RESEARCH PAPER

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Frequent promoter hypermethylation and expression reduction of the glucocorticoid receptor gene in breast tumors

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Abbreviations: GR, glucocorticoid receptor; ER, estrogen receptor; PR, progesterone receptor; Her2, human epidermal growth factor receptor 2; NR3C1, nuclear receptor subfamily 3 group C member 1; BRCA1, breast cancer 1 early onset; RACE, rapid amplification of cDNA ends; MeDIP, methylated DNA immunoprecipitation; Ct, cycle threshold; TBP, TATA-binding protein; 5′-aza-dC, 5′-aza-2-deoxycytidine; IHC, immunohistochemistry; DIN, ductal intraepithelial neoplasia; IDC, invasive ductal carcinoma; DCIS, ductal carcinoma in situ; GABPβ, GA-binding protein β; $p16^{INK4a}$, cyclin-dependent kinase inhibitor 2A; CCDN2, G1/S-specific cyclin D2; GSTP1, glutathione S-transferase gene; MSP, Methylation Specific PCR; TSG, tumor suppressor gene

Previous studies have found that expression of the Glucocorticoid Receptor (GR) is altered or reduced in various cancers, while the GR promoter has been shown to be methylated in gastric, lung, and colorectal cancers. Examining a small cohort of matched normal and breast cancer samples we found that GR levels were dramatically reduced in almost all tumors in relation to their normal tissue. The methylation status of the GR promoter was assessed to determine if this observed decrease of expression in breast tumors could be due to epigenetic regulation. While it was not methylated in normal tissue, the GR proximal promoter was methylated in 15% of tumor samples, particularly, but not exclusively, in Estrogen Receptor positive tumors. GR expression in these tumors was particularly low and loss of GR expression was specifically correlated with methylation of the proximal promoter GR B region. Overall, these results show that hypermethylation of the promoter in tumors is a frequent event and suggests that GR may act as a tumor suppressor in breast tissue.

Introduction

The glucocorticoid receptor (GR) is a steroid hormone receptor that serves as a transcriptional regulator in the human stress response when bound to its ligand, cortisol. A growing body of evidence exist that the GR promoter is methylated in human cancers. A study of gastric carcinomas found that the GR promoter was methylated in 20–30% of cases, which was 3 times higher than in normal gastric tissue.[1] Furthermore, small cell lung carcinoma cell lines showed increased promoter methylation.[2] Although not methylated in ovarian cancer,[3] GR was methylated in 25% of colorectal carcinomas and 35% of colorectal cancer cell lines, and methylation was associated with a decrease in GR gene expression.[4] Inactivation of tumor suppressors by hypermethylation is commonly observed,[5,6] suggesting that GR silencing may play a role in tumor development. In breast cancer, GR expression has been found to decrease significantly with tumor histologic grade, with one study reporting expression reduced below 50% of that of normal tissue.[7] Investigation of estrogen receptor (ER) negative and progesterone receptor (PR) negative tissues has also shown that all were negative for GR, suggesting that GR loss may occur early during breast tumorigenesis.[8] Given this evidence, epigenetic silencing of GR is one possible explanation for the observed decrease in GR expression in breast cancer.

The human GR gene (NR3C1) spans 80 kb on chromosome 5 and contains 8 coding exons (2–9) and 9 tissue-specific alternative first exons[9] located in two distinct promoter regions: the distal promoter, approximately 30 kb upstream of the translation start site, and the proximal promoter located in a 3 kb CpG island 5 kb upstream from the ATG start codon.[10,11] Cloning of the intronic regions between the alternative first exons into luciferase reporters has shown that each alternative first exon has its own unique promoter, and methylation of these promoters by SssI methyltransferase successfully reduced activity to below 10%, indicating that individual first exon promoters are susceptible to epigenetic control.[12] It has also been suggested that

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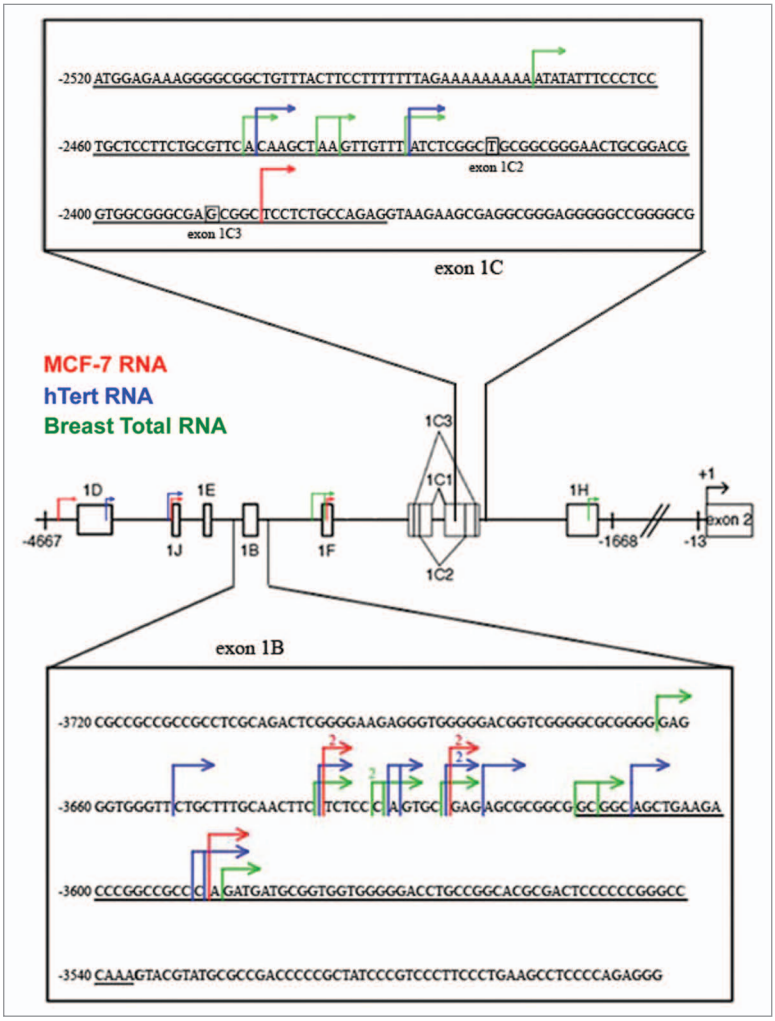

Figure 1. 5′ RACE for MCF-7, 184-hTert and Total Breast RNA. 5′ RACE was performed for MCF-7, 184-hTert and total breast RNA and the products were cloned into pBS+ vector before being transformed into DH5α electrocompetent bacteria. Positive clones were sequenced. All arrows begin at the most 5′ end of the cDNA sequence of individual samples and all analyzed sequences continue to the 3′ end of their associated alternative exon followed by the sequence of the common acceptor splice site immediately upstream of exon 2. Arrows with a number overhead indicate the number of samples sharing the same sequence. Sequence-specific information for exon 1B is underlined and shown in its entirety as well as a portion of exon 1C, including the common 3′ site for all three of its transcript variants and the splice sites for exons 1C2 and 1C3 indicated by the squared nucleotide. In total 9 MCF-7, 14 184-hTert, and 17 breast total RNA samples were sequenced. Of these, the majority (60%) were exon 1B (5/9, 10/14, and 9/17, respectively). The second most frequent exon was exon 1C1 (1/9, 2/14, and 5/17, respectively) at markedly lower abundance (20%). One sample obtained from breast total RNA mapped to exon 1A3 of the distal promoter and is not included in this figure.

methylation of these promoters may be subject to individualized, highly variable regulation.[13] Only one study has previously investigated GR promoter methylation in breast cancer (no methylation was detected),[7] but the analysis was not comprehensive as it was limited to a small region in the exon 1C promoter.

GR promoter methylation may be particularly important in the breast, as previous research has revealed a novel role for unliganded GR as a positive regulator of BRCA1 activity.[14] *BRCA1* is an important gene in breast cancer etiology as its protein product is involved in cell regulatory processes including DNA repair[15] and decreased GR expression may predispose an individual to increased risk of breast cancer due to a loss of constitutive activation and perpetually decreased *BRCA1* transcription.[16] This study investigated GR RNA levels, and characterized promoter-wide GR methylation status, in normal and tumor breast tissue to determine if epigenetic programming of GR occurs in mammary carcinogenesis and plays a role in tumor progression by decreasing GR expression.

Results

Exon 1B is the predominant alternative first exon expressed in normal and malignant breast cancer cell lines

To definitively determine which GR alternative first exons are present in breast RNA and in what abundance, 5′ RACE was conducted using normal breast RNA, as well as RNA from a non-malignant (184-hTert) and malignant (MCF-7) mammary epithelial cell line. Sequencing of the 5′ RACE products found that the majority of GR RNA sequences mapped to exon 1B for total human breast (9/17 total clones), 184-hTert (10/14 clones), and MCF-7 RNAs (5/9 clones) (**Fig. 1**). This indicates that exon 1B is the primary alternative exon used in both normal and malignant breast GR mRNA transcripts. The next most frequent was exon 1C, although at markedly lower abundance (5/17 breast total RNA, 2/14 184-hTert, 1/9 MCF-7). The rest of the start sites were in the D, J, F, and H exons, with the exception of one exon 1A3 sequence from the distal promoter, found in the normal breast RNA sample, demonstrating that in the breast the proximal promoter is predominantly responsible for GR expression. The tissue heterogeneity of the normal human breast was found not to affect the reliability of the 5′ RACE reaction, as the results for total normal breast RNA were consistent with the clonal mammary epithelial breast cell lines.

GR expression is decreased in human tumors compared with matched normal samples

Relative GR levels in normal and tumor tissue from 59 breast cancer patients (**Table 1**) were determined by qRT-PCR using a Taqman probe spanning exons 4 and 5, normalized using HPRT1, and were compared with the arbitrarily chosen normal tissue control A00235 sample. Relative GR expression in normal tissues was variable within a range of 0.15 to 16.93-fold of A00235 levels, with an average of 2.99-fold (**Fig. 2A**). In contrast, all but three tumor samples showed a decrease in GR levels with a range of 0.03 to 2.79-fold in comparison to the same normal A00235 control, with an average of 0.38-fold lower expression (**Fig. 2B**). To determine whether patients

showed any significant changes in individual gene expression the fold difference in GR expression was calculated between normal and tumor tissue. GR expression was lower in all tumors compared with normal tissue for all patients except one (D01394-fold response = 1.07) with an average decrease in GR expression of 13.81-fold (**Fig. 2C**) (standard deviation = 15.78). Based on these results, decreased GR expression appears to be a ubiquitous and important event in breast tumor development.

MeDIP-qPCR to detect GR promoter methylation

A methylation detection assay (MeDIP-qPCR) was developed to determine promoter-wide GR methylation status in human breast cell lines and primary tissues based on multiple probes to a given locus. Methylated DNA (MeDNA) was enriched using MeDIP and levels of DNA were determined by quantitative-PCR Cycle threshold (Ct) values for each specific PCR amplification probe. The percent of immunoprecipitated methylated DNA was calculated for each probe using a correction for the efficiency of methylation capture (see methods). Our 5′ RACE experiments revealed almost exclusive proximal promoter first exon usage in the breast; however, as the proximal GR promoter is complex (being comprised of multiple exons), we employed 6 primer sets spanning the GR proximal promoter to assess the methylation status of the different proximal promoter regions (**Fig. 3**).

A normal (MCF-10A) and 4 different tumor derived breast cancer cell lines were examined using our MeDIP assay. Within the 5 cell lines, only T47-D cells were methylated at the GR proximal promoter with an average percent immunoprecipitation (%IP) of 50% among all probes, compared with an overall average of 0.41% IP for the other 4 lines, with MDA-MD-231 having the next highest value (average of 0.81% IP; **Fig. 4A**). In T47-D cells, the expression of GR was 2.3-fold less than in MCF-10A cells, indicating that there was a correlation between expression and methylation status.

As ENCODE data indicates that T47-D cells are methylated in the proximal GR promoter but not in MCF-7 cells,[17,18] we wanted to further validate this assay by comparing our results to individual CpG methylation patterns. We developed Methylation Specific PCR (MSP) probes to several different regions corresponding to our MeDIP probes. Bisulphite converted DNA was subjected to MSP and the resulting product was sequenced without cloning, allowing us to determine if individual CpGs between the MSP primers were also methylated. As can be seen in **Figure 4B**, analysis of the GR proximal promoter C-H region in T47-D cells showed that all of the Cs (blue) in CpG dinucleotides (underlined) were not converted, indicating that they were all methylated, while the non-CpG C residues (red) were converted to T residues. Similar results were found for our other probes and indicate that the GR promoter is heavily methylated in T47D cells, consistent with the 50% IP level found, and that our assay correctly identified regions containing significant levels of methylation, as confirmed at the level of individual nucleotides.

Frequent GR promoter methylation of tumor but not normal breast DNA

MeDIP-qPCR analysis was performed on all tumor samples (**Fig. 5**). The average %IP of all samples and probes was 0.86—however, 8 samples showed multiple probes with a %IP over 1.

Table 1. Breast cancer patient cohort

Age	No. Samples
20–29	1
30–39	4
40–49	8
50–59	15
60–69	14
70–79	9
80–89	7
90–99	1
Hormone Receptor Status	**No. Samples**
ER+/PR+/Her2+	2
ER+/PR+/Her2-	33
ER-/PR-/Her2+	8
ER-/PR-/Her2-	4
Other (incl. UNK)	12

Matched normal and tumor breast tissue were obtained from 59 breast cancer patients from the Ontario Tumour Bank. The majority of samples were between 50 and 69 y of age and the predominant hormone receptor subtype was ER+/PR+/Her2-. Some patient samples had unknown ER, PR, or Her2 status and are included in the "Other" category.

These 8 tumors had an average of 5.5% IP for all probes, while the remaining unmethylated samples had a collective %IP for all probes of 0.05, indicating that there was a 100-fold difference in methylation levels between methylated and unmethylated tumors. The characteristics of patients with methylated tumors are summarized in **Table 2** and extensive statistical analysis did not identify an association with primary clinical characteristics, such as tumor grade—however, long-term outcome data was not available. The GR proximal promoter J-E primer set was methylated in all seven of the methylated samples and the GR proximal promoter B primer set was methylated in five. The GR proximal promoter J-E and GR proximal promoter B primer sets flank the exon 1B promoter sequence, the predominant alternative first exon in breast tissue and, thus, methylation of these regions is expected to be important for the regulation of GR transcription.

We also examined methylation of the GR promoter in matching normal tissue samples for all the tumors. We did not observe any methylation of the promoter, either in samples with methylated tumors nor in those without (data not shown). This suggests that the methylation observed in tumors is the result of the tumorigenesis process and not an endogenous modification.

Tumors methylated at the GR proximal promoter B region have lower relative GR expression than unmethylated tumors

To determine if GR promoter methylation had an effect on GR transcriptional activity, gene expression was compared between methylated and unmethylated tumor samples (**Table 2**). Initial statistical analysis revealed that there was no significant difference ($P = 0.055$) in GR expression between tumors methylated and unmethylated at the GR proximal promoter, despite the assumption that methylation would result in decreased GR

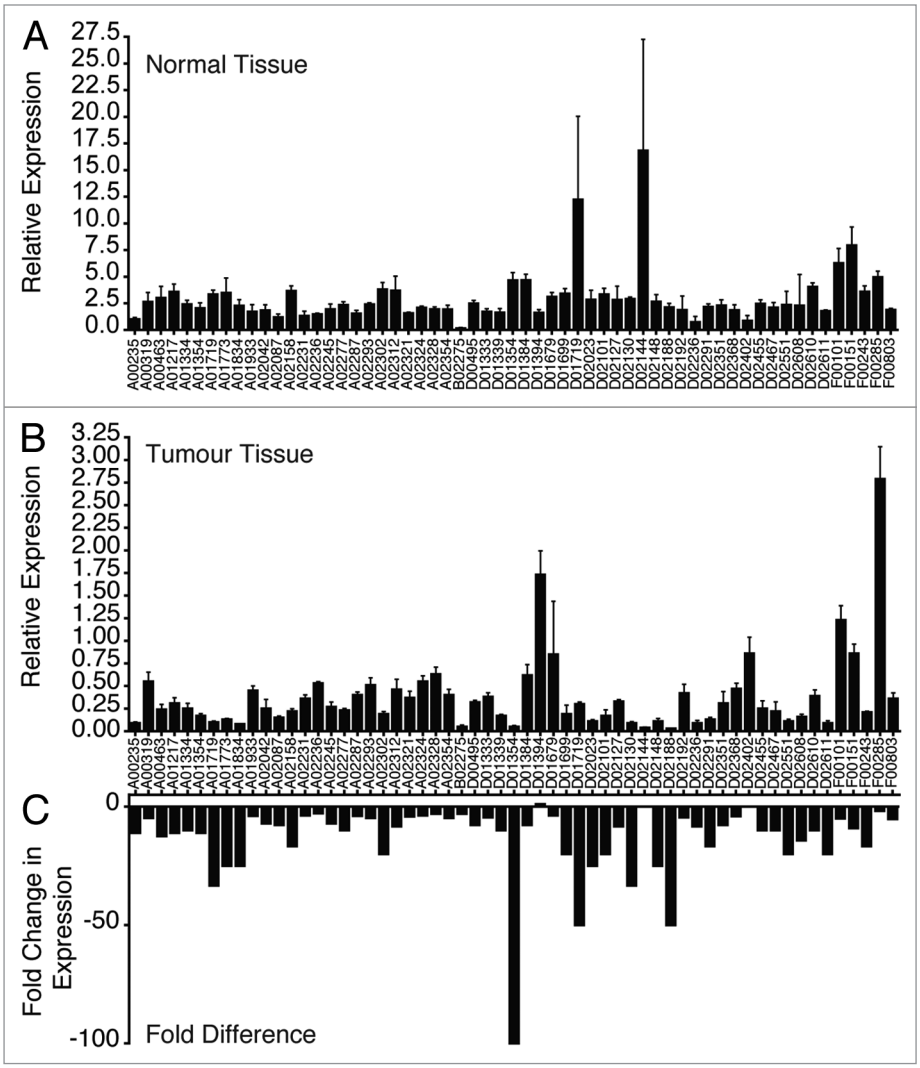

Figure 2. Fold change in gene expression from normal to tumor tissue. (**A**) GR mRNA levels in normal breast tissue were determined by Taqman assays using a probe spanning exons 4 and 5 and normalized using HPRT1 levels. Levels were expressed relative to the normal sample from patient A00235. (**B**) GR mRNA levels from breast tumor samples were determined, normalized using HPRT1 levels and expressed relative to the normal sample from patient A00235 in order to allow direct comparison between normal and tumor levels. (**C**) The difference between the levels of GR in normal verses tumor tissues for each patient was calculated. GR expression was decreased in all tumor samples, excluding sample D01394 (fold response = 1.07) with an average decrease in GR expression of 13.81-fold (standard deviation = 15.78). Sample D02144 had no GR expression in tumor tissue, calculated as an infinite fold decrease in expression, and so is not included in the average fold response calculations.

transcription. However, when tumors were segregated according to their methylation status at each of the probe sites, there was a statically significant difference for tumors methylated at GR proximal promoter B, which together showed 4.6-fold lower GR expression than tumors unmethylated at this region ($P = 4.88 \times 10^{-6}$), identifying GR proximal promoter B as being important for the regulation of GR transcription (**Table 2**).

Discussion

Immunohistochemical (IHC) analysis of normal and benign breast lesions has determined that GR expression decreases with increasing tumor histologic grade.[7] This result was also observed in a later study of normal, in situ, and invasive breast cancers, which found that GR-positive cells were present in normal breast and decreased below 50% in ductal intraepithelial neoplasia (DIN 2–3) and invasive ductal carcinomas (IDC).[8] A third study also implicated decreased positive nuclear staining of GR in breast cancer development, from 27% in ductal carcinoma in situ (DCIS) to 18% in IDC.[19] In contrast, RT-PCR in 25 archival breast tissue samples found that GR mRNA expression actually increased with tumor grade compared with controls and earlier grade cancers.[20] Although it may be that GR transcription is somehow increased while protein levels measured by IHC are decreased, our qRT-PCR analysis of normal and tumor breast samples agrees with the IHC analysis and has shown that GR mRNA levels are lower in tumor compared with normal tissue. This trend applied to all of our tumor tissues, regardless of GR methylation status. Although methylation of the GR promoter proximal B region in tumor samples was correlated with decreased GR, it may be that other cellular mechanisms, such as BRCA1-mediated changes in GR autoregulation (through the 1A and 1B promoters in breast),[21] or altered YY1 and Sp1 binding to the 1B promoter,[22] may influence its expression, predisposing it to inactivation by methylation. In the only other study that looked across the GR promoter, small cell lung cancer methylation of the 1C promoter was reported and was more closely linked to the inhibition of GR expression in a variety of cell lines—while, in contrast to our findings, exon 1B was not methylated.[2] This suggests that there are tissue-specific aspects to the regulation of GR, regardless of the tissue that is targeted for inactivation by methylation.

Increased GR promoter methylation has previously been observed in the hippocampus of rat pups in response to stress,[23] which led to several studies in humans examining the association between stress and epigenetic programming of GR. Notably, depressed maternal mood in the third trimester has been found to be associated with increased GR methylation in the cord blood of infants,[24] and suicide victims with a history of child abuse showed

decreased hippocampal GR expression of exons 1F, 1B, and 1C associated with increased methylation, when compared with non-abused suicide victims and controls.[25,26] These studies have identified a link between stress and methylation of the GR promoter. Given the general association between stress and breast cancer incidence,[27] this raises the possibility that epigenetic programming could occur locally in the breast and result in decreased GR and subsequent *BRCA1* transcription by loss of constitutive GR promoter activation, thereby increasing the risk of developing breast cancer. Analysis of 54 tumor samples using the MeDIP-qPCR assay found that a total of eight breast tumor samples (15%) were methylated at the GR proximal promoter and that methylation of the GR promoter proximal B region was particularly correlated with GR transcriptional regulation.

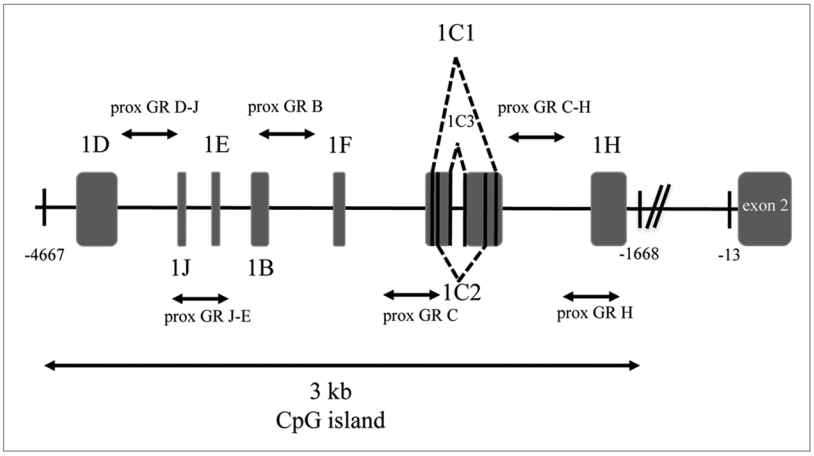

Figure 3. qPCR primers spanning the GR proximal promoter. The GR proximal promoter is located in a 3 kb CpG island approximately 5 kb upstream of the translation start site and contains seven alternative first exon sequences (gray boxes). Primers were designed spanning the proximal promoter and used for subsequent MeDIP-qPCR analysis. Relative to the translation start site, located in exon 2, the prox GR D-J primer set amplifies a 261-bp region from -4287 to -4027, prox GR J-E amplifies a 283-bp region from -4046 to -3764, prox GR B amplifies a 209-bp region from -3549 to -3339, prox GR C amplifies a 204-bp region from -2868 to -2665, prox GR C-H amplifies a 298-bp region from -2294 to -1997, and prox GR H amplifies a 222-bp region from -2017 to -1796.

The frequency of hypermethylation we have observed here is comparable to that found in other tumor types, including colon and gastric cancers, suggesting that GR methylation is a relatively common event in a wide variety of tumor types.[1,4] Other studies examining breast tumors have identified similar methylation frequencies in genes such as *p16*^{INK4a}, *CCDN2*, and *GSTP1*,[28,29] indicating that GR methylation frequency is comparable to known methylated gene promoters in breast cancer. It was expected that methylation of GR detected in tumor samples would also show promoter methylation in normal samples, indicating prior epigenetic programming previous to tumorigenesis, possibly due to stress. In this study, however, no methylation was detected in any normal breast tissues. It is possible that the original population of normal cells methylated at the GR promoter was too small to be identified in the tissue obtained or was simply not present in the location sampled.

Promoter methylation is characteristic of tumor suppressor genes (TSG) during cancer development,[30] and methylation of the *BRCA1* promoter, a known TSG, has previously been reported in sporadic breast cancer cases at frequencies ranging from 9% to 41%.[15,28-30] Based on our findings that 15% of breast tumors were methylated at the GR proximal promoter, and that these levels are comparable to methylation of other known TSGs in breast cancer, it is important to consider whether GR itself comprises a tumor suppressor gene. Glucocorticoids signal through their receptor and are normally involved in the regulation of many pathways that could contribute to cancer development, including metabolism, cell growth, apoptosis, differentiation, inflammation, vascular tone, and immunosuppressive actions.[31] One review has found tumor suppressor functions to be associated with GR in various cell lines.[32] Evidence also exists supporting a role for GR as a tumor suppressor in the breast. It has been experimentally determined that high GR expression in the breast is correlated with anti-proliferative signaling,[21] and dysregulation of this pathway is important in tumorigenesis. The effect of GR levels in breast cancer may also be tumor subtype-dependent, as evidenced by analysis of a meta-data set of early-stage primary breast tumors, which found that loss of GR expression was associated with good outcome in ER-breast cancer patients, but with significantly poorer outcome in ER+ tumors.[33] Downregulation of GR appears to be important in more aggressive ER+ tumors, which may explain our finding that methylated tumors identified in our study were predominantly ER+. In addition to being methylated, many TSGs are also mutated in cancers and three novel GR mutations have recently been identified in a study of triple-negative breast neoplasms,[34] implying that loss of GR function is a significant change promoting breast cancer development in this type of tumor. These GR mutations were found in triple negative cancers, whereas methylation of GR was predominantly observed in ER+/PR+/Her2- tumors, although one triple-negative cancer was methylated at the proximal promoter. Inactivation of GR may therefore involve mutation or methylation, depending on either the cell of origin or the path of tumor progression giving rise to the final cancer subtype. Consideration of GR as a TSG implicates its methylation as a driving factor in cancer development—however, it should be noted that based on our analysis it is also possible that methylation of the GR promoter is simply a by-product of changes in tumor cells associated with transformation. The high frequency of GR methylation in breast cancer tumors suggests that it could be part of a frequent process, such as a CpG island methylator phenotype, originally discovered in colorectal cancer.[35] Importantly, GR has not been identified as a methylation target of the methylator phenotype in colorectal cancer. In breast cancer, a hypermethylated phenotype associated with luminal B breast cancers was identified and, in addition, did not include the GR promoter.[36] These results suggest that GR methylation is likely unrelated to a general methylator phenotype. Given this, the discovery of significant mutations in

Table 2. Patients with Tumours Methylated at the GR Proximal Promoter

Sample	D-J	J-E	B	C	C-H	H	Age	Grade	Hormone Receptor Status	GR
A01719		■	■	■	■	■	85–89	I-II	ER+/PR+/Her2-	0.10
B02275	■	■	■	■	■	■	55–59	I-II	ER-/PR-/Her2-	0.05
D01354		■	■		■	■	75–79	III	ER+/PR+/Her2-	0.05
D01384		■		■		■	50–54	III	ER+/PR+/Her2-	0.62
D02130					■	■	60–64	Unk	ER+/PR+/Her2-	0.09
D02291	■	■	■	■	■	■	75–79	III	ER+/PR+/Her2-	0.13
D02368	■	■			■	■	55–59	Unk	ER+/PR_{UNK}/Her2+	0.47
D02551	■	■	■	■	■	■	65–69	III	ER+/PR+/Her2-	0.11

Eight breast cancer patients had tumors with multiple probes above 1% methylation. Gray squares indicate methylation of the indicated probe. Three tumors were methylated at every region spanned by the six primers (B02275, D02291, and D02551). Patients with methylated tumors ranged from 50 to 89 y of age and the majority were ER+/PR+/Her2- (A01719, D01354, D01384, D02130, D02291, and D02551). The Relative Expression of GR for each sample was determined by normalizing gene expression to sample A00235 normal tissue and the average relative GR expression was 0.20.

triple-negative breast cancers provides compelling evidence for a role for GR in breast cancer progression.

Materials and Methods

5′ RACE

5′ Rapid Amplification of cDNA Ends (RACE) of primary total normal breast RNA (Invitrogen) and 184-hTert and MCF-7 RNA was performed using the SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories) and a gene-specific reverse primer[9] with a BamHI cut site (GR exon $2_{[Det]}$: 5′- GGGGGATCCC AGTGGATGCT GAACTCTTGG-3′). Nested reverse primers were designed in GR exon 2 also containing BamHI cut sites (GR exon 2–2: 5′- GGGGGATCCC GACAGCCAGT GAGGGTGAAG ACG-3′ and GR exon 2–1: 5′- GGGGGATCCG GGTTTTATAG AAGTCCATCA CATCTCC-3′), and a variation of the forward SMARTer II A Oligonucleotide was designed with an XbaI cut site (SMART: 5′- GGGTCTAGAA AGCAGTGGTA TCAACGCAGA G-3′). Nested PCR amplification was performed using the following program: 94 °C for 15 min followed by 35 cycles—denaturing at 94 °C for 30 s, annealing at 68 °C for 30 s, elongation at 72 °C for 2 min, ending with 4 min at 72 °C. Products were purified using the QIAquick PCR Purification Kit (Qiagen), cloned into a pBS+ vector and plated. Colonies were selected using IPTG/X-gal color selection and processed using the QIAprep Spin Miniprep Kit (Qiagen). 5′ RACE products were sent for sequencing (ACGT Inc.).

Homogenization and DNA/RNA Extraction of Breast Clinical Samples

Matched tumor and normal breast clinical tissue samples were obtained from the Ontario Tumour Bank from 59 breast cancer patients. This work was performed with the approval of the Health Sciences Research Ethics Board of Queen's University, Canada reference number DBMS-002-11. All samples were homogenized using a 7 mm × 150 mm generator (PRO Scientific) on setting 5 for 1 min, or until completely homogenized. DNA and RNA were extracted using the AllPrep DNA/RNA Mini Kit (Qiagen) reagents and modified protocol. RNA sample quality was assessed by RNA Integrity Number (RIN) obtained using a Bioanalyzer electrophoresis assay (Agilent Technologies). Only RNA samples with RIN above 6 were used for qRT-PCR analysis. DNA samples used for MeDIP-qPCR analysis were run on 0.7% agarose gels to assess DNA quality.

qRT-PCR

Synthesis of cDNA and subsequent PCR amplification of RNA was achieved using the SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (Invitrogen) and TaqMan Gene Expression Assay probe and primer sets (Applied Biosystems) for NR3C1 (Hs00353740) using HPRT1 as an internal control. The qRT-PCR reaction was run in a Mastercycler realplex[4] (Eppendorf) using the following program—50 °C for 15 min, 95 °C for 2 min, followed by 40 cycles—denaturing at 95 °C for 15 s, annealing at 60 °C for 30 s. Quantitation of specific transcript levels was determined using the ΔΔCt Method and analysis of relative gene expression between samples was achieved by normalizing all samples to GR and BRCA1 expression in A00235 normal tissue.

Methylation-specific polymerase chain reaction

Cell line DNA was bisulfite-converted using the EpiJET Bisulfite Conversion Kit (Thermo Fisher Scientific) as described by the manufacturer. For the conversion reaction 500 ng DNA was employed and eluted in 10 μL of the provided elution buffer.

For MSP, bisulfite-converted DNA was amplified with a primer set specific for the methylated sequence. Primer sequences are as follows—GRC-M MSP 5′- GTTCGTTTTT TCGAGGTGTC-3′ and GR C-H MSP 5′-AACCAACGCT ATCACCCG-3′. Primers were designed with MethPrimer[37] and obtained from Integrated DNA Technologies (Coralville). For each 50 μL PCR, 100 ng bisulfite-converted DNA, 5 μL 10X PCR buffer (Qiagen), 2 μL dNTPs (5 mM), 4 μL each forward and reverse primer (100 ng/μL), 2 units HotStarTaq *Plus* DNA Polymerase (Qiagen), and 32.8 μL water were used. PCR conditions were 95 °C for 5 min followed by 40 cycles—denaturing at 95 °C for 30 s, annealing at 58 °C for 30 s, elongation at 72 °C for 30 s, and a final extension step at 72 °C for 7 min. Reactions were electrophoresed on a 2% agarose gel and evaluated under

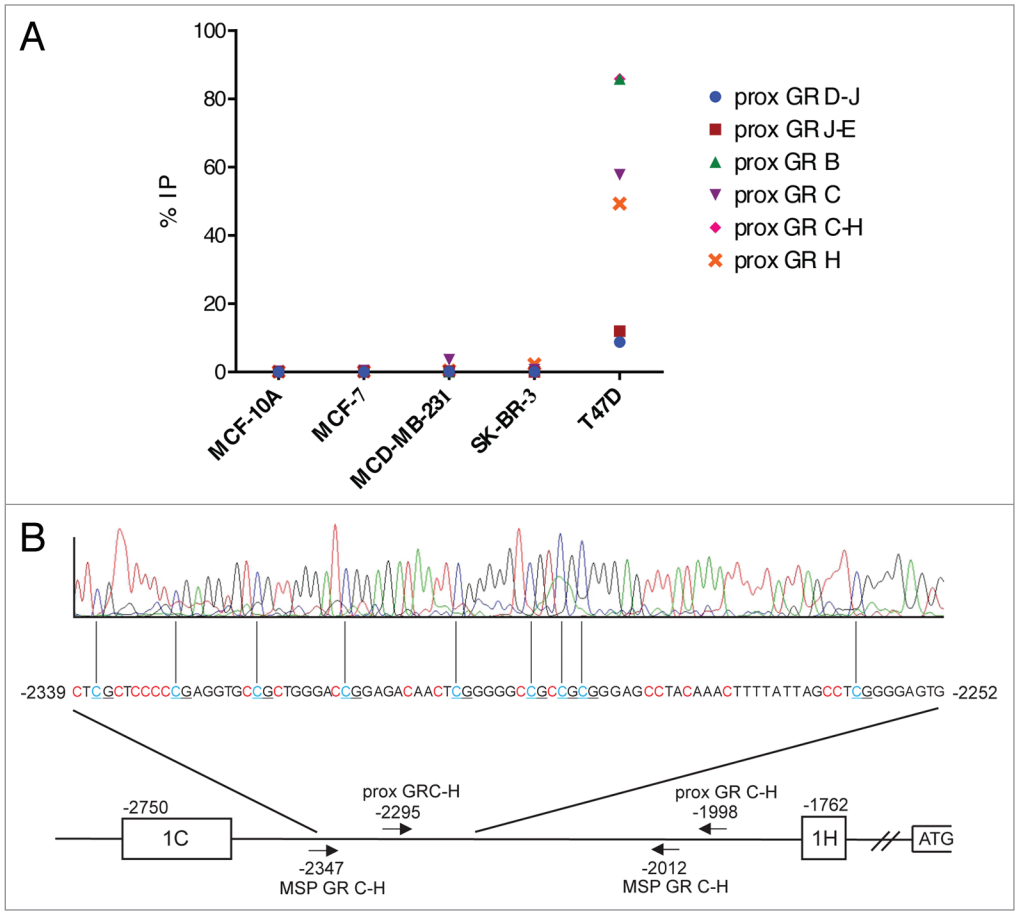

Figure 4. MeDIP Assay for characterizing GR promoter methylation. (**A**) Individual primers described in **Figure 3** were used to carry out Quantitative PCR on methylated and flow through fractions of DNA from the indicated breast cell lines. The percentage of methylated DNA precipitated (%IP) was calculated as described in the methods. (**B**) Bisulphite converted DNA was PCR amplified by a methylation specific primer set (MSP GR C-H) and the product was sequenced using the same primer. The chromatogram indicates the sequence of this product: red, T; blue, C; black, G; green, A. The sequence of the GR promoter is indicated with unconverted C residues (blue) in CpG dinucleotides (underlined) shown with a line connecting them to their position on the chromatogram. C residues which have been converted to T residues are indicated in red. A schematic of the location of the GR exons (boxes) and the positions of the MeDIP primers (prox GR C-H) and methylation specific primers (MSP GR C-H) are indicated by arrows.

UV light. MSP products were purified using the QIAquick PCR Purification Kit (Qiagen) and sent for sequencing (ACGT Inc.).

MeDIP-qPCR

Genomic DNA obtained from breast cancer patient samples was sheared using a Sonic Dismembrator Model 100 (Fisher Scientific) at setting 4 for five pulses of 30 s with a 30 s rest on ice between each pulse. Methylated DNA Immunoprecipitation was performed for 1 μg of sheared DNA using the MeDIP Kit (Active Motif) reagents and protocol. Samples were incubated with 5-methylcytosine (5mC) antibody overnight and fractionated into flow through and methylated DNA (MeDNA) fractions, which were then purified. Quantitative PCR (qPCR) was conducted using QuantiTect SYBR Green PCR Kit (Qiagen) using primers designed for the GR promoter: GR promoter primers were prox GR D-J fwd: 5′-GTCCAAGCCT TCCCGACGCG-3′, rev: 5′-CCCTCGACTC TGTGCGTTGC T-3′, prox GR J-E fwd: 5′-GCAACGCACA GAGTCGAGGG C-3′, rev: 5′-CGCCCAATGT GCTCACACTC G, prox GR B fwd: 5′-CCCCGGGCCC AAAGTACGTA TGCG-3′, rev: 5′-GCGGCTGAGC TGCGTGAGTG G-3′, prox GR C fwd: 5′-CGAGTGTGTG CGCGCCGT-3′,rev:5′-CGGCGTCTCCTTCCACCCAC-3′, prox GR C-H fwd: 5′-CCGCCGCGGG AGCCTACAAA-3′, rev: 5′-ACGAAAACGG GTGTCGGGCG-3′, prox GR H fwd: 5′-TCGCCCGACA CCCGTTTTCG-3′, rev: 5′-AACAGATAAC GCCGGCCCCG-3′. qPCR was performed in a Mastercycler realplex[4] (Eppendorf) using the following program: 94 °C for 15 min followed by 40 cycles: denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s. Cycle threshold (Ct) values for each primer set were obtained for the flow through and MeDNA fractions. The percentage of immunoprecipitated DNA (%IP) was calculated for each probe accounting for the efficiency of immunoprecipitation by the use of a positive methylation control SLC25A37, which is highly methylated in all tissues. Using the formula %IP = 2^(Ct Flowthrough Probe – [Ct Flowthrough SLC25A37 – Ct Immunoprecipitated SLC25A37] – Ct Immunoprecipitated Probe) *100 the amount of specific probe precipitated in

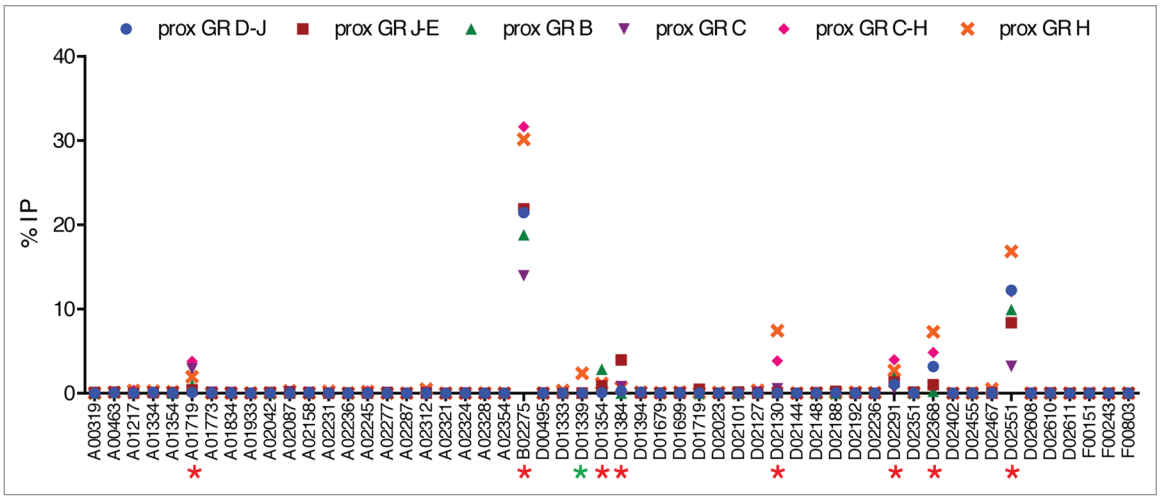

Figure 5. MeDIP-qPCR of tumor tissue for 54 breast cancer patients. Flowthrough and MeDNA extracted from 54 tumor tissues from breast cancer patients was amplified by qPCR using primers specific to the GR proximal promoter (prox GR D-J, J-E, B, C, C-H, and H) as well as SLC25A37 and TBP. The % Immunoprecipitated DNA (%IP) was calculated as defined in the methods. Eight matched tumor samples (A01719, B02275, D01354, D01384, D02130, D02291, D02368, and D02551), marked with red asterisks, were methylated at the GR proximal promoter. Sample D01339 (green asterisk) was methylated at only one probe and was not considered to be methylated.

comparison to that of the positive methylation control was calculated.

Statistical Analysis

Student's *t*-tests were performed to determine statistical significance. Correlative analysis was performed using two-tailed *P* value correlation analysis assuming a non-parametric correlation using the Spearman rank-order coefficient.

Disclosure of Potential Conflicts of Interest

The subject of this work has been the basis of a North American patent application. No business dealings are currently being performed in regards to this work.

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