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PRINCIPAL INVESTIGATOR: Kevin J. Cullen, M.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057

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13. ABSTRACT *(Maximum 200 words)*

Our hypothesis is that DNA methyl transferase expression is inversely correlated with IGF-II expression in normal and tumor tissues. That is, tumors with high DNA methyl transferase expression will imprint IGF-II, resulting in reduced IGF-II expression, while tumors with low DNA methyl transferase will have higher relative IGF-II expression. We have demonstrated that DNA methyl transferase expression can be measured readily using RNase protection assay in representative breast cancer cell lines. Sahana Kaup is continuing her graduate studies based on her findings along the outline discussed above. At the present time, her preliminary data have not confirmed her fundamental hypothesis, but further experiments which are currently underway will determine whether or not her original hypothesis remains worthy of investigation.

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Kevin JCudd   Sept 30, 1998
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PI - Signature   Date

Kevin J. Cullen, M.D.
Grant DAMD 17-94-J-4167
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November, 1998

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Project Title: The Effect of DNA Methylation on IGF-II Expression
Graduate Student: Sahana Kaup
Research Mentor: Kevin J. Cullen, M.D.

Introduction:

This will serve as an annual and final reporte for funding provided to graduate student Sahana Kaup to study of the effect of DNA methylation on IGF-II expression. Of note, this award was originally granted to Dr. Francis Kern. Dr. Kern left Georgetown University in 1997. This award was transferred to Dr. Cullen one year ago, and so this report will cover only the work done during the last year. Neither Dr. Cullen nor Sahana Kaup participated in the research funded during the three years of this funding was awarded to Dr. Kern.

The purpose of this one-year graduate student project was to determine the role of DNA methylation in the expression of IGF-II in normal and malignant stromal and epithelial cells within human breast cancers. Our hypothesis for this proposal is that abnormal methylation of IGF-II is responsible for regulation of expression of this gene product in normal and malignant breast tissue. Specifically, in the past, we have demonstrated that IGF-II expression is seen in the stroma of breast cancers under the influence of interactions with soluble factors produced by breast epithelial cells. This phenomenon is tumor specific. Malignant breast epithelial cells cultured in the presence of normal breast stromal cells produced a marked upregulation of expression of IGF-II in breast stroma.[1]

Work in other malignancies (such as pediatric Wilms tumor) has demonstrated that in those cancers, IGF-II expression is regulated through the process of DNA methylation. Specifically, in humans, during adult life, IGF-II is expressed solely from the paternal allele. The maternal allele is silenced. This appears to be the results of binding of methyl residues to specific regions within the IGF-II locus through the action of the enzyme DNA methyl transferase.

Our hypothesis is that DNA methyl transferase expression is inversely correlated with IGF-II expression in normal and tumor tissues. That is, tumors with high DNA methyl transferase expression will imprint IGF-II, resulting in reduced IGF-II expression, while tumors with low DNA methyl transferase will have higher relative IGF-II expression.

Body

The cDNA for DNA methyl transferase (DNMT) was obtained from Dr. Stephen Baylin, Johns Hopkins University, Baltimore MD. The coding sequence for DNA methyl transferase was a sub-cloned into a riboprobe vector (Promega) using standard like ligation techniques. The sequence was confirmed in the sub-cloned fragment using standard direct fluorescent sequencing methodology.

Once the sequence for the DNA methyl transferase had been confirmed in the riboprobe

vector, RNase protection assay was used to analyze DNA methyl transferase mRNA expression in a series of breast cancer cell lines. Thirty micrograms above total RNA were isolated from representative cell lines using guanidinium ultracentrifugation. The P32 labeled riboprobes for DNA methyl transferase, gamma actin (a loading control), and IGF-II were prepared as previously described.[2] The three radiolabelled riboprobes were placed in a cocktail and hybridized against thirty micrograms of total RNA. A representative result is shown in figure 1. Briefly, IGF-II expression was seen at easily detected levels in the expected cell lines (T47D, clone 8.) There was a range of DNA methyl transferase expression seen in these cell lines, but at least in the epithelial cell lines, there was no distinct correlation between IGF-II expression and DNA methyl transferase expression on and are mRNA level. Further experiments are underway at the present time to evaluate DNA methyl transferase expression in additional cell lines including stromal fibroblasts. Additionally, we are currently examining DNA methyl transferase expression in T- 61 tumors. This is an estrogen regulated human breast tumor xenograft which expresses abundant IGF-II and whose growth is dependent on IGF-II expression.[3] Previous studies have demonstrated that exposure to physiological levels of estrogen result in down regulation of IGF-II expression in these tumors and inhibition of tumor growth. We believe that this will be an ideal system to analyze DNA methyl transferase expression and its relation to IGF-II.
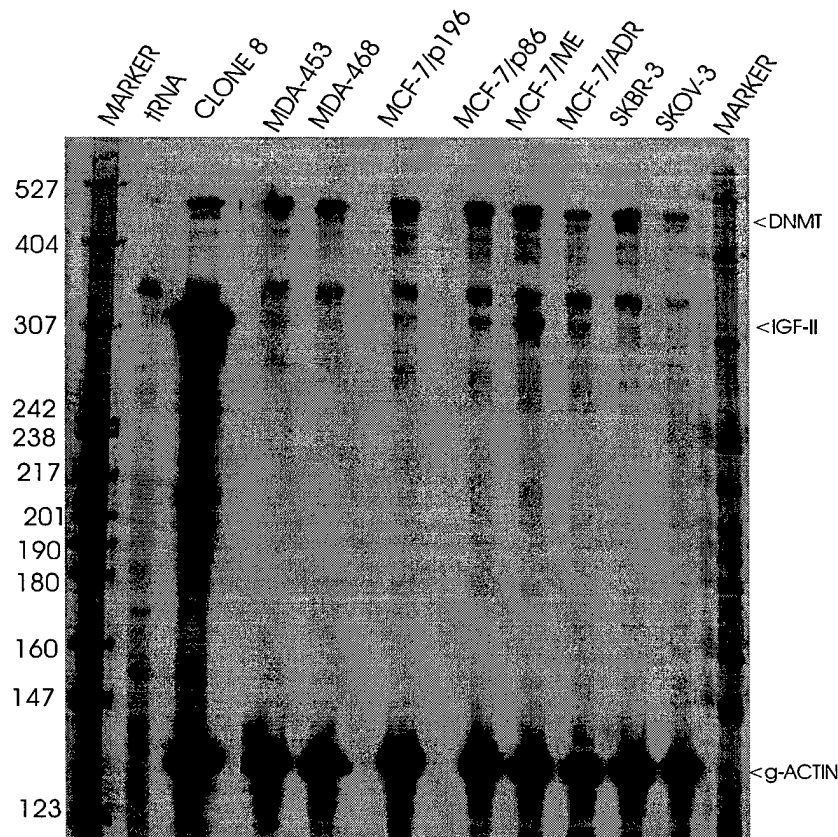

Figure 1. Detection of DNA methyltransferase (DNMT) by RNAse protection assay. A 2.5 kb fragment of DNMT cDNA cloned into a pGEX3X vector was kindly provided by Dr. Steve Baylin. A 1.4 kb

fragment was excised by PvuII restriction endonuclease and inserted into a PvuII/EcoRV site created in a pSP72 vector. The DNMT riboprobe is generated by digesting the resultant construct with HincII restriction endonuclease and transcribing from the SP6 promoter. The unprotected fragment is 468 bp long; the protected fragment is 456 bp long.

Preliminary RNAse protection assays have been done with RNA samples from various cell lines. These include MCF-7, MDA-231, MDA-453, MDA-468, SKBR-3, and T47-D breast epithelial cancer cells and SK-OV3 ovarian cancer cells. In addition, RNA was isolated from MCF-7 cells transfected with IGF-II expression plasmid (clone 8) as well as T61 tissue samples. The RNA samples were probed with the DNMT riboprobe described above, as well as IGF-II and gamma-actin riboprobes. The IGF-II riboprobe protects a 336 bp RNA fragment and the protected gamma-actin fragment is 130 bp.

The following figure is a representative 6% RNA polyacrylamide gel. The RNA samples used are T47D, MDA-453, MDA-468 and SKBR-3 breast epithelial cell lines. Four different MCF-7 breast epithelial cell lines (MCF-7/p196, MCF-7/p86, MCF-7/ME, and MCF7/ADR) as well as SKOV-3 ovarian cancer cell line were studied. The RNA sample designated "clone 8" is from a MCF-7 cell line transfected with an IGF-II expression vector.

The results demonstrate that the hybridization conditions are sufficient to ensure a legitimate estimation of DNMT, IGF-II and gamma-actin RNA levels in these RNA samples. This is shown by the similar densities of gamma-actin protected fractions in each cell line as well as the heavy band resulting from the protection of the IGF-II component in the "clone 8" sample. Since this density primarily represents the ectopic expression of IGF-II in this cell line, a direct correlation to DNMT expression can not be drawn. However, this gel shows that DNMT mRNA is expressed to commensurate levels in these cell lines. Ms Kaup is currently continuing this procedure in various cancer cell lines to optimize experiment conditions.

At the present time, the DNA methyl transferase coding sequence has been cloned into an expression vector which we have used previously in studies examining the effects of expression of IGF-II. We will use this expression vector to overexpress DNA methyl transferase in target cell lines. We expect that over expression of DNA methyl transferase will results in silenceing of IGF-II expression in appropriate target cell lines. In addition, experiments which will inhibit DNA methyl transferase action through the use of the chemotherapy agent, 5 azacytidine (a specific inhibitor of DNA methyl transferase), are underway.

Conclusion:

We have demonstrated that DNA methyl transferase expression can be measured readily using RNase protection assay in representative breast cancer cell lines. Sahana Kaup is continuing her graduate studies based on her findings along the outline discussed above. At the present time, her preliminary data have not confirmed her fundamental hypothesis, but further experiments which are currently underway will determine whether or not her original hypothesis remains worthy of investigation.

Publications supported in part by this award:

1. Cullen KJ, Kaupa SS, Rasmussen AA. Interactions between Stroma and Epithelium in Breast Cancer: Implications for Tumor Genesis, Growth and

Progression. In Manni A (ed) <u>Endocrinology of the Breast</u> Boston, Kluwer Publishers, In press, 1998

NB - Sahana Kaup was the sole person supported by this grant for the period it was assigned to Dr. Cullen (September 97 -September 98)

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