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13. ABSTRACT (Maximum 200 This proposal was designed to determine if the ataxia-telangiectasia (AT) gene acts as a tumor suppressor gene in a subset of breast cancers by looking for tumor loss of constitutional heterozygosity at chromosomal region 11q22-q23, the map position of the AT gene. The technical aims were to analyze 300 matched pairs of tumor and normal tissue from breast cancer patients for tumor loss of constitutional heterozygosity using a panel of amplification polymorphisms mapping to 11q22-q23 and, when loss of heterozygosity is detected, to map the extent of that loss. The cloning of the ATM (AT, mutated) gene reported in June 1995 allows identification of ATM mutations and polymorphisms and consequently allows direct testing of the hypothesis that women who carry these variants of ATM (AT carriers) are at greater risk for developing breast cancer. During the August 1995 to August 1996 funding period we developed antibodies directed against the ATM gene product and began development of an assay for the detection of AT carriers based on testing RNA isolated from peripheral blood lymphocytes. With the support of the Division of Radiotherapy, we are planning a large, prospective study aimed at determining if AT carrier status increases susceptibility to breast cancer or to normal tissue damage from radiotherapy.				
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FOREWORD

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Michael M. Weil 3/1/97
PI - Signature Date

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Introduction

The work proposed in this application was designed to determine if the gene responsible for ataxia-telangiectasia (AT) acts as a tumor suppressor gene in the development of breast cancer. The original approach was to survey breast cancers for tumor loss of constitutional heterozygosity in 11q22-q23, the chromosomal region where AT maps. A conceptual framework for the project was provided by epidemiological studies showing increased breast cancer risk in women who are obligate AT carriers.

The frequency of AT carriers in the U. S. general population is estimated to be 1.4% and they may compose 9 to 18% of breast cancer patients. Their putative increased breast cancer risk is based on studies of women who have AT affected children and are thus obligate carriers and blood relatives of AT patients who have an increased probability of being carriers.

Deletion of genetic material is a frequent occurrence in tumors. Generally, the loss is detected by surveying normal and tumor tissue from the same individual for a selected, mapped restriction fragment length polymorphism by Southern hybridization analysis. A finding that normal tissue has two different alleles and the tumor has lost one of these indicates a loss of genetic material and is referred to as tumor loss of constitutional heterozygosity (LOH) or reduction to hemizyosity. Consistent LOH for a chromosomal region in a tumor type implies the existence of a tumor suppressor gene at that location. At the time this proposal was made there were no comprehensive LOH studies focusing on 11q22-23 and involving large numbers of breast cancer samples reported in the literature.

During the first year of the project, 37 sets of tumor and normal tissue in pathology storage as paraffin embedded samples were screened for 14 polymorphic loci based on simple sequence repeats (SSRs) using the polymerase chain reaction (PCR). Seventeen of these tumors were found to have lost heterozygosity either in the region of the AT gene or an adjacent region that might overlap. All but one of these deletions appear to be interstitial.

In mid 1995 a partial cDNA derived from the ATM (AT, mutated) gene was cloned by Shiloh and his colleagues (Savitsky et al. 1995a). Mutations in this gene are responsible for AT. The cloning of ATM presented an opportunity to directly test breast cancer patients for mutations in this gene. During the August 1995 to August 1996 funding period we began the development of assays to detect ATM mutations. Since the entire gene was not yet cloned we started by generating antibodies directed against the ATM gene product. Once the complete coding sequence of the gene was known we began development of an assay for the detection of AT carriers based on testing RNA isolated from peripheral blood lymphocytes.

Body

Preparation of antibodies to the ATM gene product

Overview

Rabbit polyclonal antiserum was generated to a recombinant ATM protein produced in a bacterial expression system. This antiserum was tested for reactivity with ATM protein by Western blotting, immunohistology and immunoprecipitation.

Primer Design

At the initiation of this phase of the project the characterized coding region of the ATM message was about 5100 bases. Using the computer program Oligo (National Biosciences, Plymouth, MN) we have designed PCR primer sets that allowed us to amplify this region as 4 overlapping cDNAs ranging in size from 1264 to 1649 basepairs. These cDNAs encode polypeptides of 421 to 500 amino acid residues.

Molecular Cloning

Total RNA was prepared from Ficoll-Hypaque separated peripheral blood lymphocytes. The RNA was reverse transcribed using random hexamers as primers and the cDNA was PCR amplified with the four primer sets. Each cDNA was cloned, in frame, into an expression vector (pET-32, Novagen, Inc., Madison, WI) which allows it to be expressed as a fusion protein in *E. coli*. The fusion protein contains vector encoded sequences which are used to facilitate its detection, solubility and purification. Transcription of the gene encoding the fusion protein is tightly regulated and can be induced by addition of IPTG (isopropyl-B-D-thiogalactopyranoside) to the culture media.

ATM-2 Purification and Antibody Production

One of the fusion proteins, ATM-2, was purified for use as an immunogen. When expressed as a fusion protein in *E. coli* large amounts of ATM-2 accumulate as insoluble inclusion bodies in the periplasmic space. This material was partially purified from cell lysates by centrifugation and SDS polyacrylamide gel electrophoresis (PAGE). Gel slices containing ATM-2 fusion protein were emulsified in adjuvant at the M.D. Anderson Cancer Center's Department of Veterinary Science facility at Bastrop and injected twice into a rabbit. A third immunization was made using soluble, affinity purified ATM-2 fusion protein with the objective of increasing the titer of antibodies useful for immunoprecipitation. Bacteria producing ATM-2 fusion were lysed by sonication and the cell debris and fusion protein inclusion bodies were collected by centrifugation. The fusion protein was solubilized in tris-glycine buffer containing 20% methanol and affinity purified on a metal chelation resin by virtue of a vector encoded polyhistidine sequence on its amino terminus.

Affinity Purification of anti-ATM-2 Antibody

Soluble, affinity purified ATM-2 fusion protein was immobilized on a nitrocellulose membrane and used to bind anti-ATM-2 antibodies. After the filter was washed extensively with a wash buffer (10 mM Tris HCl, 0.5 M NaCl, 0.05% Tween 20, 0.5% NP40, pH 7.5), the bound

antibodies were recovered by elution with a low pH buffer (MAPS II Elution Buffer, Bio-Rad Laboratories, Richmond, CA). The eluted material was adjusted to pH 7.5 and 1 mg/ml BSA was added as a carrier protein. The affinity purified antibody was dialyzed against TBS (10 mM Tris HCl, 150 mM NaCl, pH 7.5).

Western Blot Assay

Proteins separated by SDS polyacrylamide gel electrophoresis were electrophoretically transferred to hybridization membranes (Hybond C, Amersham Corp., Arlington Heights, IL) and washed with TBS-Tween 20 (TBS containing 0.05% Tween 20). Remaining protein binding sites on the membrane were blocked with TBS-Tween 20 containing 10% fetal bovine serum. The hybridization membrane was incubated with affinity purified antibody diluted in fresh blocking solution. The membrane was washed with TBS-Tween 20 and then incubated with a secondary antibody, alkaline phosphatase conjugated mouse anti-Rabbit Ig. Chromogenic detection was accomplished by incubation of the membrane with Bromochloroindolyl Phosphate/nitro Blue Tetrazolium substrate.

Anti-ATM-2 Antibody Results

The figure in Appendix A indicates that the ATM-2 fusion protein is produced in the bacterial system (panel A) and is mostly insoluble (panel B). The purified antibodies detect the ATM fusion protein in a western blot (panel C). The purified antibodies also detect a protein of >200kd in a western blot of protein from lymphoblastoid cell lines (data not shown) however, they do not detect ATM protein in lymphocytes or lymphoblastoid cell lines by immunohistochemistry and do not immunoprecipitate ATM protein.

Development of an assay for ATM mutations and polymorphisms

Overview

Two factors motivated us to focus on developing a nucleic acid rather than immunoassay-based test for detection of ATM mutations. The first was that the complete coding sequence of the ATM message became available and that made the development of an RNA or DNA assay technically feasible (Savitsky et al. 1995b). The second was a report that suggested that frank constitutional ATM mutations may not be common in breast cancer but also revealed ATM polymorphisms that would result in amino acid substitutions in several breast cancer patients (Vorechovsky et al. 1996). These polymorphisms should be taken into account in a study examining the role of ATM in breast cancer susceptibility and they are currently only detected by RNA or DNA assays.

The detection of mutations and polymorphisms in the ATM gene presents a considerable technical challenge because of the large number of exons comprising the gene and the sheer size of the ATM transcript. The ATM gene consists of at least 65 exons and is transcribed and processed into a 12 kb mRNA of which just over 9 kb encodes amino acid sequence. Naturally

occurring variations that might result from differential splicing or a highly variable 5' UTR are not yet well characterized. In addition, the mutations described so far tend to be single base changes and small insertions or deletions that are scattered throughout the coding region.

There are no robust, inexpensive, "off the shelf" technologies that will detect random, single base pair changes in a large gene. Automated DNA sequencing is now capable of detecting single base changes in heterozygous individuals but the cost is too high for use as a screening test.

The most rigorous approaches for ATM mutation detection reported to date are polymerase chain reaction single strand conformational polymorphism (PCR-SSCP) analysis of genomic DNA and restriction endonuclease fingerprinting (REF) (Liu and Sommer 1995) of the message. Application of the PCR-SSCP to large numbers of samples of a 65 exon gene is a massive and expensive undertaking, although multiplexing and eliminating some exons as regions that could harbor consequential mutations may one day make it the method of choice, REF analysis of a 9 kb mRNA is by no means trivial but it is slightly less cumbersome than PCR-SSCP and has the advantage of a higher detection rate. A disadvantage is the use of RNA rather than DNA as the starting material, since RNA is more susceptible to degradation and cannot be retrieved from archival samples.

Over 80% of known ATM mutations result in protein truncations and this has lead to suggestions that the in vitro protein truncation test (PTT) (Telatar et al. 1996) could be used as a primary screen for ATM mutations in studies of tumor susceptibility and radiosensitivity. However, almost all of the known mutations were characterized from individuals with AT. There is no reason to assume that all ATM mutations result in AT and that those that do are the only ones that could affect cancer susceptibility or sensitivity to ionizing radiation. The PTT assay also fails to detect polymorphisms in the gene that may influence these endpoints

We have elected to use REF as a primary screen and sequencing for confirmation of any mutations and polymorphisms detected. Alternative screening strategies would employ RNA mismatch detection or CFLP (cleavase fragment length polymorphism). RNase mismatch detection has been complicated by the need to expand the sample the peripheral blood lymphocytes. CFLP is a very new technique that has not been reliable in our hands.

REF Methods

In the REF assay, the ATM message in total RNA from peripheral blood lymphocytes is reversed transcribed and the coding sequence is PCR amplified as 8 overlapping amplicons, each 1.2 to 1.6 kb in size. Each RT-PCR product is split into several aliquots which are digested with different combinations of restriction enzymes and radiolabeled with ³³P. The aliquots are then recombined and analyzed by SSCP. This procedure converts a PCR product that is larger than 1 kb into a pool of DNA fragments in a size range amenable to SSCP analysis. Since a potential mutation will be contained in several fragments (one for each restriction enzyme) rather than in a single PCR product, the detection rate approaches 100%.

The eight pairs of PCR primers were designed from the published ATM cDNA sequence. Reverse transcription and amplification of total RNA using these PCR primers results in a series of overlapping PCR products that span the length of the ATM message. The PCR products range in length from 1200 - 1600 basepairs. These amplification products are purified by agarose gel electrophoresis and recovered from the gels. The DNA is digested in separate reactions with a combination of restriction enzymes, each of which recognize and cleave DNA at a specific four base pair sequence. The combination of enzymes used is dependent on the amplification product and has been determined empirically. Restriction enzyme generated fragments are then radiolabeled by removing the 5' phosphate groups with calf intestine alkaline phosphatase and using polynucleotide kinase to replace them with ^{33}P from $[\gamma\text{-}^{33}\text{P}]\text{-ATP}$. The labeled fragments are denatured by heating in formamide, separated at room temperature or 8°C on a non-denaturing MDE gel (FMC BioProducts) in a sequencing gel formate and detected by exposure of the dried gel to a storage phosphor cassette which is scanned on a PhosphorImager (Molecular Dynamics) for computer imaging and analysis. If required, an image can also be generated using x-ray film.

REF Assay Results

Lymphoblastoid cell lines derived from individuals who are normal, obligate AT heterozygotes and AT homozygotes were used to validate the system. The results (Appendix B) provide preliminary evidence for the ability of REF to identify SSCPs in the ATM transcript. We have tested all of the 8 primer sets and screened the resulting PCR products against a battery of restriction enzymes to determine the best combination of PCR product and restriction enzymes. Other polymorphisms have been identified in additional homozygous and heterozygous cell lines using different primer pairs.

Conclusions

1. We have generated an antiserum that detects ATM protein in Western blot assays. This antiserum does not immunoprecipitate ATM protein and does not detect ATM protein in an immunohistology assay which suggests that it recognizes epitopes only on the denatured protein. In view of this limitation we see little use for the antiserum in accomplishing the goals of this project.
2. The REF assay can be used to screen for ATM mutations and polymorphisms in total RNA isolated from peripheral blood lymphocytes. Several mutations or polymorphisms have been detected and now must be confirmed by sequencing. The availability of this assay allows an epidemiological study of the association between ATM mutations and polymorphisms and susceptibility to breast cancer or susceptibility to normal tissue damage from radiotherapy.

3. The granting period for this project has been extended for one year without the allocation of additional funds. This extension will be used to developing sequencing techniques to confirm the REF results and to organize a collaborative effort to undertake the epidemiological study.

References

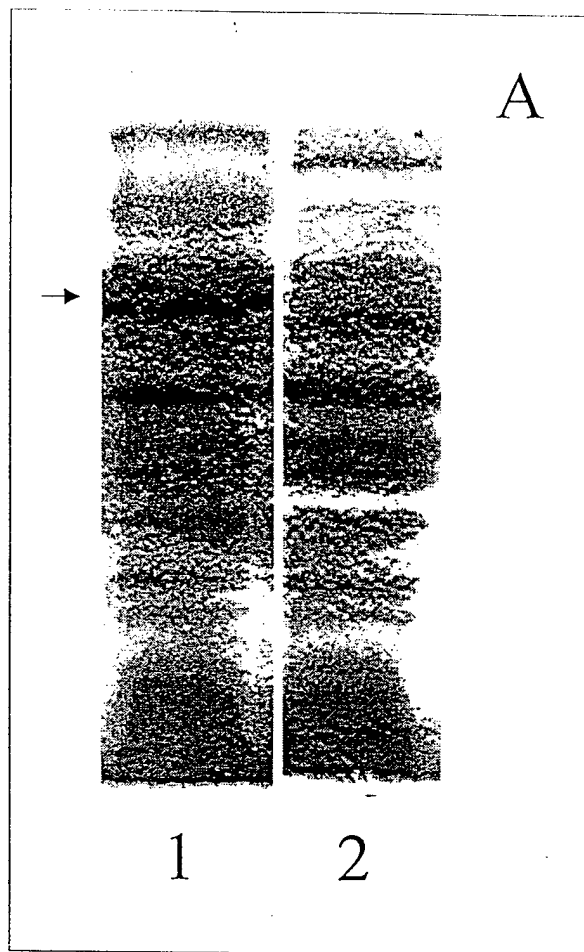
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Appendix A.

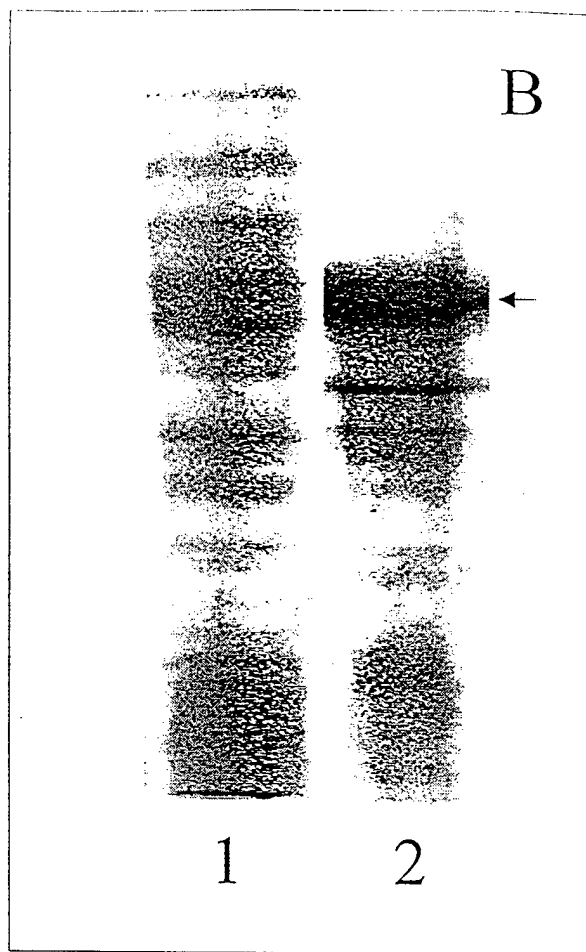
Figure legend. Panel A. Coomassie brilliant blue stained polyacrylamide gel of bacterial lysates from *E. coli* strain BL21(DE3)pLyseS carrying the pET-32 expression vector encoding the ATM-2 fusion protein (lane 1) or the same strain without the plasmid (lane 2). The position of the fusion protein is indicated by the arrow. Panel B. Following lysis by sonication and centrifugation in a microfuge (13,000 x g) the little of the ATM-2 fusion protein is located in the supernatant (lane 1), the bulk of the fusion protein is in the pellet (lane 2). Panel C. Western blot of bacterial lysate containing the ATM-2 fusion protein probed with affinity purified anti-ATM-2 antibodies and detected with alkaline phosphatase conjugate mouse anti-Rabbit Ig. Lanes contain decreasing amounts of the lysates.

Appendix A

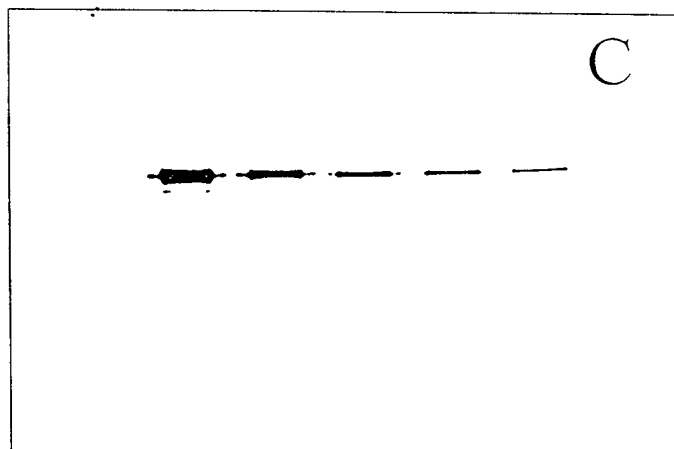
Panel A



Panel B



Panel C



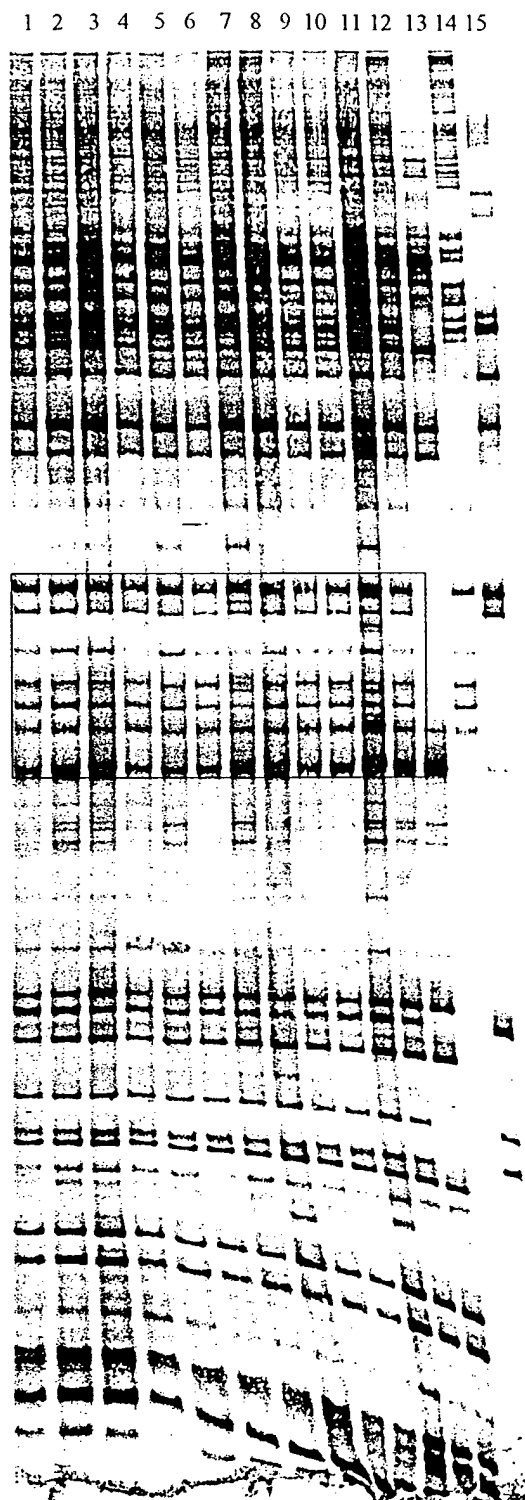


Figure Legend. Left) Computer generated image of a REF gel. Lanes 1-4 are from AT homozygotes, lanes 5-9 are from AT heterozygotes, lanes 10-12 are samples from normal cells, and lanes 13-15 are individual restriction enzyme digests from the same cells as in lane 12. Lanes 1-12 represent pooled restriction digests. Lower) Enlargement of the boxed region seen in the figure to the left. Lanes 7 and 11 contain obvious differences in the pattern of banding. Lane 7 was generated from an obligate AT heterozygote lymphoid cell line. Interestingly, lane 11 is from a blood sample taken from a "normal" volunteer whereas lanes 10 and 12 were derived from normal lymphoid cell lines.

