Expression of the Human Mismatch Repair Gene hMSH2 in Normal and Neoplastic Tissues

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Abstract

Hereditary nonpolyposis colorectal cancer is caused by inherited mutations of mismatch repair genes. We developed monoclonal antibodies to the prototype human mismatch repair gene hMSH2 and used them to detect an immunoreactive protein of Mr 100,000 in mismatch-proficient cell lines. In addition, a M, 150,000 protein coimmunoprecipitated with the hMSH2 gene product in cell lines expressing hMSH2. Immunohistochemistry demonstrated that the hMSH2 protein was exclusively nuclear. Whereas the hMSH2 protein was expressed in a variety of tissues, the most striking pattern was observed in esophageal and intestinal epithelia, where expression was limited to the replicating compartment. Neoplastic cells within benign and malignant mismatch repair-proficient tumors expressed the protein, but no hMSH2 immunoreactivity was observed in the colorectal tumors of patients with germline hMSH2 mutation. These results have implications for tumorigenic mechanisms and, potentially, for diagnosis.

Introduction

Three classes of genes causing cancer predisposition have been discovered: (a) oncogenes that positively regulate cell growth; (b) tumor suppressor genes that negatively regulate cell growth; and (c) DNA repair genes that indirectly control proliferation by limiting the rate of mutations of growth controlling genes. The first repair genes implicated in tumor predisposition were those responsible for NER and associated with xeroderma pigmentosum and related autosomal recessive inherited syndromes (1). More recently, inherited mutations of genes involved in MMR have been shown to be responsible for HNPPC (2–4). HNPPC is inherited in autosomal dominant fashion and can be caused by mutations in any one of at least four genes. The prototype human MMR gene hMSH2 (5–7) is a homologue of the bacterial mutS gene (8). Its product binds to a second mutS homologue, GTBP; both together recognize mismatched basepairs in DNA (9–11). The mutL gene products interact with mutS proteins to recruit nucleases, polymerases, and other proteins required for the repair process (8, 12, 13). Three human mutL homologues (hMLH1, hPMS1, and hPMS2) have been associated with HNPPC when inherited in mutant form (14–16).

The MMR and NER genes appear to be typical “housekeeping” genes. They are found in every prokaryotic and eukaryotic organism examined thus far, and their structure and function appear to be highly conserved (2). Tumors in patients with NER defects are largely confined to the skin. This is consistent with the idea that UV radiation is responsible for most DNA defects for which NER is indispensable (1). However, the organ specificity of tumors arising in HNPPC patients is more difficult to explain. On the basis of their housekeeping nature and their apparently ubiquitous expression patterns (at least as judged by assessment of mRNA), one might have expected that MMR deficiency would be associated with tumors of virtually every renewing cell population in the body. Nevertheless, the great majority of tumors in HNPPC patients occur in the large intestine and endometrium, with only occasional tumors elsewhere (3, 4). Continually renewing cell populations in the skin, blood, lymphoid organs, breast, lung, stomach, esophagus, small intestine, and other organs rarely develop neoplasia in these patients.

There is as yet no information on the expression of MMR proteins in higher eukaryotes. In the studies reported herein, we developed mAbs to the prototype human MMR gene hMSH2 and addressed basic questions about its expression in normal and neoplastic tissues.

Materials and Methods

CR cancer lines were obtained from American Type Culture Collection and grown to log phase in McCoy’s 5A medium containing 10% FBS. Lymphoblastoid cells were grown in RPMI 1640 containing 10% FBS. SF9 cells were grown at 27°C in Grace’s medium containing 10% FBS.

Recombinant hMSH2 Clones. The complete hMSH2 coding region was assembled from overlapping cDNA clones (6). An NcoI site was engineered at the initiating methionine using synthetic oligonucleotides to produce clone E5. An NcoI–HindIII fragment of E5 containing the first 550 amino acids of hMSH2 was then cloned into the NcoI and HindIII sites of the vector pGStag (17) to generate pGStag-MSH-N. A PstI–EcoRI fragment of hMSH2 cDNA containing the carboxyl-terminal 330 amino acids was cloned into the EcoRI and PstI sites of pGStag to generate pGStag-MSH-C. Fusion proteins were purified through a glutathione agarose resin. A baculovirus vector containing the complete hMSH2 coding region was produced. Plating density was 1–5 kb NcoI fragment of clone E5 into the vector PBluBacHis. Baculovirus was produced from this vector as described by the manufacturer, and hMSH2 protein was purified with a nickel-chelating resin (Invitrogen).

mAbs. Female BALB/c × C57B1/6 F1 mice were immunized by i.p. injection of purified pGStag-MSH-N or pGStag-MSH-C fusion proteins in Ribi adjuvant (Ribi Immunocor Research, Inc.). The fusion proteins were expressed as insoluble proteins in Escherichia coli, collected by centrifugation after cell lysis by passage through a French pressure cell (SLM Instruments), and purified by electroelution using SDS-PAGE. Hybridomas were produced.
as described (18), except that test bleeds and hybridomas were screened by ELISA for anti-MSH2 reactivity using GST-MSH-N- and GST-MSH-C-coated microtiter plates. Hybridoma supernatants that were positive by ELISA were tested by immunoblot using lysates of HCT116, LoVo, and SW480 cells. Six hybridomas reactive with the amino terminus and two reactive with the carboxyl termini of hMSH2 were isolated and subcloned twice by limiting dilution.

**IP and Western Blot Analyses.** IP was performed with extracts from [35S]methionine-labeled cells (200 μCi/ml, 6 h). Extracts were made as described (19) and preclarified by incubation with protein A-Sepharose for 60 min at 4°C, followed by microcentrifugation. The appropriate antibodies were then added to the extracts, to a final concentration of 10 μg/ml, and incubated on ice for 4–8 h. The immunoprecipitates were collected by microcentrifugation and washed 3 times, as described (19). Immunoprecipitates were analyzed by SDS-PAGE, and fluorography was performed after Enhance (Amersham) impregnation of the gels.

For Western blots, extracts from unlabelled cells were prepared as described above. After centrifugation at 14,000 × g for 5 min in a microfuge, 50 μg of protein were used in immunoblot analysis after separation through 8% SDS-PAGE. Immunodetection using enhanced chemiluminescence (Amersham) was performed following the manufacturer’s recommendations.

**Immunohistochemistry.** Frozen tissues were obtained from surgical resections and stored at −80°C until used. Cryostat sections were placed on SuperFrost slides (Fisher) and fixed in Histochoice (Ameresco) for 10 min at room temperature, then stored in PBS at 4°C until use (up to 2 weeks). Immunohistochemical analysis was performed as described (20), except that the antibodies were used at 2 μg/ml, and slides were incubated for 2 h (tissue culture cells) or overnight (cryostat sections) at room temperature. The ABC method (Vector) was used for detection, and sections were counterstained with methyl green.

**Results**

**Characterization of Antibodies.** As described in “Materials and Methods,” mAbs were generated against the amino-terminal and carboxy-terminal ends of hMSH2. Among several antibodies tested, the carboxy-terminal-directed mAb FE11 (IgG1) exhibited the best specificity and reactivity, although another COOH-terminal-specific antibody (EH12) and two NH2-terminal-specific antibodies (GB12 and CF7) yielded similar results to those shown in Figs. 1 and 2. Western blot analysis revealed that FE11 detected a Mr, 100,000 protein in extracts from Sf9 cells infected with a recombinant baculovirus expressing hMSH2 (Fig. 1). The migration of hMSH2 in SDS-PAGE was slightly slower than predicted from its sequence (Mr, 94,000). No immunoreactive protein was present in control baculovirus-infected Sf9 cells (data not shown). MMR-proficient mammalian cells (e.g., SW480, Ref. 21) also contained an immunoreactive polypeptide that migrated at the identical position. The LoVo cell line, which contains a homozygous deletion of hMSH2 (22, 23), had no immunoreactive protein (Fig. 1). The HCT116 line is MMR deficient (21) due to a homozygous nonsense mutation of hMLH1 but contained a normal-sized hMSH2 protein (Fig. 1). This result demonstrated that the synthesis and stability of hMSH2 is independent of the hMLH1-hPMS2 complex that interacts with it functionally (12, 13) because HCT116 lacks this complex (12).

We next determined whether the mutant hMSH2 gene products found in HNPCC patients could be detected by immunoblot. K. K. is an HNPCC patient with a germline splice site mutation at the 3′ end of exon 5 (24), resulting in an in-frame deletion of codons 265–314. The Mr, 85,000 protein predicted to result from this mutation was not evident in the immunoblot prepared from EBV-transformed lymphoblasts of K. K. Only the full-length protein, presumably produced from the unaffected allele, was observed (Fig. 1). Similarly, only full-length proteins were evident in four other lymphoblastoid cell lines from patients whose hMSH2 genes were predicted to contain abnormal, short polypeptides, when tested with FE11 or amino-terminal-specific mAbs (data not shown). These data suggest that aberrant hMSH2 polypeptides or the mRNAs encoding them were unstable.

IP experiments were then performed with radiolabeled cell extracts. Under the conditions used, several polypeptides were precipitated with mAb FE11, but only two proteins of 100 and 150 kb were specifically immunoprecipitated (Fig. 2). This specificity was confirmed with control antibodies (e.g., Fig. 2) and by analysis of other cell lines; three cell lines with wild-type hMSH2 genes (HCT116, RKO, SW480) each synthesized Mr, 100,000 and 150,000 polypeptides immunoprecipitated with FE11, whereas neither of these polypeptides was observed in extracts of the hMSH2-deleted LoVo cell line (data not shown).
**Immunohistochemistry.** Immunocytochemistry with FE11 revealed that the hMSH2 protein was exclusively nuclear (Fig. 3). Over 90% of unsynchronized SW480 cells in culture were intensely active, indicating that hMSH2 expression was largely independent of the cell cycle phase. The chromosomes were stained during mitosis, suggesting a relatively stable interaction of hMSH2 with chromatin.

The hMSH2 protein was expressed in a variety of human tissues, including thyroid, heart, smooth muscle, and the germinal centers of lymphoid follicles. Staining was particularly prominent in the epithelium of the digestive tract, extending from esophagus to rectum. In the esophagus, high levels of hMSH2 expression were observed in the baseal zone closest to the basement membrane, and staining gradually decreased as cells migrated through the prickle zone and differentiated (Fig. 4, A and B). In the stomach and small and large intestines, reactivity was confined to the epithelium of the bottom half of the crypts (e.g., Fig. 4, C and D). In colonic neoplasms, this compartmentalization was lost because cells throughout the neoplastic glands, extending to the luminal surface, stained intensely. From 50 to 90% of the epithelial cells from each of 10 CR adenomas and 8 CR carcinomas reacted strongly with FE11 (e.g., Fig. 4, E and F, and Fig. 5, A and B). Staining of adjacent sections with the Ki67 antibody revealed a similar but somewhat less impressive extension of the replicative compartment (data not shown).

Specificity in immunohistochemical experiments is often difficult to substantiate. The immunohistochemical specificity of FE11 was supported by the patterns observed in CR tumors from two HNPCC patients with germline hMSH2 mutations resulting in COOH-terminal truncations (23). It has been shown that in tumors arising from such patients, the wild-type hMSH2 allele inherited from the unaffected parent is usually inactivated (23, 25). In each of four tumors from these patients (an adenoma and carcinoma from each), no staining with FE11 was observed (e.g., Fig. 5, C and D). A subset of the nonneoplastic stromal cells within the same section reacted with the antibody (Fig. 5, C and D), and neoplastic epithelial cells stained with the Ki67 antibody (not shown), providing appropriate controls. In contrast, two tumors from patients with truncating mutations of hMLH1 stained normally with FE11, as did all 18 CR tumors from patients without HNPCC noted above.

**Discussion**

The results described herein demonstrate that hMSH2 is a widely expressed protein. Its expression in normal intestinal tract epithelium was limited to the replicative compartment, suggesting transcriptional or translational control analogous to other proteins involved in DNA replication. The nuclear localization of hMSH2 demonstrated here may be due to potential nuclear localization signals at codons 871–882 and 907–918 (6). The hMSH2 protein migrates slightly slower than that predicted from its sequence, both in natural form in mammalian cells and in recombinant form in insect cells (Fig. 1). Whether this difference in mobility represents posttranslational modification or unusual secondary structure is not known.

The hMSH2 protein is apparently complexed with another Mr 150,000 protein (Fig. 2). This likely represents the GT mismatch-binding protein GTBP identified by Palombo et al. (9) and Drummond et al. (10). GTBP was shown to copurify with hMSH2, suggesting that the two proteins may exist as a heterodimer (10). Our IP data support this suggestion.

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**Fig. 3. Immunocyto detection of the hMSH2 protein.** SW480 cells growing in culture were prepared for immunohistochemistry as described in “Materials and Methods.” Cells in A were stained with preimmune IgG; cells in B and C were stained with FE11. Strong nuclear staining is observed in B and C. A and B, ×100; C, ×200.
Fig. 4. Immunohistochemistry of human tissues. Cryostat sections of esophagus (A and B), small intestine (C and D), and a villous adenoma of the colon (E and F) were stained with mAB FE11 as described in “Materials and Methods.” Compartmentalized staining (black nuclei) is noted at the basal zone of the esophageal squamous cell epithelium (A and B) and crypt epithelium of the small intestine (C and D). In contrast, staining of virtually all epithelial cells is observed in the adenoma (E and F). The sections were counterstained with methyl green. A and D, ×100; B, ×400; C and E, ×20. *, basal layer of epithelium.

Approximately one-half of the hMSH2 mutations identified in HNPCC patients result in a substantially altered molecular weight of the predicted protein. We were hopeful that these altered proteins could be detected using specific mAbs, thereby facilitating presymptomatic diagnosis. Unfortunately, the mutant gene products tested seem to be unstable, either at the RNA or protein level. Instability of truncated proteins has many precedents. In particular many of the truncated APC proteins found in familial adenomatous polyposis patients are also undetectable by Western blot analysis (18).

Although immunoblot analysis did not prove useful for diagnosis,
the immunohistochemical results were promising. At least four different genes can cause HNPCC when inherited in mutant form, and the mutational spectrum within each gene is wide (3). This poses a tremendous diagnostic challenge, and screening each of these genes for mutations would be impractical. Simple methods to indicate which gene was inactivated in an individual kindred with HNPCC would considerably simplify the diagnostic strategy. The wild-type copy of MMR genes, inherited from the unaffected parent of an HNPCC patient, is usually inactivated in tumors (23, 25, and references therein), leaving the cell with no functional protein and, in particular, no protein containing carboxyl-terminal epitopes. Our results are consistent with the idea that staining with antibodies specific for the carboxyl terminus of MMR gene products could thereby provide information as to the culprit gene. Once antibodies to hMSH2 and the other MMR proteins that are reactive with formalin-fixed, paraffin-embedded samples become available, it will be possible to test this hypothesis in a larger number and variety of patients and to determine the utility of immunohistochemistry as an adjunct to genetic diagnosis.

References


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