HRad17, a Human Homologue of the Schizosaccharomyces pombe Checkpoint Gene rad17, Is Overexpressed in Colon Carcinoma

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Abstract

Using the palindromic PCR-cDNA display method, we have cloned a novel gene overexpressed by human colon carcinoma relative to normal colon. Among normal tissues examined, only testis expresses it at a high level. Sequence analysis revealed its extensive homology with checkpoint genes rad17 of Schizosaccharomyces pombe and RAD24 of Saccharomyces cerevisiae. This novel gene designated as hRad17 is localized to chromosome 5q12,13.1, a region known to be deleted in a variety of human cancers. Promoter region and one pseudogene of hRad17 have been identified. Whereas the increased expression of hRad17 by human colon carcinomas may be related to the known resistance of these cells to DNA-damaging agents during therapy, the deletion of hRad17 in a variety of cancers may predispose them to increased rate of mutation and heightened sensitivity to DNA-damaging agents, including radiation and anticancer drugs.

Introduction

Cell cycle checkpoints are essential in eukaryotes for ensuring high fidelity transmission of genetic information from one generation to the next. They include DNA damage checkpoints, DNA replication checkpoints, spindle assembly checkpoints, and cytokinesis checkpoints (1). Cell cycle checkpoints have been best studied in yeast. A series of checkpoint genes required for DNA damage and/or DNA replication checkpoints have been identified in both fission and budding yeast (2). The rad17 gene of Schizosaccharomyces pombe is one of several essential checkpoint components including rad1, rad3, rad9, rad17, hus1, and cut5/rad4 that are absolutely required for prevention of mitosis after DNA damage in fission yeast (3). Functional loss of any one of these genes abolishes the radiation-induced G2 arrest. All of these genes except cut5/ rad4 are also required for the post replication checkpoints, which prevents mitosis in response to DNA replication blocks. It has been reported that deletion of rad17 resulted in an 8-fold increase in the rate of chromosome loss (4), suggesting a role for rad17 in the maintenance of genomic integrity (5). RAD24 in Saccharomyces cerevisiae appears to be the counterpart of rad17 in S. pombe and is one of several key checkpoint genes including RAD9, RAD17, RAD24, MEC3, MEC1, TEL1, and RAD53 that are indispensable to mitotic cell cycle arrest and transcriptional activation in response to DNA damage (1). RAD24, RAD17, and MEC1 are also required for meiotic checkpoints in budding yeast (5). It is still unknown whether all of the yeast’s checkpoint proteins have structural homologues in mammalian systems or only some of them. Significant homologies between rad3 and human ATM and ATR (6), fission yeast and human chk1 (7), and human and yeast rad1 (8), as well as numerous other pairs, have been reported. However, because the most thoroughly studied mammalian checkpoint component, p53, does not have an apparent structural homologue in yeast, it remains possible that mammals share some but not all of the checkpoint machinery found in yeast. Mutations in human checkpoint proteins such as p53, ATM/ATR, and Bub1 that have been found in tumors cannot account for the majority of expected checkpoint aberrations associated with human cancer, given the multistep hypothesis of cancer etiology. We have used a novel palindromic PCR-cDNA display method to identify a human homologue of yeast checkpoint proteins rad17 of S. pombe and RAD24 of S. cerevisiae. This novel human gene, designated as hRad17, is overexpressed by colon carcinoma relative to normal colon. Its chromosomal localization at 5q12,13.1 suggests that a variety of human cancers would have a deletion in this gene. Increased expression of this gene might be responsible for increased resistance to DNA-damaging agents by cancer cells; its decreased expression, on the other hand, could lead to higher rate of mutation as well as increased sensitivity to radiation and chemotherapy.

Materials and Methods

Palindromic PCR-cDNA Differential Display. Surgical samples of human testis and prostate were placed in liquid nitrogen as soon as possible after removal. The frozen tissue was ground into fine particles, and total RNA was prepared using the guanidine isothiocyanate method of Chirgwin et al. (9). The palindromic primer TCTTAGAAC was used with the Superscript First Strand cDNA Synthesis kit (Life Technologies, Inc.) to reverse transcribe ~100 ng of RNA from human testis and prostate specimens. This was followed by PCR (94°C, 30 s; 40°C, 1 min 40 s; 72°C, 1 min; 40 cycles) using the same primer and Taq DNA polymerase with 2 mM MgCl2 and 32P-labeled dATP. The 32P-labeled cDNA was then run on a 6% polyacrylamide gel and autoradiographed. cDNA fragments of interest were then eluted from the dried gels. The gel slice was soaked in 100 μl of distilled water for 10 min, boiled for 15 min, and spun at 14,000 rpm for 2 min. The supernatant was transferred to a fresh tube, followed by the addition of 10 μl of 3 M sodium acetate, 5 μl of glycerogen (10 mg/ml), and 450 μl of 100% ethanol. The sample was then incubated at ~70°C for 30 min and spun at 14,000 rpm for 10 min. The supernatant was removed, and the pellet was washed with 200 μl of 85% ethanol. The final sample was resuspended in 10 μl of distilled water.

PCR was performed on the eluted fragments using the same conditions described above. PCR products were then run on a 1.5% agarose gel and purified using the Qiagen II Agarose Gel Extraction kit (Qiagen, Chatsworth, CA).

Northern Blot Analysis. Gel-purified hRad17 palindromic PCR fragment was converted into 32P-labeled probes for Northern analysis using a random-primed labeling kit (Boehringer). Multiple tissue Northern blots (Clonitech)

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were hybridized with the \(^{32}\)P-labeled probe as described (10). Filters were then exposed to X-ray film.

**In Situ RNA Hybridization.** In situ RNA hybridization was performed as described previously (10). A 690-bp fragment corresponding to the COOH-terminus and 3\(^\prime\) region of hRad17 was excised using Bgl II and cloned in pBluescript (Stratagene). Riboprobes were generated with T7 and T3 RNA polymerase.

**DNA Sequencing.** Reamplified PCR products were purified from an agarose gel and subcloned into TA vectors (pCR 2.1 Invitrogen). DNA was then alkaline denatured followed by neutralization and precipitation, annealing with sequencing primers and sequencing using the Sequenase Version 2.0 kit (United States Biochemical, Cleveland, OH). Sequencing was performed on both strands of the PCR products.

**cDNA and Genomic Library Screening.** The full-length clone of hRad17 was obtained by screening a human fibroblast \(\lambda\)ZAP II cDNA (Stratagene) library using standard library screening procedures. Mouse and green monkey \(r\)ad17 homologues were obtained by screening \(3T3\) and COS cell cDNA libraries, respectively. The hRad17 genomic clones were isolated from a human peripheral blood lymphocyte genomic library. The promoter region and a pseudogene were identified after DNA sequencing.

**FISH.** Mitotic chromosome spreads of human lymphocytes were prepared according to standard procedures. The Xhol/Xba hRad17 cDNA probe was biotinylated using the BRL BioNick labeling kit. Slides were baked at 55°C for 1 h. After RNase treatment, the slides were denatured in 70% formamide in 2× SSC for 2 min at 70°C, followed by dehydration in ethanol. The probe was denatured at 75°C for 5 min in 50% formamide and 10% dextran sulfate. After overnight hybridization with the probe, slides were washed, labeled with DAPI and visualized. Assignment of map position was achieved by superimposing the FISH signal with the DAPI-banded chromosomes (11).

**Generation of Antibodies and Immunohistochemical Staining.** We overexpressed full-length HRad17 as a fusion protein with GST in Escherichia coli and purified it by binding to glutathione-conjugated beads. HRad17 was released from GST-beads by treatment with thrombin, and the HRad17-GST fusion protein was eluted by excess glutathione. Mice were immunized with purified HRad17 to produce a series of monoclonal antibodies recognizing HRad17. Detailed characterization of these antibodies will be presented elsewhere.

Specimens of normal colon, colorectal carcinoma tissues, and mouse testis were fixed in 3.7% formalin and embedded in paraffin. Routine sections were cut 5 \(\mu\)m thick, dried at 40°C for 4 h, cleared in xylene, and rinsed in ethanol. The immunohistochemical staining was performed by an automated Ventana 320/ES immunohistochemistry robot. Endogenous peroxidase was quenched with methanol/peroxide solution and blocked with normal horse serum. Sections were incubated with a diluted anti-HRad17 monoclonal antibody for 20 min. A peroxidase-conjugated secondary antibody was applied and visualized with 3,3′-diaminobenzidine as a substrate. A negative control was run simultaneously with preimmune immunoglobulins.

**Results**

The differential display method typically uses an oligo-dT(NN) primer for reverse transcription, followed by PCR with the same primer and a random 10-mer (12). The resulting amplified cDNA fragments tend to represent the 3′ untranslated regions of mRNAs. We have developed a modification of this technique that uses a single palindromic primer for both mRNA reverse transcription and subsequent PCR reactions. This modification takes advantage of our observation that palindromic sequences occur more frequently within open reading frames than in untranslated regions and with a frequency 10-fold greater than would be predicted by chance alone.\(^1\) Thus, the cDNA fragments identified are much more likely to represent the protein coding regions of genes.

This technique, termed palindromic PCR cDNA differential display, was used to identify and isolate cDNA fragments expressed differentially between normal and malignant colon tissues. The mRNAs isolated from normal colon and colorectal carcinoma tissues were reverse transcribed to cDNA and amplified by PCR, all using a single palindromic primer. After electrophoresis and autoradiography, cDNA patterns derived from the testis and prostate were compared and analyzed for differences. As shown in Fig. 1A, one cDNA fragment amplified with the palindromic primer appeared to be present at significantly higher levels in colorectal carcinoma tissue as compared to normal colon (arrow). This cDNA was recovered from the gel and re-amplified by PCR with the same palindromic primer. A

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\(^1\) The abbreviations used are: FISH, fluorescence *in situ* hybridization; DAPI, 4′,6-diamidino-2-phenylindole; GST, glutathione 3-transferase; ORF, open reading frame.

\(^2\) A. Lin and L. B. Chen, manuscript in preparation.
To confirm that the expression of the gene represented by the PCR fragment is much higher in colorectal carcinoma tissues, in situ RNA hybridization on biopsies from 10 normal colon and 10 colorectal carcinoma tissues, all from different patients, was performed. High expression of the gene represented by the PCR fragment was observed in 9 of 10 colorectal carcinoma tissues (Fig. 2B), whereas very little expression was found in normal colon (Fig. 2A). These results confirmed that the PCR fragment-containing gene is indeed overexpressed by colorectal carcinoma tissues. To evaluate expression levels in other tissues, a filter containing RNAs from multiple human tissues was subjected to Northern blot analysis with the same PCR fragment. As shown in Fig. 2C, among various normal tissues examined, only testis expresses a high level of this mRNA.

Identification of the Palindromic PCR Clone as hRad17. To obtain a full-length cDNA, the PCR fragment was used as a probe to screen a human fibroblast AZAPII cDNA library. Four overlapping clones were isolated and sequenced, and one contained a complete ORF. The 2010-bp ORF encodes a 670-amino acid protein with a predicted molecular mass of about 75 kDa (GenBank accession no. AF112263). It is a basic hydrophilic protein with a putative ATP-binding site and shared limited homology with replication factor C subunits.

The sequence homology between hRad17 and S. pombe rad17 appears to be higher than that between the two yeast homologues. All three proteins are basic hydrophilic proteins with a similar size. We have also cloned the mouse and African green monkey (GenBank accession no. AF106067) homologues of the hRad17 gene. Both are very similar to the human sequences (Fig. 3A). In addition, in the course of genomic cloning and sequencing of hRad17, we have identified the promoter region (GenBank accession no. AF106065) and a pseudogene located at 7p21 (GenBank accession no. AF106066) for hRad17.

Chromosomal Localization of hRad17. A cDNA probe was used to determine the chromosomal localization of the Hrad17 gene by FISH. Fig. 3, A and B, shows chromosomes from human lymphocytes labeled with the complete XbaI/XhoI Hrad17 cDNA as a probe. Detailed analysis of the chromosomal DAPI banding patterns (11) allowed assignment of the hrad17 gene to chromosome 5, region q12–q13.2. Because a hrad17 pseudogene (GenBank accession no. AF106066), was identified during the cloning of hrad17 genomic DNA, the chromosomal localization was repeated using a 3.0-kb PCR probe from the intron located at position 1488 relative to hrad17 cDNA. This experiment confirmed the localization of hrad17 to chromosome 5q12–13.2 (data not shown). The chromosomal localization of hrad17 reported here differs from that reported previously, which has assigned the same gene to chromosome 4q13.3–21.2 (13).

Expression of HRad17 Protein in Colon, Colorectal Carcinoma, and Testis. Full-length Hrad17 was expressed as a fusion protein with GST and purified. Mice were immunized with the highly purified Hrad17, and monoclonal antibodies were generated. These monoclonal antibodies recognize Hrad17 protein by Western blot analysis as well as Hrad17 produced by in vitro transcription-translation driven by Hrad17 full-length cDNA. Characterization of these monoclonal antibodies will be presented elsewhere.

Immunohistochemistry was performed on paraffin sections of colorectal carcinoma tissues (Fig. 4A). Again, high expression of Hrad17 was detected in sections from colorectal carcinoma cells biopsies (Fig. 4A, left side of panel) when compared with normal cells (Fig. 4A, right side of panel), with staining being observed mainly in the nucleus. Hrad17 staining detected here was completely abolished when anti-Hrad17 monoclonal antibodies were first incubated with excess of highly purified Hrad17 (data not shown).

Analysis of the Hrad17 staining pattern in testis sections indicates that the protein is present primarily in the nuclei of spermatogonia, spermatocytes, and spermatids (Fig. 4, B and C). As for human colon sections, the staining again appears as distinct clusters within the...
nucleus. Interestingly, HRad17 is absent in mature spermatozoa (Fig. 4B). Thus, HRad17 is present throughout the earlier stages of spermatogenesis but not in the terminally differentiated spermatozoa. HRad17 staining described here was also completely abolished by preincubation of the antibody with purified recombinant HRad17 protein, demonstrating the specificity of the labeling (Fig. 4C).

Discussion

Conservation of cell cycle machinery across incredibly diverse eukaryotes is by now well established. Clearly, the proteins and mechanisms involved in imposing order on the process of cell division are of the highest importance and arose early in the course of evolution. Such similarities in proteins can be exploited to identify homologues of known proteins in different organisms. Recently, this strategy was used successfully to clone a human homologue of the S. pombe rad17 checkpoint gene, designated Hrad17 (13).

We identified the same protein by a different approach. Using the palindromic PCR-cDNA differential display method, we compared the mRNA expressions between normal and cancerous human colon. One of the genes we found to be expressed at much higher levels in colon carcinoma was hRad17. The basis for increased expression remains to be elucidated. We have cloned 2.0 kb of the hRad17 promoter region (GenBank accession no. AF106065) and found putative responsive elements for known oncogenic transcription factors such as c-myb and TCF-1 that are highly expressed in colon carcinoma tissues and thought to contribute to the etiology of this disease.
Whether these factors are involved in the overexpression of hRad17 in colorectal cancer remains to be determined. Although a functional role for HRad17 has not yet been established, its homology to yeast checkpoint proteins suggests it may play a part in cell cycle control. HRad17 is also abundantly expressed in the testes. The role of the testes is to produce billions of sperm cells continually. An organ with such a high rate of cell division would be expected to require high levels of proteins involved in cell cycle control. In addition, it is the only organ in adults where cells undergoing meiosis can be found. The HRad17 protein shows a striking pattern of localization within the cells of the seminiferous tubule. It is found throughout the early stages of spermatogenesis, including cells undergoing both meiotic and mitotic divisions. The only cells that lack HRad17 are the terminally differentiated spermatozoa, cells that do not divide any further. Interestingly, RAD24, the budding yeast homologue of HRad17, plays a critical role in a meiotic checkpoint as well. RAD24, in combination with RAD17 and MEC1, prevents the first meiotic division until meiotic recombination is complete (5). ATM and ATR (5), human proteins similar to MEC1, have been shown to localize to synaptonemal complexes in meiotic cells. They are also abundantly expressed in testis (16). There is ample evidence that human homologues of yeast proteins form similar complexes and function in similar pathways to those of the yeast proteins (3, 8). Thus, HRad17 may interact with ATM and/or ATR as part of a human pathway analogous to the RAD24/RAD17/MEC1 pathway in budding yeast.

The consequences of overexpression of HRad17 by human colon carcinoma are unknown. However, it may confer increased resistance of colon cancer cells to DNA-damaging agents including radiation and alkylating drugs. It has been proposed that in yeast, rad17 and RAD24 play a role in the DNA-damage checkpoint (17). Arrest of the cell cycle in G2 phase after DNA damage is believed to promote cell viability by allowing time for DNA repair before entry into mitosis. Agents that abrogate G2 arrest or mutations in genes that regulate the G2 checkpoint tend to sensitize cells to DNA-damaging agents (18, 19). If the human homologue of S. pombe rad17 acts in a similar fashion, targeting overexpressed HRad17 might render colon cancer cells more sensitive to DNA-damaging agents. It has been shown that UCN-01, a potent abrogator of the G2 checkpoint, does sensitize HT-29 colon carcinoma cells to γ-rays (20). The hRad17 gene is located on chromosome 5q12–13.1. This is in agreement with the localization of hRad17 determined by another group.5 Although no human disease has been associated with deletion of this specific chromosomal location, thousands of cases of human cancers are reported to have deletions spanning this region. Contrary to colon cancer where overexpression may be responsible for resistance to DNA-damaging agents, lack of HRad17 may contribute to the genesis of other types of both sporadic and hereditary human cancers. Because rad17 and RAD24 are essential for post-DNA damage checkpoints in yeast, HRad17 may also play a similar role in humans. If so, a loss of HRad17 could compromise checkpoint pathways responsible for the appropriate response to damaged DNA. This could result in damaged DNA going unchecked and unrepaired. Malfunctions in these pathways could constitute a critical turning point in the genesis of cancer; loss or mutation of a checkpoint gene would generate still more mutations, leading to the most intriguing aspect of human cancers, genomnic instability.

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