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Methylation Of The Brca1 Promoter And Breast Cancer In Shift Workers.

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Title

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Abstract

A search of the cancer literature reveals the strong association between long-term nighttime shiftwork and increased risk of breast cancer in female workers. Since 2007, the International Agency for Research on Cancer (IARC) has categorized shiftwork as "probably carcinogenic to humans", a Group 2A carcinogen[1]. Evidence from epigenetic studies shows that differential methylation of genes is one possible mechanism by which long-term shiftwork disrupts the expression patterns of the genes responsible for maintaining a cancer free status.

This study builds upon the work initiated by Zhu et al and investigates the association between shiftwork dependent methylation of the NBR2-BRAC1 promoter region and the risk of breast cancer in female nighttime shift workers. Understanding the effects of methylation in the NBR2-BRAC1 region is important because these genes share a bi-directional promoter that sits within a large CpG island. Unraveling the significance of methylation in this region will provide a deeper understanding of the epigenetic factors that promote breast cancer in female long-term nighttime shift workers and possibly reveal a biomarker of clinical significance.

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Introduction

Gene methylation is one of many epigenetic mechanisms by which eukaryotic cells regulate the expression of genes.[2] Methylation refers to the addition of methyl groups to the nucleic acids cytosine and adenine mediated by a family of enzymes known as DNA methyltransferases (DNMT) which occurs in genomic regions called CpG islands[2]. CpG islands are regions of 200 base pairs (bp) where the cytosine and guanine content is greater than 50% and the observed CG to expected CG ratio is more than 0.6[3]. The methylation status of a CpG sites can be inherited or influenced by exposure to environmental stressors and other behavioral factors[4,5]. The extent of methylation in the CpG regions can be quantified as hypermethylated or hypomethylated in reference to the normal methylation status of a particular region. Both hypermethylation and hypomethylation can detrimentally affect the normal balance of genomic expression and thus facilitate a cellular predisposition to cancer or other diseases. Hypermethylation is known to specifically silence the expression of genes by suppressing transcription[5]. A review of the literature finds many examples where differential methylation status is an important factor in the etiology of a variety of cancers[2,5].

Researchers have identified various environmental and behavioral factors that can disrupt the methylation status of genes and subsequently adversely affect individuals. Long-term nighttime shiftwork, marked by exposure to artificial light during night hours, is an environmental exposure suspected to be responsible for disrupting normal methylation status of genes[1,4,6]. Some of the genes thought to be

differentially methylated as a result of long-term nighttime shiftwork include those involved in the mediation of stress and in the regulation of the circadian rhythm[4,6].

An epigenetic analysis of a prospective cohort of female workers in Denmark by Zhu et al has confirmed that the methylation status of a wide variety of genes changes as a result of long-term nighttime shiftwork[6]. Zhu et al's epigenetic study of long-term nighttime shift workers revealed the differential methylation status of many genes deserving further study for possible relevance to cancer biology and other health conditions.

One interesting association observed in studies of long-term nighttime shift workers is that women are at increased risk of breast cancer compared to their daytime counterparts[6,7]. While some explanations for the increased incidence in breast cancer amongst female long-term nighttime shift workers have been suggested, no definitive mechanism has yet been discovered. This study is interested in providing insight into the possible mechanism that is responsible for increased risk of breast cancer in female long-term nighttime shift workers.

Building upon the methylation study by Zhu et al, this study further investigates the association between methylation in genes associated with breast cancer in long-term nighttime shift workers. Specifically, this study focuses on the region of the tumor suppressor BRCA1 (Breast Cancer 1, early onset) and its neighboring gene NBR2 (Neighbor of BRCA1 gene 2) because these two genes share a bi-directional promoter nested within a large CpG island[8].

The tumor suppressor gene BRCA1 lies in the 21q arm of chromosome 17 and contains 23 exons. Research has conclusively shown that BRCA1 plays a very important role in breast cancer etiology. Some of the functions that BRCA1 is responsible for are transcription, DNA repair of double stranded breaks and recombination[9]. The study of mutations and epigenetic modifications has established that disruption to the function of BRCA1 can lead to adverse effects. It is estimated that mutations in the BRCA1 gene are responsible for 50% to 85% of familial breast cancers[10]. Epigenetic studies of BRCA1 have also found evidence that methylation is accountable for cases of breast cancer[11]. A range of studies indicate that methylation of the BRCA1 promoter could be responsible for 9%-32% of sporadic breast cancers[12].

The NBR2 is a non-protein coding gene that shares a bi-directional promoter with BRCA1[13]. NBR2 is 19,531 base pairs in length and it consists of 5 exons, the fifth exon can be alternatively used[14]. The intergenic distance separating NBR2 and the transcription start site of BRCA1 is only 218 bp. The intergenic region contains cis-elements that can bind transcription factors that have the ability to function uni-directionally or bi-directionally. Thus the regulatory activity in the intergenic region can co-regulate NBR2 and BRCA1 in a spatial or temporal manner that is dependent on the availability of specific regulatory factors[14].

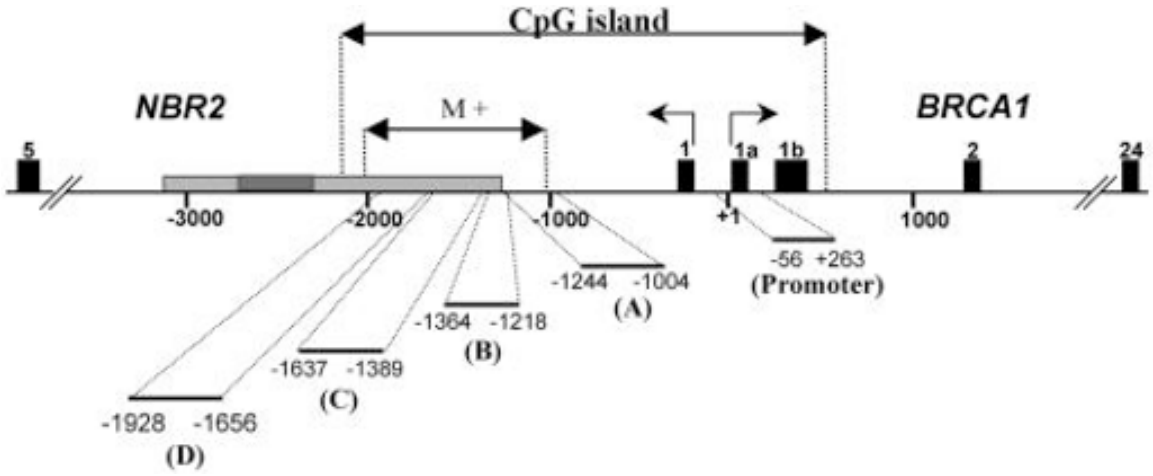

Figure 1[13]. Structure of the NBR2-BRCA1 locus. This figure shows the shared bi-directional promoter and the CpG island. M+ represents the methylated domain of the CpG island. The intergenic region between the first exon of NBR2 and the transcription start site is 218 bp. Note that the intergenic region lies within the site labeled promoter.

Interestingly, the bi-directional promoter shared by NBR2 and BRAC1 sits within a large CpG island that is found to be methylated in most human tissues[13], making this region an interesting site for epigenetic study. The CpG island spans approximately 2.8 kb in length and it extends 1810 bases downstream and 870 bases upstream from the start of the BRCA1 transcription site. However, the methylated domain (M+ in figure 1) of the CpG island lies about 1 Kb from the promoter site that regulates the expression of NBR2 and BRCA1. An in vitro study using human cell lines found that chemically induced demethylation of the CpG island increased the expression of NBR2 3.1 fold and BRCA1 1.8 fold[13]. The results indicate that methylation of the CpG island, even when not concentrated in the region of the shared promoter, can exert a degree of influence on the expression of NBR2 and BRCA1.

Understanding the regulatory effects of methylation in the region of the shared promoter of NBR2-BRCA1 is important because we have found evidence that this region is highly methylated in long-term nighttime shift workers who are also at higher risk of breast cancer than female daytime workers. It is possible that methylation of shared promoter is the mechanism by which nighttime shiftwork mediates the observed increased risk of breast caner.

Methods

Study Population[6]

Our study is based on the Danish "Diet, Cancer and Health" prospective cohort, established between December 1993 and May 1997. All female participants were born in Denmark, free of any cancer and aged between 50 and 64 years at time of invitation (now between 65 and 79 years old). The baseline questionnaire included questions on food consumption, folate intake, other lifestyle factors (e.g., tobacco smoking, alcohol habits, sun exposure, physical activity, and medical anamnesis), reproductive factors, education and occupation. All participants gave written informed consent. All collected biological samples were frozen and kept at -150 ºC[15]. The season of blood collection did not differ between night shift workers and day workers (P = 0.162). Information on the characteristics of the study subjects is provided in Table 1.

Table 1. Description of subject characteristics[1,2]

Characteristic	Day workers (*n* = 10)	Shiftworkers (*n* = 10)	*P*-value[3]
Age (years)	54.0 ± 3.3	54.8 ± 3.6	0.610
Folate intake (μg/day)	373.1 ± 108.4	349.9 ± 125.1	0.664
Tobacco smoking (years)	17.7 ± 15.2	18.0 ± 17.6	0.968
Cumulative alcohol intake (g)	93,877.4 ± 68,517.9	114,622.9 ± 62,902.0	0.490
Hormone replacement therapy (years)	2.8 ± 4.8	3.65 ± 4.0	0.673
Years of shiftwork	0 ± 0.0	21.2 ± 8.8	<0.001

[1]Tables values are given as mean ± SD.
[2]All subjects are female and of non-Hispanic ethnicity.
[3]*P*-value of independent t-test.

Collection of Shiftwork Information[6]

Night work was defined as having worked fulltime between 7 pm and 9 am. Information on night shiftwork status was obtained by telephone interview. All study subjects were classified into 3 groups: women with 10+ years of night shiftwork, women with <10 years of night shiftwork, and daytime workers.

Collection of Folate Intake Information[6]

Folate intake data were collected at baseline using a 192-item food-frequency questionnaire mailed to each participant. 12 categories, from "never" to "eight times or more per day", were used to estimate the average intake of each food item during the last 12 months. Open-ended questions were designed for supplement use on brands and doses. Information on folate content in each supplement brand was obtained from producers or distributors. For each participant, average daily folate

intake was calculated using the software program Food Calc (www.ibt.ku.dk/jesper/foodcalc) as total, dietary, and supplemental intake.

Genome-Wide CpG Island Methylation Assay[6]

10 pairs of long-term night shiftworkers and day workers were selected from the study population matched by age (±2 years) and total folate intake (±55μg/day). Genomic DNA was isolated and purified from blood samples using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's protocol. 50 ng of genomic DNA per sample were bisulfite treated using the EZ DNA Methylation Kit (Zymo research) according to the manufacturer's protocol. CpG island methylation of miRNA promoter regions was determined using the Illumina Infinium Methylation Assay, covering 255 CpG sites of 110 microRNAs. A methylation index (β) was used to estimate the methylation level of each CpG locus using the ratio of intensities between methylated and unmethylated cytosines, which is a continuous variable between 0 and 1. 0 corresponds to a completely unmethylated site, while 1 corresponds to a completely methylated site.

Methylation was calculated by use of Illumina GenomeStudio Software. The shift differential score provides a metric to judge the extent of methylation relative to a control. In this study shift workers were the comparison group and day workers were used as a reference group. The shift differential score can be converted by taking the average difference of the signal in the reference group vs. the average signal in the comparison group ($p = 10'(DiffScore*sgn(\mu cond-\mu ref)/10)$). A shift

differential score of +/- 13 is considered to be statistically significant at the alpha level of 0.05[16]. The greater the magnitude of the shift differential scores the greater the statistical significance of the results. Also, a positive shift differential score refers to hypermethylation while a negative score is indicative of hypomethylation. The results yielded a list from which the 100 most significantly hypermethylated and hypomethylated CpG sites were selected for further analysis.

From the list of the 100 most significantly hypermethylated and hypomethylated genes, we selected a smaller set of genes if they were located in relative proximity to the first exon or were located within an active promoter regions in 7 of 9 cell lines. We also selected genes with post-transcriptional regulatory function if they were located near selected methylation sites. The final list of candidate genes included NBR2 and BRCA1.

We also investigated the location of the methylated CpG sites for NBR2 and BRCA1 using the University of California Santa Cruz's (UCSC) Genome Browser. We queried the location of four significantly methylated CpG sites, 2 sites for NBR2 and 2 sites for BRCA1 using the location identifier provided by Illumina (NCBI genome assembly version 36). Table 2 provides a summary of the 4 significantly methylated probes in the NBR2-BRCA1 region.

Table 2. Methylation of NBR2 and BRCA1 CpG probes.

Gene	CpG Site	Day worker (β1)	Shiftworker (β2)	Change in Methylation Index (β2-β1)	Gene	CpG Site	Day worker (β1)	Shiftworker (β2)	Change in Methylation Index (β2-β1)
NBR2	cg20760063*	0.063	0.179	0.116	BRCA1	cg19088651*	0.108	0.162	0.054
NBR2	cg10893007*	0.042	0.101	0.059	BRCA1	cg04658354*	0.050	0.083	0.034
					BRCA1	cg19531713	0.096	0.119	0.023
					BRCA1	cg08993267	0.091	0.095	0.004
					BRCA1	cg14048487	0.784	0.784	0.000
					BRCA1	cg11964474	0.773	0.755	-0.017
					BRCA1	cg06973652	0.735	0.715	-0.020
					BRCA1	cg07054526	0.862	0.841	-0.021
					BRCA1	cg27383744	0.800	0.759	-0.042

*Significantly methylated site

The first NBR2 methylation probe (cg20760063) lies on chromosome 17, position 38,531,106, which is in the shared promoter of region BRCA1 and NBR2. The second NBR2 probe (cg1089300) was also significantly methylated, however the location of this probe was within the NBR2 gene and not within the promoter region. The two BRCA1 methylation probes (cg19088651 and cg04658354) were highly methylated but both probes lay outside of the shared promoter region.

In the initial iteration of this project we had intended to make use of public gene expression databases, however after exploring these resources we decided not to utilize them for a number of reasons. One problem was the lack of publicly available gene expression data of BRCA1 in shift workers. However, the primary problem was the inconsistent nature of the BRCA1 gene expression information available. Some studies have observed an over-expression of BRCA1, while others find it to be under-expressed, very few studies provide information on whether methylation or mutation was the mechanism affecting the expression results.

Results

The results from Zhu's methylation study showed that NBR2 and BRCA1 were hypermethylated in blood samples from female long-term shift workers in comparison to their daytime counterparts. The shift differential scores in the two NBR2 probes were 123. 744 and 48.860. Since the shift differential scores for both of the NBR2 sites exceed the value of 13 and are positive, we can be confident that both of these locations are hypermethylated relative to the reference control sample. Table 3 shows a summary of the significantly methylated NBR2 and BRCA1 probes.

Table 3. Most highly metylated CpG sites for NBR2 and BRCA1

Gene	CpG Site	Shift Differential Score	Gene	CpG Site	Shift Differential Score
NBR2*	cg20760063	123.744	BRCA1	cg19088651	25.606
NBR2	cg10893007	48.860	BRCA1	cg04658354	16.842

* Location within the shared promoter site

As we have stated previously, hypermethylation in the promoter region of genes is a mechanism that is known to silence the expression of genes. Therefore we would expect that in female long-term shift workers expression of BRCA1 would be significantly lower than its expression in daytime workers. However, we cannot offer evidence of this due to lack of expression data.

We found interesting results when we investigated the location of the NBR2 and BRCA1 CpG sites with UCSC's Genome Browser. The location of the NBR2

methylation probe (cg20760063) within the region of the shared promoter is important because this site can influence the expression of BRCA1. Some studies have found evidence that methylation in the BRCA1 promoter site can silence expression of BRCA1 in breast cancer tissues[17,11,18] and thus our finding that samples from female long-term shift workers are hypermethylated in the same region offers a possible means by which we can explain why our population of female nighttime shift workers is at higher risk of developing breast cancer than female daytime workers.

It is interesting to note that of the 9 BRCA1 CpG methylation probes, 2 were significantly methylated but not in the region of the shared promoter. The rest of the probes in the methylation panel were not significantly methylated and they did not rest within the regulatory site. Further research must be conducted to determine the significance of this finding and how the overall methylation status of the BRCA1 gene affects our hypothesis.

Discussion

While the literature regarding the specific role of NBR2 in cancer is lacking, there is sufficient evidence to warrant further study of NBR2-BRCA1 promoter region because of the sheer amount of evidence establishing the role of BRCA1 in breast cancer. Zhu and other researchers have found that female long-term nightshift workers are at higher risk of developing breast cancer compared to their daytime counterparts. Our hypothesis is that hypermethylation of the shared NBR2-BRCA1

promoter may be responsible for the increased incidence in breast cancer in female long-term nighttime shift workers.

To confirm our hypothesis that hypermethylation of the NBR2-BRCA1 promoter region mediates the risk breast cancer in female long-term nighttime shift workers we plan on investigating the methylation of the NBR2-BRCA1 region in breast tissues from female nighttime shift workers. We suspect to find similar hypermethylation in the region as we did in blood tissues.

If breast tissue samples from female long-term nighttime shift workers are confirmed to be hypermethylated relative to daytime controls it would be reasonable to expect decreased levels of expression of BRCA1 relative to controls. Other studies have confirmed that hypermethylation in the NBR2-BRCA1 shared bi-directional promoter regions leads to silencing of BRCA1 expression and we suspect to find similar results when we investigate the expression of BRCA1 in long-term nighttime shift workers.

The methylation and expression profiles in breast tissues from shift workers can be explored in Dr. Zhu's laboratory using quantitative real-time PCR (qRT-PCR) techniques. qRT-PCR is a quick and simple method for investigating the expression of selected genes relative to a stable set of controls known as housekeeping genes. If qRT-PCR finds that breast tissue samples that are hypermethylated in the NBR2-BRCA1 promoter region under-express the BRCA1 gene relative to non-

hypermethylated controls, then this would provide compelling evidence that our hypothesis is correct.

I believe that this study has found an important clue to help explain why female long-term nighttime shift workers experience higher risk of breast cancer relative to daytime workers. Hypermethylation of the NBR2-BRCA1 shared bi-directional promoter region could be the mediating factor for increased risk of breast cancer in female long-term nighttime shift workers. Further study of this region is needed to confirm our hypothesis that hypermethylation of the shared promoter may be responsible for the increased risk of breast cancer through silencing of the BRCA1 tumor suppressor gene.

It is also possible that the hypermethylation of the NBR2-BRCA1 shared promoter region observed in blood samples from female long-term shift workers could serve as an important biomarker once confirmation for the hypermethylation status of breast tissues is provided. If blood samples can serve as reliable proxies for breast tissues then our findings can provide the basis for a non-invasive diagnostic screening method.

This thesis is a continuation of professor Zhu's methylation study of long-term shift workers. We furthered his work by finding a specific locus that functions in the regulation of the tumor suppressor BRCA1 and we provide a hypothesis that hypermethylation of this region is in part responsible for mediating an increased

risk of breast cancer in female long-term nighttime shift workers. This work is an important step towards providing a mechanism to explain why long-term nighttime shift work leads to adverse health outcomes.

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