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Frequent *RASSF1A* Gene Promoter Hypermethylation in Breast Cancer

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Content

Abbreviations

AJCC	American Joint Committee on Cancer
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine Triphosphate
bp	Base pair
BRCA1	Breast Cancer Gene 1
BRCA2	Breast Cancer Gene 2
BSP	Bisulfite-Sequencing PCR
B2M	β2 Microglobulin
C	Cytosine
cDNA	Complementary Deoxyribonucleic Acid
COBRA	Combined Bisulfite Restriction Analysis
CpG	Cytosin-phospho-Guanine
dATPαS	Deoxyadenosine α-thiotriphosphate
DCIS	Ductal Carcinoma *in situ*
dCTP	Deoxycytidine Triphosphate
ddH_2O	Double distilled water
dGTP	Deoxyguanosine Triphosphate
DNA	Deoxyribonucleic Acid
DNMT	DNA methyltransferase
dNTP	Deoxy-ribonucleoside Triphosphate
dTTP	Deoxythymidine Triphosphate
EDTA	Ethylene Diaminetetraacetic Acid
EGF	Epidermal Growth Factor
ER	Estrogen Receptor
EtOH	Ethyl alcohol
FBS	Fetal bovine serum
g	Gram
G	Guanosine
HDAC	Histone Deacetylase
HER-2/neu	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
HPLC	High-performance Liquid Chromatography
HR	Hormone Receptor

IBC	Invasive Breast Carcinoma
IDC	Invasive Ductal Carcinoma
ILC	Invasive Lobular Carcinoma
kD	Kilodalton
LCIS	Lobular Carcinoma *in situ*
NCI	National Cancer Institute
ml	Milliliter
mM	Milli Molar concentration
mRNA	Messenger Ribonucleic Acid
MSO	Methylation-specific Oligonucleotide Microarray
MSP	Methylation-specific PCR
Ms-SnuPE	Methylation-sensitive Single Nucleotide Primer Extension
MTA	Methylation Target Array
NaOH	Sodium Hydroxide
ng	Nanogram
OE	Observed CpG / Expected CpG
PCR	Polymerase Chain Reaction
PMA	Pyrosequencing Methylation Assay
pmol	Picomole
PPi	Pyrophosphate
PR	Progestogen Receptor
QM-MSP	Quantitative Multiplex-methylation-specific PCR
RA domain	Ras association domain
RASSF1	Ras association (RalGDS/AF-6) domain family 1
RASSF1A	Ras association (RalGDS/AF-6) domain family 1 isoform A
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SARAH	Sav/RASSF/Hpo
T	Thymidine
TE	Tris-EDTA
TNM	Classification Tumor, Nodes, Metastasis-classification
Tris	Tris (hydroxymethyl) aminomethane
TSA	Trichostatin A
TSGs	Tumor Suppressor Genes
U	International Unit

µg	Microgram
µl	Microliter
µM	Micro molar
5-Aza-dC	5-aza-2-deoxycytidine
5mC	5-methylcytosines

1 Introduction

1.1 Breast cancer

1.1.1 Overview of breast cancer

Breast cancer is by far the most common form of cancer diagnosed in European women today, accounting for 429 900 incident cases (28.9% of total cancer) in 2006 in Europe, with the continuous increase of early diagnosed cases. Overall, breast cancer (131 900, 7.8% of total cancer death) was the third major cause of cancer death in 2006 in Europe (Ferlay et al. 2007). Because of the ageing of the European population the number of deaths from breast cancer is still rising from 130 000 in 2004 to 132 000 in 2006 in Europe (Ferlay et al. 2007).

1.1.2 Clinical classification

Table 1: The TNM classification of breast cancer (the American Joint Committee on Cancer in collaboration with the National Cancer Institute in 2002)

T-	***Refers to tumor size***
pTX:	Tumor cannot be assessed
pT0:	No evidence of primary tumor
pTis:	Carcinoma in situ, or Paget's disease of the nipple, without a detectable tumor mass
pT1:	Tumor two centimeters or less（≦ 2 cm) in greatest dimension
pT2:	Tumor more than two centimeters (> 2 cm), but less than five centimeters (≦ 5 cm), in greatest dimension
pT3:	Tumor more than five centimeters (> 5 cm) in greatest dimension
pT4:	Tumor of any size, with direct spread to chest wall or skin (includes inflammatory carcinoma and ulceration of the breast skin)
N-	***Refers to lymph node involvement***
pN0:	No regional lymph node metastasis
pN1	Metastasis to movable ipsilateral axillary lymph node(s)
pN2	Metastasis to ipsilateral axillary lymph nodes fixed to one another or to other structures
pN3	Metastasis to ipsilateral internal mammary node(s)
M-	***refers to the extent of metastasis***
MX:	The presence of distant metastasis cannot be assessed
M0:	No distant metastasis
M1:	Distant metastasis (includes metastasis to ipsilateral supraclavicular lymph node[s])

TNM classification has been traditionally used in the clinical practice to define the disease progress (Table 1).

According to the original cells and the cell growth status, it can be divided to: 1) Non-invasive carcinomas, include ductal carcinoma in situ (DCIS), lobular carcinoma in situ (LCIS), and Paget's disease; 2) Invasive carcinomas, like invasive (infiltrating) ductal carcinoma (IDC, about 75% of all invasive carcinoma), invasive lobular carcinoma (ILC, approximately 5% to 10% of all invasive breast cancer), and other three well recognized types, namely tubular cancers, medullary cancers, and mucinous cancers; 3) Inflammatory carcinoma is a very serious, rapidly spreading type of tumor that accounts for about 1% of all breast cancers; 4) A very small number of breast cancers may arise from the muscle, fat, or connective tissues of the breast. The rare types of sarcoma that occasionally are diagnosed within the breast include angiosarcoma and cystosarcoma phylloides; 5) Breast cancer in special situation, such as breast cancer in men or in pregnancy (http://www.bccancer.bc.ca).

The grading of invasive carcinoma is also important as a prognostic indicator. For the Nottingham-Bloom-Richardson system, according to the percentage of tubule formation, the degree of nuclear pleomorphism, and an accurate mitotic count using a defined field area, three grades of breast cancer histology were defined: grade 1 (the total score is 3-5, well-differentiated), grade 2 (the total score is 6 or 7, moderately-differentiated) and grade 3 (the total score is 8 or 9, poorly-differentiated) (Elston and Ellis 1991).

Steroid hormones, particularly estrogen, have long been linked to mammary carcinogenesis (Fishman et al. 1995). Estrogen receptor (ER) and progestogen receptor (PR) are two members of the steroid hormone superfamily studied in breast cancer. The presence of ER in breast tumors is a predictive marker for response to hormone therapy. However, up to one-third of breast cancers lack ER at the time of diagnosis and a proportion of cancers that are initially ER-positive lose ER during tumor progression (Hortobagyi 1998).

Three disease responsiveness categories were defined according to the steroid hormone receptor (HR): endocrine responsive (the cells express HR), endocrine non-responsive (cells have no detectable expression of HR), and endocrine

response uncertain. Features indicative of the last category include low levels of HR immunoreactivity (usually considered as < 10% of cells positive) and lack of PR (irrespective of the expression of ER) (Goldhirsch et al. 2005).

1.2 Genetic and expression aberrant of genes in breast cancer

Human cancer development is a dynamic, complex, progressive and multistep process, involving many genes and gene products that systematically, quantitatively and accumulatively affect a biological network of cellular signaling and functional pathways (Hanahan and Weinberg 2000; Michor et al. 2004). These genetic events lead to gene activation/inactivation through the mechanisms of mutation, amplification and deletion.

It is estimated that 5%–10% of all breast cancers in women are associated with hereditary susceptibility due to mutations in autosomal dominant genes, such as Breast Cancer Gene (*BRCA1* and *BRCA2*), *p53*, phosphatase and tensin homolog (*pTEN*), and serine/threonine kinase 11 (*STK11/LKB1*) (Collaborative Group on Hormonal Factors in Breast Cancer 2001; Claus et al. 1996). Another 15%–20% of female breast cancers occur in women with a family history but without an apparent autosomal dominant inheritance pattern, and are probably due to other genetic factors with environmental influence (Slattery and Kerber 1993). As a heterogeneous disease, many genetic alterations have been detected in sporadic breast cancers. For example, gene amplification at 17q23 (Sinclair et al. 2003) and 20q11-13 (Guan et al. 1996) and chromosome loss at 8p22 (Yaremko et al. 1996), 11q23 (Laake et al. 1999), and 16q22 (Iida et al. 1997) are frequently detected in breast cancer by fluorescent in situ hybridization, comparative genomic hybridization, and loss of heterozygosity studies. Alteration or changes in expression levels have been described for several other genes, including *p53*, E-cadherin, and *HER-2/neu* (Ingvarsson 1999). It is very likely that additional genes, especially some tumor suppressor genes (TSGs) contribute to the sporadic breast carcinogenesis. Determining a "genetic signature" for a tumor may prove to be a very powerful predictor of the aggressive nature of a breast cancer. Now there are nearly 70 genes whose activity patterns may help make such predictions (http://health.nytimes.com/health/guides/disease/breast-cancer/print.html).

1.3 Epigenetic alteration in tumorigenesis

Mammalian embryonic development is controlled by genetic, epigenetic and environmental mechanisms. During acquired of a progressive appearance of malignant cell behavior, many of the gene changes stem from genetic abnormalities that disrupt coding regions. However, it is becoming clear that epigenetic events, around a gene that lead to inherited alteration of gene expression without affecting the nucleotide sequence of the gene, play a fundamental role in tumor formation and progression. Two epigenetic modifications are commonly used by cells participate in this transcriptional rheostat: DNA methylation and histone modifications (Bird 2002; Yoo and Jones 2006).

1.3.1 DNA methylation

DNA methylation is one well known epigenetic mechanism and is one of the many potential causes for the abnormal growth of cancer cells. The methyl group is located in the fifth position of the ring in some cytosines within cytosine-phospho-guanine dinucleotides (CpG) in the genome of vertebrates (Vanyushin et al. 1970). The pattern of 5-methylcytosine distribution in the genome is unique for each cell type and is established and maintained by several DNA methyltransferases (DNMTs) subsequent to DNA replication in embryogenesis (Bestor et al. 1988).

Cytosine is methylated in the context of CpG dinucleotides, and most CpGs are methylated except for those on CpG islands. Most CpG dinucleotides are unevenly distributed throughout the genome and remain in short stretches or clusters (500-2000 bp), called CpG islands (Laird et al. 1996; Feltus et al. 2006; Vertino et al. 1996). These islands are located in the promoter region, generally kept unmethylated and found in half of all human genes (Baylin 2005). It has been increasingly recognized over the past 4-5 years that the CpG islands of a large number of genes, which are mostly unmethylated in normal tissues, are methylated to varying degrees in human cancers (Yang et al. 2001). In mammals, DNA methylation occurs after replication, when DNA methyltransferase can transfer a methyl group (CH3) from S-adenosyl-methionine to the 5’ position of cytosine residues in CpG dinucleotides sequence (Figure 1) (Bird 2002; Dunn 2003; Jones and Takai 2001). With the methylation of CpG islands, endonucleases degrade

foreign DNA, regulate gene expression by silencing genes normally (Farrell 2005). DNA methylation is vital during development, and aberrant DNA methylation, both hypermethylation and hypomethylation, has been associated with aging, cancer and other diseases (Jones and Baylin 2002; Issa 2000; Richardson 2003). In addition, DNA methylation inhibitors can be used to treat cancer (Santini et al. 2001). Therefore, multiple methods to study DNA methylation are important tools in biological research.

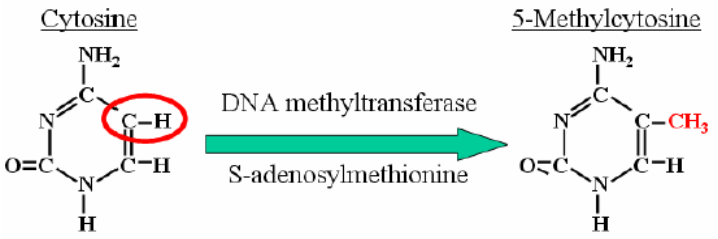

Figure 1: The pattern of DNA methylation in CpG island by DNA methyltransferase

1.3.2 Histone modifications

Histone modification, including acetylation and methylation, is another important epigenetic event in gene regulation. Histones are small, basic and lysine-rich proteins, which constitute half of the eukaryotic chromosome. Two superhelical turns of DNA are wrapped around an octamer of histone proteins: One H3-H4 tetramer and two H2A-H2B dimmers (Luger et al. 1997). These DNA-protein complexes represent the nucleosomes, which are the basic repetitive units of chromatin. Histone tails can be modified after translation by methylation, phosphorylation, acetylation, ubiquitination and ADP-ribosylation at lysine, serine and arginine residues (Wolffe 1998). The dynamics of acetylation-deacetylation of the N termini of histone is balanced by histone acetyltransferase and histone deacetylase (HDAC). The interaction between histone tails and other epigenetic marks such as DNA methylation leads to a specific chromatin conformation. Accessible euchromatin allows, and inaccessible heterochromatin inhibits gene expression (Strahl and Allis 2000).

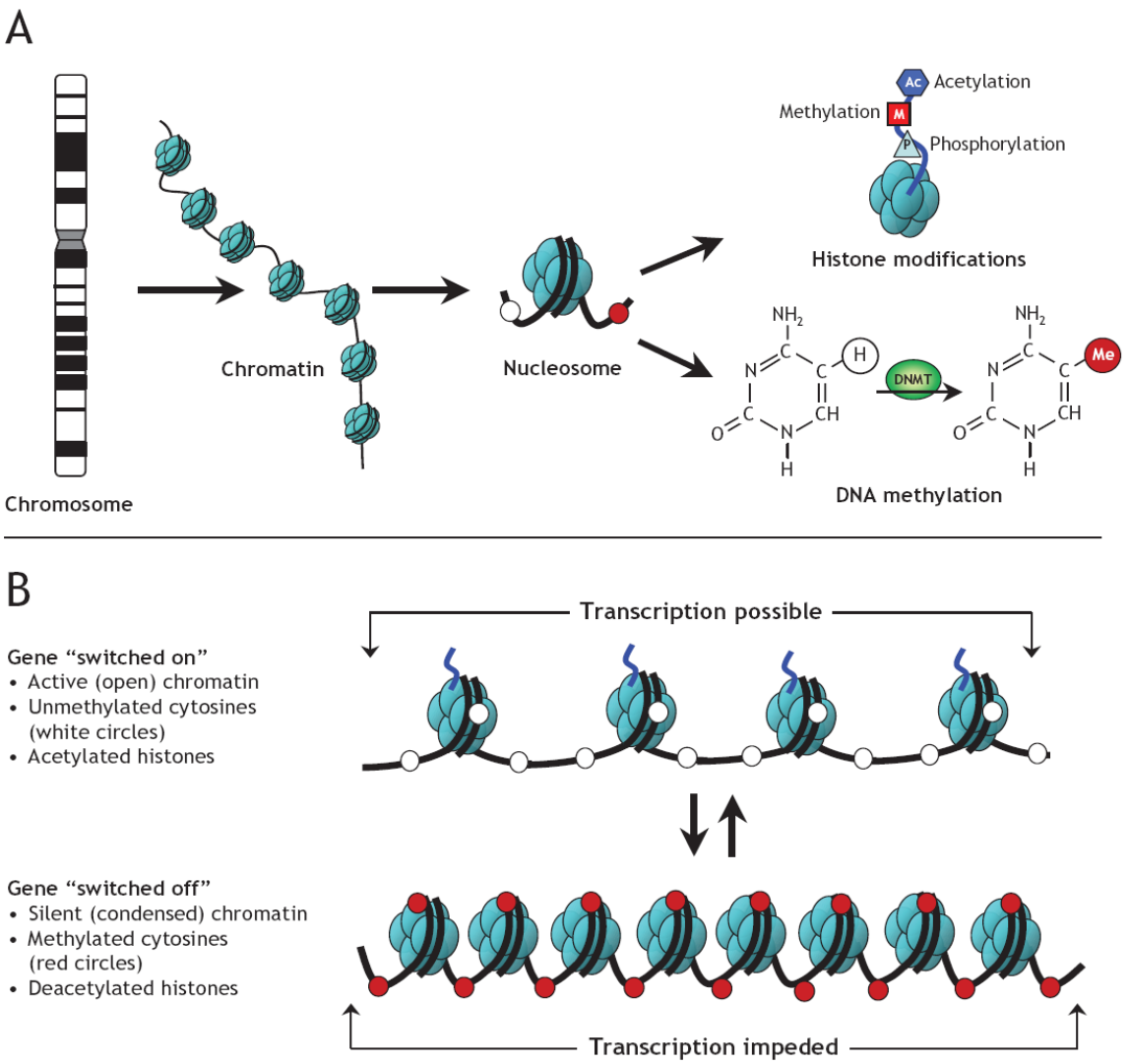

Figure 2 (Rodenhiser and Mann 2006)**: Epigenetic alteration and the influence on gene expression. A) Schematic of epigenetic modifications.** Strands of DNA are wrapped around histone octamers, forming nucleosomes. These nucleosomes are organized into chromatin, the building block of a chromosome. Reversible and site-specific histone modifications occur at multiple sites through acetylation, methylation and phosphorylation. DNA methylation occurs at 5-position of cytosine residues in a reaction catalyzed by DNMTs. Together, these modifications provide a unique epigenetic signature that regulates chromatin organization and gene expression. **B) Schematic of the reversible changes in chromatin organization that influence gene expression.** Genes are expressed (switched on) when the chromatin is open (active), and they are inactivated (switched off) when the chromatin is condensed (silent). White circles, unmethylated cytosines; red circles, methylated cytosines.

There is an intricate interplay between DNA methylation and histone modification in gene regulation during chromatin remodeling (Figure 2) (Hashimshony et al. 2003). Epigenetic information is heritable, but plasticity and epigenetic changes are

potentially reversible. Many kinds of DNMT inhibitors, like 5-azacytidine or 5-aza-2-deoxycytidine (5-Aza-dC), and HDAC inhibitors, like phenylbutyrate, trichostatin A (TSA), suberoylanalide hydrocamic acid (SAHA) and trapoxin A, have been investigated as anticancer agents, since they block the activity of DNMT or HDAC, and thus active tumor suppressor genes (Yang et al. 2001; Szyf et al.2004).

1.3.3 Hypo- and hyper-methylation

Alterations of gene expression originated by DNA methylation are usually categorized as due to hypo-methylation or hypermethylation (Figure 3). Hypomethylation and hypermethylation are terms that denote alteration of methylation status of a genome fragment in a tumor cell in comparison to a corresponding normal cell. Total DNA hypomethylation may promote carcinogenesis through activation of proto-oncogenes, genes whose expression increases metastasis and invasive potential of cells, by favoring genome instability and also through inhibition of expression of tumor suppressor genes by destruction of their imprinting (Kisseljova and Kisseljov 2005). Hypermethylation of promoter CpG islands in tumor suppressor genes or tumor-related genes is a common finding in human cancers, regardless of tissue type. The genes that are more susceptible to hypermethylation within their CpG islands are likely to be those that are involved in the regulation of cell growth, and so the cells that lack them could have a growth advantage (Costello et al. 2000). Different types of cancers are associated with methylation of tumor suppressor genes and proto-oncogenes, causing alterations in functional gene expression.

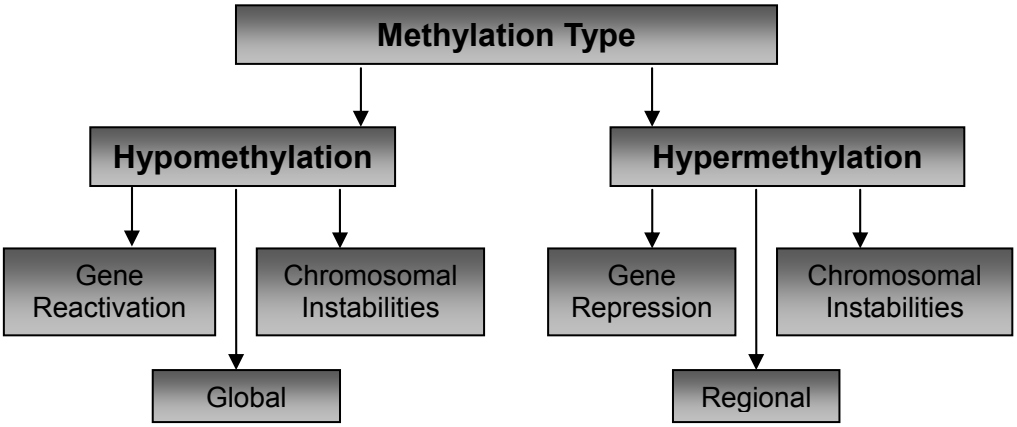

Figure 3: Two types of aberrant methylation, which elucidate certain actions

1.3.4 DNA methylation in breast cancer

In addition to chromosomal instability and gene mutation, epigenetic alteration by abnormal promoter methylation seems to be another approach to inactivate some tumor suppressor genes in breast cancer (Mielnicki et al. 2001). There are a list of well-characterized tumor suppressors, such as *BRCA1*, $p16^{INK4\alpha}$, *14-3-3δ*, *E-cadherin*, *ER* and *PR*, are targets for epigenetic silencing of breast cancer. Widschwendter and Jones summarized most of the genes with abnormal methylation status involved in breast cancer formation and progression (Table 2, 2002). All these genes fall into several broad categories including cell cycle regulating, apoptosis, steroid receptor, tumor susceptibility, carcinogen detoxification, cell adhesion and inhibitors of matrix metalloproteinases (MMPs) genes. There are also some studies demonstrated that global hypomethylation is implicated in breast carcinogenesis, like myelocytomatosis oncogene (*c-myc*), spermidine/spermine N1-acetyl transferase 2 (Sat2), and melanoma antigen (*MAGE*) (Narayan et al. 1998; Ross et al. 2003; Wischnewski et al. 2006). Both regional hypermethylation and global hypomethylation are involved in different stages of breast cancer. Global hypomethylation could be a mechanism for late stages while local hypermethylation is possible for early stages of breast cancer (Szyf 2000; Esteller and Herman 2002). Recognition of the important roles that DNA methylation play in gene expression in malignancy of breast cancer promoted to use methylation markers for detection and prognosis, and to utilize DNMT and HDAC inhibitors therapeutically to re-express silenced tumor suppressor and growth inhibitory genes.

Table 2: Genes for which direct or indirect evidence exists for involvement of methylation in breast carcinogenesis (Widschwendter and Jones 2002)

Gene	*Alternate gene name*	*Function*	*Methylation*
APAF1	Apoptosis Protease-Activating Factor 1	Activation of procaspase-9, results in initiation of a cascade involving the downstream executioners caspase-3, -6, and -7→apoptosis	indirect
APC	Adenomatous Polyposis of the Colon	Cell adhesion, signal transduction, stabilization of the cytoskeleton, regulation of cell cycle and apoptosis	direct
BCSG1	Breast Cancer Specific Gene 1; Synuclein-γ	Increases motility and invasiveness	direct
BRCA1	Breast Cancer type 1	Involved in DNA repair, recombination, checkpoint control of the cell cycle and transcription. Interacts with p53, STAT-factors, SRBC, etc.	direct
Caspase-8	Apoptosis-related cysteine protease	apoptosis	indirect
CCND2	Cyclin D2	Cell cycle regulation	direct
DAPK	Death-associated protein kinase 1	Mediator of interferon-γ induced apoptosis	direct

Gene	*Alternate gene name*	*Function*	*Methylation*
E-Cad	E-Cadherin	Epithelial cell–cell adhesion, suppresses invasion and metastasis	direct
ER	Estrogen receptor α and β	Regulation of cell proliferation, predictor of endocrine therapy	direct
FHIT	Fragile histidine triad gene	Controls proliferation and apoptosis, tumor suppressor	direct
GPC3	Glypican 3	apoptosis	direct
GSTP1	Glutathione S-transferase P1	Carcinogen detoxification	direct
H-Cad	H-Cadherin	Cell-cell adhesion	direct
HIN1	High in normal 1	Putative cytokine, inhibits cell growth	direct
HOXA5	Homeo box A5	Upregulates p53, apoptosis	direct
hTERT	Telomere reverse transcriptase	synthesizes the telomere ends of linear chromosomes, implicated in human cell immortalization	direct
IF-regulated genes	Interferon regulated genes	Interleukin 6, ICAM 1, Superoxide dismutase and Elafin are regulated by Interferons and mediate tumorsuppressive functions; involved in senescence	indirect
mac25	Insulin-like growth factor binding-related protein 1	Cell cycle regulation, apoptosis, involved in senescence	indirect
Maspin	Protease inhibitor 5	Inhibitor of angiogenesis, reduces cells' ability to induce tumors and metastasize	direct
NES1	Kallikrein 10	Inhibition of anchorage-independent growth and tumor formation	direct
Nm23-H1	Metastasis inhibition factor NM23	Metastasis suppressor activity	direct
NOEY2	Ras homolog gene family member I	Suppresses clonogenic growth; regulation of cyclin D1 and p21	direct
p16	Cyclin-dependent kinase inhibitor 2A	Cell cycle regulation, involved in senescence	direct
p21	Cyclin-dependent kinase inhibitor 1A	Cell cycle regulation	indirect
p53	Transformation-related protein 53	Apoptosis, cell cycle regulation, inhibition of growth and invasion	direct
p73	P53 related protein p73	Inhibitor of angiogenesis, apoptosis	indirect
PR	Progesterone receptor	Growth regulation	direct
Prostasin	Protease serine 8	Suppresion of invasion	direct
RAR-β	Retinoic acid receptor β	Apoptosis, involved in senescence, inhibition of proliferation	direct
RASSF1A	Ras Association domain family protein 1	Reduces colony formation, suppresses anchorage-independent growth, and inhibits tumor formation, apoptosis	direct
RFC	Reduced folate carrier	Cellular uptake of methotrexate	direct
RIZ1	Retinoblastoma protein-binding zinc finger protein	Tumor suppressor	direct
SOCS1	Suppressor of cytokine signaling 1	Suppresses growth rate and anchorage-independent growth, induction of apoptosis, regulation of STAT activation	direct
SRBC	Serum deprivation response factor (sdr)-related gene product that binds to c-kinase	Interaction with BRCA1	direct
STAT1	Signal transducer and activator of transcription	Growth regulation, induction of apoptosis, involved in senescence	indirect
SYK	Spleen tyrosine kinase	Inhibits tumor growth and metastasis	direct
TGFβRII	Transforming growth factor β receptor II	Cell cycle regulation	direct
THBS1	Thrombospondin 1	Inhibition of angiogenesis and invasion	direct
TIMP3	Tissue inhibitor of metalloproteinase-3	Suppresses tumor growth, angiogenesis, invasion and metastasis	direct
TMS1	Target of methylation induced silencing 1	apoptosis	direct
TWIST	TWIST	Inhibits oncogene- and p53-dependent cell death	direct
ZAC	Pleomorphic adenoma gene-like	Induction of apoptosis and cell cycle regulation	direct
14-3-3σ	Stratifin	Cell cycle regulation	direct

1.4 Ras association domain family 1 A (*RASSF1A*) gene

1.4.1 Ras association domain family 1 (*RASSF1*)

Cytogenetic and allelotyping studies of fresh tumors and tumor cell lines have shown that allele loss from several distinct regions on chromosome 3p, including 3p25, 3p21-22, 3p14 and 3p12-13, are the most frequent and earliest genomic

abnormalities involved in a wide spectrum of breast cancer and 30-87% genomic abnormalities of chromosome 3p and 3p21 region in breast cancer (Miller et al. 2003; Maitra et al. 2001; Yang et al. 2002). A region of minimum homozygous deletion at 3p21.3 spans approximately 120 kb and eight genes are located in this region (Sekido et al. 1998; Lerman and Minna 2000). In 2000, Dammann et al have cloned in a yeast two-hybrid screen baited with xeroderma pigmentosum complementation group A (XPA) and characterized the Ras association domain family 1 (*RASSF1*) gene, which is one of the eight genes residing in the common deletion area at 3p21.3 (Lerman and Minna 2000; Dammann et al. 2000; Burbee et al. 2001).

The *RASSF1* gene locus spans approximately 11,000 bp and contains eight exons and two different promoters give rise to eight different transcripts, *RASSF1A-RASSF1H* (Figure 4) (Donninger et al. 2007). Isoforms A and C are ubiquitously expressed, whereas isoform B is mainly expressed in cells of the hemopoietic system. Isoform D and E are specifically expressed in cardiac and pancreatic cells, respectively. They are very similar to isoform A except for slight differences in the splice sites used in exons 2αβ (*RASSF1D*) and 3 (*RASSF1E*) providing each protein with four additional amino acids (Agathanggelou et al. 2005). Two major splice variants, *RASSF1A* and *RASSF1C*, are transcribed by different independent CpG island-containing promoter usage and alternative splicing. There are approximately 2 kb apart of the start sites for them. RASSF1A is encoded by exons 1α, 2αβ and 3-6 and contains an open reading frame (ORF) of 340 amino acids with a predicated 39 kD peptide. *RASSF1C* initiates in exon 2γ and encodes a 270 amino acid protein with a calculated molecular mass of 31.2 kD (Dammann et al. 2000).

There are two CpG islands associated with the *RASSF1* promoters. A smaller, 737 bp island contains 85 CpGs (71.5% GC and OE: 0.89) and spans the promoter region of *RASSF1A*, *RASSF1D*, *RASSF1E*, *RASSF1F* and *RASSF1G*. A larger 1365 bp island, containing 139 CpGs (67.9% GC and OE: 0.88), spans the promoter region for *RASSF1B* and *RASSF1C* (Agathanggelou et al. 2005).

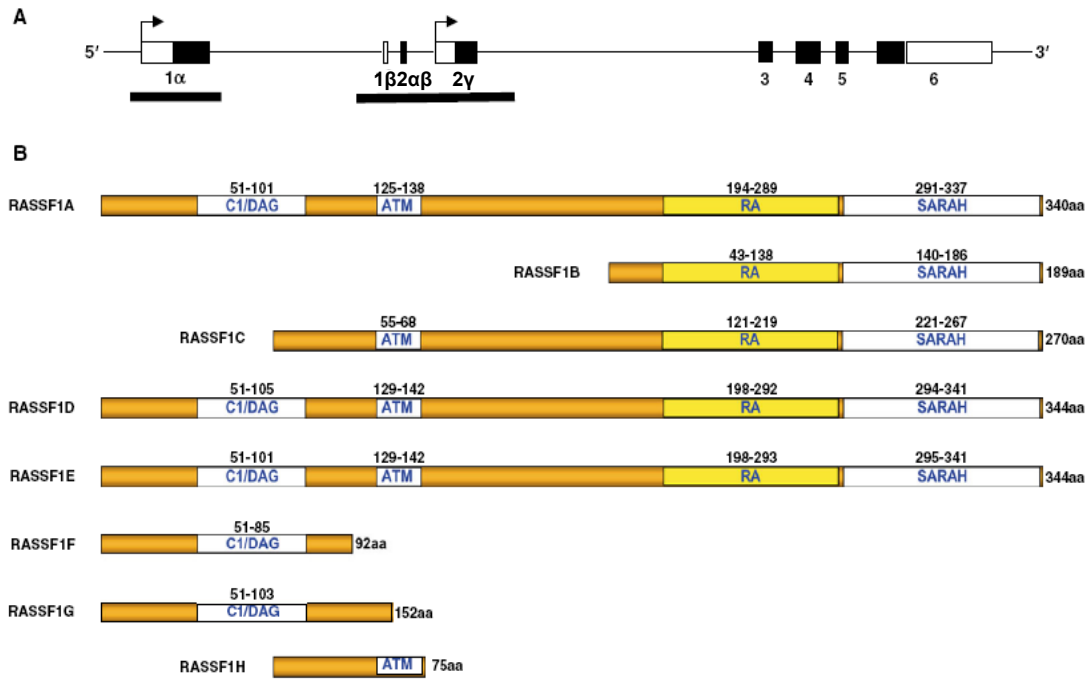

Figure 4: Ras association domain family 1 isoform A (*RASSF1A*) gene locus and domain structure of the different *RASSF1* isoforms (Donninger et al. 2007). **A)** The *RASSF1* gene locus is characterized by eight exons (boxed regions) and two different promoters (arrows) with two associated CpG islands (black bars). Black boxes represent coding regions and white boxes are non-coding regions. **B)** Schematic representation of the different RASSF1 isoforms. C1/DAG, conserved region 1 diacylglycerol-binding domain; ATM, ATM-kinase consensus phosphorylation sequence; RA, RalGDS/AF6 Ras association domain; SARAH, Sav/RASSF/Hpo interaction domain. The position of each domain (as outlined in the Swiss-Prot/TrEMBL database) is indicated above each isoform and the number of amino acids in each isoform is shown on the right.

A Ras association (RA) or RalGDS/AF-6 domain decoded by exons 4 and 5 defines the *RASSF1* gene and is located at the COOH terminus of isoforms *A-E*. This domain mediates interactions with Ras and other small GTPaese. *RASSF1A* and *RASSF1D-G* isoforms have a NH_2-terminal protein kinase C (PKC) conserved region 1 (C1) domain encoded by exons 1α and 1β, which contains a zinc finger motif (Newton 1995). A SARAH domain (Sav/RASSF/Hpo) is presented at the COOH terminus of *RASSF1A-E* and mediates heterotypic interaction between proteins as shown for Hpo/Sav and homotypic interactions as shown with Mst1. The *RASSF1* ataxia telangiectasia mutated (ATM) site, a substrate for ATM kinase, which is identified to be presented in isoforms A, C, D and E within exon 3 *in vitro* studies.

RASSF1 is a member of a Ras effectors family of six related proteins, which have been identified by the homology searches within the human genome, containing genes called *RASSF2* (20pter-p12.1), *RASSF3* (12q14.1), *Ad037* (10q11.21), *Nore1* (1q32.1) and *RASSF6* (4q21.21). Like *RASSF1*, each of these genes exhibits multiple splice variants and CpG islands are associated with their promoter regions. Each protein contains the RA and SARAH domain at the C-terminal, except for some minor splice variants. In which, *Nore1* has the most sequence identity with *RASSF1* (49%). Currently two further RA-domain-containing proteins have been identified and are now being described as RASSF7 (van der Weyden and Adams 2007) and RASSF8 (Falvella et al. 2006). The last two members contain an N-terminal RA domain and a variable C-terminus without SARAH motifs. Members of the RASSF family with the exception of RASSF3 have now been implicated as human tumor suppressors by regulating the cell cycle and apoptosis (Donninger et al. 2007).

1.4.2 RASSF1A functions

The tumor suppressor function of RASSF1A has been suggested by observations that exogenous expression of RASSF1A decreases *in vitro*-colony formation, suppresses anchorage-independent growth, and dramatically inhibited tumor formation in nude mice (Dammann et al. 2000; Burbee et al. 2001; Kuzmin et al. 2002). Additionally, two groups studied the tumor susceptibility of *RASSF1A* knockout mice independently (Tommasi et al. 2005; van der Weyden et al. 2005). The animals exhibit an enhanced tendency to develop spontaneous tumors in both cases.

Studies are in progress to investigate the biochemical function of RASSF1A and suggest it as a scaffolding protein that can assemble and modulate multiple effector protein complexes although we are far from understanding its true roles. RASSF1A is apparently involved in two pathways commonly deregulated in cancer, apoptosis and cell cycle (Shivakumar et al. 2002; Vos et al. 2000). RASSF1A can modulate apoptosis via its SARAH motif (Hwang et al. 2007) and regulate Bax activity, which is an important component of the apoptotic machinery (Vos et al. 2006). The role of RASSF1A in the cell cycle is the modulation of cyclin D1 levels at the G1-S checkpoint and inhibition of the JNK pathway as its mechanism (Whang et al. 2005).

RASSF1A localizeds to microtubules, centrosome and mitotic spindle, and can promote the stabilization of microtubules, modulate tubulin dynamics and maintain genomic stability (Liu et al. 2003; Dallol et al. 2004; Song et al. 2005). Furthermore, Dallol and colleague found that RASSF1A can control cell motility and invasion (2005). As a member of TSGs, RASSF1A may involve in the later stages of tumorigenesis as well as the early stages.

1.4.3 Tumor-associated methylation of *RASSF1A*

Aberrant promoter hypermethylation and association of loss of function is now recognized as a major mechanism of certain TSG inactivation in human cancers (Herman and Baylin 2003; Jones and Baylin 2002). Since the discovery of *RASSF1A* inactivation by DNA methylation of its promoter CpG island at a high frequency in lung cancers (Dammann et al. 2000), it is probably the most frequently methylated gene described thus far in human cancers (Table 3 and references within). There are also some reports about the re-expression of *RASSF1A* by using the DNMTs inhibitor 5-Aza-dC and/or histone HDAC inhibitor TSA treatment (Mühlisch et al. 2006; Zhang et al. 2006). Through epigenetic mechanism, the inactivation of RASSF1A played a considerable role in carcinogenesis.

Table 3: Summary of Ras association domain family 1 isoform A (*RASSF1A*) promoter methylation in primary tumors

Tumor type	*Frequency*	*References*
Breast	49% (n=39, MSP)	Burbee et al. 2001
	62% (n=45, Bisulfite Sequencing)	Dammann et al. 2001
	42 (DCIS n=12, MSP)	Honorio et al. 2003
	65% (IDC n=17, MSP)	
	58% (n=93, MTA)	Chen et al. 2003
	64% (IBC n=45, MSP)	Pu et al. 2003
	62% (DCIS n=21, MSP)	
	95% (n=40, MSP)	Yeo et al. 2005
	81% (n=151, MSP)	Shinozaki et al. 2005
	75% (n=16, ligation-based assay)	Dahl and Guldberg 2007
	50% (n=24, MSP)	Park et al. 2007

Tumor type	*Frequency*	*References*
Lung: SCLS	79%	Dammann et al. 2001
	100%	Burbee et al. 2001
Lung: NSCLS	39%	Chen et al. 2006
	63%	Burbee et al. 2001
Ovarian	40%	Yoon et al. 2001
Testicular germ cell: seminoma	40%	Honorio et al. 2003
Testicular germ cell: nonseminoma	83%	Honorio et al. 2003
Prostate	71%	Liu et al. 2002
Colorectal	20%	Miranda et al. 2006
Gastric	44%	Oliveira et al. 2005
Renal	56%	Yoon et al. 2001
Uterine cervix (adenocarcinoma)	45%	Cohen et al. 2003
Hepatocellular	93%	Schagdarsurengin et al. 2003
	95%	Zhong et al. 2003
Bladder	35%	Neuhausen et al. 2006
Pancreatic	64%	Dammann et al. 2003
Neuroectodermal: medulloblastoma	91%	Inda and Castresana 2007
Neuroectodermal: supratentorial	83%	Inda and Castresana 2007
uveal melanoma	50%	Maat et al. 2007
nasopharyngeal	82%	Zhou et al. 2005
Thyroid	71%	Schagdarsurengin et al. 2006

1.5 The aim of the study

1) To evaluate the frequency of *RASSF1A* gene promoter methylation in 181 breast cancer patients.

2) To analyze the correlation between the *RASSF1A* methylation and clinicopathological features.

3) To evaluate *RASSF1A* gene promoter methylation status in “normal” as well as in tumor breast tissues among 49 pair-matched breast cancer patients.

4) A RASSF1A hypermethylated breast cancer cell line will be used to demonstrate if the RASSF1A mRNA expression could be recovered by demethylation/deacetylation drug 5-Aza-dC/TAS.

2 Material and Methods

2.1 Material

2.1.1 Chemicals and reagents

Chemicals and Reagents	*Company*
Agarose	Sigma, St. Louis, USA
E-Gel 96 2% agarose	Invitrogen, Carlsbad, CA, USA
E-Gel 48 2% agarose	Invitrogen, Carlsbad, CA, USA
Ready-Load 100 bp DNA Ladder	Invitrogen, Carlsbad, CA, USA
dNTPs Mix	Roche, USA
Primer	Biomers.net GmbH, Ulm, Germany
Ethidium bromid	Carl Roth GmbH, Karlsruhe, Germany
Boric acid	Merck, Hohenbrunn, Germany
TRIzol® Reagent	Invitrogen, Carlsbad, CA, USA
TE buffer	Invitrogen, Carlsbad, CA, USA
Aqua ad iniectabilia Braun	B.Braun Melsungen AG, Melsungen, Genmany
Natriumhydroxid (NaOH)	Sigma-Aldrich, Seelze, Germany
Sodium Citrate	Sigma Chemical Co., St. Louis, USA
Guanidine Hydrochloride	Sigma Chemical Co., St. Louis, USA
Ethanol Absolut	VWR, Darmstadt, Germany
2-Propanol	Sigma-Aldrich, Seelze, Germany
Chloroform	Mallinckrodt Baker B.V., VA Deventer, Holland
Dodecylsulfat Natriumsalz (SDS)	Merck, Hohenbrunn, Germany
Acetic Acid	AppliChem, Darmstadt, Germany
Dimethylsulfoxide (DMSO)	Merck, Hohenbrunn, Germany
CpGenome™ Universal Methylated DNA	Chemicon, Temecula, CA
CpGenome™ Universal Unmethylated DNA Set	Chemicon, Temecula, CA
5-Aza-2'-deoxycytidine	Sigama, St.Louis, MO
Trichostatin A, From Streptomyces sp.	Sigama, St.Louis, MO
Phosphat Buffered Saline (PBS) (1×)	PAA Laboratories GmbH, Pasching, Germany
10×PBS	Invitrogen, Carlsbad, CA
RPMI1640	PAA Laboratories GmbH, Pasching, Germany
Fetal Bovine Serum	Invitrogen, Carlsbad, CA
Penicillin/ Streptomycin	Initrogen Gibco, Grand Island, USA
Trypsin-EDTA (1×)	PAA Laboratories GmbH, Pasching, Germany

Trypan Blue Stain 0,4%	GibcoBRL, Life Technologies™, USA
Streptavidin Sepharose™ High Performance beads	GE Healthcare, Uppsala, Sweden
Binding Buffer 2×	Biotage AB, Sweden
Annealing Buffer 1×	Biotage AB, Sweden
EX12 TaqMan® Gene Expression assays	Applied Biosystems, CA, USA
β2 microglobulin TaqMan® Gene Expression assays	Applied Biosystems, CA, USA

2.1.2 Enzymes

Enzymes	***Company***
HotStar Taq DNA Polymerase	Qiagen, Valencia, CA
Proteinkinase K	Qiagen, Valencia, CA

2.1.3 Solutions and buffers

Solutions and buffers	
2% Agarose gel	-3.0 g agrose
	-150 ml 1 X TBE buffer
3 M NaOH	-1.0 g NaOH
	-add 8.3 ml of ddH_2O
4 M acetic acid	-45.8 ml acetic acid
	-add ddH_2O to final volume of 200 ml
20 mM NaOH / 90% Ethanol	-900 µl of 100% Ethanol
	-6.6 µl of 3 M NaOH
	-add 93.4 µl of ddH_2O
Denaturation Solution	-8.0 g NaOH
	-add ddH_2O to final volume of 1 l
Washing Buffer	-1.21 g Tris
	-add ddH_2O to final volume of 1 l and adjust pH with 4 M acetic acid to 7.6 at 22±1℃
10 X Orange G DNA Loading Buffer	-20.0 g 40% sucrose in 40 ml ddH_2O
	-100 mg orange G in above solution to final volume of 50 ml with ddH_2O

2.1.4 Kits

Kits	*Company*
QIAGEN Genomic DNA midi kit	Qiagen, Valencia, CA
CpGenome™ DNA Modification Kit	Chemicon, Temecula, CA
Pyro Gold Reagents Kit	Biotage AB, Sweden
High-Capacity cDNA Reverse Transcription Kits	Applied Biosystems, CA, USA
TaqMan® Gene Expression Master Mix	Applied Biosystems, CA, USA
BCA™ Protein Assay Kit	Pierce, Rockford, USA

2.1.5 Equipments and softwares

Equipments and Softwares	*Company*
Analytical balance	Sartorius AG, Göttingen, Germany
RunOne™ Electrophoresis Cell	Embi Tec, San Diego, CA
E-base™ Electrophoresis	Invitrogen, Carlsbad, CA
Power PAC 3000	Bio-Rad, Hercules, CA
Flow for cell culture, DLF/RECG KL 2A	Biohazard, Germany
MP230 GLP Research pH/mV/°C Meter	Mettler Toledo, Columbus, USA
High Performance Gel Documentation and Image Analysis System	Alpha Innotech Corporation, California, USA
Thermomixer compact	Eppendorf AG, Hamburg, Germany
Shaking Water Bath 1083	GFL, Burgwedel, Germany
Water Bath Incubator	Köttermann, Hänigsen, Germany
Temperature test chambers with forced convection	Binder GmbH, Tuttlingen, Germany
Nanodrop ND-1000 spectrophotometer	NanoDrop Technologies, Wilmington, DE
Spectrophotometer DU® 640	Beckman, USA
Easia Shaker	Medgenix Diagnostics, Brussels, Belgium
SONOREX Ultrasonic cleaning devices	Bandelin, Berlin, Germany
Microscoper IX50-S8F	Olympus, Japan
GeneAmp PCR system 9600	Perkin Elmer, Norwalk, USA

PTC-100™ Programmable Thermal Controller	MJ Research INC., Watertown, USA
Cellshaker Variospeed	Malta, Italy
Power PAC 3000	Bio-Rad, Hercules, CA
Transferpipette 8-Kanal	Eppendorf AG, Hamburg, Germany
Vaccumpumpe:Vacusafe	IBS integra biosciences
Votex Genie 2™	Bender & Hobein AG, Zurich, Switzerland
Transferpipette digital (2µl , 1-10µl, 2-20µl, 20-200µl, 100-1000µl)	Gilson, Middleton, USA
CO_2 incubator	Labor-Technik-Göttingen, Göttingen, Germany
Falcon™ Tubes (15ml, 50ml)	BD., Franklin Lakes, USA
Screw-Cap micro tube 1.5ml	Sarstedt, Nümbrecht, Germany
Microtest™ Tissue Culture Plate, 96-well	Becton Dickinson France S.A., Meylan Ledex, France
6-Well Multidish	Nunc Inc., Wiesbaden, Germany
PCR Plate 96, low volume	Eppendorf Scientific, New York, USA
PCR 8-Strip Tubes	Eppendorf AG, Hamburg, Germany
MicroAmp® Optical 96-Well Reaction Plate	Applied Biosystems, CA, USA
PSQ™96 Plate Low	Biotage AB, Sweden
PSQ 96 Reagent Cartridge	Biotage AB, Sweden
PSQ 96 Sample Prep Thermoplate	Biotage AB, Sweden
Vacuum Prep Worktable	Biotage AB, Sweden
Optical Adhesive Covers	Applied Biosystems, CA, USA
Tool Filter Probes	Biotage AB, Sweden
ABI Prism 7700 Sequence Detector	Applied Biosystems, CA, USA
Heraeus® Multifuge with EASYset (Soft Touch Keypad) Multifuge 1 S-R	Thermo, NY, USA
Heraeus® Fresco 17/21 Microcentrifuges	Thermo, NY, USA
Pyrosequencing™ Assay Design Software	Biotage AB, Sweden
Pyro Q-CpG Software	Biotage AB, Sweden
AlphaImager Software	Alpha Innotech Corporation, San Leandro, CA
Sequence Detector V1.7 Software	Applied Biosystems, CA, USA

2.2 Samples

2.2.1 Tissue samples

One hundred and eighty one primary breast tumor tissues and forty nine corresponding adjacent normal breast tissues were included in this study. All patients were diagnosed and operated in Women's hospital of the University of Ulm from 1993 to 2005 and all the diagnosis were confirmed by histo-pathologists. Samples were immediately frozen and stored in -80°C or liquid nitrogen. The clinical information and follow-up data were based on chart review and reports from tumor registry service. The patients ranged in age from 30 to 91 years (median, 58 years). Histological subtypes include duct, lobular, and mixed type. None of the patient had received chemotherapy or radiation treatment before surgery. The median follow-up time was 45.47 months. The clinical characteristics of the patients in this study are summarized in Table 4. Written informed consent of the patients was obtained by the regular process.

Table 4: Patient characteristics

Clinicopathologic factors	*Number of sample*	*Censored number of sample*
Age	181	0
Median, range	58 years, 30-91 years	
Histological type	170	11
Ductal	127	
Lobular	20	
Other	23	
Tumor size (T)	173	8
T1	36	
T2	76	
T3	24	
T4	37	
Lymph node metastases (N)	174	7
N0	58	
N1	79	
N2	30	
N3	7	

Clinicopathologic factors	*Number of sample*	*Censored number of sample*
Distant metastases (M)	177	4
No	163	
Yes	14	
Stage	172	9
I	16	
II	76	
III	68	
IV	12	
Tumor grade (G)	158	23
G1	3	
G2	90	
G3	65	
Estrogen-receptor status	170	11
Negative	74	
Positive	96	
Progesterone-receptor status	170	11
Negative	102	
Positive	68	
Menopause	143	38
No	28	
Yes	115	
Relapse	166	15
No	110	
Yes	56	
Death	181	0
No	157	
Yes	24	

2.2.2 Cell lines

The cell line MDA-MB-330 and Hela were obtained from American Type Culture Collection (Manassas, VA) and were cultured in PRMI1640 containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 units/ml streptomycin at 37°C, 90% humidity, 5% CO2 concentration..

MDA-MB-330 cell line was treated by 5-Aza-2'-deoxycytidine (5-Aza-dC), trichostatin A (TSA), or in combination of the two drugs. Cells (2×10^5 / well) were

seeded in 6-well culture plates overnight. Fresh 64 μM 5-Aza-dC was prepared in dimethylsulfoxide and then was diluted from 64 μM to 0.25 μM in multiproportion with ddH_2O. TSA was dissolved in dimethylsulfoxide of 10 mM as stock solution and diluted to 0.01 mM as working solution with medium. The protocols for demethylation treatment were listed below. The medium with fresh drugs was changed every day. Control wells were set up of cells with normal medium without drug but with equivalent volumes of vehicle (medium including dimethylsulfoxide) only. All cells were washed softly with phosphate-buffered saline after treatment. One-milliliter of TRIzol reagent was used to blow the cells and to make them dissolve completely. All samples in TRIzol were stored at -80°C and processed for total DNA, RNA, and protein extraction.

Demethylation treatment *in vitro*

Protocol 1. Medium with fresh 5-Aza-dC at different final concentration from 64 μM to 0.5 μM was changed every 24 h for 72 h, and then cells were cultured in normal medium for an additional 24 h. TSA was added to the medium at a final concentration of 0.1 μM from the second day for 48 h alone or combined with 5-Aza-dC.

Protocol 2. Medium with fresh 5-Aza-dC at different final concentration from 32 μM to 0.5 μM was changed every 24 h for 72 h, then cells were harvested directly. TSA was added to the medium 18 h before the cells were harvested at a final concentration of 0.1 μM, 0.2 μM, 0.4 μM, and 1.0 μM, respectively.

2.3 Genomic DNA extraction

The genomic DNA was extracted from frozen tissue samples and stored at -20°C using the QiaAmp DNA Maxi kit (Cat.No.13362) according to the manufacturer's instructions. DNA concentration was measured at the wave lengths of 260 nm and 280 nm by Nanodrop ND-1000 spectrophotometer.

2.4 Isolation of DNA, RNA, and Protein from TRIzol-Reagent

Total DNA, RNA and protein from cell lines were extracted by using TRIzol-Reagent (Cat.No.15596-026) according to the manufacture's instruction. The quality and quantity of DNA and RNA were determined by the

spectrophotometer. The concentration of protein was detected with BCA™ Protein Assay Kit by Sunrise Remote instrument.

2.5 Bisulfite treated DNA

Bisulfite-conversion-based methods are the most widely used in recent years because they permit the rapid identification of methylated cytosine (5mC) in any sequence context. The bisulfite reaction was first described in early 1970s (Hayatsu et al. 1970; Shapiro et al. 1970), and was used in 1992 to distinguish between cytosine and 5mC in DNA (Frommer et al. 1992). As shown in Figure 5, in the bisulfite reaction, all unmethylated cytosines are deaminated and sulfonated, converting them to uracils, while 5-methylcytosines remain unaltered. The process for conversion of cytosine to uracil using the CpGenome™ kit is as following. Bases are first exposed by denaturing the DNA to its single stranded form using mild heat at an alkaline pH. Reagent I, containing a sodium salt of bisulfite ion (HSO_3^-), causes unmethylated cytosine to be sulfonated and hydrolytically deaminated, yielding a uracil sulfonate intermediate. DNA is then bound to a micro-particulate carrier (Reagent III) in the presence of another salt (Reagent II), and is desalted by repeated centrifugation and resuspension in 70% EtOH. The conversion to uracil is completed by alkaline desulfonation and desalting is repeated in 90% EtOH. The DNA is finally eluted from the carrier by heating in TE Buffer.

1 µg of DNA was used to begin the modification procedure in a screwcap 1.5-2.0 ml microcentrifuge tube. All solutions were prepared freshly and all potential sources of contamination were avoided. Firstly, 7.0 µl 3 M NaOH was added to 1.0 µg DNA in 100 µl of water (10 ng/µl) and mix followed by incubation in a heat block at 50°C for 10 min. After that, 550 µl of freshly prepared DNA Modification Reagent I was added in the DNA samples and was vortexed. Then the tubes were protected from light and the bisulfite treatment was performed at 50°C for 14 h in a heat block. Secondly, DNA Modification III was resuspended by vortexing vigorously and the suspension was drawed into and out of a 1 ml plastic pipette tip to disperse any remaining clumps. 5 µl of well-suspended DNA Modification Reagent III and 750 µl of DNA Modification Reagent II were added to the DNA solutions in turn and incubated at room temperature for 10 min after mixed briefly. The tube of DNA sample was swiveled for 10 sec at 5,000 g to pellet the DNA Reagent III and the

supernatant was discarded. The small white pellet was washed with 1.0 ml of 70% ethanol by centrifuge for 10 sec at 5,000 g three times. After the supernatant from the last wash has been removed, the tube was centrifuged at 13,000 g for 2 min and the remaining supernatant was removed with a plastic pipette tip. Thirdly, 50 µl of the 20 mM NaOH / 90% ethanol solution was added to the appropriate samples and vortexed briefly to resuspend the pellet. The tubes were incubated at room temperature for 5 min, then spinned for 10 sec at 5,000 g to move all contents to tip of the tube. The pellet was washed with 1.0 ml of 90% ethanol by centrifuge at 5,000 g for 10 sec twice and the supernatant was discarded. The sample was centrifuged at 13,000 g for 3 min and was allowed to dry for 10 to 20 min at room temperature after all of the remaining supernatant was removed with a plastic pipette tip. Finally, 50 µl TE buffer was added to dissolve the pellet followed by vortex and incubation at 50°C for 15 min to elute the DNA. After that, the sample was centrifuged at 13,000 g for 3 min and the supernatant was transferred to a new tube. The samples were processed as fresh templates or were stored at -20°C or -80°C.

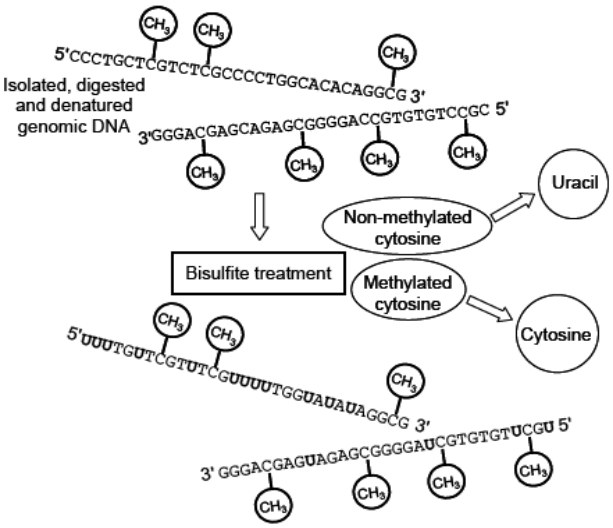

Figure 5: Conversion of cytosine molecules by bisulfite. Non-methylated cytosines within single stranded DNA are converted into uracil whereas methylated cytosines remain unchanged during bisulfite treatment.

2.6 Pyrosequencing methylation assay (PMA)

Pyrosequencing technology offers an established genetic analysis method based

on the principle of sequencing by synthesis. It was firstly applied in CpG methylation analysis in 2002 (Uhlmann et al. 2002) and was developed by Tost et al. (2003) to analyze multiple CpG sites.

2.6.1 Primer design for pyrosequencing of *RASSF1A* promoter

DNA sequences used for primer design were the previously identified *RASSF1A* promoter region (Dammann et al. 2000; Yan et al. 2003), which was about 336 bp upstream and 33 bp downstream to the start of *RASSF1A* transcription and available in the GenBank database (1000 bp upstream from NC_000003). The primers for polymerase chain reaction (PCR) amplification and sequencing were designed by Pyrosequencing™ Assay Design Software and were assumed that all cytosine molecules except CG dinucleotides were converted and appeared as thymine prior to the primer design. One primer of PCR amplification was biotin labeled. Table 5 showed details of these three pairs of PCR primers and their sequencing primers respectively.

Table 5: Details of primer pairs used for Pyrosequencing of Ras association domain family 1 isoform A (*RASSF1A*) gene

Gene	*Primer sequences*[a]	*Fragment size*	*CpG Sites*	*Primer position*[b]
RASSF1A-1	**F**: CGGCAGTATAGTAAAGTTGGTTTT	124 bp	10	-336 to -313
	R: Biot-GAAGGAGGGAAGGAAGGGCAA			-233 to -213
	S: TAAAGCTGGCCTCCAGAAAC			-325 to -306
RASSF1A-2	**F**: GAAGGAGGGAAGGAAGGGCAA	147 bp	10	-233 to -213
	R: Biot-CTGGATCCTGGGGGAGG			-103 to -87
	S: GGGAAGGAAGGGCAA			-227 to -213
RASSF1A-3	**F**: CTGGATCCTGGGGGAGG	137 bp	12	-103 to -87
	R:Biot-GAGCCTGAGCTCATTGAGCTGYGG			10 to 33
	S: GGATCCTGGGGGAGG			-101 to -87

a: Sequences for forward primer (**F**), reverse primer (**R**) and sequencing primer (**S**). They were all written from 5' to 3' end. **b:** Primer pairs were designed from the GenBank database (1000 bp upstream from NC_000003). The positions of the synthetic oligonucleotides are indicated relative to their distances from the first nucleotide position of the start codon ATG for all primers in the promoter region.

2.6.2 PCR amplification and template preparation

Amplification was carried out in a final volume of 25 µl, each containing 2.0 µl (20 ng/µl) of bisulfite-converted genomic DNA, 2.5 µl 10 × reaction buffer with 1.5 mM $MgCl_2$, 0.5 µl of each 10 x dNTPs (10 mM), 0.5 µl of each forward and reverse primer (10µM), 0.625 U HotStar Taq DNA Polymerase. The PCR reaction was performed in a GeneAmp PCR system 9600 or PTC-100™ Programmable Thermal Controller under the program conditions of a denaturing step of 15min at 95℃ followed by 45 cycles of 30s at 94°C, 30s at the annealing temperature and 30s at 72°C, with a final extension of 15min at 72°C. Water blank was used as a negative control for each PCR reaction plate. After amplification, 5 µl of the PCR products were separated by 2% agarose E-Gel. PCR products with a clear and strong band, without primer-dimers or other non-specific products were prepared to perform Pyrosequencing.

Single-stranded DNA of PCR fragments to which a sequencing primer can be annealed is needed to perform DNA analysis using Pyrosequencing™ technology. The simple and robust sample preparation process was described by Ronaghi et al. (1996), to produce high quality DNA from crude PCR reactions without prior purification. For Pyrosequencing, PyroMark Vacuum Prep Workstation (VPW) was used for preparation of single-stranded DNA prior to sequence primer annealing following the PSQ™ 96 sample preparation guide (Figure 6).

Firstly, the workstation troughs were filled with the appropriate solutions at room temperature. 20 µl denature high-performance liquid chromatography (HPLC) greed water was added to each biotinylated PCR product in a 96-well plate in total volume of 40 µl, then each well was added in 3 µl Streptavidin Sepharose™ High Performance beads in 37 µl 2 × binding buffer and mixed agitatedly for five minutes. Vacuum was applied and then the beads with immobilized PCR product were picked up by the Vacuum Prep Tool from the PCR -plate and moved to a separate trough, where 70% ethanol was aspirated through the filter probes. The Vacuum Prep Tool was then placed in a trough of denaturation Solution (sodium hydroxide), still with the vacuum pressure switched on, to denature the DNA and to filter out the released single-stranded DNA, while the 5'-biotinylated strand remained immobilized on the beads. Next, the tool was placed in a trough of washing buffer

where the strands were rinsed by aspiration. The single-stranded templates were then transferred to a previously prepared PSQ plate containing 38.4 µl 1 × annealing buffer and 1.6 µl sequencing primer (10 µM) of each well (39.6 µl 1 × annealing buffer and 0.4 µl sequencing primer for fragment II). With the vacuum pressure now switched off, a gentle shake of the Vacuum Prep Tool released the beads with attached templates into the 96- well PSQ plate. After incubation at 80°C for 2 min (for fragment II, additional incubation at 44°C for 2 min) using the PSQ 96 Sample Prep Thermoplate Low, the PSQ plate was moved to room temperature. Now, the sequencing primer was annealed and the plate was ready for analysis on the automated PSQ 96MA Pyrosequencing System.

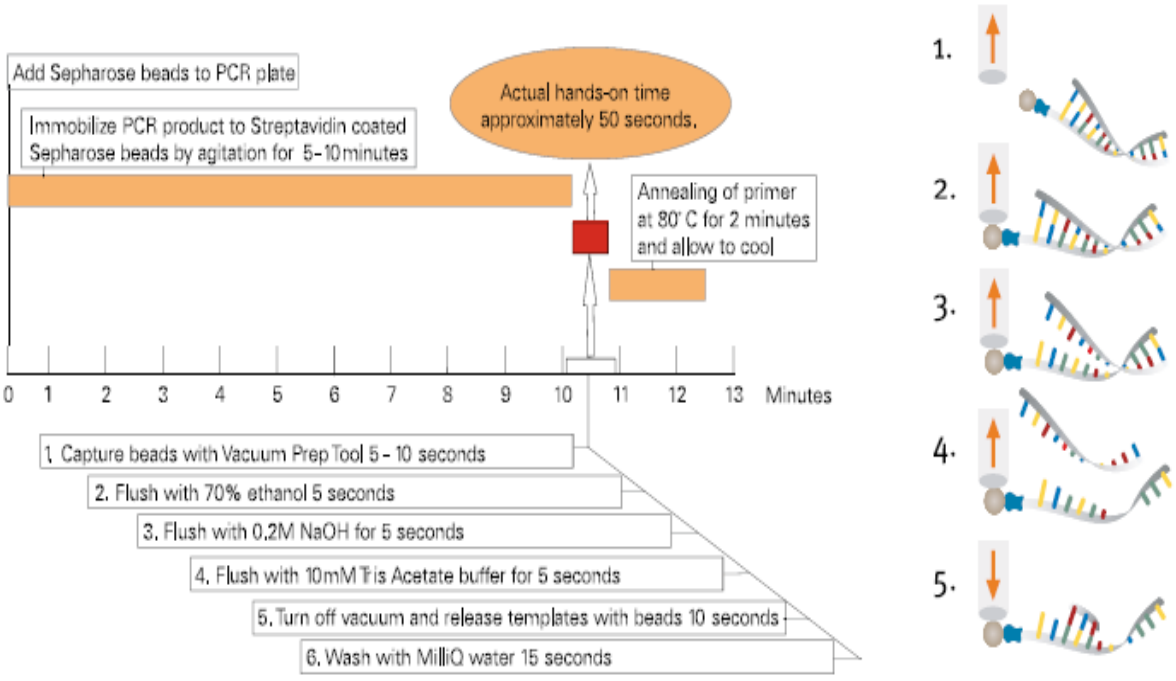

Figure 6: The workflow of templates preparation

2.6.3 Sequencing and software used

As a quantitative methylation method, Pyrosequencing could confidently measure the individual degree of methylation in adjacent CpG sites in one easy analysis, built-in quality control through sequence context and for completion of bisulfite treatment. As shown in Figure 7, Pyrosequencing analyzes single-stranded DNA templates by synthesizing complementary strands. The four nucleotides are added sequentially by a Pyrosequencing instrument to DNA templates. For every successful nucleotide incorporation, pyrophosphate (PPi) is released. PPi is

converted in enzyme-catalyzed reactions to drive light emission in a quantity that is proportional to the number of incorporations. Therefore, peak heights in the Pyrogram™ inform on homopolymeric sequences and allele frequencies. The degree of methylation is calculated from the peak heights of C and T:

$$\% \text{ methylation} = \frac{\text{C peak height} \times 100}{\text{C peak height} + \text{T peak height}}$$

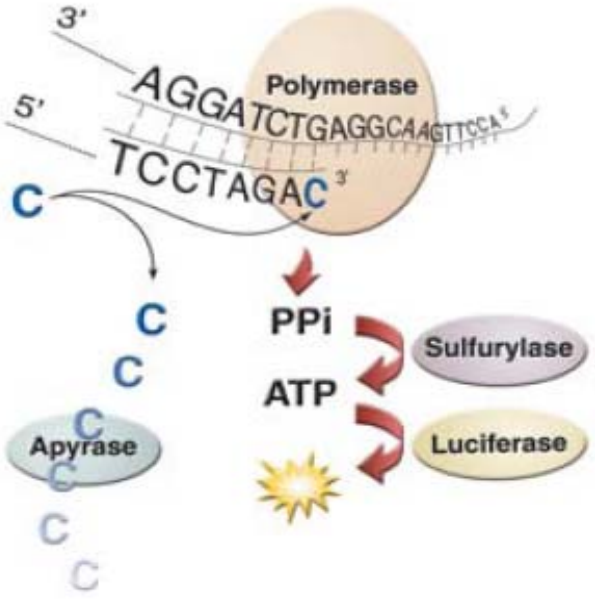

Figure 7: The principle of pyrosequencing. The Pyrosequencing reaction cascade generates light for every incorporated nucleotide, the intensity of which is proportional to the number of bases incorporated (PPi: pyrophosphate, ATP: adenosine triphosphate).

Enzyme mixture and substrate mixture from the PyroGold PSQ Reagents kit were dissolved in each 620 µl high purity water (HPLC grade), after reaching room temperature. Then, they were loaded into a special cartridge, just like each deoxynucleoside triphosphate (dATPαS, dTTP, dCTP, dGTP) from the same kit according to the volume information from the Pyro Q-CpG software. The cartridge and the sample plate were placed into the PSQ™ 96MA instrument and the analysis run automatically. The order of nucleotide dispensation was defined before, corresponding with the original template sequence. Pyrosequencing primers and templates were controlled for self-extension or primer-dimer formation. Additionally, bisulfite-conversion quality control was built in the dispensation order of each fragment to test the efficiency of bisulfite modification.

Pyro Q-CpG™ software collected the raw data of each nucleotide given in dispensation order automatically, accessed the quality for context sequencing and bisulfite modification and then analyzed the percentage of methylation in each CpG site (Figure 8).

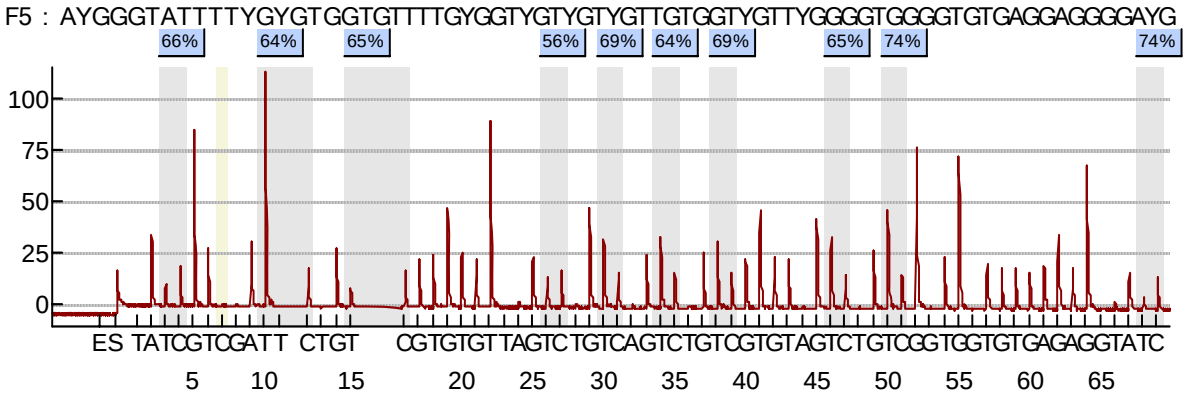

Figure 8: The detailed methylation result after software analysis. The superincumbent sequence is the original sequence of the first fragment of RASSF1A; **the underlying sequence** is the dispensation order generated by the software; **the gray colume** is the position for the quality control of bisulfite modification; **the blue colume** is each objective CpG site and the corresponding result of methylation level.

2.7 Relative quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) for *RASSF1A* gene expression

2.7.1 Reverse transcription

2 µg total RNA was reverse transcribed into 20 µl of single-stranded cDNA using the High-Capacity cDNA Reverse Transcription Kits according to the manufacturer's instructions. The composition of the 2× reaction mixture is shown in Table 6. 10 µl of 2 × RT master mix was added into an individual tube with 2 µg RNA in a total volume of 10 µl with Nuclease-free water. Reverse transcription was performed in a GeneAmp PCR system under the following conditions:

- 25°C for 10 minutes
- 37°C for 120 minutes
- 85°C for 5 seconds
- 4°C final temperature

Table 6: Composition of 2 × reaction master mix for reverse transcription

Component	*Volume per reaction (µl)*
10 × RT Buffer	2.0
25 × dNTP Mix (100mM)	0.8
10 × RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
Nuclease-free H_2O	4.2
Total per Reaction	10.0

2.7.2 Realtime reverse transcriptase polymerase chain reaction

cDNA was diluted in deionized water five times and 3.0 µl of it was used for each reaction in quantitative RT-PCR. PCR amplification was carried out in a final volume of 15 µl, each containing 7.5 µl of TaqMan Universal PCR Master Mix, 0.75 µl of the E × 12 TaqMan® Gene Expression assays, 3.75 µl deionized water. Each sample reaction was repeated twice. qRT-PCR was carried out using standard amplification conditions list in Table 7 using the ABI Prism 7700 Sequence Detector and the E × 12 TaqMan® Gene Expression assays according to the manufacturer's protocols. A house-keeping gene, β2 microglobulin (*B2M*) was used as internal standard. To relative quantify the mRNA expression of *RASSF1A*, standard curve was established for by a serial dilution of the cDNA products (Hela and SiHa cell lines) from conventional RT-PCR. The expression of *RASSF1A* was evaluated against the standard, and in a second step it was correlated with the mRNA expression of the housekeeping gene *B2M*. The formula for the calculation of amount is as follows: The input amount (ng / µl) =10 ^ [cell containing log input amount], while cell containing log input amount = ([cell containing C_T value] – [y-intercept of standard curve line]) / slope of standard curve line. The expression ratio was calculated as the quotient amount of *RASSF1A* / amount of *B2M*, *B2M* being the housekeeping gene for normalization.

Table 7: Polymerase Chain Reaction conditions

Step	***AmpliTaq Gold Enzyme Activation***	***Polymerase Chain Reaction***	
	Hold	Cycle (40 cycles)	
		Denature	Anneal/Extend
Time	10 min	15 sec	1 min
Temp	95°C	95°C	60°C

2.8 Statistical analyses

Statistical analysis was performed by use of One-Way ANOVA, Independent –Samples T test, or Paired-Samples T test. Bivariate method was used for correlate analysis. Univariate and Linear Regression models were used for analyzing *RASSF1A* gene methylation by clinic pathological factors [histological type, tumor stage, nodal status, grade, menopausal status and estrogen and progesterone receptor status].

All analyses, including univariate, multivariate, and correlate were performed by use of SPSS Windows version 11.5. All statistical tests were two-sides and the significance level for all comparisons was set at the common standard of 0.05.

3 Results

3.1 Frequent methylation of *RASSF1A* in breast tissues

Firstly, we evaluated the methylation status of breast tissue DNA from 181 breast cancer patients, including 49 match-paired samples. The mean methylation degree of normal adjacent breast tissue DNA was 8.21± 5.71% (n=49, 95% CIs 6.57% to 9.85%) and that of breast tumor tissue DNA was 32.59 ± 20.81% (n=181, 95% CIs 29.53% to 35.65%). Taking advantage of paired samples in this study, we chose the value of normal samples as the reference. If using the sample mean plus two times the standard deviation of the pooled normal samples as a cut-off point (8.21+2×5.71), there is > 93% (3/49 in normal tissues) probability that the methylation level for normal tissues will be lower than the cut-off point. It is reasonable to assume that a value larger than the cut-off point is likely to be higher than normal. Therefore, we took 19.63% as the threshold to determine hypermethylation level to analyze the methylatioin status in the tissues from breast cancer patients by pyrosequencing assays. As Figure 9 showed, *RASSF1A* gene promoter region was hypermethylated in high frequency in tumor tissues and there was a significant difference between tumor tissues and normal adjacent tissues (p=0.000, 122/181 67.40% in tumor tissue versus 3/49 6.12% in adjacent normal tissue; 33/49 67.35% in tumor tissue versus 3/49 6.12% in normal tissue in paired samples).

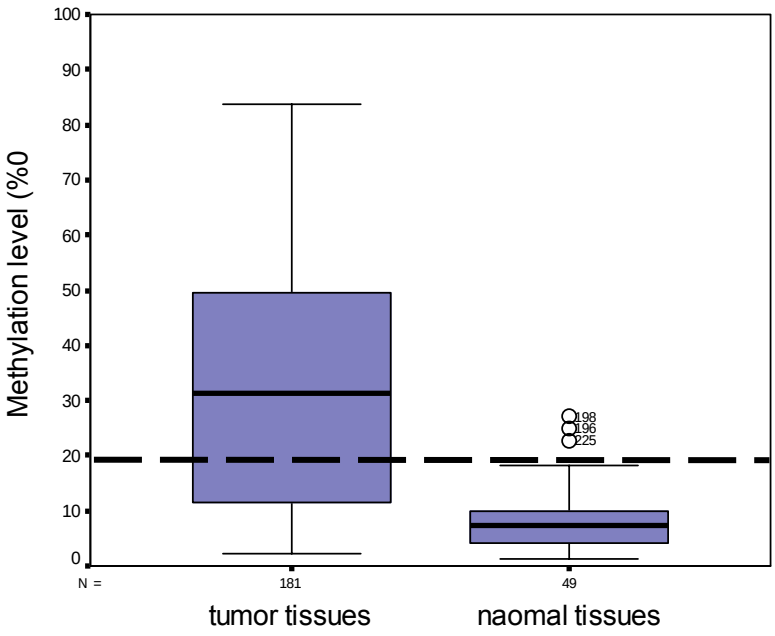

Figure 9: Results of pyrosequencing methylation analysis of Ras association domain family 1 isoform A (*RASSF1A*) gene in breast cancer. Black linea aspera illustrate for comparation of median methylation level in tumor tissue (n=181) and normal adjacent tissue (n=49) in breast cancer patients. **Black dash line** is the threshold for hypermethylation of *RASSF1A* gene promoter in breast cancer by pyrosequencing analysis.

Then we analyzed the variability of *RASSF1A* gene methylation level among each CpG site in all samples and the results showed that the differences were statistically significant, in tumor tissues as well as in normal adjacent tissues (p=0.000, One-Way ANOVA), this difference could still been observed within each fragment. In another words, different methylation level among 32 CpG islands came unlikely from the difference of amplification of three fragments. Overall, *RASSF1A* promoter methylation degree at position 18 was the lowest one (Figure 10).

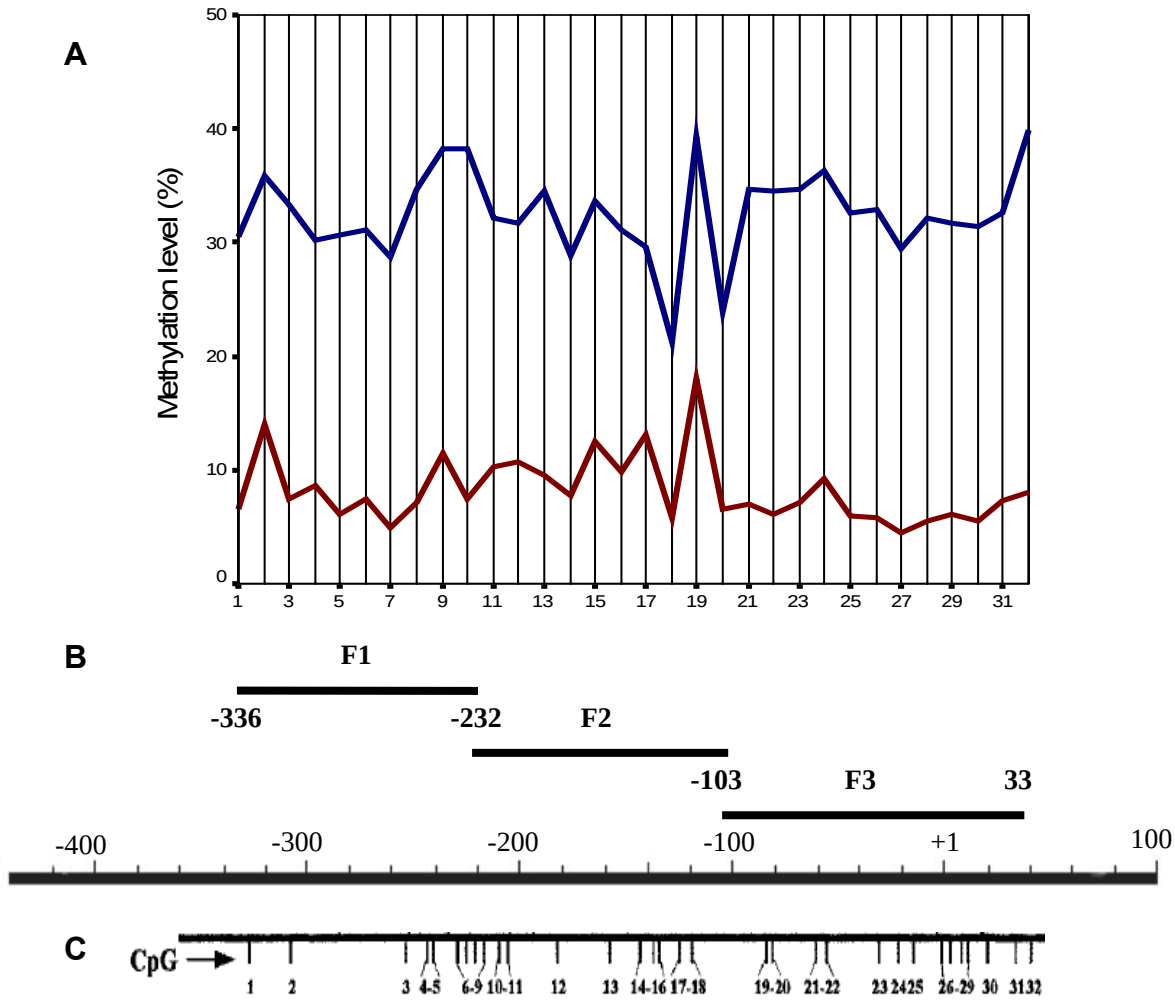

Figure 10: Compare the methylation level among all 32 CpG sites in Ras association domain family 1 isoform A (*RASSF1A*) gene promoter region. A) The blue line showed the mean methylation level of each CpG site in tumor tissues; **the rhodamine line** showed that in normal adjacent tissues from paired patients. **B)** Promoter region of the *RASSF1A* gene and the location of three fragments (F1, the first ten CpG sites; F2, the eleven to twentieth CpG site and F3, the last twelve CpG sites) of PCR products for pyrosequencing in this study are shown. Numbering indicates the nucleotide position relative to the exon 1 start site, which is designted +1. **Short vertical bars** on the bottom line show CpG sites in detail. **C)** Each CpG site in *RASSF1A* promoter region.

3.2 *RASSF1A* Methylation status in pair-matched breast samples

As Figure 11A showed, in the 49 paired patients, the methylation degree was obviously higher in the tumor tissues (mean 32.49 ± 21.79%, 95% CIs 26.2 to 38.72%) than in the adjacent normal tissues (mean 8.21 ± 5.71%, 95% CIs 6.57% to 9.85%) and the significant difference could be seen in all CpG sites (p=0.000). No statistical significant correlation existed between these two groups (Pearson's correlation coefficient R=0.261 and p=0.070). A relative increase of methylation in tumors when compared to paired normal tissue was observed in 43 (87.76%) of 49 tumors (Figure 11B and 11C). Note that six tissue paired demonstrated an apparently decreased relative methylation in tumors.

When compared the specificity for the changes of methylation degree of all the CpG sites, no statistical significance could be observed for each site in all three fragments for pyrosequencing (p=0.445, p=0.252 and p=0.995, respectively), although a relative fewer decrease could be observed on the position 18 within the second fragment in all samples.

We also compared the changes of the methylation degree in paired patients according to clinical pathologic prognostic parameters and found that no statistical significance in age (Pearson's correlation coefficient R=0.086 and p=0.556), tumor size (p=0.309), metastasis (p=0.964), grade (p=0.437), ER (p=0.891), PR (p=0.408), and death (p=0.853), respectively. Noticeable difference only existed in the methylation changes with two factors: the lymph node metastasis and recurrence. The changes of methylation level were decreased with the lymph node metastasis (p=0.014, mean level: without Lymph node metastases 38.16 ± 19.39%, n=11; with Lymph node metastases 20.55 ± 20.13%, n=37) and increased with the recurrence (mean level: relapse 37.46 ± 23.02% n=10, without relapse 18.58 ± 16.45% n=32; p=0.006), no exception for all the 32 CpG sites.

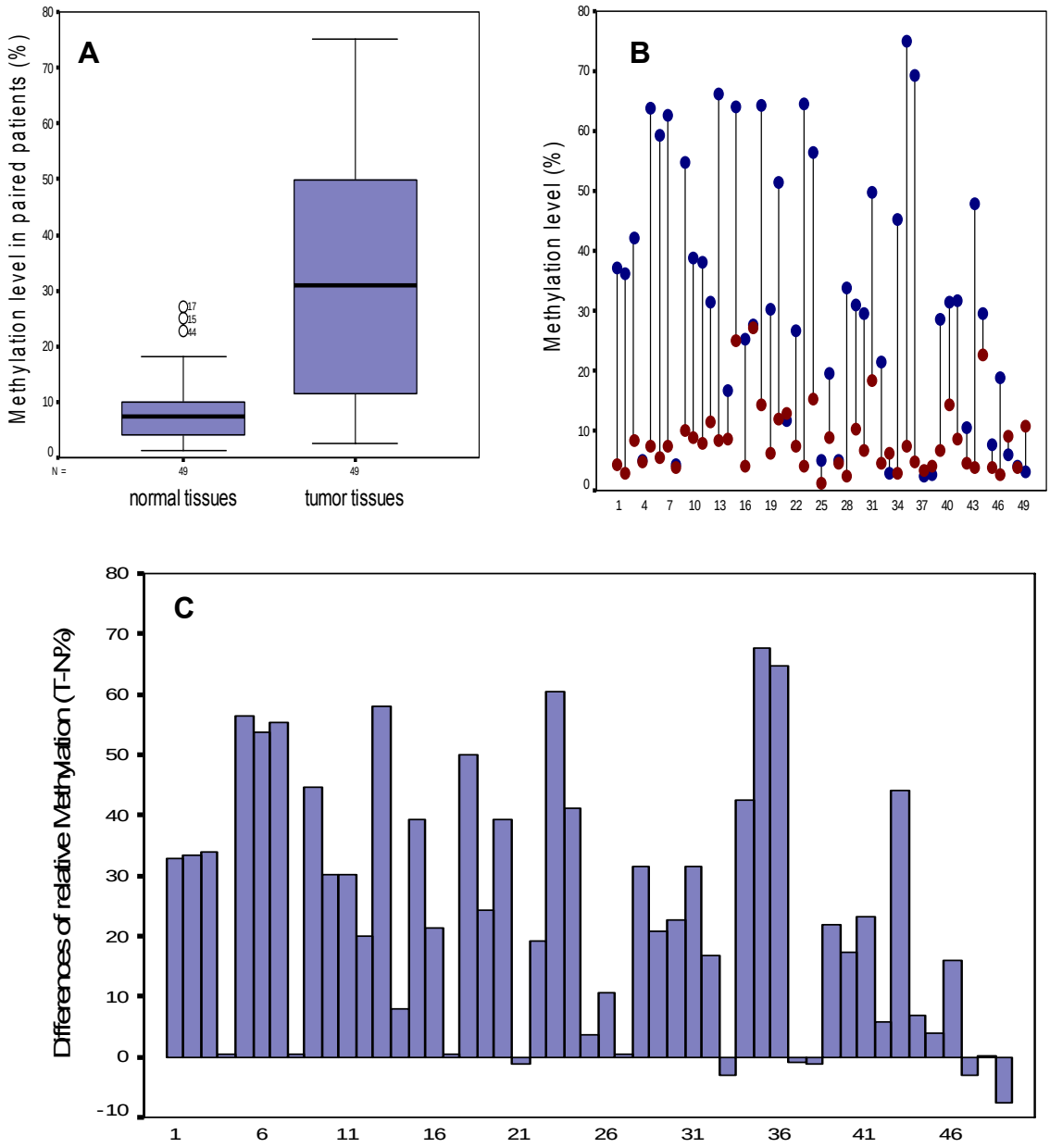

Figure 11: Results of quantitative methylation ananlysis of tissue pairs. A) Box plot illustration for in group comparison of methylation level of *RASSF1A* promoter as measured by pyrosequencing in 49 cancer and paired normal breast tissue samples. **B)** Primary data illustration for each pair-matched sample. **Blue spot** showed mean methylation level of tumor tissue and **purple spot** showed mean methylation level of normal adjacent tissue. **C)** The difference of methylation degree for each pair-matched sample. Note that most of the tumors demonstrate a substantial relative increase in methylation when compared to their corresponding normal tissue (p=0.000, n=49).

3.3 *RASSF1A* methylation and clinical parameters

The correlation between methylation status of the *RASSF1A* promoter region in breast cancer/normal tissue and clinical or pathologic parameters was analyzed.

Following parameters have been used: age the diagnosis, histological type, T stage, lymph node involvement, metastasis, grading, menopause status, hormone receptor (estrogen receptor and progesterone receptor) status, relapse, and death. All the data were summarized in Table 8.

Table 8: Ras association domain family 1 isoform A (*RASSF1A*) promoter methylation level in tumor breast tissues and clinic pathological characteristics of primary breast cancer (n=181)

Characteristics	*Number Of patients (n= 181)*	*Methylation level (mean ± sd %)*	*P*[a]
Age			0.380
<45	34	29.58 ± 20.10	
≥45	147	33.09 ± 21.06	
Histological type			0.492
Invasive ductal	127	34.25 ± 20.71	
Invasive lobular	20	31.23 ± 20.99	
Others	23	29.02 ± 21.68	
unknown	11		
Size of tumor (T)			0.083
T1	36	26.09 ± 16.12	
T2	76	32.47 ± 20.75	
T3	24	35.88 ± 24.14	
T4	37	37.79 ± 20.07	
Tx	8		
T (divided to two groups)			**0.028**
T1	36	26.09 ± 16.12	
T2+3+4	137	34.51 ± 21.17	
Lymph node metastases (N)			0.838
No	58	32.60 ± 19.61	
Yes	116	33.08 ± 21.37	
unknown	7		
Distant metastases (M)			0.501
M0	163	32.67 ± 20.60	
M1	14	36.57 ± 23.22	
unknown	4		

Characteristics	*Number Of patients (n= 181)*	*Methylation level (mean ± sd %)*	*P*[a]
Stage			0.716
I	16	35.50 ± 14.10	
II	76	31.21 ± 21.13	
III	68	33.87 ± 20.58	
IV	14	36.57 ± 23.22	
unknown	7		
Tumor grade (G)			0.115
G1	3	29.44 ± 21.29	
G2	90	35.91 ± 20.71	
G3	65	28.90 ± 20.87	
unknown	23		
G (divided to two groups)			**0.044**
G1+2	93	35.70 ± 20.64	
G3	65	28.90 ± 20.87	
Estrogen-receptor status			**0.001**
Negative	74	26.34 ± 20.97	
Positive	96	36.93 ± 19.36	
unknown	11		
Progesterone-receptor status			0.083
Negative	102	30.08 ± 21.25	
Positive	68	35.69 ± 19.50	
unknown	11		
Menopause			0.706
No	115	34.35 ± 21.11	
Yes	28	32.70 ± 18.96	
unknown	38		
Recurrence			0.624
No	110	31.50 ± 19.54	
Yes	56	33.16 ± 22.71	
unknown	15		
Death			0.484
No	157	32.85 ± 20.58	
Yes	24	29.64 ± 22.96	

[a] *P* for the comparison of mean methylation level of *RASSF1A* gene for the patients was calculated by one-way-ANOVA.

3.3.1 Correlation with age

For normal adjacent breast tissues, we found a statistical correlation between the methylation level and age (R=0.302, *p*=0.035) by Pearson's correlation analysis (see Figure 12A). The methylation level of *RASSF1A* promoter region in tumor tissues also increased along with the age of patients, but the coefficient of correlation was lower (R=0.161, *p*=0.030) by Pearson's correlation analysis (Figure 12B). No statistical significance between the mean methylation level and the patients over and under 45 years of age (*p*=0.380, mean across all patients = 58 years) was observed and no variance in each CpG site.

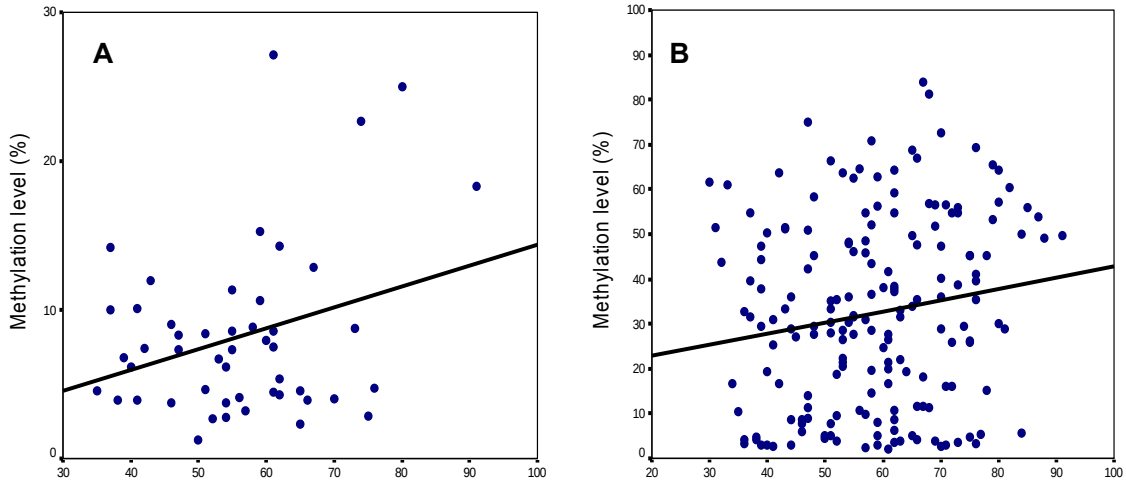

Figure 12: Correlation between patient age and the methylation level of Ras association domain family 1 isoform A (*RASSF1A*) gene. X-axis showed the age of patients. **A)** Normal adjacent tissue samples (n=49, median age 55, 95% CIs 52 to 60); **B)** Tumor tissue samples (n=181, median age 58, 95% CIs 56 to 60).

3.3.2 Correlation with the tumor size

As Figure 13A showed that the *RASSF1A* methylation level increased in accordance with the sizes and extent of the primary tumor, higher *RASSF1A* promoter methylation frequencies were found among the big tumor than the small ones (mean methylation degree: T1 of 26.09 ± 16.12%; T2 of 32.47 ± 20.75%, T3 of 35.88 ± 24.14%, and T4 of 37.79 ± 20.07%). The methylation level of T1 tumor was significant lower than that of T2 to T4 (*p*=0.028).

In normal adjacent breast tissues, an obvious difference of *RASSF1A* promoter methylation level existed in different primary tumor size (mean methylation degree:

T1 6.97 ± 6.08% n=11, T2 5.50 ± 3.11% n=13, T3 8.01 ± 3.34% n=6, and T4 11.07 ± 6.90% n=17; *p*=0.053). Moreover, the methylation level of primary tumor size 1 and 2 was much lower than that of tumor size 3 and 4 (*p*=0.009).

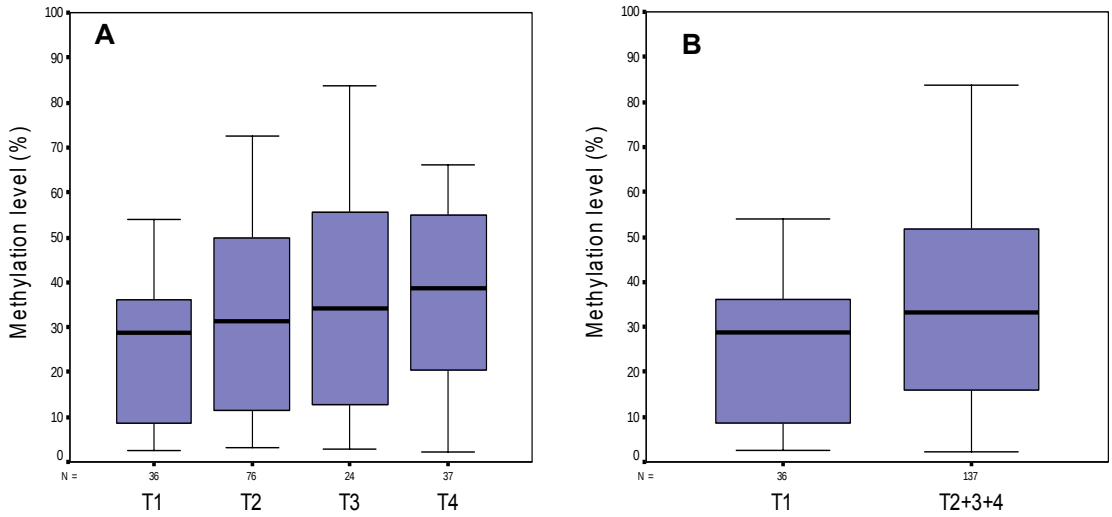

Figure 13: Methylation level of Ras association domain family 1 isoform A (*RASSF1A*) gene promoter in breast tumor tissues in different primary tumor size. T: primary tumor size (the detail of the classification of tumor size see Table 1).

3.3.3 Correlation with tumor histological grade

Considering the grade of tumor in our samples mostly localized in the grade 2 and 3, we divided the samples to two groups, one was grade 1 (only three samples in this category) plus 2 and the other one was grade 3 (Figure 14). The methylation level decreased along with the tumor grade and statistical significance could be observed between them (*p*=0.044, mean methylation degree: G1 and G2 35.70 ± 20.64% n=93; G3 28.90 ± 20.87% n=65), but there were some marked differences among different CpG sites (*p*=0.017, *p*=0.053 and *p*=0.104 for the three fragments, respectively).

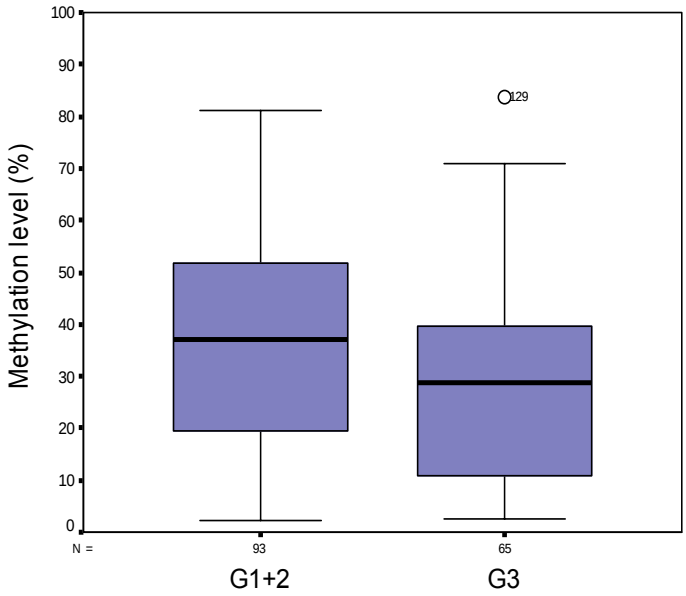

Figure 14: Methylation level of Ras association domain family 1 isoform A (*RASSF1A*) gene promoter in different tumor grade. G: tumor grade (G1+2: tumor grades I and II, G3: tumor grade III).

3.3.4 Correlation with Lymph node metastases and recurrence

For normal adjacent breast tissues, difference was observed for the *RASSF1A* methylation level by disease recurrence (mean methylation degree: relapse 11.41 ± 6.10% n=10, without relapse 7.59 ± 5.77% n=33), though it didn't reach statistical significance (p=0.077). This kind of difference could not be observed in tumor tissues. Analyze the lymph node metastases, significant difference could be observed neither in breast tumor tissues nor in adjacent normal tissues.

3.3.5 Correlation with hormone receptors

In all the clinic pathological factors, estrogen receptor (ER) and progesterone receptor (PR) are most important to influent the management strategy and the prognosis. When focusing at these two factors, cancer was considered receptor-positive if > 10% of malignant cells showed nuclear staining (score ≥ 2 /12) according to the guidelines of St. Gallen 2005 breast cancer consensus meeting (Goldhirsch et al. 2005). As shown in Figure 15A, Methylation level was lower in the receptor-negative group (mean 26.34 ± 20.97%) than that in the positive group (mean 36.93 ± 19.36%) and a statistical significance was presented (p=0.001, this p value is from the average of three fragments). The same trend for PR (Figure

15B), but it didn't reach the significant level.

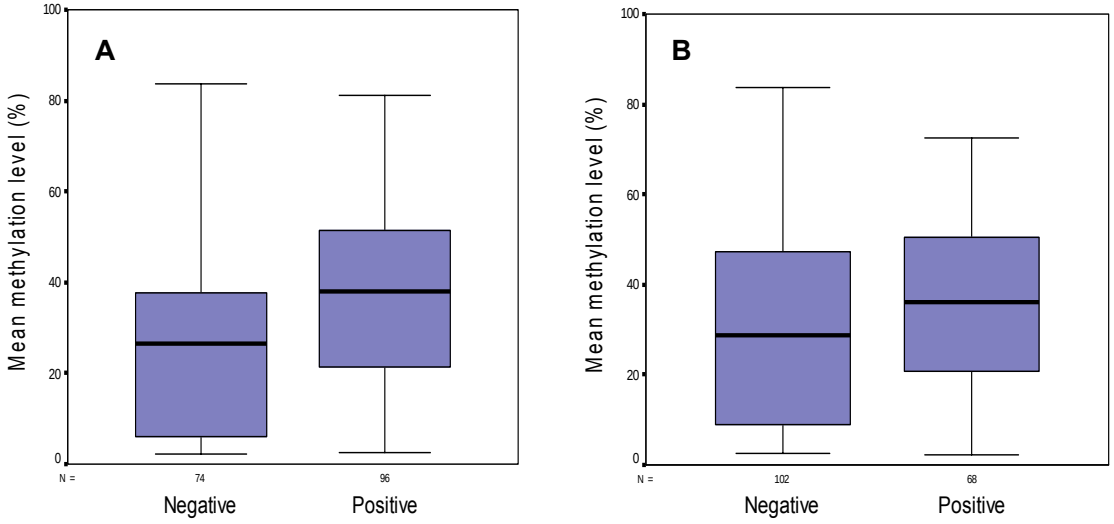

Figure 15: Methylation level of Ras association domain family 1 isoform A (*RASSF1A*) gene promoter in different group of hormone receptors. A) Methylation level between negative and positive group of estrogen receptor. **B)** Methylation level between negative and positive group of progesterone receptor.

3.4. Demethylation of *RASSF1A* in breast cancer cell line

3.4.1. Methylation status of *RASSF1A* in cell lines

The methylation state of *RASSF1A* promoter indicated aberrant high level in cell lines in our study. No methylation was detected in Hela cell line (an immortal cell line, was derived from cervical cancer cells), which express high levels of RASSF1A.

3.4.2 Effect of demethylation treatment in cell line MDA-MB-330

The analog 5-Aza-dC is a DNA methyltransferases (DNMTs) inhibitor and is one of the most commonly used demethylation drugs in assays with cultured cells. To understand the mechanism of hypermethylation/demethylation - knock-down expression/reexpression, a breast cancer cell line MDA-MB-330 was used as a model *in vitro* treated with the demethylation agent 5-Aza-dC. Theoretically, demethylation process could change the hypermethylation status of *RASSF1A* gene in this cell line and re-express *RASSF1A*.

As determined by pyrosequencing, the methylation level of *RASSF1A* promoter was significantly decreased by 5-Aza-dC (mean methylation level of 32 CpG sites, from 90.92 ± 9.42% to 49.80 ± 7.06%). For all three fragments, significant differences of the degree of 5-Aza-dC demethylation for individual CpG site could be observed in the promoter region covered by the first two fragments (p=0.000, p=0.007, and p=0.445, respectively) in our study. In another words, demethylation differed site to site. The optimal concentration of 5-Aza-dC was at the dose of 1.0 μM. Figure 16 showed the methylation level in each CpG site in MDA-MB-330 cell line for the demethylation treatment at different time point and reagent concentration in detail.

To investigate whether histone acetylation affect DNA methylation/mRNA expression, we used a histone deacetylase (HDAC) inhibitor Trichostatin A (TSA) alone or a combination with the DNMTase inhibitor 5-Aza-dC. As Figure 16 showed, to *RASSF1A* gene promoter, TSA did not show visible influence to the methylation/expression status at doses of 0.1 μM, 0.2 μM, 0.4 μM and 1.0 μM in breast cancer cell line MDA-MB-330.

3.5 Re-expression of *RASSF1A* in the breast cancer cell line

No expression of *RASSF1A* mRNA in cell line MDA-MB-330 was detected by Taqman assay and the promoter region was close to 100% methylated. As a positive control, Hela cell line was chosen due to its high expression of *RASSF1A* mRNA and low methylation at the *RASSF1A* promoter region.

The re-expression of *RASSF1A* was observed after cell line MDA-MB-330 was exposed to 5-Aza-dC treatment. The relative quantitation of *RASSF1A* mRNA expression at different doses of 5-Aza-dC and/or TSA was summarized in Table 9. In our hands, TSA did not show any effect on the re-expression of *RASSF1A* at the transcription level in this cell line. The housekeeping gene β2 microglobulin was used as an internal control.

Table 9: Relative quantitation of Ras association domain family 1 isoform A (*RASSF1A*) mRNA using the standard curve method

MM330 treatment	*RASSF1A Average* C_T	*B2M Average* C_T	$RASSF1A_T$ *Rel.to* $RASSF1A_{N72h}$
A 0.5 96h	28.42±0.35	15.98±0.08	865.01±0.24
A 0.5 96h/T 0.1 72h	29.64±0.52	16.48±0.02	530.57±0.34
A 0.5 72h	29.94±0.57	15.83±0.07	257.73±0.37
A 0.5 72h/T 0.1 18h	29.83±0.33	16.37±0.04	381.47±0.22
A 1.0 96h	28.14±0.21	15.85±0.23	946.25±0.20
A 1.0 96h/T 0.1 72h	29.03±0.10	16.79±0.11	941.06±0.10
A 1.0 72h	28.53±0.05	15.97±0.06	701.60±0.05
A 1.0 72h/T 0.1 18h	29.20±0.25	16.11±0.04	488.99±0.17
TSA 0.1 72h	40.00±0.00	16.80±0.13	0.60±0.08
TSA 0.1 18h	36.78±0.07	17.18±0.01	5.90±0.05
TSA 0.2 18h	40.00±0.00	16.87±0.38	0.55±0.24
TSA 0.4 18h	40.00±0.00	17.28±0.31	0.72±0.19
Normal control 96h	40.00±0.00	17.61±0.04	1.00±0.02
Normal control 72h	40.00±0.00	17.82±0.40	1.00±0.25

D	T	Time	CpG site																															
μM	μM	h	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
0.5		96																																
0.5	0.1	96/72																																
1.0		96																																
1.0	0.1	96/72																																
0.5		72																																
0.5	0.1	72/18																																
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	0.2	18																																
	0.4	18																																
		96																																
		72																																

Figure 16: The Ras association domain family 1 isoform A (*RASSF1A*) gene promoter methylation level in MDA-MB-330 cell line with 5-Aza-dC and/or TSA treatment at different time point and reagent concentration. D: 5-Aza-dC (μM), **A**: TSA (μM). **The area of blue sector** showed the percentage of methylation in every CpG site (100% methylation). **The area of rhodamine sector** showed the percentage of unmethylation in the same position (100% unmethylation).

4 Discussion

Aberrant DNA methylation of *RASSF1A* CpG islands and its associated gene silencing have been reported in many tumor types, including breast cancer (see Table 3 in the introduction). Although evidences have demonstrated that hypermethylation plays an important role in breast carcinogenesis, many questions have still not been answered.

In the present study, we have used pyrosequencing methylation assay (PMA) to evaluate the frequency of *RASSF1A* gene promoter methylation among 32 CpG islands in 181 breast cancer patients, including 49 paired of malignant/normal breast tissues. Our data confirmed that *RASSF1A* was frequently methylated in breast cancers but not in its neighbor normal breast tissues. By analyzing the correlation between *RASSF1A* methylation data and clinical pathologic parameters, we found significance on tumor size, histological grade and estrogen receptor, respectively. By analyzing paired of malignant/normal breast tissues, we found the significant different between tumor and normal tissues is not site specific. The change of methylation level was significantly dependent to the lymph node metastases and disease recurrence. Treatment of breast cancer cell line with 5-Aza-dC resulted in the demethylation of *RASSF1A* promoter and re-expression of *RASSF1A* mRNA.

4.1 Method for methylation study

Since the first description of bisulfite reaction in the application of studying 5mC, numerous techniques for the mapping of cytosine methylation based on the same principle have been developed. Methylation-specific PCR (MSP; Herman et al. 1996) is the most commonly used with primers specific for bisulfite modification DNA. Other method include Bisulfite-sequencing PCR (BSP; Clark et al. 1994), methylation-sensitive single nucleotide primer extension (Ms-SnuPE; Gonzalgo et al.1997), MethyLight (Eads et al. 2000), HeavyMethyl (Cottrell et al. 2004), combined bisulfite restriction analysis (COBRA; Xiong and Laird 1997; Brena et al. 2006), quantitative multiplex-methylation-specific PCR (QM-MSP; Fackler et al. 2004), Methylation Target Array (MTA; Chen et al. 2003), methylation-specific oligonucleotide microarray (MSO; Gitan et al. 2001) and pyrosequencing

methylation assay (PMA; Colella et al. 2003; Tost et al. 2003; Dupont at al. 2004). The frequently used assays, MSP or COBRA, which detect point methylation only on a few CpG island. Among them, PMA offers a semiquantitative, high-throughput, and reliable method with an inbuilt internal control for adequacy of bisulfite treatment, in which 4–25 CpG dinucleotides are individually assayed together with a non-CpG cytosine (conversion control). This technique shows low variance in replicates over a range of levels of methylation. Up to now, PMA is the one of the best method to quantitate each CpG site for gene promoter analyze, in spite of its limitation of the primer pairs, which should be specifically designed to avoid CpG dinucleotides in their sequence in order to allow the detection of the amount of methylation in a sample quantitatively.

For detection of *RASSF1A* promoter methylation, variations in the data from different groups may be because of by using different techniques. All these techniques are bisulfite and PCR based, so the quantitation is relative. Moreover, there will be some contamination of unmethylated “normal” cells and other stromal cells reside in the breast tissue samples. Analyzing the same CpG sites in *RASSF1A* promoter region with our study by using of MSO microarray, different results are existent in tissue samples but the same results in breast cancer cell lines (Yan et al. 2003).

In our study, bisulfite modification and PMA were repeated at least twice for all samples. The data have a high correlation from every experiment, so PMA has a good reproducibility for CpG methylation detection. Take advantage of the PMA quantitation for each CpG site, we can map detailed CpG methylation status of *RASSF1A* promoter and analyze site specificity. Moreover, the internal control of bisulfite modification offered a good quality control in our study.

4.2 *RASSF1A* methylation status in paired breast cancer patients

In normal cells, the majorities of promoter CpG islands are protected from epigenetic event and are unmethylated (Bird 2002; Jones and Baylin 2002). In cancer cells, it has been shown that widespread DNA methylation occurs in many promoter CpG islands, resulting in a closed, repressive chromatin configuration that disables transcription initiation of the associated genes (Jones and Baylin 2002).

Historical data indicate that *RASSF1A* promoter methylation is frequently in tumor tissues, including breast cancer (see Table 3 in the introduction). Our study also estimated a high frequency hypermethylation of *RASSF1A* promoter (122/181 67.40%) and the methylation level in tumor tissues (mean 32.59 ± 20.81%) was much higher than that in normal tissues (mean 8.21± 5.71%).

Epigenetic modifications are believed to be early events in cancer development. Nevertheless, for normal breast tissues, the results were inconsequent. Honorio et al. (2003) reported that *RASSF1A* promoter hypermethylation was detected in 65% of invasive breast cancers, in 42% of corresponding DCIS but in none of the normal breast samples. Lehmann et al. (2002) also found that no methylation in normal mammary gland epithelium. Yeo et al. (2005) gave an opposite report that *RASSF1A* promoter hypermethylation was detected in 38 of the 40 breast cancer tissues (95%) and 37 of the paired non-tumorous tissues (92.5%). Distinct with their results, we found that normal tissue showed various degrees of methylation in *RASSF1A* promoter region (mean methylation level 8.21± 5.71%), and 3 of 49 (6.12%) was hypermethylated. Consistent with ours, Dammann et al. (2001) detected some methylation in normal tissue 7.5% of the breast cancer patients. Yan et al. (2006) not only provided a comprehensive analysis of promoter hypermethylation in normal tissues adjacent to breast cancers, but also indicated that premalignant epigenetic changes radiating from the tumor epicenter could be widespread and further showed that methylation spreading from outer flanks to the promoter/first exon core (Yan et al. 2003) in normal adjacent tissues contributed to the densely methylation of *RASSF1A* promoter/first exon in primary breast cancer. Develop multiple methylation markers will be useful for predicting local recurrence in histological normal margins. Age, one of aftermentioned clinical parameters, may be another reason for the methylation of *RASSF1A* gene in normal tissues in addition.

4.3 *RASSF1A* methylation level in pair-matched breast tissues

Our study is the first to specifically address the comparison of *RASSF1A* methylation levels quantitatively detectable in paired samples of normal and tumor tissues, to analyze the variation of methylation level change in each CpG site, and to correlate this change to clinical parameters. Our methylation analysis focused

CpG sites localized directly adjacent to the start of *RASSF1A* transcription, a promoter region that has been described to cause gene silencing of *RASSF1A* (Dammann et al. 2000; Yan et al. 2003). There was no specificity of CpG site in methylation level changes. In all clinical parameters, the changes of *RASSF1A* methylation level only correlated with lymph node metastasis and disease recurrence. The methylation level was higher in both tumor tissues and normal tissues with relapse, and a high volume of methylation level change existed. On the other hand, it was lower in tumor tissues and higher in normal tissues with the former factor, and a decreased change could be find. This significant association of difference methylation degree between tumor and normal tissue with known pathologic prognostic factors may have additional clinical utility for assessing patient prognosis, especially predicting early recurrence. However, more samples need to be analyzed to provide credence of such an observation.

4.4 *RASSF1A* promoter methylation and clinical parameters

In our analysis, *RASSF1A* promoter methylation had a distinct extent of correlation with the age, tumor size (T), histological grade, recurrence and estrogen receptor (ER) in all clinical parameters. Specifically for the first two factors, both in tumor breast tissues and in normal adjacent tissues, which displayed a notable contribution to the methylation level of *RASSF1A* gene promoter.

4.4.1 Age

The risk of breast cancer increases with age. Methylation of CpG islands in non-malignant tissues increases but the total number of methylated cytosine residues decreases with age (Catania and Fairweather 1991). Individual genes are progressively methylated during aging due to chromosomal instability (Issa 1999) and this change is tissue specific. *RASSF1A* promoter hypermethylation could occur early in breast tumorigenesis (Lehmann et al. 2002). Yan et al. (2003), who found that methylated CpG sites of most of the normal breast tissue controls have been seen in the first exon but not the promoter area, considered that may be an age-related phenomenon (Issa 1999) of *RASSF1A* in normal breast tissues. Methylation occurs as part of the aging process is a phenomenon that has been described for other genes (Ahuja et al. 1998). Recently, Kwabi-Addo and his group (2007) demonstrated that several genes which are hypermethylated in prostate

tissues in an age-dependent manner, including *RASSF1A* gene.

In agreement with these findings, our data also indicates that *RASSF1A* gene methylation level increased in normal breast tissues along with patients' age and markedly increased in cancer. Because of the limitation of the source of breast tissues, we examined *RASSF1A* gene methylation in 49 normal adjacent tissues from breast cancer patients. To our knowledge, this is the first study to directly examine the relationship between methylation and age in human normal neighbor breast tissues. A large breast tissue population study, including samples from healthy individuals, is needed to confirm the age-dependent manner of *RASSF1A* gene.

4.4.2 Tumor size

Regardless of tumor breast tissues or normal adjacent tissues, we observed a striking increase of *RASSF1A* promoter methylation level along with the primary tumor size, especially with the tumor more than two centimeters (> 2 cm). The multiplication of *RASSF1A* promoter methylation level was horizontal in tumor and normal tissue from paired patient, so no statistical significance could be observed in the change of methylation level in our study.

It is known that breast cancer can recur/self-metastasize locally as well as to distant sites. Once established in premalignant tissues, the extent of such epigenetic modification will accumulate as the disease progresses. Norton (2005) proposed the self-metastasis model whereby a small tumor becomes a large tumor by becoming a conglomerate mass of tiny tumors, each may be started by a seed/stem progenitor cell. Feinberg et al. (2006) also introduced the epigenetic progenitor origin of human cancer. Certain epigenetic disruption of progenitor cells, which leads to a polyclonal precursor population of neoplasia-ready cells. Aberrant epigenetic events accumulated during the progression of primary tumor. So a piecemeal increasing of *RASSF1A* promoter methylation degree in tumor tissues accordance with the tumor size could be found. Simultaneously, augment of methylation in propotional normal tissues, derived from non-neoplastic but epigenetically disrupted stem/progenitor cells potentially, might be a crucial indicator for cancer risk and recurrence assessment.

4.4.3 Lymph node metastases and recurrence

To our knowledge, seldom researchers found that a remarkable relationship between *RASSF1A* methylation and lymph node metastasis and disease recurrence. There was only a little report about *RASSF1A* methylation considering it was associated with lymph node metastasis in other tumor types (House et al. 2003; Liu et al. 2005). We also found no statistical correlation between *RASSF1A* promoter methylation and these two factors in tumor samples, but a distinguished dependability was exist in the changes of *RASSF1A* promoter methylation with them in paired samples as mentioned above.

The natural history of breast cancer with its localized patterns of recurrence strongly suggests that the residual "normal" breast is in fact at risk for harboring occult, precancerous cells. It was notable that a higher *RASSF1A* methylation level both in tumor tissues and normal adjacent tissues with an increase change of methylation level in pair-matched samples in our study. This high degree of concordant promoter methylation in primary tumors and adjacent normal tissues suggests that the multiplication of promoter methylation level in normal tissue adjacent to tumors is more extensive than previously noted (Lewis et al. 2005), and it may be useful for predicting future local recurrence. Yan et al. (2006) also supported that frequent promoter hypermethylation of tumor suppressor loci in normal breast tissue is a harbinger for local recurrence.

4.4.4 Tumor stage and metastasis

At this point, there are some disputes in present reports. Majority of researchers though that methylation level of *RASSF1A* promoter increased in invasive tumors and took it as a biomarker (Honorio et al. 2003; Dhawan et al. 2006; Pasquali et al. 2007), but Lehmann et al. (2002) reported that *RASSF1A* methylation level had no correlation with the invasiveness. There are also some reports about *RASSF1A* promoter hypermethylation with advanced tumor stage and distance metastasis (Jo et al. 2006; Mehrotra et al. 2004)

In this report, *RASSF1A* methylation had a slight higher level in stage I than the other three stages and in M0 than in M1, but there was no apparent statistical association of *RASSF1A* methylation with the tumor stage and metastasis in breast

cancer patients. In our data, only 14 patients had metastasis and the patient numbers for stage I and IV were very less than the other two stages, this might be influent our results. A long follow-up and more samples at different stages should be involved for further study.

4.4.5 Histological grade and type

Considering gene methylation as an early event in carcinogenesis, many researchers have verified it by the high relationship between gene methylation level and histological grade (Dong et al. 2005; Dulaimi et al. 2004; Pasquali et al. 2007; Yeo et al. 2005). We detected a constant methylation frequency of *RASSF1A* in all of the different histological grades of the mammary carcinomas. Because for the grade I, there were only 3 patients in our work, we took grade I and II as one group, and the methylation level in this group was much higher than that in grade III. This result is corresponding with the result of Dammann et al. (2001) and Feng et al. (2007). Considering about hypermethylation in this two groups, the percentage of hypermethylation was also higher in grade I and II. Thus, it confirmed that methylation of *RASSF1A* was an early event during breast cancer pathogenesis. Interestingly, the three different fragments in our study had different significance to the histological grade. The long distance from the start of *RASSF1A* transcription, the more correlation with histological grade was observed. It suggests that during the progression of breast cancer, some specificity of CpG sites may be exist. This phenomenon may be explained by the so-called methylation spreading theory in which the flanking methylation barriers of a CpG island promoter are broken down in neoplastic cells, resulting in gradual invasion of methylation to the promoter core (Turker 2002; Yan et al. 2003; Strunnikova et al. 2005)

Additionally, many report said that hypermethylation of *RASSF1A* gene did not correlate with a particular histological subtype (Fackler et al. 2003; Lehmann et al. 2002). We also did not find any distinction of *RASSF1A* methylation level in different histological type. It suggests that gene silencing by promoter hypermethylation of *RASSF1A* gene is likely to be important in both groups of breast cancer.

4.4.6 Hormone receptors

Brest cancer hormone dependence is correlated with tumor progression and patient prognosis. There is evidence in the literature that the existence of an interaction between gene methylation and HR status in breast cancer cells. Widschwendter and colleagues (2004) reported significant differences in the HR status between clusters of DNA methylation profiles. The methylation of *BRCA1* strongly correlated with lack of ER/PR expression (Niwa et al. 2000). *HIN-1, RASSF1A* and *RAR-β2* gene hypermethylation was significant more common in estrogen receptor-positive breast cancer (Krop et al. 2003; Shinozaki et al. 2005; Feng et al. 2007). The methylation of *HIN-1* also associated with PR-positive subgroup (Feng et al. 2007). In our own study, the methylation level of *RASSF1A* promoter were higher both in ER-positive and PR-positive breast cancer patients, but a significant difference of methylation level only existed between ER-positive and ER-negative tumor tissues.

Estrogen is a key factor in the development of normal mammary glands in women and significant player in the development and progression of breast cancer (Clemons and Goss 2001; Clarke et al. 1997). Sixty percent of primary breast cancers are ER-positive and two thirds of advanced ER-positive breast cancers respond to therapy with antiestrogens (Hanstein et al. 2004). The loss of ER expression itself was a result of the hypermethylation of ER-α promoter (Hortobagyi 1998) and the re-expression of ER was a result of the use of DNMT and HDAC inhibitors (Yang et al. 2000). Consequently, alteration in methylation had a complicated role in transcriptional regulation of ER. Increasing of methylation in certain TSGs, such as *RASSF1A*, promote expression of ER possibly by up-regulating ER co-stimulators (Feng et al. 2007). Further studies are needed to test this hypothesis.

4.5 Re-expression of RASSF1A in breast cancer cell line

Epigenetic changes involved in cancer development, unlike genetic changes, are reversible. To date, many literatures concerned that treatment with DNMT and HDAC inhibitors resulted in the demethylation of TSGs and corresponding gene re-expression. Jung and colleagues (2007) reported that DNMT1-targeted inhibition induced the re-expression and reversed DNA methylation of *CDKN2A*,

RASSF1A, *HTLF*, *RUNX3*, and *AKAP12B*, and 5-Aza-dC reactivated and demethylated all these genes. Treatment with 5-Aza-dC along or combined with trichostatin A (TSA) could result in demethylation of the *RASSF1A* promoter and re-expression of RASSF1A transcripts in cell lines derived from melanomas, renal rhabdoid, and medulloblastoma (Reifenberger et al. 2004; Mühlisch et al. 2006). Cantor et al. (2007) found the suppression of small tumor growth and the restoration of some TSGs expression, including *RASSF1A* in lung cancer by injection of 5-Aza-dC / TSA in nude mice.

In this present study, we demonstrated *RASSF1A* mRNA expression was coincident with the *RASSF1A* promoter methylation in MDA-MB-330 cell line. For this cell line, which was close to complete methylated in *RASSF1A* promoter, displayed noticeable increasing of *RASSF1A* mRNA expression (up to 840 times) correlated to the extent of *RASSF1A* demethylation (about 50%) with 5-Aza-dC treatment alone. While HDAC inhibitor TSA, combined with 5-Aza-dC or alone, affected neither methylation nor expression.

5-Aza-dC has shown impressive effects *in vitro*, eliciting antiproliferative effects on numerous tumor cell lines, including those of the bladder, lung, breast, colon, and pancreas (Lyons et al. 2003). For HDAC inhibitor TSA, it could down-regulate DNMT1 mRNA and protein expression (Januchowski et al. 2007) in some cell lines, and associated with a significant decrease in global methylation (Ou et al. 2007). However, the genome-wide demethylation induced by TSA does not affect all methylated tumor suppressor genes equally suggesting that induction of acetylation and demethylation by TSA shows some gene selectivity. Taken together with our data, this kind of selectivity of TSA not only existed in some genes but also in some cell lines, and the influence on demethylation of TSA might be depended on the original gene methylation status and extent.

4.6 Conclusion

In our study, we detected the methylation of 32 CpG site in *RASSF1A* promoter region in 181 breast cancer patients (including 49 paired tumor-normal neighbor tissues) by using pyrosequencing methylation assay. Furthermore, we performed demethylation treatment with 5-Aza-dC and TSA in breast cancer cell line MDA-MB-330 and control cell line Hela to verify the role of methylation on the

expression of *RASSF1A* mRNA. Our conclusions were:

1) There was a high frequency of *RASSF1A* promoter hypermethylation in breast cancer tissues. The methylation level was correlated with tumor size, histological grad and estrogen receptor.

2) A certain extent of *RASSF1A* promoter methylation could be detected in normal adjacent breast cancer tissues, but it was lower than tumor tissues notably. The methylation level in normal tissues had a correlation with disease recurrence, patient age, and primary tumor size. The change of methylation level between tumor and adjacent normal tissue in each CpG site in paired patient was not site specific and relevant to the Lymph node metastases and recurrence.

3) *RASSF1A* promoter methylation might be an age-related phenomenon.

4) In cell line MDA-MB-330, the lost of *RASSF1A* expression can be recovered by 5-Aza-dC and demethylation of *RASSF1A* was confirmed.

5 Summary

We performed a retrospective survey of Ras association domain family 1 isoform A (*RASSF1A*) methylation among 181 breast cancer patients (including 49 paired tumor/normal neighbor samples) with pyrosequencing methylation assay in the promoter region.

There was a high frequency of hypermethylation in the promoter region of *RASSF1A* gene in breast tumor tissues. Significant increased methylation level in breast tumor tissues were observed in tumor > 2cm, estrogen receptor (ER)-positive tumor, and low histological grade of tumor. We also observed *RASSF1A* methylation to some degree in normal adjacent breast tissues in paired samples. The methylation level as baseline in normal adjacent tissues also increased with the patient's age and primary tumor size. The increase of methylation between paired normal neighbor tissue and tumor is significantly related to cancer recurrent and this increase is significantly more common in the lymph node negative group. No site specificity of the cytosin-phospho-guanine (CpG) islands was observed for this increase.

Cell line MDA-MB-330 which is hypermethylated at the promoter region of *RASSF1A* was used as an *in vitro* model to confirm the methylation mechanism. The lost of *RASSF1A* expression can be recovered by methylation inhibitor 5-aza-2-deoxycytidine (5-Aza-dC) after 72 hours treatment.

6 References

Agathanggelou A, Cooper WN, Latif F: Role of the Ras-association domain family 1 tumor suppressor gene in human cancers. Cancer Res 65: 3497-3508 (2005)

Ahuja N, Li Q, Mohan AL, Baylin SB, Issa JP: Aging and DNA methylation in colorectal mucosa and cancer. Cancer Res 58: 5489-5494 (1998)

Baylin SB: DNA methylation and gene silencing in cancer. Nat Clin Pract Oncol 2: S4-S11 (2005)

Bestor T, Laudano A, Mattaliano R, Ingram V: Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. J Mol Biol 203: 971-983 (1988)

Bird A: DNA methylation patterns and epigenetic memory. Genes Dev 16: 6-21 (2002)

Brena RM, Auer H, Kornacker K, Hackanson B, Raval A, Byrd JC, Plass C: Accurate quantification of DNA methylation using combined bisulfite restriction analysis coupled with the Agilent 2100 Bioanalyzer platform. Nucleic Acids Res 34: e17 (2006)

Burbee DG, Forgacs E, Zöchbauer-Müller S, Shivakumar L, Fong K, Gao B, Randle D, Kondo M, Virmani A, Bader S, Sekido Y, Latif F, Milchgrub S, Toyooka S, Gazdar AF, Lerman MI, Zabarovsky E, White M, Minna JD: Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J Natl Cancer Inst 93: 691-699 (2001).

Cantor JP, Iliopoulos D, Rao AS, Druck T, Semba S, Han SY, McCorkell KA, Lakshman TV, Collins JE, Wachsberger P, Friedberg JS, Huebner K: Epigenetic modulation of endogenous tumor suppressor expression in lung cancer xenografts suppresses tumorigenicity. Int J Cancer 120: 24-31 (2007)

Catania J, Fairweather DS: DNA methylation and cellular ageing. Mutat Res 256: 283-293 (1991)

Chen CM, Chen HL, Hsiau TH, Hsiau AH, Shi H, Brock GJ, Wei SH, Caldwell CW, Yan PS, Huang TH: Methylation target array for rapid analysis of CpG island hypermethylation in multiple tissue genomes. Am J Pathol 163: 37-45 (2003)

Chen H, Suzuki M, Nakamura Y, Ohira M, Ando S, Iida T, Nakajima T, Nakagawara A, Kimura H: Aberrant methylation of RASGRF2 and RASSF1A in human non-small cell lung cancer. Oncol Rep 15: 1281-1285 (2006)

Clark SJ, Harrison J, Paul CL, Frommer M: High sensitivity mapping of methylated cytosines. Nucleic Acids Res 22: 2990-2997 (1994)

Clarke RB, Howell A, Potten CS, Anderson E: Dissociation between steroid receptor expression and cell proliferation in the human breast. Cancer Res 57: 4987-4991 (1997)

Claus EB, Schildkraut JM, Thompson WD, Risch NJ: The genetic attributable risk of breast and ovarian cancer. Cancer 77: 2318–2324 (1996)

Clemons M, Goss P: Estrogen and the risk of breast cancer. N Engl J Med 344: 276-285 (2001)

Cohen Y, Singer G, Lavie O, Dong SM, Beller U, Sidransky D: The RASSF1A tumor suppressor gene is commonly inactivated in adenocarcinoma of the uterine cervix. Clin Cancer Res 9: 2981-2984 (2003)

Colella S, Shen L, Baggerly KA, Issa JP, Krahe R: Sensitive and quantitative universal Pyrosequencing methylation analysis of CpG sites. Biotechniques 35: 146-150 (2003)

Collaborative Group on Hormonal Factors in Breast Cancer: Familial breast cancer: collaborative reanalysis of individual data from 52 epidemiological studies including 58,209 women with breast cancer and 101,986 women without the disease. Lancet 358: 1389–1399 (2001)

Costello JF, Frühwald MC, Smiraglia DJ, Rush LJ, Robertson GP, Gao X, Wright FA, Feramisco JD, Peltomäki P, Lang JC, Schuller DE, Yu L, Bloomfield CD, Caligiuri MA, Yates A, Nishikawa R, Su Huang H, Petrelli NJ, Zhang X, O'Dorisio

MS, Held WA, Cavenee WK, Plass C: Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nat Genet 24: 132-138 (2000)

Cottrell SE, Distler J, Goodman NS, Mooney SH, Kluth A, Olek A, Schwope I, Tetzner R, Ziebarth H, Berlin K: A real-time PCR assay for DNA-methylation using methylation-specific blockers. Nucleic Acids Res 32: e10 (2004)

Dahl C, Guldberg P: A ligation assay for multiplex analysis of CpG methylation using bisulfite-treated DNA. Nucleic Acids Res 35: e144 (2007)

Dallol A, Agathanggelou A, Fenton SL, Ahmed-Choudhury J, Hesson L, Vos MD, Clark GJ, Downward J, Maher ER, Latif F: RASSF1A interacts with microtubule-associated proteins and modulates microtubule dynamics. Cancer Res 64: 4112-4116 (2004)

Dallol A, Agathanggelou A, Tommasi S, Pfeifer GP, Maher ER, Latif F: Involvement of the RASSF1A tumor suppressor gene in controlling cell migration. Cancer Res 65: 7653-7659 (2005)

Dammann R, Li C, Yoon JH,Chin PL, Bates S, Pfeifer GP: Epigenetic inactivation of a RAS association domain family protein from the lung tumor suppressor locus 3p21.3. Nat Genet 25: 315-319 (2000)

Dammann R, Takahashi T, Pfeifer GP: The CpG island of the novel tumor suppressor gene RASSF1A is intensely methylated in primary small cell lung carcinomas. Oncogene 20: 3563-3567 (2001)

Dammann R, Yang G, Pfeifer GP: Hypermethylation of the cpG island of Ras association domain family 1A (RASSF1A), a putative tumor suppressor gene from the 3p21.3 locus, occurs in a large percentage of human breast cancers. Cancer Res 61: 3105-3109 (2001)

Dammann R, Schagdarsurengin U, Liu L, Otto N, Gimm O, Dralle H, Boehm BO, Pfeifer GP, Hoang-Vu C: Frequent RASSF1A promoter hypermethylation and K-ras mutations in pancreatic carcinoma. Oncogene 22: 3806-3812 (2003)

Dhawan D, Hamdy FC, Rehman I, Patterson J, Cross SS, Feeley KM, Stephenson Y, Meuth M, Catto JW: Evidence for the early onset of aberrant promoter methylation in urothelial carcinoma. J Pathol 209: 336-343 (2006)

Dong SM, Lee EJ, Jeon ES, Park CK, Kim KM: Progressive methylation during the serrated neoplasia pathway of the colorectum. Mod Pathol 18: 170-178 (2005)

Donninger H, Vos MD, Clark GJ: The RASSF1A tumor suppressor. J Cell Sci 120: 3163-3172 (2007)

Dulaimi E, Ibanez de Caceres I, Uzzo RG, Al-Saleem T, Greenberg RE, Polascik TJ, Babb JS, Grizzle WE, Cairns P: Promoter hypermethylation profile of kidney cancer. Clin Cancer Res 10: 3972-3979 (2004)

Dunn BK: Hypomethylation: one side of a larger picture. Ann NY Acad Sci 983: 28-42 (2003)

Dupont JM, Tost J, Jammes H, Gut IG: De novo quantitative bisulfite sequencing using the pyrosequencing technology. Anal Biochem 333: 119-127 (2004)

Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, Danenberg PV, Laird PW: MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 28: E32 (2000)

Elston CW, Ellis IO: Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 19: 403-410 (1991)

Esteller M, Herman JG: Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours. J Pathol 196: 1-7 (2002)

Fackler MJ, McVeigh M, Evron E, Garrett E, Mehrotra J, Polyak K, Sukumar S, Argani P: DNA methylation of RASSF1A, HIN-1, RAR-beta, Cyclin D2 and Twist *in situ* and invasive lobular breast carcinoma. Int J Cancer 107: 970-975 (2003)

Fackler MJ, McVeigh M, Mehrotra J, Blum MA, Lange J, Lapides A, Garrett E, Argani P, Sukumar S: Quantitative multiplex methylation-specific PCR assay for

the detection of promoter hypermethylation in multiple genes in breast cancer. Cancer Res 64: 4442-4452 (2004)

Falvella FS, Manenti G, Spinola M, Pignatiello C, Conti B, Pastorino U, Dragani TA: Identification of RASSF8 as a candidate lung tumor suppressor gene. Oncogene 25: 3934-3938 (2006)

Farrell WE: Epigenetic mechanisms of tumorigenesis. Horm Metab Res 37: 361-368 (2005)

Feinberg AP, Ohlsson R, Henikoff S: The epigenetic progenitor origin of human cancer. Nat Rev Genet 7: 21-33 (2006)

Feltus FA, Lee EK, Costello JF, Plass C, Vertino PM: DNA motifs associated with aberrant CpG island methylation. Genomics 87: 572-579 (2006)

Feng W, Shen L, Wen S, Rosen DG, Jelinek J, Hu X, Huan S, Huang M, Liu J, Sahin AA, Hunt KK, Bast RC Jr, Shen Y, Issa JP, Yu Y: Correlation between CpG methylation profiles and hormone receptor status in breast cancers. Breast Cancer Res 9: R57 (2007)

Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P: Estimates of the cancer incidence and mortality in Europe in 2006. Ann Oncol 18: 581-592 (2007)

Fishman J, Osborne MP, Telang NT: The role of estrogen in mammary carcinogenesis. Ann NY Acad Sci 768: 91-100 (1995)

Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL: A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in indicidual DNA strands. Proc Natl Acad Sci. USA 89: 1827-1831 (1992)

Gitan RS, Shi H, Yan PS, Huang TH: Methylation-specific oligonucleotide microarray: a new potential for high-throughput methylation analysis. Genome Res 12: 158-164 (2001)

Goldhirsch A, Glick JH, Gelber RD, Coates AS, Thürlimann B, Senn HJ: Panel members: Meeting highlights: international expert consensus on the primary

therapy of early breast cancer 2005. Ann Oncol 16: 1569-1583 (2005)

Gonzalgo ML, Jones PA: Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). Nucleic Acids Res 25: 2529–2531 (1997)

Guan XY, Xu J, Anzick SL, Zhang H, Trent JM, Meltzer PS: Hybrid selection of transcribed sequences from microdissected DNA: isolation of genes within amplified region at 20q11-q13.2 in breast cancer. Cancer Res 56: 3446-3450 (1996)

Hanahan D, Weinberg RA: The hallmarks of cancer. Cell 100: 57-70 (2000)

Hanstein B, Djahansouzi S, Dall P, Beckmann MW, Bender HG: Insights into the molecular biology of the estrogen receptor define novel therapeutic targets for breast cancer. Eur J Endocrinol 150: 243-255 (2004)

Hashimshony T, Zhang J, Keshet I, Bustin M, Cedar H: The role of DNA methylation in setting up chromatin structure during development. Nat Genet 34: 187-192 (2003)

Hayatsu H, Wataya Y, Kai K, Iida S: Reaction of sodium bisulfite with uracil, cytosine, and their derivatives. Biochemistry 9: 2858-2865 (1970)

Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci. USA 93: 9821-9826 (1996)

Herman JG, Baylin SB: Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 349: 2042-2054 (2003)

Honorio S, Agathanggelou A, Schuermann M, Pankow W, Viacava P, Maher ER, Latif F: Detection of RASSF1A aberrant promoter hypermethylation in sputum from chronic smokers and ductal carcinoma in situ from breast cancer patients. Oncogene 22: 147-150 (2003)

Honorio S, Agathanggelou A, Wernert N, Rothe M, Maher ER, Latif F: Frequent epigenetic inactivation of the RASSF1A tumour suppressor gene in testicular

tumours and distinct methylation profiles of seminoma and nonseminoma testicular germ cell tumours. Oncogene 22: 461-466 (2003)

Hortobagyi GN: Treatment of breast cancer. N Engl J Med. 339: 974-984 (1998)

House MG, Herman JG, Guo MZ, Hooker CM, Schulick RD, Lillemoe KD, Cameron JL, Hruban RH, Maitra A, Yeo CJ: Aberrant hypermethylation of tumor suppressor genes in pancreatic endocrine neoplasms. Ann Surg 238: 423-431 (2003)

Hwang E, Ryu KS, Pääkkönen K, Güntert P, Cheong HK, Lim DS, Lee JO, Jeon YH, Cheong C: Structural insight into dimeric interaction of the SARAH domains from Mst1 and RASSF family proteins in the apoptosis pathway. Proc Natl Acad Sci USA 104: 9236-9241 (2007)

Iida A, Isobe R, Yoshimoto M, Kasumi F, Nakamura Y, Emi M: Localization of a breast cancer tumour-suppressor gene to a 3-cM interval within chromosomal region 16q22. Br J Cancer 75: 264-267 (1997)

Inda MD, Castresana JS: RASSF1A promoter is highly methylated in primitive neuroectodermal tumors of the central nervous system. Neuropathology 27: 341-346 (2007)

Ingvarsson S: Molecular genetics of breast cancer progression. Semin Cancer Biol 9: 277-288 (1999)

Issa JP: Aging, DNA methylation and cancer. Crit Rev Oncol Hematol 32: 31-43 (1999)

Issa JP: CpG-island methylation in aging and cancer. Curr Top Microbiol Immunol 249: 101-118 (2000)

Januchowski R, Dabrowski M, Ofori H, Jagodzinski PP: Trichostatin A down-regulate DNA methyltransferase 1 in Jurkat T cells. Cancer Lett 246: 313-317 (2007)

Jo H, Kim JW, Kang GH, Park NH, Song YS, Kang SB, Lee HP: Association of promoter hypermethylation of the RASSF1A gene with prognostic parameters in endometrial cancer. Oncol Res 16: 205-209 (2006)

Jones PA. Takai D: The role of DNA methylation in mammalian epigenetics. Science 293: 1068-1070 (2001)

Jones PA, Baylin SB: The fundamental role of epigenetic events in cancer. Nat Rev Genet 3: 415-428 (2002)

Jung Y, Park J, Kim TY, Park JH, Jong HS, Im SA, Robertson KD, Bang YJ, Kim TY: Potential advantages of DNA methyltransferase 1 (DNMT1)-targeted inhibition for cancer therapy. J Mol Med 85: 1137-1148 (2007)

Kisseljova NP, Kisseljov FL: DNA demethylation and carcinogenesis. Biochemistry (Mosc) 70: 743-752 (2005)

Krop I, Maguire P, Lahti-Domenici J, Lodeiro G, Richardson A, Johannsdottir HK, Nevanlinna H, Borg A, Gelman R, Barkardottir RB, Lindblom A, Polyak K: Lack of HIN-1 methylation in BRCA1-linked and "BRCA1-like" breast tumors. Cancer Res 63: 2024-2027 (2003)

Kuzmin I, Gillespie JW, Protopopov A, Geil L, Dreijerink K, Yang Y, Vocke CD, Duh FM, Zabarovsky E, Minna JD, Rhim JS, Emmert-Buck MR, Linehan WM, Lerman MI: The RASSF1A tumor suppressor gene is inactivated in prostate tumors and suppresses growth of prostate carcinoma cells. Cancer Res 62: 3498-3502 (2002)

Kwabi-Addo B, Chung W, Shen L, Ittmann M, Wheeler T, Jelinek J, Issa JP: Age-related DNA methylation changes in normal human prostate tissues. Clin Cancer Res 13: 3796-3802 (2007)

Laake K, Launonen V, Niederacher D, Gudlaugsdottir S, Seitz S, Rio P, Champème MH, Bièche I, Birnbaum D, White G, Sztan M, Sever N, Plummer S, Osorio A, Broeks A, Huusko P, Spurr N, Borg A, Cleton-Jansen AM, van't Veer L, Benitez J, Casey G, Peterlin B, Olah E, Børresen-Dale AL, et al.: Loss of heterozygosity at 11q23.1 and survival in breast cancer: results of a large European study. Breast Cancer Somatic Genetics Consortium. Genes Chromosomes Canc 25: 212-221 (1999)

Laird PW, Jaenisch R: The role of DNA methylation in cancer genetic and epigenetics. Annu Rev Genet 30: 441-464 (1996)

Lehmann U, Länger F, Feist H, Glöckner S, Hasemeier B, Kreipe H: Quantitative assessment of promoter hypermethylation during breast cancer development. Am J Pathol 160: 605-612 (2002)

Lehmann U, Celikkaya G, Hasemeier B, Länger F, Kreipe H: Promoter hypermethylation of the death-associated protein kinase gene in breast cancer is associated with the invasive lobular subtype. Cancer Res 62: 6634-6638 (2002)

Lerman MI, Minna JD: The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium. Cancer Res 60: 6116-6133 (2000)

Lewis CM, Cler LR, Bu DW, Zöchbauer-Müller S, Milchgrub S, Naftalis EZ, Leitch AM, Minna JD, Euhus DM: Promoter hypermethylation in benign breast epithelium in relation to predicted breast cancer risk. Clin Cancer Res 11: 166-172 (2005)

Liu L, Yoon JH, Dammann R, Pfeifer GP: Frequent hypermethylation of the RASSF1A gene in prostate cancer. Oncogene 21: 6835-6840 (2002)

Liu L, Tommasi S, Lee DH, Dammann R, Pfeifer GP: Control of microtubule stability by the RASSF1A tumor suppressor. Oncogene 22: 8125-8136 (2003)

Liu L, Broaddus RR, Yao JC, Xie S, White JA, Wu TT, Hamilton SR, Rashid A: Epigenetic alterations in neuroendocrine tumors: methylation of RAS-association domain family 1, isoform A and p16 genes are associated with metastasis. Mod Pathol 18: 1632-1640 (2005)

Luger K, Mäder AW, Richmond RK, Sargent DF, Richmond TJ: Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 389: 251-260 (1997)

Lyons J, Bayar E, Fine G, McCullar M, Rolens R, Rubinfeld J, Rosenfeld C: Decitabine: development of a DNA methyltransferase inhibitor for hematological malignancies. Curr Opin Investig Drugs 4: 1442-1450 (2003)

Maat W, van der Velden PA, Out-Luiting C, Plug M, Dirks-Mulder A, Jager MJ, Gruis NA: Epigenetic inactivation of RASSF1a in uveal melanoma. Invest Ophthalmol Vis Sci 48: 486-490 (2007)

Maitra A, Wistuba H, Washington C, Virmani AK, Ashfag R, Milchgrub S, Gazdar AF, Minna JD: High-resolution chromosome 3p allelotyping of breast carcinomas and precursor lesions demonstrates frequent loss of heterozygosity and a discontinuous pattern of allele loss. Am J Pathol 159: 119-130 (2001)

Mehrotra J, Vali M, McVeigh M, Kominsky SL, Fackler MJ, Lahti-Domenici J, Polyak K, Sacchi N, Garrett-Mayer E, Argani P, Sukumar S: Very high frequency of hypermethylated genes in breast cancer metastasis to the bone, brain, and lung. Clin Cancer Res 10: 3104-3109 (2004)

Michor F, Iwasa Y, Nowak MA: Dynamics of cancer progression. Nature Rev. Cancer 4: 197-205 (2004)

Mielnicki LM, Asch HL, Asch BB: Genes, chromatin, and breast cancer: an epigenetic tale. J Mammary Gland Biol Neoplasia 6: 169-182 (2001)

Miller BJ, Wang D, Krahe R, Wright FA: Pooled analysis of loss of hererozygosity in breast cancer: a genome scan provides comparative evidence for multiple tumor suppressors and identifies novel candidate regions. Am J Hum Genet 73: 748-767 (2003)

Miranda E, Destro A, Malesci A, Balladore E, Bianchi P, Baryshnikova E, Franchi G, Morenghi E, Laghi L, Gennari L, Roncalli M: Genetic and epigenetic changes in primary metastatic and nonmetastatic colorectal cancer. Br J Cancer 95: 1101-1107 (2006)

Mühlisch J, Schwering A, Grotzer M, Vince GH, Roggendorf W, Hagemann C, Sörensen N, Rickert CH, Osada N, Jürgens H, Frühwald MC: Epigenetic repression of RASSF1A but not CASP8 in supratentorial PNET (sPNET) and atypical teratoid/rhabdoid tumors (AT/RT) of childhood. Oncogene 25: 1111-1117 (2006)

Narayan A, Ji W, Zhang XY, Marrogi A, Graff JR, Baylin SB, Ehrlich M: Hypomethylation of pericentromeric DNA in breast adenocarcinomas. Int J Cancer 77: 833-838 (1998)

Neuhausen A, Florl AR, Grimm MO, Schulz WA: DNA methylation alterations in urothelial carcinoma. Cancer Biol Ther 5: 993-1001 (2006)

Newton AC: Protein kinase C. Seeing two domains. Curr Biol 5: 973-976 (1995)

Niwa Y, Oyama T, Nakajima T: BRCA1 expression status in relation to DNA methylation of the BRCA1 promoter region in sporadic breast cancers. Jpn J Cancer Res 91: 519-526 (2000)

Norton L: Conceptual and practical implications of breast tissue geometry: toward a more effective, less toxic therapy. Oncologist 10: 370-381 (2005)

Oliveira C, Velho S, Domingo E, Preto A, Hofstra RM, Hamelin R, Yamamoto H, Seruca R, Schwartz S Jr: Concomitant RASSF1A hypermethylation and KRAS/BRAF mutations occur preferentially in MSI sporadic colorectal cancer. Oncogene 24: 7630-7634 (2005)

Ou JN, Torrisani J, Unterberger A, Provençal N, Shikimi K, Karimi M, Ekström TJ, Szyf M: Histone deacetylase inhibitor Trichostatin A induces global and gene-specific DNA demethylation in human cancer cell lines. Biochem Pharmacol 73: 1297-1307 (2007)

Park SY, Kim BH, Kim JH, Cho NY, Choi M, Yu EJ, Lee S, Kang GH: Methylation profiles of CpG island loci in major types of human cancers. J Korean Med Sci 22: 311-317 (2007)

Pasquali L, Bedeir A, Ringquist S, Styche A, Bhargava R, Trucco G: Quantification of CpG island methylation in progressive breast lesions from normal to invasive carcinoma. Cancer Lett 257: 136-144 (2007)

Pu RT, Laitala LE, Alli PM, Fackler MJ, Sukumar S, Clark DP: Methylation profiling of benign and malignant breast lesions and its application to cytopathology. Mod Pathol 16: 1095-1101 (2003)

Reifenberger J, Knobbe CB, Sterzinger AA, Blaschke B, Schulte KW, Ruzicka T, Reifenberger G: Frequent alterations of Ras signaling pathway genes in sporadic malignant melanomas. Int J Cancer 109: 377-384 (2004)

Richardson B: Impact of aging on DNA methylation. Ageing Res Rev 2: 245-261 (2003)

Rodenhiser D, Mann M: Epigenetics and human disease: translating basic biology into clinical applications. CMAJ 174: 341-348 (2006)

Ronaghi M, Karamohamed S, Pettersson B, Uhlen M, Nyren P: Real-time DNA sequencing using detection of pyrophosphate release. Anal Biochem 242: 84-89 (1996)

Ross JS, Linette GP, Stec J, Clark E, Ayers M, Leschly N, Symmans WF, Hortobagyi GN, Pusztai L: Breast cancer biomarkers and molecular medicine: part II. Expert Rev Mol Diagn 3: 573-585 (2003)

Santini V, Kantarjian HM, Issa JP: Changes in DNA methylation in neoplasia: pathophysiology and therapeutic implications. Ann Intern Med 134: 573-586 (2001)

Schagdarsurengin U, Wilkens L, Steinemann D, Flemming P, Kreipe HH, Pfeifer GP, Schlegelberger B, Dammann R: Frequent epigenetic inactivation of the RASSF1A gene in hepatocellular carcinoma. Oncogene 22: 1866-1871 (2003)

Schagdarsurengin U, Gimm O, Dralle H, Hoang-Vu C, Dammann R: CpG island methylation of tumor-related promoters occurs preferentially in undifferentiated carcinoma. Thyroid 16: 633-642 (2006)

Sekido Y, Ahmadian M, Wistuba II, Latif F, Bader S, Wei MH, Duh FM, Gazdar AF, Lerman MI, Minna JD: Cloning of a breast cancer homozygous deletion junction narrows the region of search for a 3p21.3 tumor suppressor gene. Oncogene 16: 3151-3157 (1998)

Shapiro R, Weisgras JM: Bisulfite-catalyzed transamination of cytosine and cytidine. Biochem Biophy Res Commun 40: 839-843 (1970)

Shinozaki M, Hoon DS, Giuliano AE, Hansen NM, Wang HJ, Turner R, Taback B: Distinct hypermethylation profile of primary breast cancer is associated with sentinel lymph node metastasis. Clin Cancer Res 11: 2156-2162 (2005)

Shivakumar L, Minna J, Sakamaki T, Pestell R, White MA: The RASSF1A tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation. Mol Cell Biol 22: 4309-4318 (2002)

Sinclair CS, Rowley M, Naderi A, Couch FJ: The 17q23 amplicon and breast cancer. Breast Cancer Res Treat 78: 313–322 (2003)

Slattery ML, Kerber RA: A comprehensive evaluation of family history and breast cancer risk. The Utah Population Database. JAMA 270: 1563–1568 (1993)

Song MS, Chang JS, Song SJ, Yang TH, Lee H, Lim DS: The centrosomal protein RAS association domain family protein 1A (RASSF1A)-binding protein 1 regulates mitotic progression by recruiting RASSF1A to spindle poles. J Biol Chem 280: 3920-3927 (2005)

Strahl BD, Allis CD: The language of covalent histone modifications. Nature 403: 41-45 (2000)

Strunnikova M, Schagdarsurengin U, Kehlen A, Garbe JC, Stampfer MR, Dammann R: Chromatin inactivation precedes de novo DNA methylation during the progressive epigenetic silencing of the RASSF1A promoter. Mol Cell Biol 25: 3923-3933 (2005)

Szyf M: The DNA methylation machinery as a therapeutic target. Curr Drug Targets 1: 101-118 (2000)

Szyf M, Pakneshan P, Rabbani SA: DNA methylation and breast cancer. Biochem Pharmacol 68: 1187-1197 (2004)

Tommasi S, Dammann R, Zhang Z, Wang Y, Liu L, Tsark WM, Wilczynski SP, Li J, You M, Pfeifer GP: Tumor susceptibility of Rassf1a knockout mice. Cancer Res 65: 92-98 (2005)

Tost J, Dunker J, Gut IG: Analysis and quantification of multiple methylation variable positions in CpG islands by Pyrosequencing. Biotechniques 35: 152-156 (2003)

Turker MS: Gene silencing in mammalian cells and the spread of DNA methylation.

Oncogene 21: 5388-5393 (2002)

Uhlmann K, Brinckmann A, Toliat MR, Ritter H, Nurnberg P: Evaluation of a potential epigenetic biomarker by quantitative methyl-single nucleotide polymorphism analysis. Electrophoresis 23: 4072-4079 (2002)

van der Weyden L, Tachibana KK, Gonzalez MA, Adams DJ, Ng BL, Petty R, Venkitaraman AR, Arends MJ, Bradley A: The RASSF1A isoform of RASSF1 promotes microtubule stability and suppresses tumorigenesis. Mol Cell Biol 25: 8356-8367 (2005)

van der Weyden L, Adams DJ: The Ras-association domain family (RASSF) members and their role in human tumourigenesis. Biochim Biophys Acta 1776: 58-85 (2007)

Vanyushin BF, Tkacheva SG, Belozersky AN: Rare bases in animal DNA. Nature 225: 948-949 (1970)

Vertino PM, Yen RW, Gao J, Baylin SB: De novo methylation of CpG island sequences in human fibroblasts overexpressing DNA (cytosine-5-) -methyltransferase. Mol Cell Biol 16: 4555-4565 (1996)

Vos MD, Ellis CA, Bell A, Birrer MJ, Clark GJ: Ras uses the novel tumor suppressor RASSF1 as an effector to mediate apoptosis. J Biol Chem 275: 3569-3572 (2000)

Vos MD, Dallol A, Eckfeld K, Allen NP, Donninger H, Hesson LB, Calvisi D, Latif F, Clark GJ: The RASSF1A tumor suppressor activates Bax via MOAP-1. J Biol Chem 281: 4557-4563 (2006)

Whang YM, Kim YH, Kim JS, Yoo YD: RASSF1A suppresses the c-Jun-NH2-kinase pathway and inhibits cell cycle progression. Cancer Res 65: 3682-3690 (2005)

Widschwendter M, Jones PA: DNA methylation and breast carcinogenesis. Oncogene 21: 5462-5482 (2002)

Widschwendter M, Siegmund KD, Müller HM, Fiegl H, Marth C, Müller-Holzner E, Jones PA, Laird PW: Association of breast cancer DNA methylation profiles with

hormone receptor status and response to tamoxifen. Cancer Res 64: 3807-3813 (2004)

Wischnewski F, Pantel K, Schwarzenbach H: Promoter demethylation and histone acetylation mediate gene expression of MAGE-A1, -A2, -A3, and -A12 in human cancer cells. Mol Cancer Res 4: 339-349 (2006)

Wolffe AP: Packaging principle: how DNA methylation and histone acetylation control the transcriptional activity of chromatin. J Exp Zool 282: 239-244 (1998)

Xiong Z, Laird PW: COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res 25: 2532–2534 (1997)

Yan PS, Shi H, Rahmatpanah F, Hsiau TH, Hsiau AH, Leu YW, Liu JC, Huang TH: Differential distribution of DNA methylation within the RASSF1A CpG island in breast cancer. Cancer Res 63: 6178-6186 (2003)

Yan PS, Venkataramu C, Ibrahim A, Liu JC, Shen RZ, Diaz NM, Centeno B, Weber F, Leu YW, Shapiro CL, Eng C, Yeatman TJ, Huang TH: Mapping geographic zones of cancer risk with epigenetic biomarkers in normal breast tissue. Clin Cancer Res 12: 6626-6636 (2006)

Yang Q, Yoshimura G, Mori I, Sakurai T, Kakudo K: Chromosome 3p and breast cancer. J Hum Genet 47: 453-459 (2002)

Yang X, Ferguson AT, Nass SJ, Phillips DL, Butash KA, Wang SM, Herman JG, Davidson NE: Transcriptional activation of estrogen receptor alpha in human breast cancer cells by histone deacetylase inhibition. Cancer Res 60: 6890-6894 (2000)

Yang X, Phillips DL, Ferguson AT, Nelson WG, Herman JG, Davidson NE: Synergistic activation of functional estrogen receptor (ER)-alpha by DNA methyltransferase and histone deacetylase inhibition in human ER-alpha-negative breast cancer cells. Cancer Res 61: 7025-7029 (2001)

Yang X, Yan L, Davidson NE: DNA methylation in breast cancer. Endocr Relat Cancer 8: 115-127 (2001)

Yaremko ML, Kutza C, Lyzak J, Mick R, Recant WM, Westbrook CA: Loss of heterozygosity from the short arm of chromosome 8 is associated with invasive behavior in breast cancer. Genes Chromosomes Canc 16: 189-195 (1996)

Yeo W, Wong WL, Wong N, Law BK, Tse GM, Zhong S: High frequency of promoter hypermethylation of RASSF1A in tumorous and non-tumourous tissue of breast cancer. Pathology 37: 125-130 (2005)

Yoo CB, Jones PA: Epigenetic therapy of cancer: past, present and future. Nat Rev Drug Discov 5: 37-50 (2006)

Yoon JH, Dammann R, Pfeifer GP: Hypermethylation of the CpG island of the RASSF1A gene in ovarian and renal cell carcinomas. Int J Cancer 94: 212-217 (2001)

Zhang HY, Rumilla KM, Jin L, Nakamura N, Stilling GA, Ruebel KH, Hobday TJ, Erlichman C, Erickson LA, Lloyd RV: Association of DNA methylation and epigenetic inactivation of RASSF1A and beta-catenin with metastasis in small bowel carcinoid tumors. Endocrine 30: 299-306 (2006)

Zhong S, Yeo W, Tang MW, Wong N, Lai PB, Johnson PJ: Intensive hypermethylation of the CpG island of Ras association domain family 1A in hepatitis B virus-associated hepatocellular carcinomas. Clin Cancer Res 9: 3376-3382 (2003)

Zhou L, Jiang W, Ren C, Yin Z, Feng X, Liu W, Tao Q, Yao K: Frequent hypermethylation of RASSF1A and TSLC1, and high viral load of Epstein-Barr Virus DNA in nasopharyngeal carcinoma and matched tumor-adjacent tissues. Neoplasia 7: 809-815 (2005)

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