTGGTGG GAAAACCATT ATTTGATATT AAAACAAATA GGCTTGGGAT
GGAGTAGGAT GCAAGCTCCC CAGGAAAGTT TAAGATAAAA CCTGAGACTT AAAAGGGTGT
TAAGAGTGGC AGCCTAGGGA ATTTATCCCG GACTCCGGGG GAGGGGGCAG AGTCACCAGC
CTCTGCATTT AGGGATTCTC CGAGGAAAAG TGTGAGAACG GCTGCAGGCA ACCCAGGCGT
primer 2
CCCGGCGCTA GGAGGGACGA CCCAGGCCTG CGCGAAGAGA GGGAGAAAGT GAAGCTGGGA
GTTGCCGACT CCCAGACTTC GTTGGAATGC AGTTGGAGGG GGCGAGCTGG GAGCGCGCTT
GCTCCCAATC ACAGGAGAAG GAGGAGGTGG AGGAGGAGGG CTGCTTGAGG AAGTATAAGA
primer 3 +1
ATGAAGTTGT GAAGCTGAGA TTCCCCTCCA TTGGGACCGG AGAAACCAGG GGAGCCCCCC
primer 4 +99
GGGCAGCCGC GCGCCCCTTC CCACGGGGCC CTTTACTGCG CCGCGCGCCC GGCCCCCACC

and subjected to two separate asymmetric PCR reactions by primers 1 (sense primer) and 3 (antisense primer), and primers 2 (sense primer) and 4 (antisense primer). The complete DNA sequences of the amplified HER2 promoter region were obtained from the original human esophageal carcinoma (m693), from the secondary transformant 128-15:19T # 5 (m693) and from normal placenta DNA. No difference were found between the original tumor (m693), transformant, normal placenta and the reported sequences. This result showed that the HER2 promoter region essential for the full activity was not changed either in the original tumor or in its transformant.

Molecular Cloning of HER2 cDNA from Secondary Transformant Cell Line

Cytoplasmic RNA was purified from the 128-15:19T #5 (m693) cell line containing the transforming HER2 gene. A cDNA library (λgt11) constructed from this cell line was screened by a BamHI 1.8 kb genomic fragment subcloned from the EcoRI 6.6 kb fragment, which encompassed the HER2 tyrosine kinase domain. Fifty million clones were screened, of which twenty two clone were shown to be positive. Out of twelve clones analyzed further, two clones were identified containing the full size HER2 insert. Three DNA fragments, 1.3 kb, 1.4 kb and 1.6 kb were generated by EcoRI digestion of each of these two clones, a result consistent with the reported HER2 cDNA sequence (see Figure 2).

The complete cDNA sequence of this HER2 gene showed 4468 nucleotides in length, with an open reading frame coding for 1255 amino acids (Figure 14). The primary structure of HER2 cDNA has been analyzed extensively by two independent research groups (Coussens, *et al.* 1985; Yamamoto, *et al.* 1986). The analysis of the cDNA sequence of the present HER2 did not reveal significant change as compared with the reported sequences. It differed in one nucleotide (G -> A in codon 655) only from the sequence identified from a normal human placental cDNA library (Coussens, *et al.* 1985), but it was identical with the sequence identified from a gastric carcinoma cDNA library (Yamamoto, *et al.* 1986). This result, moreover, confirmed further the sequence obtained earlier from the initial genomic fragment. The transition from G to A resulted in the change of valine to isoleucine at codon 655 within the transmembrane region. The sequence data, thus, showed that the transformant cell line possessed an intact human HER2 gene with a subtle change in the transmembrane region compared to the published sequence data in a normal HER2 cDNA. This alteration was not likely to contribute to the transforming ability of HER2 because the valine and the isoleucine were structurally similar.

Preliminary Test of Tumorigenic Activity of the HER2 cDNA

During the process of analysis of the primary and secondary transformants, we sought to determine the activity

Figure 14

Complete nucleotide and amino acid sequence of HER2. Nucleotides are numbered at left side. Amino acid sequence is numbered above the sequence. The full size HER2 was isolated from a cDNA library established from the secondary transformant cell line 128-15:19T #5 (m693). The nucleotide sequence was determined by the dideoxy chain termination method. The amino acid sequence was determined according to the published sequences (Coussens, *et al.* 1985; Yamamoto, *et al.* 1986).

```
1 GCAGAAACCAGCGCAGCCCCCCGGGCAGCCCGGGCCCTCCUUCCCACGGGGCCCUUUACUGCGCCCGCCGCCCGCCCCCCACCCCUCGCAGCACCCCGCCCCCGCCCCCCCCCCAGCCGGC
                                                                1                                                  10
121 UCCAGCCGCAGCCAUGGGGCCGGAGCCGCAGUGAGCACCAUGGAGCUGGCGGCCUUGUGGCGCCUGGGGGCUCCUCCUCGCCCCCGCUCUGCCCCCCGGACCCGCGACCACCCAAGUGUGCACC
                                                     MetGluLeuAlaAlaLeuCysArgTrpGlyLeuLeuLeuAlaLeuLeuProProGlyAlaLeuThrCysGlnValCysThr
          30                  40                  50                  60                  70                  80                  90
241 GGCACAGACUGAAGCUGCGGGCUCCCUGCCAGUCCCGAGACCCACCUGGACAUGCUCCGCCACCUCUACCAGGGCUGCCAGGUGGUGCAGGGAAACCUGGAACUCACCUACCUGCCCACCAC
GlyThrAspMetLysLeuArgLeuProAlaSerProGluThrHisLeuAspMetLeuArgHisLeuTyrGlnGlyCysGlnValValGlnGlyAsnLeuGluLeuThrTyrLeuProThr
          100                 110                 120                 130                 140                 150                 160
361 AAUGCCAGCGUCUCCUUCCUGCAGGAUAUCCAGGAGGUGCAGGGCUACGUGCUCAUCGCCUCACAACCAAGUGGACCAGGCUCCUCAUGCAGAGCCUGCGGAUUCUGCAGGCACCCCAGCUC
AsnAlaSerLeuSerPheLeuGlnAspIleGlnGluValGlnGlyTyrValLeuIleAlaHisAsnGlnValArgGlnLeuProLeuGlnArgLeuArgIleValArgGlyThrGlnLeu
          170                 180                 190                 200                 210                 220                 230
481 UUUGAGGACAACUAUGGCCUGGGCCUGCUAGACAAUGGAGACCCGCUGAACAACUACCACCCCUUGCUACAGGGUCCCCCAGGAGGCCGGCGGAGCUGCAGAACCCUCAGACAGG
PheGluAspAsnTyrAlaLeuAlaValLeuAspAsnGlyAspProLeuAsnAsnTyrThrProValThrGlyAlaSerProGlyGlyLeuArgGluLeuGlnLeuArgSerLeuThrGlu
          240                 250                 260                 270                 280                 290                 300
601 AUCUUGAAAGGAGGGGUCUUGAUCCACCGAACACCCCAGCCUCGCUACCAGCACCAUGCGUCGGCUUCCUAAGAACAACCAGUCGGUCCUCACGAUAGACACCAACC
IleLeuLysGlyGlyValLeuIleHisArgAsnProGlnLeuCysTyrGlnAspThrIleLeuArgTrpPheAlaGluArgAspAsnGlnLeuAlaLeuThrLeuIleAspThrAsn
          310                 320                 330                 340                 350                 360                 370
721 CGCUCUGCGGGCCUCCUUCCUGCCUCCAGUGGUAAGGGCCUCCCGCUGCUGGGGAGAGAGUCUCGAGGAUUGCGACGCGCACUGUCCUUCCUGUGCCCGUGCCGCCGCUGC
ArgSerArgAlaCysHisProCysSerProMetCysLysGlySerArgCysTrpGlyGluSerLeuGluAspCysAspSerGlnCysLeuSerPheLeuProValProCysArgLeuLeu
          380                 390                 400                 410                 420                 430                 440
841 AAGGGGCCACUGCCCACGACUGCUCCAUGAGCAGUGGCUGCGGCUGCCCAACCACUCUGACUGCCUGGCCGCCUCACUCAACCACAGUGGCAUCUGUGAGCUCCAC
LysGlyProLeuProThrAspCysCysHisGlnCysAlaAlaGlyCysThrGlyProLysHisSerAspCysLeuAlaCysLeuHisPheAsnHisSerGlyIleCysGluLeuHis
          450                 460                 470                 480                 490                 500                 510
961 UGCCCAGCCCUGGUCACCUACACACAGCAGACGUUUGAGUCCUAUGCCCAAUCCCAGGGCCGGUGUGACUGUGGCUCCUGCAGCUGUGUGACUGUCCUUACUACACUCCUUUCUACGGAC
CysProAlaLeuValThrTyrAsnThrAspThrPheGluSerMetProAsnProGluGlyArgTyrThrPheGlyAlaSerCysValThrAlaCysProTyrAsnTyrLeuSerThrAsp
          520                 530                 540                 550                 560                 570                 580
1081 GUGGGAUCCUGCACCUUCGUCCUGCCAGCAACAAGAGGUGACAGCAGACGAUGGACAUCGCGUGGAGAAGUGCAGCCAAGCCCUGUCGCUGCGAUGGUCUGCUAUGCUGCCGCAUG
ValGlySerCysThrLeuValCysProLeuHisAsnGlnGluValThrAlaGluAspGlyThrGlnArgCysGluLysCysSerLysProCysSerAspGlyValCysTyrGlyLeuGlyMet
          590                 600                 610                 620                 630                 640                 650
1201 GAGCACUUGGCGAGAGUGAGGGACGUUACCUCCAUCGCCACUGCUGCCGUCAGAUUCCAGGAUUGUGCUGGCCUGCAGAAGCUGUUGGGACGUCUGGCCAGUGCCUGCCAGCUGCCUGCC
GluHisLeuArgGlyValArgAlaValThrSerAlaAsnIleGlnGluPheAlaGlyCysLysLysLeuPheGlySerLeuAlaPheLeuProGluSerPheAspGlyAspProAlaSer
          660                 670                 680                 690                 700                 710                 720
1321 AACACUGCCCCUGCCUCCAGCCAGAGCAGCCUCAAGUGUUUGAGACUCGGAAGACAUCACAGGUUACCUUAUCAGCUCAGCAGGCCGGACAGCCUGCCUGACCUCCAGAGCUCCUCAGAAC
AsnThrAlaProAlaSerSerGlnSerSerLeuLysCysLeuArgLeuGlyLysThrIleThrGlyTyrLeuTyrIleSerAlaLeuTrpProAspSerLeuSerValProGlnAlaSer
          730                 740                 750                 760                 770                 780                 790
1441 CUGCAAGUAACCCGGGGACACUAUCUGCACAAGUGGCCCUACUCGCUGACCUCGCAAGGCUGGCCAUCAGCUGGCUGGGGCUGCCUCACUGAGGGACUGGGCACGUGGACUGGCCCUC
LeuGlnValIleArgGlyArgIleLeuHisAsnGlyAlaTyrSerLeuThrLeuGlnGlyLeuGlyLeuSerTrpLeuGlyLeuArgSerLeuArgAspGlyLeuGlyArgGlyLeuAlaLeu
          800                 810                 820                 830                 840                 850                 860
1561 AUCCACCAUAACCCACCUCUGCUUCGUGCACGCGGUGCCCUGGGACCAGCUCUUUCGGAACCCCACCAAGCUCUGCUCCCACACUGCCAACGGCCACAGGCACGAUGUCGUUGGCCAG
IleHisHisAsnThrHisLeuCysPheValHisThrValProTrpAspGlnLeuPheArgAsnProHisGlnAlaLeuLeuHisThrAlaAsnGlyHisArgHisAspValValGlyGln
          870                 880                 890                 900                 910                 920                 930
1681 GGCCUGGCCUGCCACCAGCUGUGGCCCCGAGGGCACUGCUGGGGUCCAGGCCCACCCAGUGUGUCAACUGCAGCCAGUUCCUUCGGGGCCAGGAUGCCGUGCAGGAAUGCCCAGUACUG
GlyLeuAlaCysHisGlnLeuCysAlaArgGlyHisCysTrpGlyProGlyProThrGlnCysValAsnCysSerGlnPheLeuArgGlyGlnAspAlaValGlnGluCysAlaGlnTyrVal
          940                 950                 960                 970                 980                 990                1000
1801 CAGGGGUCCCCCAGGCAGUAUGUGAAUGCCAGGCACUGUUUGCCUGUCCACCCUGACUUCACCCGCAAUGGCUCAUGUGACUCCGGCUGCCACCGUGUGUGGCCUGU
GlnGlyLeuProArgGlnTyrValAsnAlaArgHisCysLeuProCysHisProGlnLeuThrArgAsnGlySerCysAspSerGlyCysAspSerGluCysValLeuSerCysLeuGlyPro
          1010                1020                1030                1040                1050                1060                1070
1921 GCCCACUAUAAGGACCCCUCCUUCGUGGCCGCUGCCACGCGGUGUGAAACCUGACCUUCCUCAUGCCCACUUGGAAGUUUCCAGAUGAGCGGCCGCAUCCCAGCCCUUGCCCC
AlaHisTyrLysAspProProPheValAlaAlaArgCysProSerGlyValLysProAspLeuSerTyrMetProIleTrpLysPheProAspGluGlyGlyAlaCysGlnProCysProC
          1080                1090                1100                1110                1120                1130                1140
2041 AUCAACUGCACCCACUCCUGUGUGGACGCUGGAUGCAGCUGCCCGCCGCGGACGAGCACGGCCAGCGUGCCUGCAGCUGCCAUCUGCCCUGCUCUGCCCCUGCUGUGCUGCCUGUCUUC
IleAsnCysThrHisSerCysValAspAlaGlyCysProAlaAlaGlyGlnArgAlaSerCysLeuThrSerIleSerAlaValValGlyIleLeuLeuValLeuValLeu
          1150                1160                1170                1180                1190                1200                1210
2161 GGGGUGGGCUUUGGGAUCCUCAUCAAGCAGCGGCAGCAGAAGAUCCGGAAGUACACGAUGCGGAGACUGCUGCAGGAAACGGAGCCGGUGGAGCCCCUGACACUAGCCAGACCGAGCCGAUCCC
GlyValGlyPheGlyIleLeuIleLysArgArgGlnGlnLysIleArgLysTyrThrMetArgArgLeuLeuGlnGluThrGluLeuValGluProLeuThrProSerGlyAlaGlnThrGluProIle
          1220                1230                1240                1250                1260                1270                1280
2281 AACCAGGCCGACAGUGCGGUAUCCUGAAAGACAGCGACGUAGAGAAGGUGAAGGUGCUUGGACUCGUCGGCCUUUGCGCCUACUCCAUCCAUGGGCAGAAUGCAAAGAUCGAAAUGCAAAA
AsnGlnAlaAspSerAlaValSerLeuLysGluSerAspValGluLysValLysValLeuGlyLeuValGlyLeuCysAlaTyrSerIleHisGlyGlnAsnAlaLysIleGluMetAlaAsn
          1290                1300                1310                1320                1330                1340                1350
2401 AUUCCAGUGGCCACUCAAAGUGUUGAGGGAAAACACAUCCCCCAAAGCCAACAAAGAAUCCUACGACGAAGCUACGUGAUGGUGGUUGGUGUCCCCGCUUCUCGGCCUUCUGGGC
IleProValAlaLeuLysValLeuArgGluAsnThrSerProLysAlaAsnLysGluSerTyrAspGluAlaThrValMetAlaGlyValGlySerProTyrValSerArgLeuLeuGly
          1360                1370                1380                1390                1400                1410                1420
2521 AUCUGCCUCACACUCCACGGUGCACGAGCUGGUGACACACGUUADGCCCUAUGCACGUCCGGAAAACCCGGCACGCCUGGGCUCCCGACGCCUGCUGACUGUGCUGGUGUAUG
IleCysLeuThrSerThrValGlnLeuGluValThrHisValAspProTyrAlaArgValSerGlnLeuGluAspHisValArgGlyLeuSerGlnAspLeuGluAsnTrpCysMet
          1430                1440                1450                1460                1470                1480                1490
2641 CAGAUUCCCAAGGGAUAGCUACCUGGACGAUGUGGCCGCCUACACAGGGACUUGGCCCUCUGAGUGGCUGGUCAAAUUGCACCAUCGUCCGGCUGGCU
GlnIleAlaLysGlyMetSerTyrLeuGluAspValArgLeuValAlaArgAspLeuAlaLeuSerProAsnHisValLysIleThrAspPheGlyLeuAla
          1500                1510                1520                1530                1540                1550                1560
2761 CGGCUGCUGACAUUGCACGACACGACUACCAUGCCAGUGGGGCAAGGUGCCCAUCAAGUGGAUGGCUAUUGAGUCCCUGCAGACCCAGAAGUUUACCACCAAGAGUGAUGUGUGGAGU
ArgLeuLeuAspIleAspGluThrGluTyrHisAlaAspGlyGlyLysValProIleLysTrpMetAlaLeuGluSerIleLeuArgArgArgPheThrHisGlnSerAspValTrpSer
          1570                1580                1590                1600                1610                1620                1630
2881 UAUGGGGUGACUGUGUGGGAGCUGAUGACUUUUGGGGCCAAACCUUACGAUGGGAUCCCAGCCCGGGAGAUCCCUGACCUGCUGGAAAAGGGGGAGCGGCUGCCCCAGCCCCCAUCUGC
TyrGlyValThrValTrpGluLeuMetThrPheGlyAlaLysProTyrAspGlyIleProAlaArgGluIleProAspLeuLeuGluLysGlyGluArgLeuProGlnProProIleCys
          1640                1650                1660                1670                1680                1690                1700
3001 ACCAUUGAUGUCUACAUGAUCAUGGUCAAAGCUGUGGAUCGACUCCGAUUCCGGCCAAGAUUCCGGGAGUUGGUGUCUGAAUUCUCCCGCAUGGCCAGGGACCCCCAGCCGCUUUGUG
ThrIleAspValTyrMetIleMetValLysCysTrpMetIleAspSerAspCysArgProArgAspLeuArgGluLeuValSerGluPheSerArgMetAlaArgAspProGlnArgPheVal
          1710                1720                1730                1740                1750                1760                1770
3121 GUCAUCCAGAAUGAGGACUUGGGGCCCAGCCAGUCCCUUGGACAGCACCUUCUACCGCUCACUGCUGGAGGACGAUGGGGACGCCGGUGGAUCCUGAGGACUAUCUGGUACCCCAG
ValIleGlnAsnGluAspLeuGlyProAlaSerProLeuAspSerThrPheTyrArgSerLeuLeuGluAspAspAspMetGlyAspLeuValAspAlaGluLeuTyrValProGln
          1780                1790                1800                1810                1820                1830                1840
3241 CAGGGCUUCUUCUGUCCAGCCCUGGCCCCGGGCAGGGUCUGGGGGCAUGGUCCACCACAGGCACCCCAGUCUACUCACUGACAGGAGUGGCCGUGGGCACUCUGACUCACACUGGGCUGGAGCCCCUGCAA
GlnGlyPhePheCysProAlaProAlaProGlyAlaGlyLeuGlyGlyMetValHisHisArgHisArgSerSerThrArgSerGlyLeuGlyAspLeuThrLeuGlyLeuGlyProSerGlu
          1850                1860                1870                1880                1890                1900                1910
3361 GAGCAGGCCCCCCAGGUCUCCACUGGCACCCUCCCCAAGGGCUGGCUCCCGAUGUAUUGAUGGUGGUCCAGGAAGGGGGCAGCCAAGGGCUGCUCAAAGCCCUCCCCACAUGACCCCAGC
GluGlnAlaProAlaGlyProSerAlaLeuProSerGlyLeuGlyAlaGlySerAspValPheAspGlyAspLeuGlyMetGlyAlaLeuAlaSerGlyLeuSerProThrHisAspProSer
          1920                1930                1940                1950                1960                1970                1980
3481 CCUCCUACAGCGGUACAGUCAGGACCCCACGUACCCUGCCCUCUGCCUCUGACUGAUGGCUACGUUCCCUUCCCCUGCACGCCCCCAGCCCUGGCUACUUGAUCGACCAGCCAGAUGUUCGGCCC
ProLeuGlnArgTyrSerGluAspProThrValProLeuProSerGluThrAspGlyTyrValAlaProLeuThrCysSerProGlnProGluTyrValAsnGlnProAspValArgPro
          1990                2000                2010                2020                2030                2040                2050
3601 CAGCCCCUUCCGCCCCGAGAGGGCCCCUCUGCUCGUCGCUGCCCGCCACUCUCGUGGCCACCUCUGGAAAGGCCCAAGACUUCUCCCCCAGGGAGAAGGUGGUCGUGCUGGAAAGACGUUGUUGG
GlnProProSerProArgArgGluGlyProLeuLeuValAlaAlaArgProArgSerGlyAlaThrLeuGluAlaArgProLysThrLeuSerProGlyLysGlnGlyValLysAspValPheAlaPhe
          2060                2070                2080                2090                2100                2110                2120
3721 GGGGGGCCCCGGGAGAACCCCGAGUACUUGACACCCCAGGGACGAGCUGCUCCCCUCCUCCUCAGCCCCACCCUCCUGCUGCUCAGCCCCACACUCUAUUACUGGGACACAGGACCCA
GlyGlyAlaValGluAsnProGluTyrLeuThrProGlnGlyGlyAlaAlaProGlnProHisProProProAlaPheAspAsnLeuTyrTyrTrpAspGlnAspPro
          2130                2140                2150                2160                2170                2180                2190
3841 CCAGCACGGGGGGCUCCCACCCAGCACCUCUACAAAGGGACACCUACGGCCAGACAACCCAGAGUACCUGGGCUGCAGCCGUCGACUGCAACAGAAGGCCAAGUCCGCGAUGCCCUGAUGU
ProGluArgGlyAlaProProSerThrPheLysGlyThrProTyrAlaGluAsnProGluTyrLeuGlyLeuAspValProVal
3961 GUCCCUCAGGGACAGGCAGGCAAGGCCUCGACUUCUGCCUACUACAGAGUGGGGAGCCCUCCCCACCACUUCCAGGGAACCUGCCAUGCCAGGACCAGGCUCCUAAGGAACCUUCCUUCCUGC
4081 UUGAGUUCCCAGAUGGCUGGAAGCGGUCCAGCCUCCUGCUGCAAGCAGACACGCAUCGAUGGUGCGAUUCUGAGGCCCUGCCCAUUGAGCACUCAUGGCAUGCCACAGC
4201 CCAGCTUGGCCCUUUCCUUCCAGAUCCUGGGUACUGAAAGCCUUAGGGAAGCUGGCCUCGAGAGGGGAACGGCCCUAAGCGAGUGCUCAAGAACAAAAGCGACCCAUUCAGAGACUGUCC
4321 CUGAAACCUAGUAUCUGCCCCCCCCAUGAGGAAGCAACACGCAAUGGUGCUGCAGUAUCCAGGCUUUGUCGUUUAGUUUUUACUUUUUUUUGUUUUGUUUUUUUAAAGAUGAA
4441 AUAAAGACCCAGGGGGAGAAAAAAAA
```

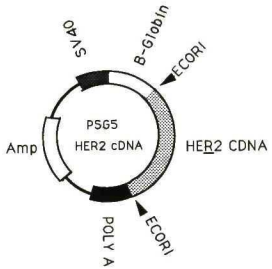
of the HER2 cDNA. Overexpression of HER2 resulting from either use of a strong promoter or by amplification was sufficient to transform NIH3T3 cells and allowing growth in nude mouse (Di Fiore, *et al.* 1987; Hudziak, *et al.* 1987). Initially, We suspected that activation of HER2 in this transfection assay was due to a qualitative change in the HER2 gene so that it can be transferred from the original tumor DNA to NIH3T3 cells through two cycles of transfection. We have also recognized the possibility that the amplification of HER2 in the transformant cell line could be a consequence of the transfection process itself. Therefore, we constructed expression plasmids to evaluate the transforming activity of the HER2 cDNA (Figure 15).

SV40-HER2: If the HER2 was activated by mutations in the coding sequence, it was likely that the SV40-HER2 would be active in transforming NIH3T3 cells. Thus, the full length HER2 isolated from the secondary transformant cell line was cloned into an expression plasmid which contained the SV40 early promoter and polyadenylation signals.

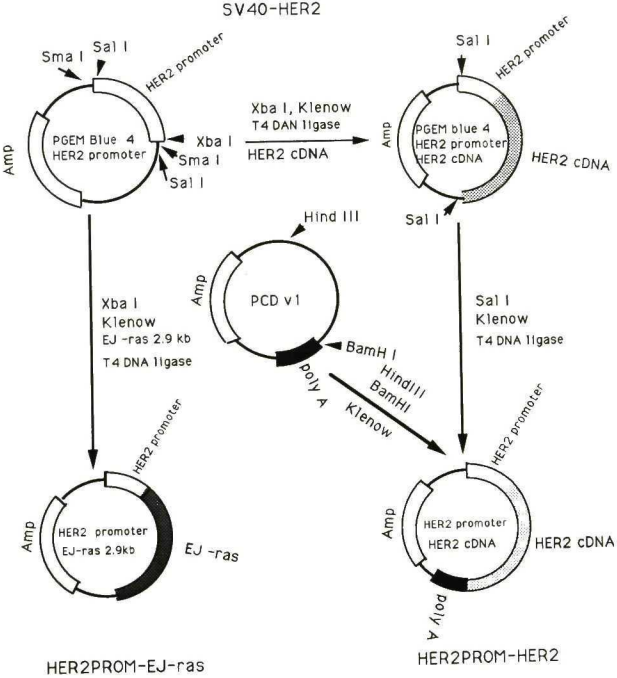
HER2 PROM-HER2: Because the HER2 promoter region showed complete activity, and because HER2 cDNA driven by its own promoter may mimic its natural function in the intact cell, the HER2 cDNA was cloned downstream from the HER2 promoter and supplemented with a polyadenylation signal from SV40. The SalI-XbaI DNA fragment of the HER2 promoter region, encompassing from -1 to -450 bp upstream to the transcription start site, was prepared via PCR. The product was blunt-end

Figure 15

Construction of the expression plasmids. The modalities of engineering are detailed in the text. SV40-HER2, full size HER2 cDNA from the transformant cell line was inserted into a expression plasmid pSG5 (Strategene). HER2 PROM-EJ-ras, 2.9 kb SacI fragment of EJ-ras gene was inserted into downstream to the HER2 promoter. HER2PROM-HER2, full size HER2 cDNA was inserted into downstream to the HER2 promoter, supplemented with a polyadenylation signal.



SV40-HER2



HER2PROM-EJ-ras

HER2PROM-HER2

ligated with SmaI digested PGEM Blue 4, and the constructs were transformed into DH5α E.coli. Clones containing the expected insert were verified by DNA sequencing. The HER2 promoter construct was digested by XbaI. A filling-in reaction with Klenow fragment was followed under condition provided by the supplier (BRL). HER2 cDNA was isolated from the secondary transformant cell line. The full size cDNA was polished at the end and ligated to the HER2 promoter construct. Together, they were cut off from the vector, cloned into the HindIII and BamHI sites of the PCDV1 vector (Okayama and Berg, 1983), which provide the polyadenylation site.

HER2 PROM-Ras: The 2.9 kb SacI fragment of EJ-ras was constructed in a similar way and designated HER2PROM-EJ-ras.

Two experiments were performed with these constructs. The results were summarized together in Table 5. HER2PROM-EJ-ras transformed the NIH3T3 cells, and produced typical transforming foci. When the transfected cells were inoculated into nude mice, four tumors developed in 10 days in all 4 mice receiving $1x10^5$ cells. This result suggested that the HER2 promoter was functional in the construct because 2.9 kb SacI fragment of EJ-ras lacked the transforming activity alone (Honkawa, *et al.* 1987). SV40-HER2 transfected cells did not induce obvious transformed foci morphologically, and only one tumor developed after 6 weeks in 8 nude mice inoculated with $1x10^6$ transfected NIH3T3 cells. HER2 cDNA was present in this tumor (Figure 11, lane 9) but the HER2 protein product was

Table 5. Tumorigenesis Study of HER2 Expression Plasmids

Expression Plasmid	Cells / Mouse	Mice Injected	Tumor
HER2PROM-EJ-ras	$1X10^5$	4	4
HER2PROM-HER2	$1X10^6$	8	0
SV40-HER2	$1X10^6$	8	1
SV40 vector	$1X10^6$	8	0

undetectable (data not shown). The HER2PROM-HER2 construct failed to induce any tumors. This result suggested that this HER2 cDNA was not sufficiently active to transform the cell under the control of a weak promoter such as its natural promoter or the SV40 early region. This result was consistent with the findings that only a highly expressed normal HER2 gene was able to transform the NIH3T3 cells. On the other hand, we could not exclude the possibility that the construct was defective in expressing the HER2 cDNA.

HER2 Alleles in Amino Acid Codon 655 within Transmembrane Region

When our HER2 cDNA sequence was compared with the two reported sequences, only one nucleotide difference presented resulting in the substitution of an isoleucine for a valine at codon 655 of amino acid (the second codon close to the extracellular domain in the transmembrane region). To address the question of whether this difference represented a mutation or was a consequence of normal polymorphism, allele specific oligonucleotide hybridizations were performed on 47 DNA samples, including normal tissue, esophageal carcinoma, cervical carcinoma and breast carcinoma. A 148 bp fragment encompassing the transmembrane region was amplified by the polymerase chain reaction, and the products were subjected to hybridization analysis. Oligonucleotide probes (16mers) were made to include this codon with one base difference in the

center, oligonucleotide A representing allele A for the valine codon originally from a normal HER2 cDNA and oligonucleotide B representing allele B for the isoleucine codon originally from a human stomach cancer HER2 cDNA. As shown in Figure 16, samples hybridized to oligo A or B were designated allele A or allele B, sample hybridized to both oligonucleotides were designated heterozygous allele A+B. Further confirmation of the results was obtained by PCR and direct sequencing of 4 selected samples.

Overall, the oligo A probe reacted with 8 DNA samples (17.06%), oligo B with 24 samples (51.06%) and both oligos reacted with 15 samples (31.91) (Table 6). A comparison of the frequency of each allele in 17 normal samples with that in 19 esophageal tumor samples did not show significant difference statistically ($P>0.05$). Also six paired DNA samples did not show any difference between peripheral lymphocytes and breast carcinoma sample from the same patient. These results suggested that the difference was probably due to a normally inherited polymorphism instead of a change occurring during the development of the malignancies. Allele B (isoleucine) appeared more frequently than allele A (valine). This might explain the high frequency of cDNA clone with isoleucine (5/6) identified to date (Coussens, *et al.* 1985; Yamamoto, *et al.* 1986; Slamon, *et al.* 1988; Di Fiore, 1988; our data). However, all five sequences were isolated from a tumor cell line or tumor tissue. Since the valine to isoleucine change was conservative, it was presumed that the

Figure 16

Allele specific hybridization of HER2 transmembrane region. HMW DNA was isolated from tumor tissue or tumor cell lines as described. PCR reactions were performed at 96°C for 30 sec., 60°C for 30 sec., and 72°C for 30 sec. for 25 cycles. One tenth of the reaction was run on 2% agarose gel, transferred to nylon membrane and was hybridized to ^{32}p labeled oligonucleotide under conditions described in Materials and Methods. The membrane first was hybridized to oligo A (Top panel) representing the valine residue at codon 655, and then to oligo B (Bottom panel) representing the isoleucine residue after stripping off the radioactivity.

1
2
3
4
oligo A
oligo B

Table 6. HER2 Alleles at Codon 655 within Transmembrane Region

Sample	Case	Allele A	Allele B	Allele A+B
Esophageal carcinoma	19	2(10.52)	13(68.42%)	4(21.05%)
Cervical Carcinoma	5	1(20.00%)	3(60.00%)	1(20.00%)
Breast carcinoma	6	2(33.33%)	2(33.33%)	2(33.33%)
Normal	17	3(17.64%)	6(35.28%)	8(47.28%)
Total	47	8(17.02%)	24(51.06%)	15(31.91%)

substitution may not have altered the HER2 function. However, the same amino acid change in APP gene (amyloid precursor protein) due to a point mutation resulting in a conservative change in the protein appeared to play a role in the pathogenesis of certain cases of hereditary Alzheimer's disease (Goate, *et al.* 1991). As indicated early, the 19 cases of esophageal carcinoma samples were collected from a high incidence area in China. Interestingly, within these samples, 10 of 11 of patients (91%) with familial history carried the isoleucine residue homozygously, whereas only 3 of 8 (37.5%) patients without a familial history had the isoleucine residue homozygously. Whether the presence of an isoleucine residue indicated a tendency to develop esophageal tumor remained unclear due to the small size of samples analyzed here.

Analysis of the Second 6.6 kb
EcoRI Genomic Fragment (S1)

Screening of the partial genomic library constructed from size fractionated transformant DNA with a human Alu probe yielded two different genomic fragments, one of which was identified as human HER2 gene. The second fragment was analyzed in a similar way as for the fragment of HER2 gene. Within the 6.6 kb EcoRI fragment, a 2.1 kb SphI fragment was found without Alu sequences. Hybridization of this fragment to the transformant DNAs produced a band of strong intensity

(Figure 17, lane 1, 7, secondary tumor DNA, lane 2, 8, the primary tumor), comparable to that yielded by the HER2 probe (see Figure 12, lane 1 and 2.). The probe also detected a mouse endogenous band about 15 kb, but with a much lesser intensity compared to that in human DNA (lane 11, NIH3T3 DNA, lane 8 secondary transformant DNA). These results suggested that this DNA fragment also contained human sequences and has been amplified in the mouse transformant (lanes 3 and 9, 4 and 10, the original tumor DNA and surrounding normal tissue DNA respectively; lane 9 and 12, human placenta DNA). The bands detected by SphI changed between the primary and secondary transformant DNA by EcoRI and SacI digestion (lanes 1, 2, and 7, 8), and were also of a size different from that of the DNA of normal placenta and the original tumor (lane 3, 6, 9, and 12). These results suggested that one end of this 6.6 kb EcoRI fragment has been modified during the transfection.

To investigate the possible relationship between this fragment and the HER2 gene, DNA samples were digested by several restriction enzymes and analyzed by Southern hybridization. The results were shown in Figure 18. All four DNA samples, including two normal and two esophageal carcinoma DNAs showed the same banding pattern when hybridized with the SphI fragment of the second 6.6 kb EcoRI fragment (S1) or with HER2 complete cDNA probe. The membrane was first hybridized to the SphI fragment, and showed a 25 kb BamHI band, 5.5 kb and 1.7 kb HindIII bands, and 8 kb and 10 kb PstI bands (Figure 18A). The membrane was stripped off and hybridized

Figure 17

Analysis of the human DNA sequence (S1 fragment) in the transformant. 10 μg of genomic DNA were digested with EcoRI lane 1-6) or SacI (7-12), electrophoresed on a 0.8% agarose gel and transferred to nylon membrane. The membrane was hybridized with a 2.1 kb SphI fragment of the 6.6 kb EcoRI fragment (S1) under conditions described in the Materials and Methods section. Sizes of molecular weight marker are shown. Lane 1 and 7, secondary tumor 128-15:19T #5; lane 2 and 8, primary tumor 97-40:44T #2; lane 3 and 9, original esophageal tumor; lane 4 and 10, original tumor adjacent tissue; lane 5 and 11, NIH3T3; lane 6 and 12, human placenta. Arrow indicates the mouse endogenous band seen in lane 11.

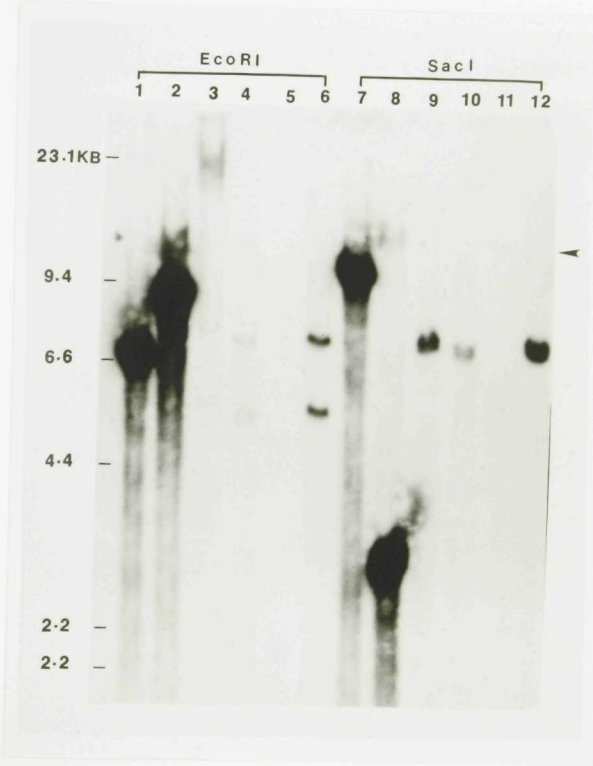
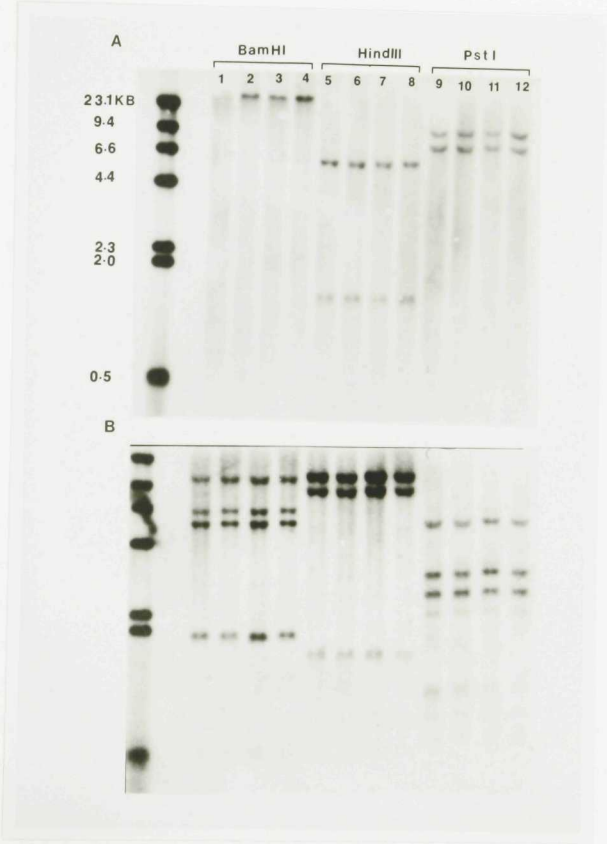

EcoRI
SacI
1 2 3 4 5 6 7 8 9 10 11 12
23.1KB
9.4
6.6
4.4
2.2
2.2

Figure 18

Comparison of the banding pattern of SphI fragment with that of HER2 cDNA by Southern analysis. 10 μg of genomic DNA from normal tissue (lane 1-2, 5-6, and 9-10) and tumor tissue (lane 3-4, 7-8, and 11-12) were digested with BamHI, HindIII and PstI, electrophoresed on a 0.8% agarose gel and transferred to nylon membrane. A. The membrane was hybridized with a 2.1 kb SphI fragment of the 6.6 kb EcoRI fragment (S1) under conditions described in the Materials and Methods. B. The same membrane was hybridized with complete HER2 cDNA probe. Sizes of the molecular weight marker are shown in kilobases.


A
BamHI
HindIII
PstI
1 2 3 4 5 6 7 8 9 10 11 12
23.1KB
9.4
6.6
4.4
2.3
2.0
0.5
B

with complete HER2 cDNA (Figure 18B)(three EcoRI fragments 1.4 kb, 1.6 kb and 1.3 kb). None of the bands shared the same size as detected by the SphI fragment (Figure 18A). Since the bands detected by the SphI probe showed differences between the primary and secondary transformant DNA (see Figure 17), it was possible that the second fragment (S1) in the transforming sequences was terminal. In addition, both ends of the HER2 gene have been shown to be altered in the transformant DNA (see Figure 10 and Figure 11). Taken together, these results suggested that the second 6.6 kb EcoRI fragment most likely was not connected immediately to the HER2 gene in the genome. Three other Alu positive EcoRI bands 20 kb, 10 kb, 3.8 kb, which were not hybridized with HER2 cDNA in the transformant, might be associated with this genomic sequence.

Partial DNA sequences were obtained at both ends of the 6.6 kb EcoRI fragment, at both ends of the subclone SphI 2.1 kb fragment and subclone AvaI 2.0 kb of the 6.6 kb EcoRI. The sequence data showed a 73.8% homology with HPVIa, HindIII restriction fragments b&c in a stretch of 42 bp, a 73.5% homology with HPV31 over 45 bp, a 82.6% homology with HPV25 in 23 bp, All of the above homologous regions were located in different sections of the 6.6 kb EcoRI fragment. Since the extent of these homologous regions was small, further study would be required to identify the entire gene structure and the possible role in the transformation of NIH3T3 cells.

Detection of p53 Mutation in Human Esophageal Carcinoma from a High Incidence Area Linxian, China

Development of a Strategy for PCR and Direct Sequencing of p53 Gene from Tumor DNA

p53 mutations have been found frequently in diverse types of human tumors. The characteristic clustering of p53 mutations within four evolutionarily conserved domains, spanning about 2 kb of genomic DNA sequence makes it possible to screen large numbers of samples for the presence of mutation via polymerase chain reaction and direct DNA sequencing. Two methods were commonly used for such analysis: (1) cloning the PCR product into a sequencing vector and then sequencing 4 to 6 independent clones by the conventional dideoxy termination sequencing method; (2) directly sequencing the purified single stranded or double stranded PCR product. Both methods had certain advantages and disadvantages. The cloning approach was time consuming and labor intensive, additional sequencing reactions were frequently required to verify accuracy due to possible errors incurred during amplification. In the second approach, the purity of the product was critical for the reaction. The leftover primer and nonspecific product often obscure the sequencing reaction. Although end-labeled sequencing primer could overcome this problem, the resolution and the sequence length were generally poorer than with the use of ^{35}S ATP, and the worker was exposed

to the higher energy of ^{32}P. In order to study large numbers of samples rapidly, a simple, more reliable strategy was needed.

To fulfill this goal, a 2.9 kb genomic fragment, encompassing the exons 4 to 9, was first amplified (Figure 19). The products of a 20 cycle reaction showed a sharp single band on the ethidium bromide agarose gels. To generate template for DNA sequencing, the primary PCR products were diluted 100 folds; 2 μl of the diluted specimen was used in the asymmetric PCR, with a new pair of primers for 30 to 35 cycles. Because the most frequent mutations were clustered in exons 5 to 8 spanning 2 kb genomic DNA, two pairs of primers were used to produce two single stranded fragments: one covered exons 5 and 6, a total 356 bp including 50 bp from the intron sequence; the other covered exons 7, 8 and 9, a total 780 bp with 350 bp of intron 7 and 62 bp of intron 8. When the PCR products were examined on ethidium bromide gel, two distinct bands were observed, one representing single strand DNA, the other representing the double stranded DNA. Regardless of such double strand products, the entire reaction mixture was filtered through a CENTRICON 30 membrane to remove unincorporated primer and dNTPs. The purified products were then subjected to a sequencing reaction without further purification. Using this strategy, readable sequence of up to 350 bp could be obtained on a 6% polyacrylamide gel as shown in figure 20. Thus, only four sequencing reactions carried out with four primers could reveal the whole sequence of exons

Figure 19

Strategy for amplifying and sequencing the "hot spot" of p53 gene. Exons of p53 gene are represented by boxes containing Roman numerals. Straight line between the boxes denotes the intron. Sizes of both exons and introns are not adjusted. Horizontal arrows denote the direction of primer extension. The reaction were performed as followings: primer 1 /primer 2, 95°C, 1'45"; 60°C, 1'; 72°C, 2'45"; primer 3 / primer 4, 95°C, 1'; 50°C, 1'; 72°C, 1'45"; primer 5 /primer 6, 94°C, 1'45"; 50°C, 1'; 72°C, 2'.

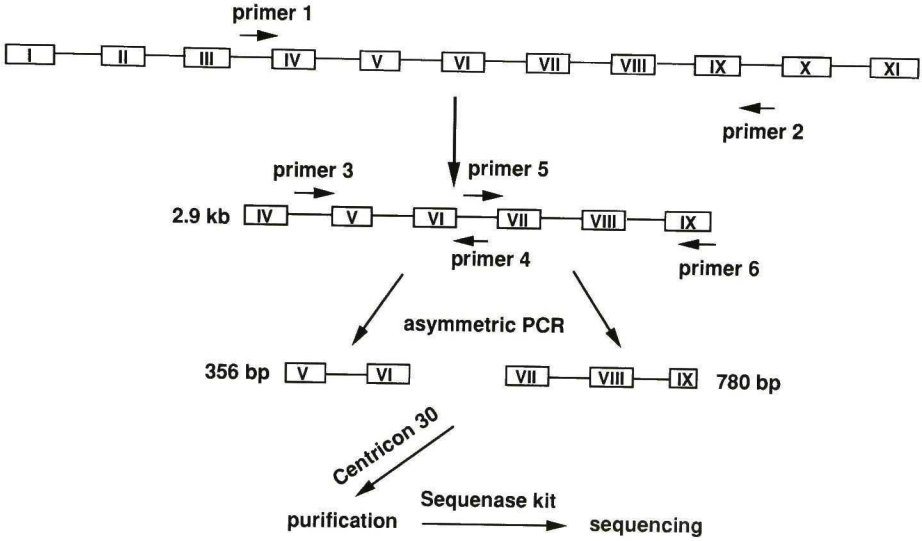
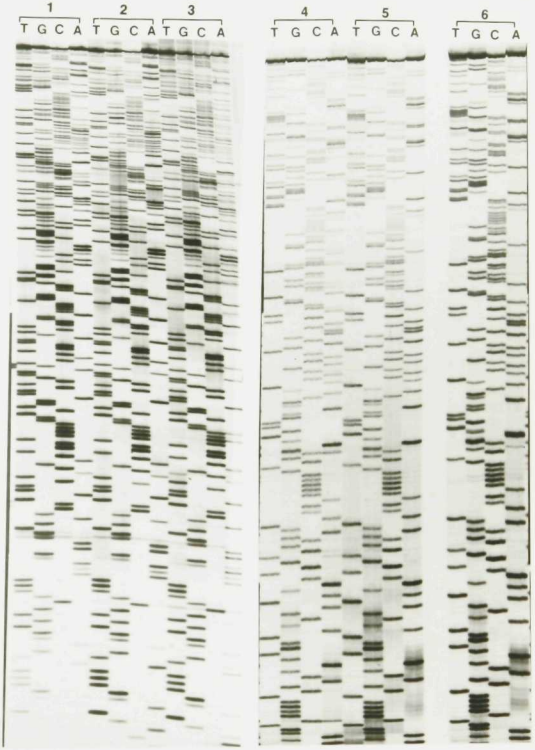

primer 1
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
primer 2
primer 3
primer 5
2.9 kb
IV
V
VI
VII
VIII
IX
primer 4
primer 6
asymmetric PCR
356 bp
V
VI
VII
VIII
IX
780 bp
Centricon 30
purification
Sequenase kit
sequencing

Figure 20

DNA sequence of exons 5 and 6 of the p53 gene. Amplification and sequencing of exons 5 and 6 were carried out as described in Figure 19. Primer 4 of p53 gene was used in the sequencing reaction. The reaction was run on 6% polyacrylamide gel at 1600 Volts for 2 hours (panel 1-3) or 6 hours (panel 4-6).


1
2
3
4
5
6
T G C A T G C A T G C A T G C A T G C A T G C A

5 to 8, as well as introns 5 and 8. To verify the sequences, the opposite strand was sequenced by a second PCR reaction in which the ratio of the pair of primers was reversed. Figure 21 shows a sample sequence obtained from exon 7 by p53 primer 5. Mutation was not observed in 18 esophageal carcinoma samples in exon 7, a frequent "hot spot" in human malignancies. However, an identical germline point mutation at codon 245 of the p53 gene was identified in 4 of the 10 members of a cancer prone family with Li-Fraumeni syndrome by this method (Srivastava, *et al*. 1990). It appeared that the current strategy was a simple, efficient, and reliable method for examine p53 mutations in human tumors.

Frequent Mutations of p53 Gene in Esophageal Carcinoma from the High Incidence Area

Eighteen esophageal carcinoma samples from the high incidence area Linxian, China were analyzed, including 17 squamous cell carcinoma and an adenocarcinoma of the gastric cardia. Of these cases, 10 had a familial history of esophageal cancer. The patients were not receiving any treatment before surgery except for two cases given radiation therapy these two cases showed no mutation). Amplification and direct sequencing of the "hot spots" regions revealed 7 out of 18 cases (39%) bearing a point mutation in the coding sequence of p53 (Table 7). Three mutations caused a termination codon (tumor m691, m692 and m701 in codon 146, 317

Table 7. p53 Mutation in Human Esophageal Carcinomas

Tumor #	Codon	Normal/ Mutant	Family History
m685	146	frame shift	yes
m691	146	TGG/TGA (Trp/Stop)	yes
m692	317	CAG/TAG (Gln/Stop)	yes
m695	163	TAC/CAC (Tyr/His)	yes
m700	175	CGC/CAC (Arg/His)	no
m701	165	CAG/TAG (Gln/Stop)	no
m702	195	ATC/ACC (Ile/Thr)	no

Figure 21

DNA sequence of exon 7 of the p53 gene. Amplification and sequencing of exon 7 were carried out as described in Figure 19. Primer 7 of the p53 gene was used in the sequencing reaction. The reaction was run on 6% polyacrylamide gel at 1600 Volts for 2 hours.

1
2
3
4
T G C A T G C A T G C A T G C A

and 165 respectively, indicating that premature products were produced in these cases. Tumor m685 had a single base deletion resulting in a frame shift change in codon 146; a heterogeneous sequencing ladder was seen after the deletion of this base, as indicated by the arrow in the Figure 22. The remaining three were missense mutations leading to an amino acid change in codon 163 for tumor m695 (Figure 23), codon 175 for m700, and codon 195 for tumor m702. Four out of seven cases showing mutations have a positive familial history for esophageal carcinoma. All seven mutations presented heterozygosity in their respective site as indicated by the equal signal intensity of normal band and the mutant band or slightly stronger normal band. There were two possible explanations for these results: first, that one allele was mutated while the other allele remained unchanged; and second, that both alleles were mutated in the tumor tissue, but that the normal band resulted from the presence of normal stromal cells and surrounding tissue. Overall, these results indicated that p53 mutations occurred frequently in esophageal cancer from high incidence areas. Either mutant proteins or premature products were produced, leading to impairment of p53 function. Because of the lack of paired normal control tissues and of tissue from other members of a proband's family, it could not be certain whether the mutations were inherited.

Figure 22

Single base deletion in the p53 gene. HMW DNA from tumor sample m685 was amplified and sequenced by the methods described in the Materials and Methods. The arrow indicates the single base deletion in one allele resulting in a sequence pattern which represents a combination of both the normal allele and the frame-shifted allele.

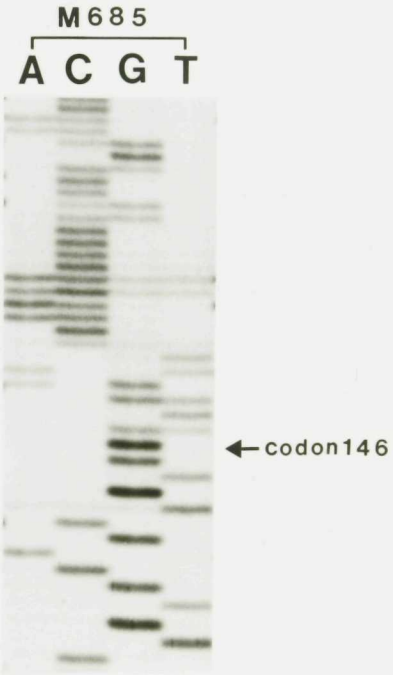
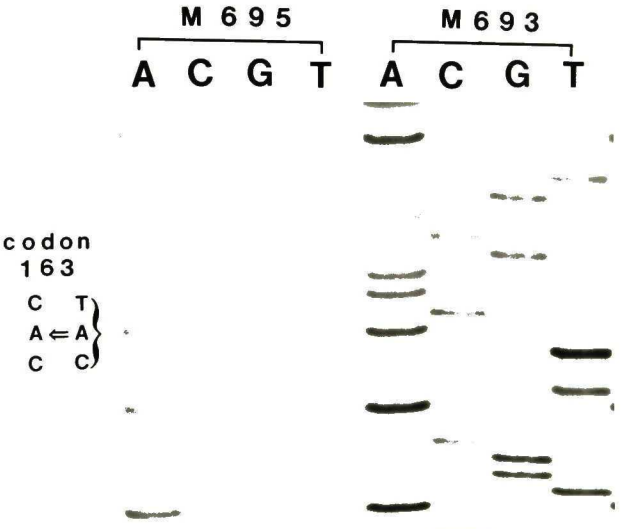

M685
A C G T
codon146

Figure 23

Missense point mutation in the p53 gene. The regions of p53 gene spanning codon 126 through 330 were amplified and sequenced as described in the Materials and Methods. Location of the mutation in one allele is shown for tumor m695. Tumor m693 shows the normal sequence of this site.


M 6 9 5
M 6 9 3
A C G T A C G T
codon
163
C T
A ⇐ A
C C

Development of a Rapid PCR Method to Clone Human Transforming Sequences from a Mouse Transformant

The DNA transfection assay is one approach to the identification of activated oncogenes which may be present in tumors tissue. Cloning of the genomic sequence, of which a chosen piece is used as a probe for subsequent cDNA cloning, was generally carried out by screening a genomic library constructed from the mouse cell line transformed by human DNA with a human Alu probe. We have designed a strategy for skipping this time consuming step by the use of the polymerase chain reaction with Alu primers. About a millon copies of the Alu repeat sequence are interspersed throughout the human genome. When a human gene was transferred into the mouse genome, it possibly carried a numbers of Alu repeats. The primers used for PCR were from the human Alu repeat sequence, designed in such a way that only the inter-Alu sequence be amplified (Figure 24).

Four DNA samples of transformed NIH3T3 cell containing known human Alu sequence (HER2/neu, N-ras, c-raf and unknown gene or genes) were tested. The results were shown in Figure 25. All four samples yielded multiple clear fragments whose size ranged from 0.1 kb to 1.3 kb. The parental NIH3T3 cellular DNA also yielded several bands which are distinguishable from those in the transformants DNA (Lane 1). Southern hybridization with the human Alu probe (Figure 26 A) revealed amplification of human sequence with size of up to

Figure 24

Schematic illustration of amplification of inter-Alu sequences. Boxed Alu denotes the human Alu repetitive sequence. Solid black lines denote the inter-Alu sequence. Horizontal arrows indicate the direction of primer extension.

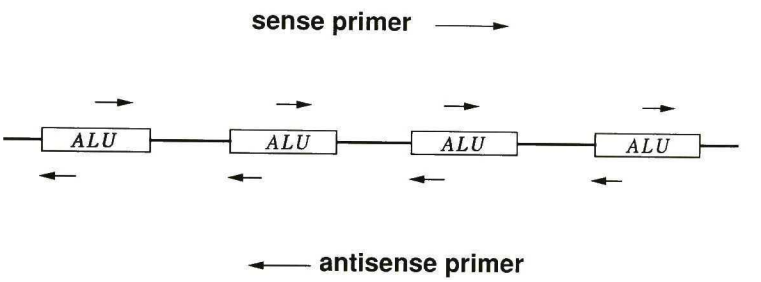

sense primer
ALU
ALU
ALU
ALU
antisense primer

Figure 25

Amplification of human genomic sequences in DNA of the mouse transformants. Four DNA samples contain N-ras gene, the HER2 gene (secondary transformant induced by human esophageal cancer DNA), C-raf gene and an unknown gene (primary transformant induced by esophageal cancer DNA) respectively. Amplification was performed at 94^{o}C, 1'45"; 52^{o}C, 1'; and 72^{o}C, 3'30" for 35 cycles. One tenth of the reaction was electrophoresed on 1.2% agarose gel. Photograph of an ethidium bromide stained gel is shown. The sizes of molecular weight marker are indicated.

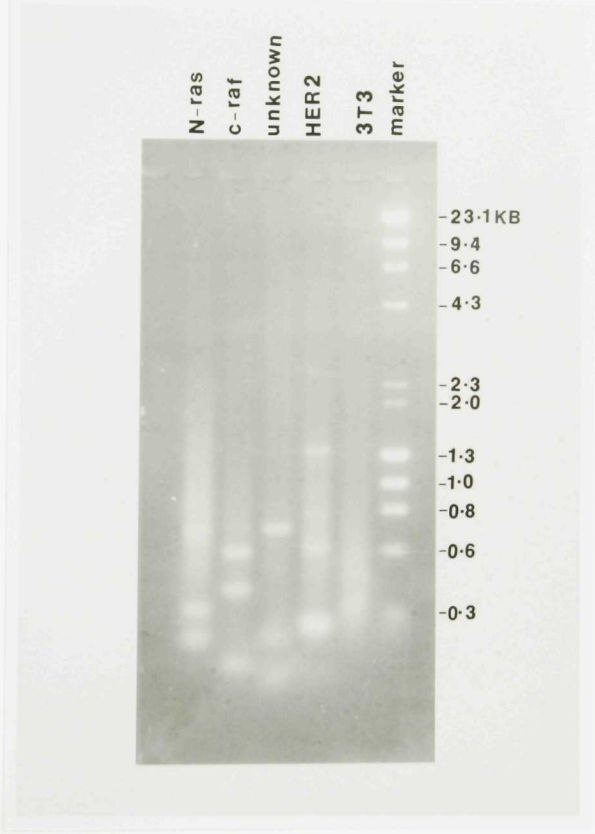
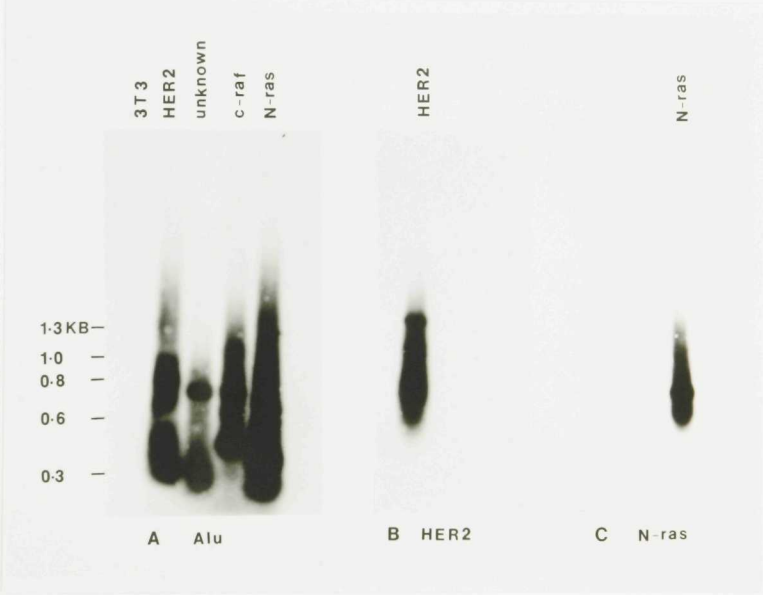

N-ras
c-raf
unknown
HER2
3T3
marker
23·1KB
9·4
6·6
4·3
2·3
2·0
1·3
1·0
0·8
0·6
0·3

Figure 26

Southern analysis of amplified human sequences from DNA of the mouse transformants. The PCR products were electrophoresed, transferred into nylon membrane and hybridized with each probe as described in the Materials and Methods. The identical membrane (Figure 25) was hybridized to the Alu probe (A), complete cDNA of HER2 (B), or N-ras cDNA respectively. The sizes of marker are shown.


3T3
HER2
unknown
c-raf
N-ras
HER2
N-ras
1·3 KB
1·0
0·8
0·6
0·3
A Alu
B HER2
C N-ras

to 2 kb (lane 2-5) while no hybridization was detected in NIH3T3 DNA (lane 1). The Alu probe had been stripped off, the very same membrane was rehybridized with cDNA probe of N-ras, HER2 or c-raf (Figure 26 B, C). The samples known to contain one of these genes showed the specific hybridization between the cDNA probe and its corresponding PCR product, HER2 and N-ras (lane 2, HER2. lane 3, unknown. lane 4, c-raf,lane 5, N-ras). C-raf probe did not reveal hybridization. These results demonstrated that the human genomic sequences could be selectively amplified from the mouse transformant DNA by using PCR primers of the Alu sequence, and also showed that the amplified DNA sequences frequently contain the coding sequence of a given gene.

Discussion

The geographical distribution of esophageal carcinoma suggests that different etiologic factors are involved in its pathogenesis. The diverse factors may act in some common way to induce genetic and epigenetic changes. The resulting genetic alterations ultimately lead to the deregulation of the molecular control of cell growth. A genetic model has been described for the progression of colon cancer (Vogelstein, *et al.* 1988; Fearon and Vogelstein, 1990). In this model, sequential genetic alterations were found at each stage from hyperplasia to metastatic carcinoma. Both allelic loss or mutation of tumor suppressor genes, including the familial adenomatous polyposis gene (FAP) in which mutation causes a hereditary predisposition to colon carcinoma, the DCC gene (deleted in colon cancer) and the p53 gene, and activation of oncogene via point mutation, such as, K-ras gene have been associated with the pathogenesis of colon cancer (Fearon and Vogelstein, 1990). However, little is known about the molecular events accompanying the genesis of esophageal cancer, whereas the concomitant pathological changes, proceeding from hyperplasia to invasive cancer, have been well described (Correa, 1982; Munoz, *et al.* 1982). In order to explore the mechanism underlying esophageal tumorigenesis, we have collected specimens of esophageal carcinoma samples from a high-incidence area in an attempt to identify the presence of abnormal genes by DNA transfection assay by using the

calcium-phosphate precipitation method and to examine the incidence of mutations in a suspected tumor suppressor gene, p53 gene.

In the present study, HER2 gene was identified in a secondarily transformed NIH3T3 cell line induced by DNA of a human esophageal tumor. An intensive search for the mechanism of activation has been performed. To date, all the oncogenes identified by transfection assays have exhibited alterations occurring either in the original tumor or during the transfection process (Bishop, 1991; Varmus, 1989). A substantial body of evidence indicated that the amplification and overexpression of HER2 significantly contributed to the genesis of breast carcinoma (Slamon, *et al.* 1987; 1989), but the gene in such cases, generally was structurally normal. In some gastrointestinal tumors, gene rearrangement was seen (Tal, *et al.* 1988; Yokota, *et al.* 1987). However, in neither of these cases has extracted DNA been reported to be capable of transforming NIH3T3 cells by the HER2 gene. Here, then, for the first time, it has been demonstrated that the human HER2 gene was transferred from the tumor DNA into the NIH3T3 cell by transfection assay. Interestingly, this HER2 originated from a human squamous cell carcinoma of the esophagus, a type of tumor in which amplification and expression is not common.

The observed 15 to 20 fold increase of copy number of HER2 in the transformed cell line, accompanied by its elevated expression, may suffice to render the transfected NIH3T3 cell

tumorigenic in nude mice. The amplification of HER2 gene in the transformant occurred as a consequence of transfection process. *In vitro* studies have shown that an increase of the normal HER2 oncogene product was capable of transforming NIH3T3 cell (Di Fiore, *et al.* 1987; Hudziak, *et al.* 1987). This, in turn, may be due to the achievement of a critical threshold of receptor-associated tyrosine kinase activity (Segatto, *et al.* 1988), producing a proliferation signal to the transduction pathway, which is persistent and independent of ligand stimulation.

Transmembrane domains function to anchor the growth factor receptors in the plasma membrane. Point mutations in the transmembrane region may cause conformational changes which result in activation of tyrosine kinase and so mimic stimulation by extracellular ligand (Bargmann, *et al.* 1986). Since the neu gene was activated by the replacement of a valine residue with a glutamic acid residue (Bargmann, *et al.* 1986), we hypothesized that the same change might also occur in the HER2 gene in the transformant. Before we had the complete cDNA clone, we first sequenced a 1 kb BamHI genomic fragment, covering the transmembrane region. The fragment was subcloned from the 6.6 kb EcoRI fragment (S3) from the secondary transformant. At amino acid codon 655 (within the transmembrane domain), an isoleucine residue was found to replace a valine residue reported for normal placental HER2 cDNA sequence (Coussens, *et al.* 1985). This substitution was further confirmed by cDNA sequencing of the HER2 gene from the

same transformant cell line. A substitution of glutamic acid for valine was not found at the equivalent position in the activated neu gene of rat, and clearly, the activation of this human HER2 did not share the same molecular mechanism as that in the neu gene. This result was consistent with the observations that point mutation at a site equivalent to that found in the mutant neu rarely occur in the human HER2.

Interestingly, HER2 genes isolated from a panel of different tumors all had the isoleucine residue at the second codon (655) of the transmembrane region close to N-terminus, whereas, a placental cDNA showed a valine residue in this same site (Yamamoto, *et al.* 1986; Di Fiore, *et al.* 1987; Slamon, *et al.* 1989; Coussens, *et al.* 1985). To determine whether this change implicated a mutation, or was attributed to genetic polymorphism, we examined 47 DNA samples by allele specific oligonucleotide hybridization; the results favored the latter interpretation. The frequency of each residue was not significantly different in normal and tumor samples, although the isoleucine was present more frequently than the valine residue. Because the same isoleucine residue was found in the DNA of peripheral leukocytes as in the tumor tissue from the same individual with breast carcinoma, it suggested an inherited event instead of a somatic mutation. Since the valine to isoleucine change is conservative, it is possible that the substitution does not materially alter the function of HER2. However, in 19 cases of esophageal cancer samples, 10 of 11 cases with a family history had the isoleucine

residue at codon 655 (two residues from the extracellular terminus), whereas only 3 of 8 cases without a family history had the isoleucine residue. But the sample size of the present study was not large enough to conclude that an isoleucine residue may predispose to tumor development, further analysis with a larger number of samples will be required to define the "disease-prone" allele in a statistically significant way.

In attempt to identify the mechanism of the HER2 activation, we analyzed the original tumor DNA and the transformant DNA by Southern blot analysis. In the original tumor DNA, the copy number of the HER2 gene was normal, and gene rearrangements were not observed in the DNA banding pattern digested with several restriction enzyme. In the transformant DNA, the HER2 sequence was amplified 15 to 20 fold. Additionally, smaller-than-normal size fragments were observed by using cDNA probes for both termini of the gene, suggesting that sequence loss had occurred during integration to the mouse genome. Both end of the gene may be linked either to cellular host sequences, or to human sequence by rearrangement. The later was indicated by the presence of human DNA sequences other than HER2 gene in the transformant. However, the expected size of the mRNA in the Northern blot indicated that the lost sequence most likely did not affect the integrity of the HER2 gene in the transformant. The cDNA sequence from the secondary transformant was also analyzed completely. No significant changes were observed except for

the polymorphic substitution at codon 655 within the transmembrane domain. Thus, we have isolated a normal HER2 gene in terms of coding sequence. The acquisition of multiple copies of HER2 gene and cognate overexpression during the transfection process may play the role in the cellular transformation of NIH3T3 cells in our experiments. The identification of this HER2 gene via transfection assay was similar to the identification hst1 gene in that no abnormality was found in the hst1 gene from the normal gastric mucosa and normal leukocytes, and that frequent amplification of hst1 is observed in human tumors (Sakamoto, *et al.* 1986; 1988).

Activation of HER2 gene, generally by amplification and overexpression, was associated to the development of adenocarcinoma. Involvement of this gene in squamous cell carcinoma of the esophagus has not been reported. Since we have isolated the HER2 gene from a transformant induced by DNA of an esophageal squamous cell carcinoma DNA, we further analyzed DNA samples from 16 esophageal squamous cell carcinoma for HER2 alteration by Southern blot. No amplification or rearrangement was observed in these tumor samples. This suggests that the identification of HER2 gene in this study may represent a fortuitous event and only suggests a genetic instability which is common to all types of tumor.

The 500 bp of HER2 promoter region was sequenced directly on PCR amplified products. It was retained normally in both the original and the transformant tumors. Since the

original tumor DNA contained a normal copy number of HER2 gene, it was likely that the expression *in vivo* was regulated at the transcriptional level by other cis-elements or trans-acting factors. *In vitro* studies have shown that the activity of HER2 promoter can be induced by a variety of agents, and this suggests that expression of HER2 can vary in response to cellular signals *in vivo* (Hudson, *et al.* 1990). In the mouse transformants, amplification and /or recombination at both ends of the HER2 gene might provide for the high transcription activity. Finally, our strategy for sequencing the HER2 promoter region may be applied to the study of other malignancies in which the HER2 gene was overexpressed without gene amplification.

Putative suppressor sequences flanking the HER2 gene might be replaced by other sequences, or a transcriptionally active sequence may have been introduced upstream to the HER2 gene during the transfection process in NIH3T3 cell. Acquisition of the transforming activity by rearrangement of upstream sequences to the coding sequence has been observed in the hst1 gene (Sakamoto, *et al.* 1988). The hst1 genes from a stomach cancer or a Koposi's sarcoma showed a variety of replacements genomic sequence at the 5' upstream to the gene (Delli Bovi, *et al.* 1987). Since the junction sites between the HER2 gene and the mouse sequence at its 5' and 3' of HER2 termini were not determined, the structural identity of these sequences remained to be analyzed. At any rate, the possible suppressor sequence located outside of the gene sequence may

have been removed. Alternatively, the integration of the human gene may also fortuitously activate proto-oncogenes or inactivate the tumor suppressor genes in the mouse genome.

The transforming activity of this HER2 was tested by placing it under the control of a SV40 early promoter and under its own promoter in constructs, SV40-HER2 or HER2-PROM-HER2 (see Figure 15). Preliminary experiments yielded inconclusive results. The only tumor induced by the SV40-HER2 construct in two experiments contained HER2 cDNA, but no product could be detected in the transformants. It seemed likely that the construct was defective in expressing detectible levels of the gene product. It has been shown that increased production of HER2 in expression plasmids under the control of an LTR was able to induce tumor formation but low level of protein expressed using the SV40 promoter was not sufficient to transform the NIH3T3 cell (Di Fiore, *et al.* 1987; Hudziak, *et al.* 1987). We initially suspected that the HER2 we isolated had undergone genetic change and it should be able to induce transformation of NIH3T3 cells, therefore, we put the HER2 gene under the control of a relatively weak promoter, SV40 early promoter, to test the tumorigenesis of this HER2. Although the tumorigenesis experiment was not informative, it was reasonable to suspect that the current normal HER2 cDNA would lack the transforming ability when it was expressed only at a low level.

In addition to the HER2 gene, another 6.6 kb Alu positive EcoRI human genomic fragment was also cloned from the

same secondary transformant tumor. Southern analysis showed that this fragment was not likely to be linked immediately next to the HER2 gene in the mouse genome. Alteration at the end of this fragment occurred during the transfection process. Its size changed between the primary and secondary transformant, and it showed substantial differences from the original DNA as well as placental DNA. HER2 also showed alteration at both termini of the gene. However, the same size of the terminal fragments were found in both primary and secondary transformants. We speculate that the recombination event during the transfection may have brought the HER2 gene and this fragment into proximity with some unidentified human sequences between them. Although the relationship between this fragment and HER2 gene in the transformant has not been determined, it was clear that they were amplified as a unit in the transformant as suggested by the comparable level of amplification in both primary and secondary tumors. No gene product has been associated with this DNA fragment in the transformant cell line by Northern analysis. Whether this fragment or its associated DNA sequences play any role in the cellular transformation of the NIH3T3 cell remains unclear.

In summary, the human HER2 gene was identified in a secondarily transformed NIH3T3 cell line induced by DNA originating from a human esophageal carcinoma. Amplification and expression of the gene appeared to cause neoplastic transformation of the NIH3T3 cells. This result was consistent with the previous reports showing that increased

amounts of a normal gene product were sufficient to transform the NIH3T3 cells.

Among the genetic changes that govern the emergence of cancer, two major classes of genes, tumor oncogene and tumor suppressor gene, have been intensively studied in a variety of tumors. Neither activation of oncogenes nor inactivation of suppressor genes are sufficient to transform a normal cell into a tumor cell. Rather, an accumulated genetic alteration leading to the deregulation of multiple cellular-growth-control pathways is apparently required for tumor progression. Since the tumor suppressor gene p53 is associated with a great many types of tumor, an investigation of the P53 gene mutation in esophageal carcinomas from endemic incidence areas should help to understand the molecular mechanisms involved in the causation of this type of tumor. PCR and direct sequencing of the hot spots of p53 gene revealed that 7 out of 18 carcinoma samples examined in the present study had p53 point mutations. Of the seven cases with mutations, four cases were from families with a history of esophageal cancer. From these results, it can be speculated that certain patients may inherit a mutant form of P53 and so be predisposed to tumor formation. The high incidence of clustering in families may result from such an inherited defect (mutant p53), or other so-far unidentified defective suppressor gene. Identification of a germ line-transmitted mutant p53 gene in cancer prone families with the Li-Fraumeni syndrome strongly supports this possibility (Srivastava, *et al.* 1990; Malkin, *et al.* 1990).

In all seven cases of esophageal cancer having point mutations in p53, none were homozygous or hemizygous. There are two explanations for this, first, that only one allele carried the mutation, and the other remained normal; second, that the mutation could be hemizygous in the tumor tissue and the DNA preparations contain normal stromal and / or contaminated with adjacent normal tissue. In this study, the DNA was not isolated from microdissected pure tumor tissue. Thus, the second explanation was more likely true. The high frequency of p53 mutation (39%) observed suggests that the p53 gene is a frequent target gene to be mutated in the high incidence areas.

The proposed dual functions of mutated or normal p53 gene products may contribute to transform the cell by the acquisition of a transforming activity or the deprivation of a tumor suppressor activity respectively (Lane and Benchimol, 1990). The high level of exposure to N-nitrosamine in this area could increase the frequency of gene mutation (Wu, 1986). DNA adducts formed by chemical carcinogens frequently cause G to T transitions in mutation assays (McMahon, *et al.* 1990). More frequent rates of sister chromotid exchanges and chromosome aberrations have been observed in this high risk population (Wu, 1986).

Aflatoxin B1, a fungal metabolite product, appeared to be strongly associated with a specific mutation in the p53 gene in hepatocellular carcinoma from the high incidence areas in China and South Africa where infection with hepatitis B

virus (HBV) and consumption of moldy food were prevalent (Hsu, *et al.* 1991; Bressac, *et al* 1991). In Linxian, frequent contamination of foodstuffs with a variety of fungi has been suspected to be an important risk factors. The mycotoxin may elicit p53 mutation through a process different from the one by aflatoxin in liver cancer. The mutations found in the present study showed a scattered distribution, they were presumably attributable to diverse etiological agents, encountered at various times during the life-span of the individuals living in the high incidence area.

In addition, the oncoproteins encoded by the DNA tumor viruses have been found to bind to the wild type p53 protein and promote the degradation of p53 (Werness, *et al.* 1990; Scheffner, *et al.* 1990). The presence of one mutant allele (its partner remaining normal) might confer one step of a genetic change, while infection of HPV could provide another step by eliminating the suppressor function of the remaining normal p53 protein. Low level of p53 expression has been observed in human cervical carcinoma cell lines which contain the HPV 16 and/or 18 genome (unpublished data). The presence of HPV genome has also been documented in esophageal carcinoma from patients of Linxian (Chang, *et al.* 1990). To test whether the tumor DNA samples analyzed in this study contain the HPV genome, we analyzed all samples with HPV specific primer by PCR (Sarkar and Crissman, 1990). Neither HPV 16 nor HPV 18 DNA sequences (which are the two subtypes most frequently encountered in genital neoplasms) were amplified.

Most likely, these viruses were not implicated in the esophageal carcinomas tested, although the possible involvement of other types of HPV could not be excluded.

In summary, p53 mutation occurred frequently in esophageal carcinoma. The mutant p53 might confer a growth advantage to cells harboring other abnormal (quantitatively or qualitatively) gene products, such as, growth factors or their receptors. Deprivation of normal p53 function resulted in the loss of growth inhibition. The functional defect of P53, as a cofactor, may play a role in the pathway to esophageal carcinoma in the Linxian area, China.

CONCLUSION

Modern technology of molecular biology provides many powerful tools for studying the molecular genetic alterations occurring in human malignancies. The understanding of these genetic alterations in neoplasia will provide theoretical knowledge leading to the treatment of this deadly disease. The geographic distribution and familial clustering of esophageal carcinoma suggests that multiple etiological factors are involved in the development of this type of cancer. Pathologically, the disease proceeds through multiple stages from chronic esophagitis to dysplasia progressing to invasive neoplasm. However, little is known about the molecular basis underlying the gross abnormalities. In general, activation of oncogenes or inactivation of tumor suppressor genes should occur during the course of esophageal cancer. In turn, multiple factors may contribute to the initiation and promotion of the process of the genetic alterations.

An initial attempt to identify transforming genes in esophageal squamous cell carcinoma from high incidence area lead to the identification of a HER2/neu oncogene from a second cycle transformed NIH3T3 cell line. Increased copy numbers of HER2 gene and its expression was identified in both primary and secondary transformant tumor; loss of the HER2 flanking sequence occurred without affecting the coding sequence. Both changes were absent in the original tumor DNA.

The analysis of the complete cDNA sequence of the HER2 isolated from the secondary transformant cell line showed no significant change except for a difference of a single amino acid at codon 655, a valine residue in a sequence of placental cDNA was replaced by a isoleucine residue in cDNA isolated from tumors. This single amino acid difference was further demonstrated to be a polymorphic change by allele specific oligonucleotide hybridization on 47 DNA samples. The frequency of each allele showed no difference between normal and tumor samples. Above all, the present study for the first time has identified the activation of HER2 oncogene by DNA transfection assay. An overexpression of the HER2 resulting from either amplification and / or recombination at the flanking sequence may cause the NIH3T3 cell transformation. Whether this reflects the activation of HER2 leading to the tumor formation in the original sample is not clear. It is likely that the HER2 activation in this assay is only a fortuitous event.

Furthermore, human sequences other than the HER2 gene appeared in the same transformant cell line. A genomic fragment cloned from the secondary transformant was not linked immediately to the HER2 gene at both ends of the gene. It was found rearranged during the transfection process. Since it showed the same extent of amplification as the HER2 gene in the transformant, this fragment was possibly cotransferred as a same unit with the HER2 gene. No expression products could be detected in the transformants by this fragment. Whether

this fragment or its related sequence play any role in the transformation requires further study.

Tumor suppressor genes have been shown to be important in the development of malignancy. Defective structure and function of tumor suppressor genes have been implicated in the pathogenesis of most human malignancies (Marshall, 1991). The present study found frequent p53 mutations in samples of human esophageal carcinoma collected in one of the highest incidence area, Linxian, China. Seven of the eighteen samples analyzed showed point mutations in the coding region of the p53 gene. Regardless of the nature of mutation, inherited or somatic, one or both alleles affected, the presence of the mutant p53 suggests that it is a frequent target susceptible to mutagenic insult. The resulting defective function of p53, as one of the many molecular events, may contribute to the development of esophageal carcinoma.

Bibliography

Ali, I.U., Campbell, G., Lidereau, R., & Callahan, R. Amplification of c-erbB-2 and aggressive human breast tumors? Science, 240:1795-1796, 1988.

Baker, S.J., Markowits, S., Fearon, E.R., Willson, J.K.V., & Vogelstein, B. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science, 249:912-915, 1990.

Baker, S.J., Preisinger, A.C., Jessup, J.M., Parasheva, C., Markowits, S., Willson, J.K.V., Hamilton, S., & Vogelstein, B. p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. Cancer Research, 50:7717-7722, 1990.

Baker, S.J., Fearon, E.R., Nigro, J.M., Hamilton, S.R., Preisinger, A.C., Jessup, J.M., VanTuinen, P., Ledbetter, D.H., Baker, D.F., Nakamura, Y., White, R., & Vogelstein, B. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science, 244:217-221, 1989.

Barbacid, M. Ras genes. Annu. Rev. Biochem., 56:779-827, 1987.

Bargmann, C.I., Hung, M.C., & Weinberg, R.A. Multiple

independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p^{185}. Cell, 45:649-657, 1986.

Bargmann, C.I. & Weinberg, R.A. Increased tyrosine kinase activity associated with the protein encoded by the activated neu oncogene. Proc. Natl. Acad. Sci. USA., 85:5394-5398, 1988.

Bastos, R.N., Vollich, Z., & Aviv, H. Messenger RNA population analysis during erythroid differentiation: a kinetical approach. Journal of Molecular Biology, 110:191-203, 1977.

Bishop, M.J. The molecular genetics of cancer. Science, 235:305-311, 1987.

Bishop, M.J. Molecular themes in oncogenesis. Cell, 64:235-248, 1991.

Bookstein, R., Shew, J.Y., Chen, P.L., Scully, P., & Lee, W.H. Suppression of tumorigenicity of human prostate carcinoma cells by replacing a mutated RB gene. Science, 247:712-715, 1990.

Bos, J.L. The ras family and human carcinogenesis. Mutat. Res., 195:255-271, 1988.

Bos,J.L., Fearon, E.R., Hamilton, S.R., Verlaan-de Vries, M., van Boom, J.H., van der EB, A.J., & Vogelstein, B. Prevalence of ras gene mutations in human colorectal cancers. Nature, 327:293-297, 1987.

Braithwaite, A.W., Sturzbecher, H.W., Addison C., Palmer, C., Rudge, K., & Jenkins, J.R. Mouse p53 inhibits SV40 origin dependent DNA replication. Nature, 329:458-460, 1987.

Bressac, B., Kew, M., Wands, J., & Ozturk, M. Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. Nature, 350:429-430, 1991.

Burgess, W.H. & Maciag, T. The heparin-binding (fibroblast) family of proteins. Annu. Rev. Biochem., 58:576-606, 1989.

Chang, F.J., Syrjanen, S., Shen, Q., J.I, H., & Kyrjanen, K. Human papilloma virus (HPV) DNA in esophageal precursor lesions and squamous cell carcinomas from China. Int. J. Cancer, 45:21-25, 1990.

Chang-Claude, J.C., Wahrendorf, J., Qiu, S.L., Yang, G.R., Munoz, N., Crespi, M., Raedsch, R., Thurnham, D.I. & Correa, P. An epidemiological study of precursor lesions of esophageal cancer among young persons in a high risk population in Huixian, China. Cancer Research, 50:2268-2274, 1990.

Chow, V., David, Y.B., Bernstein, A., Benchimol, S., & Mowat, M. Multistage friend erythroleukemia: independent origin of tumor clones with normal or rearranged p53 cellular oncogenes. Journal of Virology, 61:2772-2781, 1987.

Cline, M., Battifora, H., & Yokota, J. Protooncogene abnormalities in human breast cancer: Correlations with anatomic features and clinical course of disease. J. Clin. Oncol., 5:999-1006, 1987.

Cooper, G.M. & Neiman, P.E. Transforming genes of neoplasms induced by avian lymphoid leukosis viruses. Nature, 287:656-660, 1980.

Correa, P. Precursors of gastric and esophageal cancer. Cancer, 50:2554-2565, 1982.

Coussens, L., Yang-Feng, T.L., Liao, Y.C., Chen, E., Gray, A., McGrath, J., Seeberg, P.H., Libermann, T.A., Schlessinger, J., Francke, U., Levinson, A., & Ullrich, A. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science, 230:1132-1138, 1985.

Crawford, L.V., Pim, D.C., & Bulbrook, R.D. Detection of antibodies against the cellular protein p53 in sera from patients with breast cancer. Int. J. Cancer, 30:403-408,

1982.

Crespi, M., Munoz, N., Grassi, A., Shen, Q., Wang, K.J., & Lin, J.J. Precursor lesions of esophageal cancer in a low-risk population in China: comparison with high-risk population. Int. J. Cancer, 34:599-602, 1984.

Crespi, M., Munoz, N., Grassi, A., Aramesh, G., & Mojtabei, A. Oesophageal lesions in northern Iran; a premalignant condition? Lancet, 2:217-221, 1979.

Czosnek, H.H., Bienz,B., Givol, D., Zakut-Houri, R., Pravtcheva, D.D., Ruddle, F.H., & Oren, M. The gene and the pseudogene for mouse p53 cellular tumor antigen are located on different chromosomes. Mol. Cell. Biol., 4:1636-1640, 1984.

Delli Bovi, P., & Basilico, C. Isolation of a rearranged human transforming gene following transfection of Kaposi sarcoma DNA. Proc. Natl. Acad. Sci. USA., 84:5660-5664, 1987.

Di Fiore, P.P., Piers, J.H, Kraus, M.H., Segatto, O., King, C.R., & Aaronson, S.A. erbB2 is a potent oncogene when overexpressed in NIH3T3 cells. Science, 237:178-182, 1987.

Di Fiore, P.P., Aaronson, S.A., & Piers J.H. EGF receptor and erbB-2 tyrosine kinase domains confer cell specificity for mitogenic signaling. Science, 248:79-83, 1990.

Diller, L., Kassel, J., Nelson, C.E., Gryka, M.A., Litwak, G., Grebhardt, M. Bressac, B., Ozturk, M., Baker, S., Vogelstein, B., & Friend, S.H. p53 function as a cell cycle control protein in osteosarcomas. Mol. Cell. Biol., 10:5772-5781, 1990.

Ding, J.H. & Wu, M. Genetically determined susceptibility to esophageal cancer in high incidence areas of north China and the genetic approach to its control. Genes and Disease, (Science Press, Beijing, China) 1985.

Doerfler, W. DNA methylation and gene activity. Ann. Rev. Biochem., 52:93-124, 1983.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J., & Waterfield, M.D. Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature, 307:521-527, 1984.

Drebin, J.A., Link, V.C., Stern, D.F., Weinberg, R.A. & Greene, M.I. Down modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. Cell, 41:695-706, 1985.

Drebin, J.A., Link, V.C., & Greene, M.I. Monoclonal antibodies reactive with distinct domains of the neu oncogene

encoded p185 molecule exert synergistic anti-tumor effects. Oncogene, 2:273-277, 1988.

Drebin, J.A., Link V.C., Weinberg, R.A., & Greene, M.I. Inhibition of tumor growth by a monoclonal antibody reactive with an oncogene-encoded tumor antigen. Pro. Natl. Acad. Sci. USA., 83:9129-9133, 1986.

Eliyahu, D., Raz, A., Gruss, P., Givol, D., & Oren. M. Participation of p53 cellular tumor antigen in transformation of normal embryonic cells. Nature, 312:646-649, 1984.

Eliyahu, D., Michalovitz, D., Eliyahu, S., Pinhasi-Kimhi, O., Oren, M. Wild-type p53 can inhibit oncogene-mediated focus formation. Pro. Natl. Acad. Sci. USA., 86:8763-8767, 1989.

Fasano, O., Birnbaum, D., Edlund, L., Fogh, J., & Wigler, M. New human transforming genes detected by a tumorigenicity assay. Mol. Cell. Biol., 4:1695-1705, 1984.

Fearon, E.R., & Vogelstein, B. A genetic model for colorectal tumorigenesis. Cell, 61:759-757, 1990.

Fearon, E.R., Cho, K.R., Nigro, J.M., Kern, S.E., Simons, J.W., Ruppert, J.M., Hamilton, S.R., Preisinger, A.C., Thomas, G., Kinzler, K. W. & Vogelstein, B. Identification of a chromosome 18q gene that is altered in colorectal cancers.

Science, 247:49-56, 1990.

Fields, S. & Jang, S.K. Presence of a potent transcription activating sequence in the p53 protein. Science, 249:1046-1049, 1990.

Finlay, C.A., Hinds, P.W., & Levine, A.J. The p53 proto-oncogene can act as a suppressor of transformation. Cell, 57:1083-1093, 1989.

Finlay, C.A., Hinds, P.W., Tan, T.H., Eliyahu, D., Oren, M., & Levine, A.J. Activating mutations for transformation by p53 produce a gene product that forms on hsc70-p53 complex with and altered half life. Mol. Cell. Biol., 8:531-539, 1988.

Flanagan, J.G., & Leder, P. Neu proto-oncogene fused to an immunoglobulin heavy chain gene requires immunoglobulin light chain for cell surface expression and oncogenic transformation. Proc. Natl. Acad. Sci. USA., 85:8057-8061, 1988.

Fong, L.Y., Sivak, A., & Newberne, P.M. Zinc deficiency and methylbenzylnitrosamine-induced esophageal cancer in rats. Journal of National Cancer Institute, 61:145-150, 1978.

Friend, S.H., Bernards, R., Rogelj, S., Weinberg, R.A., Rapaport, J.M. Albert, D.M., & Dryia, T.P. A human DNA

segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature, 323: 643-646, 1986.

Fukushige, S.I., Matsubara, K.I., Yoshida, M., Sasaki, M., Suzuki, T., Semba, K., Toyoshima, K., & Yamamoto, T. Localization of a novel v-erbB-related gene, c-erbB-2, on human chromosome 17 and its amplification in a gastric cancer cell line. Mol. Cell. Biol., 6:955-958, 1986.

Gibbs, R.A. DNA amplification by the polymerase chain reaction. Anal. Chem., 62:1202-1214, 1990.

Gannon, J.V., & Lane, D.P. p53 and DNA polymerase alpha compete for binding to SV40 T antigen. Nature, 329:456-458, 1987.

Glanville, N. Unstable expression and amplification of a transfected oncogene in confluent and subconfluent cells. Mol. Cell. Biol., 5:1456-1462, 1985.

Goate, A., Chartier-Harlin, M.C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., Mant, R., Newton, P., Rooke, K., Roques, P., Talbot, C., Pericak-Vance, M., Roses, A., Williamson, R., Rossor, M., Owen, M., & Hardy, J. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature, 349:704-706, 1991.

Graham, F.L., & Van Der Eb, A.J. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology, 52:456-467, 1973.

Gross-Bellard, M., Oudet, P., & Chambon, P. Isolation of high molecular weight DNA from mammalian cells. European Journal of Biochemistry, 36:32-38, 1973.

Guerin, M., Barrois, M., Terrier, M.J., Spielmann, M., & Riou, G. Overexpression of either c-myc or c-erbB-2/neu amplification in human breast carcinomas: Correlation with poor prognosis. Oncogene Research, 3:21-31, 1988.

Gusterson, B.A., Machin, L.G., Gullick, W.J., Gibbs, N.M., Powles, T.J., Elliott, C., Ashley, S., Monaghan, P., & Harrison, S. c-erbB expression in benign and malignant breast disease. Br. J. Cancer, 58:453-457, 1988.

Gyllensten, U.B., & Erllich, H.A. Generation of single stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. Proc. Natl. Acad. Sci. USA., 85:7652-7656, 1988.

Halevy, O., Michalovits, D., & Oren, M. Different tumor derived p53 mutants exhibit distinct biological activities. Science, 250:113-116, 1990.

Harlow, E., Willianson, N.M., Ralston, R., Halfman, D.M., & Adams, T.E. Molecular cloning and *in vitro* expression of a cDNA clone for human cellular tumor antigen p53. Mol. Cell. Biol., 5:1601-1610, 1985.

Harper, P.S., Harper, R.M.J., & Howel-Evans, A.W. Carcinoma of the esophagus with tylosis. Q. J. Med., 39:317-333, 1970.

Hinds, P., Finlay, C., Frey, A., & Levine, A.J. Immunological evidence for the association of p53 with a heat shock protein,hsc70, in p53 plus ras transformed cell lines. Mol. Cell. Biol., 7:2863-2869, 1987.

Hollingsworth, R.E., & Lee, W.H. Tumor suppressor genes: new prospects for cancer research. Journal of the National Cancer Institute, 83:91-96, 1991.

Hollstein, M.C., Smits, A.M., Galiana, C., Yamasaki, H., Bos, J.L., Mandard, A., Partensky, C., & Montesano, R. Amplification of epidermal growth factor receptor gene but no evidence of *ras* mutations in primary human esophageal cancers. Cancer Research, 48: 5119-5123, 1988.

Hollstein, M.C., Metcalf, R.A., Welsh, J.A., Montesano, R., & Harris, C.C. Frequent mutation of the P53 gene in human esophageal cancer. Proc. Natl. Acad. Sci. USA., 87:9958-9961, 1990.

Honkawa, H., Masahashi, W., Hashimoto, S., & Hashimoto-Gotoh, T. Identification of the principal promoter sequence of the c-H-ras transforming oncogene: deletion analysis of the 5'-flanking region by focus formation assay. Mol. Cell. Biol. 7:2933-2940, 1987.

Hsu, I.C., Metcalf, R.A., Sun, T., Welsh, J.A., Wang, N.J., & Harris, C.C. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Nature, 350:427-428,1991.

Huang, S.S., Koh, H.A., Konish, Y., Bullock, L.D., & Huang, J.S. Differential processing and turnover of the oncogenically activated neu/erb2 gene product and its normal cellular counterpart. The Journal of Biological Chemistry, 265:3340-3346, 1990.

Hudson, L.G., Ertl, A.P., & Gill, G.N. Structure and inducible regulation of the Human c-erbB2/neu promoter. The Journal of Biological Chemistry, 265:4389-4393, 1990.

Hudziak, R.M., Lewis, G.D., Lewis, G.D., Shalaby, M.R., Eessalu, T.E., Aggarwal, B.B., Ullrich, A., & Shepard, H. M. Amplified expression of the HER2/erbb2 oncogene induces resistance to tumor necrosis factor in NIH3T3 cells. Proc. Natl. Acad. Sci. USA., 85:5102-5106, 1988.

Hudziak, R.M., Schlessinger, J., & Ullrich, A. Increases

expressing of the putative growth factor receptor $p185^{HER2}$ causes transformation and tumorigenesis of NIH3T3 cells. Proc. Natl. Acad. Sci. USA., 84:7159-7163, 1987.

Hunter, T. Cooperation between oncogenes. Cell, 64:249-270, 1991.

Ishii, S., Imamoto, F., Yamanashi, Y., Toyoshima, K., & Yamamoto, T. Characterization of the promoter region of the human c-erbB-2 protooncogene. Proc. Natl. Acad. Sci. USA., 84:4374-4378, 1987.

Jiang, W. Transforming gene in human esophageal carcinoma tissue. Chung Hua Chung Liu Tsa Chih, 10:330-334, 1988.

Kawasaki, E.S., Clark, S.S., Coyne, M.Y., Smith, S.D., Champlin, R., Witte, O., & McCormick, F.P. Diagnosis of chronic myeloid and acute lymphocytic leukemia by detection of leukemia-specific mRNA sequences amplified *in vitro*. Proc. Natl. Acad. Sci. USA., 85:5698-5702, 1988.

Kelman, Z., Prokocimer, M., Peller, S., Kahn, Y., Rechavi, G., Manor, Y., Cohen, A., & Rotter, V. Rearrangements in the p53 gene in Philadelphia chromosome positive chronic myelogenous leukemia. Blood, 75:2318-2324, 1989.

King, C.R., Borrello, I., Bellot, F., Comoglio, P., &

Schlessinger, J. EGF binding to its receptor triggers a rapid tyrosine phosphorylation of the erbB2 protein in the mammary tumor cell line SK-BR-3. The EMBO Journal, 6:1647-1651, 1988.

Kitagawa, Y., Ueda, M., Ando, N., Shinozawa, Y., Shimizu, N., & Abe, O. Significance of int-2/hst-1 coamplification as a prognostic factor in patients with esophageal squamous carcinoma. Cancer Research, 51:1504-1508, 1991.

Knudson, A.G. Mutation and cancer: statistical study of retinoblastoma. Proc. Natl. Acad. Sci. USA., 68:820-823, 1971.

Kraus, M.H., Issing, W., Miki, T., Popescu, N.C., & Aaronson, S.A. Isolation and characterization of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. Proc. Natl. Acad. Sci. USA., 86:9193-9197, 1989.

Kraus, M.H., Popescu, N.C., Amsbaugh, S.C., & King, C.R. Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. The EMBO Journal, 6:605-610, 1987.

Lane, D.P. & Crawford, L.V. T antigen is bound to a host protein in SV40-transformed cells. Nature, 278:262-263, 1979.

Lane, D.P., & Benchimol, S. Oncogene or antioncogene? Genes Dev., 4:1-8, 1990.

Lavigueur, A., Maltby, V., Mock, D., Rossant, J., Pawson, T., & Bernstein, A. High incidence of lung, bone, and lymphoid tumors in transgenic mice overexpressing mutant alleles of the p53 oncogene. Mol. Cell. Biol., 9:3982-3991, 1989.

Lee, W.H., Bookstein, R., Hong, F., Young, L.J., Shew, J.Y., & Lee, E.Y.H.P. Human retinoblastoma susceptibility gene: cloning, identification, and sequence. Science, 235:1394-1399, 1987.

Lee, J., Dull, T.J., Lax, I., Schlessinger, J., & Ullrich, A. HER2 cytoplasmic domain generates normal mitogenic and transforming signals in a chimeric receptor. The EMBO Journal, 8:167-173, 1989.

Lemoine, N.R., Staddon, S., Dickson, C., Barnes, D.M., & Gullick, W.J. Absence of activating transmembrane mutations in the c-erbB2 proto-oncogene in human breast cancer. Oncogene, 5:237-239, 1990.

Levine, A.J., & Monard, J. Tumor suppressor genes: The p53 and retinoblastoma sensitivity genes and gene products. Biochem. Biophys. Acta, 1032:119-136, 1990.

Li, M.X., & Cheng, S.J. Carcinogenesis of esophageal cancer in LinXian, China. Chinese Medical Journal, 97:311-316, 1984.

Li, G.H., & He, L.J. A survey of familial aggregation of esophageal cancer in Yangcheng county, ShanXi Province. Chin. Med. J. (Engl.Ed.), 98:749-752, 1985.

Li, J.Y., Ershow, A.G., Chen, Z.J, Wacholder, S., Li, G.Y., Guo, W., Li, B., & Blot, W.J. A case-control study of cancer of the esophagus and gastric cardia in LinXian. Int. J. Cancer, 43:755-761, 1989.

Li, M.H., Ji, C., & Cheng, S.J. Occurrence of nitroso compounds in fungi-contaminated foods: a review. Nutr. Cancer, 8:63-69, 1986.

Liechtenstein, A., Berenson, J., Gera, J.F., Waldburger, K., Martinez-Maza,O., & Berek, J.S. Resistance of human ovarian cancer cells to tumor necrosis factor and lymphokine-activated killer cells: correlation with expression of HER2/neu oncogenes. Cancer Research, 50:7364-7370, 1990.

Lizner, D.I.H. & Levine, A.J. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell, 17:43-52, 1979.

Lu, S.H., Zhang, M.S., Luo, F.C., Feng, L., & Tian, Y. O^6-methyldeoxyguanosine in DNA of the adjacent epithelium to the human esophageal cancer in Linxian county, China. J. Oncol. 8:328-331, 1986.

Lu, S.H., Camus, A.M., Tomatis, L., & Bartsch, H. Mutagenicity of extracts of pickled vegetables collected in Linhsien county , a high incidence area for esophageal cancer in northern China. Journal of National Cancer Institute, 66:33-36, 1981.

Lu, S.H., Ohshima, H., Fu, H.M., Tian, Y., Li, F.M., Blettner, M., Wahrendorf, J., & Bartsch, H. Urinary excretion of N-nitrosamino acids and nitrate by inhabitants of high- and low-risk areas for esophageal cancer in northern China: endogenous formation of nitrosoproline and its inhibition by vitamin C. Cancer Research, 46: 1485-1491, 1986.

Lu, S.H., Hsieh, L.L., Luo, F.C., & Weinstein, I.B. Amplification of the EGF receptor and c-myc genes in human esophageal cancers. Int. J. Cancer, 42:505-502, 1988.

Lu, S.H., Montesano, R., Zhang, M.S, Feng, L., Luo, F.J., Chui, S.X., Umbenhauer, R., Saffhill, R., & Rajewsky, M.F. Relevance of nitrosamine to esophageal cancer in China. J. Cell. Physiol., 54:51-58, 1986.

Malkin, D., Li, F.P., Strong, L.C., Fraumeni, J.F. Jr., Nelson, C.E., Kim, D.H., Kasse, J., Graka, M.A., Bischoff, F.Z., Tainsky, M.A., & Friend, S.H. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science, 250:1223-1238, 1990.

Marco, E.D., Pierce, J.H., Knicley, C.L., & Di Fiore, P.P. Transformation of NIH NIH3T3 cells by overexpression of the normal coding sequence of the rat neu gene. Mol. Cell. Biol. 10:3247-3252, 1990.

Marshall, C.J. Tumor suppressor genes. Cell, 64: 313-326, 1991.

McBride, O.W., Merry, D., & Givol, D. The gene for human p53 cellular tumor antigen is located on chromosome 17 short arm(17p13). Proc. Natl. Acad. Sci. USA., 83:130-134, 1986.

McGlashan, N.D., Bradshaw, E., & Harington, J.S. Cancer of the esophagus and the use of tobacco and alcoholic beverages in Transkei, 1975-76. Int. J. Cancer, 29:249-256, 1982.

McMahon, G., Davis, E.F., Huber, L.J., Kim, Y., & Wogan, G.N. Characterization of c-Ki-ras and N-ras oncogenes in aflatoxin B_1-induced rat liver tumors. Proc. Natl. Acad. Sci. USA., 87:1104-1108, 1990.

Meek, D.W., & Eckhart, W. Phosphorylation of p53 in normal and Simian Virus 40 -transformed NIH NIH3T3 cells. Mol. Cell. Biol., 8:461-465, 1988.

Mercer, W.E., Shield, M.T., Lin, D., Appella, E., & Ullrich, S.T. Growth suppression induced by wild type p53 protein is accompanied by selective down regulation of proliferation-cell nuclear antigen expression. Pro. Natl. Acad. USA., 88:1958-1962, 1991.

Mercer, W.E., Shields, M.T., Amin, M., Sauve, G.J., Appella, E., Romano, J.W., & Ullrich, S.J. Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild type p53. Pro. Natl. Acad. Sci USA., 87: 6166-6170, 1990.

Mitra, G., Pauly, G.T., Kumar, R., Pei, G.K., Hughes, S.H., Moschel, R., & Barbacid, M. Molecular analysis of O^6-substituted guanine-induced mutagenesis of ras oncogenes. Pro. Natl. Acad. Sci. USA., 86:8650-8654, 1989.

Mowat, M., Cheng, A. Kimura, N., Bernstein, A., & Benchimol, S. Rearrangement of the cellular p53 gene in erythroleukemic cells transformed by friend virus. Nature, 314:633-636, 1985.

Muller, W.J., Sinn, E., Pattergale, P.K., Wallace, R., & Leader, P. Single step induction of mammary adenocarcinoma in

transgenic mice bearing activated c-neu oncogene. Cell, 54,105-115, 1988.

Mulligan, L.M., Matlashewski, G.J., Scrable, H.J., & Cavenee, W.K. Mechanisms of p53 loss in human sarcomas. Pro. Natl. Acad. Sci. USA., 87: 5863-5867, 1990.

Mullis, K.B. & Faloona, F.A. Specific synthesis of DNA *in vitro* via polymerase catalyzed chain reaction. Methods Enzymol, 155: 335-350, 1987.

Munoz, N., Crespi, M., Grassi, A., Wang, G.Q., Shen, Q., & Li, Z.C. Precursor lesions of esophageal cancer in high risk populations in Iran and China. Lancet, 1:876-879, 1982.

Munoz, N., Wahrendorf, J., Lu, J.B., Crespi, M., Thurnham, D.I., Day, N.E, Zheng, H.J., Grassi, A., Li, W.Y., Liu, G.L., Lang, Y.Q., Zhang, C.Y., Zheng, S.F., Li, J.Y., Correa, P., O'Connor, G.T., & Bosch, F.X. No effect of riboflavin, retinol, and zinc on prevalence of precancerous lesions of esophagus. Lancet, 2:111-114, 1985.

Nigro, J.M., Baker, S., Preisinger, A.C., Jessup, J.M., Hostter, R., Cleary, K., Bigner, S.H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F.S., Weston, A., Modali, R., Harris, C.C., & Vogelstein, B. Mutations in the p53 gene occur in diverse human tumor types. Nature,

342:705-708, 1989.

Oettle, G.L., Paterson, A.C., Leiman, G., & Segal, I. Esophagitis in a population at risk for esophageal carcinoma. Cancer, 57:2222-2229, 1986.

Okayama, H., & Berg, P. A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. Mol. Cell. Biol., 3:280-289.

Ozawa, S., Ueda, M., Ando, N., Shimizu, N., & Abe, O. Prognostic significance of epidermal growth factor receptor in esophageal squamous cell carcinomas. Cancer, 63:2169-2173, 1989.

Ozawa, S., Ueda, M., Ando, N., Abe, O. & Shimizu, N. High incidence of EGF receptor hyperproduction in esophageal squamous-cell carcinomas. Int. J. Cancer, 39:333-337, 1987.

Parada, L.F., Land, H., Weinberg, R.A., Wolf, D., & Rotter, V. Cooperation between gene encoding p53 tumor antigen and ras in cellular transformation. Nature, 312:649-651, 1984.

Park, J.B., Rhim, J.S., Park, S.C., Kimm, S.W., & Kraus, M.H. Amplification, overexpression, and rearrangement of the erbB-2 protooncogene in primary human stomach carcinomas. Cancer Research, 49:6605-6609, 1989.

Paterson, M.C., Dietrich, K.D., Danyluk, J., Paterson, A.H.G., Lees, A.W., Jamil, N., Jenkins, H.H., Krause, B.E., Mcblain, W.A., Slamon, D.J., & Fourney, R.M. Correlation between c-erbB-2 amplification and risk of recurrent disease in node-negative breast cancer. Cancer Research, 51:556-567,1991.

Perucho, M., Hanahan, D., & Wigler, M. Genetic and physical linkage of exogenous sequence in transformed cells. Cell, 22:309-315, 1980.

Pimentel, E. Oncogenes (2ed), Florida, CRC Press,Inc. 291-317, 1989.

Ponder, B.A. Gene losses in human tumors. Nature, 335:400-402, 1988.

Press, M.F., Cordon-Cardo, C., & Slamon, D.J. Expression of The HER2/neu proto-oncogene in normal human adult and fetal tissues. Oncogene, 5:953-962, 1990.

Qiu, S.L., & Yang, G.R. Precursor lesions of esophageal cancer in high risk population in henan province, China. Cancer, 62:551-557, 1988.

Raycroft, L., Wu, H., & Lozano, G. Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene. Science, 249:1049-1051, 1990.

Rogel, A., Popliker, M., Webb, C.G., & Oren, M. p53 cellular tumor antigen: Analysis of mRNA levels in normal adult tissues embryos and tumors. Mol. Cell. Biol. 5:2851-2855, 1985.

Sager, R. Tumor suppressor genes: the puzzle and the promise. Science, 246:1406-1412, 1989.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., & Erlich, H.A. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, 239:487-491, 1988.

Saiki, R.K., Scharf, F., Faloona, F.A., Mullis, K.B., Horn, G.T., Erlich, H.A., & Arnheim, N. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science, 230:1350-1354, 1985.

Sakamoto, H., Mori, M., Taira, M., Yoshida, T., Matsukawa, S., Shimizu, K., Sekiguchi, M., Terada, M., & Sugimura, T. Transforming gene from human stomach cancers and a noncancerous portion of stomach mucosa. Proc. Natl. Acad. Sci. USA., 83:3997-4001, 1986.

Sakamoto, H., Yoshida, T., Nakakuki, M., Odagiri, H., Miyagawa, K., Sugimura, T., & Terada, M. Cloned hst gene from normal human leukocyte DNA transforms NIH3T3 cells.

Biochemical and Biophysical Research Communications, 151:965-972, 1988.

Sarkar, F.H. & Crissman, J.D. Detection of human papilloma virus DNA sequences by polymerase chain reaction. BioTechniques, 9:180-185, 1990.

Schechter, A.L., Stern, D.F., Vaidyanthan, L., Decker, S.J., Drebin, J.A., Greene, M.I., & Weinberg, R.A. The neu oncogene: an erbB-related gene encoding a 185,000-M_r tumor antigen. Nature, 312:513-516, 1984.

Scheffner, M., Werness, B.A., Huibregtse, J.M., Levine, A.J., & Howley, P.M. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of P53. Cell, 63:1130-1136, 1990.

Segatto, O., King, C.R., Pierce, J.H., Di Fiore, P.P., & Aaronson, S.A. Different Structural alterations upregulate *in vitro* tyrosine kinase activity and transforming potency of the erbB-2 gene. Mol. Cell. Biol., 8:5570-5574, 1988.

Semba, K., Kamata, N., Toyoshima, K., & Yamamoto, T. A v-erbB-related protooncogene, c-erbB-2, is distinct from the c-erbB-1/EGF receptor gene and is amplified in a human salivary gland adenocarcinoma. Proc. Natl. Acad. Sci. USA., 82:6497-6501, 1985.

Shih, C., Padhy, L.C., Murray, M., & Weinberg, R.A. Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. Nature, 290:261-263, 1981.

Shu, Y.J. The cytopathology of esophageal carcinoma: precancerous lesions and early cancer. New York masson pub.USA. Chapter 2, 1985.

Singer, G.M., Ji, C., Roman, J., Li, M.H., & Lijinsky, W. Nitrosamine and nitrosamine precursors in foods from Linxian, China, a high incidence area for esophageal cancer. Carcinogenesis, 7:733-736, 1986.

Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A., & McGuire, W.L. Human breast cancer:Correlation of relapse and survival with amplification of the HER2/neu oncogene. Science, 235:177-182, 1987.

Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A., & Press, M.F. Studies of the Her2/neu proto-oncogene in human breast and ovarian cancer. Science, 244:707-712, 1989.

Soussi, T., deFronmentel, C.C., Mechali, M., May, P., & Kress, M. Cloning and characterization of a cDNA from Xenopus Laevis coding for a protein homologous to human and murine p53.

Oncogene, 1:71-78, 1987.

Soussi, T., Fromentel, C.C.D., & May, P. Structural aspects of the p53 protein in relation to gene evolution. Oncogene, 5:945-952, 1990.

Southern, E.M. Detection of specific sequence among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology, 98:503-517, 1975.

Sporn, M.B., & Roberts, A.B. Role of retinoids in differentiation and carcinogenesis. Cancer Research, 74:1291-1297, 1983.

Srivastava, S., Zou, Z.Q., Pirollo, K., Blattner, W., & Chang, E.H. Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. Nature, 348: 747-749, 1990.

Stanbridge, E.J. Genetic analysis of human malignancy using somatic cell hybrids and monochromosome transfer. Cancer Surv., 7:317-324, 1988.

Sturzbecher, H.W., Brain, R., Maimets, T., Addison, C., Rudge, K., & Jenkins, J.R. Mouse p53 blocks SV40 DNA replication *in vitro* and downregulates T antigen DNA helicase activity. Oncogene, 3:405-413, 1988.

Takahashi, T., Nau, M.M., Chiba, I., Birrer, M.J., Rosenberg, R.K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A.F., & Minna, J. p53 : a frequent target for genetic abnormalities in lung cancer. Science, 246:494-494, 1989.

Tal, M., King, C.R., Kraus, M.H., Ullrich, A., Schlessinger, J., & Givol, D. Human Her2(neu)promotor: evidence for multiple mechanisms for transcriptional initiation. Mol. Cell. Biol., 7:2597-2601, 1987.

Tal, M., Wetzler, M., Josefberg, Z., Deutch, A., Gutman, M., Assaf, D., Kris, R., Shiloh, Y., Givol, D., & Schlessinger, J. Sporadic amplification of the HER2/neu protooncogene in adenocarcinomas of various tissues. Cancer Research, 48:1517-1520, 1988.

Tsuda, T., Tahara, E., Kajiyama, G., & Sakamoto, H. High incidence of coamplification of hst-1 and int-2 genes in human esophageal carcinoma. Cancer Research, 49:5505-5508, 1989.

van de Vijver, M.J., Mooi, W.J., Wisman, P., Peterse, J.L., & Nusse, R. Immunohistochemical detection of the neu protein in tissue sections of human breast tumors with amplified neu DNA. Oncogene, 2:175-178, 1988.

Varmus, H. An historical overview of oncogenes. Oncogenes and the Molecular Origins of Cancer, New York: Cold spring

Harbor Laboratory Press, 3-44, 1989.

Ventor, D.J., Tuzi, N.L., Kumar, S. & Gullick, W.J. Overexpression of the c-erbB-2 oncoprotein in human breast carcinomas: Immunohistological assessment correlates with gene amplification. lancet, 2:69-72, 1987.

Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, A.C., Leppert, M., Nakaura, Y., White, R., Smits, A.M.M, & Bos, J.L. Genetic alterations during colorectal-tumor development. N. Engl. J. Med., 319:525-530, 1988.

Wagata, T., Ishizaki, K., Imamura, M., Shimada, Y., Ikenaga, M., & Tobe, T. Deletion of 17p and amplification of the int-2 gene in esophageal carcinoma. Cancer Research, 51:2113-2117, 1991.

Wahrendorf, J., Munoz, N., Lu, J.B., Thurnham, D.I., Crespi, M., & Bosch, F.X. Blood retinol, zinc and riboflavin status in relation to precancerous lesions of the esophagus: findings from a vitamin intervention trial in People's Republic of China. Cancer Research, 48:2280-2283, 1988.

Wahrendorf, J., Claude, J.C., Qiu, S.L., Yang, G.R., Munoz, N., Crespi, M., Raedsch, R., Thurnham, D., & Correa, P. Precursor lesions of esophageal cancer in young people in a high risk population in China. The Lancet, 2:1239-1240, 1989.

Wang, E.H., Friedman, P.N., & Prives, C. The murine p53 protein blocks replication of SV40 DNA *in vitro* by inhibiting the initiation functions of SV40 large T antigen. Cell, 57:379-392, 1989.

Wang, Y., You, M., Reynolds, S.H., Stoner, G.D., & Anderson, M.W. Mutational activation of the cellular Harvey *ras* oncogene in rat esophageal papillomas induced by methyl benzylnitrosamine. Cancer Research, 50:1591-1595, 1990.

Wang, T., Miller, K.W., Tu, Y.Y. & Yang, C.S. Effects of riboflavin deficiency on metabolism of nitrosamines by rat liver microsomes. Journal of National Cancer Institute, 74:1291-1297, 1985.

Werness, B.A., Levine, A.J., & Howley, P.M. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science, 248:76-83, 1990.

Whang-Peng, J., Banks-Schlegel, S.P., & Lee, E.C. Cytogenetic studies of esophageal carcinoma cell lines. Cancer Genet. Cytogenet., 45:101-120, 1990.

Wigler, M., Pellicer,A., Silverstein, S. & Axel, R. Biochemical transfer of single copy eukaryotic genes using total cellular DNA as donor. Cell, 14:725-734, 1978.

Wildenhain, Y., Pawson, T., Blackstein, M.E., & Andrulis, I.L. $p185^{neu}$ is phosphorylated on tyrosine in human primary breast tumors which overexpress neu/erbB-2. Oncogene, 5:879-883, 1990.

Wu, Y.Q. Genetic etiology of esophageal cancer--III. The spontaneous and mitomycin-c (MMC) induced sister chromatid exchanges (SCE) and chromosome aberrations in cultured lymphocytes from members of "high risk and "low risk esophageal cancer families in LinXian County. Chung Hua Chung Liu Tsa Chih, 8:23-25, 1986.

Wu, M., Hu, N., & Wang, X. Genetic factors in the etiology of esophageal cancer and the strategy for its prevention in high-incidence areas in Northern China. Genetic Epidemiology of Cancer. Florida, CRC Press, Inc. 187-202, 1989.

Wu, Y.C., & Ran, S.Z. Genetic etiology of esophageal cancer:Cytogenetic study of individuals in five"cancer families" in Linxian. Acta Genet.Sin., 6:3-7,1979.

Yamamoto, T., Ikawa, S., Akiyama, T., Semba, K., Nomura, N., Miyajima, N., Saito, T., & Toyoshima, K. Similarity of protein encoded by the human c-erbB-2 gene to epidermal growth factor receptor. Nature, 319:230-234, 1986.

Yamamoto, T., Kamata, N., Kawano, H., Shimizu, S., Kuroki, T.,

Toyoshima, K., Rikimaru, K., Nomura, N., Ishizaki, R., Pastan, I., Gamous, S., & Shimizu, N. High incidence of amplification of the epidermal growth factor receptor gene in human squamous carcinoma cell lines. Cancer Research, 46:414-416, 1986.

Yang, C.S. Research on esophageal cancer in China: A review. Cancer Research, 40:2633-2644, 1980.

Yokota, J., Yamamoto, T., Miyajima, N., Toyoshima, K., Nomura, N., Sakamoto, H., Yoshida, T., Terada, M., & Sugimura, T. Genetic alteration of the c-erbB-2 oncogene occur frequently in tubular adenocarcinoma of the stomach and are often accompanied by amplification of the v-erbA homologue. Oncogene, 2:283-287, 1987.

Yoshida, K., Kyo, E., Tseda, T., Tsujino, T., Ito, M., Niimoto, M., & Tahara, E. EGF and TGF-alpha, the ligand of hyperproduced EGFR in human esophageal carcinoma cells, act as autocrine growth factors. Int. J. Cancer, 45:131-135, 1990.

Yuasa, Y., & Sudo, K. Transforming genes in human hepatomas detected by a tumorigenic assay. Jpn. J. Cancer Res., 78:651-654, 1987.

Zhou, D., Battifora, H., Yokata, J., Yamamoto, T., & Cline, M.J. Association of multiple copies of the c-erbB-2 oncogene with spread of breast cancer. Cancer Research, 47:6123-6125,

1987.

Zhou, D.J., Ahuja, H., & Cline, M.J. Proto-oncogene abnormalities in human breast cancer: c-erbB2 amplification does not correlate with recurrence of disease. Oncogene, 4:105-108, 1989.