Defects of DNA Mismatch Repair in Human Prostate Cancer


ABSTRACT

Loss of mismatch repair (MMR) function leads to the accumulation of errors that normally occur during DNA replication, resulting in genetic instability. Germ-line mutations of MMR genes in the patients with hereditary nonpolyposis colorectal cancer lead to inactivation of MMR protein functions, and the defects of MMR are well correlated to the high rate of microsatellite instability in their tumors. Previous studies (T. Uchida, et al. Oncogene, 10: 1019–1022, 1995; S. Egawa, et al. Cancer Res., 55: 2418–2421, 1995; J. M. Cunningham, et al. Cancer Res., 56: 4475–4482, 1996; X. Gao, et al. Oncogene, 9: 2999–3003, 1994; H. Rohrbach, et al. Prostate, 40: 20–27, 1999) have shown that genetic instability (chromosomal and microsatellite instability) is detectable in human prostate cancer. To elucidate the role of MMR genes in the tumorigenesis of prostate cancer, we evaluated the expression of these genes in human cancer cell lines and in tumor specimens. Using Western blot analysis, we detected loss among MSH2, MLH1, PMS2, and PMS1 proteins in DU145, LNCaP, p69SV40T, M2182, and M12 cells. In addition, genomic instability in the prostate cell lines including DU145, PC3, LNCaP, p67SV40T, M2182, and M12 was detected by a microsatellite mutation assay. Significantly, immuno-histochemical analysis of prostate tissue revealed the reduction or absence of MMR protein expression in the epithelium of prostate tumor foci compared with normal adjacent prostate tissue. In contrast to hereditary nonpolyposis colorectal cancer, characterized by defects predominantly in MLH1 and MSH2, the samples we examined showed more tumor foci with loss of PMS1 and PMS2. PMS1, which is only expressed in the basal cells in normal glands, is conspicuously absent in most prostate cancer. From these results, we conclude that there are defects of MMR genes in human prostate cancer.

INTRODUCTION

Prostate cancer is the most prevalent cancer in American males and the second leading cause of their cancer deaths (1). Many factors contribute to its etiology, including age, race, dietary fat, androgens, and environmental and genetic factors. The development of many tumor types progresses from normal → dysplasia → carcinoma in situ → localized primary tumor → tumor with metastases. However, the development of prostate tumors is a more stochastic process. The progression of prostate cancer varies considerably among patients. Indolent prostate cancers may remain localized for decades, whereas aggressive prostate cancers can metastasize rapidly to lymph nodes and/or by hematogenous routes. Human prostate cancers are heterogeneous in appearance and genetically unstable. Multiple genotypes from the same primary prostate tumor in phenotypically similar foci are identified frequently (2).

MMR3 is one of the genome surveillance mechanisms that recognizes and repairs misincorporated nucleotides during DNA replication. In Escherichia coli, protein products from MMR genes including mutS, mutL, and mutH are involved in the recognition and correction of mismatched bp that result from replication errors (3–5). The repair is directed to the newly synthesized DNA strand that lacks post-synthetic modification of adenine-methylation at the d(GATC) sequences (6). MutS recognizes and binds to mismatched DNA (3, 7). Then mutL interacts with this complex to activate a latent endonuclease associated with mutH protein. MutH initiates the DNA MMR by cleaving the unmethylated strand at a d(GATC) site (6, 8–10). Post-replication repair in the eukaryotes is very similar to that in prokaryotes. Homologues of bacterial mutS and mutL have been identified in eukaryotes. In eukaryotes, the MSH gene products (MSH1–6) are homologous to bacterial mutS (11, 12), whereas MLH1, PMS2, and PMS1 are homologous to bacterial mutL (13, 14). Among the six products of MSH genes, MSH2 forms heterodimers with either MSH6 (previously called GTBP or p160) or MSH3. The MSH2/ MSH6 (MutSα) complex binds most base/base mismatches and small IDLs, whereas the MSH2/MSH3 (MutSβ) complex preferentially binds to small and large IDLs (15–17). Eukaryotic homologues of MutL also form heterodimers in the process of MMR. The MLH1/ PMS2 (MutLα) heterodimer predominates over the MLH1/PMS1 (MutLβ) heterodimer in HeLa cells (known to have normal MMR activity) because of the lower amount of PMS1 protein (18).

Mutations of hMSH2, hMLH1, hMSH6, hPMS2, and hPMS1 have been found in most cases of the HNPCC and a subset of sporadic colon tumors (11, 13, 14, 19–23). The mutations in MSH2 and MLH1 account for the majority of HNPCC (92.7%), whereas mutations in MSH6 are uncommon (6.6%) and mutations in PMS2 and PMS1 are rare (24–26). Defects of MMR genes also have been identified in other kinds of tumors, such as pancreatic cancer (27), gastric cancer (28), uterine sarcoma (29), endometrial cancer (30), and ovarian cancer (31). Loss of MMR function has been linked to genetic instability that results in high mutation rates, especially among repetitive sequences such as microsatellites. Microsatellite sequences are highly unstable in tumor cells with defective DNA MMR. Therefore, such MSI is a marker for a mutator phenotype and a measure of defects in the DNA MMR pathway.

MSI has been observed in a number of tumors, including most cases of the HNPCC (20, 32–35), sporadic colon cancer not related to HNPCC (34, 35), lung cancer (36), breast cancer (37), stomach cancer (38), pancreatic cancer (36–39), uterine sarcoma (29), endometrial cancer (30), and ovarian cancer (29–31). Defects in MMR allow accumulation of mutations in cancer-related genes, leading to carcinogenesis and/or tumor progression (40). Frameshift mutations attributable to MSI have been reported in at least nine genes including TGFβ-RII (41), APC (42), BAX (43), MSH3, MSH6 (44), the transcription factor E2F4 (45), BRCA1, BRCA2 (46), and PTEN (47).

MSI has also been detected in prostate cancer. The frequency of the MSI reported in different studies (48–52) varies greatly and depends on the number and type of microsatellite markers used in the assay.

Received 10/20/2000; accepted 3/19/01.
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Supported by Grant N00014-96-1-1298 from the Department of Defense, Navy. This paper is dedicated to the memory of our friend and mentor, Dr. Takis S. Papas.

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2 The abbreviations used are: MMR, mismatch repair; HNPCC, hereditary nonpolyposis colorectal cancer; MSI, microsatellite instability; RT-PCR, reverse transcriptase-PCR; PIN, prostate intraepithelial neoplasia; IDL, insertion/deletion loop, FBS, fetal bovine serum, OCT, optimal cutting temperature media, DAB, 3,3’-diaminobenzidine.
Nonetheless, the overall mutation rate reported in prostate cancer is lower than in HNPPC (20, 32–34, 48–52). MSI might also be associated with poorly differentiated tumors in some prostate cases but not always correlated to the clinical stage (48, 51). In addition, an in-frame deletion of CAG repeats encoding 6 of 24 glutamine residues of the androgen receptor has been reported in a human prostate cancer (53). Thus, the presence of MSI in prostate cancer suggests that MMR may be deficient in these tumor cells.

In the present study, we evaluated the expression of MMR protein in human prostate cell lines. Loss of the expression of MMR genes was detected in prostate cancer cell lines. We have demonstrated loss of MMR function in prostate cell lines by analyzing microsatellite stability using a β-galactosidase reporter system. We also examined MMR protein expression in human prostate cancer samples and found decreases or loss of MMR proteins in the tumor cells. Significantly, we found that PMS1 and PMS2 proteins are more frequently lost than the MLH1 and MSH2 proteins in the prostate tumors analyzed. The results of our experiments demonstrate that the spectrum of defects of MMR genes in human prostate cancer is different from that in HNPPC, suggesting that an altered spectrum of genetic disruption may occur in prostate cancer.

MATERIALS AND METHODS

Cell Lines. Human prostate cancer cell lines PC3, DU145, and LNCaP (metastatic cells) were purchased from the American Type Culture Collection (Manassas, VA) and were grown in RPMI 1640 medium (Life Technologies, Inc., Gaithersburg, MD) containing 10% FBS (Life Technologies, Inc.). The 267B-1 cell line (SV-40 T-antigen immortalized neonatal prostate epithelium) was generously provided by Dr. J. Rhim (Bethesda Naval Hospital, Bethesda, MD) and cultured in P–4F medium with 2% serum (Biological Research Faculty & Facility, Inc., Jamsville, MD). The TSU-PR1 cell line (metastatic cells), cultured in RPMI 1640 medium with 10% FBS, was a gift from Dr. J. T. Isaac’s laboratory (Johns Hopkins Oncology Center, Baltimore, MD). p69SV40T (SV-40 T-antigen immortalized adult prostate epithelium; passage 39), M2182, and M12 cell lines (derivatives of p69SV40T with different tumorigenicity in nude mice; passage 13 and 10, respectively) were kindly provided by Dr. J. L. Ware (Medical College of Virginia, Richmond, VA). These cells were cultured in the media as described previously (54). HeLa cell line (from American Type Culture Collection) was cultured in DMEM medium with 10% FBS and was used as an experimental control in this study. All of the cells were cultured at 37°C in a humidified incubator with 5% CO2.

Human Prostate Tumor Samples. Human prostate cancer samples were obtained from the Tumor Bank of Hollings Cancer Center, Medical University of South Carolina. All of the patients in this study had clinically localized, pathologically documented prostate cancer and were treated at the Medical University of South Carolina Department of Urology from 1998 to 1999. The average age of these patients was 57.3 ± 8 years old. The prostate samples were either formalin-fixed, paraffin-embedded (7 tumor specimens) or snap-frozen in OCT (13 tumor specimens). Frozen tumor specimens were maintained at −80°C. All of the samples had a Gleason grade between 5 and 7. A total of 20 specimens from 11 patients were scored for MLH1, MSH2, and PMS2 expression; 13 specimens from 6 patients were scored for PMS1 expression.

Western Blot Analysis and Immunohistochemical Staining. For Western blot analysis, nuclear extracts of cultured cells were prepared as described previously (55). Fifteen μg of protein/sample were used in Western blot analyses. The primary antibodies including polyclonal antihuman MSH2 (N-20), MLH1 (C-20), PMS1 (K-20), PMS2 (E-19), and MSH6 (N-20) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated goat antirabbit IgG (Amersham, Arlington Heights, IL) was used as the secondary antibody. Enhanced chemiluminescence system (Amersham) was used for final immunoblot detection. For immunohistochemical staining of the formalin-fixed, paraffin-embedded prostate cancer sections, the epitope site was retrieved by heating slides twice in antigen-unmasking solution (Vector Laboratories, Burlingame, CA) for 5 min in a microwave at high power. For frozen prostate cancer samples, sections on the slides were fixed in 100% acetone (4°C) for 10 min before immunostaining. Immunohistochemical staining was performed according to manufacturer’s protocol (Vector Laboratories). The primary monoclonal antibodies used recognized MSH2 (Ab-1), MLH1 (Ab-1), PMS2 (Ab-1; Oncogene Research Products, Cambridge, MA), and high molecular weight cytokeratin (clone DE-SQ, like-34JE12; NeoMarkers/Lab Vision, Corp., Fremont, CA) in formalin-fixed samples. Polyclonal antibody to PMS1 (K-20; Santa Cruz Biotechnology, Inc.) was used on frozen sections. Vectastain Elite ABC and complete substrate kit containing DAB (Vector Laboratories) were used to develop the color reaction. Sections were counterstained with hematoxylin. The prostate epithelial cells (luminal or basal cells) with brown nuclei were scored as positive, whereas negative staining cells had blue nuclei from hematoxylin counterstaining. Two pathologists (M. M. F. and J. M.) identified the prostate tumor loci, and one person (Y. C.) scored the fraction of MMR protein detected in the prostate cancer cells by examining the entire tumor area under the microscope (at least five ×100 fields). The following scoring system reflecting four main categories of observation was used: (a) all of the tumor cells are positive and the staining intensity is the same as normal adjacent glands; (b) no staining in up to one-third of the tumor field and positive-staining intensity is equal to or less than normal adjacent glands; (c) no staining in greater than one-third of the tumor area, but some positive-staining cells remain with intensity equal to or less than normal adjacent glands; (d) tumor area is completely negative for immunohistochemical stain. Staining intensities for positive cells in categories b and c were nearly always reduced compared with normal adjacent glands.

Sequenceing of MLH1 cDNA from DU145 Cell Line. RT-PCR was used to obtain a fragment containing exons 1 and 2 of MLH1 cDNA from DU145 and HeLa cells. The primers used in the PCR are: forward primer, 5′-GAGACTC-GAGCTAGACGTTTCCTTGGCTCTTC-3′ and reverse primer, 5′-GAGGCG-GCCGCTACATTTTCTATGGCTTTTCTCA-3′. This fragment of cDNA encodes codons 1 to 235 of MLH1. The cDNA fragment was sequenced by direct cycle sequencing using an ABI 373 automated sequencer.

MMR Functional Analysis. Two pCAR reporter vectors (pCAR-OF and pCAR-IF) were generously provided by Dr. B. Vogelstein (Johns Hopkins Oncology Center and Howard Hughes Medical Institute, Baltimore, Maryland; Ref. 56). These two pCAR reporter vectors were separately transfected into each cell line by using Superfect reagent (Qiagen, Valencia, CA). The cells were selected in hygromycin for 3 weeks (concentration of hygromycin was 100 μg/ml for DU145, PC3, 261-B1, and TSU-PR1 cells; 75 μg/ml for p69SV40T, M2182, and M12; and 50 μg/ml for LNCaP cells). The protein lysates from pools of hygromycin-resistant clones were harvested and analyzed by the β-galactosidase enzyme analysis system (Promega, Madison, WI). Protein (20 μg) from each cell lysate was used in our assay. The incubation time for detection is 30 min. The β-galactosidase activity from pCAR-IF served as a parallel control for transfection efficiency. Relative β-galactosidase activity was expressed as the ratio of the β-galactosidase activity in pCAR-OF-transfected cells to that in the pCAR-IF-transfected cells.

RESULTS

Expression of MMR Genes in Human Prostate Cell Lines. Most cases of HNPPC have mutations in MLH1 and MSH2, whereas mutations in PMS2 and PMS1 are rare (11, 13, 14, 19–23). About 50 to 75% of the HNPPC mutations result in protein truncation. To determine whether any of the major MMR proteins were truncated or not expressed in human prostate cell lines, we prepared the nuclear extracts from 267B-1, DU145, PC3, LNCaP, TSU-PR1, p69SV40T, M2182, and M12 cells and performed Western blot analysis. HeLa cells, which have normal MMR function (57), were used as a control. Fig. 1 shows that MLH1, MSH2, PMS1, and MSH6 proteins could be easily detected in the HeLa cells. Similar results were observed from the proteins prepared from 267B-1, TSU-PR1, and PC3 cells. However, loss of one or more MMR proteins was detected for all of the other cell lines. MLH1 protein was absent in the DU145 cell line (the faint band at M, 75,000 is most likely a cross-reactive protein; see “Discussion”). MSH2 protein could not be detected in LNCaP cells. PMS1 protein was greatly reduced in p69SV40T cells and absent in DU145, M2182, and M12 cells. Furthermore, PMS2 protein could not be detected in any of these four cell lines. Greatly reduced expression levels of MSH6 were observed in p69SV40T and

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its derivatives (M12 and M2182) and in LNCaP cells. This initial survey revealed that five of eight prostate cancer cell lines have lost wild-type expression of at least one MMR gene, with PMS1 and PMS2 lost most frequently.

Mutations of MLH1 in DU145 Cells. A mutation located in the splice acceptor between exons 1 and 2 of the MLH1 gene of DU145 cells was reported previously (57). This mutation alters the sequence of the splice acceptor for exon 2 from TTTGCCAG to TTTGCCGT. We predicted that this mutation might result in aberrant mRNA splicing between exon 1 and exon 2 of the MLH1 gene and lead to the loss of full-length MLH1 protein observed by Western blot analysis (see Fig. 1). To test this possibility, we cloned and sequenced a fragment of MLH1 cDNA from DU145 cells and from HeLa cells (harboring only wild-type MLH1 genes). The DNA sequence of RT-PCR product cloned from HeLa cells was identical to the wild-type sequence published previously (data not shown; Ref. 20). In contrast, the MLH1 cDNA fragment prepared from DU145 mRNA contained several nucleotide changes, including the loss of five coding nucleotides, resulting in a frameshift that produced a TGA stop at codon 39 (Fig. 2A). This is likely to have been caused by the use of alternate splice acceptor 5 nucleotides downstream from the mutated acceptor (Fig. 2B). This mutation is consistent with the absence of a full-length MLH1 protein from our Western blot analysis (Fig. 1).

MMR Function in Cell Lines. Loss of MMR protein expression will lead to defects in MMR function, causing genetic instability that is often revealed by MSI. However, loss-of-function mutations that do not alter electrophoretic mobility would not be detected by our Western blot analysis. Furthermore, loss of other proteins involved in MMR may occur in prostate cancer. To further assess loss of MMR function, we performed an assay to test the stability of microsatellites in prostate cell lines by transfecting a plasmid with a cryptic splice acceptor. As a result, the pCAR-OF-reporter does not generate β-galactosidase activity unless a frame-restoring mutation arose in the transfected cells that changed the length of (CA)n repeat by nucleotide insertion or deletion. Thus, restoration of enzyme activity from the pCAR-OF-transfected cells indicates that the microsatellite repeats were not stable in that cell line. Cells transfected with pCAR-IF are positive for β-galactosidase activity because the poly (CA) tract inserted within the β-galactosidase gene maintains the correct reading frame of the enzyme. The ratio of β-galactosidase activity in the pCAR-OF-transfected cells to that in the pCAR-IF-transfected cells (relative β-galactosidase activity) reflects the status of MSI in the prostate cell lines. The two pCAR reporter vectors were transfected separately into each prostate cell line, and cell lysates from a pool of resistant clones (>100 clones/pool) were harvested after 3 weeks of hygromycin selection. For HeLa, 267B-1, and TSU-PR1 cells, the β-galactosidase activity was detected in the cells transfected with pCAR-IF but not pCAR-OF vector, indicating that MMR function is intact in these cell lines. For DU145, p69SV40T, M2182, and M12 cells (these demonstrate loss of at least one MMR protein), the β-galactosidase activity could be detected in both pCAR-IF-transfected and pCAR-OF-transfected cells. The relative β-galactosidase activity was 0.7% for p69SV40T cells, 6.4% for M2182 cells, 10.6% for M12 cells, and 32% for DU145 cells (Fig. 3). Interestingly, although MLH1, MSH2, PMS2, PMS1, and MSH6 protein could be detected in PC3 cells (see Fig. 1), these cells showed significant β-galactosidase activity derived from the pCAR-OF vector. Thus, the (CA)n microsatellite was not stable in PC3 cells, suggesting that there are other MMR defects or defects attributable to a nontruncating mutation in one or more of the five genes tested. pCAR-OF-transfected LNCaP cells (loss of MSH2 protein) also showed some β-galactosidase activity; however, the enzyme activity was very low. We could not detect enzyme activity when we performed a standard 30-min reaction with 20 μg of LNCaP-pCAR-OF cell lysate. The enzyme activity was barely detectable over background in a reaction incubated overnight with 300 μg of cell lysate (data not shown). This suggested that the pCAR microsatellite assay was not sensitive to the MMR defects in LNCaP cells. Two other cell lines, 267-B1 and TSU-PR1, had no measurable β-galactosidase activity unless a frame-restoring mutation arose in the transfected cells.

Fig. 1. Western blot analysis of MMR protein expression in human prostate cancer cell lines. Fifteen μg of the nuclear protein extract/sample were used in Western blot analysis. The primary antibodies used were polyclonal antihuman MLH1, MSH2, PMS1, PMS2, and MSH6. Horseradish peroxidase-conjugated goat antirabbit IgG was used as secondary antibody, and the enhanced chemiluminescence system was used for detection.

Fig. 2. Partial sequence of MLH1 cDNA and proposed splicing event in DU145 cells. RT-PCR products were used as templates for direct sequencing of partial MLH1 cDNA. The sequence was analyzed on an ABI automated sequencer. A, alignment of partial MLH1 cDNA sequence in DU145 cells with wild-type sequence. B, possible mechanism of RNA splicing at intron 1 of MLH1 in HeLa and DU145 cell lines. The five deleted nucleotides are shown in parentheses. The AG3 dinucleotide (in italics) is part of the cryptic splice acceptor.
tosidase activity, even when the enzyme reaction was incubated overnight with 300 μg of each lysate. These have stable microsatellites, an observation concordant with the presence of full-length MMR proteins (see Fig. 1). From these results, we conclude that microsatellite (CA)₆ repeats are unstable in p69SV4OT, M2182, M12, DU145, and PC3 and minimally unstable in LNCaP cell lines. A summary of MMR protein expression and function for the eight prostate cancer cell lines tested is shown in Table 1.

Expression of MMR Genes in Human Prostate Cancer Samples. From the previous results, we conclude that there are defects of DNA MMR in several human prostate cancer cell lines. The defects were reflected either by the loss of MMR protein expression (predominantly PMS1 and PMS2) and/or by the presence of ectopic MSI. To evaluate their potential role in human prostate cancer, we next investigated the protein expression of four MMR genes (MLH1, MSH2, PMS1, and PMS2) by immunohistochemical staining. Monoclonal antibodies to MLH1, MSH2, and PMS2 with all of the samples and polyclonal anti-PMS1 antibody with frozen sections of prostate samples were investigated. We found that in normal prostate, MLH1, MSH2, and PMS2 were predominantly detected in the nuclei of glandular luminal epithelium, basal cells, and some stromal cells of the prostate gland. However, PMS1 protein expression was present in the nuclei of normal basal cells and absent in luminal cells. PMS1 expression was significantly reduced in all of the tumor specimens examined (Fig. 4). In the malignant glands of prostate tumor specimens, we observed variable patterns of staining among different tumor sections. The intensity of nuclear immunostaining for the MMR proteins varied from similar to normal tissue, to reduced (less than normal tissue), to negative. Significantly, the staining in the tumor cells was generally less intense than the normal adjacent cells, and all of the tumors had some foci with decreased expression of MMR proteins. Furthermore, even when MMR proteins are detected by immunohistochemistry in tumor cells, it is still possible that the protein derives from a mutant gene and may lack function. There were no observations of increased staining in tumor relative to normal adjacent tissue. Therefore, the immunohistochemical assay represents a minimum estimate of loss of MMR function.

We also observed variable patterns of staining within the same tumor sections; e.g., in one case, we found that MSH2 or PMS2 protein was detected in almost all of the tumor cells (data not shown). However, we found that in a specific area of the tumor, the staining of MSH2 was negative (Fig. 5C), whereas staining of PMS2 was positive (Fig. 5D). In another tumor region just adjacent to the previous one, staining of MSH2 was positive (Fig. 5C), whereas staining of PMS2 was negative (Fig. 5D). Positive MLH1 staining was found in both of these areas (Fig. 5B). This pattern demonstrates that the staining of the MLH1, MSH2, and PMS2 protein in malignant cells of the same case of prostate cancer is heterogeneous.

PMS1 protein expression, however, was present in the nuclei of normal basal cells and absent in luminal cells. PMS1 expression was significantly reduced in all of the tumor specimens examined (Fig. 4), with 86% of the tumor cells completely lacking PMS1 (derived from the immunohistochemical staining used in Table 2). Such a significant decrease in PMS1 expression may be expected because cancerous glands are generally regarded as lacking basal cells; however, this concept is still a matter of some debate (see “Discussion”). Interestingly, we found that PMS1 is present in the nuclei of 14% of prostate tumor cells. The residual expression of PMS1 in these 14% of the cancer cells does suggest that a subpopulation of basal cells can contribute to cancer or that luminal cells in cancerous glands express PMS1 (see “Discussion”).

Twenty specimens from 11 different patients were scored for MMR protein expression where significant areas (at least five ×100 fields) of both tumor and normal adjacent tissue were present in the same section (Table 2). Generally, the staining in the tumor cells was less intense than the normal adjacent cells, and all of the tumors had some foci with decreased expression of MMR proteins. Also, the tumor region generally had a lower percentage of cells with any expression of the MMR proteins tested, although there was variation among the samples examined. In Fig. 6 and Table 2, we show that PMS1 was most likely to be absent or
reduced in the tumor cells with the four MMR proteins following the hierarchy MLH1 > MSH2 > PMS2 > PMS1.

PIN is the presumed precursor lesion to prostate cancer (58, 59). In one paraffin-fixed section, we found that although MLH1 protein could be detected in the PIN area, there was little or no expression of MSH2 or PMS2 compared with protein levels in nearby normal glands (Fig. 7, D–F). Basal cell-specific PMS1 expression is also largely absent in PIN, although expression of high molecular weight cytokeratin persists in the basal cells (Fig. 7, H and I, respectively). In PIN, generally MLH1 expression is similar to normal levels, MSH2 expression is present but reduced, and PMS2 and PMS1 are absent or significantly reduced (data not shown). Thus, the results we obtained from this study show a consistent loss of some MMR protein expression in human prostate cancer as well as in PIN, suggesting that loss of MMR function can occur early in human prostate neoplasia.

Fig. 4. Immunohistochemical staining of MMR protein in human prostate cancer sections. Normal regions (A, C, E, and G) and adjacent cancer regions (B, D, F, and H) are paired from the same section. A and B are stained for MLH1; C and D are stained for MSH2; E and F are stained for PMS2; G and H are stained for PMS1. Tissue sections in A–F are formalin-fixed, paraffin-embedded tumor tissues. Tissue sections in G and H are frozen samples. DAB was used as a substrate in final detection, and hematoxylin was used for counterstaining. Positive-staining and negative-staining nuclei are indicated with black and white arrowheads, respectively. All of the microscopic magnifications are ×400, and the bar in H is 100 μm.
DISCUSSION

In human prostate cancer, heterogeneity is a common phenomenon that includes different histological grade among different tumor foci and different genotypes among phenotypically similar foci in one primary tumor (2). Human prostate cancer, like other cancers, is also genetically unstable. Several laboratories have observed abnormalities on chromosomes 7, 8, 10, 13, 16, 17, and 18 (50) and MSI (48–52). Any number of these genetic alterations may be involved in prostate tumor development leading to inactivation of tumor suppressor genes and activation of oncogenes. These observations support a stochastic model for tumor progression that could explain the heterogeneity within a single prostate tumor sample.

The linkage between MSI and MMR deficiency was first found in HNPCC (32, 60–62) and later found in many other types of human cancer (29–31). It has also been revealed that genomic instability in colon cancers with MSI promotes the inactivation of other genes, such as APC, TGFβ-RII, BAX, hMSH3, and hMSH6 (40). Thus, MSI generally indicates deficiency of MMR. Because genetic instability of microsatellites is found in prostate cancer, it is important to study the involvement of MMR genes in human prostate cancer.

This is the first report of MMR protein expression and function in prostate cancer cell lines as well as primary prostate tumors. Loss of expression of at least one MMR gene was detected in most of the prostate cancer cell lines tested. Among these eight prostate cell lines, and...
one cell line lost the expression of MLH1, one cell line lost expression of MSH2, two cell lines had reduced levels of MSH6 protein, three cell lines lost expression of PMS1, and four cell lines lost expression of PMS2. A recent report (63) has documented a homozygous deletion of exons 9–16 of the \textit{MSH2} gene in LNCaP cells and a concomitant loss of MSH2 protein expression in Western blots. The same report also described loss of MLH1 protein expression from DU145 cells. Our Western blot analyses are in agreement with their report. Furthermore, our study confirms loss of MMR function in which the microsatellite (CA)\textsubscript{n} repeat assay also detected MSI in all of the prostate cell lines with loss of at least one MMR protein (summarized in Table 1). Interestingly, very high levels of MSI were detected in PC3 cells (Fig. 3), although no loss of MLH1, MSH2, PMS1, PMS2, and MSH6 was observed (Fig. 1). Although these MMR proteins can be detected by Western blot analysis, mutations could still exist in some of the MMR genes that would cause defects in protein function without altering their electrophoretic mobility. Furthermore, we have not assessed the expression of MSH3, MSH4, MSH5, or DNA polymerase \( \delta \) in PC3 cells. Alternatively, it is possible that there are defects in other, unknown genes that might affect the function of the MMR pathway in PC3 cells causing the high rate of MSI observed here.

Several other interesting results were obtained in this study. First, in DU145 cells, we observed MSI and a loss of full-length MLH1 protein. This observation is consistent with a reported splice site mutation between exon 1 and 2 (57) and the more recent report of loss of protein expression mentioned above (63). The deletion of five nucleotides observed in the MLH1 cDNA from DU145 cells is predicted by this mutation. This differently spliced mRNA encodes a stop codon \textit{TGA} at the end of exon 1 leading to truncation of the MLH1 protein after amino acid 38 (Fig. 2B). DU145 cells have a modal chromosome number of 64 and are triploid for chromosome 3 (64). The location of \textit{MLH1}. Both genomic sequencing (57) and our own cDNA analysis (Fig. 2) indicate that DU145 cells contain and transcribe only the mutant allele, whether present on one, two, or all three chromosomes. Therefore, the faint protein band detected in the Western blot of DU145 cells (Fig. 1) is likely to be a cross-reactive protein and not MLH1.

A second result derives from our analysis of a series of human prostate epithelial cell lines that display progressively neoplastic
phenotypes from rarely tumorigenic to fully metastatic cells (54). The parental cell line p69SV40T was originally derived from an adult prostate epithelium that was immortalized by SV40 T-antigen. When this cell line was injected into nude mice, tumor development required at least 4 months (54). However, when a series of prostate epithelial cells were isolated from these tumors and reinjected into mice, those cells developed tumors faster than p69SV40T cells. M2182 and M12 are two of these sublines exhibiting increased tumorigenicity. The M12 cell line is also metastatic (54). This series of cells provides a model to study the relationship between tumor progression and function of MMR protein. We found in this study that p69SV40T had reduced expression of PMS1 and no expression of PMS2, and microsatellite (CA)$_n$ repeats were not stable. These results suggest that the p69SV40T cell line itself has defects in MMR. Thus, it may explain why this parental cell line is susceptible to genetic instability that eventually leads to tumor development. Furthermore, we found that both M2182 and M12 cells have lost protein expression of PMS1 and PMS2, and that these sublines have greater MSI than p69SV40T cells. This deficiency of MMR in these two sublines is likely to have contributed further to tumor progression.

The third result is the reduction or loss of MMR protein expression in human prostate cancer. The levels of immunostaining of MMR proteins in the prostate tumor cells were heterogeneous among the samples, varying from positive (normal level) to reduced or negative in the nuclei of tumor cells. However, the observed reduction or loss of MLH1, MSH2, PMS2, and PMS1 protein expression in prostate tumor foci was quite frequent. The loss of MSH2, PMS2, and PMS1 protein can occur as early as in the PIN lesion, a precancerous stage of prostate cancer. Previous reports (48, 49, 51) have correlated increased MSI with poorly differentiated and late stage (nodal involvement and distant metastases) prostate cancer. To our knowledge, we describe here the first evidence of loss of MMR protein in PIN. We also observed that the majority of cases showed the greatest reduction or loss of PMS1 and PMS2 protein expression. This result contrasts with HNPPC where the great majority of cases (>90%) have mutations in the MLH1 and MSH2 genes and a few cases have mutations in MSH6, and mutations in PMS1 and PMS2 are very rare. In contrast, our data show that the spectrum of mutations in patient samples as well as in the established cell lines indicates that the predominant MMR mutations in prostate cancer are in the PMS1 and PMS2 genes. Therefore, prostate cancer reflects a different spectrum of MMR mutations than HNPPC. We will further confirm and extend this finding by assessing a larger collection of tumor samples at different clinical stages and histological grade. If this is the case, our results may provide clues as to the heterogeneity of prostate cancer.

Our fourth observation is that PMS1 expression is restricted to the basal cells in normal prostate glands and is the most frequently lost in PIN and prostate cancer. In contrast to the complete loss of basal cell-specific cytokeratins in our samples, PMS1 expression was retained in some prostate cancer cells. However, others (65) have reported that prostate cancer cells coexpress both basal and luminal cell cytokeratins. Although loss of PMS1 expression may simply reflect the loss of basal cells in prostate cancer, there are still conflicting data on this issue that have implications for models of neoplastic progression (regardless of the reason for PMS1 loss in cancer, it is clearly lost from basal cells in PIN; see Fig. 7f). In one of the models, neoplastic prostate epithelial cells are postulated to arise from a proliferating subpopulation of stem cells present in the basal compartment in normal glands as well as during malignant transformation (66). This subpopulation of androgen-independent stem cells also gives rise to androgen-dependent luminal cells that are terminally differentiated (67, 68). Consistent with this hypothesis is the observed expression of a putative prostate stem cell antigen mRNA in a subset of normal basal cells as well as in the basal and luminal cells of PIN and in prostate cancer cells (69). Similarly, expression of the c-met and Bcl-2 proteins is basal cell-specific in normal prostate, yet both are detected in prostate cancer cells (70, 71). Furthermore, it has been hypothesized that during malignant transformation, some proliferative capacity shifts from basal cells to luminal cells (66). We show in normal prostate that all of the basal cells (including the proliferating stem cell subpopulation) express MLH1, MSH2, PMS2, and PMS1, whereas terminally differentiated luminal cells express the former three proteins but not PMS1 (Fig. 7 and data not shown). In precancerous PIN and cancer, PMS1 expression is generally absent, whereas PMS2, MSH2, and MLH1 expression is reduced or absent; therefore, the proliferating cell subpopulation may be replicating its DNA without the benefit of MMR function. This places the proliferating cells at a greater risk of accumulating mutations. Another model of neoplastic progression suggests that benign prostate luminal cells maintain some limited self-renewal capacity (72, 73). If these cells contribute to the cycling population in PIN and cancer, they also replicate their DNA with decreased or no MMR protein. Therefore, in either scenario, proliferating cells with decreased or no MMR function are at risk of accumulating mutations that lead to transformation.

Previous studies (reviewed in Ref. 74) have shown that the MutS heterodimers have preferred binding specificities. The MSH2/MSH6 heterodimer recognizes and binds most base/match mistakes and small IDLs, whereas the MSH2/MSH3 heterodimer preferentially binds to small and large IDLs (15–17). In cells with normal MUtL function, the MLH1/PMS2 heterodimer provides the majority of repair activity, whereas the MLH1/PMS1 heterodimer is much less abundant (18). It has also been shown that MutL heterodimer binding to MutS heterodimers is required for efficient MMR (74). Furthermore, the MLH1/PMS1 heterodimer fails to restore MMR activity for base/mismatch mistakes and small IDLs in extracts deficient in MLH1 or PMS2 (18). This demonstrates that PMS2 has at least some functions that, if lost, cannot be compensated by PMS1. However, the precise role of PMS1 remains unclear.

Loss of PMS2 expression is more directly associated with disease. Although rare in HNPPC, two families have been identified with PMS2 mutations (14). A family with Turcot’s syndrome had severe MSI and concomitant mutations in APC and TGFβ-RII in which the only mutated MMR gene was PMS2 (75). Although the APC mutation (a T insertion) did not fall within any nucleotide repeat region, the TGFβ-RII frameshift mutation (a single nucleotide deletion) was in the mononucleotide (A)$_n$ tract. This tract was found previously (41) to be a target of deficient MMR in colon cancer cells with MSI. Loss of PMS2 causes MSI and MMR deficiency in HEC-1A endometrial cancer cells, but genomic stability is restored upon transfection with the wild-type PMS2 gene (76, 77). Therefore, PMS2 appears to play a critical role in normal MMR activity in some tissues. The types of mutations associated with loss of PMS2 include multiple base substitutions and frameshifts (attributable to replication slippage errors), plus a lesser amount of single nucleotide changes (78).

In animal models, Msh2 knockout mice display MSI and are prone to develop lymphomas (79, 80). They also exhibit promiscuous recombination between divergent DNAs and methylation tolerance (79). Msh6-deficient mice are prone to develop gastrointestinal tumors as well as lymphomas (81). However, they do not develop MSI; rather, they fail to repair only single nucleotide mismatches. Mice deficient in Mlh1, Pms2, or Pms1 were found to have different spontaneous mutation rates, mutation patterns, and tumor susceptibility (82). The Mlh1 and Pms2 knockout mice showed highly MSI in both mononucleotide and dinucleotide repeats. Pms1-deficient mice had a low rate of alteration, restricted to mononucleotide repeats. Mlh1-deficient mice predominantly developed lymphomas, intestinal adenomas, and
adenoacarcinomas. Pms2-deficient mice developed lymphomas and carcinomas, whereas Pms1-deficient mice did not develop any tumors. These differences suggested that these proteins have different biological functions and/or tissue preferences reflected by their unique downstream MMR targets. Mlh1 mutants also have defective crossing over during meiosis apparently because of abnormal chiasma formation (83, 84). Pms2 mutants also have defective meiosis because of abnormal chromosomal synapse (85). However, it is not clear if Pms1 participates with Mlh1 during meiosis. Interestingly, the level of mononucleotide MSI in Mlh1 mutants is 2–3 times higher than in Pms2 mutants, raising the possibility that heterodimers between Mlh1 and other MLL family members such as Pms1 or Mlh3 (86) can partly compensate for loss of Pms2 in vivo (87). This may partially explain why most prostate cancers are indolent (with PMS1 mutations), becoming aggressive when mutations occur in additional MMR genes (PMS2, MSH2, and MLL1) or other epistatic targets. Together, these observations imply that the loss of “strong” mutator genes (MLH1 and MSH2) in HNPCC may target one spectrum of genes (such as APC, TGFβ-R1, BAX, hMSH3, and hMSH6), whereas the loss of “weak” mutator genes (PMS1 and PMS2) in prostate cancer may result in a different but overlapping (APC, TGFβ-R1, and possibly AR) spectrum of secondary gene mutations. The biological impact of defects in “weak” mutants such as PMS1 remains largely unknown. One challenge will be to identify important secondary mutations that lead to aggressive prostate cancer.

Our overall conclusions from this study are that loss of protein expression in PMS1 and PMS2 are the predominant MMR mutations in both prostate cancer tissues and cell lines, that loss of MMR protein expression, like prostate neoplasia themselves, are heterogeneous, and that the PMS1 protein is distinct from PMS2, MSH2, and MLH1 in that its expression in normal prostate glands is limited to the basal epithelium.

ACKNOWLEDGMENTS

We thank Dr. J. S. Rhim for providing 267B1-cell line, Dr. J. T. Isaacs for TSU-Pr1 cell line, Dr. J. L. Ware for p69SV40T, M2182, and M12 cell lines, and Dr. B. Vogelstein for providing pCAR-OF and pCAR-IF plasmids. We also thank Kelly Henderson for DNA sequencing. Margaret Romano from the Hollings Cancer Center Tumor Bank for collecting and preparing the prostate cancer specimens, James Nicholson from the Image Analysis Core in the Department of Pathology for expert help with the digital photomicroscopy, and the Hollings Cancer Center DNA Sequencing Facility.

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DNA MISMATCH REPAIR IN PROSTATE CANCER


Defects of DNA Mismatch Repair in Human Prostate Cancer

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