Award Number: DAMD17-03-1-0466

TITLE: Expression and Promoter Methylation of p16Ink4A During Estrogen-Induced Mammary Carcinogenesis in the ACI Rat

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Breast cancer is one of the leading causes of death for women in the United States and estrogen exposure has been hypothesized to be involved in the development of this cancer. Our lab is studying the ACI rat, an estrogen-induced breast cancer animal model to begin to elucidate estrogen’s role in breast cancer. The ACI rat develops mammary cancer after prolonged exposure to 17β-estradiol, while the BN and genetically related COP rats do not. We have identified several polymorphisms in the promoter region of $p16^{ink4a}$ between the ACI rat and either the BN or COP rats. Several of these polymorphisms are in key regions of transcriptional control for $p16^{ink4a}$ and similar polymorphisms have been identified in the human. In addition, we have shown in the ACI rat that the $p16^{ink4a}$ message levels are down regulated and that this down regulation is due to hypermethylation of the promoter region. Further research will focus on the determination of the role of these polymorphisms in the down regulation and methylation of $p16^{ink4a}$ and the timing of this down regulation and methylation.
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The ACI rat is uniquely susceptible to estrogen (E2) induced mammary carcinogenesis while the BN and genetically related COP rats are not as susceptible. Genetic studies utilizing these strains have allowed us to identify a region on rat chromosome 5, containing \( p16^{cdkn2a} \), that confers susceptibility to E2 induced mammary cancer. \( p16^{cdkn2a} \) has been identified as a tumor suppressor gene that functions as an inhibitor of CDK4 and CDK6 and controls the G1/S transition of the cell cycle (1). Loss of \( p16^{cdkn2a} \) expression has been shown to occur in human breast cancer and is most commonly reported to be due to hypermethylation (1). We have previously demonstrated that the expression of \( p16^{cdkn2a} \) is dramatically down regulated at the protein level at an early stage of E2-induced mammary carcinogenesis, whether or not this occurs at the mRNA level and the mechanism behind this down regulation has yet to be identified. Our hypothesis is that the mRNA levels of \( p16^{cdkn2a} \) decrease upon exposure to E2 in the ACI strain in the focal regions of atypical hyperplasia, carcinoma in situ, and invasive carcinoma. In addition, we hypothesize that the mRNA levels in the BN and COP strains will not decrease upon the same time of exposure to E2. Furthermore, we hypothesize that this loss of expression is due to methylation of the promoter and exon1 of \( p16^{cdkn2a} \) in mammary tissue of the ACI, COP and BN strains, to determine the methylation pattern of the promoter and exon1 of \( p16^{cdkn2a} \) in the ACI, COP and BN strains of rats in normal, lobular hyperplasia, atypical hyperplasia, carcinoma in situ, and invasive carcinoma.

Identification and Characterization of Promoter Polymorphisms in 4 inbred strains of rats. As planned in Task 3 in the statement of work, we sequenced the 5'UTR and promoter region of the ACI/SegHsd, BN/ssNHsd, and COP strains of rats. In addition, we sequenced this same region from the DA/OlaHsd strain of rat. In total we sequenced 1900 bp 5' of the translational start site and have deposited these sequences in GenBank (Accession No AY376712, AY376713, AY376714, and AY376715). Table 1 contains the polymorphisms that have been identified between these three strains of rats (ACI, BN and COP).

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In addition, we compared known binding sites for transcription factors that have been shown to regulated \( p16^{cdkn2a} \) in the mouse and human. We identified conserved transcription factor binding sites for Ets1 (3), Sp1 (4, 5), RBAR1 (6), RNA helicase A (7), INK4a transcription silence element (8), and E47 (9, 10) in the rat, mouse and human. We also used several transcription factor binding site prediction programs and identified possible binding sites for C/EBPa, HNF-4, PAX4 and ER.
transcription factors. Further studies will need to be done to determine if these factors do indeed regulate $p16^{c6kn2a}$.

The transcriptional start site for $p16^{c6kn2a}$ in the rat or mouse has yet to be experimentally identified. However, six transcriptional start sites have been identified in the human (2). Comparison of the sequences around these start sites was done. We found homology near three of these sequences in rat and mouse. In addition, Abe et al. (11) identified a putative transcription start site in this same region for the rat.

One important regulatory mechanism that has been identified in the human is hypermethylation of the promoter region. It has been reported that the human $p16^{c6kn2a}$ 5'UTR and promoter has a CpG island (12-14). We identified a CpG island located within the rat 5'UTR and promoter using CpGProD (http://pbil.univ-lyon1.fr/software/cpgprod.html). We repeated this analysis on human and mouse and in both cases found that they reside in the same portion of the $p16^{c6kn2a}$ 5'UTR and promoter region. The identified CpG island includes the translational and transcriptional start sites and conserved transcriptional factor binding sites.

Using the above homologies to mouse and human regions with known and predicted function we chose next to compare the location of the polymorphisms we identified in the rat in relation to these sites. The -48 C/A polymorphism is located near the Sp1 transcription factor binding site. In addition, comparing $p16^{c6kn2a}$ promoter and 5'UTR sequences in human we identified a similar polymorphism located in the same position relative to the Sp1 site. In addition, the -393 and -396 G/C polymorphisms lie within the predicted transcriptional start site of the rat and the -393 G/C polymorphism is the residue that has been predicted to be the transcriptional start site in rat. This polymorphism may change the location of the transcriptional start site in the ACI rat relative to the other three strains. This polymorphism also alters the location of a CpG site which also may affect transcriptional initiation. A similar polymorphism exists in human. In addition, the BN sequence has an additional two CpG sites (-400 C/T and -1010 C/T polymorphisms) than does the ACI, COP and DA rat strains, however only the -400 C/T polymorphism lies within the predicted CpG island.

We have begun to prepare a manuscript that reports the comparison of these four sequences to each other. In addition, we plan to include the comparison of the rat sequences to mouse and human and discuss the polymorphisms which fall in similar regions of transcriptional control in this manuscript.

**mRNA expression of $p16^{c6kn2a}$ is reduced in tumor tissues in comparison to nontumor tissues.**

We have started analyzing mRNA expression and methylation of the promoter as outlined in Task 2 and Task 4 in the Statement of work in mammary tissue samples currently available in the Shull Lab. This was done to help develop the experimental approaches necessary to complete these tasks before we begin the animal experiments outlined in Task 1, 2, 4 and 5. More importantly, this will establish if whether $p16^{c6kn2a}$ message is down regulated in tumor tissues in comparison to nontumor tissue from the same rats.

To determine whether the observed changes in protein expression was due to a change in $p16^{c6kn2a}$ gene expression we used RT-PCR to probe for expression levels of the gene. RNA was isolated from adjacent non-tumor and tumor mammary tissues derived from the same set of E2 treated ACI rats where possible. In total we isolated RNA from 6 non tumor samples from four rats and 24 tumor samples from 18 rats. In some cases multiple non-tumor and /or tumor tissue were analyzed from the same rat. We used primers that amplified from exon 1 of $p16^{c6kn2a}$ to exon 3 and were specific for $p16^{c6kn2a}$. Qualitative RT-PCR as shown in Figure 1A and C indicates that expression levels of $SI5$, a
Figure 1. RT-PCR analysis of \( p16^{cdk2a} \) in non-tumor and tumor mammary tissue for 6 rats and tumor tissues from 12 additional rats. \( S^t \) message was used as a positive control (non-tumor, panel A and tumor, panel B). Samples from the non-tumor mammary tissue expressed \( p16^{cdk2a} \) (panel C) while those from tumor samples from the mammary tissue of the same rats did not (Panel D). M is the molecular weight marker. Numbers above lanes refer to the number of rat used. The same number above multiple lanes indicates that multiple tissue samples from the same rat were used. Sample 1 is control RNA from mouse provided by Ambion for use with the Reverse Transcript RTPCR kit. Sample 2 is spleen tissue from an ACI rat.

Differences in Methylation of DNA from non-tumor and tumor tissue from E2 treated ACI rats. DNA was isolated from six tumor and four non-tumor samples from five E2 treated ACI rats using Trizol (Gibco-Brl). Four of the rats had DNA isolated from tumor and non-tumor tissue and the fifth rat had DNA isolated from two different tumors.

DNA (1.35 ug) was bisulfite treated using the EZ DNA Methylation Kit (Zymo Research). Primers were used to amplify two overlapping portions of the \( p16^{cdk2a} \) promoter region including the putative transcriptional start site and known transcription factor binding sites. As a positive control for methylation, DNA was treated with the enzyme Sss1 Methylase (Promega) that methylates all CpG sites. As a negative control, DNA from spleen tissue from each of the rats was used.

The results from the methylation analysis of the promoter and 5'UTR region of \( p16^{cdk2a} \) was obtained from at least ten clones from three separate bisulfite treatment reactions. Our results indicate that tumor samples were more methylated than the corresponding nontumor sample from the same rat. In addition, the putative transcriptional start site (sites 4 and 5) was not methylated in the nontumor samples, but was methylated in tumor samples (Figure 2). However, in other areas of the promoter we show little difference between nontumor and tumor samples (Figure 2, sites 1, 2, and 3). The positive control and negative controls for these experiments gave the expected results (data not shown).

We are currently using statistical software to analyze this data to determine which differences in methylation are significance. We are also comparing what is known about \( p16^{cdk2a} \) methylation in humans and in other rat tumors to our results in order to prepare a manuscript on these findings.
Key Research Accomplishments
- Sequenced 1900 bp of the 5′UTR and promoter region of $p16^{cdkn2a}$ in four strains of rats.
  - Identification of 16 polymorphisms in four strains of rats.
  - Compared rat sequence to mouse and human
  - Compared location of polymorphisms between strains.
- Used RT-PCR to determine expression levels of $P16^{cdkn2a}$ in nontumor and tumor tissue
- Used Bisulfite sequencing to determine methylation levels and pattern in nontumor and tumor tissue.

Reportable Outcomes
1. Genebank Submissions (See Appendix)
   - Accession No. AY376712. Rattus norvegicus strain ACI/SegHsd p16 cyclin dependent kinase inhibitor 2a (Cdkn2a) gene, promoter region and partial cds.
   - Accession No. AY376713. Rattus norvegicus strain BN/ssNHsd p16 cyclin dependent kinase inhibitor 2a (Cdkn2a) gene, promoter region and partial cds.
   - Accession No. AY376714. Rattus norvegicus strain COP p16 cyclin dependent kinase inhibitor 2a (Cdkn2a) gene, promoter region and partial cds.
   - Accession No. AY376715. Rattus norvegicus strain DA/OlaHsd p16 cyclin dependent kinase inhibitor 2a (Cdkn2a) gene, promoter region and partial cds.

2. Abstracts
   - Beckerbauer, LM, B. Xie, LK Buckles, JD Shull. Potential Early Role of $p16^{cdkn2a}$ in Estrogen-Induced Mammary Carcinogenesis in the ACI Rat. 2003 Proceedings of the American Association for Cancer Research. (See Appendix)

3. Manuscripts
Conclusions
We have sequenced the $p16^{cdkn2a}$ promoter in four strains of rats and identified 16 different polymorphisms between the strains. Comparing these sequences to known mouse and human sequences we are able to make predictions about the regulation of this gene, which can be further investigated. In addition, a few of the polymorphisms identified in rat have been localized to regions where polymorphisms have been identified in human and therefore may indicate sites in human where differences in regulatory regions may play a role in the etiology of mammary cancer. Several of the polymorphisms add and change the location of CpG sites which may methylate differently and affect the down regulation of $p16^{cdkn2a}$. In addition, our data shows that $p16^{cdkn2a}$ is down regulated in mammary tumors from E2 treated ACI rats. This down regulation is similar to that found in human mammary tumors (1). In addition, data from bisulfite sequencing shows that hypermethylation occurs on the $p16^{cdkn2a}$ promoter and 5’UTR. This could then lead to the down regulation of $p16^{cdkn2a}$.

Further research (Parts of tasks 1, 2, 4 and 5) will focus on determining the timing of this down regulation, we plan on studying the expression and methylation status of $p16^{cdkn2a}$ at early time points of tumor formation, especially in lobular hyperplasia and atypical hyperplasia. In addition to studying the ACI rat we plan on doing the same assays in tissues derived from COP and BN rats. We can then make a correlation between the polymorphisms and methylation and expression status. This will allow us to determine if in the rat these polymorphisms have a functional consequence. If a functional consequence is determined, this may then hold true for E2 induced mammary cancer in humans.

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LOCUS       AY376715                        2060 bp DNA linear ROD 01-MAR-2004
DEFINITION  Rattus norvegicus strain DA/OlaHsd p16 cyclin dependent kinase
            inhibitor 2a (Cdkn2a) gene, promoter region and partial cds.
ACCESSION  AY376715
VERSION     AY376715.1 GI:39653670
KEYWORDS    .
SOURCE      Rattus norvegicus (Norway rat)
            Rattus norvegicus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae;
            Rattus.
REFERENCE   1 (bases 1 to 2060)
            Bartsch,L.M. and Shull,J.D.
            Identification of polymorphisms in the p16Cdkn2a 5'UTR and promoter
            sequence in four inbred rat strains
            JOURNAL   Unpublished
            REFERENCE  2 (bases 1 to 2060)
            Bartsch,L.M. and Shull,J.D.
            Direct Submission
            JOURNAL   Submitted (29-AUG-2003) Genetics, Cell Biology and Anatomy,
            University of Nebraska Medical Center, 985805 Nebraska Medical
            Center, Omaha, NE 68198, USA
FEATURES   Location/Qualifiers
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IDENTIFICATION AND CHARACTERIZATION OF 5'UTR AND PROMOTER POLYMORPHISMS IN P16^{cdkn2a} IN FOUR STRAINS OF INBRED RATS

Bartsch, Lois M, and Shull, James D.
Department of Genetics, Cell Biology, and Anatomy. Eppley Cancer Institute. University of Nebraska Medical Center, Omaha NE.

We have shown that the ACI rat is highly susceptible to 17B-estradiol (E2)-induced mammary carcinoma, while the genetically related COP and the unrelated BN rat are not. We have also mapped to rat chromosome 5, Emcal, a genetic determinant of E2-induced mammary cancer in crosses between the ACI and either the COP or BN rats. P16^{cdkn2a}, a cell cycle regulator of the G1-S transition, maps within Emcal. The expression of p16^{cdkn2a} is commonly down regulated in breast cancer in humans. Previous data from our lab has shown that p16^{cdkn2a} protein is down regulated in focal regions of atypical hyperplasia. To begin to study any possible changes in gene regulation of p16^{cdkn2a} between the ACI, BN and COP rats, we sequenced the 5'UTR and promoter region in four inbred rat strains. In total we sequenced 1900 bp of sequence that encompasses the 5'UTR and promoter of p16^{cdkn2a} in the ACI, BN, COP and DA rats. We identified 16 polymorphisms between the four strains. We hypothesize that one or more of these polymorphisms alter the regulation of this gene in different strains of rats through changes in the frequency or timing of methylation. We have compared these sequences to the known mouse and human sequences to identify regions of the 5'UTR and promoter regions for similarities. In addition, we have used one of these polymorphisms to correlate p16^{cdkn2a} genotype with the incidence of E2-induced mammary carcinoma in a phenotypically defined (BN x ACI) F2 population. We have also begun to investigate how these polymorphisms may explain the phenotype differences in the susceptibility of the different strains of rats to E2-induced breast cancer with regard to transcriptional initiation and methylation of the promoter. Together, these data, strongly suggest that p16^{cdkn2a} plays an early and contributory role in E2-induced mammary carcinogenesis in the ACI rat. Supported by NIH grant R01-CA77876, training grant DAMD17-00-1-0361 from the DOD, DOD grant DAMD17-03-1-0466.
Potential Early Role of \( p16^{cdkn2a} \) in Estrogen-Induced Mammary Carcinogenesis in the ACI Rat

Lois M Beckerbauer, Benjamin Xie, Linda K Buckles, James D. Shull, Eppley Cancer Institute, University of Nebraska Medical Center, Omaha, NE

We have shown that the female ACI rat is highly susceptible to mammary carcinoma after prolonged exposure to 17\( \beta \)-estradiol (E2), while the genetically related Copenhagen (COP) rat and the unrelated Brown Norway (BN) rat are not. More recently, we have mapped to rat chromosome 5, \textit{Emca1}, a determinant of susceptibility to E2-induced mammary cancer in F2 progeny from crosses between the ACI and COP or BN strains, \textit{Cdkn2a} maps within \textit{Emca1}. \( p16^{cdkn2a} \), one of at least two distinct products of \textit{Cdkn2a}, regulates the G1 to S transition, and expression of this protein is commonly lost in breast cancers. Data from our laboratory indicate that expression of \( p16^{cdkn2a} \) is significantly diminished in both focal regions of atypical hyperplasia and mammary cancers induced in ACI rats by E2, compared to the adjacent epithelium. To define further the role of \( p16^{cdkn2a} \) in E2-induced mammary carcinogenesis, we are sequencing this gene from the ACI, COP and BN rat strains. Although no nucleotide polymorphisms have been identified within the coding region, several have been identified within the 5' flanking region. We hypothesize that these putative promoter polymorphisms may impact transcriptional regulation of \( p16^{cdkn2a} \) and/or the probability that this gene will be transcriptionally silenced due to promoter methylation. Preliminary data suggest that \( p16^{cdkn2a} \) mRNA expression is altered during E2-induced mammary carcinogenesis and that the promoter becomes methylated. A large phenotypically defined F2 population from a cross between BN females and ACI males was genotyped at one of the \( p16^{cdkn2a} \) polymorphisms. Whereas 100\% of F2 rats homozygous for ACI alleles at \( p16^{cdkn2a} \) were mammary cancer positive following 196 days of E2 treatment, only 30\% of those rats homozygous for BN alleles were tumor positive at this time point. Together, these data strongly suggest that \( p16^{cdkn2a} \) plays an early and contributory role in E2-induced mammary carcinogenesis in the ACI rat. Supported by NIH grant RO1-77876 and training grant DAMB17-00-1-0361 from the DOD.