ANALYSIS OF 45S rDNA PROMOTER METHYLATION AND EXPRESSION OF rRNA TRANSCRIPTS IN BREAST CANCER

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By

Gurbet Karahan

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We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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ABSTRACT

ANALYSIS OF 45S rDNA PROMOTER METHYLATION AND EXPRESSION OF rRNA TRANSCRIPTS IN BREAST CANCER

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Ph.D. in Molecular Biology and Genetics
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July, 2015

Ribosome biogenesis has a central role in cell growth and proliferation that is usually disrupted in tumor cells by the inactivation of tumor suppressor genes and activation of oncogenes. Ribosomal RNA (rRNA) gene expression is one of the most important factors regulating ribosome production, which is controlled by CG rich 45S ribosomal DNA (rDNA) promoter. The effect of DNA methylation at 45S rDNA promoter on rRNA gene expression is a subject of controversy in the literature. In this thesis, a 434 bp region (-380 bp to +54 bp) spanning both upstream control element (UCE) and core promoter located in 45S rDNA promoter containing 54 CpGs was analysed in breast cancer. We also analysed the related rRNA expression levels in the same samples in order to clarify the role of 45S rDNA promoter methylation on rRNA gene expression.

45S rDNA promoter region was highly methylated (74%-96%) in all cell lines including non-tumorigenic breast cell line (MCF10A). Even though 45S rDNA promoter region of breast cancer cell lines are extensively methylated, rRNAs (18S, 28S, 5.8S and 45S ETS) were expressed independent of the heavy methylation. Expression levels of rRNAs are assessed either using housekeeping genes (ACTB, TBP, ACTB&TBP) or geometric mean of rRNAs (GM-rRNAs). We propose GM-rRNA normalization as a new method to identify relative expression differences between rRNA transcripts.

Epigenetic drugs 5-Aza-2'-deoxycytidine (5-AZA) and Trichostatin A (TSA) were used to determine the effect of DNA methylation and histone acetylation on rRNA expression. Demethylation with 5-AZA resulted in an unexpected decrease in the expression of all rRNA. TSA treatment did not lead to any significant expression difference in cell lines.

To better evaluate the effect of DNA methylation on the expression of rRNA transcripts we analysed the methylation status of 19 breast tumor and matched normal frozen tissue samples. The results showed that majority of the tumors (13/19) have significantly higher methylation levels than their normal pairs. Using the GM-rRNA as reference helped us to determine significant differences in the proportionate expression of rRNAs in these tissue samples. The 5.8S rRNA ratio was significantly lower whereas the 18S rRNA ratio was significantly higher in breast tumor samples. Furthermore, the 45S rDNA promoter methylation levels in normal breast tissue samples were negatively correlated with the18S rRNA ratio but this correlation was disrupted in breast tumors. Similarly, rRNA transcript levels were significantly correlated with each other in normal samples, were lost in tumor samples. It is clear that, there is a dysregulation both in rDNA methylation levels and spliced rRNA transcripts specific to breast tumor samples, which was not observed in normal breast tissues. rRNA gene expression is controlled by mechanisms other than promoter DNA methylation. Tumorigenesis may cause disruption of many control mechanisms that are required for proper rRNA expression, splicing and maturation, resulting in a dysregulation of the correlation between spliced rRNA expression levels, which should be investigated further.

Keywords: Breast Cancer, DNA methylation, 45S rDNA promoter, rRNA gene expression

ÖZET

MEME KANSERİNDE 45S rDNA PROMOTÖR METİLASYON VE rRNA TRANSKRİPTLERİNİN İFADE ANALİZİ

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Moleküler Biyoloji ve Genetik Doktora Tezi
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Temmuz, 2015

Tümör hücrelerinde genellikle tümör baskılayıcı genlerin inaktivasyonu ve onkogenlerin aktifleştirilmesi ile bozulan ribozom biyogenezi hücre büyümesi ve bölünmesinde merkezi bir rol oynar. Ribozomal RNA (rRNA) gen ifadesi ribozom üretimini düzenleyen en önemli faktörlerden biridir ve CG'ler bakımından zengin 45S ribozomal DNA (45S rDNA) promotörü tarafından kontrol edilir. 45S rDNA promotörünün rRNA ifadesi üzerindeki etkisi literatürde tartışmalı bir konudur. Bu tezde, 54 CpG içeren, 45S rDNA promotöründe bulunan yukarı kontrol elementini (Upstream control element, UCE) ve çekirdek promotörü (Core promoter, CP) kapsayan 434 bç lik bir bölge meme kanserinde bisülfit dizileme yöntemi kullanılarak analiz edilmiştir. 45S rDNA promotör metilasyonunun rRNA gen ifadesindeki rolünü netleştirmek için aynı örneklerde ilgili rRNA ifade düzeylerini de analiz ettik.

45S rDNA promotör bölgesi tümörijenik olmayan meme hücre hattı (MCF10A) da dahil olmak üzere tüm meme kanseri hücre hatlarında oldukça metillenmiştir. Her ne kadar 45S rDNA promotör bölgesi hücre hatlarında yoğun biçimde metillenmiş olsa da rRNA'lar (18S, 28S, 5.8S ve 45S ETS) bu ağır metilasyondan bağımsız olarak ifade olmuşlardır. rRNA'ların ifade düzeyleri ya referans genleri (ACTB, TBP, ACTB&TBP) yada rRNA'ların geometrik ortalaması (GM-rRNAs) kullanılarak

değerlendirilmiştir. GM-rRNA normalizasyonunu rRNA'lar arasındaki göreceli farklılıkları tanımlamak için yeni bir yöntem olarak öneriyoruz.
DNA metilasyonu ve histon asetilasyonunun rRNA ifadesi üzerindeki etkisini belirlemek için epigenetik ilaçlar 5-Aza-2'-deoksisitidin (5-AZA) ve Trikostatin A (TSA) kullanılmıştır. 5-AZA ile demetilasyon TBP, ACTB veya ACTB&TBP normalizasyonları ile tüm rRNA türlerinde beklenmedik bir azalmayla sonuçlanmıştır. TSA muamelesi hücre hatlarında anlamlı bir ifade farkına sebep olmadı.
DNA metilasyonunun rRNA transkriptlerinin ifadesi üzerindeki etkisini daha iyi değerlendirmek için 19 dondurulmuş meme tümörü ve eş normal doku örneklerinin metilasyon durumları analiz edildi. Sonuçlar tümörlerin çoğunun (13/19) normal eş dokularına göre metilasyon seviyelerinin anlamlı olarak daha yüksek olduğunu gösterdi. Referans gen olarak GM-rRNA'yı kullanmamız bu doku örneklerinde rRNA'ların ifadesindeki anlamlı oransal farklılıkları belirlemek için bize yardımcı olmuştur. Meme kanseri örneklerinde 5.8S rRNA oranı anlamlı olarak düşük iken 18S rRNA oranı anlamlı olarak yüksektir. Ayrıca normal meme dokularında 45S rDNA promotör metilasyon seviyeleri ile 18S rRNA oranı negatif olarak korele ederken bu korelasyon meme tümörlerinde bozulmuştur. Benzer şekilde normal doku örneklerindeki rRNA transkriptlerinin seviyeleri birbirleri ile korele ederken tumor hücrelerinde bu kaybolmuştur. Hem rDNA promotör metilasyon seviyelerinde hem de kırpılan rRNA transkriptlerinde meme tümör örneklerine özgü normal meme dokularında gözlemlenmeyen bir bozulma olduğu açıktır. rRNA gen ifadesinin meme kanserinde promotör DNA metilasyonundan başka mekanizmalar tarafından kontrol edilmektedir. Tümörigenez, düzgün rRNA ifadesi, kırpılması ve olgunlaşması için gerekli birçok kontrol mekanizmasının bozulmasına neden olabilir ve bu kırpılmış rRNA ifade seviyelerindeki korelasyonun bozulmasıyla sonuçlanan durumun daha iyi araştırılması gerekir.

Anahtar sözcükler: Meme kanseri, DNA metilasyonu, 45S rDNA promotörü, rRNA genlerinin ifadesi

I would like to dedicate my thesis to my beloved mother whose love, support and prayers enable me to get through all the challenges of life...

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Abbreviations

rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
45S ETS	45S External transcribed spacer
5-AZA	5-Aza-2'-deoxycytidine
TSA	Trichostatin A
GM-rRNA	Geometric mean of rRNAs (18S, 28S, 5.8S and 45S ETS)
ER	Estrogen receptor
PR	Progesterone receptor
GWAS	Genome-wide association studies
TNM	Tumor-Node-Metastasis
SBR	Scarff-Bloom-Richardson
NORs	Nucleolar organizing regions
Pol I, II and III	RNA Polymerase I, II and III
IGS	Intergenic spacer region
UCE	Upstream control region
CP	Core Promoter
ITS	Internal transcribed spacer
PIC	Pre-initiation complex
UBF	Upstream binding factor
TBP	TATA-binding protein
TAF	TBP-associated factors
TTF-1	Transcription termination factor
CpG	Cytosine followed by a guanine
CGI	CpG island
SAM	*S*-adenosyl L-methionine
DNMT	DNA methyltransferases
HDAC	Histone deacetylase
HAT	Histone acetyltransferase

1 INTRODUCTION

1.1 Breast Cancer

Breast cancer is a type of cancer that develops in breast tissue. Remarkable advances have been made in the diagnosis, prevention and treatment of breast cancer through the clinical application of scientific developments in recent years. Yet breast cancer still affecting the lives of millions worldwide.

Breast cancer is the most common type of cancer among women and is responsible for 25% of all new cases. It was the most frequent cause of cancer deaths in women living in less developed regions (14.3 % of all cancer deaths) and the second most common cause of cancer deaths in more developed regions (15.4% after lung cancer) in 2012 (Figure 1.1) (Ferlay J et al. 2013). Breast cancer is derived either from the lining of ducts carrying milk (ductal carcinoma) or from the milk glands called lobules (lobular carcinoma). Breast carcinoma more frequently arises from ductal tissue and is then called ductal carcinoma (invasive and in situ). Breast cancer occurs in both genders but male breast cancer is very uncommon (http://www.cancer.gov/cancertopics/types/breast).

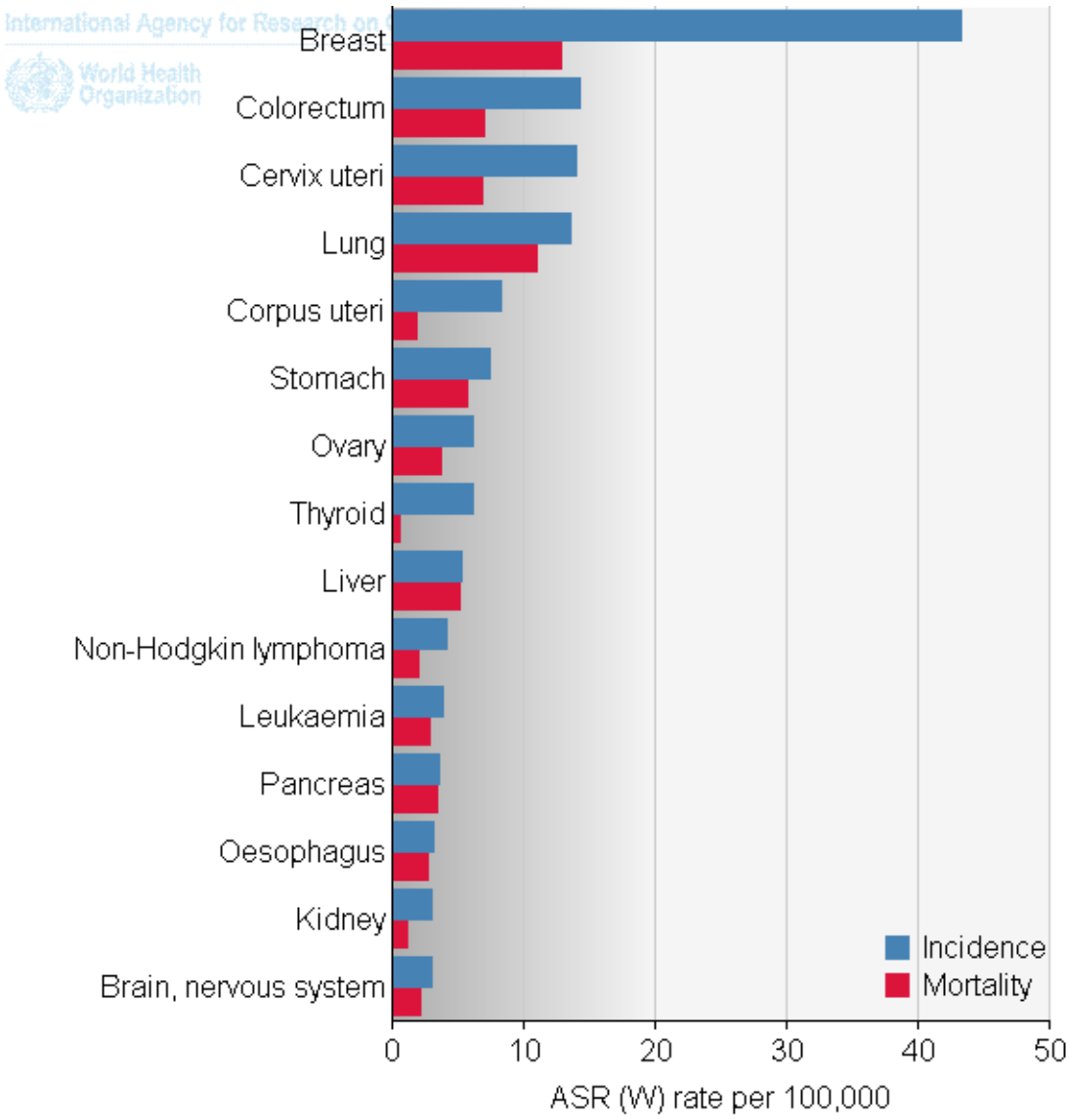


Figure 1.1. Estimated age-standardized incidence and mortality rates of different cancer types in women according to the Globocan 2012 (Ferlay J et al. 2013). Figure taken from (Ferlay J et al. 2013), Copyright (2015) IARC.

1.1.1 Epidemiology and Risk Factors of Breast Cancer

Breast cancer is associated with several risk factors such as age, age of menarche and menopause, age of first pregnancy, life style and family history. The breast cancer incidence increases with age (McPherson et al. 2000).

Menarche at young age and late menopause increase the time of exposure to estrogen as well as the risk of getting breast cancer. Parity and age at first childbirth are also important factors. The breast cancer risk is twice as high in women who had their first child after the age of 30 compared to women who had their first child before the age of 20 (McPherson et al. 2000). Breastfeeding was suggested to have a protective role against breast cancer, breastfeeding for one year is estimated to lead to a 4.3% reduction in breast cancer risk (Anon 2002). Exposure to estrogen is an important regulator of cell proliferation of breast cells but breast cancer pathogenesis does not solely depend on estrogen and estrogen receptors. Some of the patients are ER (estrogen receptor) positive but do not respond to anti-estrogen treatment (Higa 2009). Patients with proliferative breast disease also have a higher risk of breast cancer compared to the general population (Hartmann et al. 2005).

Lifestyle risk factors of breast cancer includes alcohol consumption, obesity, physical inactivity and radiation exposure (Danaei et al. 2005).

Familial or somatic mutations of *BRCA1* and *BRCA2* are well-known high risk factors for breast cancer formation (Lalloo & Evans 2012). Breast cancer history in the first degree relatives increase the risk of breast cancer (Higa 2009) and familial cases constitute 10% of all the breast cancer cases (McPherson et al. 2000). Hereditary breast and ovarian cancers can arise from constitutional mutations in *BRCA1* gene. *BRCA1* gene is involved in double-strand break recognition mechanism during DNA repair but also implicated in several other cellular functions such as; chromatin remodelling, transcriptional regulation, cell cycle checkpoint control and genomic stability (Campeau et al. 2008). The initial studies claimed that *BRCA1* and *BRCA2* mutations are the main cause of the familial cases, however recent studies identified that mutations at these two genes consist only 25-28% of the hereditary cases (Gerdes et al. 2006; Melchor & Benítez 2013). Other genes are also implicated in increased risk factors for breast cancer such as *TP53, CDH1, PTEN, RAD51C, STK11* and *RAD51D* with high penetrance and *CHEK2, ATM, PALB2* and *BRIP1* low or moderate penetrance (reviewed in (Vargas et al. 2011)). Mutations in *TP53* and *PTEN* genes, both of which encode proteins that function as key regulators of cell division, account for only a minority of inherited breast cancers (Higa 2009). Genome-wide association studies (GWAS) identified breast cancer susceptibility loci

including 2q33, 2q35, 5q11, 5p12, 8q24, 10q26, 11p15 and 16q12 (Cox et al. 2007; Easton et al. 2007; Stacey et al. 2007). Despite intensive studies performed with GWAS and next generation exome sequencing, no single high penetrance allele was identified to explain remaining large fraction of breast cancer cases (Smith et al. 2006; Rosa-Rosa et al. 2008; Snape et al. 2012; Gracia-Aznarez et al. 2013). Remaining susceptibility is more likely to depend on the involvement of multiple low penetrance genes.

1.1.2 Breast Cancer Progression

Progression of breast cancer is similar to Vogelstein's model for colon cancer development (Vogelstein et al. 1988), a multi-step process including changes from normal (terminal duct lobular unit) to hyperplasia, carcinoma in situ, invasive carcinoma and metastasis (Figure 1.2) (Wellings et al. 1975). The traditionally accepted multi-step progression of breast cancer schematized in Figure 1.2, is only based on morphological and epidemiological studies.

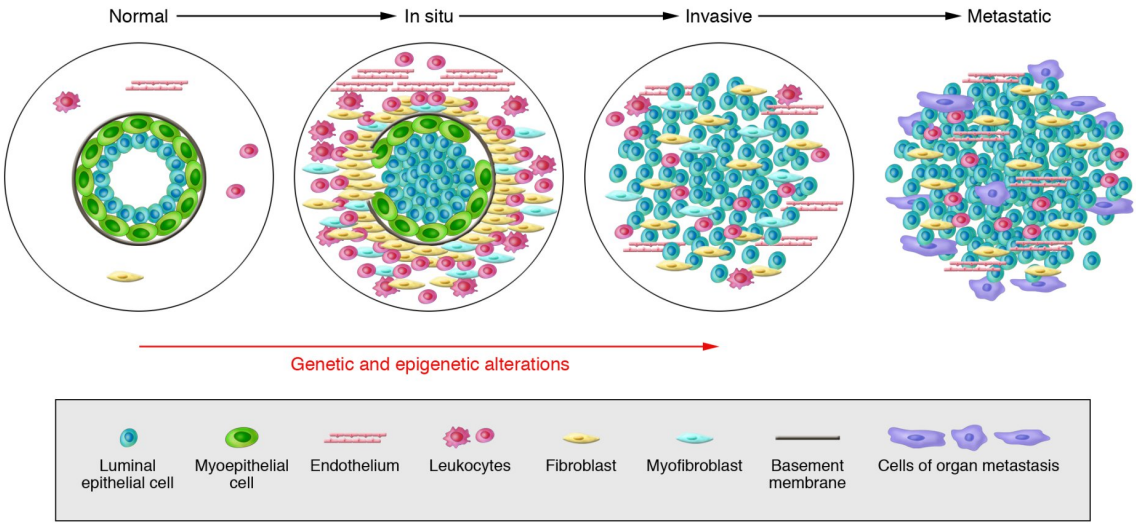


Figure 1.2. Breast cancer progression model (Polyak 2007). Figure taken from (Polyak 2007), Copyright (2015), The Journal of Clinical Investigation.

The improvements in molecular biology, immunohistochemistry, microarray technology and next-generation sequencing have changed our understanding about this progressive

breast cancer model. Now we know that progression is not a very straight path but it is rather complex and cumulative events in different pathways towards the invasive breast cancer (Simpson et al. 2005).

The molecular mechanisms involved in tumor initiation and progression constitute an important challenge in the breast cancer field. Developmental signalling pathways regulating mammary development have found to be disrupted in cancer. Developmental signalling pathways such as IGF, integrin, Notch, NF-κB, STAT, TGF-β and Wnt pathways are deregulated in breast cancer. Development and branching of the ductal epithelial tree during puberty requires amphiregulin/EGFR signalling, while overexpression of both the ligand and the related receptor ErbB2, is associated with poor prognosis in breast cancer. Estrogen is an important factor in breast cancer initiation and progression, and is also implicated as a risk factor. ER α together with ER β and several different signalling factors are central operators of breast cancers (Thompson et al. 2008).

1.1.3 Clinical Grading and Staging of Breast Cancer

Grading of breast cancer is the first most important step that helps clinicians to decide the treatment options of the patients because it is a measure of tumor aggressiveness. Grading refers to the appearance of the cancer cells under the microscope. “Low grade” tumors are less aggressive than “high grade” tumors. Breast cancer grading depends on three factors: nuclear grade (change in the cell size, shape and uniformity), tubule formation (percent of the cells with the tubular formation) and mitotic rate (rate of cell division) (Rank et al. 1987). Each variable is scored from 1 to 3 (1 is given for the best and 3 for the worst). Scores from each component are added together to determine the “grade”. This grading system is called the Scarff-Bloom-Richardson (SBR) grading system. The lowest possible score is 3 and given to the tumors with low cell proliferation rate and higher level of differentiation, the highest possible score is 9 and given to the highly proliferative and undifferentiated tumors. There are three grades in this system; grade 1 (low-grade, score: 3-5), grade 2 (moderate or intermediate grade, score: 6,7) and grade 3 (high-grade, score: 8,9) (Bloom & Richardson 1957).

Cancer stage is scored based on the spreading state of the tumor. TNM (Tumor-Node-Metastasis) staging considers three aspects of tumor: tumor size, lymph node involvement and invasiveness of the tumor and whether it has spread away from the breast. Staging helps to classify cancer into groups depending on the different variables and characteristics of the cancer, which further improves the treatment decision. Early stages are stage 0, stage I, stage II, some stages of stage III and late or advanced stages are stage III and IV (Elston & Ellis 1991). Stage II is divided into two groups as IIA and IIB, while stage III is divided into three groups as IIIA, IIIB and IIIC.

Breast cancer is a complex disease with different subtypes having different properties and various clinical outcomes are associated with these different subtypes (Elston & Ellis 1991). The most important determinants of these subtypes are ER and progesterone receptor (PR) status of tumor cells and the amplification, overexpression of the HER2 oncogene. Classification of this heterogeneous disease required for preventive and therapeutic approaches. Many genes play crucial roles in breast cancer and the heterogeneous nature of the disease interferes with grouping of these subtypes. Recent molecular studies using microarray technology with large group of tumor sets clustered breast cancer depending on the gene expression profiles into five major molecular subtypes: luminal A, basal-like, normal breast-like, luminal B and $HER2^{+}/ER^{-}$ (Sørlie et al. 2001; Perou et al. 2000; Hu et al. 2006).

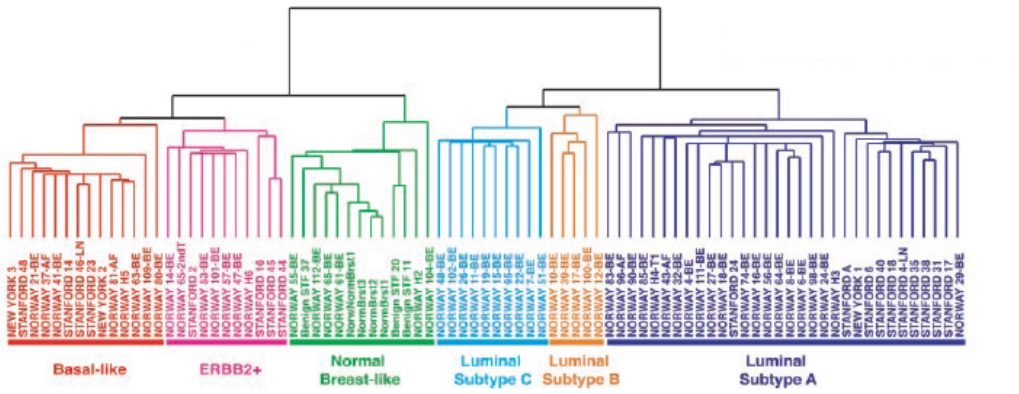


Figure 1.3. Hierarchical clustering of 85 samples (78 carcinomas, 4 normal tissues and 3 benign tumors) depending on the gene signatures acquired using microarray data (Perou et al. 2000). Figure taken from (Perou et al. 2000), Copyright (2015) Macmillan Publishers Limited.

As seen in Figure 1.3 tumors are clustered into two main groups. The first group contains already characterized basal-like, ErbB2+ and normal-breast like subgroups with low or no expression of ER. Basal-like subtype defined by high expression of certain keratins (5 and 17), laminin and fatty acid binding protein 7, while ErbB2 characterized by high expression of *ErbB2* and *GRB7* and normal-like breast showed a similar expression pattern to adipose tissue and non-epithelial cell types. The second branch of the dendogram contains luminal ER+ subtypes (two or possibly three different groups). Luminal subtype A is defined with the highest expression of ER α gene GATA binding protein 3, X-box binding protein 1, trefoil factor 3, hepatocyte nuclear factor 3 a, and estrogen-regulated *LIV*-1. The other two groups, luminal subtype B and C, both show expression of genes including the ER cluster and those that are specific to the luminal phenotype. Luminal C subtype also shows expression of a novel set of genes with unknown functions (Perou et al. 2000).
The classification of breast cancer using new strategies is important for finding the missing pieces between genotype and phenotype. It can provide insights into the progression of disease from normal to invasive carcinoma. Identification of new subtypes can provide new prognostic parameters and personalized care for the patients.

1.2 Ribosomal RNA Genes

Ribosomes are essential organelles that are required to support cell growth. rDNA transcription regulates the ribosome biogenesis that has a central role in cell cycle progression (Brown & Szyf 2008). Ribosome synthesis is a very complex process that is closely related to cell metabolism. Nucleoli form in the nucleus wherever the rRNA genes are transcribed. Ribosome biogenesis is tightly correlated with rRNA synthesis. The human genome contains about 300-400 copies of rRNA genes but only a fraction of these genes are actively transcribed depending on the cell type, external signals and cell stage while the rest of the genes remain inactive (McKnight & Miller 1976).

1.2.1 Organization of rDNA Genes

rRNA genes are organized as tandemly repeated arrays within the nucleolar organizing regions (NORs), located on the short arms of five human acrocentric chromosomes 13, 14, 15, 21 and 22 NORs can be observed as secondary constrictions in the chromosomes during metaphase (Schmickel 1973; Henderson et al. 1972) (Figure 1.4). Location of rDNA genes on the short arms of chromosomes insulate rDNA genes from Pol II and Pol III transcribed genes, this isolation was further reinforced by surrounding repetitive satellite DNA.

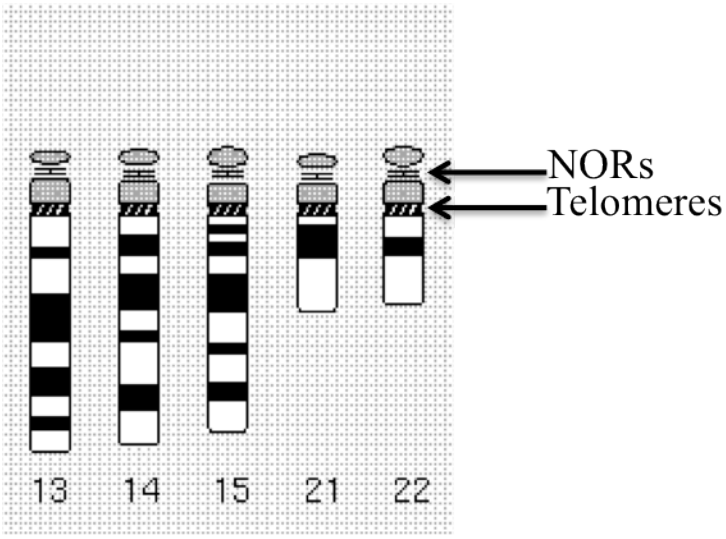


Figure 1.4. NORs are located at secondary constrictions of five human acrocentric chromosomes (Henderson et al. 1972). Figure adapted from (McStay & Grummt 2008). Ideograms were taken from the University of Washington, Department of Pathology website (http://www.pathology.washington.edu/research/cytopages/idiograms/human/). Copyright (1994) David Adler.

rDNA repeats were thought to organized as only head to tail direction, but recently the molecular combing technique has revealed that rDNA repeats exist in both head to tail and tail to head direction in the NORs (Caburet et al. 2005).

Mammalian rDNA transcription units are approximately 43 kb long and each rDNA repeat is interrupted with long (~ 30kb) intergenic spacer region (IGS) (Gonzalez & Sylvester 1997). The entire promoter region of rRNA genes is contained in the IGS

region. The promoter region of rRNA consists of two important elements; core promoter (CP) and upstream control element (UCE). CP is located between -50 bp to +20 bp and is essential for basal transcription whereas UCE is located 150-200 bp upstream of the transcription start site and required for efficient pre-initiation complex formation (Paule & White 2000; Learned et al. 1986) (Figure 1.5).

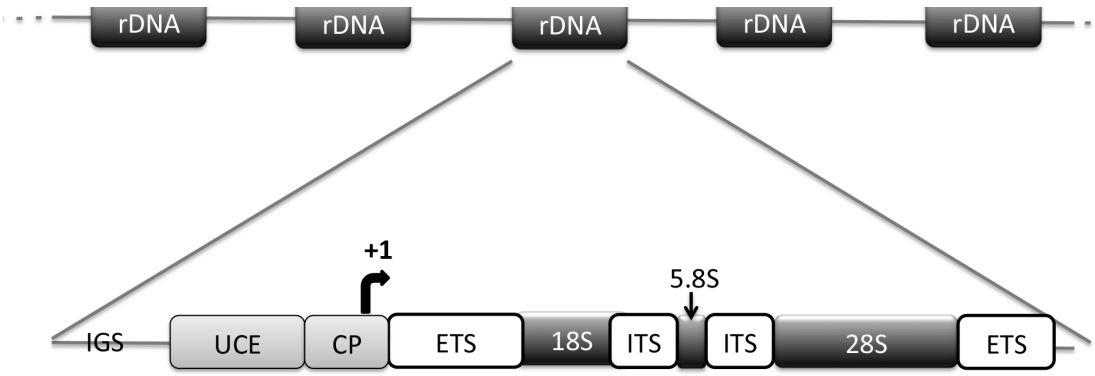


Figure 1.5. Structural organization of mammalian rDNA transcription unit. Adapted from (Paule & White 2000; McStay & Grummt 2008). IGS: Intergenic spacer, UCE: Upstream control element, CP: Core promoter, ETS: External transcribed spacer, ITS: Internal transcribed spacer.

rRNA genes (except 5S, which is transcribed by RNA Polymerase III) are transcribed from 45S rDNA promoter by RNA polymerase I (Pol I) as a long precursor known as 45S pre-RNA that is rapidly spliced into the 18S, 28S and 5.8S rRNA transcripts.
These rRNA transcripts are the catalytic and structural components of the ribosomes and are processed, modified and assembled into respective ribosomal subunits in the nucleolus.

1.2.2 Regulation of rDNA genes

Since approximately 80% of the total RNA of a proliferating cell consists of Pol I products, rRNA gene expression should be tightly regulated in order to avoid energy loss or unwanted growth of the cell. Therefore, the transcription of rRNA genes is regulated at every step of the road; pre-initiation complex (PIC) formation, initiation, promoter

escape, elongation, termination, re-initiation, RNA processing and post-transcriptional modifications (Russell & Zomerdijk 2005).

There are two essential transcription factors identified in mammals; upstream binding factor (UBF) and the selectivity factor (SL1 in humans and TIF-IB in mouse), cooperative binding of these two factors to the promoter region is required for Pol I recruitment (Learned et al. 1986; Clos et al. 1986). UBF contains several HMG boxes and this motif is known to bend DNA and enable UBF interaction with the minor groove of DNA (Putnam et al. 1994). UBF has two major roles in the rDNA transcription: stabilizing SL1/TIF-IB on the rDNA promoter and competing with non-specific DNA binding proteins such as H1, which prevents the binding of SL1/TIF-IB (Kuhn & Grummt 1992). SL1/TIF-IB is a protein complex, which contains TATA-binding protein (TBP) and five Pol I specific TBP-associated factors (TAF_I 48, TAF_I 68, TAF_I 35, TAF_I 12 and TAF_I 95/110), TAF_Is are responsible for the promoter recognition (Comai et al. 1992; Heix et al. 1997; Zomerdijk et al. 1994; Gorski et al. 2007; Denissov et al. 2007). TAF_Is also interact with a basal regulatory factor TIF-IA which associate with transcriptionally active Pol I subpopulation (Pol Iβ) (Miller et al. 2001; Yuan et al. 2002).

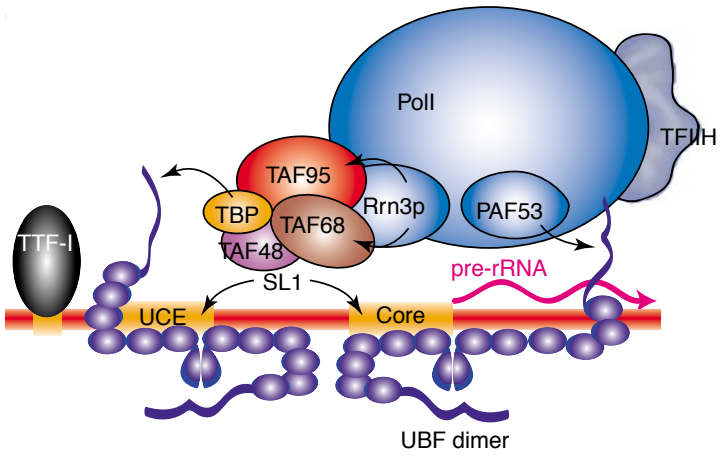


Figure 1.6. Recruitment of Pol I to the pre-initiation complex by UBF and SL1. Figure taken from (Moss 2004), Copyright (2004) Elsevier Limited.

Transcription from 45S rDNA promoter by Pol I was found to be a dynamic process. Components of transcription machinery are rapidly shuffled between nucleoplasm and fibrillar centers (rDNA transcription centers) while subunits of transcription machinery

enter the nucleolus apart from each other rather than as a preassembled protein complex (Dundr et al. 2002). Transcription terminator sequences are found both the 5'and 3'end of the rDNA transcription unit. Termination sequences at the 3'end are obviously important for transcriptional termination and are bound by TTF-I (transcription termination factor) and PTRF (polymerase and transcript release factor) for proper transcription termination but terminator sequence at the 5'end serves for a different function (Jansa & Grummt 1999; Grummt et al. 1986; Grummt et al. 1985; Bartsch et al. 1987). It is bound by TTF-I and required for transcription initiation and recruitment of chromatin remodelling complexes such as NuRD, NuRC and CSB (Strohner et al. 2001; Santoro et al. 2002; Yuan et al. 2007; Xie et al. 2012).

Psoralen crosslinking experiments revealed that rDNA clusters exist in two distinct configurations in the nucleolus, active NORs are uncondensed (Heliot et al. 1997) and are occupied by Pol I and Pol I-specific transcription factors (UBF, SL1 and TTF-I) during mitosis (Roussel et al. 1993; Roussel et al. 1996). Meanwhile inactive NORs are packaged into a heterochromatin structure and lack Pol I as well as specific transcription factors.

rRNA synthesis is thought to be controlled either by changing the number of active rRNA copies or changing the Pol I activity and regulating the initiation frequency on already active genes (Grummt 2003; Moss 2004). Both mechanisms are shown to be operating under different circumstances in yeast but similar to vertebrates proliferating yeast cells increase the transcription initiation on already active rDNA genes instead of transforming inactive copies to active copies (Reeder 1999). Active and inactive rDNA copies are maintained through the cell cycle and are independent from rRNA levels (Conconi et al. 1989). Epigenetic modifications also support active and inactive rDNA states but will be discussed in a further section.

rDNA synthesis can be regulated by any protein that is essential for Pol I transcription, such as phosphorylation of UBF C terminus at different serine residues by casein kinase II is required for UBF transactivation and its hypophoshorylation is associated with transcriptional inactivity in terminally differentiated cells (O'Mahony et al. 1992; Voit et al. 1992; Voit et al. 1995; Tuan et al. 1999).

Post-transcriptional modifications also have very important regulatory roles. As previously mentioned, rRNA genes are transcribed as 45S pre-RNA which then is rapidly spliced into the 18S, 28S and 5.8S rRNA transcripts (Eichler & Craig 1994). These transcripts undergo extensive chemical modification, sometimes even before they are cleaved. There are three major types of rRNA modifications: 2'-*O*-methylation (Nm), pseudouridylation (ψ) and base methylation at various positions (Smith & Dunn 1959; Davis & Allen 1957; Wagner et al. 1967). Small nucleolar RNA-protein complexes (snoRNPs) direct the modification of rRNAs in eukaryotes. Two families of snoRNPs; box C/D and H/ACA are assigned for most prevalent modifications of rRNAs; 2'-*O*-methylation and pseudouridylation, respectively (Tyc & Steitz 1989; Balakin et al. 1996; Kiss et al. 1996; Tycowski et al. 1998; Ganot et al. 1997). RNA component of the snoRNPs selects the site for modification through base pairing (Bachellerie et al. 1995). Protein component of box C/D fibrillarin and pseudouridine synthase of box H/ACA snoRNPs dyskerin catalyse the modification reaction (Tollervey et al. 1993; Zebarjadian et al. 1999). Besides their role in chemical modification of rRNAs both family of snoRNPs play role in rRNA processing and also help rRNA folding (Henras et al. 2008; Phipps et al. 2011). Processed and modified rRNA transcripts assembled into respective ribosomal subunits in the nucleolus (Trapman et al. 1975; Venema & Tollervey 1999).

1.2.3 rRNA Genes and Cancer

The association of nucleolus and cancer has been long known. Abnormal morphology of the nucleolus in cancer cells has drawn attention of tumor pathologists as early as the 19th century. However the molecular biology of rRNA synthesis and ribosome biogenesis of cancers cells have recently begun to be explored.

Nucleoli is visible as a result of numerous macromolecules that are required for rRNA transcription, nucleoli disappears if rRNA synthesis is reduced whereas enlarges if Pol I activity is increased as observed in cancer cells.

The cell growth defines an increase in the cell mass whereas proliferation is an indicator of increase in cell number. Cell division can not occur without a proper cell growth

(Killander & Zetterberg 1965; Johnston et al. 1977). Ribosome biogenesis is highly correlated with both of the processes.

Several tumor suppressors and oncogenes have been demonstrated to affect Pol I dependent transcription (Zhai & Comai 2000; Poortinga et al. 2004; Arabi et al. 2005; Voit et al. 1997). There are number of pathways known to regulate rRNA synthesis such as mammalian target of Rapamycin (mTOR), phosphatidyl inositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) which are usually deregulated in cancer cells (Grummt 2003; Moss 2004). c-Myc, overexpressed in various cancers, is essential for cell cycle entry and directly enhances Pol I activity by recruiting SL1 to the rDNA promoter (Arabi et al. 2005; Grandori et al. 2005). However, only a part of the rRNA genes are transcribed even in the highly proliferative cancer cells. This fact illustrates that various epigenetic mechanisms in addition to the signalling pathways take part in the regulation of rRNA genes.

1.2.4 Normalization of rRNA gene expression in cancer

For interpretation of quantitative gene expression measurements in clinical tumor samples, a normalizer is necessary to correct expression data for differences in cellular input, RNA quality, and RT efficiency between samples. In the literature, rRNA gene expression is usually determined by normalizing to genes such as; GAPDH, TBP, ARPP P0 (acidic ribosomal phosphoprotein) and TFIIB (Raval et al. 2012; Uemura et al. 2011; Grandori et al. 2005) yet all these reference genes are transcribed by Polymerase II. However the output of such analysis is only help the researcher to identify rRNA expression levels relative to the polymerase II transcribed mRNA levels. Total RNA is represented mostly by rRNA (>80%), even a small decrease in rRNA expression may lead to a disproportional increase in the assessment of mRNA levels. We propose using geometric mean of four rRNA transcripts (18S, 28S, 5.8S and 45S ETS) to eliminate any bias introduced by using mRNA levels. This novel approach is also essential for identification of relative changes of rRNAs with regard to each other within a total rRNA pool.

1.3 Epigenetics

"The branch of biology which studies the causal interactions between genes and their products, which brings the phenotype into being" said Conrad Waddington (1905-1975) who is attributed to have created the term of epigenetics in 1942 (http://epigenome.eu/en/1,1,0). Epigenetic, as its name implies (epi- means over, above in Greek), does not involve changes in the DNA sequence itself but rather effects the gene expression or phenotype. Non-genetic factors effect the gene expression differently without effecting the DNA sequence (Bird 2007).
DNA methylation, histone modifications and nucleosomal remodelling constitute interacting branches of epigenetic regulation. These processes regulate chromatin structure to form euchromatin or heterochromatin, and in turn activate or silence the gene expression. Micro RNAs (miRNAs) are also accepted as epigenetic regulators of gene expression but in contrast to other mechanisms miRNAs do not affect the chromatin structure and rather affect the mRNAs levels.

1.3.1 DNA Methylation

DNA methylation is the covalent addition of a methyl group to the 5^{th} carbon of the cytosine ring. Even though mammalian DNA methylation is thought to be restricted to the cytosine followed by a guanine (CpG sites), recent studies have revealed that embryonic stem cells have methylation at non-CpG sites (Lister et al. 2009; Ramsahoye et al. 2000; Woodcock et al. 1987). CpG frequency of the human genome (2-5%) is less than expected (Josse et al. 1961; Swartz et al. 1962), since they are hotspots for mutation (Coulondre et al. 1978) thus CG dinucleotides are decreased during evolution. They are not randomly dispersed through the genome but mostly found as clusters either at promoter regions of the genes (known as CpG islands) or at the repetitive sequences (such as rDNA, satellite sequences, or centromeric repeats). The formal definition of a CpG island (CGI) is "a region with at least 200 bp and with a GC percentage that is greater than 50% and with an observed/expected CpG ratio that is greater than 60%" (Gardiner-Garden & Frommer 1987). More than 70% of the CpGs sites in the vertebrate

genome are methylated (Cooper & Krawczak 1989). CGIs are usually located around transcription start sites of genes (Takai & Jones 2002). DNA methylation at the promoter region is associated with gene silencing (Bird 2002).

The best-known DNA methylation instance is found at the X chromosomes of females. One of the X chromosomes (randomly selected in each cell) is hypermethylated during early development and enables monoallelic gene expression while providing dosage compensation (Panning & Jaenisch 1998). Imprinting is also ensured by DNA methylation. Some of the genes should be expressed maternally or paternally, one of the allele from the other parent should be silenced for normal development of the organism (Feil 1999). CGI methylation is also important in carcinogenesis as well as silencing of intragenomic mobile elements (Yoder et al. 1997).

DNA methyltransferases (DNMTs) mediate the transfer of the methyl group from *S*-adenosyl L-methionine (SAM) to cytosine of CpG dinucleotide and generation of 5-methylcytosine (Brenner & Fuks 2006). Known DNMTs are DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L. DNMT3a, DNMT3b and DNMT3L are responsible for the *de novo* methylation whereas DNMT1 is required for maintenance of methylation pattern during cell division (Okano et al. 1999; Tajima & Suetake 1998).

1.3.2 Histone Modifications and Chromatin Remodelling

The chromatin consists of nucleosomes. A nucleosome is a protein-DNA complex containing an octamer of core histone proteins (H2A, H2B, H3 and H4) wrapped with 146 bp long DNA (Kornberg & Thomas 1974). N-tails of core histones exposed to different covalent modifications such as acetylation, methylation, sumoylation, ubiquitination, phosphorylation and ADP-ribosylation. These modifications regulate chromatin structure as well as gene expression at the affected regions which in turn can alter the cell behaviour (Shiio & Eisenman 2003; Schübeler et al. 2004; Shilatifard 2006; Bradbury 1989).

Histone modifications first started to draw attention by the identification of first histone deacetylase (HDAC) and histone acetyltransferase (HAT) as transcriptional coactivator and corepressor, respectively (Brownell et al. 1996; Taunton et al. 1996). Histone

acetylation mainly occurs in lysine residues of H3 and H4. Acetylation of lysine residues are associated with open chromatin structure and active gene expression whereas deacetylation causes condensation of chromatin (Schübeler et al. 2004). Histone phosphorylation is observed in different histone residues such as serines, tyrosines, threonines and phosphorylation is not limited to the N-terminus of histones (Banerjee & Chakravarti 2011). Phosphorylation and dephosphorylation reactions are catalysed by kinases and phosphatases which are also regulated by phosphorylations (Bodenmiller et al. 2010; Zorina et al. 2011). Both histone acetylation and phosphorylation modifications are changeable and dynamic events (Jackson et al. 1975; Barth & Imhof 2010). Histone methylation can occur on lysine and arginine residues as mono-, di-, tri-methylation and is regulated by histone methyltransferases (HMTs) and their action balanced by histone demethylases (Rice et al. 2003; Tsukada et al. 2006).

Histone modifications have been considered to affect the chromatin structure through changes in histone-histone and histone-DNA interactions for a long time (Wolffe & Hayes 1999; Hansen et al. 1998). However, the vast variety of modifications and association of histone patterns with specific biological functions have led to the idea that histone modifications might be referred as a "histone code" and this code is read by other proteins and protein complexes (Mizzen et al. 1998; Turner 1993; López-Rodas et al. 1993; Loidl 1994; Tordera et al. 1993).

Chromatin associated proteins recognize and bind to the sites of histone modifications with their dedicated domains (acetylated lysine residues recognized by bromodomains, methylated lysine residues recognized by chromodomains) and mediate chromatin remodelling and regulate gene expression (Zeng & Zhou 2002; Daniel et al. 2005; Martin & Zhang 2005).

Some of the aforementioned histone modification enzymes are also parts of chromatin remodelling complexes, which together regulate chromatin configuration as well as gene expression.

Profiling histone modifications revealed that cancer cells display loss of mono- and tri-methylated forms of H4 and a general deacetylation of histones observed as a result of inactivation of HATs (Jones & Baylin 2002). Deregulation of histone modifications at tumor suppressor genes and proto-oncogenes has great effect on the cancer progression.

DNA methylation and chromatin modifications are closely associated with each other during the packaging of DNA. Recent studies have revealed that methyl-CpG binding proteins associate with HDACs, DNMTs and HMTs to alter chromatin structure and cause gene silencing. Nucleosomal remodelling complexes such as Sin3A, NuRD, SUV39H, NCoR/SMRT, CoREST, and SWI/SNF have also been found to be partners with methyl-CpG binding proteins. Epigenetic cancer studies have revealed that methyl-CpG binding proteins are found mostly at hypermethylated promoters of cancer related genes. Nucleosomal remodelling complexes are recruited to the methylated DNA bound by methyl-CpG binding proteins to reorganize chromatin to the repressive heterochromatin state causing gene silencing. Moreover, chromatin-remodelling complexes can further alleviate binding of the methyl-CpG binding proteins to the methylated DNA, generating a never-ending cycle between two mechanisms. Methyl-CpG binding proteins link the DNA methylation to repressive chromatin modification and remodelling by interacting multiple chromatin related proteins (reviewed in (Lo & Sukumar 2008)). There are many intersecting pathways that are engaged in the epigenetic regulation of gene expression in this emerging field, yet many others remain to be uncovered.

1.3.3 DNA Methylation and Cancer

CpG islands at the promoter regions of genes are unmethylated and they are actively expressed in normal cells. Genes with CpG islands became susceptible to methylation when cell start to transform from normal cell to cancer cell. Methylation patterns are inverted in the genome of cancer cells compared to normal cells (Baylin & Ohm 2006). For example: repetitive sequences normally methylated to protect the genome from mobile elements become unmethylated in transformed cells whereas normally unmethylated CpG islands at the promoter regions of the genes become methylated in cancer cells (Manel Esteller 2007; Jones & Baylin 2002). Both of the mechanisms are advantageous for cancer cells to survive; hypermethylation of the CpG islands at promoters of the tumor suppressor genes lead to the survival of the cancer cells whereas hypomethylation of the repetitive elements increase genomic instability in cancer cells

and so they can accumulate more genomic aberrations (Manel Esteller 2007). Change in the DNA methylation profile is one of the characteristics of almost all human cancers, including breast cancer.

The two-hit model was purposed by Knudson states that inactivation of a tumor suppressor gene requires both of the copies to lose their function (Knudson 2000). Methylation of a tumor suppressor gene can be the second hit to an already mutated tumor suppressor gene. *p16INK4a, APC* and *BRCA1* are, well-known tumor suppressor genes, inactivated in the germ line by mutations and functional allele is inactivated through hypermethylation of the DNA (Foster et al. 1998; Virmani et al. 2001; Birgisdottir et al. 2006). Hypermethylated tumor suppressor can be related to many biological functions and many of them have already been identified. Examples include genes playing role in cell cycle regulation, angiogenesis, cell adhesion, apoptosis, DNA repair, invasion, hormone regulation, and cellular growth-inhibitory signalling and the list is growing everyday (Szyf et al. 2004).

1.3.4 rDNA Methylation and Cancer

As mentioned earlier, psoralen crosslinking experiments revealed that rDNA clusters exist in two distinct configurations in the nucleolus as active and inactive (Heliot et al. 1997). Epigenetic modifications support the active and inactive states of rDNA gene clusters. Increase in histone acetylation levels are known to associated with active genes (Tazi & Bird 1990). MeCP2 protein is known to associate with histone deacetylases, has also high affinity towards methylated DNA (Meehan et al. 1992). DNA methylation and histone acetylation as well as other histone marks are together involved in epigenetic regulation of gene expression and chromatin structure.

Methylation of CpGs at the promoter (Stancheva et al. 1997), repressive histone codes such as; methylated H3K9me3, H4K20 and H3K27me3 are indicators of inactive rRNA copies (Santoro et al. 2002), whereas acetylated histones H4, H3 and H3K4me3 and unmethylated rDNA promoter region associates with active copies of rDNA (Earley et al. 2006; Lawrence et al. 2004; Zhou et al. 2002).

45S rDNA promoter region and transcribed regions have an unusual CpG composition, they are very rich in CG dinucleotides however much longer than a regular CpG island (Worton et al. 1988). CpG islands in the genome of cancer cells are known to be subjected to hypermethylation and eventually loose the gene expression. Several studies have been reported about rDNA promoter methylation status in cancer in the literature but rRNA expression levels and their relations with rDNA promoter methylation have been overlooked in these studies.

Bisulfite sequencing of clonal DNA is frequently used in rDNA promoter methylation studies. It has been reported that 45S rDNA promoter region methylation decreases rRNA gene expression in hepatocellular carcinoma (Ghoshal et al. 2004) and similar results were reported in the $CD34^{+}$ cells of patients with myelodysplastic syndromes (Raval et al. 2012) . Some other studies showed no relation or positive correlation between promoter methylation and rRNA transcription. Loss of rRNA promoter methylation was shown to inhibit both the synthesis and the processing of rRNA proved a contrary effect of rDNA methylation on rRNA production. Further investigation in the same study suggested that this inhibition was the result of cryptic RNA polymerase II transcription of the rRNA genes (Gagnon-Kugler et al. 2009). In another study, the authors demonstrated that in prostate cancer specimens and prostate cancer cell lines increased rRNA levels do not correlate with hypomethylation of rDNA promoter (Uemura et al. 2011; Yan et al. 2000). A more recent study showed that 45S rDNA promoter including 5' regions of 18S and 28S rDNA were hypermethylated in breast cancer tissues compared to paired normal tissues and identified a correlation between methylation levels of these regions and nuclear grade and nuclear size (Bacalini et al. 2014).

In addition to bisulfite sequencing of clonal rDNA promoters, CpG methylation of rDNA promoter was also evaluated using methylation specific restriction techniques. Three studies have identified CpG methylation of rDNA promoters as a prognostic factor in ovarian cancer, endometrial cancer and breast cancer using this technique (Chan et al. 2005; Yan et al. 2000; Powell et al. 2002).

Our motivation to analyse 45S rDNA promoter methylation region came from the study of a former lab member. The aim of the study was to find suitable reference genes for

breast cancer by using matched breast tumor and normal samples. All of the reference gene candidates showed a relatively higher expression in tumor samples than in normal counterparts (Gur-Dedeoglu et al. 2009). Since 80% of the total RNA from a sample is rRNAs, even the small changes with regard to rRNA expression can influence the mRNA ratio of the total sample. Differential expression of reference genes drove our attention to mRNA/rRNA ratio. Quantification of 18S rRNA with reference genes ACTB and SDHA in 13 tumor and normal pairs revealed that 18S rRNA was down regulated in most of the tumor samples (9/13) whereas ACTB and SDHA expressions were higher in tumor samples (Gur-Dedeoglu et al. 2009).

In our knowledge, although many studies have been conducted to analyse rDNA promoter methylation levels in cancer, the relationship between rRNA transcript levels and rDNA promoter methylation levels have not been investigated in breast cancer.

1.4 Aim and Hypothesis

rRNA genes are the one of the most important genes for a cell to grow, proliferate and survive since they are the core elements of protein factories of the cells, ribosomes. Since cancer cells harbor mutations to overcome proliferation restricting mechanism they have to alter the ribosome biogenesis in order to keep growing and dividing. Many tumors are known to increase ribosome biogenesis through increasing the transcription level of rRNA genes. However expression of rRNA transcripts remain largely unexplored in breast cancer.

18S and 28S rRNA genes had been used as housekeeping genes along with the classical housekeeping genes such as GAPDH and β-actin because they are thought to have stable expression (Murthi et al. 2008; Pérez et al. 2008). In contrast to common belief about the stable expression of rRNA genes, in recent studies it has been shown that 18S rRNA is differentially expressed in breast tumor and normal samples and it is not an appropriate reference gene (Gur-Dedeoglu et al. 2009; Tricarico et al. 2002; de Kok et al. 2005). Underlying mechanism for this differential expression was recently identified to be rDNA methylation in some cancer types such as hepatocellular carcinoma, patients with myelodysplastic syndromes (Ghoshal et al. 2004; Raval et al. 2012) although some other

studies found an opposite or no correlation between rDNA methylation and rRNA gene expression. rRNA gene regulation through 45S rDNA methylation is controversial in the literature.

rRNA transcripts (18S, 28S and 5.8S) are transcribed from the same promoter and more than 80% of the total RNA in a cell is comprised from these essential transcripts. Any dysregulation regarding to the expression or splicing of these transcripts might have major impact on cancer development and progression. rRNA gene expression is commonly analysed by using reference genes such as; ACTB, TBP and GAPDH. However, using mRNA levels for rRNA expression analysis do not identify whether the ratios of spliced products of the 45S precursor were differentially expressed between tumor and normal pairs in breast cancer. We purpose using geometric mean of all four rRNA products (18S, 28S, 5.8S and 45S ETS) to normalize rRNA gene expression to identify relative changes of rRNAs within a pool of rRNA transcripts.

DNA methylation is one of the important mechanisms contributing to the tumorigenesis. Our aim was to find whether abnormal DNA methylation extends to an essential gene promoter, 45S rDNA promoter and how the level of rDNA methylation affects the rRNA expression and proportions of different rRNA trasncripts in the rRNA pool in breast cancer and normal breast tissues.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 General Laboratory Reagents and Kits

General chemicals (such as ethanol, methanol, isopropanol, chloroform…) were all analytical grade and mainly purchased from Sigma Aldrich Sigma-Aldrich (St. Louis, MO, USA) or Merck (Darmstadt, Germany). A full list of reagents, chemicals and kits are catalogued in Table 2-1.

Table 2-1. List of reagents and kits used in this study

Reagents	Catalogue No	Company
4-Bromoanisole	BN 191	Molecular Research Center, USA
Agar (microbiology grade)	05039-500G	Fluka, Sigma Aldrich, USA
Agarose-Basica Le	BHE500	Prona, Spain
Ampicillin	A0839.0025	Applichem, Germany
Bacto-tryptone	1612	Conda, Spain
$CaCl_2$. $2H_2O$	433381	Carlo Erba, Italy
D(+)-Glucose	16325	Riedel-de Haen, Sigma Aldrich, USA
DEPC	A0881.0100	Applichem, Germany
dNTP Set	R0182	Fermentas, Thermo

		Scientific, USA
DyNAmo HS SYBR Green qPCR Kit	F-410L	Thermo Scientific, USA
EDTA	A3562.1000	Applichem, Germany
EpiTect Bisulfite Kit	59104	Qiagen, Germany
Etidium Bromide	17898	Thermo Scientific, USA
Gene ruler 50 bp ladder	SM373	Thermo Scientific, USA
Glacial acetic acid	27225-2.5L-R	Sigma Aldrich, USA
HyPure Molecular Biology Grade water	SH30538.01	Hyclone, Thermo Scientific, USA
IPTG	A4773.0005	Applichem, Germany
KCl	12636.1KG	Sigma Aldrich, USA
KOH	1.05012.1000	Merck, USA
MessageClean Kit	M601	GenHunter, USA
$MnCl_2.\ 4H_2O$	M3634-100G	Sigma Aldrich, USA
NaCl	31434	Sigma Aldrich, USA
NaOH	6203	Sigma Aldrich, USA
Nucleospin RNA	740955.5	Macherey Nagel, Germany
Nucleospin Tissue	740952.5	Macherey Nagel, Germany
pGEM-T Easy vector system	A1360	Promega, USA

Phenol: Chloroform: Isoamyl alcohol, 25:24:1, pH 6.6	AM9730	Ambion by Life Sciences, USA
Pipes	A1079.0100	Applichem, Germany
PureLink Quick Plasmid Miniprep Kit	K210011	Invitrogen, Thermo Scientific, USA
QIAquick Gel Extraction kit	28704	Qiagen, Germany
RevertAid First Strand cDNA Synthesis Kit	K1622	Thermo Scientific, USA
Taq DNA Polymerase	EP0402	Fermentas, Thermo Scientific, USA
TRI Reagent RT	RT 111	Molecular Research Center, USA
Trizma Base	T1503-1KG	Sigma Aldrich, USA
X-Gal	R0404	Fermentas, Thermo Scientific, USA
Yeast extract	1702	Conda, Spain

2.1.2 Tissue Culture Reagents

All reagents used in tissue culture are listed in Table 2-2.

Table 2-2. List of reagents and chemicals used in tissue culture

	Catalogue No	Company
5-aza-2' deoxycytidine	A3656	Sigma Aldrich, USA
DMEM HIGH Glucose	SH30243.01	Hyclone, Thermo Scientific, USA
DMEM Low Glucose	SH30021.01	Hyclone, Thermo Scientific, USA
DMEM/F-12	SH3002301	Hyclone, Thermo Scientific, USA
DMEM/Low Glucose	SH30021.01	Hyclone, Thermo Scientific, USA
EGF	E9644	Sigma Aldrich, USA
FBS	CH30160.03	Hyclone, Thermo Scientific, USA
Hydrocortisone	H4001	Sigma Aldrich, USA
Insulin	I9278	Sigma Aldrich, USA

L-glutamine	SH3003401	Hyclone, Thermo Scientific, USA
McCoy's 5A	SH30200.01	Hyclone, Thermo Scientific, USA
Non-Essential Amino Acids	SH30238.01	Hyclone, Thermo Scientific, USA
PBS	SH30256	Hyclone, Thermo Scientific, USA
PBS (Ca -Mg free)	SH30256.01	Hyclone, Thermo Scientific, USA
Penicillin/Streptomycin	SV30010	Hyclone, Thermo Scientific, USA
RPMI 1640	SH30027.01	Hyclone, Thermo Scientific, USA
Sodium Pyruvate	11360-070	Gibco, Thermo Scientific, USA
Trichostatin A	T8552	Sigma Aldrich, USA
DMSO	A1584.0100	Applichem, Germany
Trypsin/EDTA	SV3003101	Hyclone, Thermo Scientific, USA

2.1.3 Instruments

All equipment used in this study are listed in Table 2-3.

Table 2-3. List of instruments used in this study

Name	Company
Applied Biosystem PCR Machine	Applied Biosystems, Life Sciences, USA
AutoFlow NU-8500 Water Jacket CO2 Incubator	NuAire, USA
AxioCam MRc5 image capture device	Carl Zeiss, Germany
Centrifuges 5810 and 5810 R	Eppendorf, Germany
Stratagene Mx3005P Real-Time PCR System	Agilent, USA

2.1.4 Primers

All primers used in this study are listed in Table 2-4.

Table 2-4. Primers used in this study

Primers	Sequence	Product Size (bp)	Efficiency Value
45S BSP forward	5'-GAGTCGGAGAGCGCTCCCTGAG -'3	434	-
45S BSP reverse	5'-CTGGAGAGGTTGGGCCTCCG-'3		

18S rRNA forward	5’-AAACGGCTACCACATCCAAG-’3	154	1.95
18S rRNA reverse	5’-CCTCCAATGGATCCTCGTTA-’3		
28S rRNA forward	5’-CAGGGGAATCCGACTGTTTA-’3	151	1.85
28SS rRNA reverse	5’-ATGACGAGGCATTTGGCTAC-’3		
5.8S rRNA forward	5’-CTCTTAGCGGTGGATCACTC-’3	155	2.0
5.8S rRNA reverse	5’-GACGCTCAGACAGGCGTAG-’3		
45S ETS forward	5’-CGATCTGAGAGGCGTGCCTT-’3	87	1.93
45S ETS reverse	5’-GGCAGCGCTACCATAACGGA-’3		
ACTB forward	5’- CCAACCGCGAGACGATGACC-’3	124	2.03
ACTB reverse	5’- GAGTCCATCACGATGCCAG-3		
TBP forward	5’-TGCACAGGAGCCAAGAGTGAAAT-’3	134	2.2
TBP reverse	5’-CACATCACAGCTCCCCACCA-’3		

BSP: Bisulfite Sequencing Primers

2.2 Solutions and Media

2.2.1 General Solutions

50X Tris-Acetate-EDTA (TAE) Buffer

242 g Trizma base

18.61 g Disodium EDTA

57.1 ml Glacial Acetic Acid

Bring volume up to 1 litre with double distilled water (ddH_2O).

Lysogeny Broth (LB) Medium

10 g tryptone

5 g yeast extract

10 g NaCl in 950 mL double distilled water

pH of the medium is adjusted to 7.0 using 1N NaOH and volume is brought up to 1 litre with ddH_2O and autoclaved.

Add Ampicillin (to a final concentration of 100 μg/ml) when LB is cooled down to 55°C.

LB Agar plates with IPTG/X-Gal/ Ampicillin

10 g tryptone

5 g yeast extract

10 g NaCl

15 g Microbiology grade Agar in 950 mL ddH_2O

pH of the medium is adjusted to 7.0 using 1N NaOH and volume is brought up to 1 litre with ddH_2O and autoclaved.

Add Ampicillin (100 μg/ml) IPTG (0.5 mM) and X-Gal (80 μg/ml) when LB is cooled down to 55°C and pour into sterile petri dishes. Let them cool down, then invert and store at +4°C.

Transformation Buffer

10.88 g $MnCl_2.4H_2O$

2.2 g $CaCl_2.2H_2O$

18.65 g KCl

20 ml 0.5M Pipes (pH 6.7 adjusted with KOH) and the volume is adjusted to 1 litre with ddH_2O. Transformation buffer is filter sterilized and stored +4°C.

SOC Medium

20 g tryptone

5 g yeast extract

2 ml of 5M NaCl

2.5 ml of KCl

10 ml of 1M $MgCl_2$

10 ml of 1M $MgSO_4$

20 ml of 1M glucose

Adjust the volume to 1liter with ddH_2O and autoclave.

2.3 Methods

2.3.1 Cell Culture Techniques

Growth conditions of breast cancer cell lines and non-tumorigenic cell line MCF-10A are listed in Table 2-5.

Table 2-5. Growth medium ingredients of cell lines used in this study

Cell line	Growth Medium
MCF7	10% FBS (Hyclone, Thermo Scientific, USA) and 1% Penicillin / Streptomycin (P/S) (Hyclone) supplemented low glucose Dulbecco's Modified Eagle Medium (DMEM) (Hyclone)
MDA-MB-453	10% FBS and 1% Penicillin / Streptomycin (P/S) supplemented low glucose DMEM

MDA-MB-468	10% FBS and 1% Penicillin / Streptomycin (P/S) supplemented low glucose DMEM
BT20	10% FBS and 1% Penicillin / Streptomycin (P/S) supplemented low glucose DMEM
MDA-MB-231	10% FBS and 1% Penicillin / Streptomycin (P/S) supplemented low glucose DMEM
CAMA-1	10% FBS and 1% Penicillin / Streptomycin (P/S) supplemented low glucose DMEM
ZR-75-1	10% FBS, 1% P/S and 2mM glucose (Sigma-Aldrich, USA) supplemented RPMI-1640 medium (Hyclone)
BT474	10% FBS, 1% P/S and 10µg/ml insulin (Sigma-Aldrich)
MDA-MB-157	10% FBS, 1mM sodium pyruvate (Gibco, Invitrogen, USA) and 1% P/S supplemented low glucose DMEM
MDA-MB-361	10% FBS, 1mM sodium pyruvate and 1% P/S supplemented low glucose DMEM
HCC-1937	10% FBS, 1% P/S and 1mM sodium pyruvate supplemented RPMI-1640 medium
MCF10A	10% FBS, 1% P/S, 10µg/ml insulin, 20 ng/ml EGF (Sigma-Aldrich) and 0.5 mg/ml hydrocortisone (Sigma-Aldrich) supplemented DMEM/Ham's F-12 (1:1) medium (Biochrome, Merck Millipore, Germany)
SKBR-3	10% FBS and 1% P/S supplemented McCoy's 5A medium (Hyclone)

CAL-51	20% FBS and 1% P/S supplemented high glucose DMEM

All cells were grown in 5% CO_2 and 95% air at 37°C cell culture incubator.

All cell lines except MCF-10A were purchased from ATCC while MCF-10A was kindly provided by Assist. Prof. Dr. A. Elif Erson (Middle East Technical University).

2.3.1.1 Passaging of cell lines

All cell lines used in this study were adherent cell lines. Cell lines were passaged every 2-3 days before they became confluent, depending on the growth rate of the cell line. Growth medium was discarded using a sterile glass pipette and cells were washed with 1X PBS (Hyclone). After discarding PBS, Trypsin/EDTA solution (Hyclone) was added onto cells until it covers the surface of the cell monolayer. In order to increase the enzymatic activity of the Trypsin/EDTA solution, the cell culture dish was placed in a cell culture incubator at 37°C for 3-5 minutes. When cells dissociated from the flask, cells were diluted with fresh medium and transferred to new flasks.

2.3.1.2 Cryopreservation and thawing of the cells

Cells were passaged one day prior to cryopreservation to increase the viability of the cells during cryopreservation process. Similar to passaging, old medium was discarded and cells were washed with 1X PBS. Trypsinization was performed; cells were collected with fresh medium and placed into a 15 ml falcon tube. Cells were centrifuged at 1500 rpm for 5 minutes. The medium was discarded and the cell pellet was gently resuspended with 1.5 ml freezing medium (10% DMSO and 90% FBS). Cells were transferred into properly labelled cryotube vials and left in -20°C for 1-2 hours. Then cryotube vials were taken from -20°C and replaced into -80°C for overnight. After overnight at -80°C, vials are placed in liquid nitrogen for long-term storage.
For thawing of the cells, vials were taken from liquid nitrogen tanks and transported the cell culture room in a liquid nitrogen container. Vials were taken from the liquid nitrogen

and immediately put in 37°C water bath. Vials were allowed to stand until half of the cell mixture was thawed. Half defrosted cells were added on top of 5 ml of medium in 15 ml of falcon tube and centrifuged at 1500 rpm for 5 minutes. Supernatant was discarded to get rid of the unwanted DMSO, cell pellet was gently resuspended in 5 ml of fresh medium and transferred to sterile T25 tissue culture flask and placed into the cell culture incubator.

2.3.1.3 Collection of the cells for RNA or DNA isolation

Cells were either collected before becoming confluent or after a particular treatment (5-AZA, TSA etc.). First, used medium was discarded and cells were washed with 1X PBS. After trypsinization, cells were collected with fresh medium into a 15 ml falcon. Falcon tubes were immediately placed into ice and centrifuged at 1500 rpm for 5 minutes at +4°C. After discarding the medium, pellet was washed with cold 1X PBS and replaced back into centrifuge and centrifuged at the same conditions. PBS was discarded; tubes were snap frozen in liquid nitrogen and stored at -80°C until use.

2.3.1.4 Treatment of cell lines with 5-AZA and/or TSA

MCF7, MDA-MB-231, MDA-MB-453, MDA-MB-468, BT-474, ZR-75-1, BT-20, MDA-MD-361, SKBR-3, CAL-51 and MCF-10A cell lines were treated with 5-AZA and TSA separately.
750.000 cells were plated in 100 mm cell culture dish and treated with 5μM 5-AZA (Sigma-Aldrich) or DMSO (at the same amount used to solubilize 5-AZA). 5-AZA/DMSO and medium changed everyday for four days and cells were collected at the end of day four. For TSA treatment, cells were treated with either 400 nM TSA or DMSO (at the same amount used to solubilize TSA) 24 hour after the cell seeding and collected at the end of 48^{th} hour.
MCF7, MDA-MB-231, MDA-MB-453, MDA-MB-468, BT-474, ZR-75-1, BT-20, SKBR-3, CAL-51 and MCF-10A were treated with both 5-AZA and TSA together.

Similar to single treatments, 5μM of 5-AZA put into the medium at the day of seeding and 5-AZA was changed along with the medium everyday. 400nM TSA was added three days after the seeding (24 hours before the collection of cells) cells were collected at day four to analyze the effect of 5-AZA and TSA together.

2.3.2 Patients and Tissue Samples

19 primary breast tumors and matched normal tissues were collected from Ankara Numune Research and Teaching Hospital (see Table 2-6). The Research Ethics Committee of Ankara Numune Research and Teaching Hospital approved the use of collected clinical tissue samples and patient consent was obtained in agreement with the Helsinki Declaration.

Both breast tissues and matched normal tissues resected during surgery and were immediately frozen in liquid nitrogen and stored at -80°C. Hematoxylin and eosin staining was used for determining the pathological characteristics. For tumor samples, only samples compromised of more than 80-90% of tumor cells were involved in the study.

Table 2-6. Clinical/Pathological characteristics of patient samples

Patients	Age	ER	PR	Diagnosis	Lym. Node	Grade	Stage	DM	Month of the DM
113	63	+	-	IDC	+	1	IIIB	+	20
115	57			Papillary carcinoma	-	3	IIA	-	
96	39	-	+	IDC	-	2	IIA	-	
116	74	-	-	IDC	+	2	IIA	-	
137	42	-	+	Medullary	-	3	IIB	-	
146	49	+	+	ILC	+		IIB	-	
148	70	+	-	IDC	+	2	IIIA	+	15
154	32	-	-	IDC	+	3	IIIB	-	
159	30	-	+	Metaplastic	-	2	IIA	-	
161	41	-	-	IDC	-	2	IIB	+	47
164	74	+	+	IDC	-	2	IIB	-	
166	55	-	+	IDC	+	2	IIA	-	
168	44	-	+	IDC	+	2	IIB	-	
170	60	-	-	IDC	+	2	IIIB	-	
176	49	+	+	IDC	+	2	IIA	-	
177	47	-	+	IDC	+	2	IIIA	+	48
181	44	-	-	IDC	+	2	I	-	
133*									
173*									

* Patients with missing information
ER: Estrogen receptor status, PR: Progesterone receptor status, IDC: Infiltrating ductal carcinoma, Lym. Node: Lymph node status, DM: Distant metastasis status.

2.3.3 DNA Extraction

Breast cancer cell lines as well as clinical breast cancer and matched normal tissue samples were resuspended in Proteinase K containing solution and placed onto a heat block at 56°C overnight in order to increase the DNA yield of the isolation. The manufacturer's instructions of the Nucleospin Tissue (Macherey Nagel, Germany) DNA

extraction kit was followed. Extracted DNA samples were measured with Nanodrop Spectrophotometer (Thermo Scientific).

2.3.4 Generation of Competent *E. coli* DH5α

E. coli DH5α glycerol stock was streaked on an agar plate (without antibiotics) using a sterile loop and left in 37°C bacteria incubator overnight. 2-3 colonies were selected and grown in 15 ml LB overnight in a shaker incubator at 37°C. Overnight culture from one colony was added into 200 ml LB in a 1000 ml flask until the optical density (OD) reaches 0.2. The bacteria mixture was placed in a shaker incubator at 25°C and grown until the OD at 600 nm becomes 0.5-0.6. The bacteria mixture was transferred into a 465 ml ultracentrifugation tube and left on ice 10 minutes. Tubes were centrifuged at 2500 g for 10 minutes at +4°C. The supernatant was discarded and bacteria pellet is resuspended with 64 ml ice-cold transfer buffer. The mixture was transferred into two cold 30 ml ultracentrifugation tubes and left on ice for 10 minutes. The tubes were centrifuged again at 2500 g for 10 minutes at +4°C. The supernatant was discarded and bacteria were resuspended in 8 ml ice-cold transfer buffer containing 7% DMSO. This mixture was aliquotted as 200 µl per 1.5 ml eppendorf tube, frozen immediately with liquid nitrogen and stored at -80°C until use.

2.3.5 Bisulfite Sequencing

2.3.5.1 Bisulfite treatment of genomic DNA

1 µg of genomic DNA was used for sodium bisulfite treatment of DNA, which was used to convert unmethylated cytosine residues to uracil and leaves methylated cytosine residues as cytosine. EpiTect Bisulfite Kit (Qiagen, Germany) was used to perform sodium bisulfite treatment of DNA. Manufacturer's instructions were followed. Elution was performed using 20 µl of elution buffer.

2.3.5.2 Bisulfite specific PCR for 45S rDNA promoter region

Bisulfite converted DNA (1µl) was amplified with Taq DNA Polymerase (Fermentas, Thermo Scientific, USA) using bisulfite DNA specific primers targeting 45S promoter (sequences of 45S BSP forward and reverse were listed in Table 2-4.
45S BSP PCR reaction mixture was prepared as follows:

10X Taq Buffer	2.5 µl
$MgCl_2$ (25 mM)	3 µl
dNTP (10 mM)	1.5 µl
45S BSP Forward Primer (10 mM)	1 µl
45S BSP Reverse Primer (10 mM)	1 µl
Bisulfite DNA	1 µl
ddH_2O	14.7 µl
Taq Polymerase	0.3 µl

Thermal cycler condition for 45S BSP primers is as follows: initial denaturation at 95°C for 5 minutes, 45 cycles of denaturation (30 seconds at 95°C), annealing (30 seconds at 56°C) and extension (30 seconds at 72°C) and PCR was finished with a final extension at 72°C for 5 minutes. At the end of the BSP PCR, 5 µl of 6X DNA loading dye (Thermo Scientific) was added into PCR products and all samples were loaded into 1.5% agarose gel. Agarose gel was briefly visualized with AxioCam MRc5 image capture device.

2.3.5.3 Gel extraction, ligation and transformation

45S BSP PCR bands at correct size (436 bp) were quickly excised from the gel under UV light. PCR products were extracted from the gel by using QIAquick Gel Extraction kit (Qiagen). Purified products were cloned into pGEM-T Easy vector by using pGEM-T Easy vector system (Promega, USA). Ligation mixture was prepared for each PCR product and for controls described in Table 2-7.

Table 2-7. Preparation of ligation mixture

	Standard Reaction	Positive Control	Background Control
2X Rapid Ligation Buffer	5 µl	5 µl	5 µl
pGEM - T Easy Vector	1 µl	1 µl	1 µl
PCR product	3 µl	-	-
Control Insert DNA	-	2 µl	-
T4 DNA Ligase	1 µl	1 µl	1 µl
Deionized water	-	1 µl	3 µl

Ligation mixtures were allowed to stand at room temperature for 1 hour then placed into +4°C for overnight before transformation.

The transformation protocol was performed according to the pGEM-T Easy vector system manual using competent *E. coli* DH5α. Bacteria plated on LB-agar containing 100 µg/ml Ampicillin (Applichem, Germany), 0.5 mM IPTG (Fermentas) and 80 µg/ml X-Gal (Fermentas).

2.3.5.4 Selection and sequencing of the bisulfite clones

Five colonies from each cell line and 10 colonies from tissue samples (positive, white colonies) were randomly selected from the plates and inoculated in 3ml LB with Ampicillin (100 µg/ml) at 37°C in a shaker incubator overnight.

Small-scale isolation of plasmid DNA (mini-prep) was performed with Nucleospin Plasmid Isolation (Macherey Nagel) kit according to the manufacturer's instructions. Plasmids containing the cloned inserts confirmed with PCR using T7 and SP6 universal primers.

PCR reaction mixture for T7 and SP6 primers was prepared as follows:

10X Taq Buffer	2.5 µl
$MgCl_2$ (25 mM)	1µl
dNTP (10 mM)	1 µl
T7 Primer (10 mM)	1 µl
SP6 Primer (10 mM)	1 µl
Plasmid DNA	1 µl
ddH_2O	17.3 µl
Taq Polymerase	0.2 µl

PCR was placed into a thermal cycler and conditions were set as follows; initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation (30 seconds at 95°C), annealing (30 seconds at 47°C) and extension (30 seconds at 72°C) and PCR was finished with a final extension at 72°C for 5 minutes. At the end of the PCR, 5 µl of 6X DNA loading dye (Thermo Scientific) was added into PCR products and 15 µl from each plasmid PCR product was loaded into 1.5% agarose gel. Agarose gel was visualized with the AxioCam MRc5 image capture device. Positive clones with the correct insert size were selected and sequenced with SP6 primers using dideoxy chain termination method (by Iontek Company, Turkey).

2.3.6 RNA Isolation and cDNA synthesis

2.3.6.1 RNA isolation from frozen tissue samples

4-5 slices from frozen tumor samples and 20-25 slices for normal tissue samples were dissected into 60-µm-thick sections and RNA isolation was performed. Tissue samples were homogenized with a homogenizer in 1 ml TRI Reagent RT (Molecular Research Center, USA) and a 21-gauge needle was used for fine homogenization of the samples. Samples were incubated at room temperature for 5 minutes and 50 µl of 4-Bromoanisole (Molecular Research Center) was added for every ml of TRI reagent used. Samples were vigorously shaken for 15 seconds using vortex and allowed to stand at room temperature for 2-3 minutes. Centrifugation of the samples was performed at 12000xg for 15 min at

4°C and then the upper aqueous phase was collected into a new eppendorfa tube. 0.5 ml of isopropanol was added into the RNA containing clear phase. After 10 minutes of incubation at room temperature, the mixture was centrifuged at 12000xg for 15 min at 4°C for to recovery of the RNA. The supernatant was discarded without disturbing the pellet. The pellet was washed twice with 75% ethanol and centrifuged at 7500xg for 5 min at 4°C. Lids of the tubes left open in the fume to air-dry the pellets for 10 minutes. RNA pellets were dissolved in 30-50 µl (depending on the visibility of the RNA pellet) of Diethylpyrocarbonate (DEPC) treated H2O. RNA concentrations were determined with the Nanodrop Spectrophotometer.

2.3.6.2 RNA isolation from cell lines

RNA isolation was performed from collected breast cancer cells using NucleoSpin RNA (Macherey Nagel, Germany) kit following manufacturer's instructions.

2.3.6.3 DNase I treatment of RNA samples

DNase I treatment was performed with MessageClean Kit (GenHunter, USA) in order to eliminate DNA contamination of total RNA that are obtained from tissue samples. 10 µg of total RNA diluted in 5.7 µl of 10X reaction buffer, 1 µl of DNaseI I added and volume increased to 56.7 µl with nuclease free water. Samples were mixed and incubated at 37°C for 30 minutes. Then, RNA was cleaned with phenol: chloroform: isoamyl alcohol extraction.

An equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) mixture was added into the incubated mixture and vortexed for 30 seconds. The sample was incubated on ice for 10 minutes and centrifuged 5 minutes at 13000 rpm at +4°C. Aqueous phase was transferred into a new 1.5 ml tube, an equal volume of chloroform was added and vortexed for 30 seconds. The sample was again incubated on ice for 10 minutes and centrifuged for 5 minutes at 13000 rpm at +4°C. The aqueous phase was transferred into a new 1.5 ml Eppendorf tube and 5 µl of 3M NaoAc pH: 5.2 and 200 µl of ice-cold

ethanol added. The samples were incubated at -80°C overnight. Samples were centrifuged 20 minutes at 13000rpm at +4°C after overnight incubation and supernatant was discarded. The pellet was washed with 1 ml of 70% EtOH and centrifuged for 5 minutes at 13000 rpm at room temperature. EtOH was removed and the pellet was air-dried. The pellet was resuspended with 10-20 µl DEPC treated water.

2.3.6.4 Random primed cDNA synthesis from RNA samples

500 ng of total RNA from the tissue samples and 1 µg of total RNA from the cell lines were used in random primed cDNA synthesis with RevertAid First Strand cDNA Synthesis Kit (Fermentas).
The specified amount of RNAs (500 ng for tissues and 1 µg for cell lines) was taken and prepared in two different tubes (1 tube for +RT and 1 tube for – RT). 1 µl of random hexamer primers were added and mixture is completed to 12 µl with DEPC H_2O. Samples were mixed, spun down, incubated at 65°C for 5 minutes in a thermal cycler and then samples chilled on ice. 4 µl of 5X reaction buffer, 1 µl of RiboLock RNase Inhibitor and 2 µl of 10 mM dNTP mix added in both of the tubes. 1 µl of RevertAid RT is added only to +RT tubes. Samples are mixed, spun down, incubated at 25°C for 5 minutes, followed by 42°C for 60 minutes and reaction was terminated at 70°C for 5 minutes in a thermal cycler. cDNA products were kept at -80°C for long-term storage.

2.3.7 Real-Time PCR

2.3.7.1 Real-Time PCR

Real-time PCR was performed with primers targeting 45S ETS, 18S, 28S and 5.8 rRNA transcripts. All primer sequences were listed in Table 2-4. Random primed cDNAs from both cell lines and frozen tissue samples were diluted at 1/5 ratio. 1µl of diluted cDNA was used in every reaction containing 10 µl of Dynamo SYBR Green qPCR Kit (Thermo Scientific) and 10 pmol of forward and reverse primers in 20 µl of final volume. Thermal cycling conditions are as follows: initial denaturation 5min at 95°C, 40 cycles of 30 s at

95°C, 30 s at 60°C and 30 s at 72°C followed by melting curve. All reactions were set as duplicates. The Stratagene Mx3005P Real-Time PCR System (Agilent, USA) was used for real-time PCR experiments.

2.3.7.2 Real-Time PCR data analysis

The Stratagene Mx3005P Real-Time PCR System automatically calculates the threshold line and cycle threshold (Ct) values are calculated depending on the threshold line. We used the logarithmic scaling to manually determine the threshold line to increase the sensitivity of each run. The automatic and manual threshold lines were presented for 5.8S rRNA as an example (Figure 2.1). New threshold lines enabled us to determine more subtle changes between samples while eliminating the noise signal.

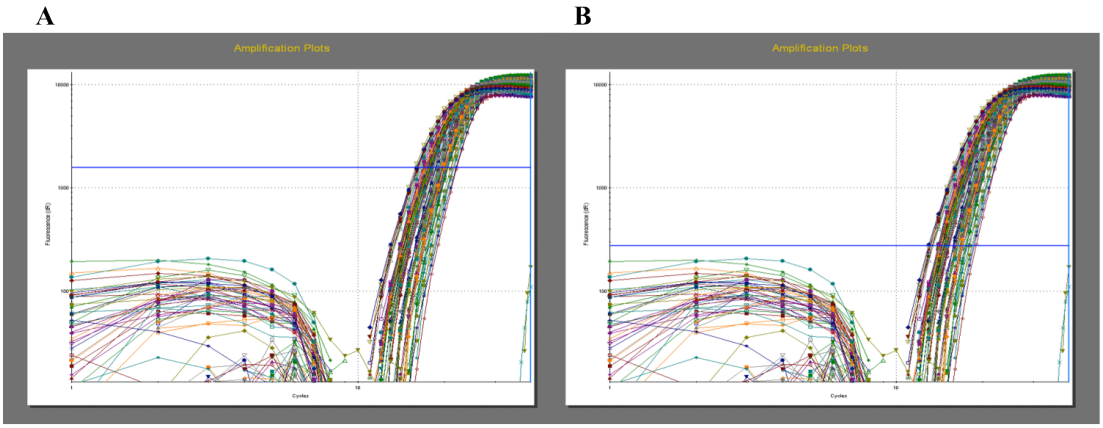


Figure 2.1. The threshold line of qRT-PCR results were changed from automatically calculated threshold line (A) to manually determined threshold line (B) by using log transformed Ct values for more sensitive analysis.

The average of the duplicates were calculated if the duplicates are consistent with each other. Relative expression levels of rRNAs (18S, 28S, 5.8S and 45S ETS) were evaluated using Log2 ($2^{-\Delta Ct}$) calculation.

TBP, ACTB and *ACTB&TBP* genes were used as reference genes for cell lines and *TBP* was used as a reference gene in breast tumor and paired normal tissue samples to assess the amount of cDNA. However both genes are known to be fluctuating between breast

tumor and normal samples, we purposed the use of geometric mean of rRNA transcripts (GM-rRNAs) to identify relative changes of rRNAs in the rRNA pool. Accordingly, we also used GM-rRNA values to determine relative rRNA expression values again using the Log2 ($2^{-\Delta Ct}$) calculation method.

2.3.8 Statistical Analyses

2.3.8.1 Statistical analyses of methylation data

Raw bisulfite sequencing data were aligned with genomic DNA sequence of amplified region (rDNA promoter region from -380 to +53 bp) using Quantification of Methylation Analysis (QUMA) tool which is a web-based quantification tool for methylation analysis (http://quma.cdb.riken.jp) (Kumaki et al. 2008). Raw data was trimmed with reference to genomic DNA sequence using QUMA. Analysing the unconverted cytosine residues in non-CG sites in raw sequencing data used to determine the bisulfite conversion rate of the clones. A 95% bisulfite conversion rate was determined as the threshold and clones with lower conversion rates were excluded. Clones from each sample were displayed as lollipop graphs using QUMA program.
The Wilcoxon signed rank test was used to test paired differences between breast tumor and matched normal methylation levels instead of the Mann-Whitney U test offered by the QUMA tool.

2.3.8.2 Pattern similarity analyses in tissue samples

We used methylation percentages of every CpG position in a tissue sample and correlated it with its pair to identify whether there is a correlation in the methylation pattern of pairs. We performed the same analysis with unmatched tumor and normal pairs to find out whether the methylation pattern is patient specific or tissue specific. We used Pearson correlation to test the pattern similarity analysis.

2.3.8.3 Analyses of clinical variables with methylation difference values

Methylation difference in each CpG was calculated as methylation % tumor sample - methylation % of matched normal sample as shown in Table 2-8. Only 12 CpGs from two paired samples were shown.

Table 2-8 Calculation of percent methylation difference in each CpG

CpG Number	1	2	3	4	5	6	7	8	9	10	11	12…
113N	40	70	60	70	90	40	60	60	70	70	40	50
113T	40	40	30	50	30	50	60	60	50	40	40	40
Meth Dif. (T-N)	0	-30	-30	-20	-60	10	0	0	-20	-30	0	-10
146N	20	20	10	60	50	10	10	30	30	10	20	40
146T	100	90	90	100	90	90	80	100	100	90	70	70
Meth Dif. (T-N)	80	70	80	40	40	80	70	70	70	80	50	30

Green and squares indicate less and more methylated CpGs in tumors compared to normal tissue pair, respectively.

Percent methylation differences of 54 CpGs of all the clones for each sample were used to generate Figure 3.6 and Figure 3.7. Both CpG positions and samples were grouped by their methylation pattern using Cluster 3.0 program.

Cluster 3.0 is a publicly available program (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm), generally used in the analysis of expression arrays. We used unsupervised hierarchical clustering of Cluster 3.0 program with breast tumor and normal paired samples based on their methylation differences to group the patient samples. Java Treeview program (http://jtreeview.sourceforge.net/) was used to visualization of the clustering data.

Two groups (methylated and unmethylated) were identified. Fisher's exact test was used to identify any association between these groups and clinical variables (categorical variables).

2.3.8.4 Correlation analyses of clinical variables and methylation levels

Methylation differences might not be reflected in the patient characteristics. We used only methylation levels (%) of the tumor and normal samples to test the association with patient features: age, ER, PR, distant metastasis, lymph node and metastasis status. Spearman correlation was performed this time since it is better at testing different types of data (methylation and clinical variables in our case) by using a ranking system compared to Pearson that can evaluate linear relationships.

2.3.8.5 Relationship between methylated CpGs and clinical variables

Multivariate analysis of variance (MANOVA) is a statistical test used for comparing mean values of multiple variables in different groups. We used MANOVA to determine the effect of the independent variables (clinical variables) on methylation levels of each CpG position (dependent variable).

2.3.8.6 Expression analyses of rRNA genes

We used ACTB, TBP, ACTB&TBP and GM-rRNA normalization methods to test the effect of normalization on expression changes in rRNA transcripts. Expression differences of rRNAs between treated and untreated cell lines or tumor and matched normal samples were performed using paired t-test.

2.3.8.7 Analysis of clinical variables and rRNA expression levels

Multiple regression analysis was used to predict clinical variables based rRNA expression values. We also used Spearman correlation to identify the association between rRNA levels and clinical features of the patients.

2.3.8.8 Correlation analyses of rRNA expression and rDNA methylation levels

rDNA methylation levels and rRNA gene expression levels were tested using Spearman correlation to determine whether rDNA promoter methylation has an effect on rRNA gene expression in breast cancer cell lines as well as in tissue samples. The same correlation analysis is also used to identify the correlation between CpG methylation levels and rRNA gene expression.

Ct values of rRNA species (18S, 28S, 5.8S and 45S ETS) were used in Spearman correlation test to see whether there is a correlation between spliced rRNA products in tissue samples.

3 RESULTS

3.1 Methylation Analysis of 45S rDNA Promoter Region in Breast Cancer

3.1.1 Methylation Analysis of 45S rDNA Promoter Region in Breast Cancer Cell Lines

Bisulfite sequencing primers targeting a 434 bp region spanning both Upstream Control Element (UCE) and core promoter (CP) region (from -381 bp to +53 bp) were used to amplify bisulfite treated DNA from ten breast cancer cell lines (MCF7, MDA-MB-231, MDA-MB-453, MDA-MB-468, BT-474, ZR-75-1, BT-20, MDA-MD-361, SKBR-3 and CAL-51) and a non tumorigenic breast cell line (MCF-10A). Five clones were randomly selected and sequenced using SP6 primers. Raw data was aligned, trimmed and analysed using the QUMA tool (Kumaki et al. 2008).

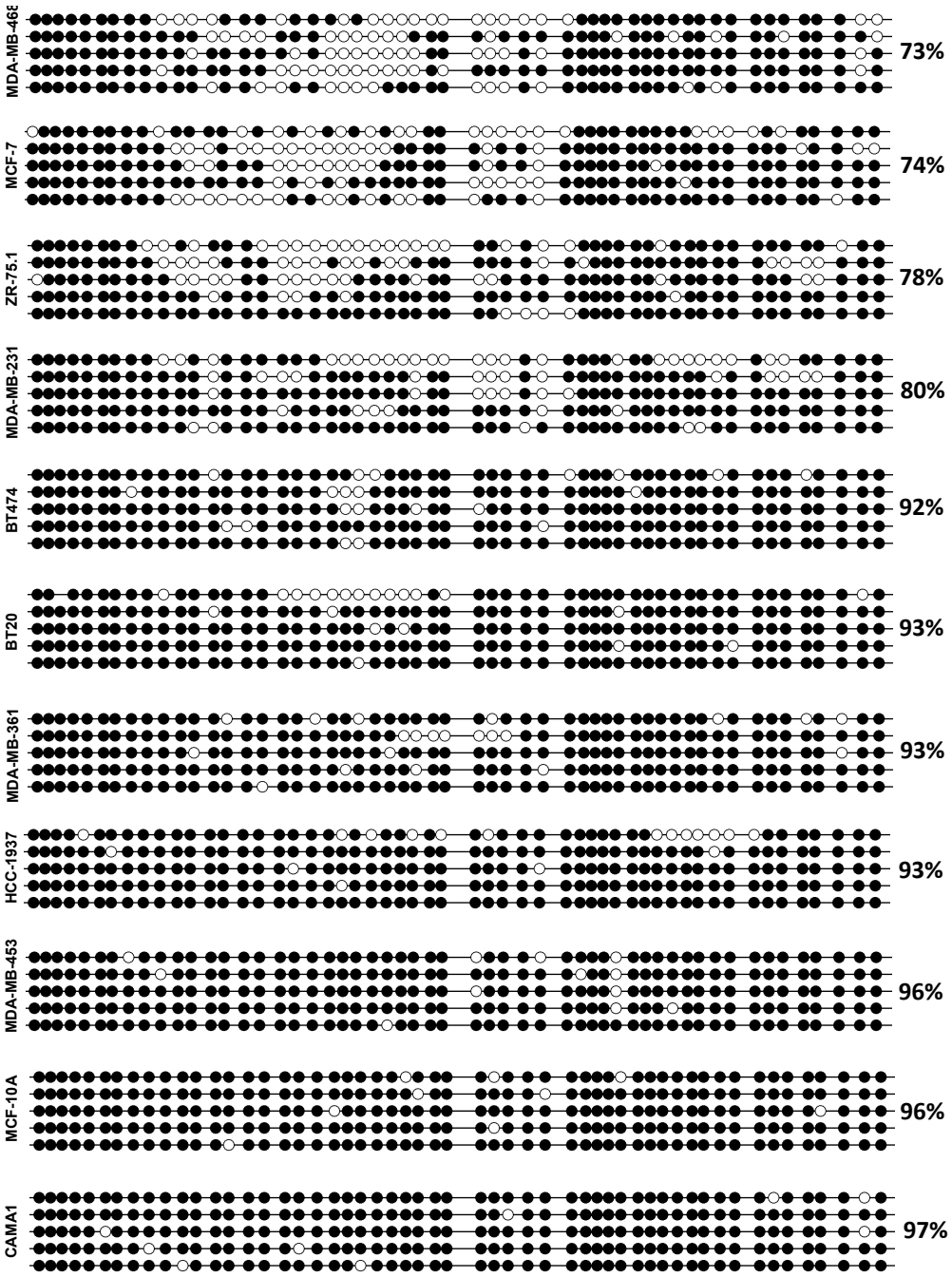


Figure 3.1. The methylation status of the 45S rDNA promoter in breast cancer cell lines and a non-tumorigenic breast cell line (MCF-10A). A total of 54 CpGs in a region spanning -381 bp to +53 bp was analysed by the bisulfite sequencing method. Each row corresponds to the sequence analysis of one clone and each column represents the CpG positions. The filled and empty circles stand for methylated and unmethylated CpGs respectively. Average methylation percentages of the clones for each sample are indicated at the right of the graph.

45S rDNA promoter region was heavily methylated (ranging from 74% to 96%) in all breast cancer cell lines (Figure 3.1).

3.1.2 Methylation Analysis of 45S rDNA Promoter in Breast Tumor and Matched Normal Tissue Samples

Nineteen breast cancer tissue and matched normal tissues (Table 2-6) were used to analyse 45S rDNA promoter methylation status with the same bisulfite sequencing method. Randomly selected ten clones were sequenced for each sample.

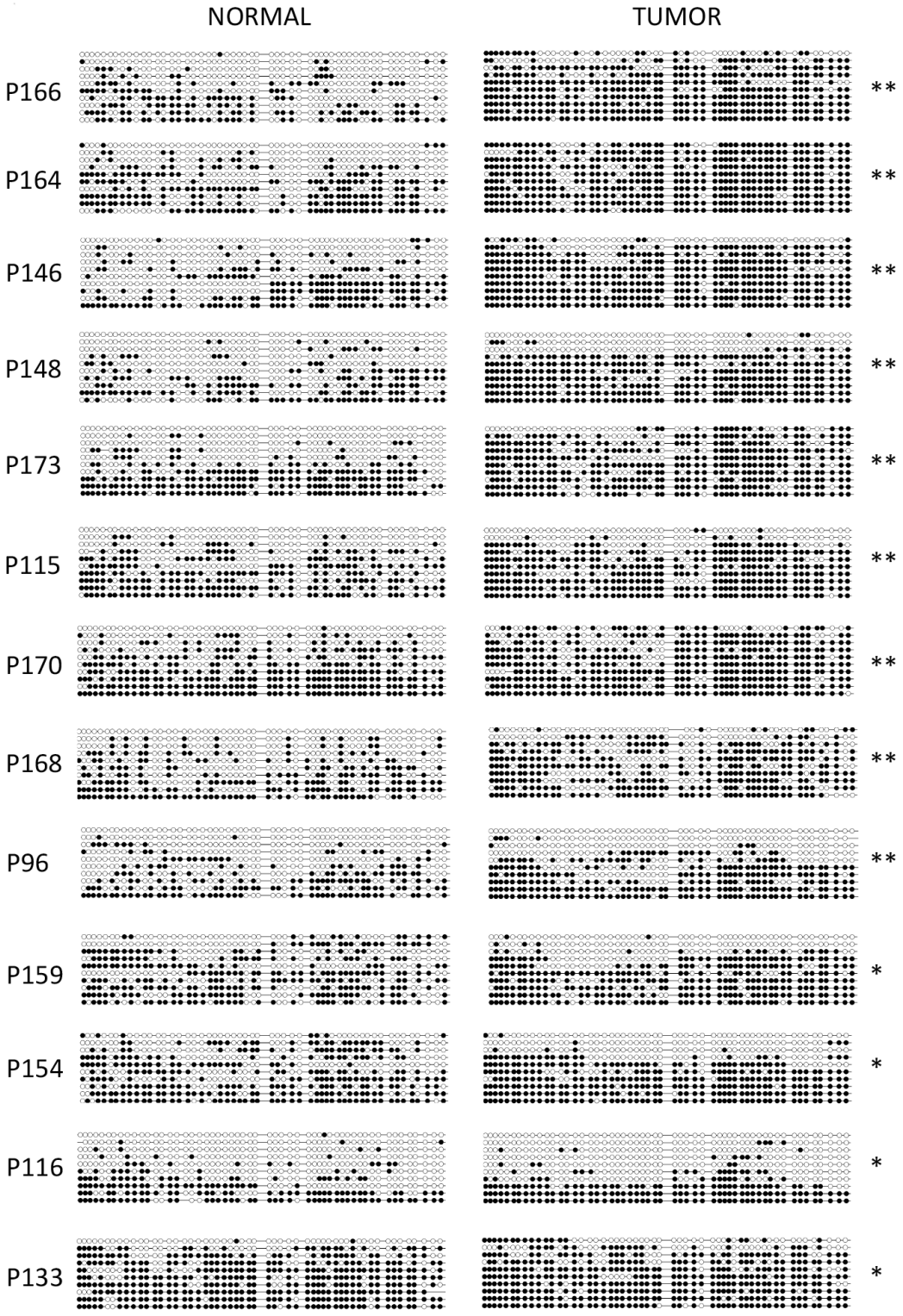


Figure 3.2. Methylation levels of the 45S rDNA promoter region in clinical breast cancer are significantly higher than their matched normal tissue samples in thirteen pairs. Ten randomly selected clones were sequenced from each sample. Wilcoxon Signed-Rank test was used to identify methylation differences between paired samples. (for $p<0.0001$ and * for $p<0.05$).**

Thirteen breast tumor samples (68.4%) have significantly higher methylation levels than their matched normal samples at the 45S rDNA promoter region (Figure 3.2).

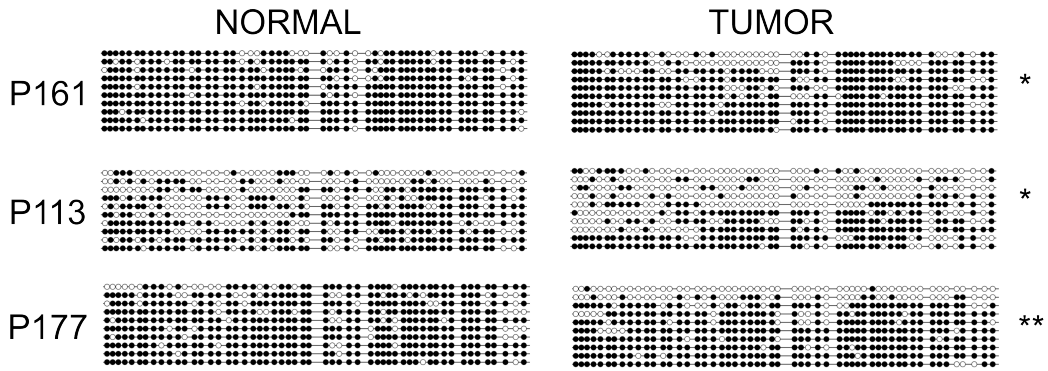


Figure 3.3. Methylation levels of the 45S rDNA promoter region in normal breast tissues are significantly higher than their matched breast tumor tissue samples in three pairs. Ten randomly selected clones were sequenced from each sample. Wilcoxon Signed-Rank test was used to identify methylation differences between samples. (for $p<0.0001$ and * for $p<0.05$).**

Three normal breast samples have significantly higher methylation levels than their matched tumor tissue at the 45S rDNA promoter region (Figure 3.3).

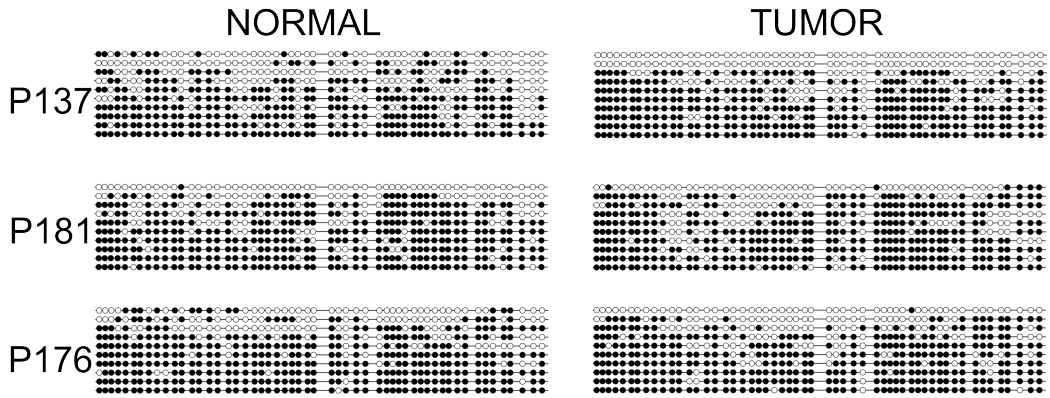


Figure 3.4. Methylation status of the 45S rDNA promoter region in clinical breast cancer and matched normal tissues are at similar levels in three pairs. Ten randomly selected clones were sequenced from each sample. Wilcoxon Signed-Rank test was used to identify methylation differences between samples.

No significant methylation difference was observed between three pairs of tissue samples (Figure 3.4).

Our results revealed that 13 out of 19 (68.4%) breast cancer tissue samples had higher methylation levels at 45S rDNA promoter region (Figure 3.2). On the other hand, three samples (15.8%) showed significantly higher methylation levels in normal samples compared to their tumor pairs (Figure 3.3), whereas there was no significant difference between promoter methylation levels of breast tumor and matched normal tissues in the remaining three samples (15.8%) (Figure 3.4). Normal samples were not fully unmethylated and instead showed a mosaic methylation pattern, a relatively common observation for human rDNA promoters (Ghoshal et al. 2004).

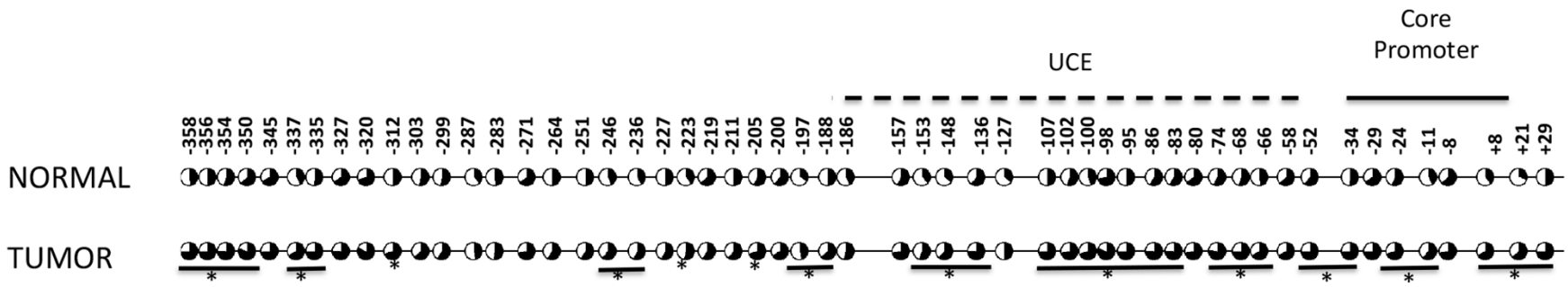


Figure 3.5. Methylation levels of every CpG in clones from breast tumor and paired normal tissues were compared to determine differentially methylated CpGs. Methylation percentages of every CpG are presented as a pie chart. Significantly ($p<0.05$) methylated CpGs in tumor samples compared to normal samples were indicated with "*".

The analysis of individual CpGs in all tumor and normal tissue samples using Wilcoxon matched pairs signed rank test, revealed significant methylation differences at certain CpGs (Figure 3.5).

3.1.3 Methylation Pattern Similarity Between Breast Tumor and Matched Normal Tissues 45S rDNA Promoter Region

We used methylation levels in all CpGs of a tumor and its normal pair in order to see whether a patient (individual) specific methylation pattern exists in this region.

Table 3-1. Correlation of methylation between tumor and matched normal samples

	Pearson r	Pearson p	Significant
96	0.199	0.149	
113	0.328	0.015	*
115	0.352	0.009	*
116	0.335	0.013	*
133	0.339	0.012	*
137	0.196	0.156	
146	0.346	0.010	*
148	0.474	<0.0001	*
154	0.398	0.003	*
159	0.300	0.028	*
161	0.552	<0.0001	*
164	0.204	0.138	
166	0.121	0.382	
168	0.511	<0.0001	*
170	0.201	0.145	
173	0.081	0.561	
176	0.272	0.047	*
177	0.348	0.010	*
181	0.259	0.058	

Pearson r: Pearson correlation coefficient, Pearson p: Pearson correlation significance, *Significant correlation $p<0.05$

Twelve pairs out of nineteen pairs (63%) showed significant correlation for their methylation patterns (Table 3-1). Unmatched tumor and normal samples were also analysed to ensure that this pattern similarity is patient specific but not tissue specific.

Table 3-2. A representative of Pearson correlation between unmatched tumor and normal breast tissue samples

		96T	113T	115T	116T	133T
96N	Pearson r	0.199	0.497	0.316	0.163	0.435
	Pearson p	0.149	p<0.001*	0.02*	0.24	0.001*
113N	Pearson r	0.346	0.328	0.501	0.179	0.412
	Pearson p	0.01*	0.015	p<0.001*	0.195	0.002*
115N	Pearson r	0.226	0.343	0.352	0.039	0.611
	Pearson p	0.1	0.011*	0.009	0.778	p<0.001*
116N	Pearson r	0.303	0.279	0.324	0.335	0.421
	Pearson p	0.026*	0.041*	0.017*	0.013	0.002*
133N	Pearson r	0.004	0.233	0.422	0.239	0.339
	Pearson p	0.978	0.09	0.001*	0.081	0.012

Pearson r: Pearson correlation coefficient, Pearson p: Pearson correlation significance, *Significant correlation $p<0.05$

All unmatched tumor and normal tissue samples were evaluated with Pearson correlation test. Table 3-2 shows correlation results between a few tumors and normal samples. Significantly ($p<0.05$) correlated methylation levels between unmatched tumor and normal samples were indicated with "*". Unpaired tumor and normal samples were analysed with Pearson correlation and 48.8% (167/342) of the unpaired samples showed significant correlation. This data indicates that pattern similarity might be tissue specific rather than patient specific.

3.2 Analysis of Clinical Variables with Promoter Methylation Levels

3.2.1 Classifying Patients According to Methylation Difference

Cluster 3.0 program was used for unsupervised hierarchical clustering of breast tumor and normal paired samples based on their methylation differences.

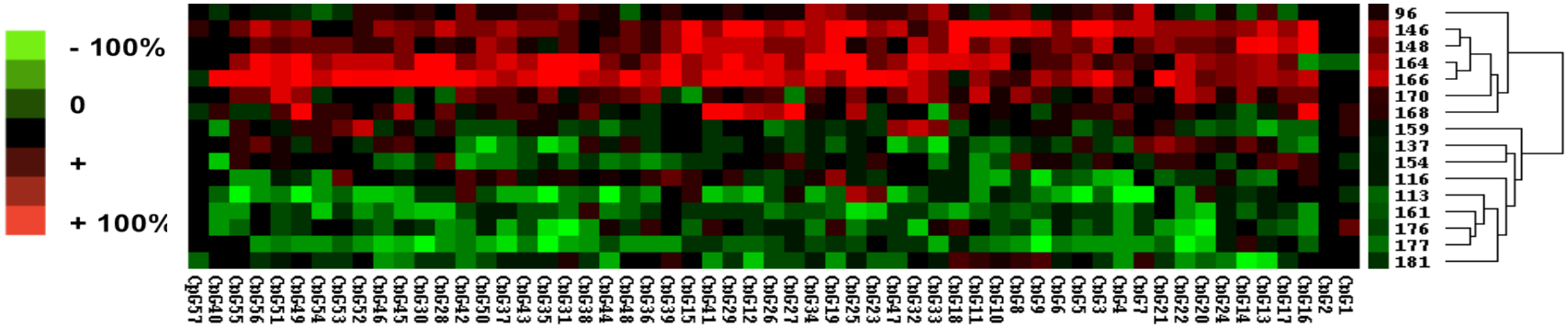


Figure 3.6. CpG methylation differences were calculated (tumor methylation % - normal methylation %) in each pair and unsupervised hierarchical clustering was performed using Cluster 3.0 program and Treeview program. Reddish squares indicated the CpG positions with higher methylation in tumor samples whereas greenish squares showed CpG position with higher methylation in normal pairs.

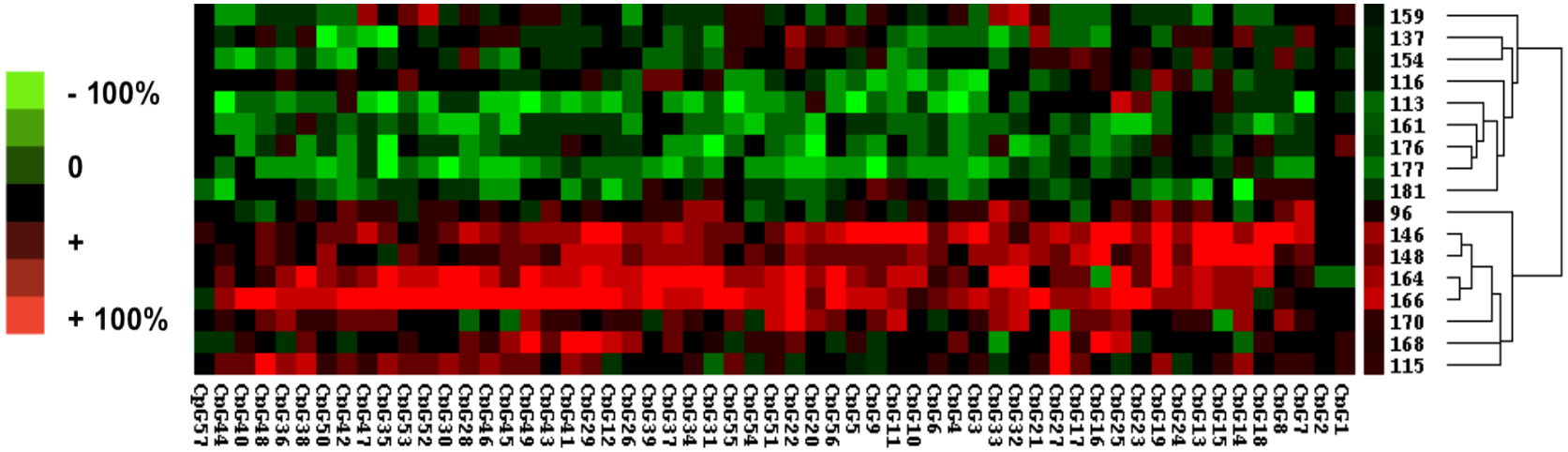


Figure 3.7. CpG methylation differences were calculated (tumor methylation % - normal methylation %) in each pair (including sample 115) and unsupervised hierarchical clustering was performed using Cluster 3.0 program and Treeview program. Reddish squares indicated the CpG positions with higher methylation in tumor samples whereas greenish squares showed CpG position with higher methylation in normal pairs.

.

Unsupervised hierarchical cluster images were generated using percent methylation differences in each CpG of tumor and normal paired samples. Dendogram divided patients into two main groups depending on their methylation patterns.
Samples were grouped by their methylation differences using the values given in Table 2-8 upper part of the Figure 3.6 includes highly methylated tumor samples 96, 146, 148, 164, 166, 170 and 168 (Group 1, more methylated in tumors compared to matched normal tissues) whereas the lower part contains lower methylation of tumor samples including 159, 137, 154, 116, 113, 161, 176, 177 and 181 (Group 2, less methylated in tumors compared to matched normal tissues). This grouping of the samples by their methylation differences can be used for further analysis with their ER (Estrogen Receptor) and PR (Progesterone Receptor) status. Samples 133 and 173 were excluded from this analysis since we do not have any patient information for these two samples and sample 115 was excluded from the first clustering in Figure 3.6 since we do not have its ER and PR information of the patient 115.
When sample 115 was included in hierarchical clustering, the picture did not change significantly much as seen in Figure 3.7 but sample 115 was grouped with more methylated in tumor samples group (Group 1).
The cluster image in Figure 3.7 is similar to the cluster in Figure 3.6, but sample 115 was clustered with highly methylated tumor samples. This grouping of the samples by their methylation differences can be used for further analysis with distant metastasis and pathological lymph node status of the patient samples.

3.2.2 Analysing Clinical Characteristics of the Patients with Methylation Difference Classifications

Methylation data can be linked to the clinical variables and it can be statistically tested whether methylation differences or methylation patterns can explain any of the clinical features.
In the Figure 3.6 two groups were identified depending on their methylation differences.

Group 1, methylated in tumors, including samples 96, 146, 148, 164, 166, 170 and 168. Group 2, unmethylated in tumors, including samples 159, 137, 116, 113, 161, 176, 177 and 181.

Patterns of methylation differences between tumor and normal pairs grouped the patients into two groups as explained above and this data could be used to analyse association between methylation groups and the clinical variables of the patients. A simple statistical test known as Fisher's exact test was used to examine the association (contingency) between the two kinds of classification. We generated the contingency tables using hierarchical clustering groups and ER, PR, metastasis and lymph node status.

Table 3-3. Contingency table is generated using hierarchical clustering groups and ER status of the patients

	ER +	ER-	Total
Group 1	3	4	7
Group 2	2	7	9
	5	11	16

No statistically significant (p=0.5962) association was found between hierarchical clustering groups of methylation (group 1 and group 2) and ER status (+,-).

Table 3-4. Contingency table is generated using hierarchical clustering groups and PR status of the patients

	PR +	PR-	Total
Group 1	5	2	7
Group 2	4	5	9
	9	7	16

No statistically significant (p=0.3575) association was found between hierarchical clustering groups of methylation (group 1 and group 2) and PR status (+,-).

As shown in Figure 3.7 sample 115 was included and hierarchical clustering was performed again, sample 115 was identified in the Group 1.

Table 3-5. Contingency table is generated using hierarchical clustering groups and distant metastasis status of the patients

	Metastasis +	Metastasis -	Total
Group 1	1	7	8
Group 2	3	6	9
	4	13	17

No statistically significant (p=0.5765) association was found between hierarchical clustering groups of methylation (group 1 and group 2) and the distance metastasis status.

Table 3-6. Contingency table is generated using hierarchical clustering groups and lymph node involvement of the patients

	Lym. Node +	Lym. Node -	Total
Group 1	5	3	8
Group 2	6	3	9
	11	6	17

No statistically significant (p=0.600) association was found between hierarchical clustering groups of methylation (group 1 and group 2) and the lymph node status. Methylation differences based hierarchical clustering has generated two groups, these two groups analysed with Fisher's exact test in order to find a relation between clinical parameters and methylation levels yet no significant result was obtained.

3.2.3 Correlation Analysis Between Clinical Variables and 45S rDNA Promoter Methylation Levels in Breast Cancer Tissues

Analysis using methylation differences between tumor and paired normal samples did not identify any significant association with the clinical variables using Fisher's exact test. So we used using Spearman correlation (two-tailed) analysis to determine whether DNA methylation levels of 45S rDNA promoter in tissue samples have a more direct association with the clinical variables (Table 3-7).

Table 3-7. Spearman correlation analysis of methylation levels and clinical features of the tumor and normal samples

		Age	ER	PR	Dist. Met.	Lym. Node	Grade	Stage
Methylation of Normal Samples	Spearman ρ	-0.368	-0.12	0.346	0.084	0.139	-0.096	-0.031
	Spearman p	0.24	0.726	0.297	0.796	0.666	0.779	0.924
	N	12	11	11	12	12	11	12
Methylation of Tumor Samples	Spearman ρ	-0.091	0.12	0.289	-0.084	0.251	0.064	-0.047
	Spearman p	0.778	0.726	0.389	0.796	0.432	0.852	0.885
	N	12	11	11	12	12	11	12

ρ ; Spearman correlation coefficient, p Spearman correlation significance

None of the clinical variables showed correlation with rDNA promoter methylation levels of breast tumor and matched normal pairs.

3.2.4 Analysis of Methylation Differences at Certain CpG Positions and their Relation to Clinical Parameters by Using MANOVA

In the previous analysis, total methylation percentage of each sample was used to analyse the effect of total methylation at the rDNA promoter region. Multiple ANOVA (MANOVA) test was used to analyse the effect of clinical variables on methylation levels of each CpG position.

Table 3-8. CpG positions showing significant methylation difference depending on clinical parameters.

	Dependent Variable	Mean Square	F	Sig.
ER	CpG10	4439.264	7.35	0.017
	CpG13	3114.409	4.709	0.048
	CpG15	2753.059	6	0.028
	CpG18	4597.878	13.228	0.003
	CpG19	3178.7	4.862	0.045
PR	CpG4	4145.589	6.58	0.022
Distant Metastasis	CpG7	3204.935	7.861	0.013
	CpG30	4626.812	6.069	0.026
	CpG36	1748.355	4.853	0.044
	CpG43	1921.188	4.724	0.046
	CpG45	3201.889	5.448	0.034
	CpG46	3553.622	5.939	0.028
	CpG51	1980.609	4.643	0.048
	CpG52	3390.332	7.71	0.014
	CpG55	2317.119	5.093	0.039
Lymph Node	No CpG			

F: Representative of the degree of difference in the dependent variable generated by the independent variable, it also considers covariance of the variables. Sig: Significance

Sequential CpGs CpG 10, 13, 15, 18 and CpG 19 can be important and can be analysed further to see whether this part of the promoter contains any estrogen responsive elements.

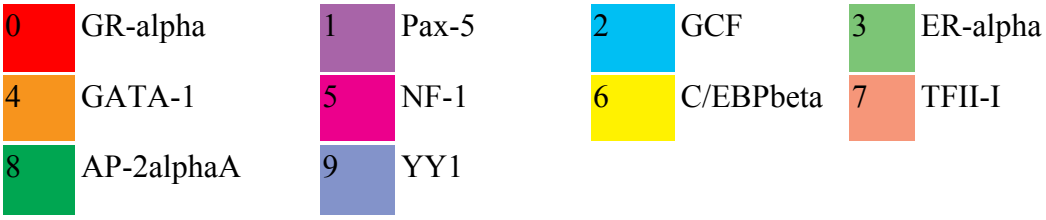
Distant metastasis status was also found to be associated with several CpG positions, some of them are sequential, such as: CpG 43, 45 and CpG 46 or CpG 51, 52 and CpG 55.

ER status and distant metastasis status was found to be associated with more than one CpG position (almost all found to be sequential) whereas PR was found to be related to only one CpG position (CpG 4) and there was no relationship found between lymph node status and differential methylation of CpG positions.

3.3 Transcription Factor Binding Site Analysis of 45S rDNA Promoter with Promo 3.0

Transcription factor binding site prediction tool PROMO 3.0 was used in order to see whether any of the CpGs in the analysed 45S rDNA promoter region is important for the binding of potential transcription factors.

PROMO is a virtual laboratory for the identification of putative transcription factor binding sites (TFBS) in DNA sequences from a species or groups of species of interest. TFBS defined in the TRANSFAC database are used to construct specific binding site weight matrices for TFBS prediction (Messeguer et al. 2002; Farré et al. 2003).



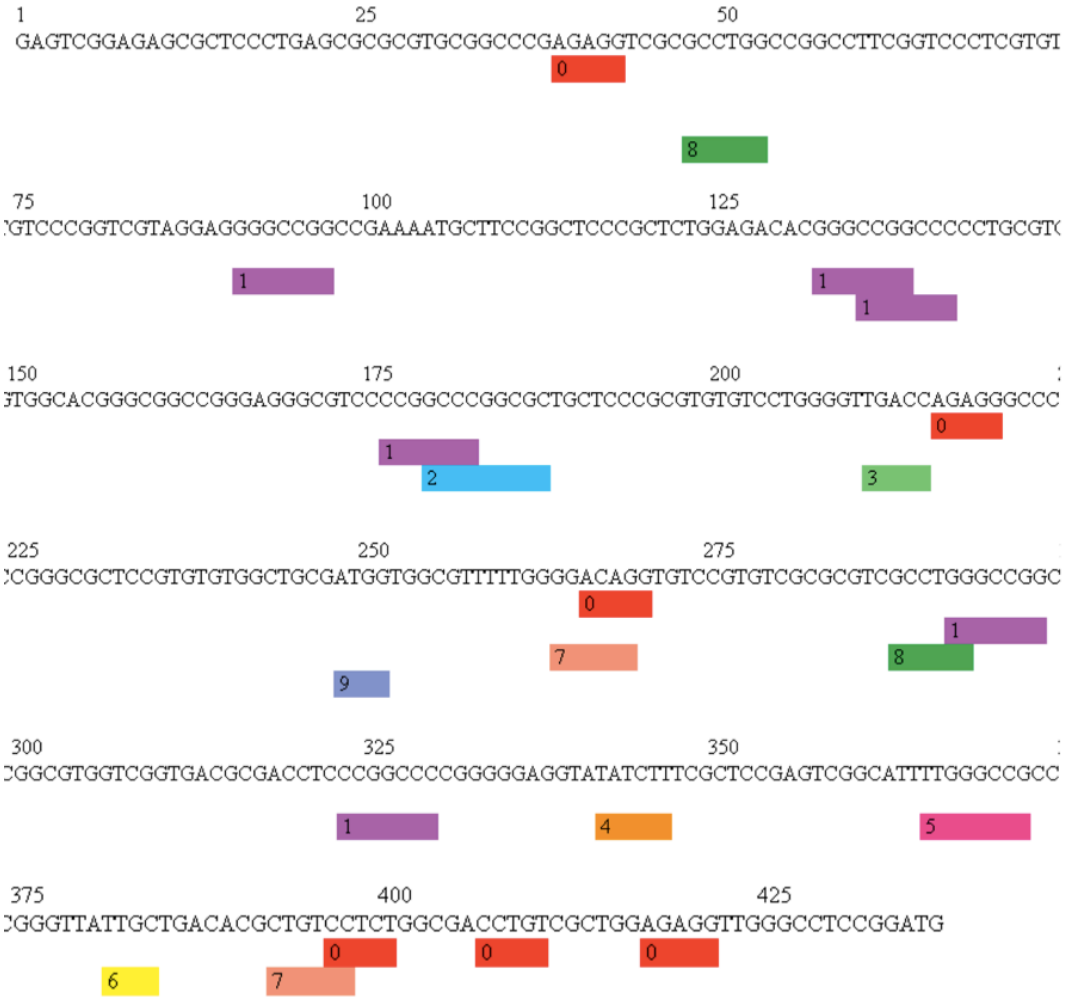


Figure 3.8. Promo 3.0 program is used to predict potential transcription factor binding sites at the 45S rDNA promoter region.

Nine different types of transcription binding sites were identified in this region. All sites predicted have a dissimilarity rate less or equal to 1%.

Some of the TFBS predicted to have more than one binding site (GR-alpha, Pax-5, TFII-I and AP-2 alpha A).

Table 3-9. Transcription binding sites in 45S promoter sequence predicted by PROMO 3.0

TFBS	Sequence	Start bp	End bp	CpG Status
	AGAGG	38	42	No CpG
	AGAGG	215	219	No CpG
	ACAGG	265	269	No CpG
	CCTCT	395	399	No CpG
	CCTGT	405	409	No CpG
GR-alpha	AGAGG	416	420	No CpG
	GGGCCGG	90	96	CpG 15
	GGGCCGG	131	137	**CpG 20**
	CCGGCCC	134	140	**CpG 20**
	CCGGCCC	175	181	**CpG 26**
	GGGCCGG	291	297	**CpG 41**
Pax-5	CCGGCCC	322	328	CpG 47
GCF	GCCCGGCGC	178	186	CpG 27 and **CpG 28**
ER-alpha	TGACC	210	214	No CpG
GATA-1	TATCTT	340	345	No CpG
NF-1	TTGGGCCG	364	371	**CpG 52**
C/EBPbeta	TTGC	380	383	No CpG
	GGACAG	263	268	No CpG
TFII-I	CTGTCC	391	396	No CpG
	GCCTGG	47	52	No CpG
AP-2 alpha A	GCCTGG	287	292	No CpG
YY1	ATGG	247	250	No CpG

Significantly methylated CpGs in tumor samples were shown with bold characters.

Predicted transcription binding sites may contain CpGs (such as Pax-5, GCF and NF-1) and CpG methylation at these sites might play a key role in the expressional regulation. As shown in Table 3-9, Pax-5 TFB sites overlap with 5 CpG positions; 3 of them (CpG 20, 26 and 41), written with bold characters, were found to be significantly methylated in

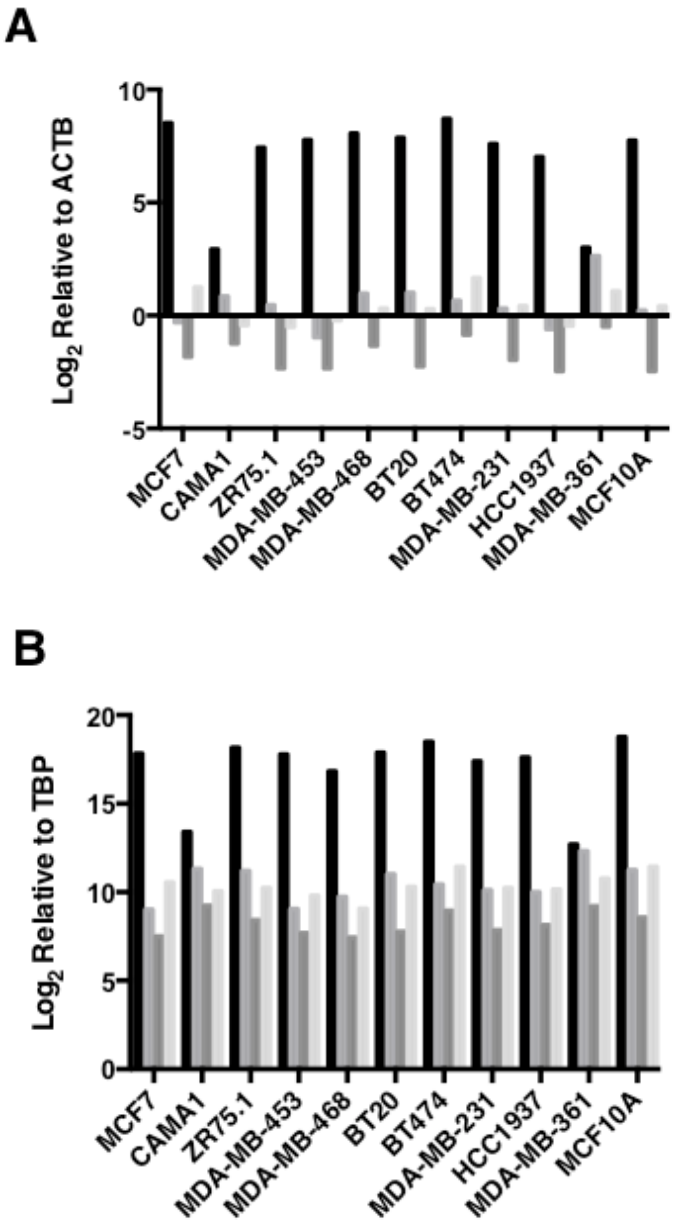
tumor samples compared to normal pairs. GCF sites overlap with two CpG positions and only one of them was identified as significantly methylated in tumors whereas the NF-1 binding site contains one CpG position (CpG 52), which is significantly methylated in tumor samples compared to normal samples.

3.4 Expression Analysis of rRNA Transcripts in Breast Cancer

There are three ribosomal RNAs (18S, 28S and 5.8S) synthesized from the 45S rDNA promoter. They are synthesized as a long precursor 45S pre-RNA including ETS (external transcribed spacer) and ITS (internal transcribed spacer) regions and then rapidly processed, modified and assembled into respective ribosome subunits. Expression analyses of 18S, 28S, 5.8S rRNAs and 45S ETS region (which has a relatively short half life) was performed with the qRT-PCR primers listed in Table 2-4.

3.4.1 Expression Analysis of rRNA Transcripts in Breast Cancer Cell Lines

It is known that promoter DNA methylation has a repressive effect, especially on Pol II transcribed genes in cancer and increased methylation levels are implicated in decreased levels of rRNA transcription (M Esteller 2007; Esteller et al. 2001; Ghoshal et al. 2004; Raval et al. 2012). Thus we hypothesized that rRNA transcription levels might also be down regulated in these breast cancer cell lines with the hypermethylated 45S rDNA promoter (Figure 3.1). Total RNA was analysed with primers targeting Pol I products; 18S, 28S, 5.8S and 45S ETS region in cell lines by qRT-PCR.
TBP, ACTB and *ACTB&TBP* genes were used as reference genes for cell lines to assess the amount of cDNA. We also used the geometric mean of rRNA transcripts (GM-rRNAs) to identify relative changes of rRNAs in the rRNA pool.


A
Log2 Relative to ACTB
10
5
0
-5
MCF7
CAMA1
ZR75.1
MDA-MB-453
MDA-MB-468
BT20
BT474
MDA-MB-231
HCC1937
MDA-MB-361
MCF10A
B
Log2 Relative to TBP
20
15
10
5
0
MCF7
CAMA1
ZR75.1
MDA-MB-453
MDA-MB-468
BT20
BT474
MDA-MB-231
HCC1937
MDA-MB-361
MCF10A

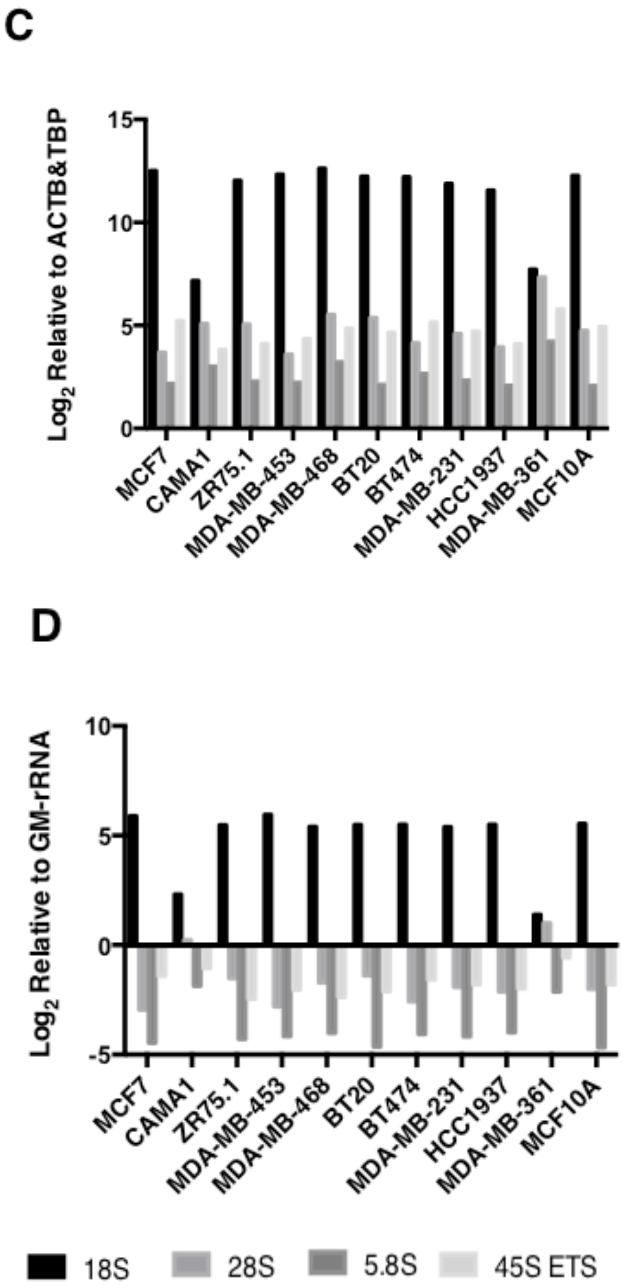
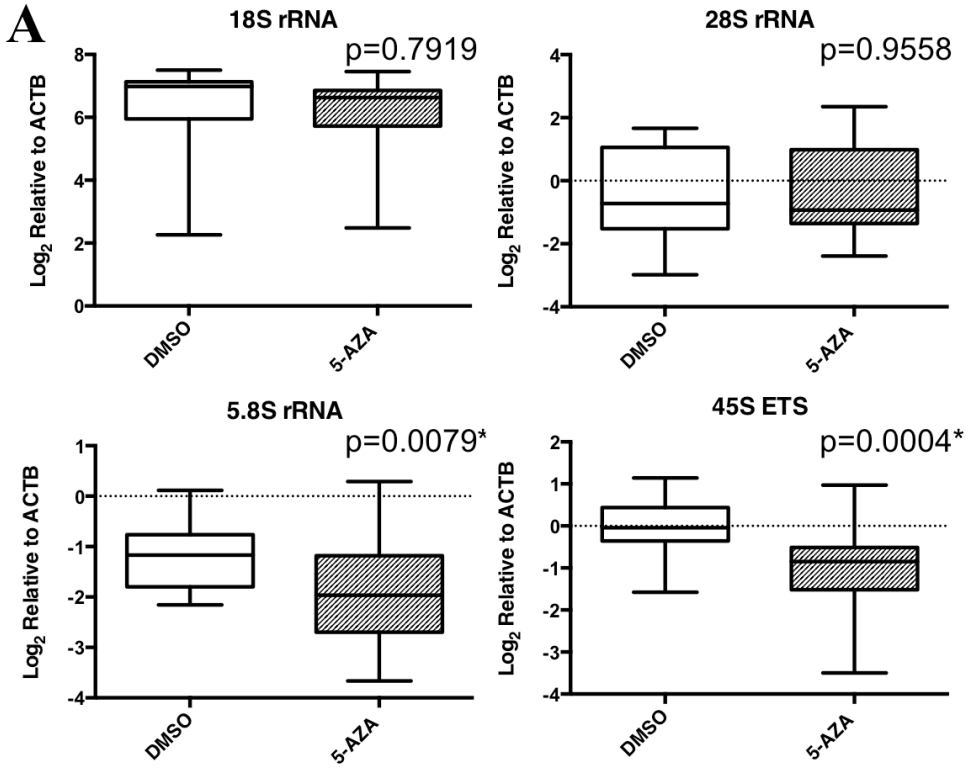


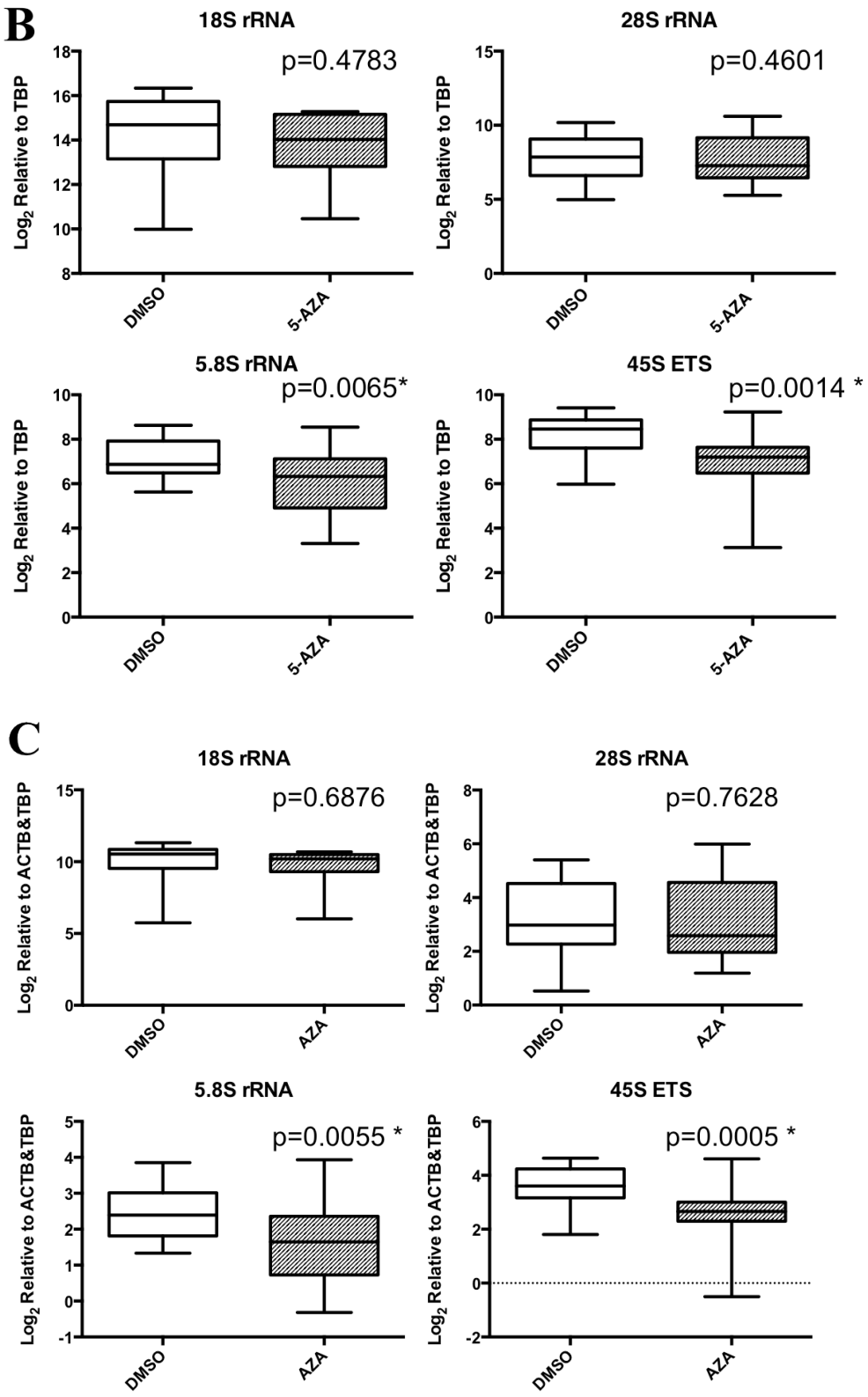
Figure 3.9. rRNA transcript levels in breast cancer cell lines normalized with reference genes (A) ACTB transcript level (B) TBP transcript level (C) geometric mean of ACTB&TBP (D) GM-rRNA.

All of the rRNA transcripts were expressed at different levels among cell lines when normalization was performed with ACTB, TBP and ACTB&TBP expression values (Figure 3.9 A, B and C). Similar results were obtained when we used GM-rRNA to determine changes in the ratio of rRNA transcripts (Figure 3.9 D).

3.4.2 Expression Analysis of rRNA Transcripts in Breast Cancer Cell Lines Treated with Epigenetic Drugs 5-AZA and/or TSA

To further establish the relationship between 45S rDNA promoter methylation and rRNA expression levels, we used the hypomethylating agent, 5-Aza-2'deoxycytidine (5-AZA), which prevents DNA methylation by inhibiting DNA methyltransferases and leads to increased RNA transcription (Ballestar & Esteller 2008). Expression analyses of rRNA transcripts were determined using ACTB, TBP, geometric mean of ACTB&TBP (ACTB&TBP) or geometric mean of rRNAs (GM-rRNA).




B
18S rRNA
p=0.4783
28S rRNA
p=0.4601
5.8S rRNA
p=0.0065*
45S ETS
p=0.0014 *
Log2 Relative to TBP
DMSO
5-AZA
C
18S rRNA
p=0.6876
28S rRNA
p=0.7628
5.8S rRNA
p=0.0055 *
45S ETS
p=0.0005 *
Log2 Relative to ACTB&TBP
DMSO
AZA

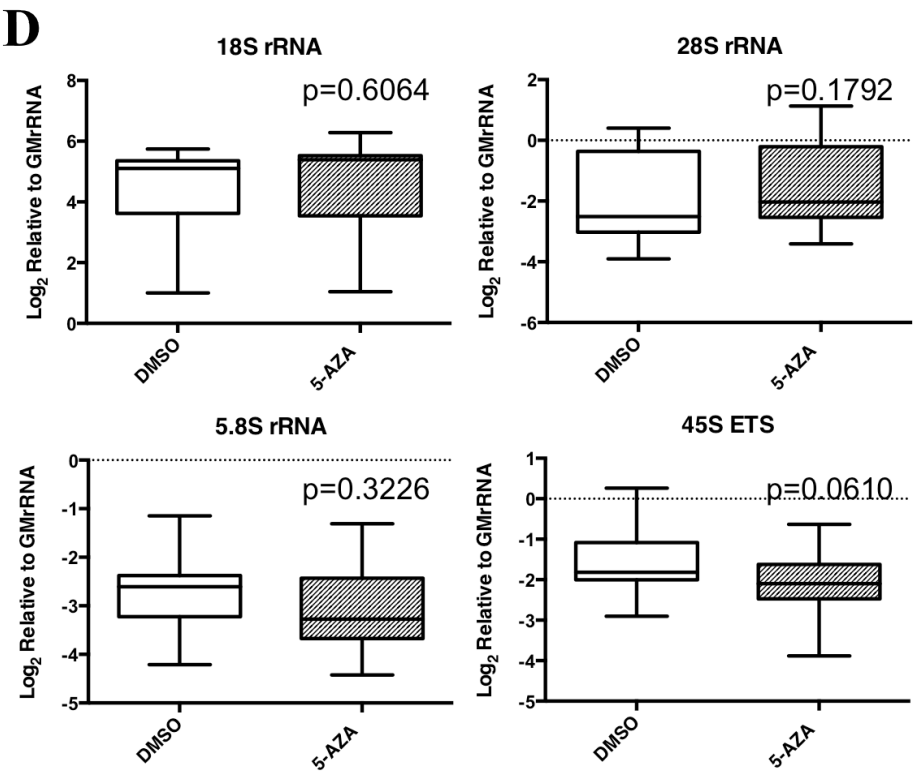
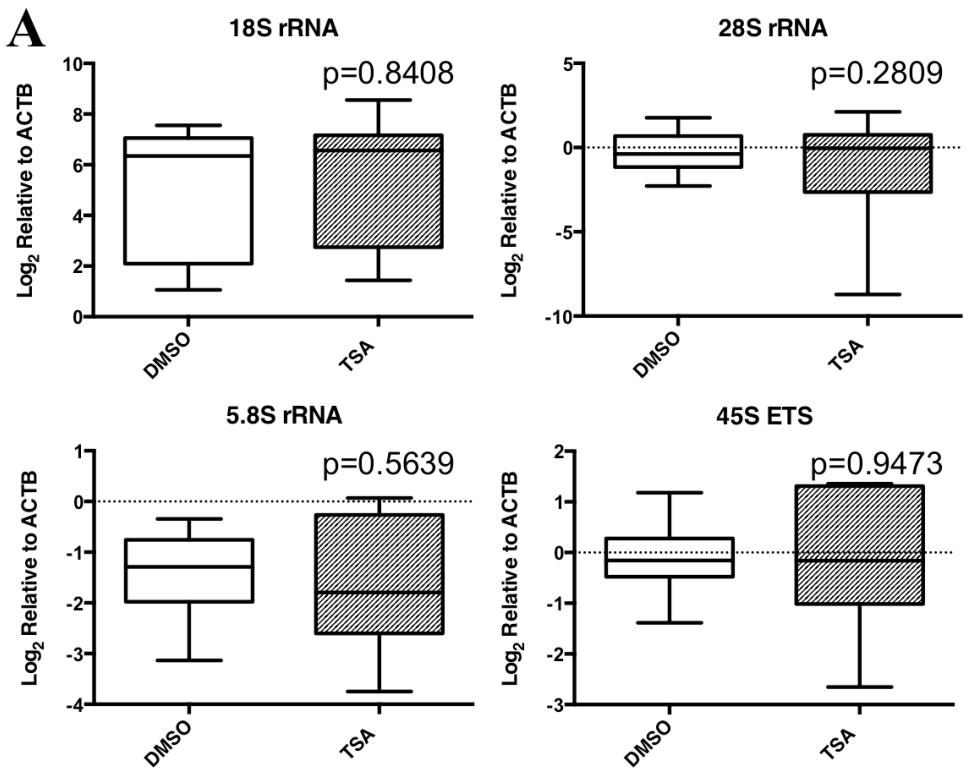


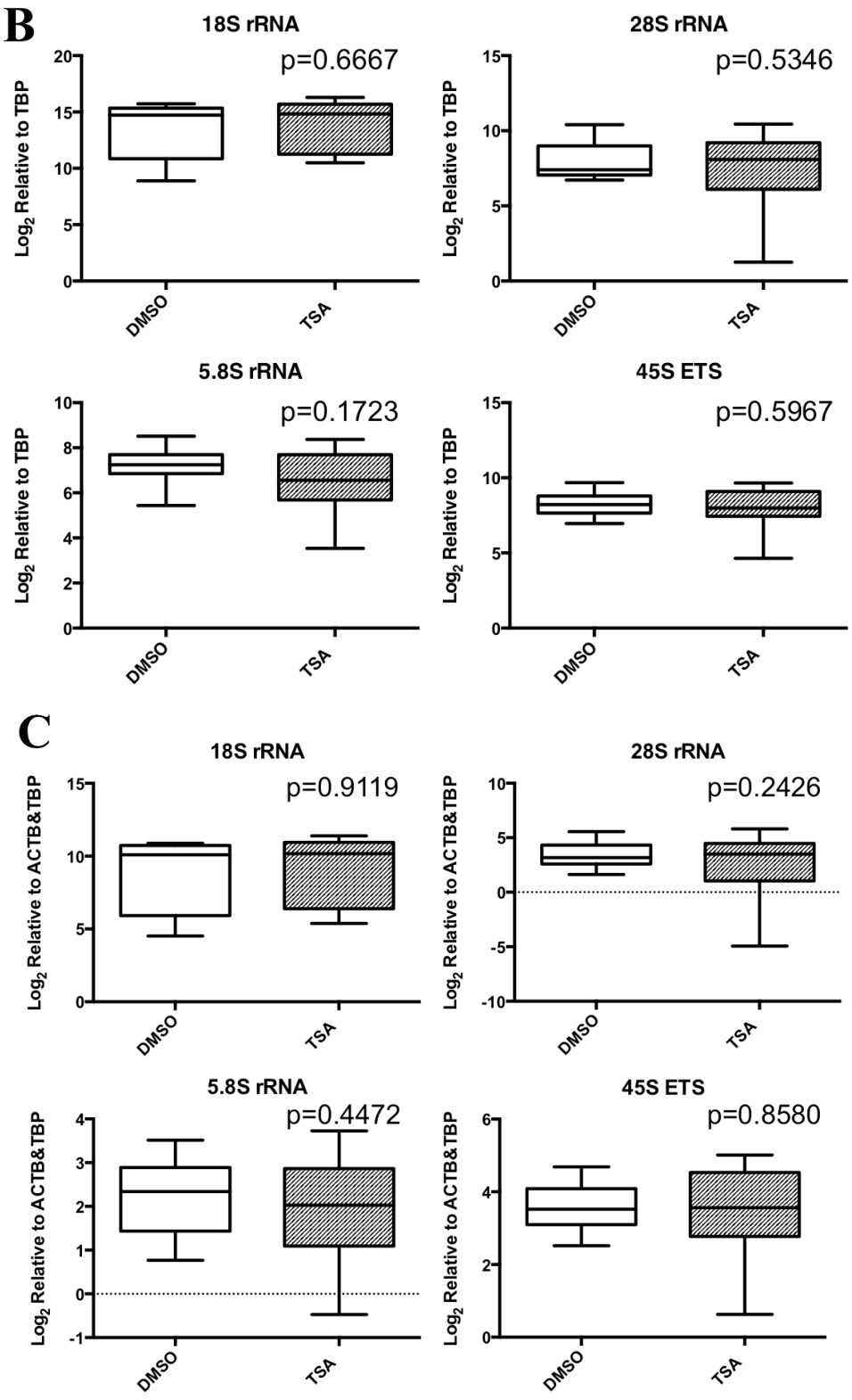
Figure 3.10. rRNA transcript levels in 5-AZA and DMSO (control) treated cell lines. Expression levels of rRNA transcripts in the 5-AZA or DMSO treated breast cell lines were normalized to (A) ACTB transcript level (B) TBP transcript level (C) geometric mean of ACTB&TBP (D) GM-rRNA. Box plots indicate relative expression levels of rRNA transcripts in DMSO and 5-AZA treated cell lines. Significant (* $p<0.05$) rRNA expression differences between DMSO and 5-AZA treated cell lines were determined using paired t-test.

ACTB, TBP and ACTB&TBP normalized expression levels of 5.8S and 45S ETS transcripts were significantly decreased upon 5-AZA treatment (Figure 3.10 A, B and C). However, proportion of rRNA transcripts did not exhibit significant differences between 5-AZA treated and DMSO treated samples (normalization with GM-rRNA) (Figure 3.10 D).

TSA is a non-specific histone deacetylase (HDAC) inhibitor. TSA treatment of cells affects the acetylation status of H3 and H4 and thus TSA indirectly upregulates gene expression by dispersion of the chromatin structure (Ballestar & Esteller 2008). Therefore, TSA was used to determine whether other mechanisms (such as histone acetylation) were involved in rRNA synthesis besides DNA methylation.



TSA treatment alone did not significantly alter the expression levels or the relative proportions of rRNA transcripts when normalized with ACTB, TBP, ACTB&TBP or GM-rRNA, respectively (Figure 3.11 A, B, C and D).


B
18S rRNA
p=0.6667
28S rRNA
p=0.5346
5.8S rRNA
p=0.1723
45S ETS
p=0.5967
Log2 Relative to TBP
DMSO
TSA
C
18S rRNA
p=0.9119
28S rRNA
p=0.2426
5.8S rRNA
p=0.4472
45S ETS
p=0.8580
Log2 Relative to ACTB&TBP

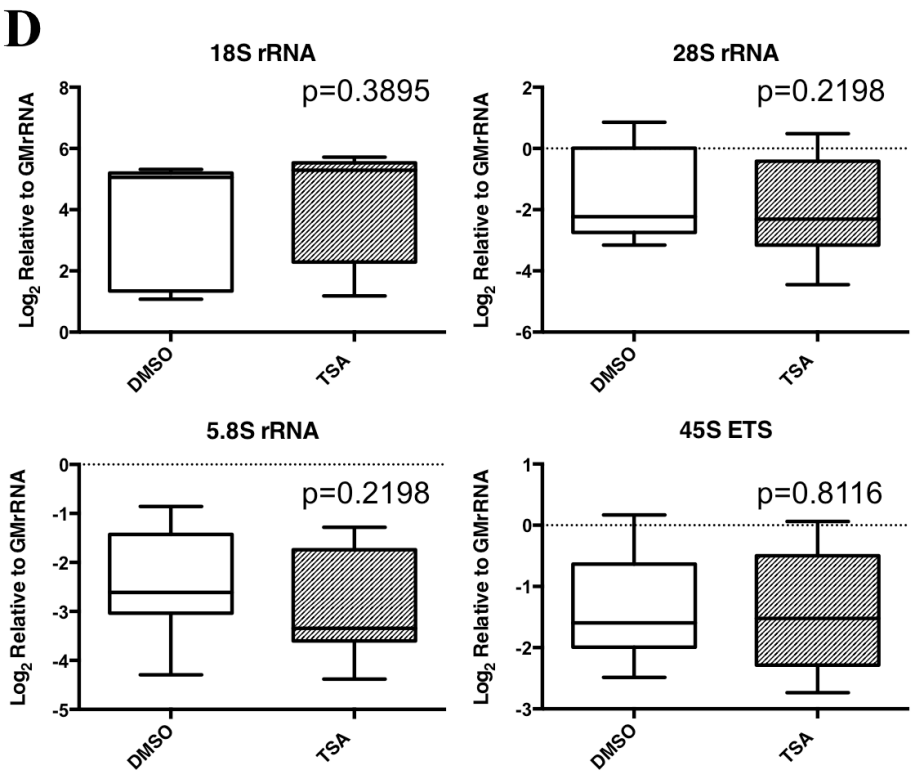
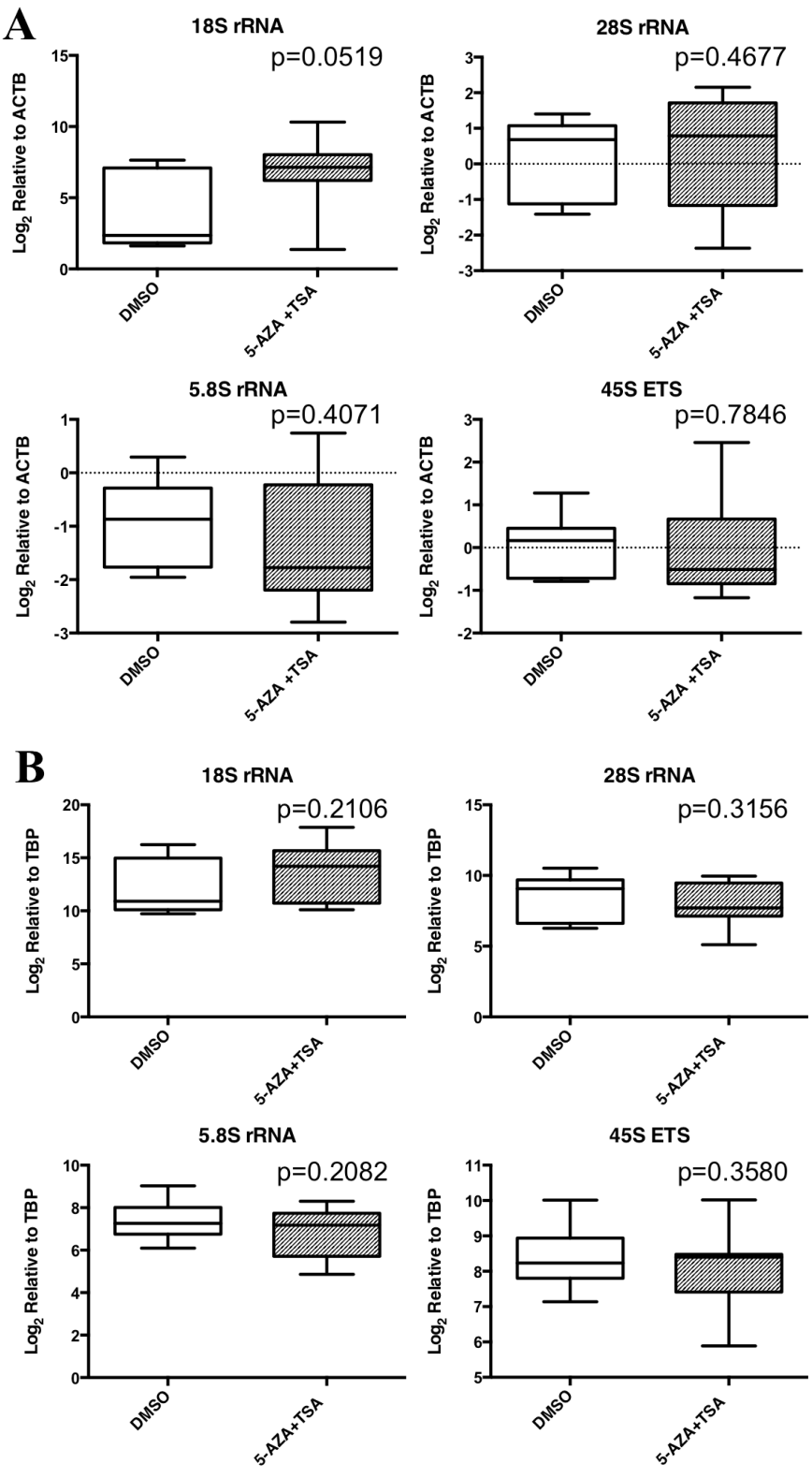


Figure 3.11. rRNA transcript levels in TSA and DMSO (control) treated cell lines. Expression levels of rRNA transcripts in the TSA or DMSO treated breast cell lines were normalized to (A) ACTB transcript level (B) TBP transcript level (C) geometric mean of ACTB&TBP (D) GM-rRNA. Box plots indicate relative expression levels of rRNA transcripts in DMSO and TSA treated cell lines. Significant (* $p<0.05$) rRNA expression differences between DMSO and TSA treated cell lines were determined using paired t-test.

In order to identify whether these two epigenetic mechanisms (DNA methylation and histone acetylation) work together to regulate the rRNA expression, 5-AZA and TSA were used together to treat cell lines.


A
18S rRNA
p=0.0519
28S rRNA
p=0.4677
5.8S rRNA
p=0.4071
45S ETS
p=0.7846
Log2 Relative to ACTB
DMSO
5-AZA +TSA
B
18S rRNA
p=0.2106
28S rRNA
p=0.3156
5.8S rRNA
p=0.2082
45S ETS
p=0.3580
Log2 Relative to TBP
DMSO
5-AZA+TSA

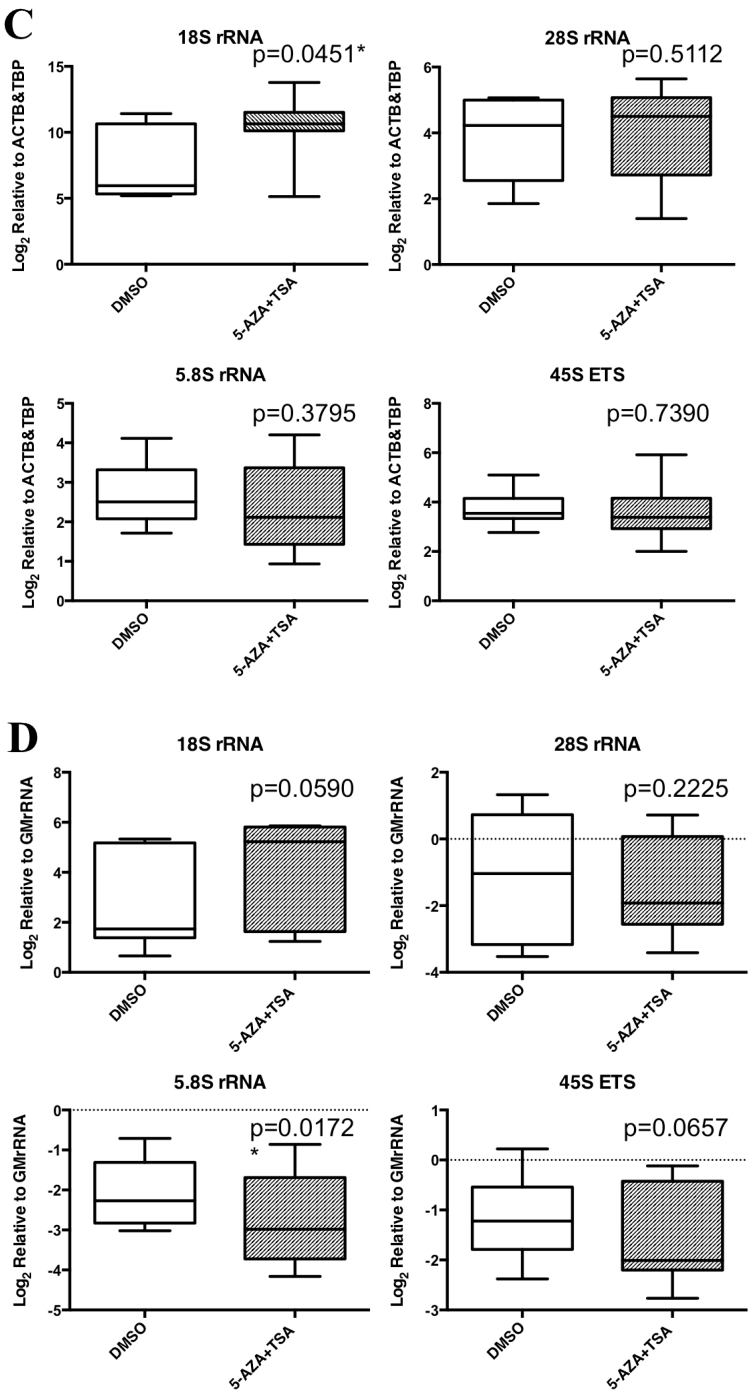


Figure 3.12. rRNA transcript levels in 5-AZA+TSA and DMSO (control) treated cell lines. Expression levels of rRNA transcripts in the 5-AZA+TSA or DMSO treated breast cell lines were normalized to (A) ACTB transcript level (B) TBP transcript level (C) geometric mean of ACTB&TBP (D) GM-rRNA. Box plots indicate relative expression levels of rRNA transcripts in DMSO and 5-AZA+TSA treated cell lines. Significant (* $p<0.05$) rRNA expression differences between DMSO and 5-AZA+TSA treated cell lines were determined using paired t-test.

Treatment with 5-AZA and TSA together (5-AZA+TSA) did not significantly effect the ACTB or TBP normalized expression levels of rRNA transcripts (Figure 3.12 A, B), but it significantly increased 18S rRNA expression when normalization was performed with ACTB&TBP (Figure 3.12 C). However, the 5.8S proportion of rRNAs was significantly decreased in 5-AZA+TSA treated samples compared to DMSO treated samples (Figure 3.12 D).

3.4.3 Expression Analysis of rRNA Genes in Breast Tumor and Matched Normal Tissue Samples

TBP, GAPDH and ACTB have been used as reference genes to determine rRNA expression levels in many studies (Raval et al. 2012; Uemura et al. 2011; Brown & Szyf 2008) but these RNA Polymerase II (Pol II) transcribed genes are variably expressed in many cancer types (Guo, Liu & Sun 2013; Guo, Liu, Wang, et al. 2013).
We performed an expression analysis with the potential reference genes using Oncomine expression database (https://www.oncomine.org/resource/main.html) to be able to choose the most stably expressed reference gene between breast tumor and normal samples. There were 10-13 available breast cancer tissue datasets in Oncomine acquired through microarray or next-generation sequencing technology, we showed only 2 of them (the most variable datasets).

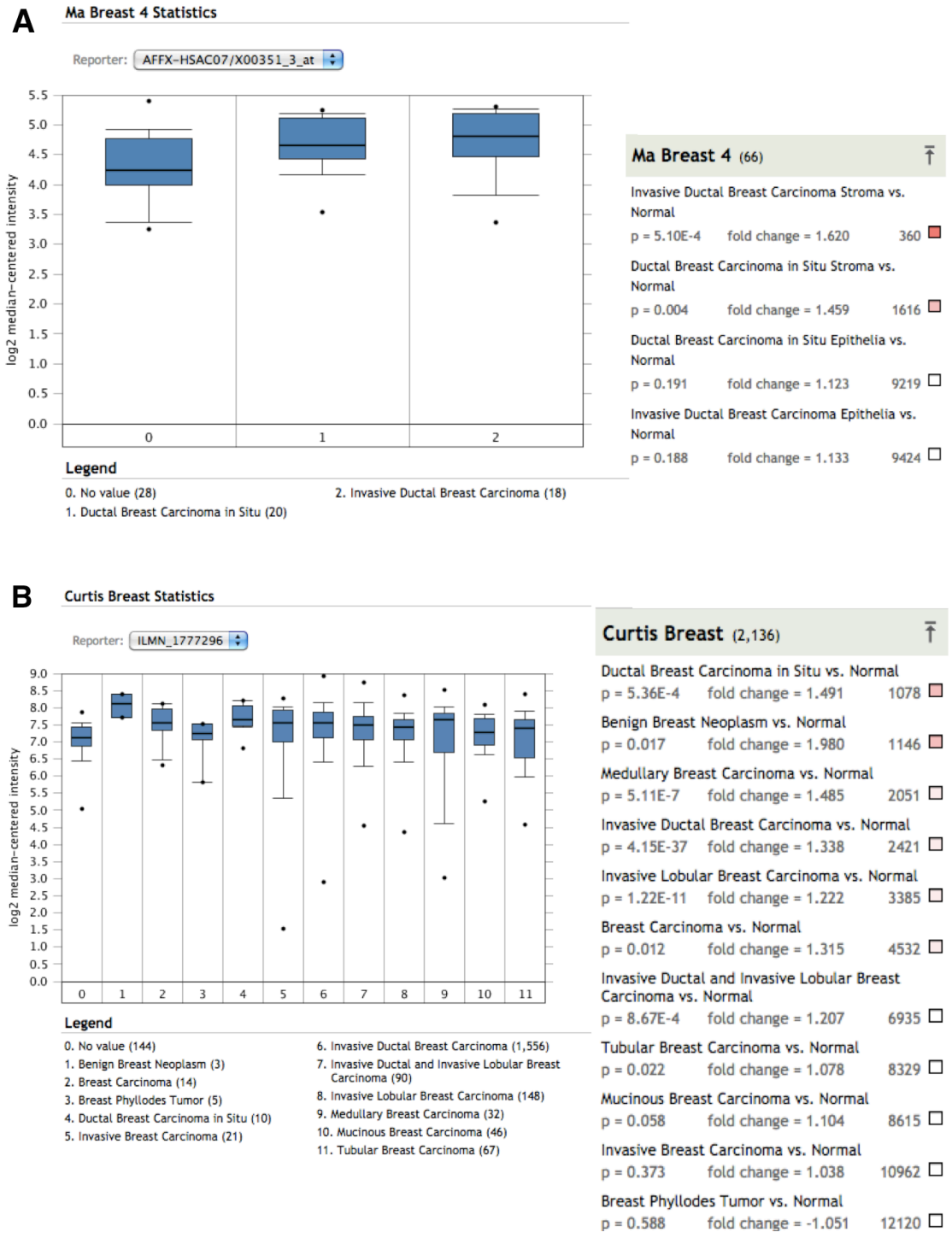
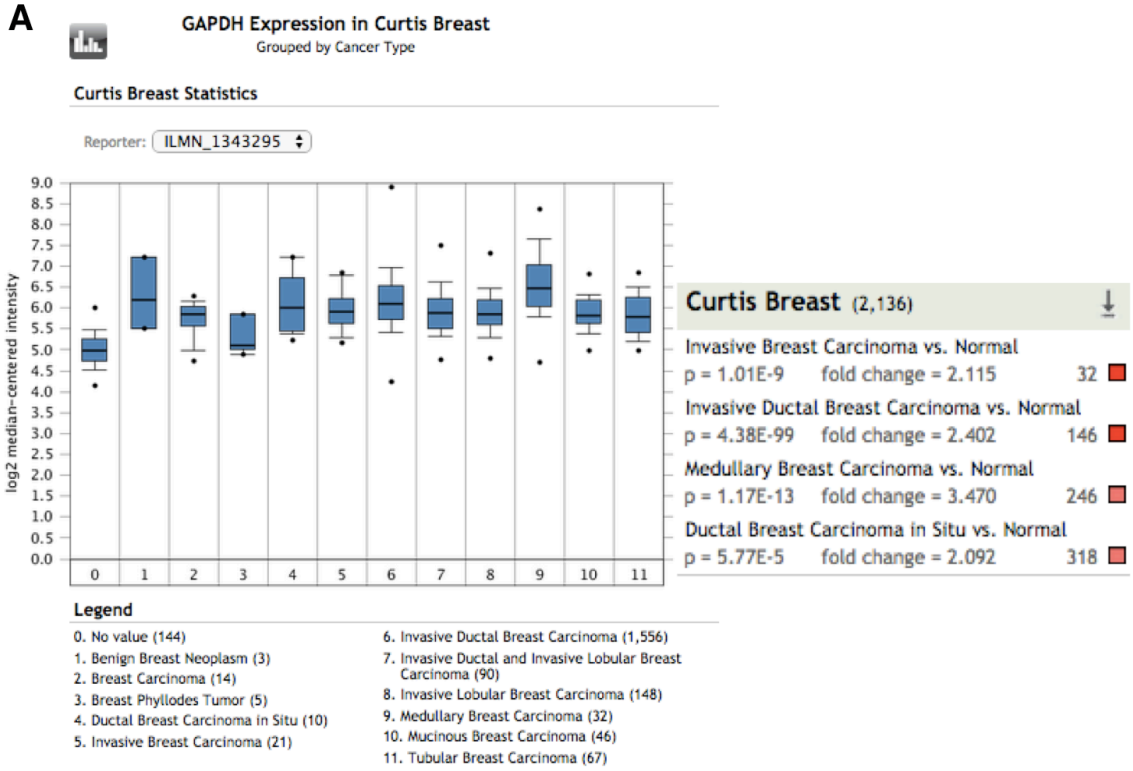


Figure 3.13. Expression analysis of ACTB expression levels in Oncomine datasets. (A) ACTB expression analysis using Ma Breast (Ma et al. 2009) dataset in Oncomine (B) ACTB expression analysis using Curtis Breast (Curtis et al. 2012) dataset in Oncomine. No value: Normal breast tissue samples.

As shown in Figure 3.13 ACTB levels were variably expressed between normal breast tissue and breast cancer samples. The difference of ACTB levels was statistically significant between normal and cancer in different studies. The ACTB gene is therefore not a suitable reference gene to normalize breast tumor-normal tissues.



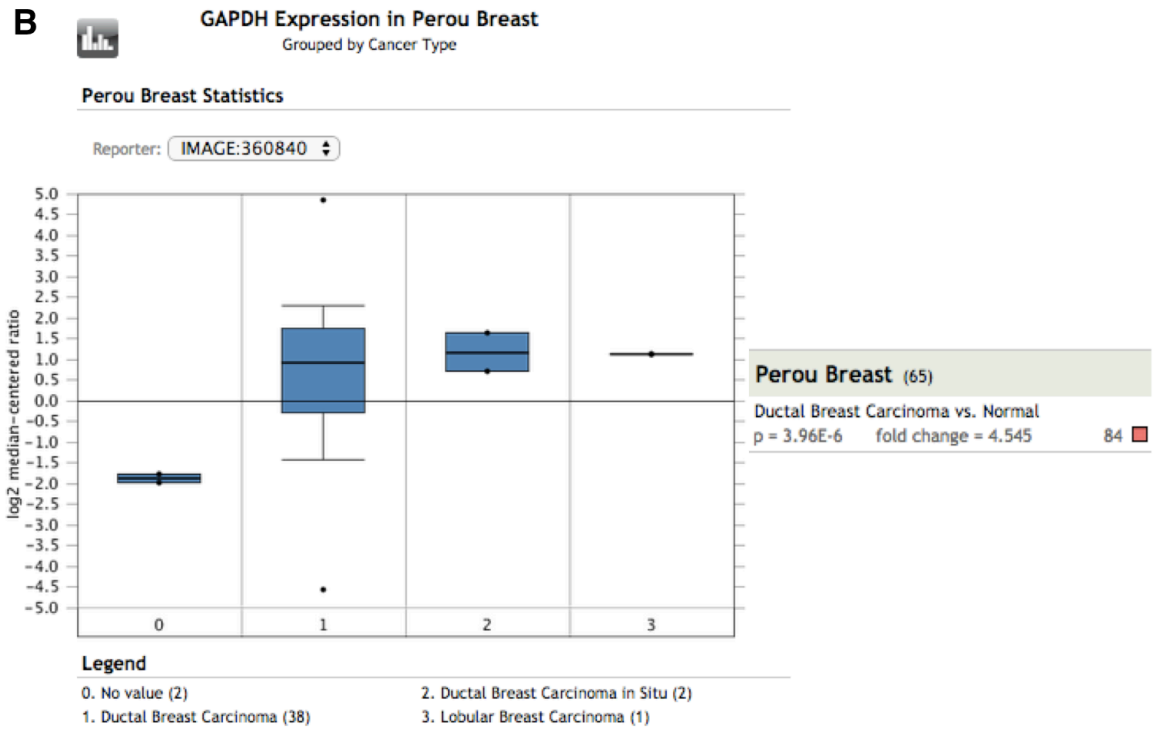


Figure 3.14. Expression analysis of GAPDH expression levels in Oncomine datasets. (A) GAPDH expression analysis using Curtis Breast (Curtis et al. 2012) dataset in Oncomine (B) GAPDH expression analysis using Perou Breast (Perou et al. 2000) dataset in Oncomine. No value: Normal breast tissue samples.

The variability of GAPDH expression levels was more significant than of ACTB levels. GAPDH was therefore not the most suitable reference gene.

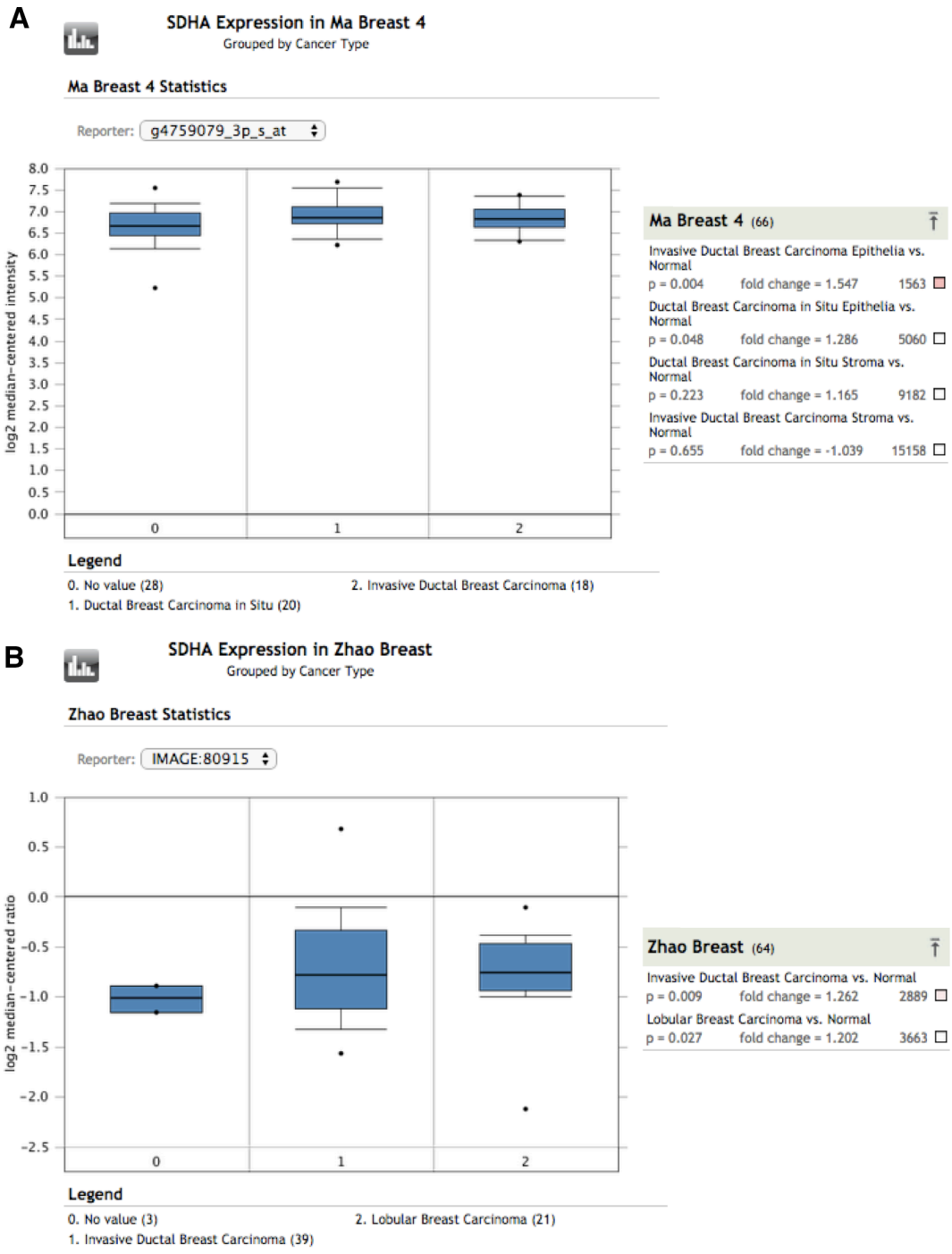
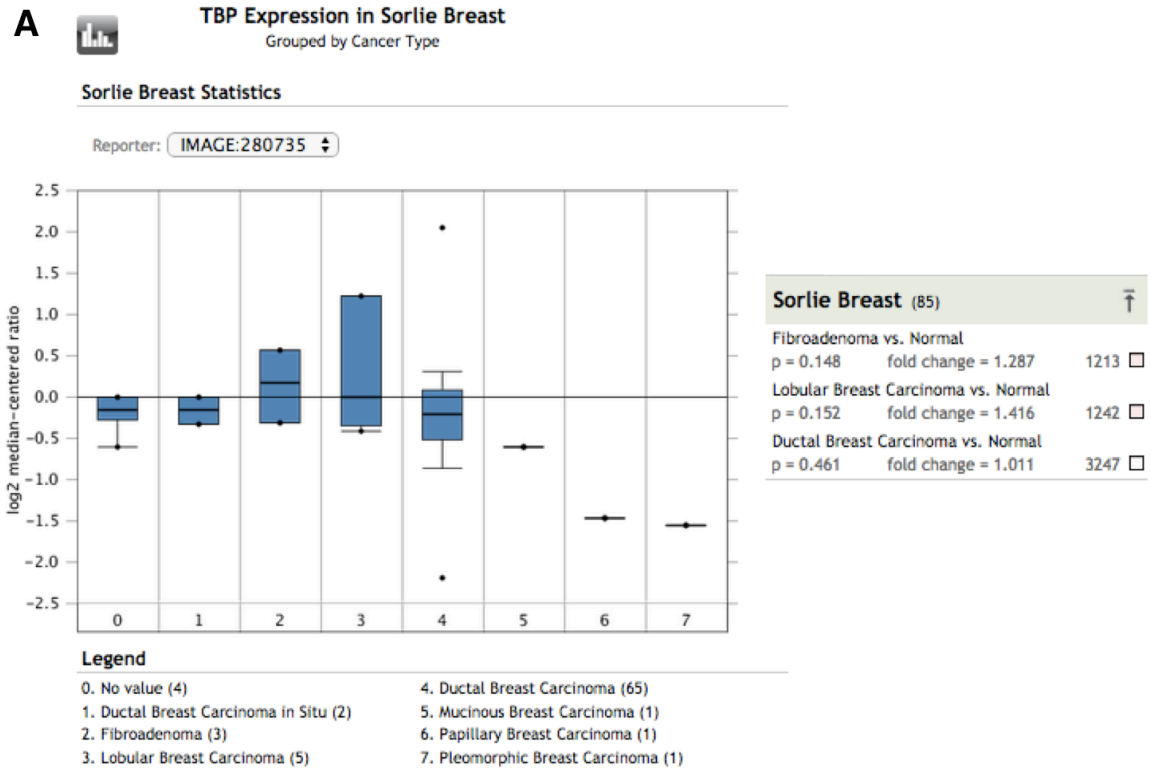


Figure 3.15. Expression analysis of SDHA expression levels in Oncomine datasets. (A) SDHA expression analysis using Ma Breast (Ma et al. 2009) dataset in Oncomine (B) SDHA expression analysis using Zhao Breast (Zhao et al. 2004) dataset in Oncomine. No value: Normal breast tissue samples.

SDHA expression levels were more stable than ACTB and GAPDH but SDHA was still significantly lower in normal breast tissue samples compared to breast cancer tissues in both studies.



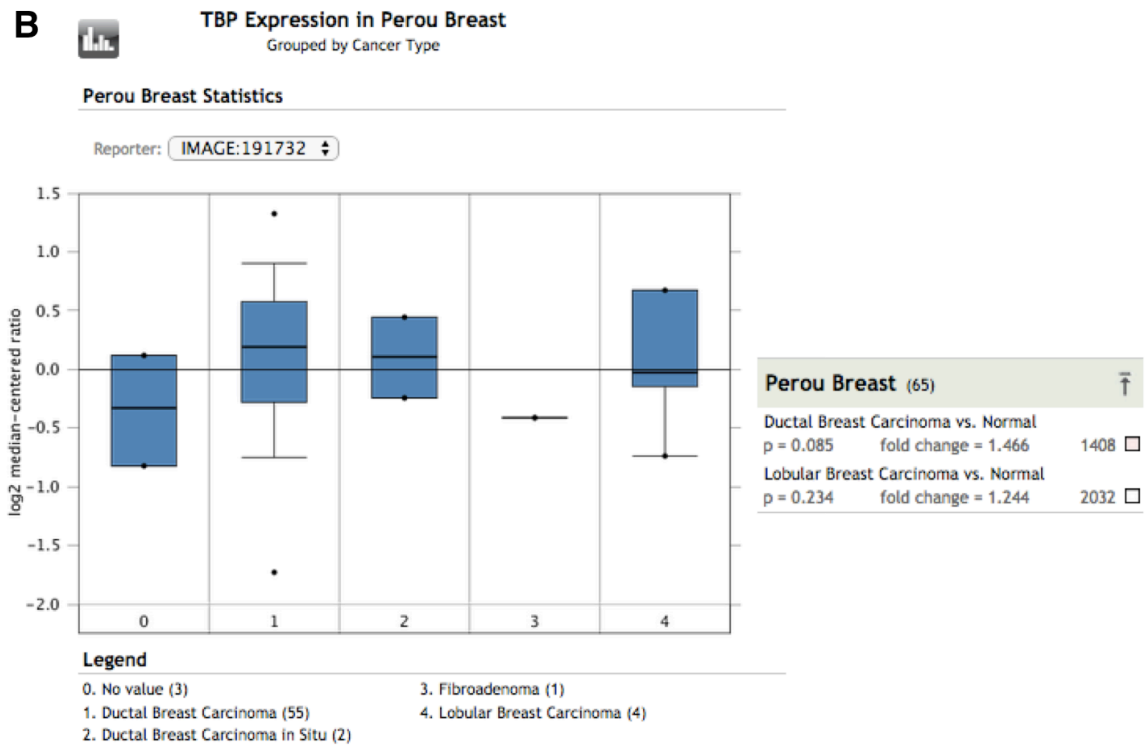


Figure 3.16. Expression analysis of TBP expression levels in Oncomine datasets. (A) TBP expression analysis using Sorlie Breast (Sørlie et al. 2001) dataset in Oncomine (B) TBP expression analysis using Perou Breast (Perou et al. 2000) dataset in Oncomine. No value: Normal breast tissue samples.

As we can see from Figure 3.16 TBP levels were very stable between normal breast and breast cancer tissues in different studies especially compared to the other reference gene candidates.

3.5 Expression Analysis of rRNA genes in Breast Tumor and Matched Normal Tissue Samples using TBP as a Reference Gene

Since, we found that TBP gene is relatively stable compared to other reference genes between breast tumor tissues and normal breast tissue samples we used TBP as a reference gene to identify absolute rRNA expression levels.

We tested whether increased levels of methylation of the 45S promoter in tumor samples led to repressed expression levels of rRNA transcripts. RNA isolation was performed

from the same tissue samples used in methylation analysis (only 14 of 19 paired tissue samples had enough tissue for RNA isolation).

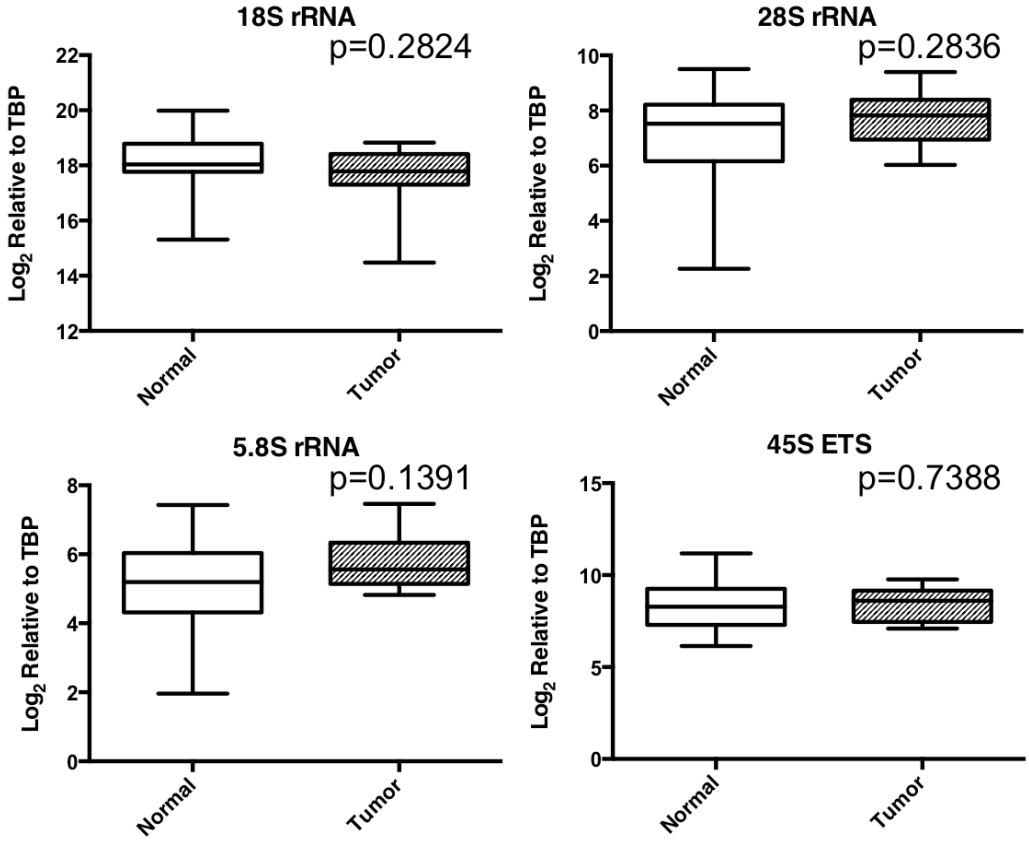


Figure 3.17. Expression analysis of rRNA transcripts in clinical breast tumor and normal tissue pairs relative to TBP transcript levels. Relative expression levels of rRNA transcripts are shown with box plots for clinical breast cancer and matched normal samples. Significant (* $p<0.05$) expression differences between normal and tumor samples were determined using paired t-test.

Expression levels did not differ between tumors and corresponding normal tissues for any of the rRNA transcripts when expression was analysed using TBP as seen in Figure 3.17.

3.5.1 Multiple Regression Analysis of rRNA Expression Levels (TBP normalization) and Clinical Variables in Breast Cancer Tissue Samples

Ribosome biogenesis and rRNA synthesis are implicated in cancer progression. Thus rRNA expression levels were used to predict clinical variables in breast cancer tissue samples.

A multiple regression was run to predict clinical variables (age, ER, PR, lymph node, distant metastasis status, grade and stage of the tumors in patients) from rRNA expression values (TBP normalized) of tumor samples.

Table 3-10. Multiple regression analysis of clinical variables and expression values of rRNAs in tumor samples

Dependent variable	Independent variable	Mean Square	ANOVA F	ANOVA Sig.
Age	TBP nor.	269.88	2.645	0.124
ER	TBP nor.	0.281	1.188	0.404
PR	TBP nor.	0.06	0.146	0.958
Distant Metastasis	TBP nor.	0.024	0.077	0.987
Lymph Node	TBP nor.	0.214	1.075	0.436
Grade	TBP nor.	0.505	1.876	0.234
Stage	TBP nor.	0.105	0.173	0.945

ER; estrogen receptor, PR; progesterone receptor, nor; normalization, ANOVA F; representative of the degree of difference in the dependent variable generated by the independent variable, it also considers covariance of the variables

(Note: Stages were merged due to low number of samples. For example; IIA and IIB samples were analysed as stage II).

None of the variables significantly predicted clinical characteristics of the tumors samples.

3.5.2 Correlation Analysis of rRNA Expression Levels (TBP normalization) and Clinical Variables in Breast Cancer Tissue Samples

We have performed Spearman correlation to identify whether there are any correlation between rRNA expression levels and clinical variables of the patients.

Table 3-11. Spearman correlation analysis of clinical variables and expression levels of rRNA genes (rRNA/TBP)

		Normal				Tumor			
		18S	**28S**	**5.8S**	**45S ETS**	**18S**	**28S**	**5.8S**	**45S ETS**
ER	ρ	-0.418	0.06	-0.179	-0.538	0.239	-0.418	-0.06	-0.478
	p	0.2	0.861	0.598	0.088	0.479	0.2	0.861	0.137
	N	11	11	11	11	11	11	11	11
PR	ρ	-0.289	0.173	-0.115	-0.289	0.058	0.115	0.404	-0.115
	p	0.389	0.611	0.735	0.389	0.866	0.735	0.218	0.735
	N	11	11	11	11	11	11	11	11
Dist. met	ρ	-0.195	0.028	0.028	-0.195	-0.028	0.084	-0.084	-0.028
	p	0.543	0.931	0.931	0.543	0.931	0.796	0.796	0.931
	N	12	12	12	12	12	12	12	12
Lym. node	ρ	0.084	0.195	0.139	-0.028	0.307	-0.195	-0.084	-0.084
	p	0.796	0.543	0.666	0.931	0.332	0.543	0.796	0.796
	N	12	12	12	12	12	12	12	12
Grade	ρ	0.554	0.011	0.107	0.650	-0.32	0.384	0.235	-0.043
	p	0.077	0.975	0.755	0.03*	0.338	0.244	0.488	0.901
	N	11	11	11	11	11	11	11	11
Stage	ρ	0.008	-0.331	0.023	-0.171	0.202	0.132	0.019	0
	p	0.981	0.294	0.943	0.595	0.528	0.682	0.952	1
	N	12	12	12	12	12	12	12	12

ρ ; Spearman correlation coefficient, p; Spearman correlation significance, N; Sample size. * Significant correlations ($p<0.05$)

45S ETS expression levels (relative to TBP) in normal pairs of the tumor were positively correlated with the tumor grade.

3.6 Expression Analysis of rRNA genes in Breast Tumor and Matched Normal Tissue Samples using GMrRNA as a Reference Value

Several studies advise against using rRNA levels to determine mRNA levels (Gur-Dedeoglu et al. 2009; Tricarico et al. 2002), since they are variable between breast and normal tissue samples. Accordingly, using mRNA levels to normalize rRNA levels have a similar drawback as shown in Figure 3.13, Figure 3.14 and Figure 3.15. But TBP gene is the most suitable gene among the other candidates. Therefore we used TBP as reference gene for rRNA expression analyses in tissue samples.

In this study, the geometric mean of expression from an rRNA pool (18S, 28S, 5.8S and 45S ETS) synthesized by Pol I, GM-rRNA, was also used to analyse the relative changes of rRNAs with respect to each other between tumor and normal samples.

TBP normalization and GM-rRNA normalization have helped us to identify two different aspects of the rRNA expression levels. TBP normalization is used to find absolute rRNA expression levels and GM-rRNA normalization is used to assess whether rRNA transcript ratios were affected within rRNA pool.

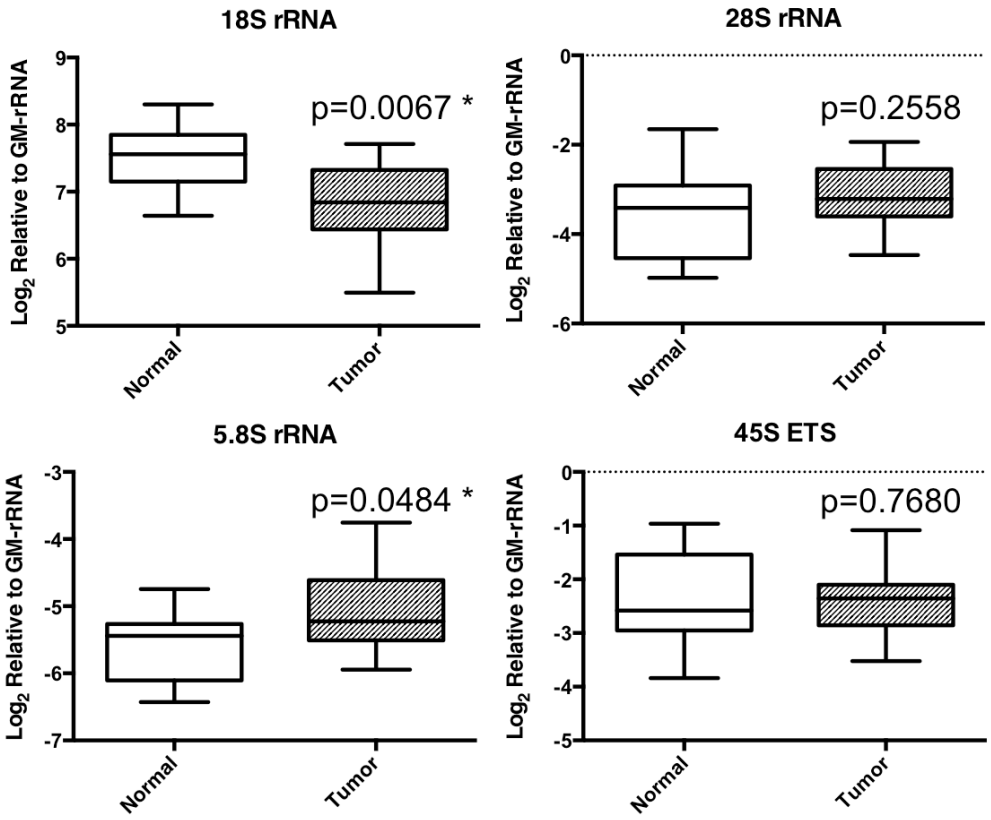


Figure 3.18. Expression analysis of rRNA transcripts in clinical breast tumor and normal tissue pairs relative to GM-rRNA value. Relative expression levels of rRNA transcripts are shown with box plots for clinical breast cancer and matched normal samples. Significant (* $p<0.05$) expression differences between normal and tumor samples were determined using paired t-test.

When normalized with GM-rRNA the proportion of 5.8S rRNA was significantly increased in tumor samples whereas that of 18S was significantly decreased (Figure 3.18). This indicates a dysregulation of the spliced rRNA products in tumor rRNA pool.

3.6.1 Multiple Regression Analysis of rRNA Expression Levels (GMrRNA normalization) and Clinical Variables in Breast Cancer Tissue Samples

A multiple regression was run to predict clinical variables (age, ER, PR, lymph node, distant metastasis status, grade and stage of the tumors in patients) from rRNA expression values (GM-rRNA normalized) of tumor samples.

Table 3-12. Multiple regression analysis of clinical variables and expression values of rRNAs in tumor samples

Dependent variable	Independent variable	Mean Square	ANOVA F	ANOVA Sig.
Age	GM-rRNA nor.	94.935	0.47	0.757
ER	GM-rRNA nor.	0.352	1.861	0.237
PR	GM-rRNA nor.	0.082	0.206	0.926
Distant Metastasis	GM-rRNA nor.	0.116	0.456	0.766
Lymph Node	GM-rRNA nor.	0.256	1.459	0.31
Grade	GM-rRNA nor.	0.57	2.52	0.15
Stage	GM-rRNA nor.	0.294	0.59	0.681

ER; estrogen receptor, PR; progesterone receptor, nor; normalization, ANOVA F; representative of the degree of difference in the dependent variable generated by the independent variable, it also considers covariance of the variables

(Note: Stages were merged due to low number of samples. For example; IIA and IIB samples were analysed as stage II).

None of the variables significantly predicted clinical characteristics of the tumors samples.

3.6.2 Correlation Analysis of rRNA Expression Levels (GMrRNA normalization) and Clinical Variables in Breast Cancer Tissue Samples

We have performed Spearman correlation to identify whether there are any correlation between rRNA expression levels and clinical variables of the patients.

Table 3-13. Spearman correlation analysis of clinical variables and expression levels of rRNA genes (rRNA/GM-rRNA)

		Normal				Tumor			
		18S	**28S**	**5.8S**	**45S ETS**	**18S**	**28S**	**5.8S**	**45S ETS**
ER	ρ	0	0.359	0.12	-0.418	0.538	-0.418	0	-0.239
	p	1	0.279	0.726	0.2	0.088	0.2	1	0.479
	N	11	11	11	11	11	11	11	11
PR	ρ	-0.231	0.173	0.115	0	0.058	0.231	0.289	-0.346
	p	0.494	0.611	0.735	1	0.866	0.494	0.389	0.297
	N	11	11	11	11	11	11	11	11
Dist. met	ρ	-0.084	0.307	0.084	-0.362	0.139	-0.084	0.028	0.139
	p	0.796	0.332	0.796	0.247	0.666	0.796	0.931	0.666
	N	12	12	12	12	12	12	12	12
Lym. node	ρ	-0.195	0.418	-0.195	-0.418	0.53	-0.53	-0.418	-0.307
	p	0.543	0.176	0.543	0.176	0.077	0.077	0.176	0.332
	N	12	12	12	12	12	12	12	12
Grade	ρ	0.181	-0.437	-0.213	0.33	-0.0714	0.725	0.586	-0.107
	p	0.594	0.179	0.529	0.321	0.014*	0.012*	0.058	0.755
	N	11	11	11	11	11	11	11	11
Clin. Stage	ρ	0.342	-0.148	0.089	-0.288	0.245	-0.012	0.074	-0.043
	p	0.276	0.647	0.782	0.364	0.443	0.971	0.819	0.895
	N	12	12	12	12	12	12	12	12

ρ; Spearman correlation coefficient, p; Spearman correlation significance, N; Sample size. * Significant correlations ($p<0.05$)

28S/GM-rRNA ratio of tumor samples was positively correlated while the 18S/GM-rRNA ratio was negatively correlated with the grade of the tumors.

3.7 Correlation Analysis of rDNA methylation levels and rRNA expression levels in breast cancer

rDNA methylation levels as well as rRNA expression levels were determined in both breast cancer cell lines and breast cancer and matched normal tissue samples. Since rDNA methylation levels were implicated in decreased rRNA expression levels we

performed Spearman correlation analysis between rDNA methylation levels and rRNA expression levels.

3.7.1 Correlation Analysis of rDNA Methylation Levels and rRNA Expression Levels in Breast Cancer Cell Lines

Both 45S rDNA promoter methylation levels and rRNA expression levels were determined in ten breast cancer cell lines and MCF-10A. Total rDNA methylation levels and rRNA expression levels (normalized with ACTB, TBP, ACTB&TBP or GM-rRNA) were analysed with Spearman correlation analysis.

Table 3-14. Spearman correlation analysis of 45S rDNA promoter methylation levels and rRNA expression levels (ACTB normalization) in breast cancer cell lines

	18S rRNA	28S rRNA	5.8S rRNA	45S ETS
Spearman ρ	-0.5012	-0.1058	-0.2299	-02805
Spearman p	0.1078	0.7261	0.4680	0.3768

ρ ; Spearman correlation coefficient, p: Spearman correlation significance

Table 3-15. Spearman correlation analysis of 45S rDNA promoter methylation levels and rRNA expression levels (TBP normalization) in breast cancer cell lines

	18S rRNA	28S rRNA	5.8S rRNA	45S ETS
Spearman ρ	-0.0413	0.4736	0.5564	0.0299
Spearman p	0.8744	0.1432	0.0794	0.9308

ρ ; Spearman correlation coefficient, p: Spearman correlation significance

Table 3-16. Spearman correlation analysis of 45S rDNA promoter methylation levels and rRNA expression levels (ACTB and TBP Normalization) in breast cancer cell lines

	18S rRNA	28S rRNA	5.8S rRNA	45S ETS
Spearman ρ	-0.4368	-0.0505	-0.2207	-0.3678
Spearman p	0.1652	0.8530	0.4854	0.2461

ρ ; Spearman correlation coefficient, p: Spearman correlation significance

Table 3-17. Spearman correlation analysis of 45S rDNA promoter methylation levels and rRNA expression levels (GM-rRNA Normalization) in breast cancer cell lines

	18S rRNA	28S rRNA	5.8S rRNA	45S ETS
Spearman ρ	0.0459	0.1885	0.1793	0.3035
Spearman p	0.8960	0.5773	.5965	0.3615

ρ ; Spearman correlation coefficient, p: Spearman correlation significance

No significant correlation was identified between rDNA promoter methylation levels and rRNA expression levels in breast cancer cell line panel. This indicates that rRNA expression levels are independent from the 45S rDNA methylation status in cell lines.

3.7.2 Correlation Analysis of rDNA Methylation Levels and rRNA Expression Levels in Breast Cancer and Matched Normal Tissue Samples

Even though no significant correlation was identified between 45S rDNA promoter methylation levels and rRNA expression levels in breast cancer cell lines, a correlation analysis was also performed for tissue samples since cell lines and actual cancer cells might act differently.

Table 3-18. Spearman correlation analysis of 45S rDNA promoter methylation levels and rRNA expression levels (TBP Normalization) in primary breast tumor and matched normal samples

		18S rRNA	28S rRNA	5.8S rRNA	45S ETS
Normal	Spearman ρ	-0.0901	0.3582	0.1253	0.1473
	Spearman p	0.7616	0.2090	0.6706	0.6158
Tumor	Spearman ρ	0.3011	-0.2352	-0.2044	-0.0725
	Spearman p	0.2951	0.4175	0.4827	0.8083

ρ ; Spearman correlation coefficient, p: Spearman correlation significance

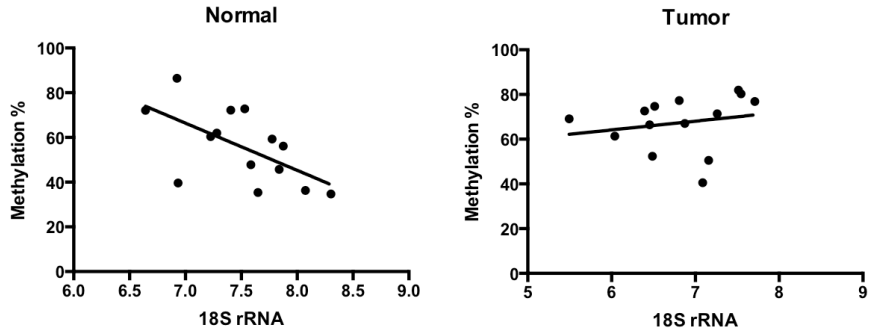
No correlation was identified between rDNA promoter methylation levels and rRNA expression levels with TBP normalization.

Table 3-19. Spearman correlation analysis of 45S rDNA promoter methylation levels and rRNA expression levels (GM-rRNA Normalization) in primary breast tumor and matched normal samples

		18S rRNA	28S rRNA	5.8S rRNA	45S ETS
Normal	Spearman ρ	-0.6571	0.3495	-0.3099	-0.1473
	Spearman p	0.0128*	0.2210	0.2806	0.6158
Tumor	Spearman ρ	0.3934	-0.3055	-0.3451	-0.1253
	Spearman p	0.1652	0.2878	0.2272	0.6706

ρ ; Spearman correlation coefficient, p: Spearman correlation significance
Significant ($p<0.05$) correlations were shown with "*".

The rDNA promoter methylation levels and 18S/GM-rRNA ratio was found to be negatively correlated in normal breast tissues but no correlation was identified in matched tumor samples.



Spearman ρ	**-0.6571**	0.3934
Spearman p	**0.0128***	0.1652

Figure 3.19. Correlation graph of 45S rDNA promoter methylation levels and 18S rRNA ratios (18S/GM-rRNA) in breast tumor and matched normal tissue samples. ρ; Spearman correlation coefficient, p: Spearman correlation significance. Spearman correlation analysis results are given in the chart below. * Significant correlation p<0.05

Significant correlation between rDNA promoter methylation levels and 18S/GM-rRNA ratio is more visible in Figure 3.19.

3.7.3 Correlation Analysis of CpG DNA Methylation and Expression Levels in Breast Cancer and Matched Normal Tissue Samples

In the previous analysis we identified a negative correlation between 18S/GM-rRNA ratio and 45S rDNA promoter methylation levels in normal breast tissue samples. We performed a correlation analysis with methylation levels of CpG positions and rRNA/GM-rRNA ratio in breast tumor and matched normal tissue samples in order to see whether methylation levels in any CpG position contribute to this correlation.

Table 3-20. Spearman correlation analysis of CpG methylation levels and rRNA proportions in normal breast tissues samples

	18S rRNA		28S rRNA		5.8S rRNA		45S ETS	
Correlation	ρ	**p**	ρ	**p**	ρ	**p**	ρ	**p**
CpG1	**-0.619**	**0.018***	0.445	0.111	-0.485	0.079	-0.16	0.584
CpG2	**-0.603**	**0.023***	0.421	0.134	-0.417	0.138	-0.119	0.686
CpG3	-0.461	0.097	0.206	0.48	-0.359	0.208	-0.089	0.763
CpG4	**-0.790**	**0.001****	0.521	0.056	-0.402	0.154	-0.184	0.529
CpG5	-0.521	0.056	0.389	0.169	-0.23	0.429	-0.382	0.177
CpG6	-0.525	0.054	0.372	0.191	-0.523	0.055	-0.092	0.753
CpG7	-0.261	0.367	0.167	0.569	0.029	0.921	-0.239	0.411
CpG8	-0.403	0.153	0.101	0.732	-0.356	0.212	0.02	0.946
CpG9	-0.269	0.353	0.091	0.757	**-0.631**	**0.016***	0.089	0.763
CpG10	**-0.613**	**0.02***	0.339	0.236	-0.435	0.12	-0.085	0.773
CpG11	-0.509	0.063	0.291	0.313	-0.504	0.066	0.051	0.862
CpG12	-0.484	0.08	0.233	0.423	-0.417	0.138	-0.002	0.994
CpG13	-0.269	0.353	-0.053	0.856	-0.36	0.206	0.193	0.508
CpG14	-0.415	0.14	0.033	0.91	-0.368	0.196	0.178	0.542
CpG15	-0.428	0.127	0.105	0.72	-0.372	0.19	0.072	0.808
CpG16	-0.371	0.192	0.002	0.994	**-0.545**	**0.044***	0.269	0.352
CpG17	-0.528	0.052	0.172	0.556	-0.164	0.576	-0.013	0.964
CpG18	-0.385	0.174	0.069	0.815	-0.047	0.874	0.06	0.838
CpG19	-0.317	0.269	0.114	0.698	-0.042	0.885	-0.089	0.761
CpG20	-0.527	0.053	0.346	0.225	-0.206	0.481	-0.154	0.599
CpG21	-0.525	0.054	0.215	0.46	-0.072	0.806	-0.061	0.836
CpG22	-0.461	0.097	0.193	0.509	-0.002	0.994	-0.172	0.555
CpG23	-0.393	0.165	0.153	0.601	-0.166	0.569	-0.087	0.769
CpG24	**-0.749**	**0.002****	0.44	0.115	-0.257	0.375	-0.212	0.466
CpG25	**-0.691**	**0.006****	0.425	0.129	-0.223	0.444	-0.068	0.819
CpG26	-0.44	0.116	0.217	0.457	-0.161	0.583	-0.1	0.733
CpG27	**-0.678**	**0.008****	0.418	0.137	-0.342	0.231	-0.209	0.474
CpG28	**-0.615**	**0.019***	0.384	0.175	-0.313	0.276	-0.177	0.546
CpG29	**-0.656**	**0.011***	0.409	0.147	-0.184	0.528	-0.28	0.332
CpG30	**-0.691**	**0.006****	0.573*	0.032	-0.098	0.739	-0.439	0.116
CpG31	-0.459	0.098	0.183	0.532	-0.283	0.327	-0.018	0.952
CpG32	**-0.616**	**0.019***	0.362	0.204	-0.281	0.33	-0.192	0.511
CpG33	-0.414	0.141	0.209	0.473	-0.313	0.277	0.06	0.839
CpG34	**-0.680**	**0.007****	0.395	0.163	-0.314	0.274	-0.172	0.557

CpG35	**-0.625**	**0.017***	0.293	0.31	-0.293	0.31	-0.129	0.661
CpG36	**-0.567**	**0.035***	0.376	0.186	-0.398	0.159	-0.1	0.734
CpG37	-0.358	0.209	0.121	0.681	0.143	0.626	-0.358	0.209
CpG38	-0.376	0.185	0.19	0.515	-0.514	0.06	-0.005	0.988
CpG39	**-0.733**	**0.003****	0.458	0.1	-0.28	0.332	-0.193	0.508
CpG40	-0.505	0.065	0.186	0.524	-0.425	0.13	0.133	0.651
CpG41	**-0.691**	**0.006****	0.421	0.134	-0.236	0.416	-0.252	0.385
CpG42	**-0.687**	**0.007****	0.472	0.088	-0.389	0.169	-0.277	0.337
CpG43	**-0.533**	**0.05***	0.349	0.221	-0.029	0.921	-0.309	0.283
CpG44	**-0.568**	**0.034***	0.455	0.102	-0.277	0.337	-0.259	0.371
CpG45	-0.478	0.084	0.236	0.417	-0.26	0.369	-0.024	0.934
CpG46	-0.423	0.132	0.248	0.394	-0.331	0.248	-0.054	0.855
CpG47	**-0.644**	**0.013***	0.35	0.22	-0.155	0.597	-0.144	0.624
CpG48	**-0.595**	**0.025***	0.281	0.331	-0.308	0.284	-0.049	0.868
CpG49	**-0.550**	**0.041***	0.398	0.158	-0.107	0.715	-0.177	0.546
CpG50	-0.488	0.076	0.355	0.214	-0.185	0.526	-0.156	0.594
CpG51	**-0.605**	**0.022***	0.468	0.092	-0.029	0.922	-0.315	0.273
CpG52	**-0.539**	**0.047***	0.395	0.162	0.142	0.629	-0.423	0.132
CpG53	-0.361	0.204	0.301	0.296	-0.164	0.576	-0.323	0.26
CpG54	**-0.585**	**0.028***	0.438	0.118	-0.107	0.715	-0.411	0.145

ρ ; Spearman correlation coefficient, p: Spearman correlation significance. ** Correlation is significant at the 0.01 level, * correlation is significant at the 0.05 level (2-tailed).

Table 3-21. Spearman correlation analysis of rRNA proportions with CpG methylation levels in breast tumor tissue samples

	18S rRNA		28S rRNA		5.8S rRNA		45S ETS	
Correlation	ρ	**p**	ρ	**p**	ρ	**p**	ρ	**p**
CpG1	0.016	0.956	0.002	0.994	0.051	0.863	-0.223	0.444
CpG2	0.177	0.546	-0.034	0.908	-0.104	0.723	-0.242	0.404
CpG3	-0.09	0.759	-0.016	0.957	-0.023	0.939	-0.127	0.666
CpG4	0.098	0.739	-0.155	0.597	-0.002	0.994	-0.255	0.378
CpG5	0.224	0.441	-0.22	0.451	-0.358	0.208	-0.096	0.743
CpG6	0.308	0.284	-0.315	0.273	-0.256	0.377	-0.278	0.335
CpG7	0.37	0.193	-0.516	0.059	-0.297	0.302	-0.043	0.883
CpG8	0.013	0.964	-0.148	0.613	-0.027	0.927	0.034	0.909
CpG9	0.469	0.091	-0.373	0.189	-0.42	0.135	-0.186	0.524
CpG10	0.504	0.066	-0.052	0.861	-0.193	0.509	**-0.600**	**0.023***
CpG11	0.246	0.397	0.025	0.932	0.084	0.775	-0.403	0.153
CpG12	**0.542**	**0.045***	-0.176	0.546	-0.167	0.567	-0.342	0.232
CpG13	0.171	0.56	0.023	0.938	-0.228	0.432	-0.404	0.152
CpG14	0.115	0.695	0.217	0.456	-0.389	0.169	-0.109	0.712
CpG15	0.209	0.474	0.155	0.597	-0.025	0.933	-0.254	0.381
CpG16	0.048	0.871	-0.154	0.598	-0.098	0.74	-0.143	0.626
CpG17	0.195	0.503	0.076	0.795	-0.088	0.766	-0.002	0.994
CpG18	0.105	0.72	0.004	0.988	0.04	0.891	0.094	0.749
CpG19	-0.09	0.759	0.174	0.552	0.213	0.466	-0.038	0.896
CpG20	0.301	0.296	0.124	0.672	-0.383	0.177	-0.179	0.541
CpG21	0.427	0.127	-0.22	0.451	-0.392	0.166	-0.241	0.407
CpG22	0.521	0.056	-0.265	0.36	-0.505	0.065	-0.123	0.674
CpG23	0.273	0.344	-0.031	0.916	-0.411	0.144	-0.116	0.694
CpG24	0.278	0.335	-0.083	0.778	-0.321	0.263	-0.166	0.57
CpG25	0.145	0.62	0.105	0.721	-0.018	0.952	-0.246	0.396
CpG26	0.025	0.934	0.178	0.542	0.087	0.768	-0.35	0.22
CpG27	0.082	0.78	-0.049	0.868	-0.049	0.868	-0.151	0.606
CpG28	0.061	0.835	-0.045	0.877	-0.023	0.939	-0.161	0.582
CpG29	0.331	0.248	-0.34	0.235	**-0.631**	**0.015***	0.034	0.908
CpG30	0.447	0.109	-0.385	0.174	-0.519	0.057	0.002	0.994
CpG31	0.507	0.064	-0.471	0.089	-0.518	0.058	-0.129	0.66
CpG32	0.205	0.482	-0.339	0.236	-0.288	0.318	0.111	0.707
CpG33	0.027	0.927	-0.321	0.264	-0.408	0.147	0.419	0.136
CpG34	0.112	0.704	-0.344	0.229	-0.172	0.557	0.248	0.393

CpG35	-0.007	0.982	-0.129	0.659	-0.163	0.578	0.105	0.721
CpG36	-0.273	0.346	-0.016	0.957	-0.038	0.897	0.207	0.477
CpG37	0.333	0.245	-0.353	0.216	-0.211	0.468	0.007	0.982
CpG38	-0.212	0.467	-0.119	0.684	-0.068	0.818	0.178	0.543
CpG39	0.257	0.376	-0.199	0.496	0.029	0.922	-0.159	0.588
CpG40	0.273	0.346	-0.216	0.458	-0.437	0.118	0.167	0.569
CpG41	0.065	0.825	-0.155	0.597	-0.249	0.39	0.11	0.708
CpG42	-0.145	0.621	-0.071	0.808	0.317	0.269	0.134	0.648
CpG43	0.313	0.275	-0.127	0.666	-0.242	0.405	-0.053	0.857
CpG44	0	1	-0.286	0.322	-0.021	0.944	0.16	0.585
CpG45	0.332	0.247	-0.011	0.97	-0.369	0.194	-0.089	0.762
CpG46	0.048	0.871	-0.485	0.079	-0.082	0.781	0.43	0.125
CpG47	0.321	0.263	-0.303	0.292	-0.222	0.447	-0.009	0.976
CpG48	0.498	0.07	**-0.550**	**0.042***	-0.289	0.316	0.044	0.88
CpG49	**0.542**	**0.045***	-0.296	0.305	-0.36	0.206	-0.191	0.513
CpG50	0.054	0.854	-0.169	0.563	0.056	0.848	-0.005	0.988
CpG51	**0.545**	**0.044***	-0.392	0.166	-0.22	0.451	-0.267	0.356
CpG52	0.128	0.663	-0.516	0.059	-0.146	0.618	0.158	0.59
CpG53	-0.196	0.503	-0.04	0.891	0.121	0.679	0.236	0.417
CpG54	0.351	0.219	**-0.535**	**0.049***	-0.207	0.477	-0.034	0.908

ρ ; Spearman correlation coefficient, p: Spearman correlation significance. ** Correlation is significant at the 0.01 level, * correlation is significant at the 0.05 level (2-tailed).

As we can see in Table 3-20, methylation levels in several CpGs (CpG 1, 2, 4, 10, 24, 25, 27, 28, 29, 30, 32, 34, 35, 36, 39, 41, 42, 43, 44, 47,48, 49, 51, 52, 54) were found to negatively correlate with the 18S rRNA/GM-rRNA ratio in normal breast tissue samples, yet methylation levels of only two CpGs (CpG 9 and 16) were correlated with the 5.8S rRNA/GM-rRNA ratio. Other rRNA proportions (28S/GM-rRNA and 45S ETS/rRNA) did not show any correlation with methylation levels of CpGs. In the tumor samples, only three CpGs (CpG 12, 49 and 51) were positively correlated with the 18S/GM-rRNA ratio. Two CpGs (CpG 48 and 54) were negatively correlated with the 28S/GM-rRNA ratio, CpG 29 negatively correlated with the 5.8S/GM-rRNA ratio. CpG 10 was also negatively correlated with the 45S ETS/GM-rRNA ratio in breast tumor tissue samples (Table 3-21).

3.7.4 Correlation Analysis of rRNA Species Expression Levels in Breast Tumor and Matched Normal Tissue Samples

rRNA species are transcribed as 45S rRNA precursor and spliced into 18S, 28S and 5.8S rRNA transcripts. If they were spliced simultaneously, one would expect them to be expressed relatively equally. However, as shown in Figure 3.18, rRNA proportions were dysregulated in tumor samples, which indicates possible disruption of rRNA maturation mechanism. Spearman correlation analysis was performed to see whether expression of rRNA species were correlated with each other in breast tumor and matched normal tissue samples.

Table 3-22. Spearman correlation analysis between rRNA transcripts in tumor and normal samples

		28S rRNA	5.8S rRNA	45S ETS
Normal	**18S rRNA**	0.837 (p<0.01*)	0.824 (p<0.01*)	0.818(p<0.01*)
	28S rRNA		0.833 (p<0.01*)	0.674(p=0.012*)
	5.8S rRNA			0.57 (p=0.033*)
Tumor	**18S rRNA**	0.042 (p=0.887)	-0.051 (p=0864)	0.288 (p=0.318)
	28S rRNA		0.521 (p=0.056)	0.349 (p=0.221)
	5.8S rRNA			0.543 (p=0.045*)

Shown are Spearman correlation coefficients ρ and p values in brackets

Expressions of rRNA transcripts were significantly correlated with each other in normal breast tissue samples whereas this correlation was lost in matched tumor samples. Only Ct values of 5.8S rRNA and 45S ETS transcripts were correlated with each other in tumor samples whereas all rRNA transcripts were correlated with each other in normal samples Table 3-22.

4 DISCUSSION

Ribosome biogenesis is the essential requirement for a cell to grow and divide but it is limited to the transcription of rRNA genes. Highly proliferative cancer cells are expected to increase rRNA expression and thus ribosome biogenesis, but there are only a limited number of studies analysing the rRNA expression levels (only 18S rRNA) in breast cancer tissues and matched normal samples. These previous studies tested the variation in 18S rRNA expression in breast cancer and paired normal tissues to use it as a reference gene (Gur-Dedeoglu et al. 2009; Tricarico et al. 2002). One of these studies found that 18S rRNA was expressed at higher levels in breast tumors compared to matched normal tissues and the other study found just the opposite but none of them identified whether this expression difference of 18S rRNA was the result of the 45S rDNA promoter methylation (Gur-Dedeoglu et al. 2009; Tricarico et al. 2002). They also did not use any other rRNA transcripts (28S, 5.8S or 45S ETS) to see whether these rRNAs are also differentially expressed within the tumor or between tumor and normal pairs.

DNA methylation of the CpG islands in promoter regions is known to repress transcription by interrupting the binding of Pol II to the promoter; considering both Pol I and Pol II have common features and transcription factors 45S rDNA promoter methylation may interfere with rRNA gene expression from the promoter by Pol I (Sentenac 1985; Sharp 1992; Comai et al. 1992; Eden & Cedar 1994).

In this study, we investigated 45S rDNA promoter methylation levels and expression levels of rRNA transcripts (18S, 28S, 5.8S and 45S ETS) together with their relation to each other in both breast cancer cell lines and clinical breast cancer tissues. We used ten breast cancer cell lines (MCF7, MDA-MB-231, MDA-MB-453, MDA-MB-468, BT-474, ZR-75-1, BT-20, MDA-MD-361, SKBR-3 and CAL-51) and a non-tumorigenic breast cell line (MCF-10A). The investigated region (-381 bp to +53 bp) was profoundly methylated (74%-96% methylation) in all cell lines (Figure 3.1). In accordance with our results, transformed cell lines with different origins (Jurkat, CEM, HeLa, KB, NIH 3T3, HEK293 were also found to exhibit high levels of 45S rDNA promoter methylation in the literature (Kochanek et al. 1996; Németh et al. 2008; Brown & Szyf 2007). Another possible explanation for the high levels of methylation in rDNA promoter of breast

cancer cell lines is long-continued culturing after all rDNA gene locus was identified to be one of the loci sensitive to accumulate methylation with aging in rat liver cells and spermatozoa (Oakes et al. 2003). Some studies in the literature identified rDNA promoter methylation clones to consist of two populations: one with hypermethylated and one with hypomethylated promoter (Ghoshal et al. 2004; Raval et al. 2012; Uemura et al. 2011; Gagnon-Kugler et al. 2009), nevertheless we were unable to identify these two populations in breast cancer cell lines. However this result might be due to low number of bisulfite sequencing clones (five clones from each cell line) analysed in cell lines.

In order to further analyse 45S rDNA promoter methylation levels in breast cancer we used frozen breast tumor and matched normal tissue samples collected and immediately frozen during surgery. We used 19 breast tumor and matched normal tissue samples, clinical characteristics of tissues were detailed in Table 2-6 45S rDNA promoter methylation levels were significantly higher in most of the breast tumor samples (13/19) compared to their normal counterparts (Figure 3.2). We also identified many consequent CpG sites in the 45S rDNA promoter to be significantly methylated in tumor samples compared to normal pairs (Figure 3.5). 45S rDNA promoter region was analysed with a transcription factor binding prediction tool (PROMO 3.0) to find overlapping sequences between PROMO and sequentially methylated CpG sites. Methylation at certain CpG sites in 45S rDNA promoter region may affect the bindings of transcription factors to their predicted binding sites. GCF, is a repressor of GC-rich promoters (Tohgi et al. 1999), which binds to GCF transcription factor binding site also found in 45S rDNA promoter and overlaps with two CpG position one of them (CpG 28) was significantly methylated in tumor samples. Nuclear factor-1 binding site also found to be located in 45S promoter and contains one CpG (CpG 52), which is also identified as significantly methylated in tumor samples compared normal samples. However, methylation at NF-1 site and its effect on methylation has not been studied yet. Pax-5 is TFBS located in 45S promoter known to require unmethylated CpG to be bound by Pax-5 transcription factor (Maier et al. 2003). Pax-5 TFB sites overlap with five CpG positions in the 45S rDNA promoter; three of them (CpG 20, 26 and 41), were significantly methylated in tumor samples compared to normal pairs. No relationship has been shown between Pax-5 binding to 45S promoter or whether it is required for transcription by RNA polymerase I

but it should be considered as a regulatory mechanism. Before further analysing Pax-5 binding to rDNA promoter we wanted to make sure that 45S rDNA promoter methylation has a significant effect on rRNA transcription.

Normal breast tissue samples exhibited a mosaic methylation pattern, which is rather frequently observed at 45S rDNA promoters (Ghoshal et al. 2004; Uemura et al. 2011). Analysis of methylation patterns between breast tumor and normal pairs revealed that 63% of the tumor-normal pairs methylation patterns were significantly correlated with each other (Table 3-1) that indicates a possible individual specific methylation pattern at 45S rDNA promoter in breast. To make sure that this correlation is individual specific rather than tissue specific, we performed the same correlation analysis with unmatched tumor and normal samples, many of the (48.8%) unmatched tumor and normal samples showed significant correlation as well (a representative of the results were given in Table 3-2). Determining the methylation pattern of 45S rDNA promoter region in different tissues may help to reveal whether it is tissue specific or not, considering different loci are differentially methylated in different tissues (Muangsub et al. 2014).

rDNA methylation levels were implied to have relationship with different clinical characteristics in different cancer types (Chan et al. 2005; Powell et al. 2002; Yan et al. 2000), so we sought a relationship between 45S rDNA promoter methylation levels and clinical features of breast cancer samples used in our study. In order to take advantage of using paired samples, we took into account the methylation differences between paired breast tumor and normal samples in each CpG and used this methylation difference to cluster (unsupervised hierarchical clustering) samples in to two groups (Group 1: methylated in tumors, Group 2: unmethylated in tumors). We used Fisher's exact test to analyse these two categorical groups with other categorical variables; ER, PR, lymph node status and metastasis status. However, no statistically significant association was found between hierarchical clustering groups of methylation (group 1 and group 2) and ER, PR, lymph node status and metastasis status. This method uses the methylation difference between paired samples but maybe clinical parameters associate with tumor rDNA promoter methylation levels of the tissues rather than paired methylation difference. To test this hypothesis we used Spearman correlation analysis and tested the correlation between clinical variables (including grade and stage) and 45S rDNA

promoter methylation levels (%) of tumor and normal samples separately. As seen in Table 3-7, this approach also failed to identify any association between 45S rDNA methylation levels and clinical variables of the patients. Similar to our findings, another study on paired breast tumor and normal tissue samples also failed to identify any significant correlation between rDNA promoter, 5' regions of 18S and 28S rDNA methylation levels and any clinicopathological features, except nuclear size and grade (Bacalini et al. 2014). Using a larger sample size might be more effective for identifying an association between rDNA methylation and clinical parameters in breast cancer, if there is any.

Our initial motivation to analyse 45S rDNA methylation level was to identify its effect on rRNA gene expression, so we performed expression analysis of rRNA transcripts (18S, 28S, 5.8S and 45S ETS) in both breast cancer cell lines, non-tumorigenic breast cell line (MCF-10A) and breast tumor and matched normal tissue samples. To identify rRNA expression levels in cancer, different studies used different housekeeping genes for normalization; the most commonly used ones are ACTB, GAPDH and TBP (Raval et al. 2012; Uemura et al. 2011; Brown & Szyf 2008). ACTB, TBP and ACTB&TBP levels were used as reference genes to analyse expression levels in cell lines. We used TBP (RNA polymerase II transcribed genes) as a reference gene for tissue samples since it was the most stably expressed reference gene between breast normal and breast cancer tissues among other reference genes (ACTB, SDHA and GAPDH) according to the Oncomine analysis (Figure 3.13, Figure 3.14, Figure 3.15 and Figure 3.16). We also propose that using the geometric mean (GM) of rRNAs (18S, 5.8S, 28S and 45S ETS) to normalize rRNA expression might be useful in identifying relative changes of rRNAs within the rRNA pool. GM-rRNA is calculated from the rRNA transcripts synthesized by Pol I and it might be less susceptible to alterations compared to genes transcribed by Pol II. Normalization of rRNA expression with reference genes (ACTB, TBP, ACTB&TBP in cell lines, TBP in tissue samples) enabled us to identify relative change of rRNAs compared to mRNA levels whereas using GM-rRNA (geometric mean of 18S, 28S, 5.8S and 45S ETS) for normalization has helped us to identify relative changes of rRNA transcripts within their own rRNA pool.

As we have seen in Figure 3.1 all breast cancer cell lines as well as non-tumorigenic MCF-10A cell line were hypermethylated at the 45S rDNA promoter region. Breast cancer cell lines with hypermethylated 45S rDNA promoter expressed rRNA transcripts at different levels (Figure 3.9) and independent from 45S rDNA promoter methylation levels (Table 3-14, Table 3-15, Table 3-16 and Table 3-17). These results imply that expression from 45S rDNA promoter might not be the major determinant of rRNA expression in breast cancer cell lines. Consistent with our results, *Xenopus leavis* oocytes are able to transcribe rRNA from transfected fully methylated (*Xenopus leavis* sperm DNA) and unmethylated rDNA promoter constructs equally efficient (Macleod & Bird 1983).

To further investigate whether rRNA transcription is really independent of 45S rDNA promoter methylation, we treated breast cancer cell lines with 5-Aza-2'-deoxycytidine (5-AZA), which is a cytosine analogue, incorporated into DNA during replication and blocks DNA methylation by inhibiting DNMTs (Christman et al. 1983; Creusot et al. 1982; Taylor & Jones 1982). 5-AZA treatment of cell lines is expected to decrease CpG methylation in all genome along with CG rich 45S rDNA promoter and increase expression of rRNA genes. Surprisingly, all rRNA transcripts were relatively downregulated in 5-AZA treated group compared to control group and the downregulation of 5.8S and 45S ETS rRNA transcripts were significant (normalization with reference genes). Nonetheless no change was observed in the relative rRNA transcript ratios (GM-rRNA normalization) (Figure 3.10 D). Consistent with our results, another study used 5-AZA treatment in HCT116 (colon cancer) cell line and identified a downregulation in rRNA synthesis, which was caused by cryptic transcription via RNA Pol II from unmethylated 45S rDNA promoter that prevents proper processing and stability of rRNA transcripts (Gagnon-Kugler et al. 2009). Decreased methylation of 45S rDNA promoter in breast cancer cell lines upon 5-AZA treatment might have decreased the stability of rRNA transcripts and we found significant downregulation of both 5.8S rRNA and 45S ETS products in these cell lines compared to the control group. This cryptic transcription by RNA polymerase II, which acts similar to a negative feedback mechanism, might be a way for cell to achieve a fine balanced expression of these

essential rRNA genes and also a mechanism to protect cell from energy loss in the absence of CpG methylation at 45S rDNA promoter.

We used another epigenetic drug Trichostatin A (TSA) to determine whether other regulatory mechanisms such as histone acetylation play a more determinant role in the regulation of rRNA gene expression. TSA is a potent HDAC inhibitor (Vigushin et al. 2001), treating cell lines with TSA indirectly increases H3 and H4 lysine acetylation by inhibiting HDACs, which also leads to dissociation of the chromatin hence upregulation of many genes in the genome (Ballestar & Esteller 2008). Treatment of breast cancer cell lines only with TSA did not significantly alter the rRNA expression or the ratio of rRNA transcripts but a significant increase in 18S rRNA/ACTB&TBP level and a significant decrease in 5.8S/GM-rRNA ratio was observed in 5-AZA+TSA treatment group compared to the control (DMSO treated) group. It is plausible that transcription of rRNAs might be mainly regulated by other mechanisms (PIC formation, initiation, promoter escape, elongation, termination, re-initiation, RNA processing and post-transcriptional modifications) instead of epigenetic modifications in breast cancer cell lines. It is also critical to acknowledge that using epigenetic drugs affect hundreds of genes at once in the genome along with rDNA genes. The variation between mRNA normalization and rRNA normalization might be due to the fact that mRNA genes are subjected to the direct or indirect effects of these drugs.

We had already identified a significant difference in rDNA promoter methylation levels between breast tumors and matched normal tissue samples, and we also analysed expression levels of rRNA genes in these tissue samples to see whether the significant methylation of rDNA promoter in breast tumor samples was reflected in rRNA expression levels. TBP normalization analysis did not reveal a significant difference between breast tumors and matched normal samples (Figure 3.17). When we used GM-rRNA for normalization, we found that 18S and 5.8S rRNA ratios were significantly down- and up -regulated, respectively, in breast tumor samples compared matched normal pairs (Figure 3.18). These results demonstrate that 18S and 5.8S rRNA proportions shift in the reverse direction within the same total rRNA pool even though total rRNA amounts might be rather equal. Supportingly, normal breast tissue samples exhibited a perfect correlation between rRNA transcripts but this correlation was

vanished in breast tumor tissue samples (Table 3-22). To identify whether rRNA expression levels or ratios are correlated with promoter methylation levels we performed a correlation analysis and found that TBP normalized expression levels were not significantly correlated with 45S rDNA promoter methylation levels in breast tumor and matched normal samples (Table 3-18). However, rDNA promoter methylation levels of normal samples (which display varied methylation pattern) and 18S/GM-rRNA ratio were negatively correlated in normal breast tissues but this correlation was disappeared in matched tumor samples (Figure 3.19). As far as we know, this is the first study to show that methylation status of the promoter might affect the expression of one or more rRNA transcripts but not all of them.

rRNA expression levels were also used to predict clinical variables in breast cancer tissue samples. None of the variables significantly predicted clinical characteristics of the tumors with multiple regression analysis. Using spearman correlation analysis identified 45S/TBP and 28S/GM-rRNA to be positively correlated with the grade of the tumors. Breast cancer grading uses nuclear pleomorphism as a criteria that groups breast cancer according to the size and shape of the nucleoli (Egner 2010). Increased expression or ratio of certain rRNA transcripts might be responsible for the morphological abnormalities observed in the nucleoli of higher-grade breast tumor samples.

Post-transcriptional modifications are also important players in gene regulation. In some cases, promoter-sharing polycistronic mRNAs and miRNAs were shown to exist at unequal levels due to post-transcriptional regulation in plants (Jia & Rock 2013; Malik Ghulam et al. 2013). As reported by other studies, promoter-sharing genes might be differentially expressed by other mechanisms not only by basal transcription machinery. Mixed methylation patterns observed in normal tissues (moderate methylation levels) might still be coordinating the expression of 18S rRNA but high methylation levels observed in tumor samples lost this coordination, as seen in Figure 3.19. On the other hand, methylation of promoter indirectly influences splicing, modification and stabilization of rRNA transcripts (Gagnon-Kugler et al. 2009). Correlation between rRNA transcripts (Table 3-22) observed in normal tissue samples were lost in tumor samples indicating that hypermethylated promoter of 45S rDNA might influence the processing of rRNAs.

Ribosome maturation is a multistep and complex process facilitated through orchestration of many factors (~200) (Kiss et al. 2006; Terns & Terns 2006). Splicing, processing and modification of rRNAs are mainly coordinated by snorNAs and changes in snoRNA levels might be reflected in the rRNA ratios. Special modifications are required for proper folding and stabilization of rRNAs. Two specific sites in 28S RNA should be modified with 2'-O-methylation modification via U50 (box C/D snoRNA). U50 was found to be altered through mutations, deletions and somatic rearrangements in various cancer types such as prostate cancer, breast carcinoma, B-cell lymphoma and colon cancer (Dong et al. 2008; Dong et al. 2009; Tanaka et al. 2000; Pacilli et al. 2013). Decreased expression of GAS5 and/or its snoRNAs have been displayed in head and neck squamous cell carcinoma, glioblastoma multiforme and breast cancer (Gee et al. 2011; Lee et al. 2006; Mourtada-Maarabouni et al. 2009). It is hard to interpret the exact role of rDNA promoter methylation in rRNA expression as there are many players in rRNA gene regulation but increased methylation seems to affect rRNA modification and processing as well, which requires further studies.

Finally, rRNA transcription in breast cancer cell lines was found to be independent of hypermethylation at the 45S rDNA promoter region. Yet tissue samples did not support this result, as the 18S rRNA/GM-rRNA ratio was significantly correlated with methylation of the 45S rDNA promoter region in normal breast tissue samples. As indicated earlier, both breast cancer cell lines and breast cancer tissue samples have heavily methylated 45S rDNA promoter regions and promoter methylation of 45S rDNA promoters might have a different role than regulating rRNA gene expression. It might for example be required for protecting these essential genes in all conditions. Like many repeats in the genome, rDNA repeats have also been implicated in stability of the genome and decreased genomic stability has also been associated with 45S rDNA promoter hypomethylation (Kobayashi 2014; Peng & Karpen 2007; Kobayashi 2008). Hypermethylation of 45S rDNA promoter in tumors might be a strategy used by the cell to restore the disrupted genomic integrity. Further research is required to unravel the possible cause of dysregulation in rRNA transcripts in cancer as well as its association with rDNA promoter methylation.

5 FUTURE PERSPECTIVES

In order to, better establish the relationship between 45S rDNA promoter methylation and rRNA expression levels in breast cancer sample size should increase.

rRNA gene expression is tightly regulated since it codes for one of the most essential genes in the genome and these genes exist in all forms of life, even the most primitive. There are many regulatory mechanisms that are evolved for the optimal rRNA gene expression starting with tandemly repeated rDNA genes and continues with PIC formation, initiation, promoter escape, elongation, termination, re-initiation, RNA processing and post-transcriptional modifications.

We focused on the effect of the rDNA methylation on rRNA gene expression in breast cancer but transcriptional and post-transcriptional regulation of rRNAs should be molecularly dissected to be able to better comprehend the whole regulatory mechanism of rRNA gene expression.

Transcription factors binding to the 45S rDNA promoter region (GR-alpha, Pax-5, GCF, ER-alpha, GATA-1, NF-1, C/EBPbeta, TFII-I, AP-2, alpha A YY1) can be further analysed using chromatin immunoprecipitation (ChIP). Artificially methylated (Sss1 methyltransferase treated) and unmethylated (DNA from 5-AZA treated or DNMT knockout cells) rDNA products can also be used in immnuoprecipitation with these transcription factors to identify whether there is a difference in binding efficiencies of transcription factors to methylated and unmethylated rDNA promoter.

Active and inactive histone marks can be used in order to further identify the effect of 45S rDNA promoter methylation on rRNA gene expression. Chromatin immunoprecipitation with active histone marks (such as H3K9me3, H4K20 and H3K27me3) and inactive histone marks (such as acetylated histones H4, H3 and H3K4me3) followed by a bisulfite sequencing targeting 45S rDNA promoter can reveal the true nature of DNA methylation states of active and inactive rDNA promoter.

Promoter associated RNA (pRNA) is a non-coding DNA transcribed from the intergenic spacer ~2 kb upstream of the rRNA transcription start site and is associated with rRNA gene silencing (Mayer et al. 2008; Mayer et al. 2006). pRNA levels could be determined

to see whether it has any effect on dysregulation of rRNAs in cancer cells compared to normal cells.

Post-transcriptional modifications of rRNAs play a major role in maturation, ribosome assembly and accuracy of the decoding. There are three major types of rRNA modifications; 2'-*O*-methylation (Nm), pseudouridylation (ψ) and base methylation at various positions (Smith & Dunn 1959; Davis & Allen 1957; Cohn 1960; Wagner et al. 1967). Detection of changes in post-transcriptional modifications using chromatography and mass spectrophotometry based methods is vital for understanding the deregulated expression of rRNA genes in cancer.

Until recently, the only known epigenetic mark of DNA itself was 5-methylcytosine (5mC). Hydroxymethylation of cytosine residues at 5^{th} position was first characterized in Purkinje neurons and embryonic stem cells (Kriaucionis & Heintz 2009; Ficz et al. 2011). 5-hydroxymethylcytosine (5-hmC) is generated through the oxidization of 5-mC by TET family of enzymes (Ito et al. 2010; Tahiliani et al. 2009). These reports provided new insights into the mechanism of active DNA demethylation, suggesting that a hydroxylated methyl group could be an intermediate for oxidative demethylation. Recently, it has been shown that hydroxymethylation levels are also altered in various types of cancer including breast cancer (Kroeze et al. 2014; Kraus et al. 2012; Ko et al. 2010; Haffner et al. 2011). Furthermore, many standard methods used for detecting methylated DNA, such as bisulfite conversion, cannot distinguish between 5-mC and 5-hmC. 45S rDNA promoter hydroxymethylation status could also be analyzed to identify if the region contains any 5-hmC along with 5mC.

New technologies such as whole genome bisulfite sequencing and next generation sequencing methods (instead of Sanger sequencing) can be used to unravel the complete picture of the DNA methylation states of 45S rDNA.

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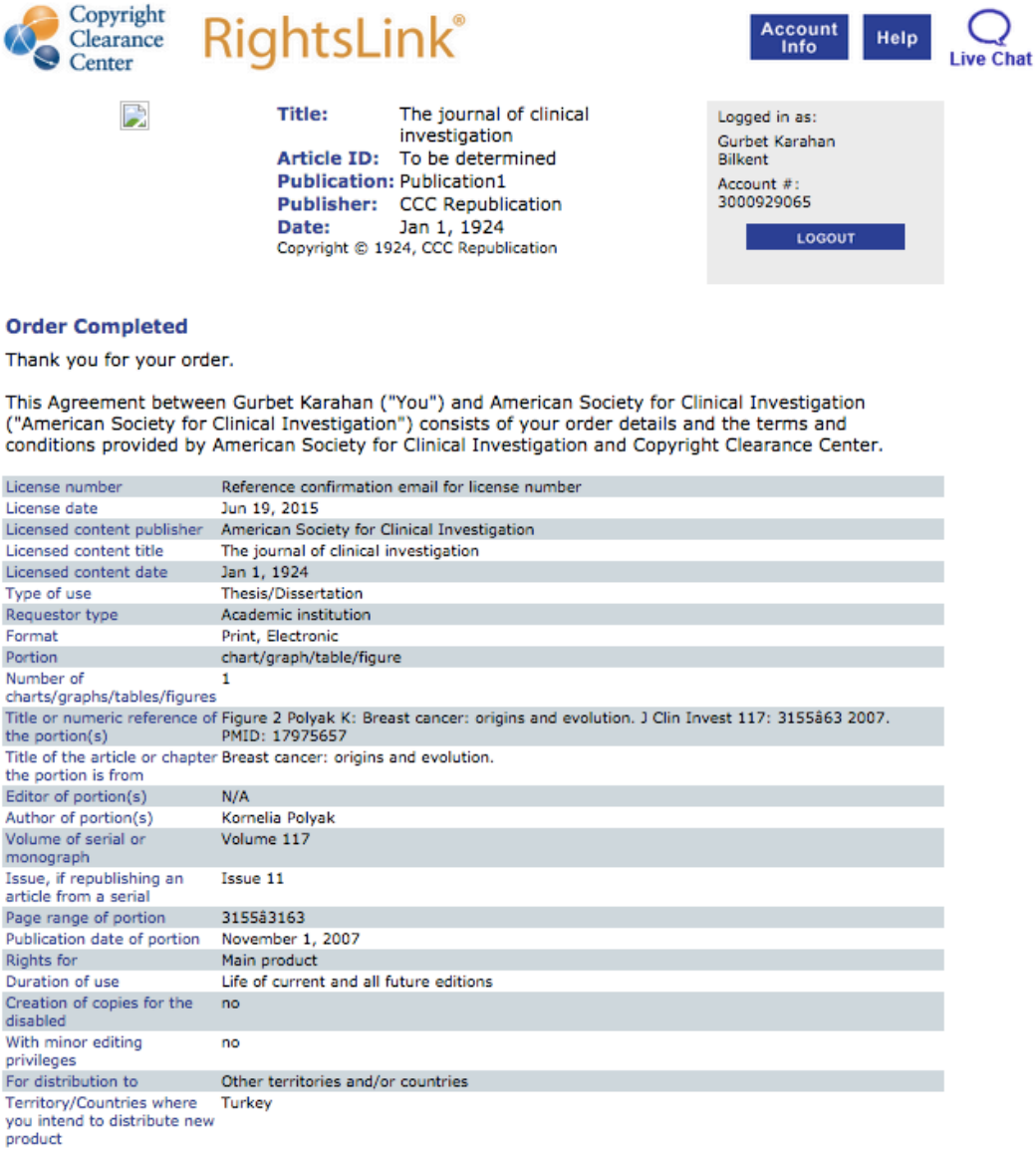

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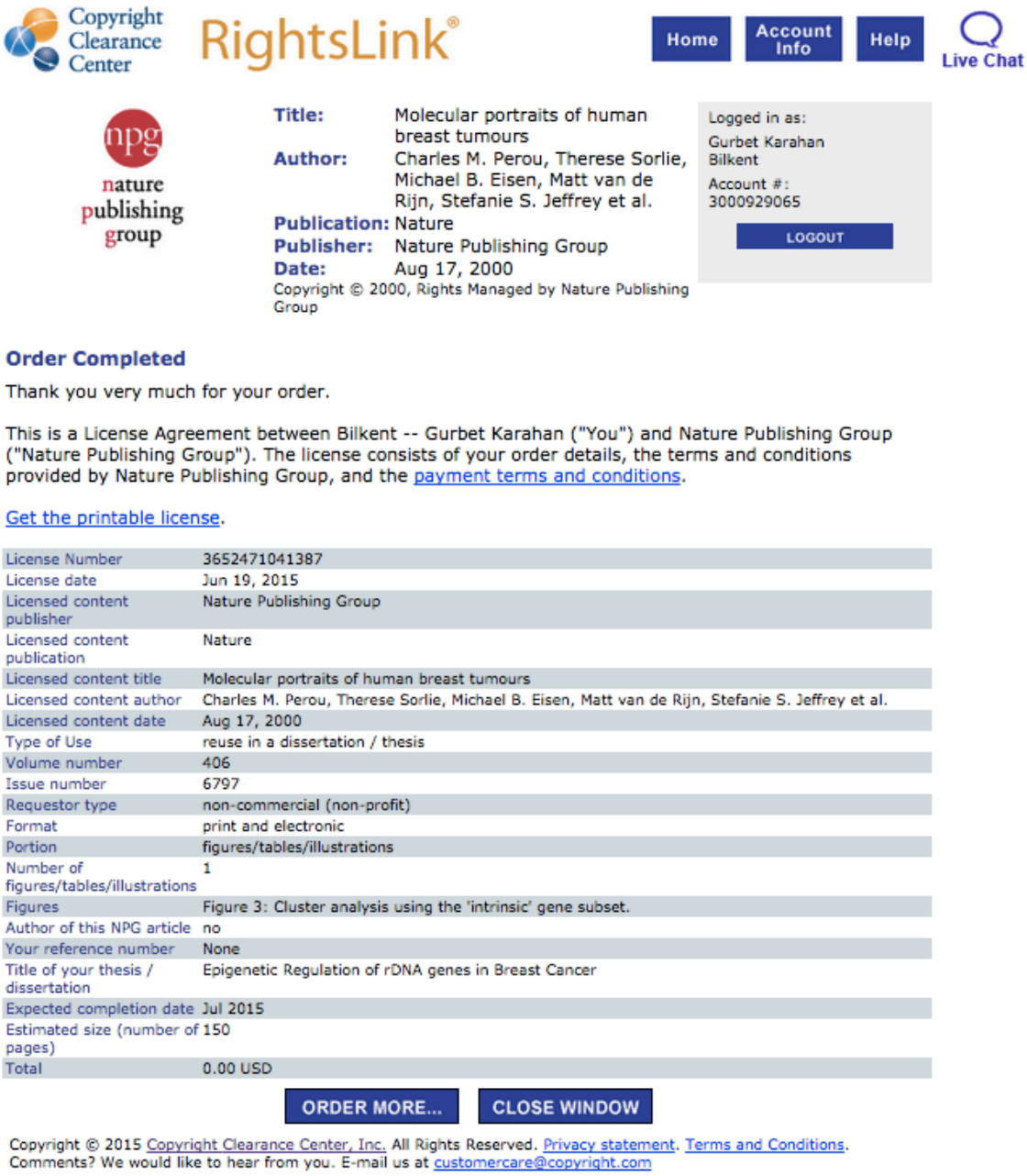

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Appendix B

MIQE: Minimum Information for Publication of Quantitative Real-Time PCR Experiments Check List

ITEM TO CHECK	IMPORTANCE	CHECKLIST
EXPERIMENTAL DESIGN		
Definition of experimental and control groups	**E**	✓
Number within each group	**E**	14
Assay carried out by core lab or investigator's lab?	D	Investigator's lab
Acknowledgement of authors' contributions	D	✓
SAMPLE		
Description	**E**	Total RNA from tissue
• Volume/mass of sample processed	D	60-µm-thick sections (4-5 slice from tumor and 20-25 slices from normal tissues)
• Microdissection or macrodissection	**E**	Macrodissection
Processing procedure	**E**	Snap frozen with liquide nitrogen
• If frozen - how and how quickly?	**E**	Immediately, during surgery
• If fixed - with what, how quickly?	**E**	NA
Sample storage conditions and duration (especially for FFPE samples)	**E**	-80°C, 4-6 years
NUCLEIC ACID EXTRACTION		
Procedure and/or instrumentation	**E**	Trizol
• Name of kit and details of any modifications	**E**	TRI Reagent RT (Molecular Research Center), no modification
• Source of additional reagents used	D	Sigma
Details of DNase or RNAse treatment	**E**	MessageClean Kit (GenHunter)
Contamination assessment (DNA or RNA)	**E**	-RT
Nucleic acid quantification	**E**	✓
• Instrument and method	**E**	Nanodrop Spectrophotometer (Thermo Scientific)

• Purity (A260/A280)	D	1.7-2.1
• Yield	D	500-2000 ng
RNA integrity method/instrument	**E**	Agilent RNA 6000 Nano Kit
• RIN/RQI or Cq of 3' and 5' transcripts	**E**	RIN: 4-7
• Electrophoresis traces	D	NA
Inhibition testing (Cq dilutions, spike or other)	**E**	NA
REVERSE TRANSCRIPTION		
Complete reaction conditions	**E**	✓
• Amount of RNA and reaction volume	**E**	500 ng and 20µl
• Priming oligonucleotide (if using GSP) and concentration	**E**	Random hexamer primer and 24 µg at 100 µM concentration
• Reverse transcriptase and concentration	**E**	RevertAid Reverse Transcriptase (200 U/µL)
• Temperature and time	**E**	1 hour at 42°C
• Manufacturer of reagents and catalogue numbers	D	Thermo Scientific (K1691)
Cqs with and without RT	D*	8-10 and 30-32
Storage conditions of cDNA	D	'-80°C
qPCR TARGET INFORMATION		
If multiplex, efficiency and LOD of each assay.	**E**	NA
Sequence accession number	**E**	✓
Location of amplicon	D	✓
• Amplicon length	**E**	✓
• *In silico* specificity screen (BLAST, etc)	**E**	✓
• Pseudogenes, retropseudogenes or other homologs?	D	Identical repeated genes
• Sequence alignment	D	✓
• Secondary structure analysis of amplicon	D	✓
Location of each primer by exon or intron (if applicable)	**E**	✓

• What splice variants are targeted?	**E**	No splice variants
qPCR OLIGONUCLEOTIDES		
Primer sequences	**E**	✓
RTPrimerDB Identification Number	D	NA
Probe sequences	D**	NA
Location and identity of any modifications	**E**	None
Manufacturer of oligonucleotides	D	Iontek Company (Turkey)
Purification method	D	Standard desalination
qPCR PROTOCOL		
Complete reaction conditions	**E**	✓
• Reaction volume and amount of cDNA/DNA	**E**	✓
• Primer, (probe), Mg++ and dNTP concentrations	**E**	✓
• Polymerase identity and concentration	**E**	✓
• Buffer/kit identity and manufacturer	**E**	DyNAmo qPCR kit, Thermo Scientific
• Exact chemical constitution of the buffer	D	✓
• Additives (SYBR Green I, DMSO, etc.)	**E**	✓
Manufacturer of plates/tubes and catalog number	D	Bioplastics, B70501
Complete thermocycling parameters	**E**	✓
Reaction setup (manual/robotic)	D	Manual
Manufacturer of qPCR instrument	**E**	Stratagene
qPCR VALIDATION		
Evidence of optimisation (from gradients)	D	✓
Specificity (gel, sequence, melt, or digest)	**E**	Gel
For SYBR Green I, Cq of the NTC	**E**	35-No Cq

Standard curves with slope and y-intercept	**E**	✓
• PCR efficiency calculated from slope	**E**	✓
• Confidence interval for PCR efficiency or standard error	D	NA
• r2 of standard curve	**E**	✓
• Linear dynamic range	**E**	✓
• Cq variation at lower limit	**E**	0.9 Cq
• Confidence intervals throughout range	D	NA
Evidence for limit of detection	**E**	NA
If multiplex, efficiency and LOD of each assay.	**E**	NA
DATA ANALYSIS		
qPCR analysis program (source, version)	**E**	Excel for Mac 2011, Version 14.5.3
• Cq method determination	**E**	Delta Ct
• Outlier identification and disposition	**E**	NA
Results of NTCs	**E**	✓
Justification of number and choice of reference genes	**E**	✓
Description of normalisation method	**E**	TBP and Geometric Mean of rRNAs
Number and concordance of biological replicates	D	NA
Number and stage (RT or qPCR) of technical replicates	**E**	2
Repeatability (intra-assay variation)	E	NA
Reproducibility (inter-assay variation, %CV)	D	NA
Power analysis	D	NA
Statistical methods for result significance	**E**	$p<0.05$
Software (source, version)	E	IBM SPSS Statistics, Version 21
Cq or raw data submission using RDML	**D**	NA

MIQE checklist for authors, reviewers and editors. All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available. If using primers obtained from RTPrimerDB, information on qPCR target, oligonucleotides, protocols and validation is available from that source.

*: Assessing the absence of DNA using a no RT assay is essential when first extracting RNA. Once the sample has been validated as RDNA-free, inclusion of a no-RT control is desirable, but no longer essential.

**: Disclosure of the probe sequence is highly desirable and strongly encouraged. However, since not all commercial pre-designed assay vendors provide this information, it cannot be an essential requirement. Use of such assays is advised against.

NA: Not Available

✓ : Either given in the thesis or performed

Relative expression of rRNA transcripts and 45S rDNA promoter methylation status are dysregulated in tumors in comparison with matched-normal tissues in breast cancer

GURBET KARAHAN[1], NILUFER SAYAR[1], GOKCEN GOZUM[1], BETUL BOZKURT[2], OZLEN KONU[1] and ISIK G. YULUG[1]

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Abstract. Ribosomal RNA (rRNA) expression, one of the most important factors regulating ribosome production, is primarily controlled by a CG-rich 45S rDNA promoter. However, the DNA methylation state of the 45S rDNA promoter, as well as its effect on rRNA gene expression in types of human cancers is controversial. In the present study we analyzed the methylation status of the rDNA promoter (-380 to +53 bp) as well as associated rRNA expression levels in breast cancer cell lines and breast tumor-normal tissue pairs. We found that the aforementioned regulatory region was extensively methylated (74-96%) in all cell lines and in 68% (13/19 tumor-normal pairs) of the tumors. Expression levels of rRNA transcripts 18S, 28S, 5.8S and 45S external transcribed spacer (45S ETS) greatly varied in the breast cancer cell lines regardless of their methylation status. Analyses of rRNA transcript expression levels in the breast tumor and normal matched tissues showed no significant difference when normalized with TBP. On the other hand, using the geometric mean of the rRNA expression values (GM-rRNA) as reference enabled us to identify significant changes in the relative expression of rRNAs in the tissue samples. We propose GM-rRNA normalization as a novel strategy to analyze expression differences between rRNA transcripts. Accordingly, the 18S rRNA/GM-rRNA ratio was significantly higher whereas the 5.8S rRNA/GM-rRNA ratio was significantly lower in breast tumor samples than this ratio in the matched normal samples. Moreover, the 18S rRNA/GM-rRNA ratio was negatively correlated with the 45S rDNA promoter methylation level in the normal breast tissue samples, yet not in the breast tumors. Significant correlations observed between the expression levels of rRNA transcripts in the normal samples were lost in the tumor samples. We showed that the expression of rRNA transcripts may not be based solely on promoter methylation. Carcinogenesis may cause dysregulation of the correlation between spliced rRNA expression levels, possibly due to changes in rRNA processing, which requires further investigation.

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Abbreviations: rRNA, ribosomal RNA; rDNA, ribosomal DNA; GM-rRNA, geometric mean of rRNA expression values; 5-AZA, 5-aza-2'-deoxycytidine; TSA, trichostatin A

Key words: epigenetics, DNA methylation, ribosomal DNA, ribosomal RNA expression, breast cancer

Introduction

Breast cancer, the most common type of cancer among women, was also the primary and secondary cause of cancer-related deaths among women living in less developed (14.3% of all cancer-related deaths) and more developed regions (15.4% after lung cancer) in 2012, respectively (1). Familial or somatic mutations of BRCA1, BRCA2 and TP53 (alias p53) are well-known high risk factors for breast cancer formation while others (PALB2, BRIP1, ATM, CHEK2, PTEN and CDH1) have been estimated to have moderate or weak effects (2).

Contrary to mutations that modify the DNA sequence itself, epigenetic alterations affect gene expression via DNA methylation, histone modifications and chromatin remodeling. DNA methylation, the frequently studied epigenetic modification in the context of embryogenesis, X chromosome inactivation and imprinting (3-5), is also important for the protection of genome integrity and hence cancer. Global hypomethylation of the genome, commonly observed in multiple cancers, increases genome instability and activates proto-oncogenes while hypermethylation of promoter CpG islands silences the expression of tumor suppressor genes (6-8). Promoter DNA methylation, identified at the promoter region of many genes, contributes to breast tumorigenesis; however, DNA methylation of the rDNA region has been overlooked in DNA methylation studies related to breast cancer.

Ribosome synthesis is closely related to the cell metabolism involved in cell growth and proliferation, and is tightly correlated with ribosomal RNA (rRNA) synthesis (9). The

human genome contains ~300-400 copies of rRNA genes but only a fraction of these genes are actively transcribed depending on the cell type, external signals and the cell stage, while the rest of the genes remain inactive (10). rRNA genes are organized in tandem repeated arrays within nucleolar organizing regions (NORs) located on the short arms of five human acrocentric chromosomes: chromosome 13, 14, 15, 21 and 22 (11). rRNA genes (except 5S, which is transcribed by RNA polymerase III) are transcribed from the 45S rDNA promoter by RNA polymerase I (Pol I). Since ~60% of the total RNA of a cell consists of Pol I products (12), rRNA genes are regulated tightly at different levels that include pre-initiation complex (PIC) formation, initiation, promoter escape, elongation, termination, re-initiation, RNA processing and post-transcriptional modifications (13).

The entire promoter region of rRNA genes is contained in an intergenic spacer region (IGS) between rDNA units. The promoter region of rDNA repeating unit consists of two important elements: the core promoter and upstream control element (UCE). The core promoter is located between -50 to +20 bp and is essential for basal transcription, whereas the UCE is located 150-200 bp upstream of the transcription start site and is required for efficient pre-initiation complex formation (14) (Fig. 1A). Cooperative binding of the HMG1 box containing upstream binding factor (UBF) and the selectivity factor (SL1 or TIF-IB) to the promoter region is required for Pol I recruitment (15,16). rRNA genes are transcribed as long precursors known as 45S pre-RNA which are then rapidly spliced into the 18S, 28S and 5.8S rRNA transcripts (17). Processed and modified rRNA transcripts are assembled into respective ribosomal subunits in the nucleolus (18,19).

The association between the nucleolus and cancer has long been known. Abnormal morphology of the nucleolus in cancer cells has drawn the attention of tumor pathologists since the 19th century. However, only recently has the molecular biology of rRNA synthesis and ribosome biogenesis in cancer cells begun to be explored.

CpG island methylation at the promoters of tumor suppressor genes is known to be an important factor in the formation and progression of many types of cancer (20). The promoter and transcribed regions of rRNA genes are rich in CG dinucleotide yet they are longer than regular 1 to 2 kb CpG islands (21). A limited number of studies analyzing the DNA methylation status of the rDNA promoter region in cancer have focused on the relationship between rDNA promoter methylation and the expression levels of rRNA genes.

Methylation at the 45S rDNA promoter region decreased the expression of rRNA genes in hepatocellular carcinoma (22) and in $CD34^+$ cells of patients with myelodysplastic syndromes (23). On the other hand, other studies have shown no relationship or a positive correlation between promoter methylation and rRNA transcription (24,25).

Although rRNA genes and particularly 18S RNA are frequently used in qRT-PCR as housekeeping genes, recent studies have shown that 18S is differentially expressed in breast tumor and normal samples (26-28). Furthermore, changes in the relative amount of spliced rRNA products from 45S have not been tested in the context of breast cancer.

CpG methylation of rDNA has been identified as a prognostic factor in ovarian, endometrial and breast cancer (29-31). A recent study also revealed that the 45S rDNA promoter as well as the 5' regions of 18S and 28S rDNA are hypermethylated in breast cancer tissues compared to paired normal tissues. Notably, methylation levels of these regions exhibited a correlation with nuclear grade and nuclear size values (32). However, none of the previous breast cancer studies examined the ratio of rRNA transcript levels and rDNA promoter methylation levels in tumors and normal tissues comparatively.

In the present study, we analyzed the methylation levels of the 45S rDNA promoter in breast cancer cell lines as well as in primary breast tumor tissues and matched normal samples. We also determined the expression levels of rRNA transcripts in the same samples in order to understand the role of rDNA promoter methylation on rRNA gene expression in breast cancer. We showed for the first time that the relative expression ratio of 18S and 5.8S rRNA was differentially modulated in tumors in comparison to adjacent normal tissues. In addition, relative rRNA expression in normal tissues was significantly and negatively correlated with the methylation status but this was not observed in the breast tumors. Furthermore, the high correlation between expression of rRNA transcripts in normal breast tissue was lost in tumors. Our findings suggest a significant dysregulation of relative rRNA expression in conjunction with promoter methylation.

Materials and methods

Cell culture, 5-aza-2'-deoxycytidine (5-AZA) and trichostatin A (TSA) treatments. MCF7, MDA-MB-453, MDA-MB-468, BT20, MDA-MB-231 and CAMA-1 breast cancer cell lines were grown in 10% fetal bovine serum (FBS) (HyClone, Thermo Scientific, USA) and 1% penicillin/streptomycin (P/S) supplemented with low glucose Dulbecco's modified Eagle's medium (DMEM) (both from HyClone). ZR-75-1 cell line was grown in 10% FBS, 1% P/S and 2 mM glucose (Sigma-Aldrich, USA) supplemented with RPMI-1640 medium (HyClone). BT-474 cell line was propagated in 10% FBS, 1% P/S and 10 μg/ml insulin (Sigma-Aldrich) supplemented with low glucose DMEM. MDA-MB-157 and MDA-MB-361 cell lines were grown in 10% FBS, 1 mM sodium pyruvate (Gibco, Invitrogen, USA) and 1% P/S supplemented with low glucose DMEM. HCC-1937 cell line was propagated in 10% FBS, 1% P/S and 1 mM sodium pyruvate supplemented with RPMI-1640 medium. MCF10A was grown in 10% FBS, 1% P/S, 10 μg/ml insulin, 20 ng/ml EGF and 0.5 mg/ml hydrocortisone (both from Sigma-Aldrich) supplemented with DMEM/Ham's F-12 (1:1) medium (Biochrome, Merck Millipore, Germany). SKBR-3 cell line was grown in 10% FBS and 1% P/S supplemented with McCoy's 5A medium (HyClone). CAL-51 cell line was propagated in 20% FBS and 1% P/S supplemented with high glucose medium. All cells were grown in 5% CO_2 and 95% air at 37˚C in a cell culture incubator. All cell lines except MCF-10A and CAL-51 cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). MCF-10A and CAL-51 were kindly provided by Assistant Professor Dr A. Elif Erson (Middle East Technical University). Short tandem repeat profiling was used to verify the authenticity of all cell lines.

A total of 10 breast cancer cell lines (MCF7, MDA-MB-231, MDA-MB-453, MDA-MB-468, BT-474, ZR-75-1, BT-20, MDA-MD-361, SKBR-3 and CAL-51) and one non-tumor-

igenic breast cell line (MCF-10A) were treated with 5-AZA or TSA. Cells seeded at a density of 750,000/100 mm were treated with either 5 μM 5-AZA (Sigma-Aldrich) or dimethyl sulfoxide (DMSO) (same amount used to solubilize 5-AZA). Drugs were changed every day along with the medium, and cells were collected on day 5. 400 nM TSA or DMSO (same amount used to solubilize TSA) was administered to the cells 24 h after cell plating, and the cells were collected after 48 h.

A total of 10 cell lines (MCF7, MDA-MB-231, MDA-MB-453, MDA-MB-468, BT-474, ZR-75-1, BT-20, SKBR-3, CAL-51 and MCF-10A) were treated with both 5-AZA and TSA (combination treatment). 5-AZA (5 μM) was added at the day of seeding and 400 nM TSA was added 72 h later in combination treatment (5-AZA+TSA); the cells were collected on day 5.

Patients and tissue samples. Primary breast tumors and matched normal tissues were obtained from 19 patients at Ankara Numune Research and Teaching Hospital (Table I). Clinical tissue samples were used with the approval of the Research Ethics Committee of Ankara Numune Research and Teaching Hospital, and consent was obtained from the patients according to the Helsinki Declaration.

Tissues acquired from patients during surgery were immediately frozen with liquid nitrogen and stored at -80˚C until RNA or DNA extraction was performed. Pathological examinations were carried out with hematoxylin and eosin staining. Only the tumor samples identified by pathological examination consisting of >80-90% tumor cells were included in the present study.

DNA extraction and bisulfite treatment. Genomic DNA was extracted from the breast cancer cell lines as well as the clinical breast cancer and matched normal tissue samples using the NucleoSpin Tissue DNA extraction kit (Macherey-Nagel, Germany) following the manufacturer's instructions.

Sodium bisulfite treatment of DNA, which converts unmethylated cytosine residues to uracil leaving methylated cytosine residues unaffected, was performed with 1 μg genomic DNA using the EpiTect Bisulfite kit (Qiagen, Germany). Elution was performed using 20 μl of elution buffer.

Bisulfite-specific PCR, gel extraction and bisulfite genomic sequencing. Bisulfite-converted DNA (1 μl) was amplified with *Taq* DNA polymerase (Fermentas, Thermo Scientific, USA) using bisulfite DNA-specific primers (22) targeting the 45S rDNA promoter (45S bisulfite sequencing forward and reverse primer sequences are listed in Table II). PCR products were extracted from the gel using the QIAquick Gel Extraction kit (Qiagen). Purified products were cloned into the pGEM-T Easy Vector using the pGEM-T Easy Vector system (Promega, USA). The transformation protocol was performed according to the pGEM-T Easy Vector system manual using competent *E. coli* DH5α. Bacteria were plated on LB-agar containing ampicillin (AppliChem, Germany), IPTG and X-Gal (both from Fermentas) and positive clones (five colonies from cell lines and 10 colonies from tissue samples) were randomly selected.

Small-scale isolation of plasmid DNA (mini-prep) was performed with the NucleoSpin Plasmid Isolation kit (Macherey-Nagel) according to the manufacturer's instructions. Plasmids containing the cloned inserts were confirmed with PCR using T7 and SP6 universal primers. The insert-containing plasmids were sequenced with SP6 primers using the dideoxy chain-termination method (Iontek, Turkey).

Methylation analysis. Raw bisulfite sequencing data were analyzed using the QUantification tool for Methylation Analysis) (QUMA), a web-based quantification tool for methylation analysis (http://quma.cdb.riken.jp) (33). Bisulfite conversion rates of raw sequencing data were determined by analyzing unconverted cytosine residues in non-CG sites. Clones with a bisulfite conversion rate of <95% were excluded. Clones from each sample were trimmed, aligned and displayed as lollipop graphs using QUMA.

RNA isolation and cDNA synthesis. The frozen tumor (4-5 slices for each sample) and normal (20-25 slices for each sample) tissue samples were cut into 60-μm sections and used for RNA isolation. Tissue samples were lysed in 1 ml TRI reagent RT (Molecular Research Center, USA) with a homogenizer and passed through a 21-gauge needle several times. After a 5-min incubation at room temperature, 50 μl of 4-bromoanisole (Molecular Research Center) was added/ml of TRI reagent. Tubes were vortexed for 15 sec and incubated at room temperature for 2-3 min. After incubation, the mixture was centrifuged at 12,000 x g for 15 min at 4˚C and then the aqueous phase was collected into a clean tube. Isopropanol (0.5 ml) was added to the aqueous phase/1 ml of TRI reagent used. The mixture was incubated at room temperature for 10 min and then centrifuged at 12,000 x g for 15 min at 4˚C to recover the RNA. The supernatant was removed, and the pellet was washed with 75% ethanol twice and centrifuged at 7,500 x g for 5 min at 4˚C. The pellet was air-dried and dissolved in diethylpyrocarbonate (DEPC)-treated H_2O. In order to avoid DNA contamination of the total RNA acquired from the tissue samples, DNase I treatment was performed with the Message Clean kit (GenHunter, USA) according to the manufacturer's instructions. Total RNA (500 ng) was used in random primed cDNA synthesis with the RevertAid First Strand cDNA Synthesis kit (Fermentas).

RNA isolation from the cell lines was performed using the NucleoSpin RNA II RNA isolation kit (Macharey-Nagel) following the manufacturer's protocol. Total RNA (1 μg) was used in random primed cDNA synthesis with the RevertAid First Strand cDNA Synthesis kit.

Real-time PCR. Real-time PCR was performed with primers targeting 45S ETS, 18S, 28S and 5.8 rRNA transcripts. All primer sequences are listed in Table II. Randomly primed cDNAs from both cell lines and frozen tissue samples were diluted in a 1/5 ratio. Diluted cDNA (1 μl) was used in every reaction containing 10 μl of DyNAmo SYBR-Green qPCR kit (Thermo Scientific) and 10 pmol of forward and reverse primers in a final volume of 20 μl. Thermal cycling conditions were as follows: initial denaturation for 5 min at 95˚C, 40 cycles of 30 sec at 95˚C, 30 sec at 60˚C and 30 sec at 72˚C followed by melting curve. All reactions were set as duplicates. The Stratagene Mx3005P Real-Time PCR System (Agilent, USA) was used for real-time PCR experiments.

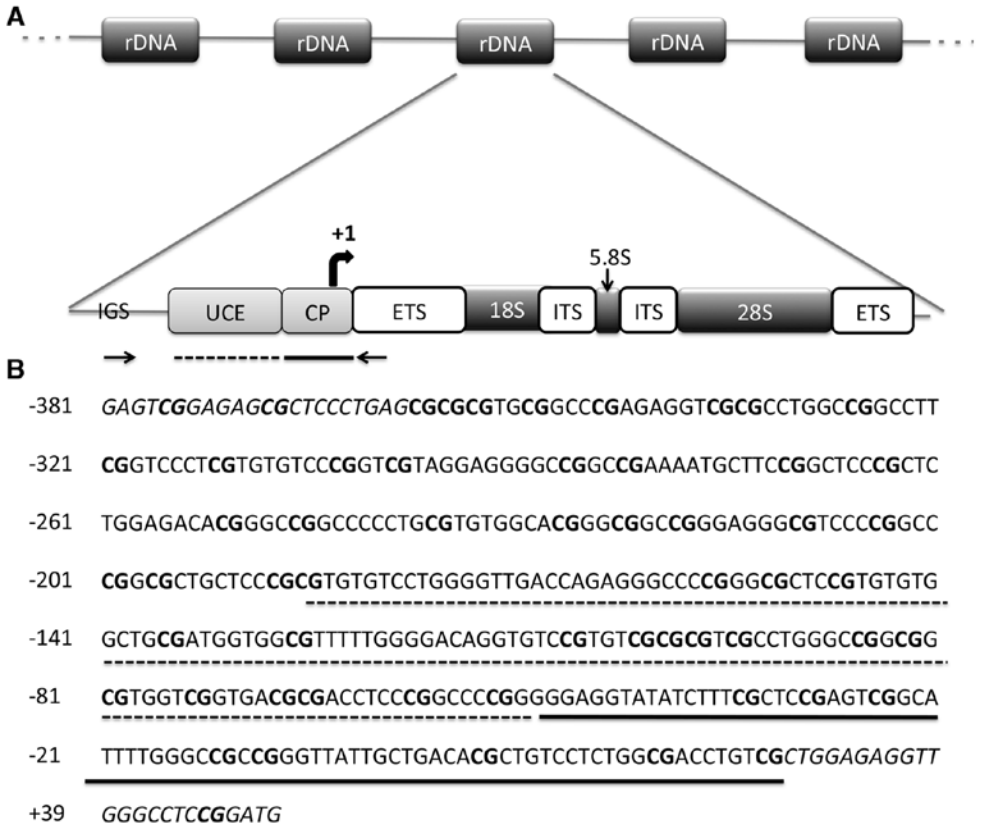

Figure 1. The human rDNA promoter. (A) Structural organization of human rDNA repeating unit. Bisulfite sequencing primers are shown by inward pointing arrows. IGS, intergenic spacer; UCE, upstream control element; CP, core promoter; ETS, external transcribed spacer; ITS, internal transcribed spacer. (B) Sequence of the amplified PCR product, spanning the upstream core element (marked with dashed line) and the core promoter (straight line). There are 54 CpGs in the 434-bp region analyzed; the transcription start site is indicated with a curved arrow. Primers are shown with italicized characters and CG-dinucleotides are indicated with bold characters. rDNA, ribosomal DNA.

The relative expression levels of rRNAs were evaluated using log2 ($2^{\Delta Ct}$) calculation. TBP was used as the reference gene both for cell lines and clinical tissue samples to assess the amount of cDNA. The geometric mean of the rRNA expression values (GM-rRNAs) (18S, 28S, 5.8S and 45S ETS) was also used as a reference gene in tissue samples in order to understand the relative changes of rRNAs with respect to each other.

Statistical analysis. Raw bisulfite sequencing data were aligned, trimmed and quality checked using QUMA. Lollipop graphs and pie charts of the methylation status were also generated using QUMA (33). The Wilcoxon matched pairs signed rank test was used to assess both sample-wise and CpG-wise significant methylation differences between breast tumor and matched normal samples. Significant expression differences were determined using the paired t-test. Correlations between 45S rDNA promoter methylation and rRNA expression levels, as well as rRNA transcripts with each other were analyzed using Spearman correlation.

The association of rDNA promoter methylation and rRNA expression with clinical variables was evaluated with Spearman correlation. $p<0.05$ was accepted as statistically significant for all statistical analyses. All statistical analyses of methylation and expression data were performed using IBM SPSS software version 21.0 or GraphPad Prism 6.0.

Results

45S rDNA promoter is highly methylated in breast cancer cell lines. To identify the methylation levels of the 45S rDNA promoter region in breast cancer *in vitro*, we performed bisulfite genomic sequencing for the 45S rDNA promoter region in 10 breast cancer cell lines and a non-tumorigenic breast cell line (MCF-10A). Bisulfite sequencing primers were obtained from a previous study (22), which amplified a 434-bp region spanning two important elements: UCE and the core promoter (34) (Fig. 1B). Isolated DNAs from the cell lines were treated with sodium bisulfite reagent, allowing for integration of epigenetic information (DNA methylation) into genetic information. Five randomly selected clones from each cell line were sequenced, aligned and analyzed using QUMA (33). All cell lines, including a non-tumorigenic breast cell line, MCF-10A, exhibited very high levels of methylation (varying between 74 and 96%) in their 45S rDNA promoter regions (Fig. 2).

Breast tumors are heavily methylated compared to their normal matched tissues in the 45S rDNA promoter region. We analyzed 19 breast tumor and matched normal frozen tissues (Table I) using the same bisulfite genomic sequencing method to test whether methylation levels of the 45S rDNA promoter region in patient samples were similar to those of the cell lines. Ten randomly selected clones were sequenced,

Table I. Clinicopathological characteristics of the patients.

Patient no.	Age (years)	ER	PR	Diagnosis	Path lymph node	Grade	Clinical grade	DM	-DM month
113	63	+	-	IDC	+	1	Grade 3B	+	20
115	57			Papillary carcinoma	-	3	Grade 2A	-	
96	39	-	+	IDC	-	2	Grade 2A	-	
116	74	-	-	IDC	+	2	Grade 2A	-	
137	42	-	+	Medullary	-	3	Grade 2B	-	
146	49	+	+	ILC	+		Grade 2B	-	
148	70	+	-	IDC	+	2	Grade 3A	+	15
154	32	-	-	IDC	+	3	Grade 3B	-	
159	30	-	+	Metaplastic	-	2	Grade 2A	-	
161	41	-	-	IDC	-	2	Grade 2B	+	47
164	74	+	+	IDC	-	2	Grade 2B	-	
166	55	-	+	IDC	+	2	Grade 2A	-	
168	44	-	+	IDC	+	2	Grade 2B	-	
170	60	-	-	IDC	+	2	Grade 3B	-	
176	49	+	+	IDC	+	2	Grade 2A	-	
177	47	-	+	IDC	+	2	Grade 3A	+	48
181	44	-	-	IDC	+	2	Grade 1	-	
133[a]									
173[a]									

[a]Patients with missing information. ER, estrogen receptor status; PR, progesterone receptor status; IDC, infiltrating ductal carcinoma; Path lymph node, pathological lymph node status; DM, distant metastasis status.

Table II. Primers used in the present study.

Primer		Sequence	Product size (bp)	Efficiency value
45S bisulfite sequencing primers	Forward	5'-GAGTCGGAGAGCGCTCCCTGAG-3'	434	-
45S bisulfite sequencing primers	Reverse	5'-CTGGAGAGGTTGGGCCTCCG-3'		
18S rRNA	Forward	5'-AAACGGCTACCACATCCAAG-3'	154	1.95
18S rRNA	Reverse	5'-CCTCCAATGGATCCTCGTTA-3'		
28S rRNA	Forward	5'-CAGGGGAATCCGACTGTTTA-3'	151	1.85
28SS rRNA	Reverse	5'-ATGACGAGGCATTTGGCTAC-3'		
5.8S rRNA	Forward	5'-CTCTTAGCGGTGGATCACTC-3'	155	2.00
5.8S rRNA	Reverse	5'-GACGCTCAGACAGGCGTAG-3'		
45S ETS	Forward	5'-CGATCTGAGAGGCGTGCCTT-3'	87	1.93
45S ETS	Reverse	5'-GGCAGCGCTACCATAACGGA-3'		
TBP	Forward	5'-TGCACAGGAGCCAAGAGTGAAAT-3'	134	2.20
TBP	Reverse	5'-CACATCACAGCTCCCCACCA-3'		

rRNA, ribosomal RNA; ETS, external transcribed spacer.

aligned and analyzed from each of the breast tumor and normal pairs using QUMA (33). We used the Wilcoxon signed rank test for testing the paired differences instead of the Mann-Whitney U test offered by the QUMA tool. Our results revealed that 13 out of 19 (68%) breast cancer tissue samples had higher methylation levels of the 45S rDNA. On the other hand, three samples showed significantly higher methylation levels in normal samples compared to their tumor pairs, whereas there was no significant difference between promoter methylation levels in breast tumor and matched normal tissues

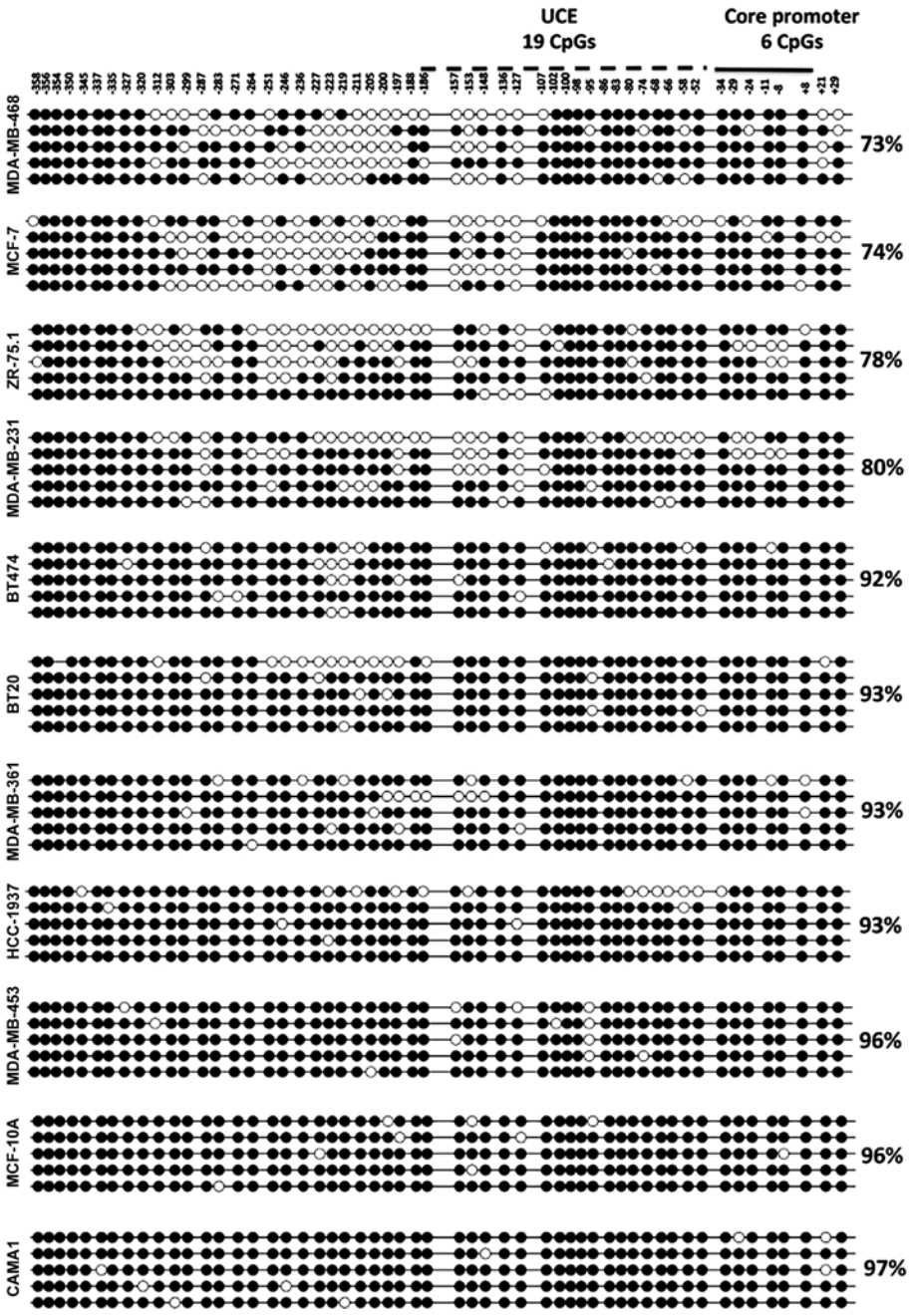

Figure 2. The methylation status of the 45S rDNA promoter in breast cancer cell lines and a non-tumorigenic cell line. A total of 54 CpGs in a region spanning -381 to +53 bp was analyzed by the bisulfite sequencing method. Each row corresponds to the sequence analysis of one clone and each column represents the CpG positions. The filled and empty circles stand for methylated and unmethylated CpGs, respectively. Average methylation percentages of the clones for each sample are indicated at the right of the graph. UCE, upstream control element.

in the remaining three samples (Fig. 3A). Normal samples were not fully unmethylated and instead showed a mosaic methylation pattern, a relatively common observation for human rDNA promoters (22). Methylation patterns of tumor and normal pairs showed a significant correlation with each other. To test whether this correlation was due to patient-specific methylation of rDNA promoters, we performed a correlation analysis between randomly selected tumor and normal samples; and these showed similar degrees of correlation (data not shown). In addition, the analysis of individual CpGs in tumor and normal pairs revealed significant methylation levels in different CpGs identified with the Wilcoxon matched pairs signed rank test (Fig. 3B).

No significant correlation was identified between rDNA promoter methylation levels and patient clinical variables.

Expression levels of rRNA transcripts in breast cancer. TBP, GAPDH and ACTB have been used as reference genes to determine rRNA expression levels in several studies (23,24,35) but these RNA polymerase II (Pol II) transcribed genes are variably expressed in numerous types of cancer (36,37). However, several studies advise against using rRNA levels to determine mRNA levels (26,27). Accordingly, using mRNA levels to normalize rRNA levels may have a similar drawback. Herein, we propose that GM-rRNA, the geometric mean of expression from an rRNA pool (18S, 28S, 5.8S and 45S ETS)

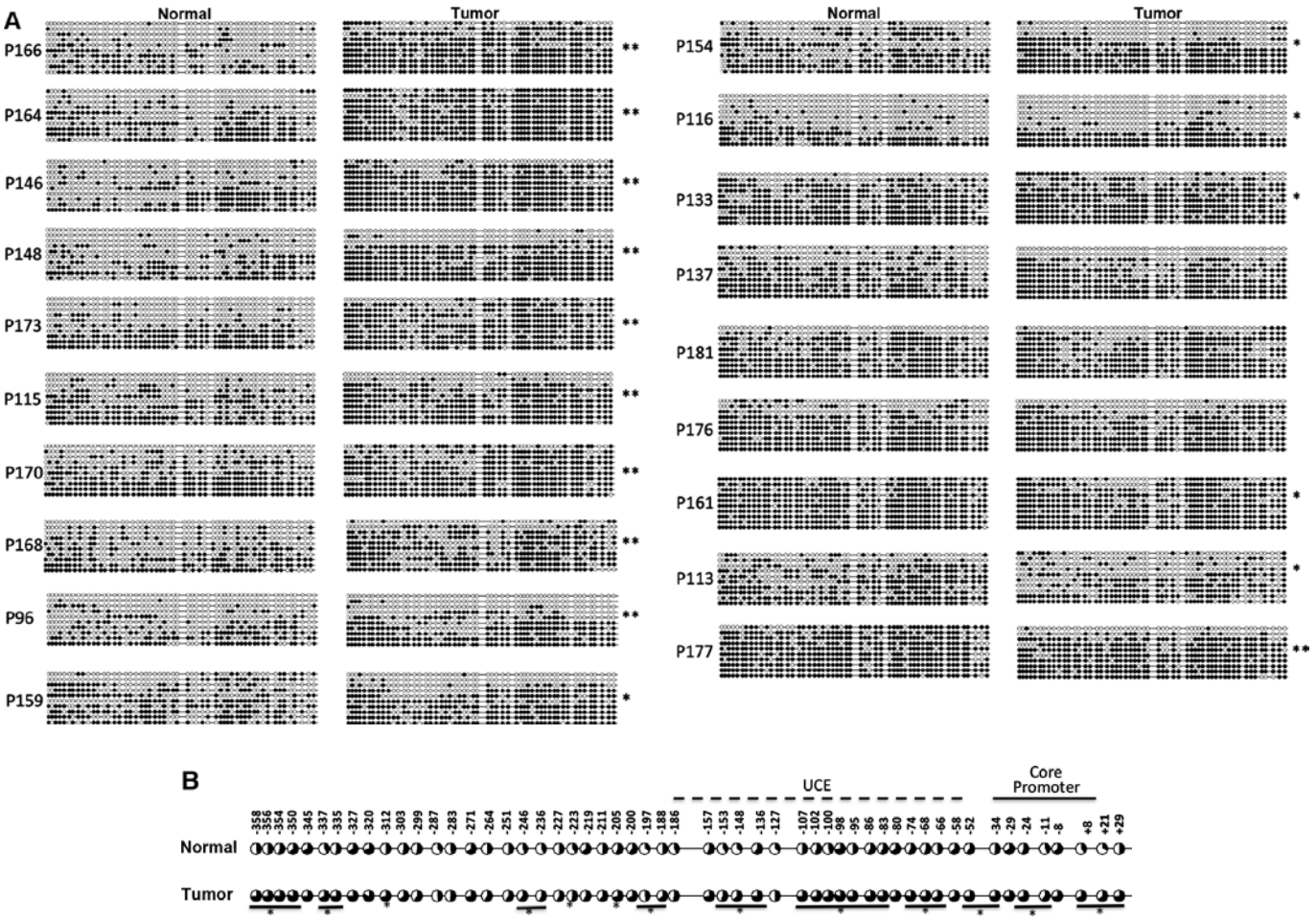

Figure 3. Methylation status of the 45S rDNA promoter region in clinical breast cancer and matched normal tissues. (A) The methylation status of each CpG dinucleotide in the region spanning -381 to +53 bp was analyzed in 19 breast cancer and normal pairs. Ten randomly selected clones were sequenced from each sample. Significant ($^{**}p<0.0001$ and $^{*}p<0.05$) methylation difference between tumor and matched normal samples. (B) Methylation levels of every CpG in all tumor clones and normal clones were compared to determine differentially methylated CpGs. Methylation percentages of every CpG are presented as a pie chart. Significantly ($^{*}p<0.05$) methylated CpGs in tumor samples compared to normal samples.

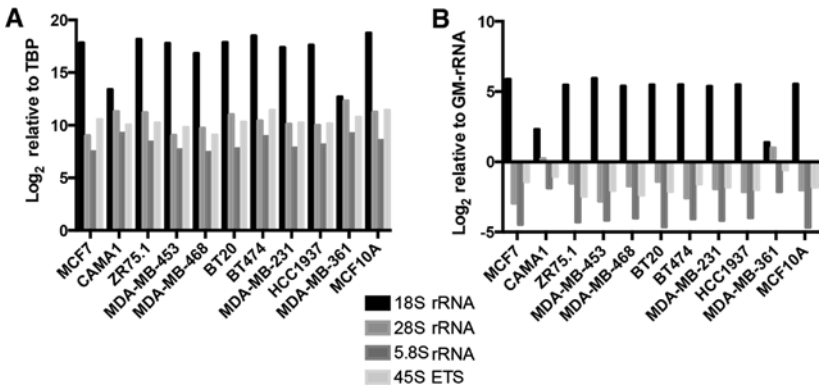

Figure 4. rRNA transcript levels in breast cancer cell lines (A) Relative expression levels of rRNA transcripts in the breast cancer cell line panel, normalized to the TBP transcript level. (B) Relative proportions of rRNA transcripts in the breast cancer cell line panel, normalized to GM-rRNA.

synthesized by Pol I, can be used to analyze relative changes in rRNAs with respect to each other between tumor and normal samples, as well as in cell lines. We performed our analyses using both TBP and GM-rRNA normalization to test the effect of normalization on expression changes in rRNA transcripts.

Expression of rRNA transcripts is highly variable in breast cancer cell lines. It is known that promoter DNA methylation has a repressive effect, particularly on Pol II transcribed genes in cancer (20,38) and increased methylation levels are implicated in decreased levels of rRNA transcription (22,23). Thus, we hypothesized that rRNA transcription levels may also be downregulated in these breast cancer cell lines with a hypermethylated 45S rDNA promoter. Total RNA was isolated and tested in cell lines with qRT-PCR using primers targeting

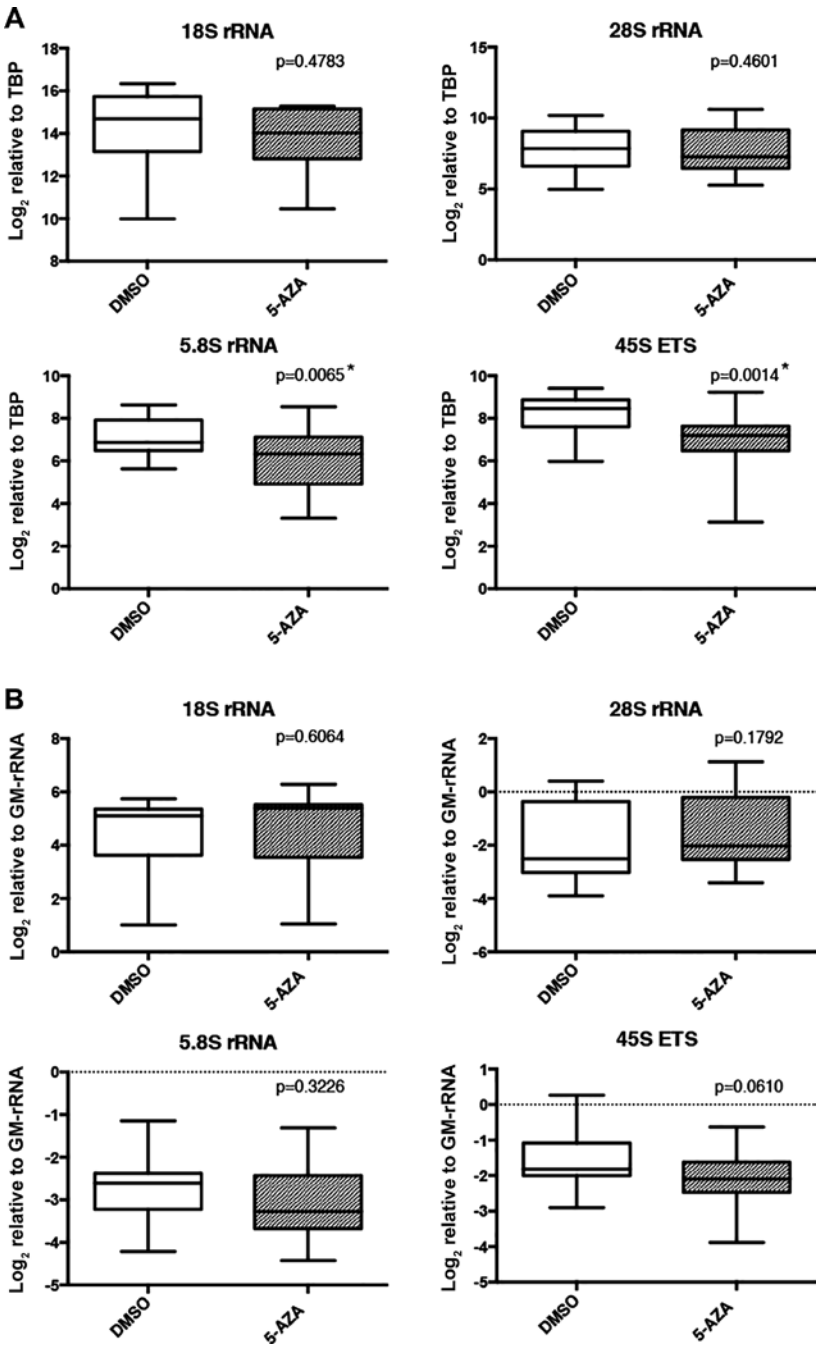

Figure 5. rRNA transcript levels in 5-AZA- and DMSO (control)-treated cell lines. (A) Expression levels of rRNA transcripts in 5-AZA- or DMSO-treated breast cell lines. Expression levels of rRNA transcripts were normalized to the TBP transcript level. (B) Relative proportions of rRNA transcripts in the 5-AZA- or DMSO-treated breast cell lines. Expression levels of rRNA transcripts were normalized to GM-rRNA. Box plots indicate relative expression levels of rRNA transcripts in the DMSO- and 5-AZA-treated cell lines. Significant ($^{*}p<0.05$) rRNA expression differences between DMSO- and 5-AZA-treated cell lines were determined using the paired t-test. rRNA, ribosomal RNA; 5-AZA, 5-aza-2'-deoxycytidine; DMSO, dimethyl sulfoxide; GM-rRNA, geometric mean of rRNA expression values.

Pol I products 18S, 28S, 5.8S and 45S external transcribed spacer (ETS) region.

All of the rRNA transcripts were expressed at varying levels among the cell lines when normalization was performed with the TBP gene (Fig. 4A). Similar results were obtained when we used GM-rRNA to determine changes in the ratio of the rRNA transcripts (Fig. 4B).

Epigenetic drugs 5-AZA and 5-AZA+TSA modulate expression of rRNA transcripts. To further establish the relationship between 45S rDNA promoter methylation and rRNA expression, we used the hypomethylating agent, 5-AZA, which prevents DNA methylation by inhibiting DNA methyltransferases (39) and leads to increased RNA transcription. TBP normalized expression levels of 5.8S and 45S ETS transcripts were significantly decreased upon 5-AZA treatment (Fig. 5A). However, proportion of rRNA transcripts did not exhibit significant differences between 5-AZA- and DMSO-treated samples (normalization with GM-rRNA) (Fig. 5B).

TSA is a non-specific histone deacetylase (HDAC) inhibitor. TSA treatment of cells affects the acetylation status of

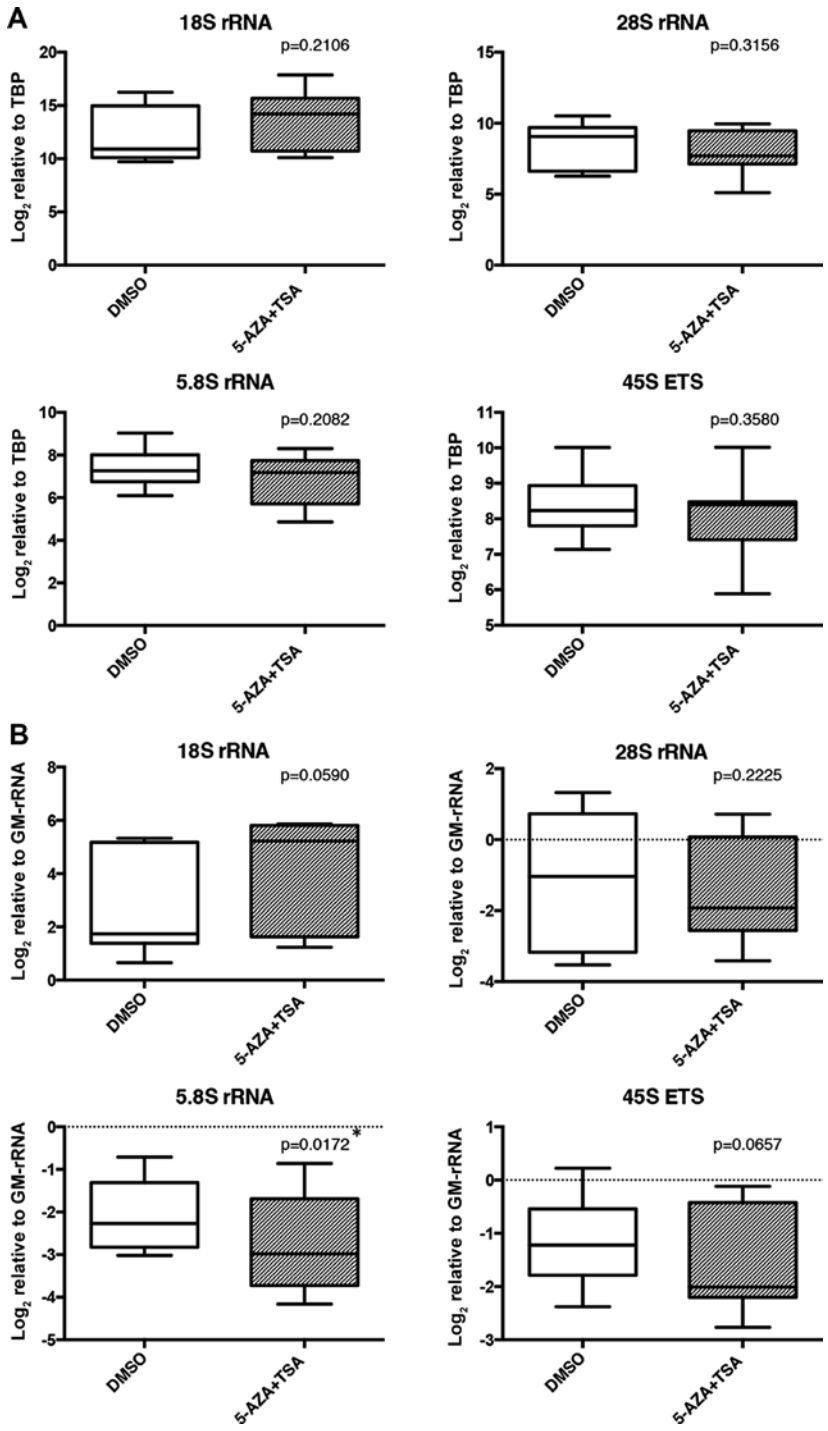

Figure 6. rRNA transcript levels in the 5-AZA+TSA- and DMSO (control)-treated cell lines. (A) Expression levels of rRNA transcripts in the 5-AZA+TSA- or DMSO-treated breast cell lines. Expression levels of rRNA transcripts were normalized to the TBP transcript level. (B) Relative proportions of rRNA transcripts in the 5-AZA+TSA- or DMSO-treated breast cell lines. Expression levels of rRNA transcripts were normalized to GM-rRNA. Box plots indicate relative expression levels of rRNA transcripts in the DMSO- and 5-AZA+TSA-treated cell lines. Significant ($^{*}p<0.05$) rRNA expression differences between DMSO- and 5-AZA+TSA-treated cell lines were determined using the paired t-test. rRNA, ribosomal RNA; 5-AZA, 5-aza-2'-deoxycytidine; TSA, trichostatin A; DMSO, dimethyl sulfoxide; GM-rRNA, geometric mean of the rRNA expression values.

H3 and H4 and thus TSA indirectly upregulates gene expression by dispersion of the chromatin structure (39). Therefore, TSA was used to determine whether other mechanisms (such as histone acetylation) are involved in the rRNA synthesis besides DNA methylation. TSA treatment alone did not significantly alter the expression levels or the relative proportions of the rRNA transcripts when normalized with TBP or GM-rRNA, respectively (data not shown). Treatment with 5-AZA and TSA together (5-AZA+TSA) did not significantly affect the TBP normalized expression levels of the rRNA transcripts (Fig. 6A). However, the 5.8S proportion of rRNAs was significantly decreased in the 5-AZA+TSA-treated samples compared to the DMSO-treated samples (Fig. 6B).

Relative expression levels of 18S and 5.8S transcripts are altered in breast tumors. Next, we tested whether increased levels of methylation of the 45S rDNA promoter in tumor

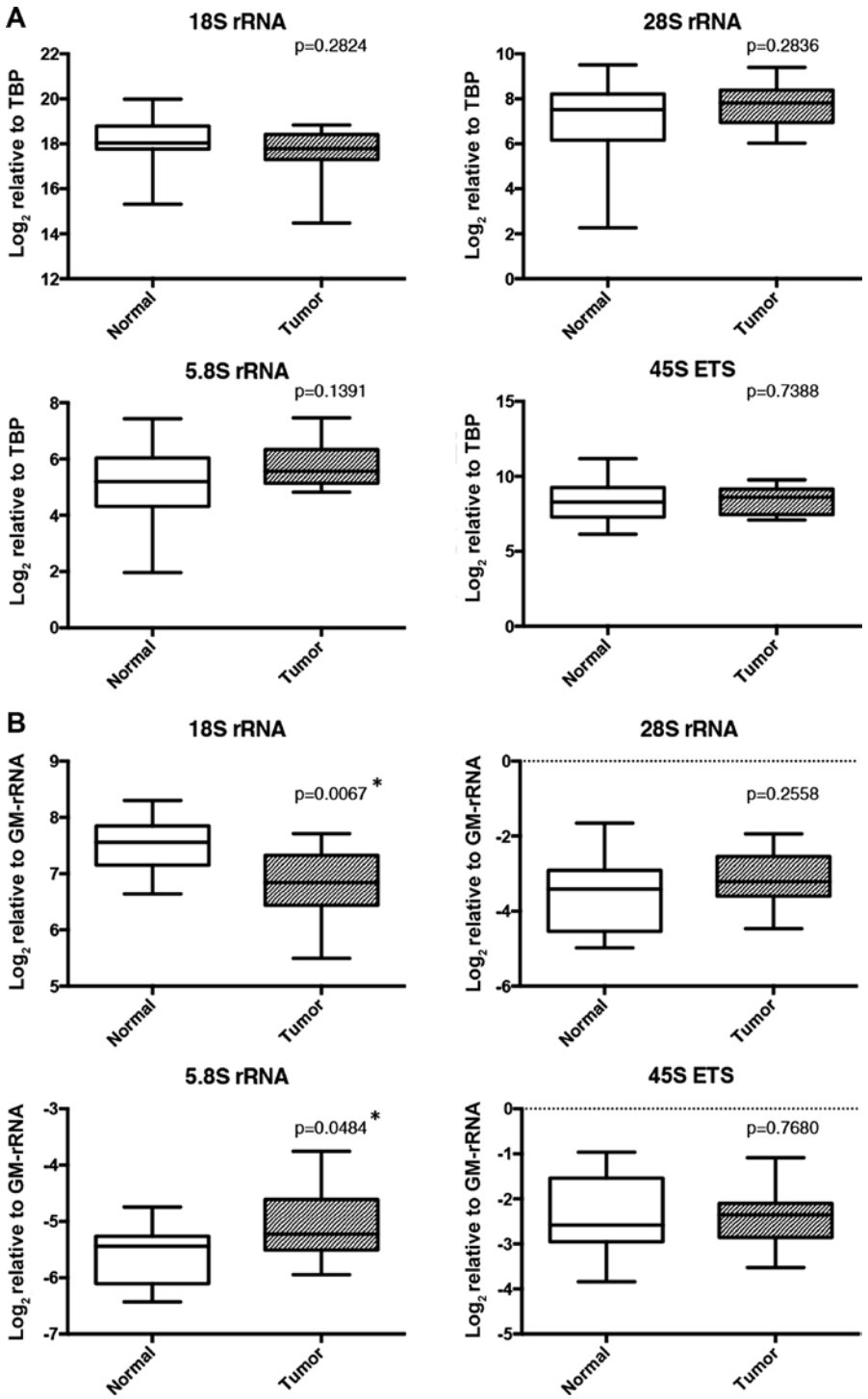

Figure 7. Expression analysis of rRNA transcripts in clinical breast cancer and normal pairs. Relative expression levels of rRNA transcripts are shown with box plots for the clinical breast cancer and matched normal samples. (A) Expression levels of rRNA transcripts were normalized to the TBP transcript level. (B) Expression levels of rRNA transcripts were normalized to GM-rRNA. Significant ($^{*}p<0.05$) expression differences between normal and tumor samples were determined using paired t-test. rRNA, ribosomal RNA; GM-rRNA, geometric mean of the rRNA expression values.

samples led to repressed expression levels of rRNA transcripts. RNA isolation was performed from the same tissue samples used in the methylation analysis (only 14 of 19 paired tissue samples had enough tissue for RNA isolation). Expression levels, when analyzed using TBP as a reference gene, did not differ between the tumors and corresponding adjacent normal tissues for any of the rRNA transcripts (Fig. 7A). However, when normalized with GM-rRNA, the proportion of 18S was significantly decreased in the tumor samples whereas that of 5.8S rRNA was significantly increased (Fig. 7B).

rRNA transcripts are expressed independent from 45S rDNA promoter methylation levels in breast cancer cell lines. There was no significant correlation of rDNA methylation levels with rRNA expression levels or rRNA ratios in the breast cancer cell lines or in the MCF10A cells (non-tumorigenic cell line).

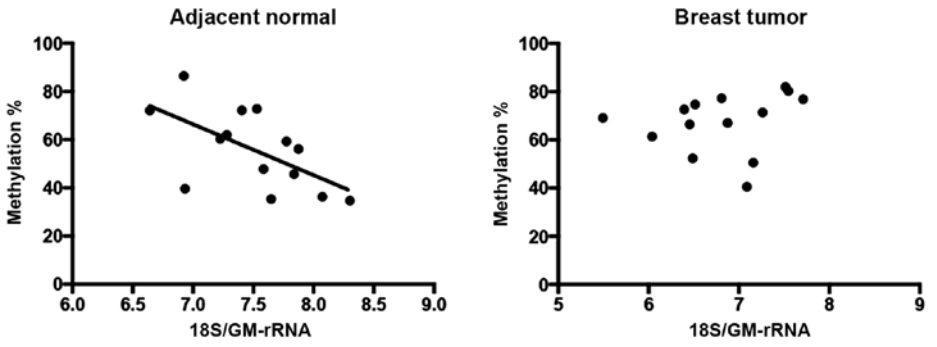

Figure 8. Correlation graph of 45S rDNA promoter methylation levels and 18S rRNA ratios in breast tumor and matched normal tissue samples. A significant correlation (*p<0.05) was identified using Spearman correlation analysis.

Table III. Correlation analysis between rRNA transcripts in tumor and normal samples.

Sample	Transcript	28S rRNA	5.8S rRNA	45S ETS
Normal	18S rRNA	0.837 (p<0.01)[a]	0.824 (p<0.01)[a]	0.818 (p<0.01)[a]
	28S rRNA		0.833 (p<0.01)[a]	0.674 (p=0.012)[a]
	5.8S rRNA			0.57 (p=0.033)[a]
Tumor	18S rRNA	0.042 (p=0.887)	-0.051 (p=0864)	0.288 (p=0.318)
	28S rRNA		0.521 (p=0.056)	0.349 (p=0.221)
	5.8S rRNA			0.543 (p=0.045)[a]

Significant correlation ([a]p<0.05) was identified using Spearman correlation coefficients (r) and p-values. rRNA, ribosomal RNA; ETS, external transcribed spacer.

rRNA expression levels, as well as relative rRNA proportions in cell lines were found to be independent of their promoter methylation levels (data not shown). These results indicate that rRNA transcripts were expressed even in the presence of heavy methylation at the 45S rDNA promoter.

Correlation between 45S rDNA promoter methylation and 18S rRNA expression is disrupted in breast tumors. Next, Spearman's correlation analysis was performed to test whether the 45S rDNA promoter methylation levels in the breast tumor and matched normal samples were correlated with either rRNA expression levels or rRNA proportions in the rRNA pool. When rRNA expression levels were normalized with TBP expression, no correlation between expression and methylation was observed (data not shown). The increase in methylation of the rDNA promoter levels in the normal samples was inversely correlated with the 18S rRNA/GM-rRNA expression ratio (Spearman r=-0.6571 and p=0.0128), yet promoter hypermethylation did not exhibit any correlation with 18S rRNA/GM-rRNA ratios in the tumor samples (Fig. 8).

Furthermore, Ct values of rRNAs transcribed from the 45S rDNA promoter were highly correlated with each other in the normal samples, yet this correlation was lost in the tumor samples (Table III).

rRNA expression levels were correlated with the grade of breast cancer. Expression of the 45S ETS region (TBP normalization) in tumor samples (n=11) was positively correlated with the grade of breast cancer (Spearman r=0.650, p=0.03). Additionally, the 28S/GM-rRNA ratio of tumor samples was also positively correlated (Spearman r=0.725, p=0.012) while the 18S/GM-rRNA ratio was negatively correlated with the grade (Spearman r=-0.714, p=0.014).

Discussion

A high expression of rRNA transcripts characterizes cancer cells but only a few studies have analyzed the expression of 18S rRNA in breast cancer tissues and matched normal samples. Previous studies have mostly focused on testing whether rRNA genes are suitable as reference genes (26,27). One of these studies found that 18S rRNA was expressed at lower levels in breast tumors compared to matched normal tissues in contrast to the general acceptance of higher rRNA expression in tumors (26). None of the studies, however, investigated whether the expression difference between breast cancer and normal pairs was due to rDNA promoter methylation or whether the ratios of spliced products of the 45S precursor were differentially expressed between tumor and normal pairs in breast cancer.

DNA methylation is a well-known phenomenon that inactivates transcription by interfering with Pol II binding to the

promoter (40); since Pol I and Pol II share common features and transcription factors (41-43), DNA methylation in the 45S rDNA promoter may have a similar effect on expression of rRNA genes.

We used both breast cancer cell lines and clinical breast cancer tissues to investigate the methylation levels of rDNA promoters and expression differences of rRNA transcripts, as well as their relationship with each other in breast cancer. We found that breast cancer cell lines were hypermethylated (74-96% methylation) at the 45S rDNA promoter region (Fig. 2). High methylation levels of rDNA promoters are frequently identified in many transformed cell lines such as Jurkat, CEM, HeLa, KB (44), NIH 3T3 (45) and HEK293 (46). A genome-wide analysis of aberrant methylation changes with aging in mammals identified the rDNA gene locus in which methylation levels increased age-dependently in both spermatozoa and rat liver cells (47). If the rDNA locus is sensitive to accumulating random methylation over time, the high methylation levels found in breast cancer cell lines may be explained with long-continued culturing, a common characteristic of cancer cell lines.

We could not identify two populations of alleles (one population with a hypermethylated promoter, the other population with a hypomethylated promoter; Fig. 2) in breast cancer cell lines as proposed earlier by other studies (22-25). Considering the repetitive nature of rRNA genes, this result may be due to the low number of clones (5 clones) analyzed for each cell line. Breast cancer cell lines with hypermethylated 45S rDNA promoters did not repress or alter the ratio of rRNA transcripts (Fig. 4). rRNA expression levels and proportions of rRNA transcripts were found to be independent of the rDNA promoter methylation levels; this result may indicate that the methylation of the 45S rDNA promoter may not be solely responsible for rRNA expression or proportion changes in breast cancer cell lines. Completely methylated (*Xenopus leavis* sperm DNA) and unmethylated rDNA constructs were found to be transcribed with equal efficiency when transfected to *Xenopus leavis* oocytes (48). This is consistent with our results in which expression of rRNA transcripts was found to be relatively independent of their rDNA promoter methylation levels.

Upon 5-AZA treatment of breast cancer cell lines, expression of some forms of mature rRNA transcripts (significant for 5.8S RNA and 45S ETS) unexpectedly decreased compared to the DMSO-treated group when normalized with TBP (Fig. 5A), while rRNA proportions were not significantly altered within the rRNA pool (Fig. 5B). A recent study demonstrated that the loss of CpG methylation of the rDNA promoter (either with 5-AZA treatment or DNMT knockout) regions caused cryptic transcription of RNA polymerase II from 45S rDNA promoters. Cryptic transcription from rDNA promoters by RNA polymerase II significantly correlates with reduced modification and processing of rRNA transcripts (25). Loss of CpG methylation at rDNA promoter regions in 5-AZA-treated breast cancer cell lines may be affected by this cryptic RNA polymerase II transcription, which explains the downregulation of 5.8S and 45S ETS rRNA transcripts in the 5-AZA-treated cell lines. This mechanism, which acts as a negative feedback loop, may be a strategy developed by cells to achieve a balanced expression of rRNA transcripts and prevent energy loss in cells in the absence of CpG methylation.

Gene expression is usually regulated by a combination of DNA methylation, histone modifications and the activities of chromatin remodeling complexes (49). TSA treatment alone and 5-AZA+TSA treatment did not result in a significant increase in rRNA levels (Fig. 6A), yet the 5.8S rRNA ratio was decreased in the 5-AZA+TSA treated group (Fig. 6B). rRNA transcription may predominantly be regulated by other transcriptional or post-transcriptional mechanisms rather than epigenetic regulatory processes (at least DNA methylation and histone acetylation) in breast cancer cell lines.

The discrepancy between rRNA expression levels (TBP normalization) and rRNA ratios (GM-rRNAs) in the 5-AZA- and 5-AZA+TSA-treated groups, compared to their controls, may result from expression changes of TBP upon drug treatment. The change may also be due to some indirect effect of the drugs through other genes as both drugs affect several other genes along with the rDNA genes.

Further analysis of rDNA promoter methylation with the Wilcoxon matched-pairs signed rank test in tissues showed that most of the breast tumors (13/19) had significantly higher methylation levels than their normal counterparts (Fig. 3A). Our findings indicated similarities of methylation patterns within tumors and between tumor and adjacent normal tissues, indicating tissue- and/or locus-specificity of methylation. Methylation analysis of the same region in different tissues and types of cancer may reveal whether the methylation pattern of this region is tissue-specific, cancer-specific or neither, since different tissues display different methylation patterns at different loci (50).

Previous studies have identified rDNA methylation as a prognostic factor in some cancer types (31,51) including breast cancer (30). However, the correlation analysis of methylation levels with clinicopathological characteristics (as described in Table I) of the patients used in the present study did not show any significance (data not shown). Similarly, a breast carcinoma study on 45 paired breast tumor and normal samples could not identify any significant associations between methylation of rDNA promoters, 5' regions of 18S and 28S rDNA and ER, PR, grade and other clinicopathological features, except nuclear size and grade (32). The use of larger sample sizes may help clarify the clinical importance of rDNA methylation in breast cancer. rRNA transcript expression (45S/TBP and 28S/GM-rRNA) in tumors on the other hand showed a positive correlation with the grade of the tumor. Nuclear pleomorphism is one of the criteria used in the grading of breast cancer, which includes classification of tumors by the size and the shape of the nucleoli (52). An increase in the expression or the ratio of rRNA transcripts may be responsible for the abnormal appearance of nucleoli in higher grade breast cancer samples.

Expression analyses of rRNA transcripts with TBP and GM-rRNA normalizations revealed different sides of the same coin. While the former enables measurement of expression with respect to a stable mRNA pool, use of the latter reflects changes in the relative ratios of rRNA transcript levels. Our data accordingly revealed that 5.8S and 45S rRNA transcripts were downregulated in the 5-AZA-treated cell lines with TBP normalization (Fig. 5A). However, 5-AZA treatment did not affect rRNA ratios in the cell lines (Fig. 5B). Unlike the cell line results, expression analysis of rRNA genes in the breast tumor and matched normal tissues showed no significant

difference when normalized with TBP (Fig. 7A) while 18S and 5.8S rRNAs were proportionally altered in the breast tumor tissue samples (Fig. 7B). As stated earlier, the disparity between TBP and GM-rRNA normalizations could be due to the fact that they analyze separate aspects of rRNA expression.

A recent study with results supporting our findings demonstrated that two rRNA forms (5.8S and 45S precursors) were overexpressed (TBP normalization) in clinical prostate cancer tissues compared to matched-benign tissues. However, methylation levels of the 45S rDNA promoter in the same prostate tumor and normal pairs were not significantly different (24). Another study showed that loss of CpG methylation at the rDNA promoter surprisingly decrease rRNA transcript levels by disrupting rRNA synthesis and processing via activating cryptic transcription of rRNA genes by Pol II (25).

Different studies have used various reference genes, TBP and ACTB being among the most common, to determine rRNA expression levels in cancer (23,24,35). We used TBP (a RNA polymerase II transcribed gene) to normalize rRNA gene expression and found no significant differences between the breast tumor and matched normal samples. ACTB also failed to identify such differences in our cohort (data not shown). We propose that the geometric mean (GM) of rRNAs synthesized by RNA polymerase I (18S, 5.8S, 28S and 45S ETS) to normalize rRNA expression can be used to detect relative changes in rRNAs with each other. GM-rRNA may be less prone to changes than Pol II gene transcripts since it is calculated from the rRNA transcripts synthesized by Pol I. When GM-rRNA was used for normalization, 5.8S and 18S rRNA expression levels were significantly upregulated and downregulated, respectively, in the tumor samples compared to the normal pairs (Fig. 7B). Our data indicate that the proportion of 18S and 5.8S rRNA in the total rRNA pool changed in the opposite direction while total rRNA levels may be relatively constant. We found that methylation levels of normal samples (which exhibit mixed methylation patterns) showed a negative correlation with the 18S rRNA/GM-rRNA expression level but this correlation was disrupted in the tumor samples (Fig. 8). As far as we are aware, this is the first study to show that the methylation status may be reflected in the expression of one or more rRNA transcripts but not all.

Some forms of polycistronic mRNAs and miRNAs that are expressed from the same promoter have been shown to be post-transcriptionally regulated and exist at different levels in plants (53,54). The SNRPN-SNURF gene, possessing a biscistronic structure and sharing a common promoter, for example, is differentially expressed, as identified by both northern blot and microarray analysis in mammalian cells (55,56). As reported in other studies, genes that are expressed from the same promoter can be differentially expressed by other mechanisms apart from the effect of basal transcription machinery.

The maturation of ribosomes is a complex process assisted by multiple factors (~200) that need to be orchestrated in harmony (57,58). Alteration in the methylation levels of rDNA promoters may have an effect on rRNA stabilization, which could leads to this non-proportional change in rRNA transcripts. Moderate levels of rDNA promoter methylation (as observed in normal samples) may still be regulating 18S rRNA levels but this correlation is disrupted in tumor samples, possibly due to the high methylation levels found in the 45S rDNA promoter. Another possibility is that methylation levels can indirectly affect splicing, post-transcriptional modifications and stabilization of rRNA transcripts (25). The fact that normal samples showed a higher correlation between rRNA transcript expression while this correlation was disrupted in tumor samples supported this possibility (Table III), indicating that the methylated promoter of 45S rDNA in tumors may have an effect on the processing of rRNAs.

Since rRNA processing and modification are largely dependent on snoRNAs, any change in snoRNA levels globally may be reflected in the rRNA ratios. U50 is a box C/D snoRNA that is required for 2'-O-methylation of two specific positions in the 28S rRNA and was shown to be altered by somatic rearrangements, mutations and deletions in prostate cancer (59), breast carcinoma (60), B-cell lymphoma (61) and colon cancer (62). Another snoRNA, GAS5, was also found to be downregulated in breast cancer (63). Increased methylation levels of rDNA promoters and their effect on rRNA modification and processing need to be better analyzed in future studies.

In conclusion, we found that rRNA transcripts were expressed independently of the hypermethylated 45S rDNA promoter region in breast cancer cell lines. However, the 18S rRNA/GM-rRNA ratios were significantly correlated with methylation levels in the normal samples but not in the tumor samples. Promoter methylation of rDNA promoters appears to have a different role than regulating the expression of rRNA transcripts in breast cancer. It may be used as a mechanism to stabilize and protect these essential genes under any circumstances. rDNA repeats have been proposed to be responsible for genomic stability (64) and hypomethylation of rDNA promoter has been implicated in decreased genomic stability (65,66). Increased methylation of the rDNA promoter in tumor cells may be an indicator of the tumor cell effort to restore impaired genomic stability. Future research is needed to evaluate the cause of relative expression changes observed among rRNA transcripts in tumors and their relationship with rDNA promoter methylation.

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