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REGULATION OF *INK4* GENE EXPRESSION IN BREAST CANCER

by

P.Thirukkumaran

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ABSTRACT.

Thirty-five breast cancer tissues were analyzed for their mRNA expression patterns. No significant difference was detected in the expression patterns of *p15* ,*p18*, *p19* and *pRb* mRNA in these tissues. However, *p16* was found to be poorly expressed (< 0.0005% of mRNA) in breast cancer tissues and variable between specimens.

Gene structural alterations such as deletions, point mutations and methylation of the *p16* gene were examined in order to correlate the altered mRNA expression. No deletions were detected in *p16* gene. Gene alterations were found in the region of exon 3 in two tumor tissues (tissue #5, and #24). These alterations were more likely polymorphisms than mutations. No significant differences in the methylation patterns were found at various sites of the *p16* -5' regions of the variably expressing specimens. The low *p16* mRNA expression most likely was associated with methylation of various CpG sites at the promoter of the gene.

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This thesis is dedicated

to

MY DEAR FAMILY: CHANDINI, MENEKA AND VINOTH

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LIST OF ABBREVIATIONS

A	adenine
aa	amino acid
bp	base pair
C	cytosine
Ci	curie
^{0}C	degrees centigrade
CDGE	constant denaturing gradient gel electrophoresis
cDNA	complementary deoxyribonucleic acid
CpG	dinucleotide (cytosine- guanine)
cpm	counts per minute
Dal	Dalton
DGGE	denaturing gradient gel electrophoresis
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	nucleotide triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylene diamine tetraacetic acid
G	guanine
g	gram

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HPV	human papilloma virus
kb	kilobase
kDal	kilo Dalton
LB	Luria-Bertani
M	molar
met	methionine
ml	milliliter
mM	millimolar
nt	nucleotide
PCR	polymerase chain reaction
PEG	polyethylene glycol
poly(A)$^+$	polyadenylated (mRNA)
RNA	ribonucleic acid
S6	ribosomal protein gene
SDS	sodium dodecyl sulfate
SSCP	single stranded conformational polymorphism
ssDNA	single stranded deoxyribonucleic acid
SV40	simian virus 40
T	thymine
TE	Tris/EDTA (buffer)
Tm	melting temperature

Tris	tris(hydroxymethyl)aminomethane
pg	picogram
μg	microgram
μM	micromolar

1. INTRODUCTION

Breast cancer is one of the most common malignant diseases among women. Breast cancers that arise in glandular epithelial tissues are classified as adenocarcinomas. Based on the histological types they are classified as either ductal or lobular. The identification of specific molecular changes in these tumors may lead to development of novel tumor specific preventive and treatment strategies (Dhingra *et al.*, 1995).

1.1. Cell cycle regulation:

DNA replication in the eukaryotic cell cycle is a highly regulated process. Interphase (the time in between two mitoses) can be split into three intervals: G1, the gap between mitosis and the onset of DNA replication; the S phase, the period of DNA synthesis; and G2, the gap between S and M phases. The entry into S phase, the G1-S transition, is regulated by a family of protein kinases known as cyclin-dependent kinases(CDKs) (Elledge, 1996). A critical target of CDK enzymes is the retinoblastoma tumor suppressor protein (pRb). An overview of the cell cycle and the pRb pathway is summarized in Figure 1.

The development of human cancer is frequently associated with the inactivation of two major tumor suppressor (p53 or pRb) pathways (Weinberg, 1995 ; Sherr, 1996). p53 is induced after DNA damage by ionizing radiation. p53 induces p21, which is a universal CDK inhibitor and as a result cell cycle arrest occurs at the G1-S checkpoint.

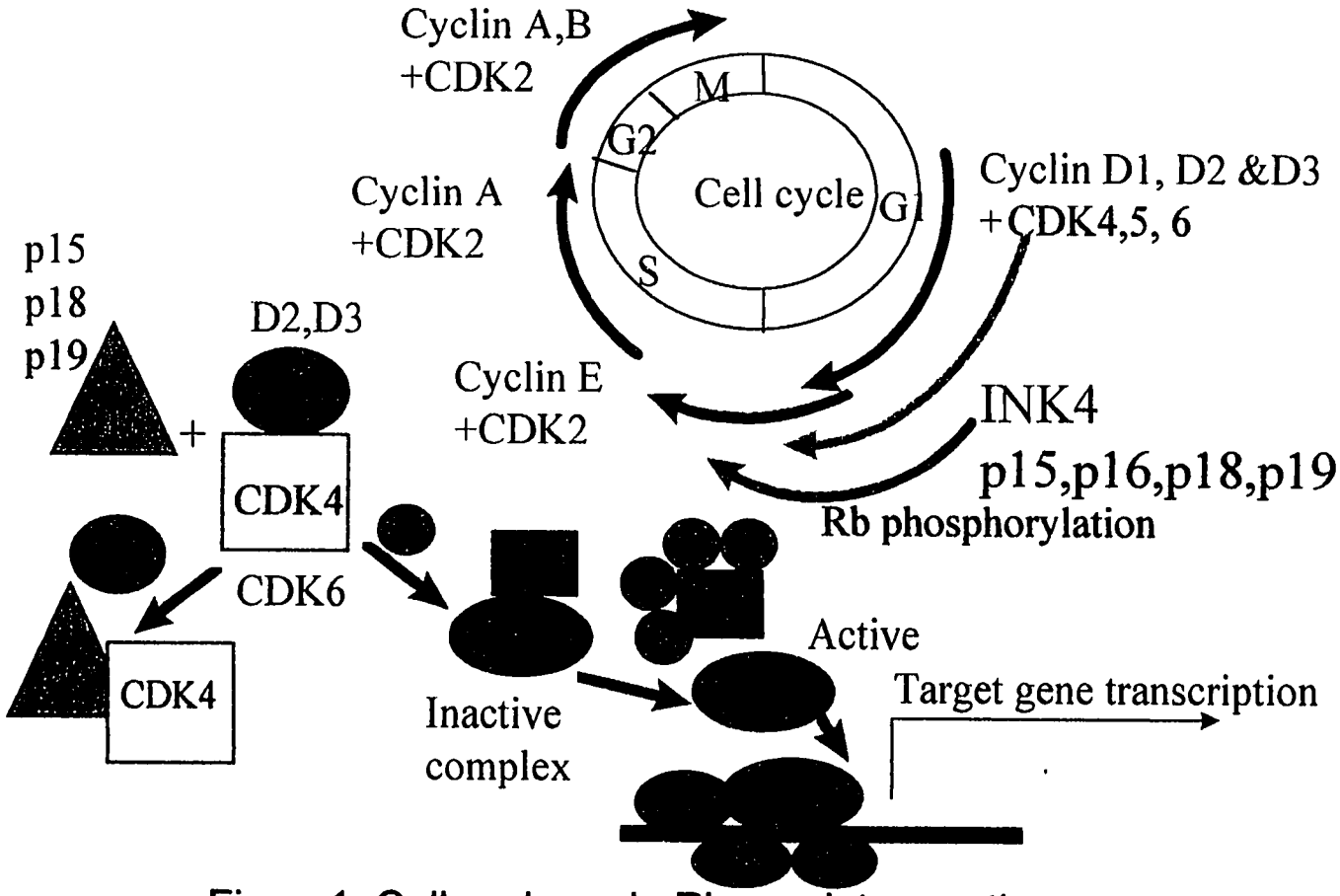


Figure1: Cell cycle and pRb regulatory pathway

This also slows the S phase and blocks progression in G2 allowing additional time for the repair of chromosomes before entry into mitosis (Sherr, 1994 ; Sherr, 1996). In the present study the pRb regulatory pathway was examined in detail.

1.2. pRb PATHWAY:

The retinoblastoma tumor suppressor gene product (pRb) is a nuclear protein of 110kDa. pRb binds to many other cellular proteins and plays a variety of roles in cell proliferation and differentiation. One of its key functions is to be an integrator of cellular signals connecting the major pathways involved in controlling the progression of cells through the cell cycle (Weinberg, 1995 ; Taya, 1997). Phosphorylation of pRb is a key step in its regulation during G1-S transition. Cyclins D (D1, D2, D3), E and A complexed with their catalytic partners, CDKs (CDK4, and CDK6), are prominently involved in the phosphorylation of pRb (Elledge *et al.*, 1996). The CDKs are integrated into diverse signaling pathways and are regulated by many mechanisms. Such mechanisms include cyclin abundance (Sherr, 1994 ; Sherr, 1996), positive and negative-acting phosphorylation of the kinase subunit and the actions of the inhibitory proteins Cycle Kinase Inhibitors (CKIs) or CyclinDependent Kinase Inhibitors (CDIs) (Hunter and Pines, 1994 ; Morgan, 1995).

Cyclin D1 is located on human chromosome 11q13 and is overexpressed in many human cancer (Inaba *et al.*, 1992 ; Xiong *et al.*, 1992). The amplification of this region 11q13 is frequent in a broad spectrum of common adult cancers, which includes 43% of squamous cell carcinomas of the head and neck, 34% of esophageal carcinomas, 15% of

bladder cancer and 13% of primary breast carcinoma. In breast cancer even though the amplification frequency is low (13%), more than 50% of the amplified products overexpress the protein(Sherr, 1996). This can lead to pRb hyper-phosphorylation and eventual tumor formation.

The gene encoding CDK4 is located on chromosome 12q13 (Demetrick *et al.*, 1996) and is also amplified in sarcomas and gliomas (Hall and Peters, 1996). Among several other potential oncogenes that are mapped to this region, a protooncogene *mdm2* is also frequently amplified in many human cancers. It has been reported that in 40% of human sarcomas tested by Oliner *et al.*(1992) the *mdm2* gene is amplified. MDM2 protein interacts with p53 and blocks the p53 mediated transactivation (Chen *et al.*, 1995) and targets the p53 for rapid degradation (Haput *et al.*, 1997).

Two classes of CKIs have been identified based on their sequence similarity, specificity of targets, and mechanisms of inhibition. The INK4 family (p16, p15 p18, and p19) is specific for CDK4 and CDK6 (Elledge *et al.*, 1996). Studies by (Serrano *et al.*, 1993) have shown that the activity of the CDK4 kinase complex is downregulated by p16, a specific inhibitor. The second group of the Kinase Inhibitor Protein(KIP) composed of three members, p21(also identified as Cip), p27(also identified as Kip1), p57 (Kip2). This CIP-KIP family proteins effectively inhibits multiple classes of kinases including CDK2, CDK3,CDK4, and CDK6 (Sherr and Roberts, 1995). pRb and other related proteins such as p130, p107 (Chellappan *et al.*, 1991) of this family control gene expression mediated by a family of heterodimeric transcriptional regulators is called the

E2Fs. pRB in its active (unphosphorylated) form binds to a variety of E2Fs such as E2F1, E2F2, and E2F3. Phosphorylation of pRb results in releasing these E2F transcription factors. These transcription factors have the ability to recognize the specific sequences in the promoter regions of a variety of genes and activate transcription of *c-myc* , *N-myc, c-myb, B-myb,* and *cdc2* which play prominent roles in cell growth control (Weinberg, 1995). The E2F family of transcription factors play a key role in G1-S progression. Five members in this family have been identified to date. As a result of phosphorylation of pRb, E2F is released from the complex. The free E2F is present as a heterodimer with its binding partner DP1 or DP2 and this active transcription factor promotes the transcription of E2F target genes (Knudsen and Wang, 1997). Sp1 is a Zinc finger DNA-binding protein. It has largely been recognized as a basal factor for which there are binding sites in TATA-less promoters. It has been reported by Lin *et al.*(1996) that Sp1 and E2F1 both functionally and physically interact. Therefore, Sp1 and E2F may regulate transcription of genes containing binding sites for either or both factors (Lin *et al.*, 1996).

The p16/pRb tumor suppressive pathway is abrogated in many human tumors. An inverse correlation between pRb and p16 tumor-suppressor proteins has been reported for many tumors(Okamoto *et al.*, 1994).

Yeager *et al.*,(1995) examined *p16* expression in human uroepithelial cell lines (HUC) and reported significant differences among HUC cell lines, while not detecting *p16* DNA mutations. However, an inverse correlation between elevated p16 and loss of

pRb function was observed. Ten samples with normal pRb showed low or undetectable p16 levels, while seven samples with known pRb alterations showed abundant p16 but nevertheless grew vigorously in culture. These results support the hypothesis that p16 mediated cell cycle inhibition, as well as p16 regulation, occurs via pRb dependent pathways (Yeager *et al.*, 1995). In another study, Shapiro *et al.*, (1998) demonstrated that non-small cell lung cancer cells, which retain pRb, and lack p53, do not arrest in G1 following DNA damage. However, engineered expression of p16 at levels compatible with cell proliferation restores a G1 arrest checkpoint in response to treatment with gamma-irradiation, topoisomerase I and II inhibitors and cisplatin. The authors concluded that, in response to DNA damage p16 expression is required for the reduction of CDK4 and CDK6 mediated pRb kinase activity. During tumor progression, loss of p16 expression may be necessary for cells with wildtype pRb to bypass this G1 arrest checkpoint and attain a fully transformed phenotype (Shapiro *et al.*, 1998).

1.3. INK4 proteins and their functions:

p16 and *p15* are tightly linked genes in the chromosome 9p21 region (Serrano *et al.*, 1993 ; Hannon and Beach 1994 ; Kamb *et al.*, 1994a). *p18* is located at 1p32 (Guan *et al.*,1994) and *p19* at 19p13 (Guan *et al.*, 1996). The *p16* locus can produce two transcripts p16α and p16β (also known as p16 and p19 ^{Arf}) that are derived from two distinct promoters (Quelle *et al.*, 1995 ; Serrano *et al.*, 1993 ; Serrano *et al.*, 1996). Both p16α and p16β differ only in the first exon (E1 α and E1 β) but contain the same exon 2 and exon 3. The alternative transcription product p19 ^{Arf} (p16β) blocks cell cycle

progression at both G1 and G2 phases, but the actual mechanism by which p16β accomplishes has been not elucidated (Elledge *et al.*, 1996).

Recently, two groups (Pomerantz *et al.*, 1998 ; Zhang *et al.*, 1998) reported that in mice p16β binds to MDM2, which is a proto-oncogene protein. The physical binding of the N-terminal domain of p16β and the C-terminal domain of the MDM2 forms a complex. This complex formation blocks the MDM2 induced transcriptional activity of p53. Deletion of the INK4 (p16α and p16β) locus resulted in functional abrogation of both pRb and p53 pathways.

In studies conducted by Zindy *et al.*(1997) the expression patterns of the four *INK4* genes during mouse development and aging showed both p18 and p19 to be widely expressed during mouse embryogenesis. However, p15 and p16 were not readily detected prenatally. Four weeks after birth, p15, p18, and p19 were detected in many tissues. However, p16 expression was restricted to lung and spleen of older mice with more pronounced mRNA expression with aging. When mouse embryos were disrupted and cultured as mouse embryo fibroblasts(MEFs) the levels of p16 and p18 increased as cultures approached senescence. Similar observations were not detected for p15 and p19. After multiple passages of these cell lines, three out of four became polyploid and expressed higher levels of functional p16. One cell line sustained a bi-allelic deletion of p16 but initially remained diploid. The authors concluded that p18 and p19 may regulate pre-and post-natal development, while p16 more likely plays a checkpoint role during cell senescence which underscores its function as a tumor suppressor (Zindy *et al.*, 1997).

Zindy *et al.*(1997) demonstrated in another study that *p18* and *p19* expression is specific to the central nervous system. No expression of the other two family members (*p15*and *p16*) were identified in this study. Precise expression of p18 was observed at these developmental stages when neuroblasts switch from a symmetric to an asymmetric pattern of cell division with concomitant increases in their G1 interval. p19 was detected from embryonic day 11.5 onward at higher levels than p18. Distinct expression of p19 was observed in dorsal root ganglia, spinal cord, and focally throughout the brain, but primarily in postmitotic neurons.

The above studies examplified that even though the INK4 family proteins have many similarities (Guan *et al.*, 1996); the expression pattern varies among tissues. Furthermore, the expression pattern in one particular tissue could also change during aging. Other studies that implicate p16 up-regulation and tumorigenesis indicate that p16 RNA and protein accumulate as human cells approach their finite lifespan in tissue culture (Hara *et al.*, 1996). As reported by Xiong *et al.*(1993), when human fibroblasts were transformed by SV40, higher levels of p16 expression were detected.

1.4. IN VIVO FUNCTION:

The most convincing evidence to support the hypothesis that the *INK4* locus contains the suppressor function came from a study by Serrano *et al.*(1996). A targeted deletion of the *INK4a* locus created in mice affects both *p16*α and *p16*β transcripts. Although the mice were viable and developed normally, they developed spontaneous

tumors at an early age. *INK4a* deficient primary fibroblasts proliferated rapidly and had a high colony -formation efficiency. Introduction of activated H-*ras* in these fibroblasts resulted in neoplastic transformation (Serrano *et al.*, 1996). The results of these experiments suggest that during development, restriction point control does not critically depend on pRb, D1 or p16, but it may be governed by families of redundant pRb-like proteins (p130, p107) complicated by the known *p16*β deletion, which itself is a tumor suppressor.

In a study conducted by Lene (1997) a cell line which had a homozygous deletion of the p16 locus was utilized in the development of stable clones with the tetracycline repressible vector system. Upon induction, p16 was expressed in these cell lines. The expression of p16 caused a G1 arrest and enlargement of the cells similar to that of senescent cells. This phenotype was reversed upon reintroduction of tetracycline suppression (Lene *et al.*, 1997).

In ovarian cancer cell lines, Fang *et.al.*(1998) showed higher levels of pRb mRNA and Rb proteins but lack of the *p16* gene products. Overexpression of *p16* by an adenovirus vector mediated system in these cell lines induced transcriptional down-regulation of the *Rb* gene. This was confirmed by nuclear runoff assays. (Fang *et al.*, 1998). These findings indicate that a pRb-p16 mediated inducible system may be useful in future gene therapy studies.

1.5. Genetic alterations and cancer:

Frequent homozygous deletions of the human chromosome 9p21 region (*p16*) in bladder cancers have been detected by Fluorescence In Situ Hybridization (FISH) (Balazs *et al.*, 1997). *p16* is a tumor suppressor gene involved in the development of various types of tumors such as gliomas (Castresana *et al.*, 1997). Since their initial detection in bladder cancers, somatic mutations of *p16* have been observed in many other cancers. Germ line alteration of *p16* and resultant melanoma has also been reported (Foulkes *et al.*, 1997 ; Lairmore and Norton, 1997 ; Kees *et al.*, 1997). Analysis of gene alteration in pancreatic carcinomas examined by Schutte *et.al.*, (1997) showed that 49 out of 50 carcinomas (98%) had inactivated *p16* genes. All 49 carcinoma tissues indicated normal expression level of pRb and no amplification of cyclin D1 or CDK4 genes. Furthermore, immunohistochemical analysis of these tumors showed that all had normal levels of pRb. In this study the inactivation of the *p16* gene consisted of mutations, homozygous deletions and methylations.

Zariwala *et al.*(1996) examined the genomic status of *p15* and *p18* in 15 normal and 73 tumor-derived cell lines. The tumor cell lines were established from 23 different tissues as well as 26 invasive primary breast cancers and with 20 acute myelogenous leukemias. Their results indicated *p15* to be homozygously deleted in 22% of the tumor derived cell lines. However, no point mutations were found either in the cultured cells or in the two types of primary tumors with the exception of a single breast cancer cell line. No deletions or mutations were found in the *p18* gene in either cultured cell lines or primary tumors. These results indicate that mutation of the *p18* gene is rare in human

tumors. Thus, while they share a very similar biochemical mechanism of inhibiting the kinase activity of CDK4 and CDK6, members of the *p16* gene family likely play different roles in controlling cell proliferation and suppression of tumor growth. (Zariwala *et al.*, 1996). Intragenic mutations of *p15, p16* and *p18* genes were looked for in 71 non-small cell lung cancer (NSCLC) samples by Rusin *et al.*(1996). No intragenic mutations were found in *p15* or *p18* genes. Six somatic mutations were detected in the *p16* gene and of these, 3 were DNA polymorphisms. The authors concluded that in NSCLC cancers, the involvement of *p16* is uncommon and *p15* and *p18* are rare.

Molecular analysis of all *INK4* genes conducted in 100 human cancer cell lines by Gemma *et al.*(1996) showed homozygous deletions of the *p15* gene in 29 cancer cell lines. Thirty-five homozygous deletions and 7 intragenic mutations of the *p16* gene were also detected in this study. However, neither homozygous deletions nor intragenic mutations of the *p18* and *p19* genes were found except in one ovarian cancer cell line harboring a single base pair deletion in exon one of *p19*. Therefore, these authors concluded that the INK4 family may be divided into two groups. One group includes *p15* and *p16* in which gene alterations are common and might contribute to the development of many types of cancers. The other group includes *p18* and *p19*, in which somatic mutations are uncommon in many types of human cancers (Gemma *et al.*, 1996).

A few cases of gene alterations in *p18* and *p19* have been reported more recently Miller *et al.*(1997) reported alterations in the *p19* gene in five out of 67 (i.e.,7%) samples from patients with osteosarcomas. No alterations were found in other types of

sarcomas or in lung cancers. These cases were confirmed by sequencing. Therefore, the authors concluded that even though alterations affecting the *p19* gene are rare, they may be significant in osteosarcomas.

1.6. Methylation and transcriptional silencing of genes:

As reported by Richard *et al.*(1992), *de novo* methylation of the promoter region has been associated with transcriptional inactivation of many genes. The presence of DNA methylation at gene promoters can inhibit transcription in two possible ways. One model proposes that CpG methylation can interfere with transcription directly by modifying the binding site (through methylation) of transcription factors so that they can no longer bind their cognate sequences. Alternatively, there are factors in the nucleus which specifically bind methylated DNA and thereby deny transcription factors. Furthermore, two methyl-CpG binding proteins (MeCPs) (MeCP1 and MeCP2) have been identified and which favors the second method of transcriptional repression (Richard *et al.*, 1992).

Further to these findings, repression of transcription by methylation associated has been reported for several genes. Extensive DNA methylation of the *Rb* promoter region has been noted in retinoblastomas (Clare *et al.*, 1997). In renal cancers, *VHL* gene methylation has been shown (Herman *et al.*, 1994).

Methylation of the *p16* gene has been noted in several common cancers and CpG island methylation associated with transcriptional silencing has also been reported.

(Merlo *et al.*, 1995). Herman *et al.*(1995) reported that *de novo* methylation of of the *p16* gene (CpG island) in 31% of primary breast tumors and in 40% of colon tumors. Methylation of the 5' CpG island of the *p16* gene has been shown to correlate well with gene silencing (Gonzalez-Zulueta *et al.*, 1995). In a study conducted by Joseph *et al.*(1996) of gliomas, partial methylation of the CpG islands was detected only in 10 out of 42 tissues. In normal brain cells methylation was not present. Exposure of these partially methylated cells to 5-aza-2-deoxycytidine (directly binds and inhibit DNA methyl transferase) resulted in a dramatic increase in the promoter accessibility and induction of *p16* expression. In a study of tumors from 21 patients with esophageal adenocarcinomas, 8 out of 21 had promoter hypermethylation and Loss of Heterozygosity (LOH) of the region 9p21. This suggest that promoter hypermethylation with LOH may be a common mechanism for inactivation of *p16* (David *et al.*, 1997).

In an elegant study conducted by Otterson *et al.* (1995) 33 tumor cell lines showed hypermethylation of a G:C-rich region within exon1 of the *p16* gene in all samples with wild-type pRb expression. These cell lines had no detectable *p16* mutations. Treatment for at least 4 hours with the demethylating agent 5-aza -2'-deoxycytidine induced the expression of the p16 protein in each of these samples following a discrete 24-48 hour lag period. These findings suggest, (1) that hypermethylation of the *p16* gene is a tumor-specific mechanism for gene inactivation, and (2) there is a link between DNA methylation and the p16:pRb tumor suppressor pathway.

Thirty cases of sporadic cutaneous melanoma tissues were analyzed for methylation status of the *p16* gene by Mark *et al.*(1997). A significant level of methylation was observed in 3 of the 30 tissues. Two of these three tumors were also positive for LOH on 9p21, implying that both *p16* alleles were inactivated via deletion. This suggests that in melanoma methylation associated transcriptional silencing of *p16* is not a common mechanism (Mark *et al.*, 1997).

Extensive methylation of several genes has been reported in human breast cancer. These include estrogen receptor gene (Ottaviano *et al.*, 1994), E-cadherin gene (Graff *et al.*, 1995), BRCA1 gene (Dobrovic and Simpfendorfer, 1997), a candidate tumor suppressor gene S100A2 (Wicki *et al.*, 1997), and another candidate tumor suppressor gene HIC-1 (Fujii *et al.*, 1998). These results suggest that gene methylation may be a common mechanism of inactivating tumor suppressor genes in breast tissues.

1.7. SUMMARY OF LITERATURE REVIEW:

The pRb protein binds to a variety of other cellular proteins and controls cell proliferation and differentiation. Development of human cancers is frequently associated with alterations in either the *Rb* tumor suppressor gene itself or other genes associated with the pRb regulatory pathway such as cyclin, CDK's or *INK4*. Alteration of *Rb* has been reported in about 20% in breast cancers.

Frequent other modes of inactivation, such as homozygous deletion, of the *INK4* genes, specifically *p15* and *p16*, have been reported in cell lines. Alterations in *p18* and

p19 are not common. Methylation of the promoter region and as a result *p16* transcriptional silencing, have been reported in many human cancers (31% of breast cancer and 40% of colon cancer). However, the role of other INK4 family proteins such as p15, p18 and p19 in breast cancer is not well elucidated. Therefore, the present study was initiated to examine the role of *INK4* expression in human breast tissues.

1.8. Hypothesis and objectives:

HYPOTHESIS:

Downregulation of one or more *INK4* (*p15,p16,p18,p19*) genes by mutation, deletion or *de novo* methylation is a common mechanism of abrogating pRb blockade of G1 progression in breast cancer cells.

OBJECTIVES:

The main objectives of the project are to study the expression pattern of *INK4* in normal human tissues, to examine altered expression patterns in human breast cancer tissues and to correlate any alterations in expression detected with genetic alterations such as homozygous deletions, point mutations, or methylation of the promoter regions.

2. NORMAL AND ALTERED mRNA EXPRESSION OF *INK4* GENES.

2.1. AIMS

The aims of this present study were:

1. To conduct a survey of the expression patterns of *INK4* genes in normal human tissues.
2. To quantify *p16* mRNA in a HeLa cell line and human tumor tissues.
3. To investigate the expression patterns of *INK4* genes and the *Rb* gene in breast tumor tissues.

A survey of the mRNA expression patterns of all *INK4* genes in normal human tissues was conducted. The analysis of normal expression levels of *INK4* genes would indicate whether these genes are expressed uniformly or tissue specifically. RNA from HeLa cells (defective pRb protein activity as a result of HPV viral infection) usually serves as a control for *p16* analysis. Quantification of *p16 mRNA* from this cell line and breast tumor tissues was therefore carried out. In order to study the expression of *INK4* and *Rb* in 35 breast tumor tissues, RT-PCR method was utilized.

2.2. MATERIALS AND METHODS

In order to determine the normal expression patterns of *INK4* genes, a commercially available RNA master blot (details of the master blot are given under section 2.2.1) was used in the experiment. For *p15*, *p18* and *p19*, cDNA probes were used for expression analysis. The *p16α* and *p16β* expression analysis was done by using

an exon specific 20 bp oligonucleotide. Hybridization was carried out with radiolabelled *INK4* specific probe as described under Materials and Methods section 2.2.8 and 2.2.9.

p16 mRNA quantitation was carried out using a dot blot analysis(details under section 2.2.2). A *p16* α exon1 specific oligonucleotide was used as a probe in this experiment. The labelling and hybridization procedures were given under section 2.2.8 and 2.2.9.

The mRNA analysis from 35 breast tumor tissues was performed by first isolating the total RNA from the tissues as described under section 2.2.1 and normalizing the RNA concentration. The normalization of the RNA was carried out with either *GAPDH* or *S6* gene expression. All mRNA expression analyses were performed by RT-PCR protocols described below (section 2.2.5). All experimental conditions for RT-PCR were calibrated by using RNA from cell lines. The cDNA amplification primers are listed in Table 3.

2.2.1 Human RNA Master Blot (CLONTECH ,U.S.A).

The Human RNA Master Blot is a positively charged nylon membrane to which poly A^+ RNA (100-500ng) from several human tissues have been immobilized in separate dots. The RNA samples on the Master blot have been normalized to the mRNA expression levels of eight different "housekeeping" genes. Hybridization of a cDNA or an exon specific oligonucleotide probe to this blot will ensure an accurate assessment of mRNA abundance in different tissues.

2.2.2 RNA dot blot for mRNA quantitation

A dot blot was prepared as shown in Figure 3. The first row contains 10μg of heat denatured total RNA from a cell line (HeLa). The second row contains denatured total RNA (10 μg) from six breast tumor tissues. The third row contains the series of cDNA dilutions with known concentrations.

2.2.3 Collection of breast tissue samples.

Thirty five breast cancer tissues (primarily epithelial cells) were obtained from the Foothills Provincial Hospital in accordance with the policies of the tissue bank (Dr. D. J. Demetrick, Director of Research Tissue Bank of the Foothills Hospital, Department of Histopathology). Frozen tumor tissues were carefully dissected out by Dr. L. DiFrancesco with minimum contamination (<20%) of normal tissues. In addition, ten normal (primarily connective tissues and non cancerous) tissues were also obtained to be used as controls in some experiments.

2.2.4 Total RNA isolation from cell lines and breast tissues.

In all RNA experiments, in order to inhibit RNase activity, all solutions were prepared using sterile deionized water that had been treated with diethylpyrocarbonate (DEPC) and autoclaved. Breast tissues (50-100 mg) were homogenized in one ml of TRIzol(GIBCO BRL, Life technologies) using a power homogenizer. Cell lines which were grown in monolayer needed no homogenization. Therefore, one ml of TRIzol was added to a 3.5 cm culture dish following the protocol recommended by the manufacturer. Homogenized samples were incubated for 5 minutes at 30°C. One ml of chloroform was

added to the homogenized sample, mixed and centrifuged at 12,000 g for 15 min. at 4°C. The aqueous phase was transfered into a fresh tube and 0.5 ml of isopropyl alcohol was added and RNA was precipitated at 12,000g for 10 min. The RNA pellet was washed with 75% ethanol, air dried and dissolved in RNase free water. RNA was stored frozen at -70°C further to the determination of concentrations.

2.2.5 Reverse transcription.

Five µg of total RNA was utilized to reverse transcribe and synthesize the cDNA. This was done following the protocol recommended by the manufacturer (Gibco BRL Life Technologies). Briefly, a 12 µl mixture containing (1-5µg) total RNA and 1µl of random hexamers $pd(N)_6$ (Pharmacia Biotech) (500 µg/ml) was prepared. This mixture was heated at 70 °C for 10 min and quickly chilled on ice. The following components were added to the chilled RNA mixture. 4µl of 5x first strand buffer, 2 µl of 0.1M DTT, 1µl 10mM dNTP mix and 1µl (200 units) of Super Script II (Gibco BRL Life Technologies). These reactions were incubated at 42 °C for 50min. The reaction was stopped by heat inactivation at 70 ° C for 15 min. The cDNA was used in PCR amplification of specific genes.

2.2.6 The polymerase chain reaction.

PCR amplifications were carried out with gene specific primers (Table 3). One hundred ng of cDNA was used as the template. The standard reaction contained the following components. Ten µl of 10x PCR buffer (200 mM Tris-HCL and 500 mM KCL), 1µl of 10 mM dNTP mixture, 1.5 µl of 50 mM $MgCl_2$,1µl of primer mix (10 µM

of each), 1.5 µl of 6% DMSO,10 µl of template DNA, 0.5 µl of *Taq* DNA polymerase (5U/µl) (Gibco BRL Life Technologies) and water to 100 (µl). The amplification was carried out 35-40 cycles in a thermocycler (M&J PTC-200) with a initial denaturing step of 96°C for 2 min. and 45 cycles consists of a denaturing at 96°C for 30 sec, annealing step at 60 °C for 30 sec, and an extension at 72°C for 50 sec. The final extension step was at 72°C for 3 min.

2.2.7 Oligonucleotide primers.

Specific primers were designed from the cDNA sequences. The primers were specifically designed from the intron spanning exons. These primers will not amplify genomic DNA because of the presence of large introns in the middle. This excludes the amplification of contaminating genomic DNA. The primers used in the present study are summarized in Table 3.

2.2.8 Gel electrophoresis.

PCR products were electrophoresed through 1% agarose and visualized with ethidium bromide staining. Radioactivity (^{32}P dCTP) incorporated into *p16*-PCR amplified products was analyzed on 6% acrylamide gels followed by autoradiography.

2.2.9 Radio labeling of c-DNA and end labeling of oligo primers.

Random labeling was carried out with 25ng of denatured cDNA using Random primers DNA labelling system (Gibco BRL Life Technologies). This was done following the protocol recommended by the manufacturer. The denatured cDNA was mixed with 2µl of dATP, 2µl of dGTP, 2µl of dTTP, 15µl random primers buffer mixture, 5µl [α-^{32}P]

dCTP (3000 Ci/mmol), 1μl Klenow enzyme, and distilled water to a total volume of 50μl. This reaction was incubated at room temperature for 1 hour and stopped by adding 5μl of stop buffer. The specific activity of the cDNA probes were >$3.0x10^8$ cpm/μg. End labeling was carried out in a final volume of 25μl by mixing 10 pmol of 20 mer oligonucleotide with 2.5μl of 10X kinase buffer, 10 μl [γ^{32}P]dATP (6000 Ci/mmol) and 5 units of T4 polynucleotide kinase. This reaction was incubated at 37 °C for 30 min. The reaction was stopped by adding 1μl of 0.5M EDTA(pH7.5). The specific activity of the probes were >$1.9x10^8$ cpm/μg.

2.2.10 Hybridization.

Church and Gilbert (CG) solution (Church,1984) which is routinely used in our laboratory, was utilized for all hybridizations. The CG solution contains the following components: 0.25 M sodium phosphate (pH 7.2), 7%SDS , and 1mM EDTA. Northern hybridization was carried out at 65°C for cDNA probes. Blots were washed with 0.1XSSC and 0.1% SDS at room temperature for 10 minutes. The second wash contained 0.1XSSC and 0.1% SDS and this was performed at 65°C for 30 min. The third and fourth washes contained the same components and were for 40 mins each. The Oligo probes for *p16* (αand β) were hybridized at 48°C and the blots were washed at 40°C. The washed blots were kept wet, wrapped in plastic-wrap and autoradiographed. The autoradiography was carried out with intensifying screen and Kodak BioMax-ms Film(#829 4985) for 72hrs at -70°C. The blots was re-hybridized after stripping by boiling (10 min) in 10mM Tris pH 8.0. Second hybridizations were carried out after cooling them for 10min.

Breast tissue	Diagnosis	*p16* mRNA expression
	GRADE I	
1	IDC, medullary	+++++
4	ILC, Scanty tumor	+
7	IDC, Gr. I	++++
9	IDC, Gr. I (colloid)	++
12	IDC, Gr. I (tubular)	+++
10	IDC	++
11	IDC	+++
18	IDC,	+
19	ILC	++++
21	IDC, Gr. I (tubular)	+
23	IDC, Gr. I	++
24	ILC	+
28	DCIS, IDC, Gr.I	+
29	DCIS, IDC	+
34	IDC, Gr. I	+++
	GRADE II	
2	IDC, Gr. II	++
3	IDC, Gr. II	+
5	IDC, Gr. II	+++++
8	IDC, Gr. II	+++++
31	IDC, Gr. II	+
32	DCIS, IDC, Gr. II	+
	GRADE III	
6	IDC, Gr. III	+++++
13	IDC, Gr. III	+
22	IDC, Gr. III	+
25	IDC, Gr. III	++
26	DCIS, IDC, Gr. III	+
30	IDC, Gr. III	+++
33	DCIS, IDC, Gr. III	++++
35	DCIS, IDC, Gr. III	+

Table 1: Breast tumor grade (for available samples) and *p16* mRNA expression.
IDC = Infiltrating Ductal Carcinoma.
DCIS = Ductal Carcinoma In Situ.
ILC = Infiltrating Lobular Carcinoma.
+++++ = *p16* mRNA expression (radioactive signal intensity is high).
\+ = *p16* mRNA expression (radioactive signal intensity is low).

Table2: p16α mRNA quantitation from a cell line from Figure3.

Sample	Optical Density	Background	Differences	Average
Row1 sample1	167.4 289.4	107.3 247.7	60.11 41.68	50.895
control cDNA (#7).	117.9 81.32	112.9 55.0	5.02 26.3	15.66

Control : sample optical intensity ratio is =1: 3.25 .

control cDNA is approximately 16 pg. Therefore, the HeLa cell line contains approximately 16X3.25 = 52pg of p16α mRNA in 10 μg of total RNA.

p16α mRNA content in the cell line is 0.00052 % of the total RNA.

Table 3: cDNA amplification primers

#	Gene	Sequence (5'---3')	Annealing Temperature
1	p16-α	TGGAGCCTTCGGCTGACTGG GCCCTCCCGGGCAGCGTCGT	60 °C
2	p16-β	ACCCTCCGGATTCGGCGCGCG GCCCTCCCGGGCAGCGTCGT	60 °C
3	p15	CGCGAGGAGAACAAGGGCATG TGCAGCACCACCAGCGTG	60 °C
4	p18	CTGATCGTCAGGACCCTAAAG AGCAAAGTCTGTAAAGTGTCCAG	60 °C
5	p19	GAGCTGGTGCATCCCGACG GGGCAGGAGAAACAAGAAGAAAG	62 °C
6	GAPDH	CGGAGTCAACGGATTTGGTCGTAT AGCCTTCTCCATGGTGGTGAAGAC	60 °C
7	S6	GTTATGTGGTCCGAATCAGTGG GCTCTCTTCGCAATTTGTTCCTG	60 °C
8	Rb	GAACGCCTTCTGTCTGAGCAC AATGTCTCCTGAACAGCATGAG	60 °C

Tissues	*p16*(α)	*p16*(β)	*p18*	*p19*	Tissues	*p16*(α)	*p16*(β)	*p18*	*p19*
whole brain	x	2x	x	x	pancreas	4x	4x	x	x
amygdala	x	x	x	x	pituitary gland	2x	2x	x	x
caudate nucleus	x	x	x	2x	adrenal gland	2x	2x	x	x
cerebellum	x	x	x	x	thyroid gland	2x	2x	x	x
cerebral cortex	x	x	x	x	salivary gland	4x	4x	x	x
frontal lobe	x	x	x	x	breast	x	x	>4x	x
hippocampus	x	x	x	x	kidney	4x	x	x	4x
medulla oblongata	x	x	x	x	liver	2x	4x	x	2x
occipital lobe	x	x	x	2x	small intestine	x	x	x	x
putamen	x	x	x	2x	spleen	x	x	x	2x
substantia nigra	x	x	x	2x	thymus	x	x	>4x	2x
temporal lobe	x	x	x	x	peripheral leukocyte	x	x	x	4x
thalamus	2x	x	x	x	lymph node	x	x	4x	x
subthalamic nucleus	x	x	x	x	bone marrow	x	x	4x	4x
spinal cord	x	x	2x	x	appendix	x	x	x	x
heart	2x	2x	x	x	lung	2x	x	x	x
aorta	x	x	x	x	trachea	2x	2x	x	x
skeletal muscle	x	x	x	x	placenta	x	2x	x	x
colon	2x	x	x	x	fetal brain	x	x	x	4x
bladder	x	x	x	x	fetal heart	x	x	x	x
uterus	x	x	x	x	fetal kidney	x	x	x	x
prostate	2x	x	x	x	fetal liver	x	2x	x	2x
stomach	2x	x	x	x	fetal spleen	x	x	x	2x
testis	x	2x	x	x	fetal thymus	x	x	4x	2x
ovary	x	x	2x	x	fetal lung	x	x	x	x

Legend for approximate expression levels by inspection :

x - low (Basal).
2x - Medium.
4x - High.
>4x - very high.

Figure 2: mRNA expression pattern of *p18*, *p19*, and *p16* (α and β) genes in normal human tissues.

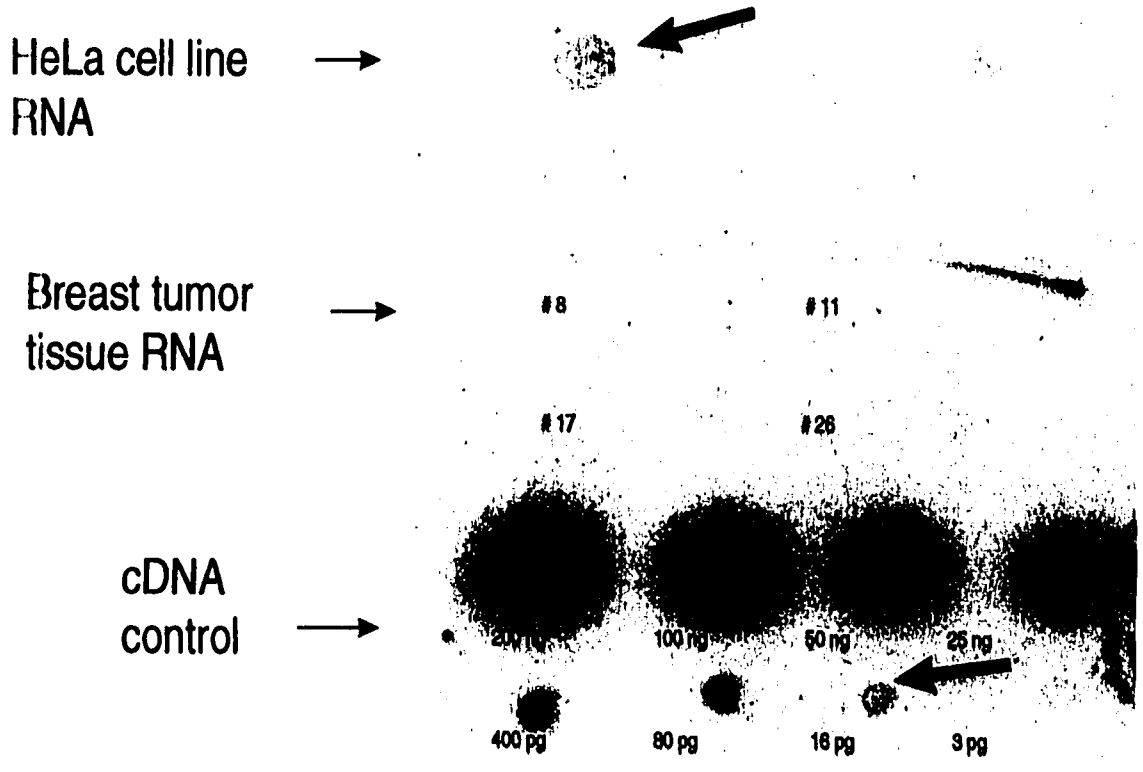


Figure 3: A dot blot analysis for *p16* mRNA quantitation in HeLa cell line and breast tumor tissues.

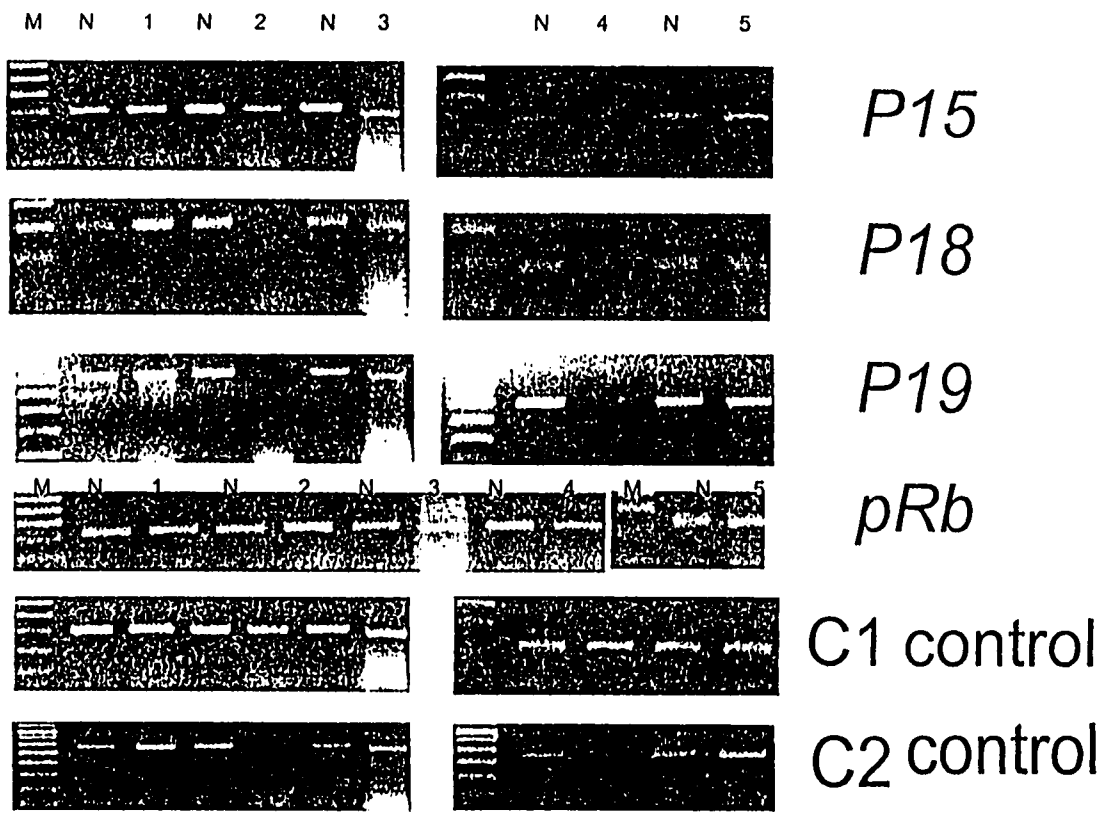


Figure 4: RT- PCR analysis of mRNA expressed in breast tumors(1-5).
1- 5 = Tumor tissues (primarily epithelial cells).
N = Corresponding control tissue (primarily connective tissue).
C1 = GAPDH C2= S 6 (control) M =marker.

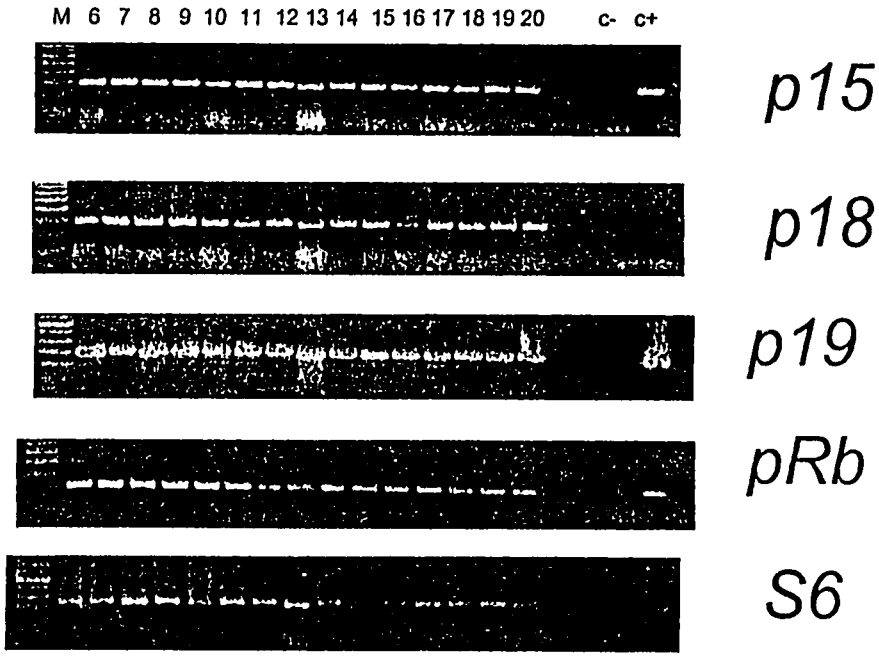


Figure 5: RT- PCR analysis of mRNA expressed in breast tumors (6-20).

C- = Negative control.

C+ = Positive control.

M = Marker.

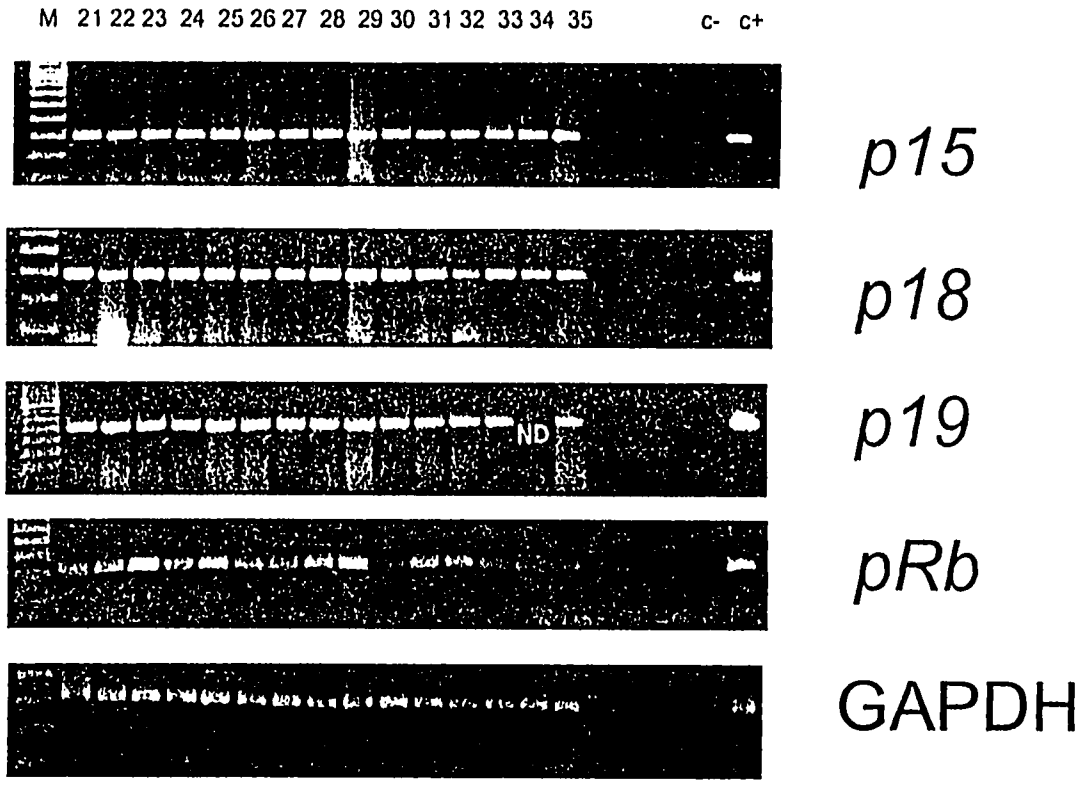


Figure 6: RT- PCR analysis of mRNA expressed in breast tumors (21-35).
C- = Negative control.
C+ = Positive control.
M = Marker.
ND=Not done (insufficient quantity)

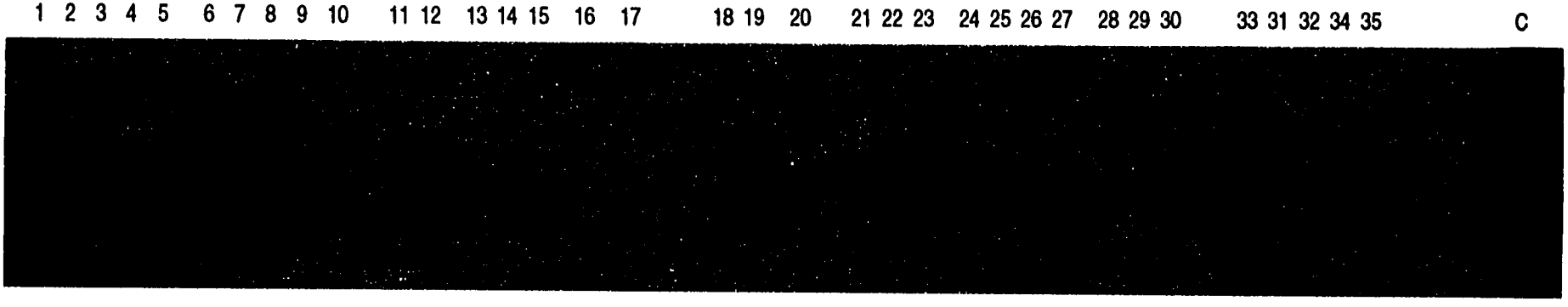


Figure 7: Radioactive RT - PCR analysis of p16 mRNA from breast tumors (1-35).
C = Control c-DNA.

2.3. RESULTS

The normal expression patterns of *p16*, *p18*, and *p19* are summarized in Figure 2. The *p16*α and *p16* β expression is ubiquitous and a low basal level of expression is found in all tissues analyzed. Some tissues (thalamus, heart, colon, prostate, stomach, pituitary gland, adrenal gland, thyroid gland, liver, lung, and trachea) had a moderately increased level of expression. High levels of *p16* α mRNA expression were also found in the pancreas, salivary gland, and kidney. The p16β expression is quite similar in pancreas and salivary glands. Higher levels of p16β are evident in liver than the *p16* α form. The p16β form is moderately expressed in brain, heart , testis, pituitary gland, adrenal gland, thyroid gland, trachea, placenta and fetal liver.

The expression patterns of *p18* and *p19* show high tissue specificity. *p18* is highly expressed in mammary glands, thymus, and moderately expressed in bone-marrow, lymph node and fetal thymus. *p19* is highly expressed in fetal brain, bone marrow, peripheral leukocyte and kidney and moderately expressed in caudate nucleus, occipital lobe, putamen, substantia nigra, liver, spleen, fetal liver, spleen, and thymus. The *p15* expression signals were weak. This is because several hybridizations and washes were carried out of the same blot. Therefore, these data were not included in the analysis.

The quantitation analysis revealed that the levels p16 mRNA in breast tumor tissues are very low < 0.0005% in comparison to a HeLa cell line (Figure 3 and Table 2.) The accurately detectable level of poly A+ RNA is 0.01%, which is approximately equal

to 100 target mRNA molecules/cell (CLONE TECH ,USA manual). Therefore, the *p16* expression could only be analyzed by radioactive RT-PCR and the results are shown in Figure 7.

The mRNA expression analysis of INK4 in breast tumor tissues is shown in Figures 4, 5, 6 and 7. Two tumor tissues (samples 2 and 4 in Figure 4) resulted in a loss of *p18*, and *p19* mRNA expression. In Figure 6 sample 34 is not included in *p19* RNA analysis because of insufficient quantity.

2.4. DISCUSSION.

In order to investigate the normal INK4 expression pattern in human normal tissues, a commercial dot blot was used. There were 3 advantages in using this blot. Firstly, tissues of varying ages could be analyzed. Secondly, the RNA on the blots were normalized to eight house keeping genes. Therefore, tissue specific variations were minimized and more accurate information about normal expression levels of the gene of interest could be obtained. Finally, many tissues could be analyzed in one blot.

Dot blot analysis revealed that *p16*α and *p16*β are expressed ubiquitously in all the tissues examined with some specific expression. Expression of the *p16*β form is similar to the *p16*α form except in liver. The *p16*β form expression is higher in liver than the *p16*α form. However, *p18* and *p19* showed tissue specific expression patterns. In the present survey high expression of *p18* was detected in mammary glands, thymus, lymph node and bone marrow. However, higher levels of *p18* expression appear to have

been reported in human skeletal muscle Guan *et al.*(1994) than our findings. They were also able to detect moderate amounts in human skeletal muscle, pancreas and heart

In the present study, expression of p19 at high levels was detected in peripheral leukocyte and in fetal brain, kidney and bone marrow. Similarly, Guan *et al.*(1996) also detected high levels of p19 expression in peripheral leukocyte, thymus and brain.

The expression pattern of another family of inhibitory proteins such as p21, and p27 were examined in many normal human tissues by Polyak *et al.*(1994). The results from these analyses revealed uniform expression patterns in the many human tissues examined. In comparison, the results from the present analysis clearly demonstrate that *INK4* family expression is quite variable in normal human tissues.

The quantitative analysis of p16 levels in 10 μg of total RNA from tumor tissues revealed the amount of *p16* mRNA to be extremely low (<0.0005%). This precludes the use of in situ mRNA detection.

The mRNA expression pattern of the *INK4* genes (Figures 4, 5, 6) in breast cancer specimens clearly shows that there are no major expression differences of *INK4* genes (*p18*, *p19*, *p15*) and *pRb*. mRNA expression of *p18* and *p19* in two samples (2, and 4) was not detected. However, loss of expression was also detected in control S6 expression as well. Therefore, most likely there could be degradation of the RNA in these tumor samples. The expression of *p16* is not detectable in ethidium bromide stained gels, it is

detectable only by radioactive RT- PCR as shown in Figure 7. Viewing of the radioactive *p16* RT-PCR blot reveals differences in signal intensity between samples. From the mRNA expression analysis it is not clear whether the signal differences represent true quantitative differences in expression or the amplification of differences of low level constitutive expression. Furthermore, these expression patterns had no correlation with the histological grade or histological type as shown in Table 5. However, 10 tissues had relatively high levels of expression of *p16* mRNA. Therefore, further genomic analysis of *p16* was carried out in determining gene structural alterations associated with the poorly expressed specimens.

3. GENE ALTERATION (Mutations and Deletions)

3.1 AIMS:

The specific aim of this experiment was to identify whether homozygous deletions or point mutations were responsible for the altered expression of *p16* discussed in the previous chapter (see Figure7).

Genomic alterations such as deletions and mutations directly influence the mRNA expression of genes. If the exons were deleted, there would be no mRNA expression of the gene. However, if any point mutations or small deletions were present the defective mRNA will accumulate in the cells. The genomic analysis of *p16*, *p15* and *p18*, deletions and mutations were carried out as described under 3.2.

3.2 MATERIALS AND METHODS

Exon deletion analysis of cell lines:

The genomic DNA was first extracted from tumor cell lines and microdissected tumor tissues as described below (section 3. 2.1). Genomic PCR amplification of the *p15* and *p16* exons were carried out with the cell line genomic DNA(section 3.2.2) and the amplicons were analyzed on agarose gels.

Mutation analysis :

Breast tumor DNA was utilized in this experiment. The mutation analysis was carried out by PCR amplification of the exons *p16* exon1, 2, and 3 and *p18* exon 1 was carried out with 100 ng of genomic DNA with radioisotopes (0.1µl /reaction) of [α-^{32}P]dCTP (3000 ci/mmol)as described under section 3.2.2. The PCR products were analyzed by either Single Stranded Conformational Polymorphism (SSCP) or Constant

Denaturing Gradient gel Electrophoresis (CDGE) as described under section 3.2.3 and 3.2.4. The SSCP analysis technique involves denaturing double- stranded DNA, followed by electrophoresis on a non denaturing polyacrylamide gel. Single base changes may alter the secondary structure of the ss DNA and usually causes differential migration. The CDGE analysis technique involves the formation of heteroduplexes. This is achieved by first mixing the mutant and the wild type PCR fragments in a microfuge tube and denaturing the DNA by heating and allowing them to re-anneal. The heteroduplex contains a mismatch in the double strand that causes distortion in its usual shape. This leads to the separation of DNA strands at a lower concentration of the denaturant. The heteroduplex bands always migrate more slowly than the normal homoduplex bands.

Mutation confirmation:

Mutations were confirmed by cloning and sequencing of the respective exons from tumor and corresponding normal tissue DNA as described under section 3.2.5, 3.2.6, and 3.2.7. The sequence analysis of the normal and tumor tissues would confirm mutations.

3.2.1: Genomic DNA isolation:

DNA was isolated from the 35 microdissected breast cancer samples and from 13 cell lines (Table 4) using DNAzol (GIBCO BRL Life Technologies) reagents following the protocol recommended by the manufacturer (Gibco BRL Life Technologies). One ml of DNAzol was added to the cell lines grown in monolayer. In the case of tissue samples, they were homogenized with a hand held homogenizer. The homogenate was centrifuged at 10,000xg at 4°C and the viscous supernatant was transferred to a fresh tube.

DNA was precipitated by addition of 0.5ml of 100% ethanol. The pellet was washed twice with 95% ethanol and the DNA was dissolved in 100 µl of 8mM NaOH. The pH was adjusted to pH 7.0 with the addition of 4.2 µl of 1 M HEPES. The DNA was quantitated and used in the experiments. DNA(100ng) was used for gene mutation and deletion analysis by PCR.

3.2.2. Polymerase chain reaction.

A standard PCR amplification identifies specific exons. 100 ng of genomic DNA was mixed with a 100µl of master mix containing 10 µl of 10xPCR reaction buffer, 3.0 µl of 50mM $MgCl_2$, 5µl of each primers(10 µM), 2µl of 10mM dNTPs, 7µl of DMSO and 0.5 µl of *Taq* DNA polymerase. PCR amplification was carried out in a thermal cycler (The DNA Engine, MJ research PTC-200) with an initial denaturing step of 96°C for 2 min, and 45 cycles consists of a denaturing step at 96°C for 30 sec, an annealing step at 60 °C for 30 sec, and an extension step at 72°C for 50 sec. The final extension step was at 72°C for 3 min.

3.2.3. SSCP analysis:

Exons from individual tumor tissues were amplified with 0.1µl /reaction [α-^{32}P] dCTP (3000Ci/m mol) in a 25 µl volume. From the PCR product, 5 µl were mixed (1:1) in formamide loading buffer (95% formamide, 0.09% bromphenol blue, 0.09% xylene cyanol FF) and denatured by heating the sample at 96°C for 10 minutes, then cooling the sample on ice. 4µl of the ice cold sample was electrophoresised on 6% polyacrylamide and 15% glycerol gels. The gels were run overnight at constant power at 18 Watts and were autoradiographed.

3.2.4. CDGE analysis:

The PCR amplification of the *p16* exons were carried out in the same way as SSCP analysis. Ten µl of the PCR products products were heated at 95°C for 10 minutes at 65°C for an hour and the denatured ss-DNA was cooled at 24°C overnight to form heteroduplexes. The samples were electrophoresed on a 16x16 cm gel with 10% acrylamide and 56 % denaturant (5.28 ml of formamide and 2.32 g of urea) at 130V for 3 hrs at 57°C . The gels were autoradiographed.

3.2.5 Cloning and selection of positive clones.

PCR products were directly cloned into the pBluescript vector at the EcoRV sites modified by adding poly dT at the ends. (Short Protocol in Molecular Biology 1995). The dT-addition was carried out in a 100 µl reaction consisting of 5 µg of blunt ended vector, 20µl of 5x amplification buffer, 20 µl of 5mM dTTP, 10 µl of 50mM of $MgCl_2$ and 5units of *Taq* DNA polymerase. This was incubated for 2hrs at 75°C. Ligation was carried out by simply mixing PCR products with the dT -overhang vector, ligation reaction buffer and ligase enzyme over night at 15°C. The constructs were transformed into XL1-Blue *E.coli* strain. This was carried out by heat shock transformation of competent cells. The cloned PCR products were confirmed by mini plasmid isolation and restriction enzyme digestion. The miniprep DNA was analyzed on agarose gel for the correct size insert and the positive clones were selected and used for the isolation of ss-DNA.(Single-Stranded DNA).

3.2.6 Single-Strand DNA Isolation:

A single colony of identified clones was grown overnight in LB media containing ampicillin (75μg/ml). A 50 μl culture was inoculated into 2ml LB broth ampicillin media and 2μl of VCSM13 (helper phage). The pBluescript phagemid is derived from pUC 19 vector and contains f1 filamentous phage origin of replication allowing recovery of the sense strand of the *lac* Z gene when a host strain contain the pBlue script phagemid is co-infected with helper phage (Stratagene 1998). The cultures were grown at 37°C for 1 hour and kanamycin was added to 70 μg/ml (selection for infected cells). The cultures were grown for 16hrs. 1.5ml cultures were centrifuged and 1 ml of supernatant were removed and transfered into a separate tube. 150 μl of 20% PEG/2.5M NaCl was added and phage particles were precipitated on ice for 15minutes. The PEG pellet was precipitated by centrifuging and the pellet was resuspended in 150 ml of 0.3M NaOAc at pH6.0. The solution was extracted with phenol/chloroform and centrifuged for 1-2 minutes. The aqueous phase was transfered to a fresh tube and 500 ml of ethanol was added. DNA was precipitated and the pellet was dissolved in 25 ml of TE.

3.2.7 Sequencing and analysis:

Two μl of ssDNA was used with M13 -21 primer in ABI PRISM Dye Terminator cycle sequencing (Perkin Elmer) reaction protocol recommended by the manufacturer. Individual clones were sequenced at the University of Calgary DNA sequencing facilities. The sequences were analyzed in the laboratory by the "Lasergene" sequence analysis software 1997 (DNASTAR inc. U.S.A.) for windows 95 operating system computers.

Table 4: List of cell lines

#	Cell lines	Origin
1	G361	Human melanoma
2	Mewo	Human melanoma
3	RPMI4445	Human melanoma
4	Bewo	Human chorio carcinoma
5	Hep3B	Human hepatocellular carcinoma
6	RPMI7272	Human melanoma
7	T24	Human bladder carcinoma
8	HTB20	Human breast carcinoma
9	HTB19	Human breast carcinoma
10	HTB132	Human breast carcinoma
11	HTB75	Human breast carcinoma
12	HTB30	Human breast carcinoma
13	MCF7	Human breast carcinoma

Table 5: Genomic DNA amplification primers

#	Gene	Sequence(5'----3')	Annealing Temperature
1	p16 αExon 1	CGGAGAGGGGGAGAGCAG TCCCCTTTTTCCGGAGAATCG	60 °C
2	p16 αExon 2	GCTCTACACAAGCTTCCTTTCC GGGCTGAACTTTCTGTGCTGG	60 °C
3	p16 αExon3	AGGAATTCGGTAGGGACGGCAAGAGAGG GAAGCTTGGGGGAAGGCATATATCTACG	60 °C
4	P16-β	AGTGGCGCTGCTCACCTC TCTTCTAGGAAGCGGCTGCTG	60 °C
5	p15 Exon1	TTAAGTTTACGGCCAACGGTGGAT TGTACAAATCTACATCGGCGATCTA	60 °C
6	p15 Exon2	TCTTTAAATGGCTCCACCTGCCTT TCCCCCTTGGCAGCCTTCATTCATCGA	60 °C
7	p18 Exon 1	ATGGCCGAGCCTTGGGGGAACGAGTT CAACATTATTGACTTGTTTTCCCCAC	60 °C
8	p18 Exon 2	AGGATTCTACCATTTCTACTTCTTT TTATTGAAGATTTGTGGCTCCCCCA	60 °C
9	p19 Exon1	TTTGCAGGCCGCCAGTGTC GTTCGATCCTCATCCCGCTTAGCC	60 °C
10	p19 Exon2	CTGATCCTCTGTCCCTCAAAC GGGCAGGAGAAACAAGAAGAGAAAG	60 °C

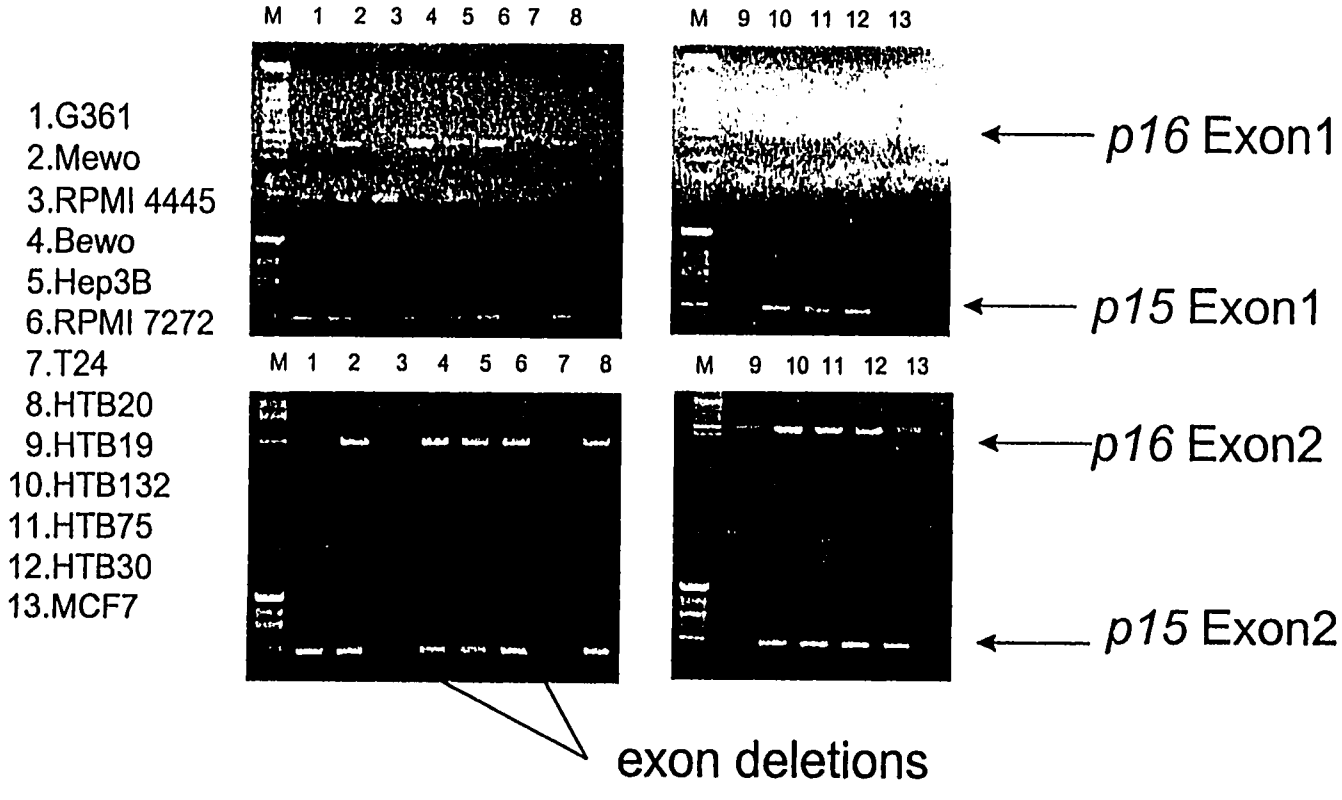


Figure 8: Exon deletion analysis in cell lines (1-13).
PCR amplified products were electrophoresed on agarose gels and visualized with ethidium bromide.
M=Marker

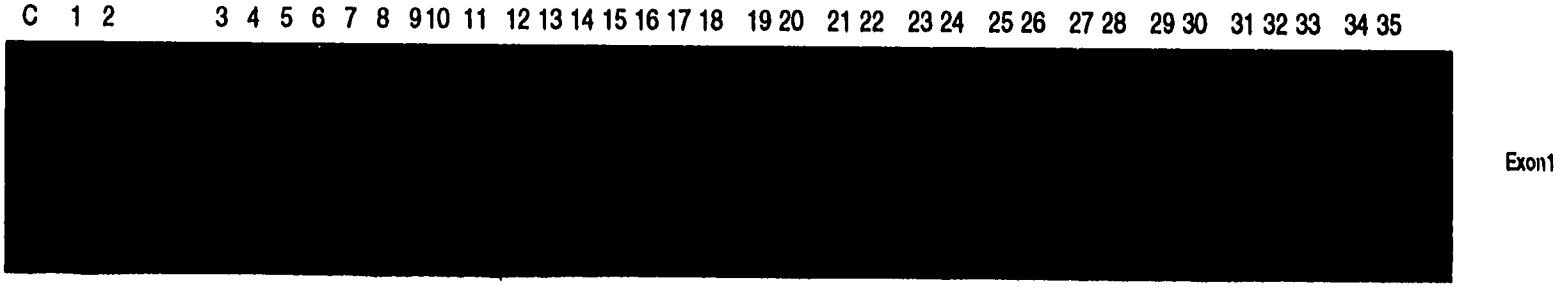


Figure 9: SSCP analysis of *p18* Exon1 from breast tumors (1-35), electrophoresed through 5% acrylamide and 10% glycerol gels at 18 watts for 22hrs.
C= control.

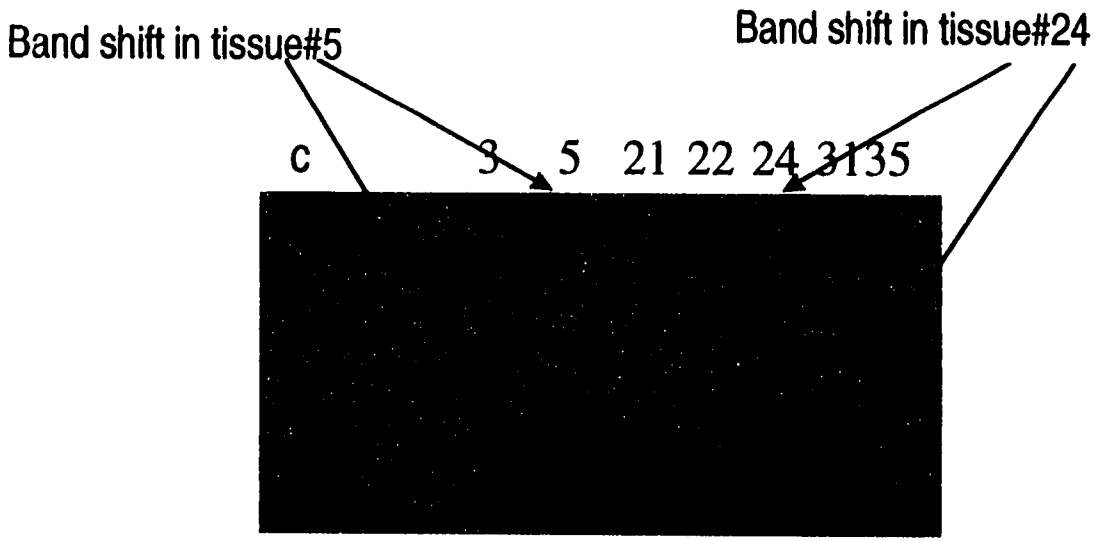


Figure 10: SSCP analysis of *p16* exon3 .
Detailed analysis for breast tumor samples 3, 5, 22, 24, 31 and 35.
Note alteration of band position in tissue 5 and 24.
C= control.

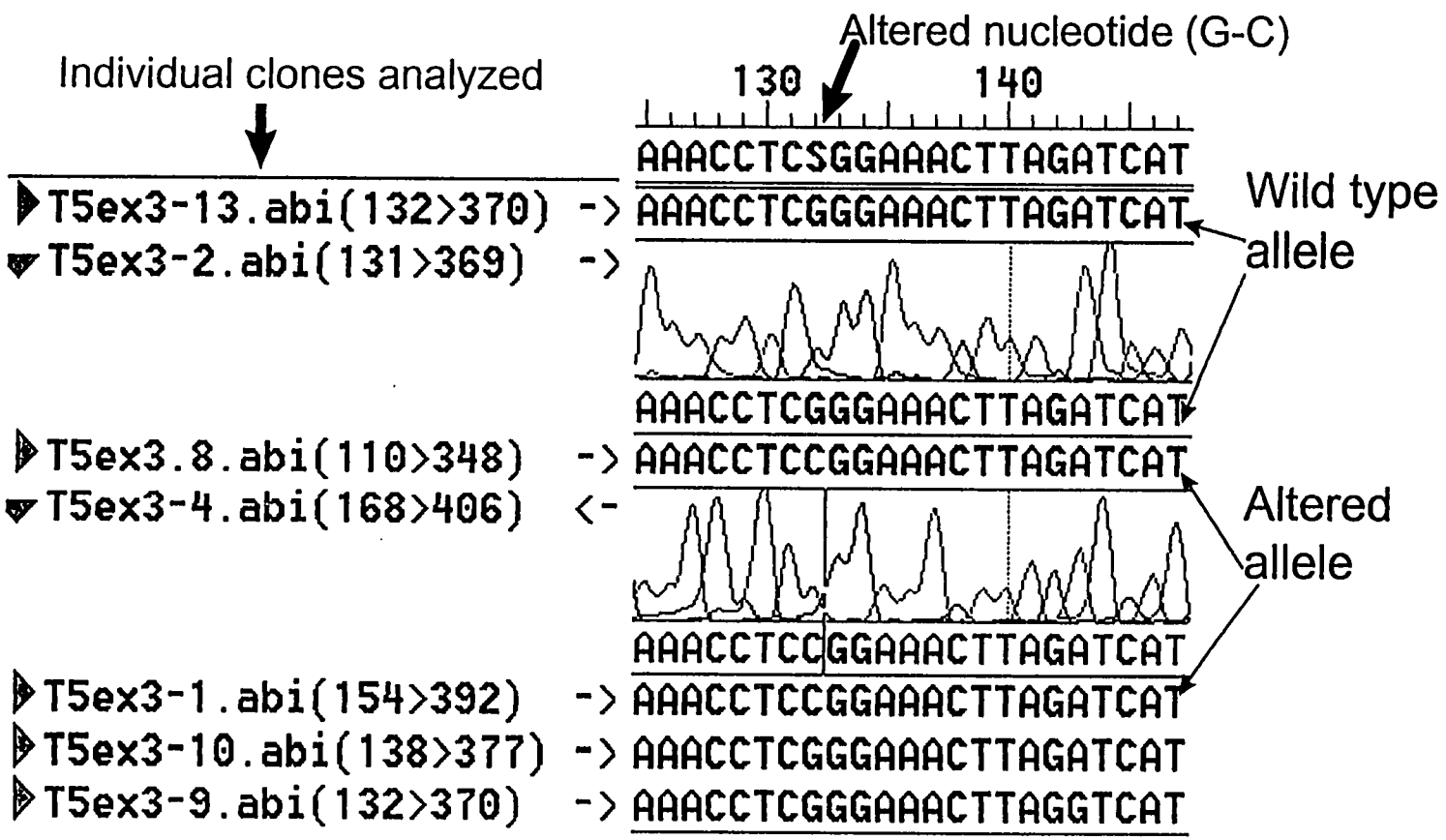


Figure 11: *p16* exon3 sequence variation detected in breast tumor *5*

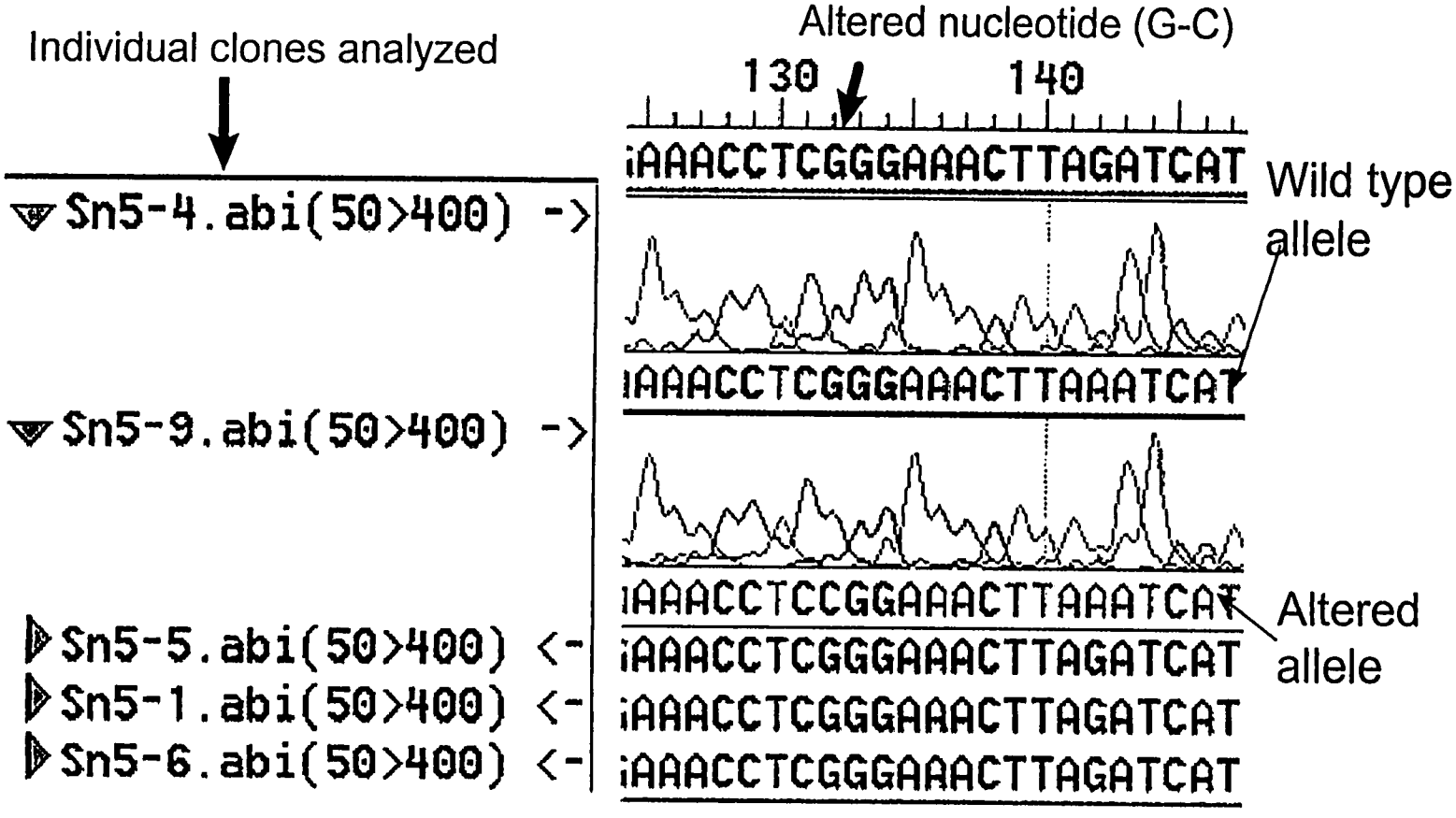


Figure 12: *p16* exon3 sequence variation detected in normal tissue 5.

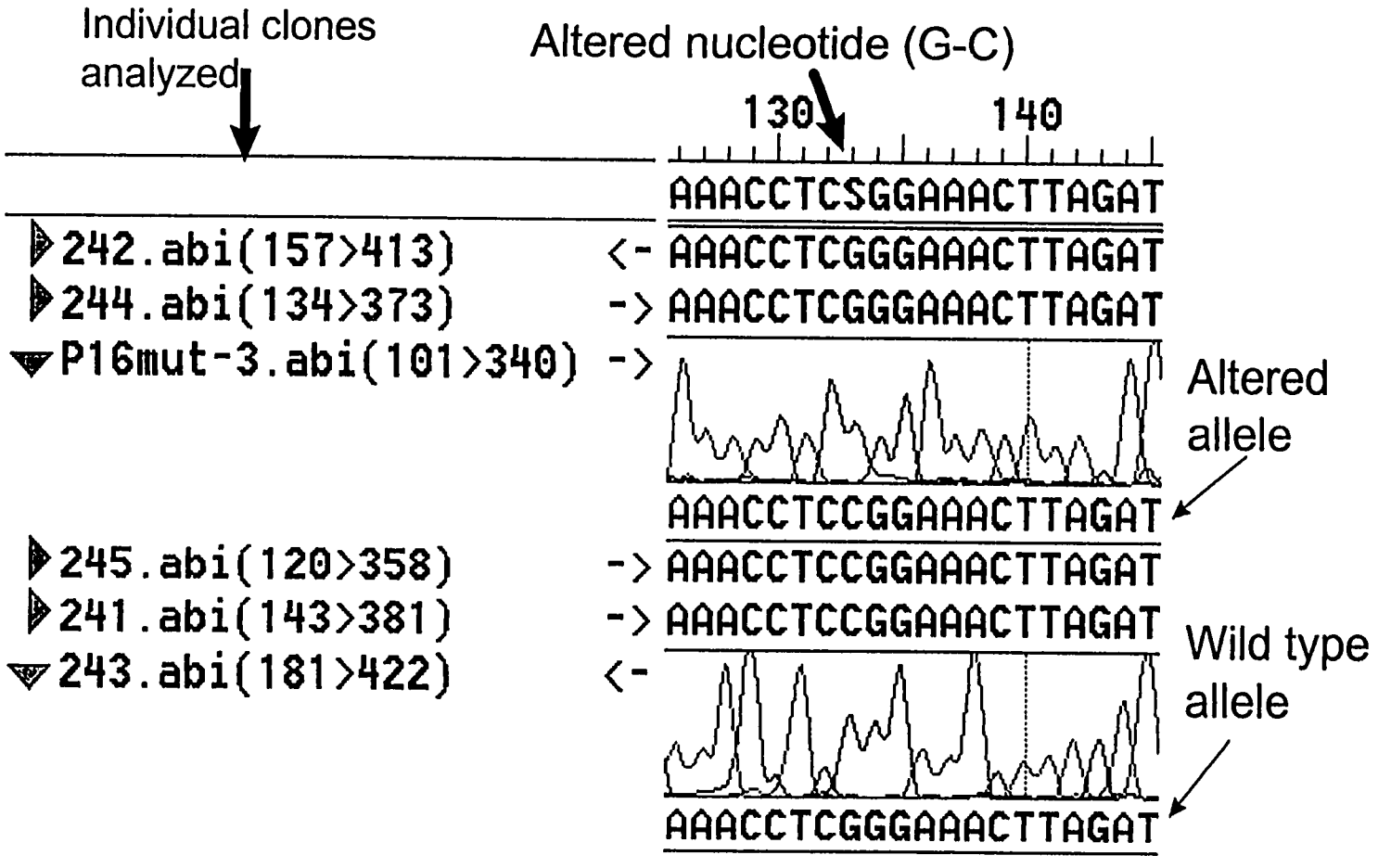


Figure 13: *p16* exon3 sequence variation (1) in breast tumor 24.

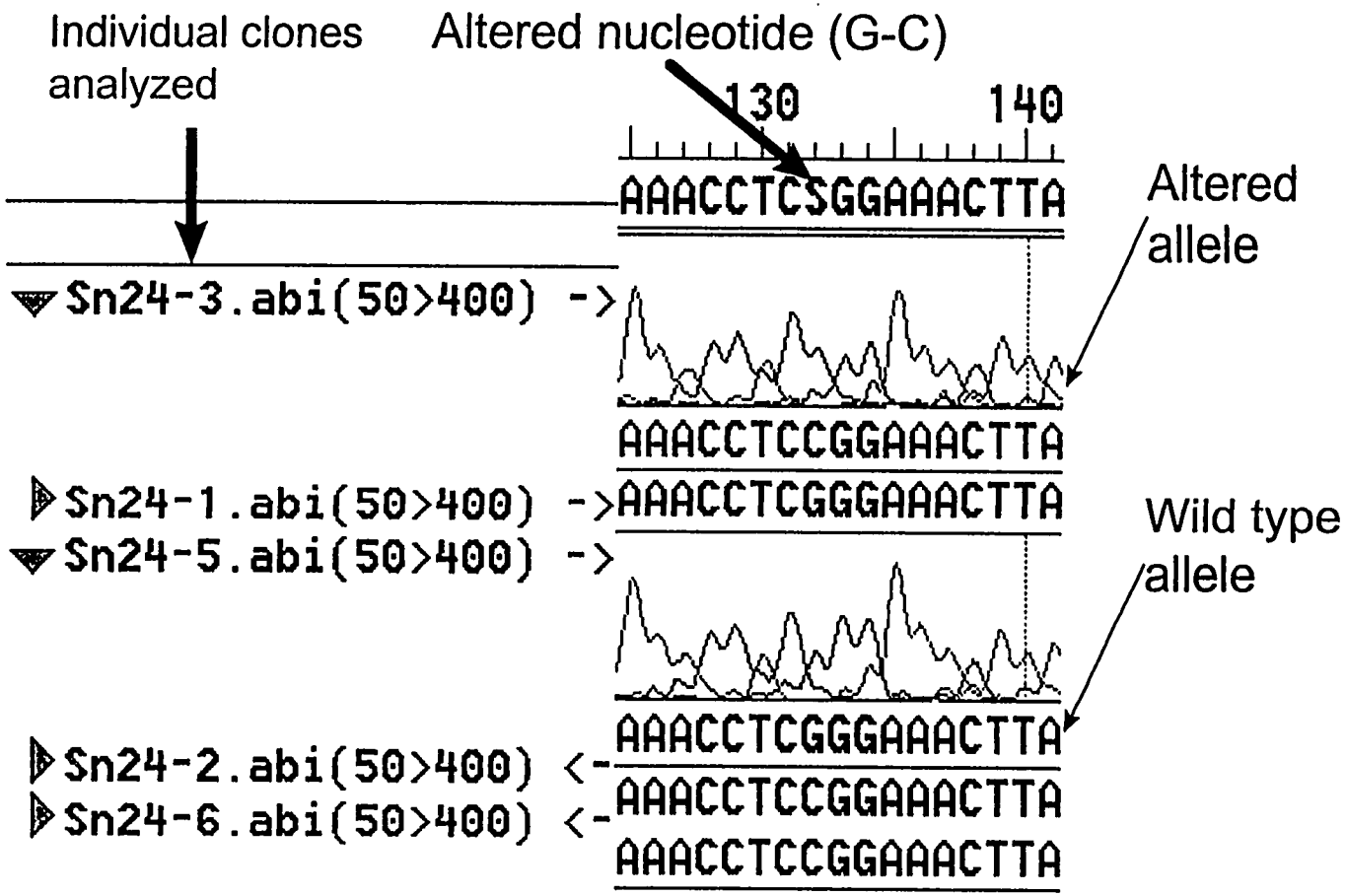


Figure 14: *p16* exon 3 sequence variation (1) in normal tissue 24.

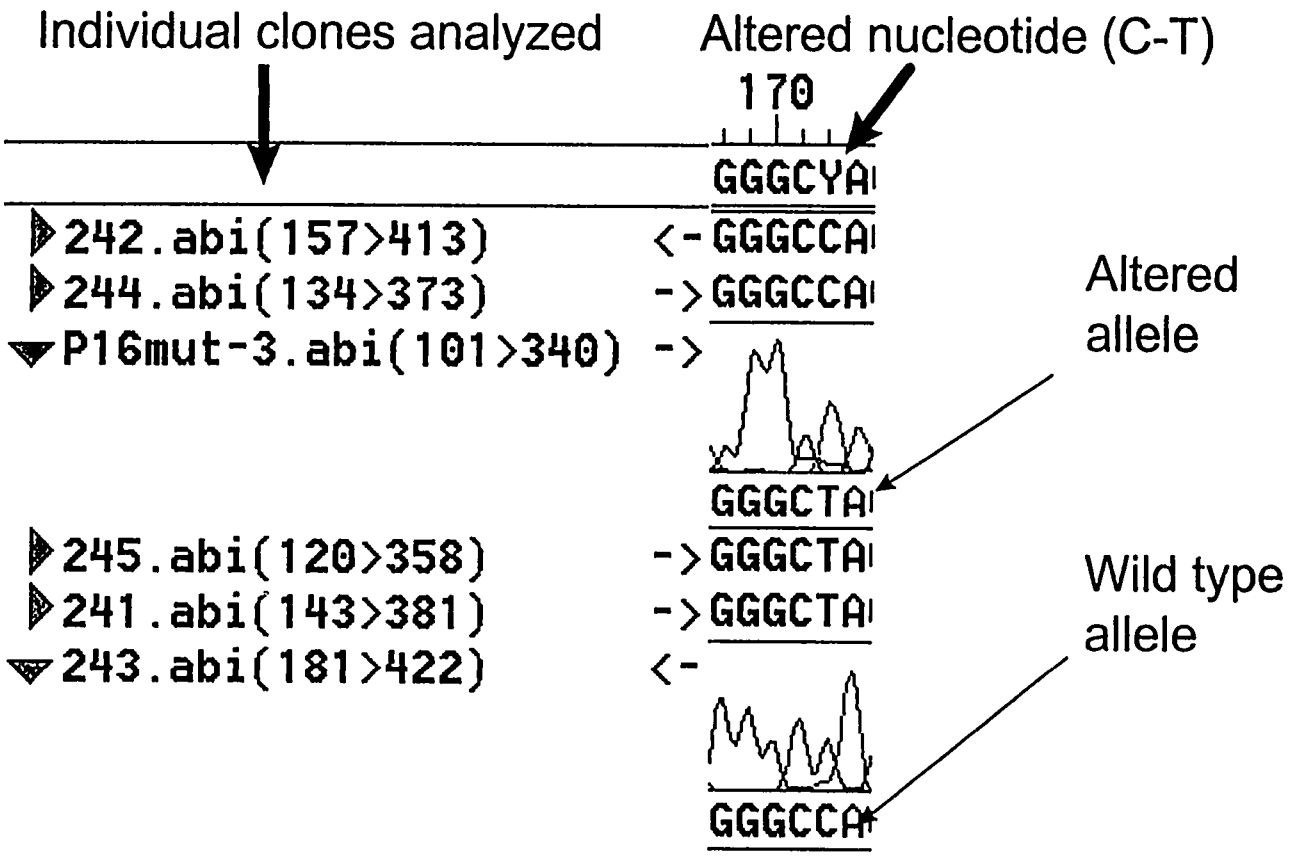


Figure15: *p16* exon3 sequence variation (2) in tumor tissue 24.

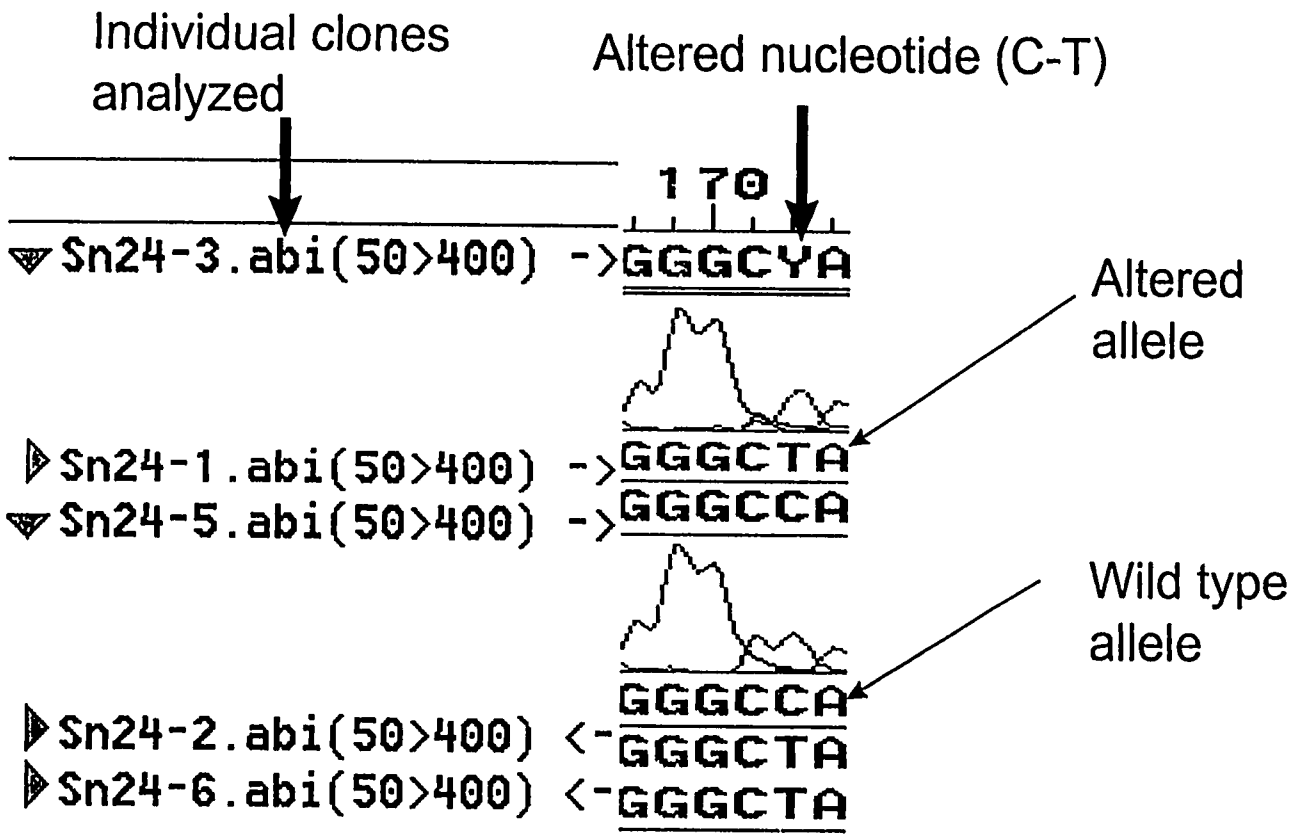


Figure 16: *p16* exon3 sequence variation (2) in breast normal tissue 24

3.3. RESULTS

The genomic deletion analysis of cell lines revealed deletions of exon1 and exon 2 of the *p15* and *p16* genes (Figure 8). *p16* exon 1 deletions were found in melanoma cell lines G361, RPMI 4445, bladder carcinoma cell line T24, breast carcinoma cell line HTB19, MCF7. *p16* and *p15* exon1 and exon 2 were co-deleted in a melanoma cell line RPMI4445. SSCP analysis of *p18* genes of breast tumor tissues revealed that there were no single base-pair alterations in exon1(Figure 9) or the presence of homozygous deletions in the breast tumor tissues. The analysis of *p16* exon1 and 2 by SSCP also revealed that no *p16* alterations were present (not shown). However, alterations in the exon 3 of the *p16* gene were found. A detail SSCP analysis of breast tumor samples 3, 5, 22, 24, 31 and 35 were shown in Figure 10. Alterations in two breast tumor tissues (tissue # 5 and #24) were detected when using the SSCP analysis. However, the CDGE method failed to reveal any mutations in *p16*.

In order to confirm whether these alterations detected in p16 were due to mutations or polymorphisms, exon3 regions from normal tissue of the same patients were also amplified. The amplified PCR products were cloned into a pBluescript vector. These constructs were transformed into *E.coli* (XL1-Blue) strain. These inserts were confirmed by restriction analysis and individual clones were sequenced and analyzed.

The sequence analysis of individual clones from breast tumor and the corresponding normal tissues resulted in two kinds of sequences (different alleles). One of these was a wild type sequence (wild type allele) and the other was with the altered

sequence (altered allele). The alterations (G-C) was found in the *p16* gene 3'region. The wild type allele and the altered allele found in the sequencess of individual clones from the breast tumor tissues #5 was shown in Figure11. The wild type and the altered allele were also found in corresponding normal tissue #5 shown in Figure12.

Cloning and sequencing analysis of tissue #24 also showed the same alterations(G-C) in tumor and normal tissues (Figures13, and 14 sequence position 130-140). In addition, tumor tissue 24 and its corresponding normal tissue had one additional alteration(C-T) found in one allele of the *p16* gene sequence 3' region position at 170 (Figure 15 and 16).

3.4 DISCUSSION

Tumor suppressor genes are intimately involved in the development of cancer. In normal tissues, tumor suppressor genes control cellular growth but this protective function is lost as cancer is initiated or sometime during the process. The loss of this protective function can be due to mutation, deletion or methylation of promoter regions of the specific genes.

Analysis of cell lines have shown that *p15* and *p16* are frequently deleted in cell lines (Figure 8). Gemma *et al.*(1996) and several other groups have also analyzed gene alterations in cancer cell lines and shown that homozygous deletions of the *p15* and *p16* genes are a frequent event. These results clearly support our findings.

In this study 35 breast cancer specimens were analyzed and no homozygous deletions of *p16* α, *p16* β or *p18* were found. A study conducted by Xu *et al.*(1994) also reported no homozygous deletions or mutations of the *p16* gene found in 37 human breast carcinomas. However, higher incidences of homozygous deletion of *p16* were found in bladder cancers (Balazs *et al.*, 1997).

Mutation analysis:

The denaturing gradient gel electrophoresis(DGGE) was used as an alternate method to detect point mutations. There are two advantages of using DGGE over SSCP analysis. First, larger fragments (greater than 500 bp), can be analyzed by DGGE. In SSCP the sensitivity decreases with larger fragments. Secondly, DGGE provides rapid analysis (less than 4 hours) and the use of radioisotopes is not essential. The disadvantages of this method are that an optimum denaturing condition (temperature and denaturant concentration) needs determination prior to actual experimentation. In these experiments a constant denaturant of 57% (5.28 ml of formamide and 2.32 g of urea) and 57 ^{0}C was used. Unfortunately two sequence variants of *p16* were not detected with this technique. In this experiment, the heteroduplex formation requires an equal ratio of wild type and mutant DNA. Since this experiment was carried out with only PCR products, this may have contained an unequal ratio of wild type and variant DNA and this may have resulted in the failure of mutant detection.

Exon 3 alterations of *p16* genes were identified in both tumor and somatic tissues. These alterations were found in the 3' untranslated region of the *p16* gene. Two tissues

revealed alterations of G to C in the 130-140 base pair regions (#5 and #24) of one allele of the gene. The second allele (wild type) was normal and found in some clones from the normal and tumor tissues. A second alteration of C to T at 170 bp of the *p16* exon 3 also detected in tumor tissue #24. This alteration was also found in both normal and tumor tissues of one allele. These alterations were found in the 3'untranslated region of the *p16* gene. Formation of new splice sites requires a GT-AG at 5'-3'in the introns. Therefore, these alterations found in the 3' untranslated region from G to C and C to T does not create any new splice sites at end of the 3' region.

The genomic alteration analysis from the breast tumor tissues reveals that:

(1) These alterations were found in both normal and tumor tissues in one allele.

(2) These alterations were outside the coding regions.

(3) These alterations do not create alternate splice sites.

Therefore, these alterations likely represent DNA polymorphisms rather than mutations that are significant to gene expression.

As discussed above, altered mRNA expression of *p16* detected in the breast tumor tissues was not associated with homozygous gene deletions or mutations. Thus, the changes in mRNA expression associated with methylation of the promoter region had to be considered. Methylation analysis of the 5'- region of the *p16* gene is discussed in the following section.

4 . METHYLATION AND ALTERED m-RNA EXPRESSION OF *p16.*

4.1 AIMS.

Specific aims of this chapter were

(1).To investigate whether the 5' region of the *p16* gene is methylated in breast tissues.

(2).To verify whether specific methylation patterns of the *p16* gene 5' region in the breast tumor DNA contributes to the altered *p16* mRNA pattern as detected earlier in this study (see Figure 7).

Experiments were carried out with five breast cancer tissues (with varying *p16* expression levels) and a cell line genomic DNA in the methylation analysis.

4. 2. MATERIALS AND METHODS

In order to identify the genomic DNA methylation patterns, a method described by Clark *et al.*(1994) was used in this project. The basic principal behind the conversion reaction relies on the ability of sodium bisulfite to efficiently convert cytosine residues of a linear ss DNA to uracil. If the cytosine is methylated to 5-methylcytosine (5-MeC) it remains non-reactive in single stranded DNA without any conversion. The DNA sequence under investigation is amplified with specific primers and sequenced. In the sequence analysis all uracil and thymine residues are amplified as thymine. 5-MeC residues remains as C.

The bisulfite conversion reaction involves the following steps: First the addition of bisulfite forms an adduct across the 5-6 double bond of cytosine (cytosine -SO_3). In

the second step, the hydrolytic deamination of the cytosine-bisulfite derivative takes place in order to give a uracil-bisulfite derivative. In the final step, an alkali treatment removes the sulphonate group to yield uracil-U. The steps involved in the biochemical reaction are described in Figure 17.

Two experiments were carried out in the bisulfite genomic sequencing analysis. One of these was to optimize the bisulfite reaction conditions. The second was to analyze the breast cancer genomic methylation pattern of the 5' region of *p16* .

Experiment 1:

In this experiment bisulfite conversion reaction was carried out with 10μg of DNA from a cell line (HTB - 20) and a breast tumor tissue. The linear genomic DNA preparation, and the ss DNA were isolated out as described under section 4.2.1. Sodium bisulfite treatment was carried out as in section 4.2.2. However, in this experiment the bisulfite reaction time and the incubation temperature were modified as follows:

Treatment a (T_2): bisulfite conversion reaction at 55°C - 24 hours.

Treatment b (T_3): bisulfite conversion reaction at 62°C - 12 hours.

After the final step of the reaction, the modified DNA was used in the PCR amplification reactions. The PCR amplification was carried out at the region D *p16* - 5'region as described in table 6. The PCR amplified products were cloned into pBluescript vector as described under materials and methods 4.2.4 and 4.2.5. The construct was transformed into XL1-Blue *E.coli* strain and several individual clones were

picked. All clones were tested for the correct size of the insert by restriction analysis. Individual clones were sequenced and analyzed.

Experiment 2:

The second experiment was performed with 10μg DNA from four breast tumor tissues. The linear genomic DNA preparation, and the ss DNA were isolated as described in section 4.2.1. Sodium bisulfite treatment was carried out as discussed in section 4.2.2 at 55°C for 14 hours. Further to the bisulfite conversion reaction (see section 4.2.3 for details), the PCR amplification of the 5'region of the *p16* was carried out with specific primers designed at region C and D (Table 6 gives primers for regions C and D). The PCR amplified products were cloned into pBluescript vector as described earlier (see section 3.2.5) and the individual clones were sequenced and analyzed (see setion 3.2.6 and 3.2.7).

4.2.1 Genomic DNA digestion.

The bisulfite conversion reaction is efficient in linear and ssDNA. In order to linearize the genomic DNA, enzymatic digestion is preferred. Proper selection of the restriction enzyme is important in this procedure as the enzyme should not cut within the target sequence of interest. In this study, *EcoR* I produces fragments of genomic DNA that do not cut the 5' region of *p16* in the area to be analyzed (Merlo, 1995).

4.2.2 Bisulfite treatment.

Linearized (*EcoR* I digested) genomic DNA was denatured with 3N NaOH at 37°C for 15 min. The reaction was carried out by adding 3.6M sodium bisulfite and 20 mM hydroquinone to a final concentration of 3.1 M and 0.5mM. The reaction was overlayed with mineral oil and incubated at 55°C for 12 -14 hrs. Desalting DNA of the reaction was carried out using Promega Wizard Prep and DNA was recovered in a small volume (<100µl). Desulphonation was carried out adding 3N NaOH to yield a final concentration of O.3 N NaOH and Incubating at 37°C for 15 min. DNA was ethanol precipitated by adding 10 M NH_4OAc to a final 3M concentration and 2 volume of ethanol. The pellet was dried and resuspended in 100µl H_2O.

4.2.3 Polymerase chain reaction.

Specific primers were designed for the bisulfite converted 5' region of the top strand of the *p16* gene (5'-3' direction) Table 6. PCR amplifications were carried out in a standard reaction containing 1X reaction buffer(BRL),1.5 mM Mg, dNTP mixture 0.2mM , and 0.2µM of each primer and 1 unit of *Taq* DNA polymerase. The PCR reaction was carried out in a thermal cycler (The DNA Engine PTC-200, MJ Research). The cycles contained an initial denaturing step of 96 °C for 3 minutes and 40 cycles of 95°C denaturing for 30 Sec, 50°C annealing step for 30sec and 72°C primer extension for 50 sec. The final extension step contained a 5 minutes extension at 72°C.

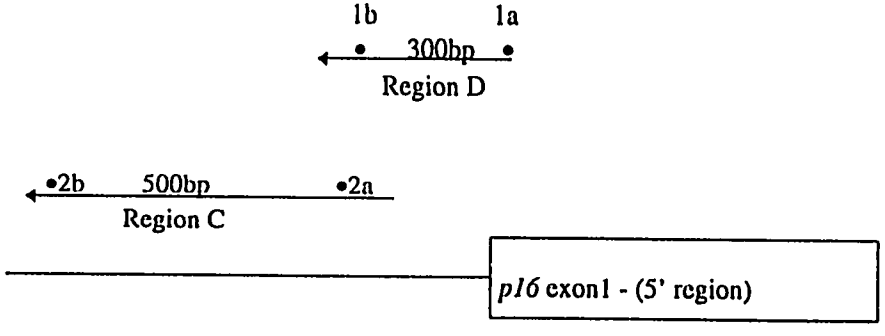
4.2.4 Cloning and selection of positive clones.

PCR products were analyzed in agarose gels for the detection of the correct size inserts. The correct size fragments were excised and cloned into the pBluescript plasmid at an EcoR V site modified with dT as described in chapter 3. The constructs were transformed into XL1-Blue *E. coli* strain. Positive clones were selected and confirmed by restriction analysis for the correct size and sequenced as described in chapter 3.

4.2.5 Sequencing and analysis.

Single stranded DNA was isolated and the ABI sequencing analysis was carried out as described in chapter 3.

Table 6: Bisulfite genomic sequencing primers for the 5' region of *p16* gene



#	*p16*—5'	Sequence	Annealing Temperature
1-a	(Region D)	ATCA/GA/GCCTCCA/GACCA/GTAACTATTC	50 °C
1-b	(Region D)	GAAAGATAT/CT/CGT/CGGTT/CT/CT/CTT/CT/CAGAGG	50 °C
2-a	(Region C)	GTCCCTCAAATCCTCTA/GA/GAA/GA/GA/GACCA/GACCA/GC	50 °C
2-b	(Region C)	TTTT/CTAGTT/CGTAT/CAGGTGATTTT/CGATT	50 °C

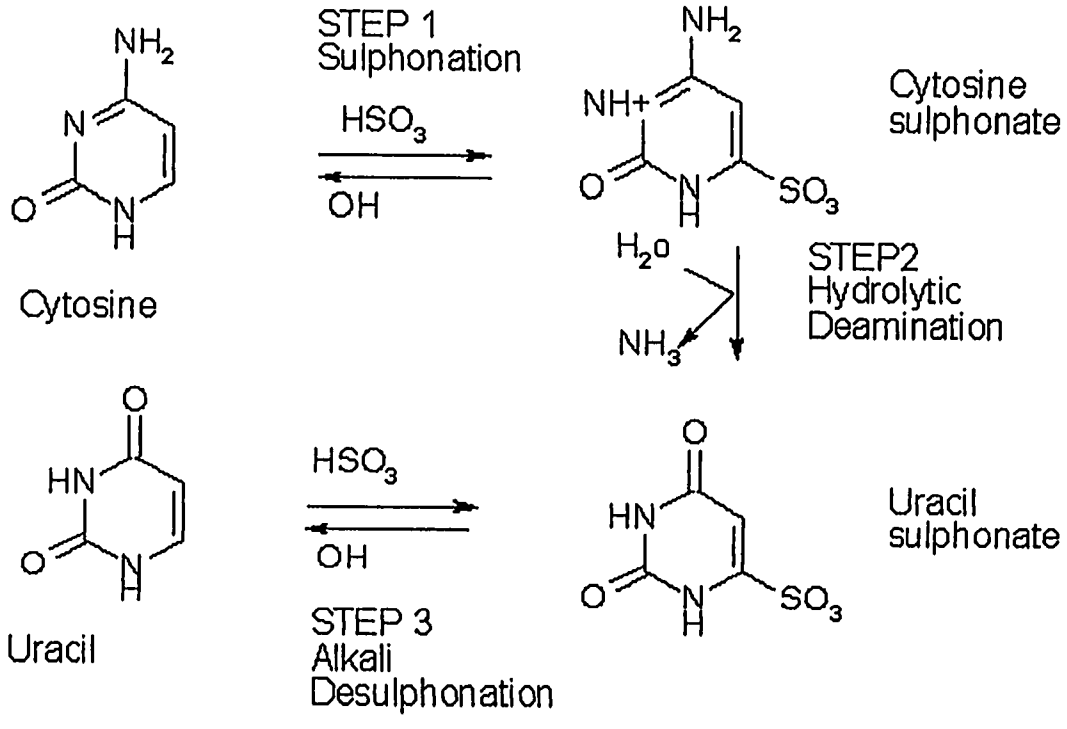


Figure 17: A schematic diagram of the bisulfite conversion reaction.

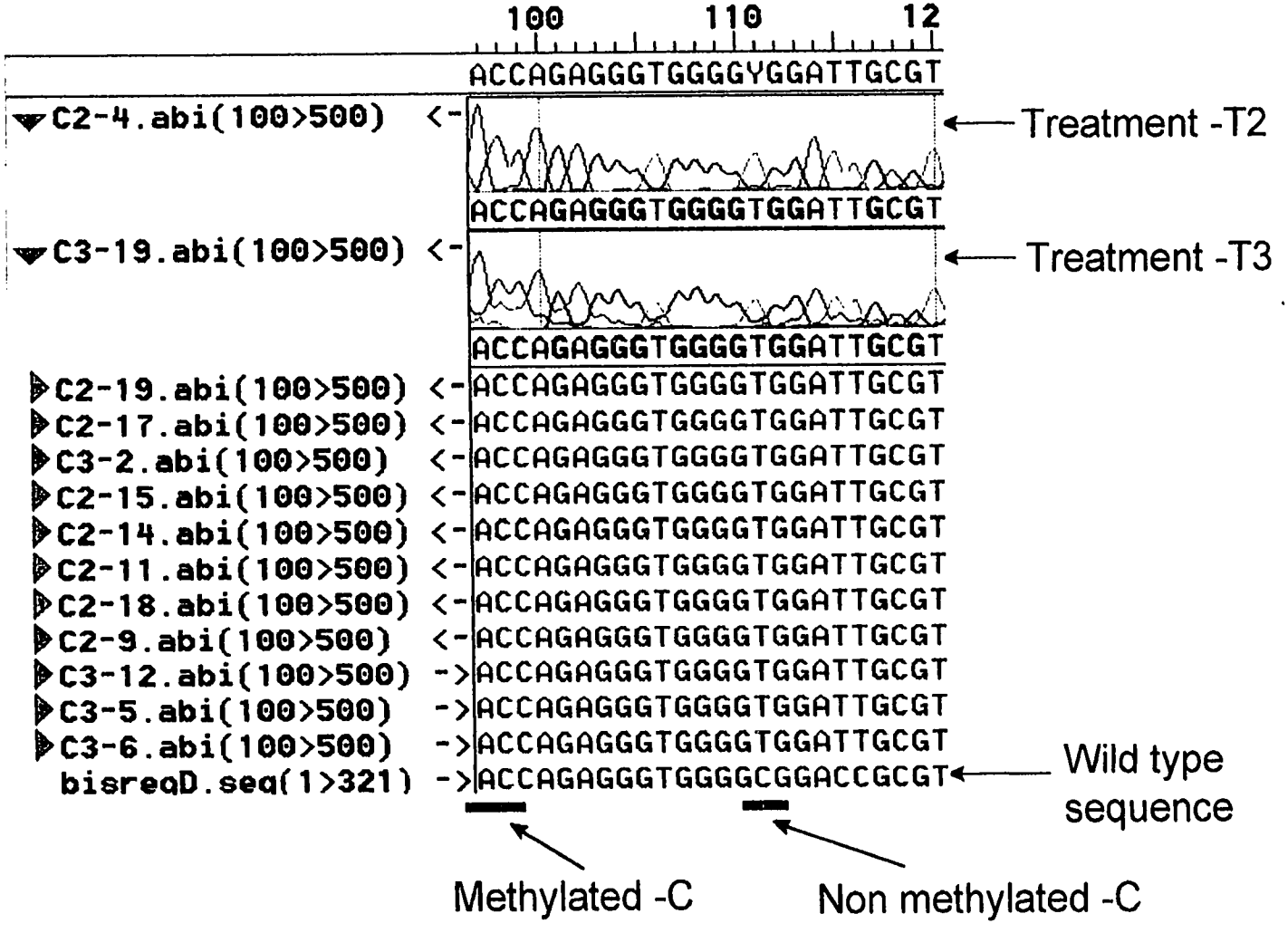


Figure18(a) : Methylation analysis of the 5' region of *p16* from a cell line (HTB20) DNA
T2= 55 ^{0}C treatment for 24hrs. T3= 62 ^{0}C treatment for 12hrs

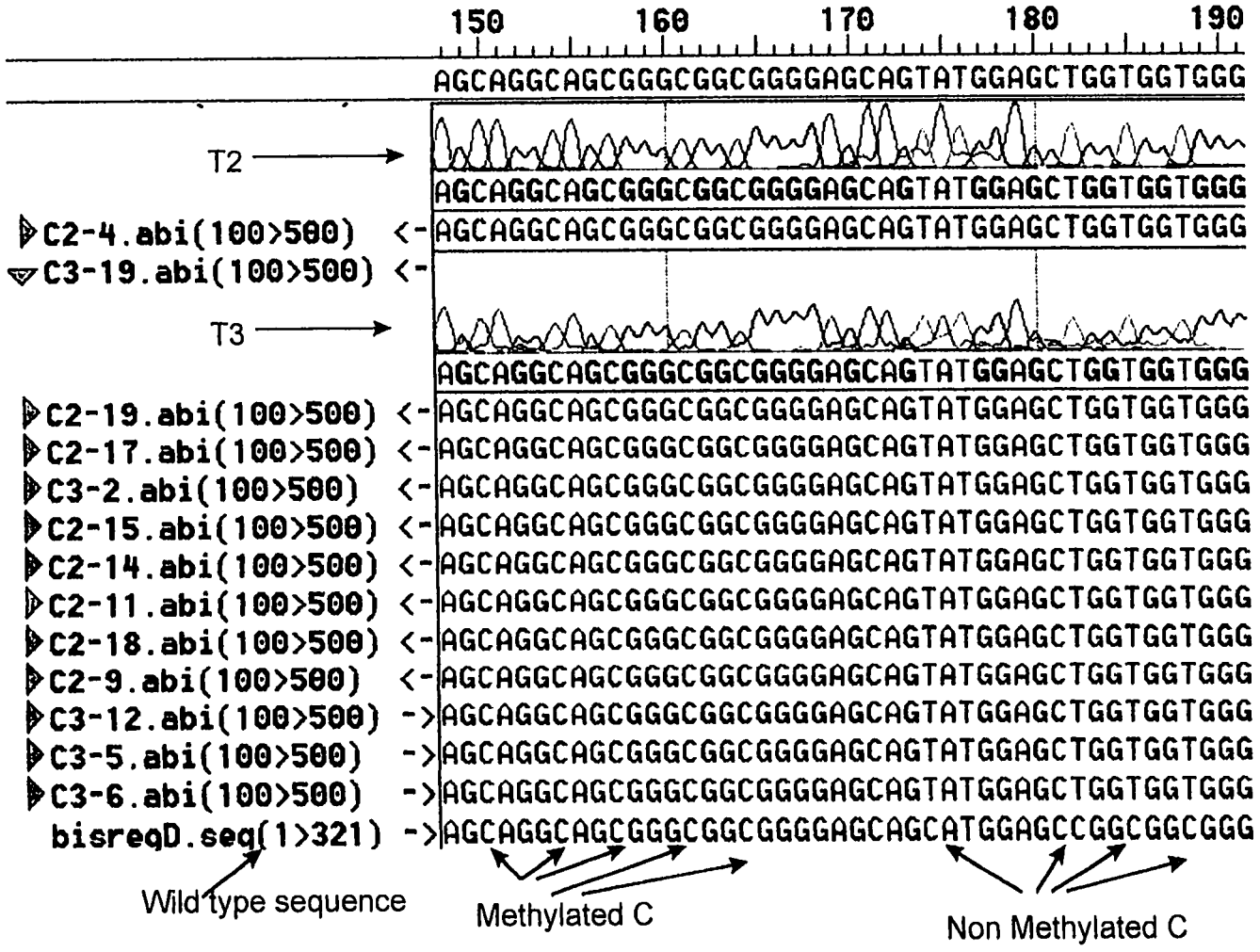


Figure18(b): Methylation analysis of the 5' region of *p16* from a cell line (HTB 20) DNA..
T2= 55°C treatment for 24hrs. T3=62°C for 12hrs.

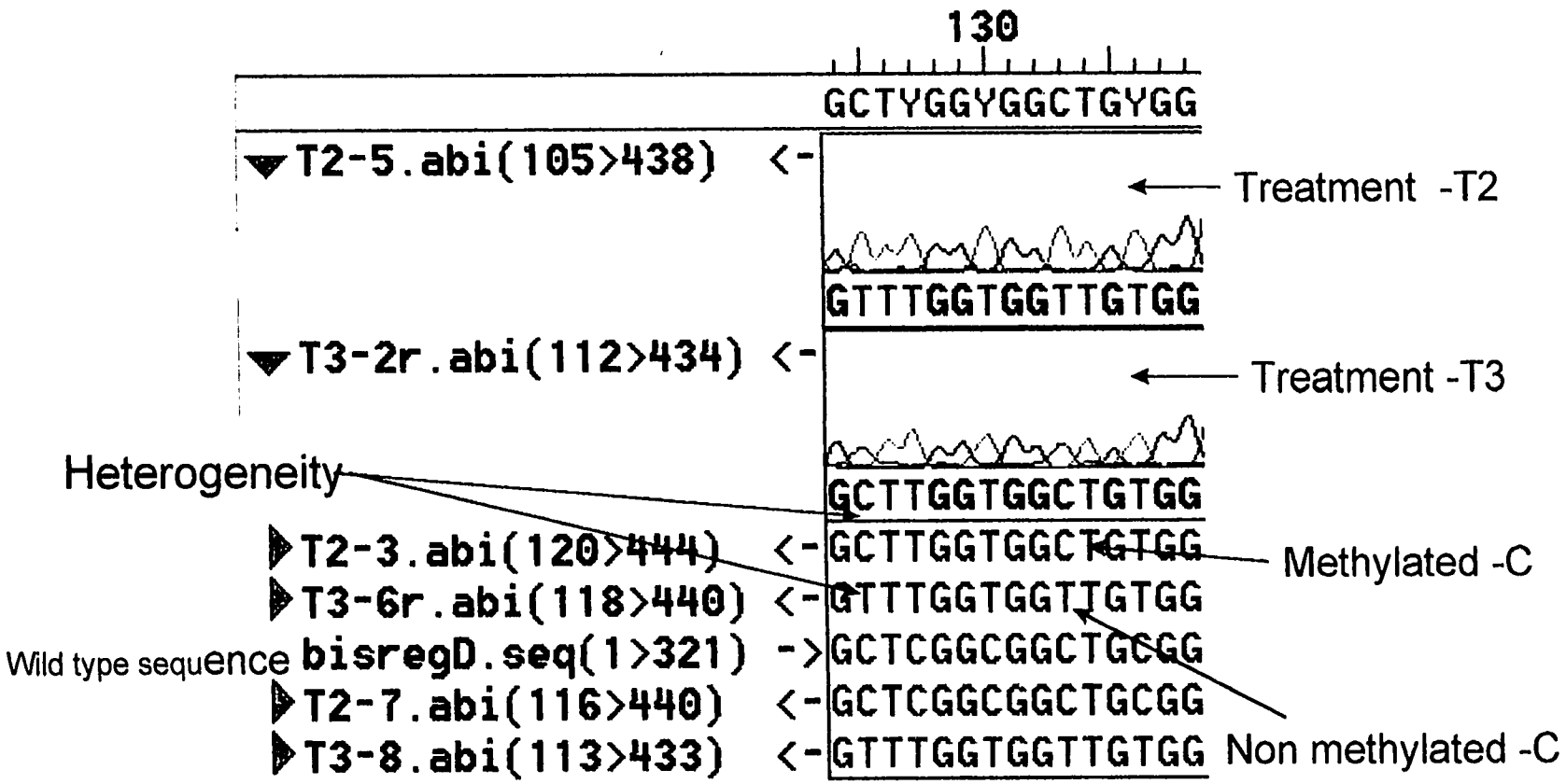


Figure 19(a) : Methylation analysis of the 5' region of *p16* from a tumor tissue.
T2= 55 ^{0}C treatment for 24hrs. T3= 62 ^{0}C treatment for 12hrs

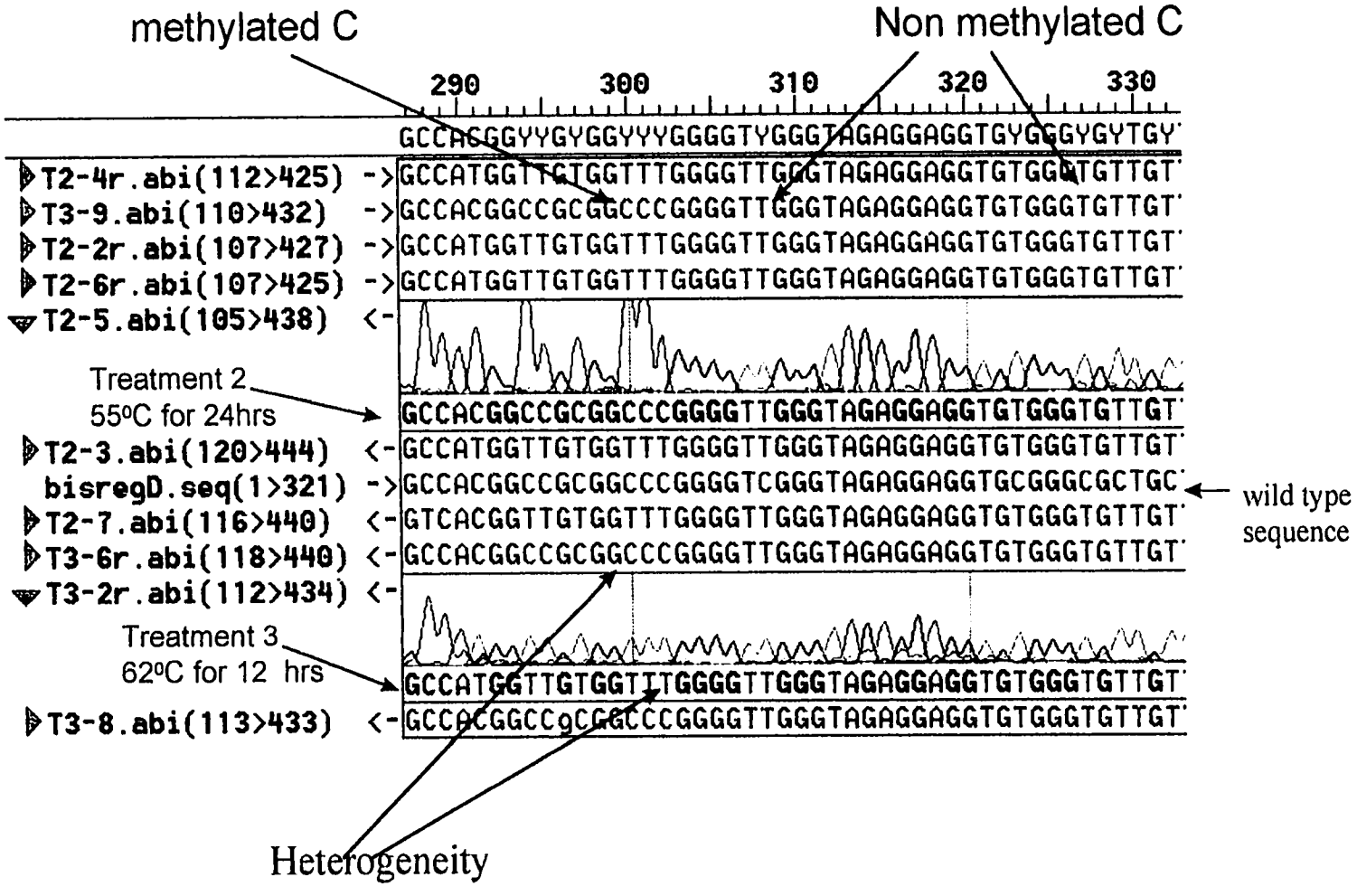


Figure 19(b): Methylation analysis of the 5' region of p16 from a tumor tissue

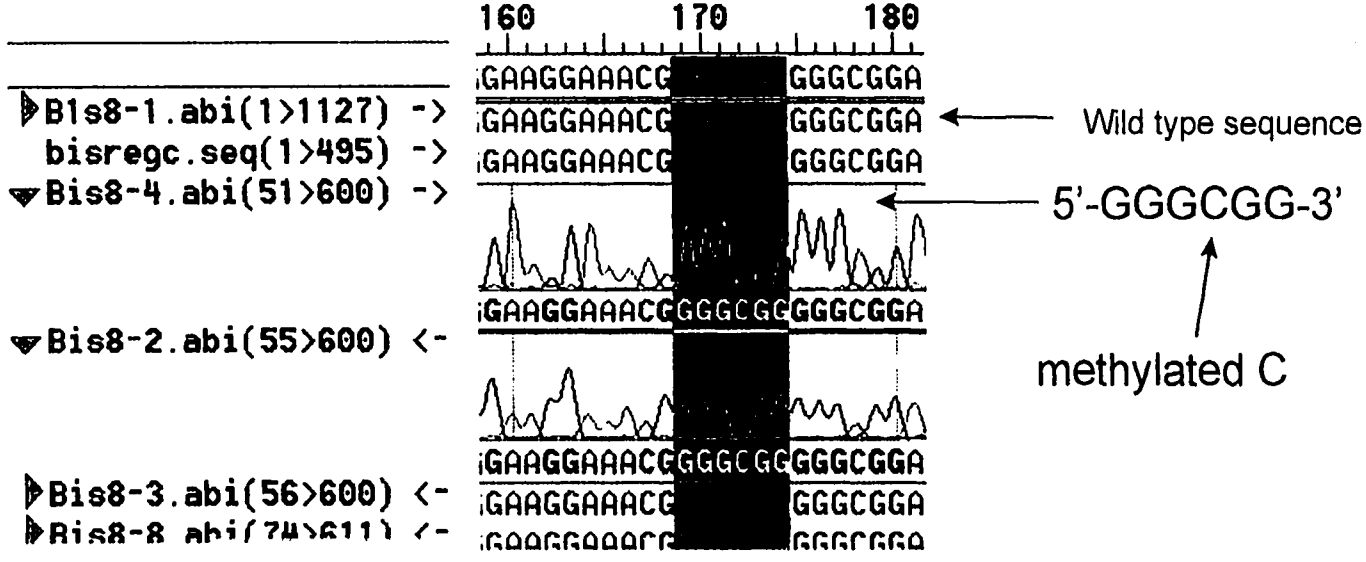


Figure 20(a): DNA methylation analysis of the sp1 binding site of *p16* from breast tumor tissue.

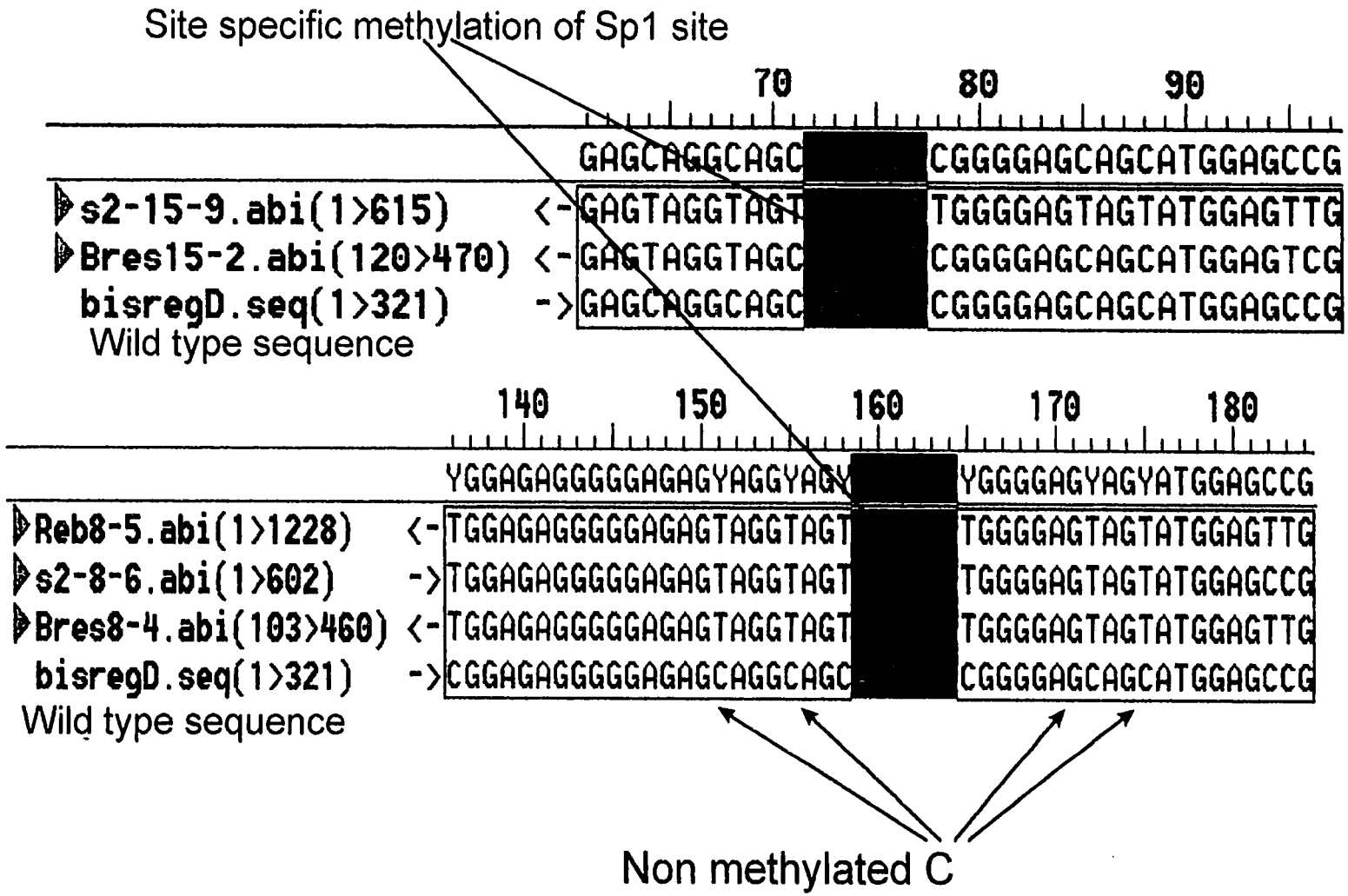


Figure 20(b): Site specific DNA methylation of Sp1 region from breast tumor tissue.

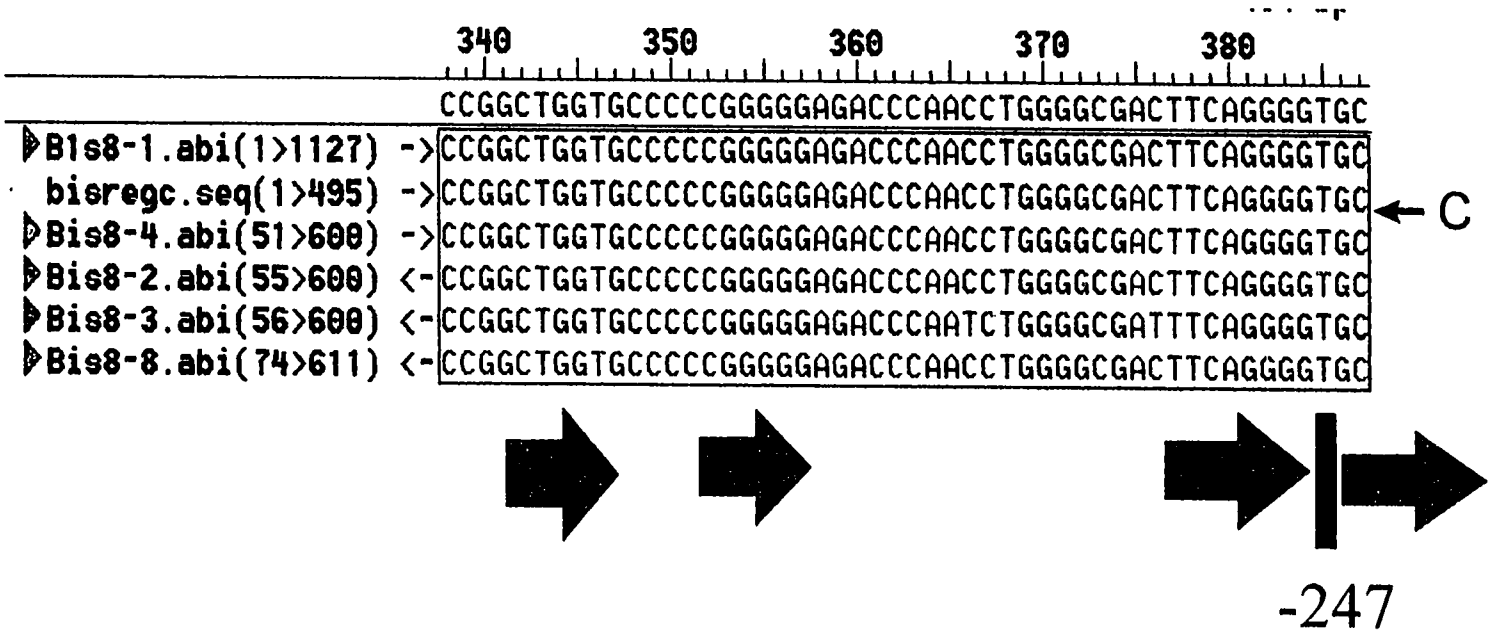


Figure 21: DNA methylation at transcriptional initiation site (-247 bp at the 5'region of *p16*. Hara et al.,1996).

C= Wild type sequence

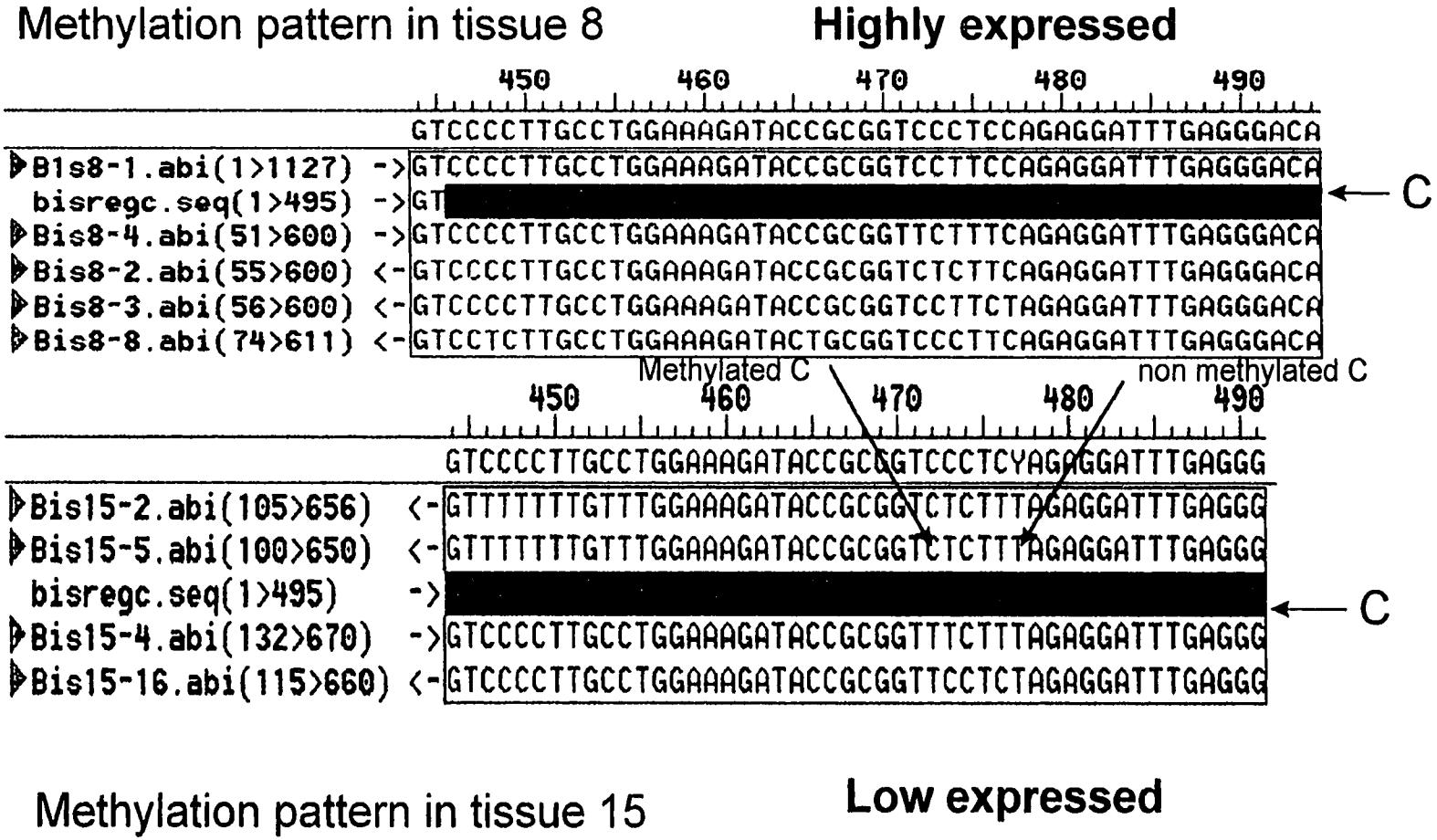


Figure 22(a): Methylation pattern in 5' upstream region of *p16* from breast tumor DNA. Possible altered methylation pattern in sample 8 and 15.
C= Wild type sequence

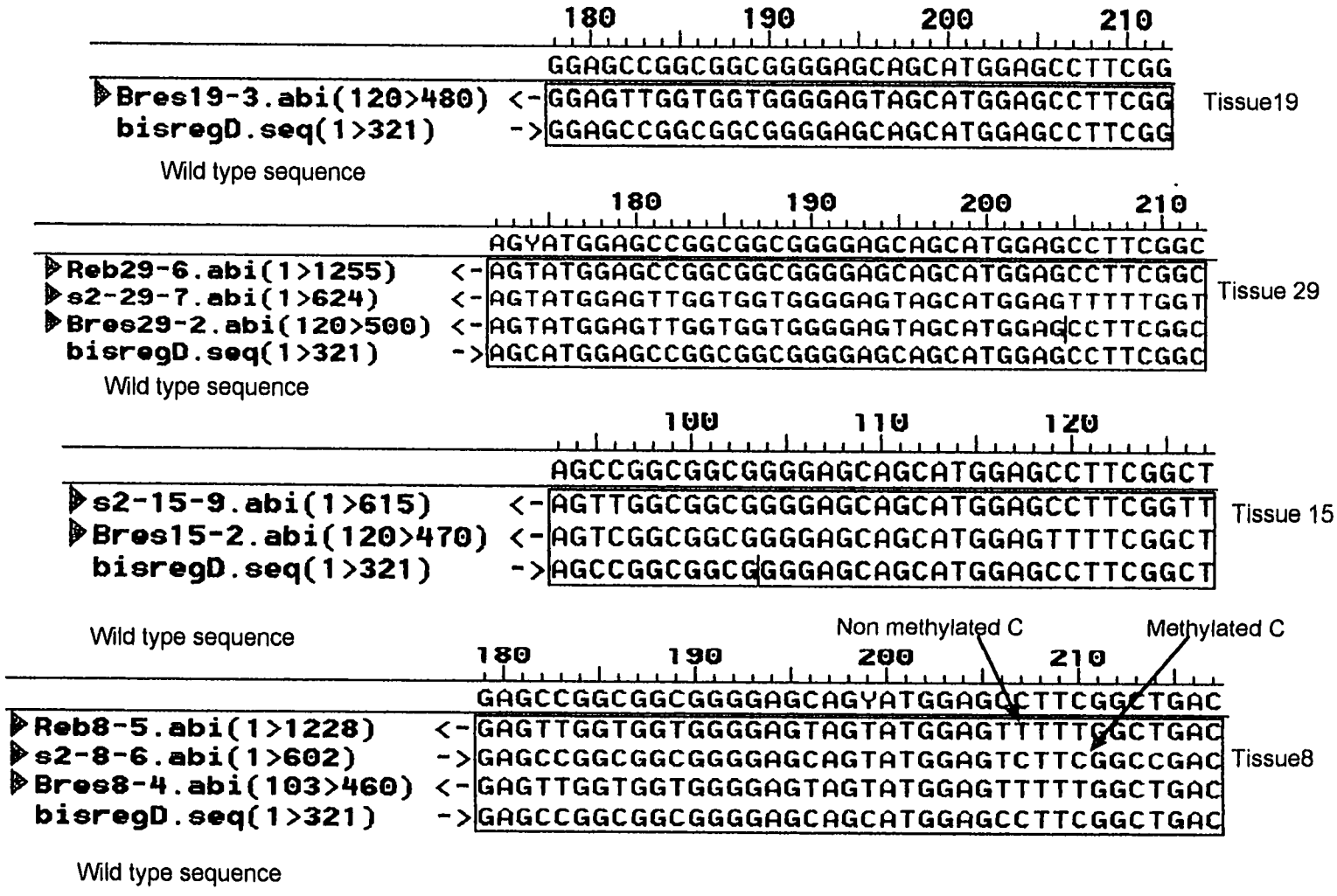


Figure22(b): Methylation pattern in 5' upstream region of *p16* from breast tumor DNA
Possible altered methylation pattern in sample 8, 15, 19 and 29.

4.3 RESULTS.

Experiment 1:

Individual clones sequence analysis obtained from the cell line (HTB20) genomic bisulfite treated DNA (3.1M sodium bisulfite) shows, that the conversion of the C residue was uniform among the clones analyzed (Figure18a, 97-120 bp and Figure18b, 150-190 bp). No differences were detected in samples that were incubated longer (24hrs) or at higher temperature (62°C). However, in the breast tumor tissue sample the conversion pattern was different amongst the individual clones analyzed in both treatments (Figure 19a, 124-138 bp and Figure 19b, 287-332 bp). These experiments indicate that there is heterogeneity in the breast tumor DNA and not due to insufficient reaction time.

Experiment 2:

The bisulfite genomic sequence analysis from breast tumor DNA revealed the following:

1. In breast tumor tissue the 5' region of the *p16* gene contains methylated and unmethylated regions. (Figure 19a, 290-310 bp and Figure 19b, 307-302bp).
2. Site specific methylation of Sp1 binding sites are shown (Figure 20a and Figure 20b).
3. Methylation at possible transcription initiation sites in the -200 to -247 regions (Figure 21) was detected.
4. No specific methylation differences (Figure 22a) were found in specimens that exhibited variable p16 mRNA expression (tumor tissue #8 with high mRNA and tumor tissue #15 with low level of mRNA see Figure 7).

In Figure 22b methylation patterns of the *p16* gene 5'region 180-220 bp were shown for four tumor tissues (#8, #15,# 19,#29).

4.4. DISCUSSION.

In the first part of the experiment, genomic DNA from a cell line and a breast tumor tissue was used. The genomic sequence analysis of individual clones resulting from the bisulfite conversion clearly demonstrated that both cell line DNA and the breast tumor DNA are methylated in the 5' region of *p16*. However, in cell lines conversion of the C was uniform among the clones analyzed. No differences were found among different treatments (high temperature, at 62°C or long incubation, 24 hour at 55°C). This was due to a uniform cell population and as a result the DNA used in this experiment was pure and the methylation patterns from individual clones were uniform. In breast tumor tissues, the conversion of C was not uniform. There was heterogeneity in conversion of the C. This is probably due to a mixed population of tissue cells (normal and tumor) present in the tumor DNA and not due to insufficient bisulfite conversion. The heterogeneity in the tumor tissues makes the interpretation of the data difficult. Merlo *et al.*, (1995) also reported the difficulty in precise assessment of tumor samples which are usually comprised a heterogeneous mixture of normal and neoplastic cells.

Five breast tumor tissues were examined for the methylation status of the 5'-region of the *p16*. The results revealed that the tumor tissues are methylated at various sites. Only minor methylation differences were detected in the 5'-region (800 bp) among the highly expressed and low expressed breast tumor tissues. No distinct methylation

patterns were found between the variably expressing breast tissues. Therefore, it is very unlikely that these minor changes in the methylation pattern would have contributed to the expression differences.

The results from methylation analysis of the breast tumor tissues, indicate abnormal hypermethylation of the CpG islands that encompass the 5' region of the *p16* gene. Specific methylation of the Sp1 sites (Figure 20a and Figure 20b) were detected in this experiment. Many other methylated sites were found in the 5' region of *p16*. These methylated sites may be associated with the binding of other transcription factors. The most likely explanation for the variable *p16* expression found (Figure 7) is due to artificial amplification introduced with the PCR reaction.

5. CONCLUSIONS AND FUTURE PERSPECTIVES.

5.1. Conclusions:

Breast cancer is one of the most common malignant diseases among women which lead to death (Canadian Cancer statistics 1996). The development of human cancer is frequently associated with the inactivation of one or more cell cycle regulatory pathways. The phosphorylation of the pRb protein and entry into S phase is controlled by CDK/cyclin kinase complexes. The specific CDK4 and CDK6 inhibitors INK4 proteins (p15, p16, p18, p19) can directly block the activity of CDKs or the CDKs could be activated by D-type cyclins (D1 ad D2) (Sherr and Roberts 1994 ; Sherr 1995). The *INK4* genes *p15, p16, p18* and *p19* share considerable similarities at the amino acid level such as ankyrin repeats and secondary structure Guan *et al.*(1994). In spite of their similarities, the function of each *INK4* genes in breast cancer has not been elucidated. Therefore, in the present study a hypothesis was formulated as to whether the down regulation of one or more *INK4* genes abrogate the pRb blockade of G1 progression in breast cancer.

In the first part of this study a survey of the normal expression pattern of the *INK4* genes were examined in a variety of human tissues. The results have shown that the *INK4* family genes are expressed in distinct tissue specific patterns. This suggests that the tissue specific expression is a special characteristic of the INK4 family. The results of Guan *et al.*(1994 ; 1996) who also found tissue specific expression of *INK4* family genes corroborate with the findings of the present study. In the present study *p16* is expressed in basal amounts in human mammary tissue.

In the second part of this study, thirty-five microdissected specimens of breast cancer were examined for altered expression patterns of *INK4* genes. The expression pattern of *INK4* in breast tumor tissues show no alterations in the expression of p15, p18, p19 or pRb but the expression of p16 was found to be low in breast tumor samples. Radioactive RT- PCR analysis of *p16* mRNA in breast tissues revealed low and variable expression. A low expression level of the *p16* gene was found in all of the breast tumor tissues examined and was most likely due to methylation of the promoter region. The methylation analysis of the 5' region of the *p16* gene from the present study has shown (1) the existence of methylation at the 5' region, (2) specific methylation of Sp1 sites, and (3) specific methylation at transcription initiation sites. The variable expression showed no correlation with tumor grade, homozygous deletions, single base pair mutations or even specific altered methylation patterns.

In conclusion, the results from the experiments support the major hypothesis of the present study. In breast cancer tissues, downregulation of *p16* may be the common mechanism control of the pRb pathway. However, the role of other family members (p15, p18, and p19) appear not to be very prominent in breast tissues. The major down regulation mechanism for *p16* in breast tumor tissues is most likely due to methylation of the promoter region. Alternate mechanisms such as homozygous deletions or point mutations are not common in breast cancer tissues. The data from this study (1) support the existence of p16: pRb tumor suppressor pathway in breast cancers and (2) indicate that the mode of inactivation of *p16* gene is most likely due to methylation. Other studies

in breast carcinomas have reported similar findings. No *p16* gene mutations were found in 37 breast tumors by Xu *et al.*(1994). However, 24 primary breast carcinomas were analyzed by Brenner and Aldaz (1995) for gene alterations of *p16* and the chromosome 9p region in breast cancer. A high frequency of loss of heterozygosity affecting the subregion of 9p21-22 was found. Further analyses have shown only one mutation in *p16*. This implies that a possible existence of another tumor suppressor in this region (Brenner, 1995).

Methylation analysis of the 5' region of the *p16* gene was carried out in a variety of cell lines and primary tumors by Herman *et al.*(1995). The methylation sensitive restriction digest (EagI, SacII or SmaI) of the genomic DNA and followed by a southern hybridization was used in this experiment. Even though the sample size ranged from 5-26 the results from Herman(1994) revealed methylation at these specific restriction sites in several cell lines, (33% in breast cancer cell line, 60% in prostate cancer cell line, 23% in renal cancer cell line and 92% in colon cancer cell line). In primary tumors about 31% of breast tumors and 40% of colon cancer tumors displayed *de novo* methylation. No homozygous deletions were detected in these samples. Therefore, the authors concluded that CpG island methylation is a common mode of inactivation of *p16* in a variety of human cancers.

A study on lung cancer cell lines by Merlo *et al.*(1995) reported a distinct methylation of CpG islands in the 5' region of *p16* and, as a result, transcriptional

silencing of the gene. As expected, addition of de-methylating agents such as 5-deoxyazacytidine reversed the transcriptional blockade.

The *p16* and *p15* genes are located close to each other. Methylation analysis in bladder cell lines by Gonzalez-Zulueta *et al.*(1995) reported that only the *p16* gene was specifically methylated and as a result transcription of *p16* repressed. No association was found between *p15* CpG methylation and expression (Gonzalez-Zulueta *et al.*, 1995).

A major problem associated with the current conventional method of microdissection of tissue to obtain a pure population of tumor cells is contamination. Since tumor samples are usually composed of a heterogeneous mixture of normal and neoplastic cells they can pose some difficulty in precise assessment of specific gene alterations in tumors. A level of 20% contamination from the surrounding normal cells is common. The new improved technology of "Laser Capture Microdissections" (LCM) Emmert-Buck *et al.*(1996) will eliminate the contamination problems and will improve correct assessment of tumor sample analysis.

5.2 Future directions:

INK4 mRNA expression analysis in breast cancer tissues have shown that *p16* may be an important gene in the development of breast cancer. Therefore, the role of p16 in the development of cancer should be examined in detail in the future. The LCM can be used to define the expression of *p16* in normal breast epithelium and compare to the neoplastic epithelium more directly. The use of inducible expression of *p16* in cell

lines which lack the p16 function may be used in examining the specific function. These inducible models include the use of reteroviral constructs, conditional inducible systems (Tetracycline constructs) and demethylating agents such as 5-aza-2'-deoxycytidine.

A detailed analysis of the *p16* promoter region and the associated transcription factors would be helpful in order to pinpoint the methylation associated transcriptional repression of *p16*.

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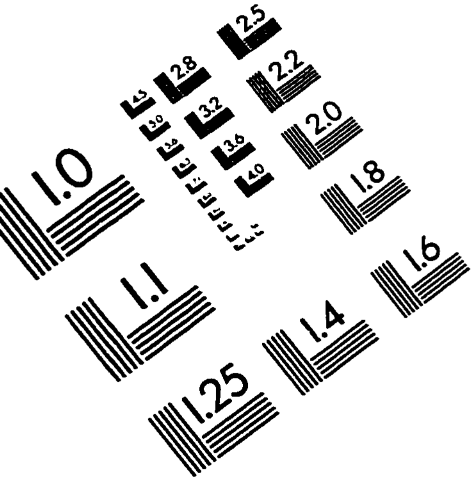
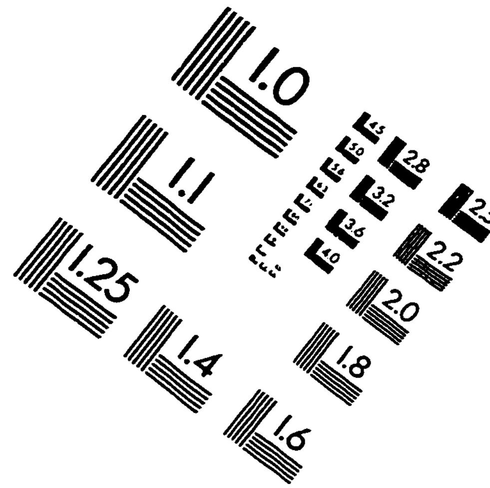
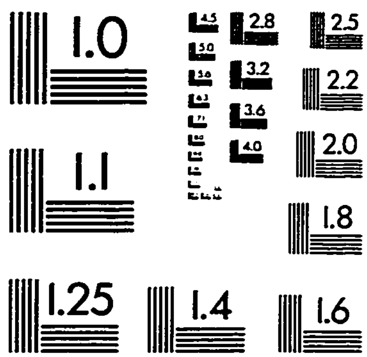
IMAGE EVALUATION TEST TARGET (QA-3)







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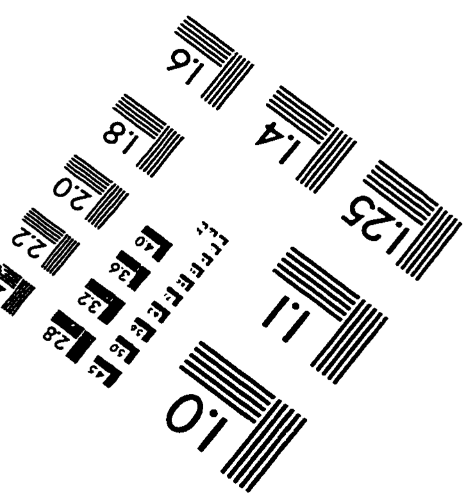

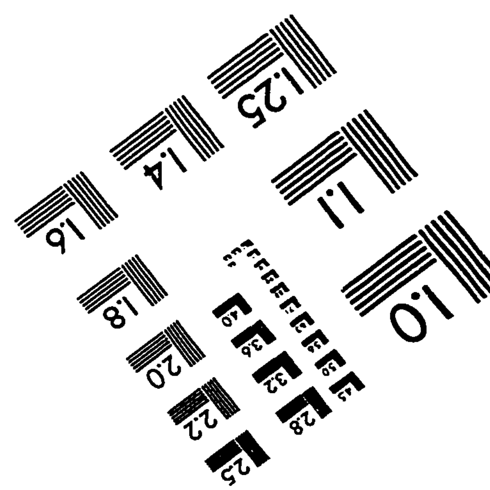

APPLIED IMAGE . Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989