Cancer-Associated Expression of Minichromosome Maintenance 3 Gene in Several Human Cancers and Its Involvement in Tumorigenesis

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ABSTRACT

Purpose: The purpose of our study was to identify an unique gene that shows cancer-associated expression, evaluates its potential usefulness in cancer diagnosis, and characterizes its function related to human carcinogenesis.

Experimental Design: We used the differential display reverse transcription-PCR method with normal cervical, cervical cancer and metastatic tissues, and cervical cancer cell line to identify genes overexpressed in cancers.

Results: We identified a minichromosome maintenance 3 (MCM3) gene that was overexpressed in various human cancers, including leukemia, lymphoma, and carcinomas of the uterine cervix, colon, lung, stomach, kidney and breast, and malignant melanoma. Western blot and immunohistochemical analyses also revealed that MCM3 protein was elevated in most of human cancer tissues tested. We compared the MCM3 protein expression levels in human cancers with conventional proliferation markers, Ki-67 and proliferating cell nuclear antigen. MCM3 antibody was the most specific for multiple human cancers, whereas proliferating cell nuclear antigen was relatively less effective in specificity, and Ki-67 failed to detect several human cancers. The down-regulation of MCM3 protein level was examined under serum starvation in both normal and cancer cells. Interestingly, MCM3 protein was stable in MCF-7 breast cancer cells even up to 96 hours after serum starvation, whereas it was gradually degraded in normal BJ fibroblast cells. Nude mice who received injections of HEK 293 cells stably transfected with MCM3 formed tumors in 6 weeks.

Conclusions: Our study indicates that determination of MCM3 expression level will facilitate the assessment of many different human malignancies in tumor diagnosis, and MCM3 is involved in multiple types of human carcinogenesis.

INTRODUCTION

Increasing knowledge on the molecular mechanisms involved in regulating cellular proliferation has contributed to development of proteins as biomarkers to detect various human malignancies. The use of these markers to identify proliferating fraction within the tumors has proven valuable as proliferative fraction is associated with the degree of malignancy.

The most widely used conventional proliferation markers include Ki-67 and proliferating cell nuclear antigen (PCNA). Expression of Ki-67 has been particularly useful in a diagnosis of several human cancers such as breast cancer, soft tissue sarcoma, meningiomas, prostate cancer, and non-Hodgkin’s lymphoma (1–4). Despite of its usefulness, the exact function of Ki-67 still remains unclear although report suggests that it might be required for the ribosome biosynthesis during cell proliferation instead of being directly associated to the cell cycle (5). Furthermore, there is evidence indicating that Ki-67 is not essentially required for cell proliferation (6). Although useful, Ki-67 provides only a limited information on cell cycle state (7–9). Likewise, PCNA, a valuable marker for assessing human neoplasms, is known to act as an auxiliary factor for DNA polymerase 6 and is, thus, involved in DNA repair mechanisms, as well as replication (10, 11). In this respect, the relationship of PCNA expression to proliferation has been controversial (12).

The minichromosome maintenance (MCM) proteins are essential replication initiation factors originally identified as proteins required for MCM in Saccharomyces cerevisiae (13, 14). Similar classes have been found in Xenopus, murine, and human cells, with significant conservation of gene sequences (15–17). The best known among these are the MCM2–7 proteins, a family of six conserved proteins that are the key components of the replication initiation complex that initiates DNA synthesis in all eukaryotes (18, 19). The assembly of ORC, MCMs, and Cdc6 at the replication origins makes the chromatin competent or licensed for replication activities (20). At the G1 to S-phase transition, DNA replication is initiated by S-phase-promoting cyclin-dependent kinases and the Cdc7 kinase. The MCM proteins gradually dissociate from chromatin as S-phase advances (21). This dissociation of Cdc6 and MCM proteins from chromatin ensures that replication occurs only once per cell cycle (22–24).

The mRNA levels of MCMs are dramatically induced at the G1-S boundary, coupled with a significant increase in protein
levels upon growth stimulation (25). Thus, in mammalian cells, the transcriptional mechanism of the MCM genes plays an important role in the regulation of MCM activities. In contrast, MCM proteins are relatively constant and stable throughout the cell cycle but rapidly disappear after entry into quiescent cells (G0) and terminal differentiation stage in tissues (26, 27). This makes MCMs specific markers of the cell cycle state suitable, and thus, anti-MCM antibodies have recently been investigated as diagnostic tools for detecting proliferating cells in various human neoplasms and dysplasia. Although most of studies are done with anti-MCM2 and MCM5 antibodies, all MCMs appear to have the similar distribution (28, 29).

However, the MCMs are also expressed in normal proliferating cells and the proliferative basal layers of normal tissues. The expression level in normal cells seems to be lower than in cancer cells, suggesting that the expression level of MCMs is differently regulated in cancer compared with normal cells (30). E2F can induce DNA replication in otherwise quiescent cells, suggesting that genes involved in DNA replication are subject to control by E2F (31–33). These include genes for enzymatic activities. In the present study, we report that antibody against MCM3 is a powerful marker for cell proliferation. We also compared the expression level of MCM3 in various human cancers to that of PCNA and Ki-67. Our results indicate that MCM3 might be proven valuable in the diagnosis of various human cancers.

MATERIALS AND METHODS

Tissues and Cell Lines. For differential display of mRNA, a normal exoccervical tissue specimen was obtained from uterine myoma patients during hysterectomy, and untreated primary cervical cancer tissues and metastatic lymph node tissues were obtained during radical hysterectomy. Patient consent was obtained from each individual and the use of tissue samples was approved by the ethics committee of our institution. The cervical cancer cell line used in differential display was CUMC-6, which was isolated in our laboratory and maintained as described previously (37). Mammalian cell lines described below were all obtained from the American Type Culture Collection (Manassas, VA): HEK 293, BJ, and IMR-90 are a human embryonic kidney cell line, a human fibroblast from normal foreskin, and a human lung cell fibroblast, respectively. MCF-12F is an epithelial cell line from normal mammary gland. A549 is a human lung cancer cell line, and MCF-7 is a human breast cancer cell line. Finally, HeLa is a human cervical cancer cell line.

HEK 293, BJ, and IMR-90 cell lines were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (fetal bovine serum). MCF-12F was grown in medium containing a 1:1 mixture of DMEM and Ham’s F-12 medium with low Ca2+ (0.04 mmol/L), 1-glutamine (2.5 mmol/L) adjusted to contain 1.5 g/L, and sodium bicarbonate supplemented with epidermal growth factor (20 ng/mL), insulin (0.01 mg/mL), and hydrocortisone (500 ng/mL). Cancer cell lines HeLa, A549, and MCF7 used in this study were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. For serum starvation experiments, cells were grown to an 85% confluence and deprived of serum from the media. Cells were maintained serum-free, harvested at 0, 24, 48, 72, and 96 hours after serum starvation, and used for experiments.

Clinical Specimen. Formalin-fixed, paraffin-embedded human tissues and tissue samples used in Northern and Western analyses were obtained from diagnostic biopsy or resection specimens from patients at Kangnam St. Mary’s Hospital (Seoul, South Korea). The tissue was used in agreement with ethical guidelines approved by the ethics committee of our research institute.

Differential Display Reverse Transcription-PCR. Total RNA was extracted from tissues with an RNA extraction kit (RNasy total RNA kit, Qiagen, Inc., Valencia, CA), and 0.2 μg of total RNA were used to generate cDNA in a reverse transcription reaction (RNAimage kit, GenHunter, Nashville, TN). With the use of the differential display kit (RNAimage kit), we performed PCR with oligo-dT primers and arbitrary sequences, each 13 bases in length according to the manufacturer’s recommendations. After cDNA of mRNAs was generated, the PCR products were separated by electrophoresis on a 6% denaturing polyacrylamide gel. Bands representing cDNA of interest were excised from dried sequencing gel. The cDNA was eluted in distilled water by boiling for 15 minutes and then was reamplified without [α-35S]dATP and with 20 μmol/L deoxynucleotide triphosphates instead of 2 μmol/L deoxynucleotide triphosphates. From the films, a 292-bp partial cDNA (referred to as CG262) was identified, which was expressed in the primary cervical cancer tissue, metastatic lymph node, and CUMC-6 cervical cancer cell line but not in normal cervical tissue. CG262 was identified by the use of 5′-arbitrary primer H-AP26 (5′-AAGCTTGCCATGG-3′) and 3′ H-T1G anchored primer (5′-AAGCTTTTTTTTTTTTTG-3′; GenHunter). CG262 was then subcloned into the pGEM-T easy vector with the use of the TA-cloning system and subjected to an automatic sequencing analysis.

cDNA Library Screening. To isolate the full-length cDNA clone that contained the partial CG262 partial sequence, a bacteriophage λgt11 human lung embryonic fibroblast cDNA library was screened by plaque hybridization with 32P-labeled CG262 partial cDNA probe.

Construction of Expression Vector and DNA Transfection. To generate an eukaryotic expression construct of MCM3, we amplified the coding region of MCM3 by PCR, which was then subcloned into pcDNA3.1 plasmid (pcDNA3.1 Directional TOPO expression kit, Invitrogen) according to the manufacturer’s recommendations. To express MCM3 in HEK 293 cells, we seeded 3 × 105 cells per 60-mm tissue culture dish (Costar, Cambridge, MA). The next day, the cells were incubated with 4 mL of serum-free DMEM, which contained 15 μL of Lipofectamine reagent (Life Technologies, Inc., Rockville, MD) and 5 μg of pcDNA3.1-MCM3 and pcDNA3.1 vector alone. After a 10-hour incubation, the cells were cultured in DMEM supplemented with 10% fetal bovine serum for HEK
293 cells containing 0.6 mg/mL G418 (Life Technologies, Inc.). HEK 293 cells were selected for resistance to 0.6 mg/mL G418 for 3 to 4 weeks. After 4 weeks, the surviving cells were detached with trypsin and cloned by limiting dilution in 96-well culture plates at a concentration of 0.5 cell per well. The HEK 293 cells were maintained in DMEM containing G418 (0.4 mg/mL). Thirty-five HEK 293 clones were selected and screened for the expression of MCM3 by Western blot analysis.

Antibodies. Rabbit polyclonal antibody was raised against the product of the MCM3 gene. A plasmid expressing a whole MCM3 fused to a 6× His tag was constructed by ligating a PCR product of MCM3 into a pBAD/TOPO expression vector (pBAD/TOPO thioufision expression kit, Invitrogen). Induction of Escherichia coli carrying the resulting plasmid with arabinose produced a fusion protein that was purified with the use of a Ni-nitrilotetraacetic acid-agarose column (ProBond purification system, Invitrogen). Antibody was raised against the purified protein in New Zealand White rabbits. The resulting rabbit serum was affinity-purified with MCM3 protein covalently cross-linked to CNBr-activated Sepharose beads (Amersham Biosciences, Piscataway, NJ).

The mouse monoclonal Ki-67 and PCNA antibodies were purchased from Oncogene Research Products (San Diego, CA). The mouse vimentin and cytokeratin antibodies were purchased from Dako (Glostrup, Denmark). Finally, secondary antirabbit antibody conjugated to FITC for immunofluorescence staining was obtained from Jackson Immunoresearch (West Grove, PA).

Northern and Western Blot Analyses. Total RNA was extracted from frozen human tissues with TRIzol reagent (Invitrogen). Northern blot analysis was carried out in which 20 μg of denatured total RNA were electrophoresed on a 1.0% formaldehyde agarose gel and transferred to a nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany). The mRNA expression of MCM3 was also assessed in normal human tissues and a variety of human cancer cell lines with the use of membrane commercially available from Clontech (Pal Alto, CA), which was processed as recommended by the supplier. Human β-actin cDNA control probe provided by Clontech was used as a loading control. All blots were hybridized with the randomly primed [32P]-labeled MCM3 partial cDNA probe (the CG262 fragment).

For Western blot analysis, tissues were homogenized in a lysis buffer [20 mmol/L Tris (pH 7.4), 1% NP40, 5 mmol/L EDTA, 10% glycerol, 0.1% SDS, and 150 mmol/L NaCl] containing protease inhibitor mixture (Sigma, St. Louis, MO), and the homogenated was clarified by centrifugation at 5000 × g for 10 minutes. Equivalent volumes of cell lysates containing 20 μg of total protein were loaded on 10% SDS-polyacrylamide gels and separated by electrophoresis. Protein samples were then transferred to nitrocellulose membranes. The blots were incubated with polyclonal anti-MCM3 serum and developed with the enhanced chemiluminescence detection kit (Pierce, Rockford, IL).

Immunofluorescence. Cells incubated under serum starvation conditions were fixed by incubation with 3% paraformaldehyde in PBS for 15 minutes at room temperature. The cells were then washed with PBS and permeabilized in 0.2% Triton X-100 in PBS for 5 minutes. After then, samples were blocked by incubation with 1% BSA (Sigma) in PBS for 30 minutes at room temperature. Incubation of the cells with anti-MCM3 antibodies was done for 2 hours at room temperature in the above blocking solution. The cells were washed with the same blocking solution and incubated with antirabbit IgG conjugated to FITC for 1 hour. Then, they were washed with the blocking solution and photographed with fluorescence microscopy (AX-70, Olympus, Tokyo, Japan).

Immunohistochemistry and Scoring Immunohistochemistry data. For immunohistochemistry, paraffin sections (5-μm thick) of human cancer tissues were used. The sections were deparaffinized with xylene and ethanol. After washing with tap water, the sections were treated with methanolic H2O2 for 30 minutes. Before incubation with primary antibody, the sections were permeabilized by incubation in 0.5% Triton X-100 in PBS for 15 minutes and then blocked with normal goat serum for 15 minutes. The sections were incubated with affinity-purified anti-MCM3 antibody for 2 hours at room temperature. After three washes with PBS, the sections were sequentially incubated with secondary antibody conjugated to horseradish peroxidase (Zymed Laboratories, Inc., San Francisco, CA) for 1 hour at room temperature according to the manufacturer’s recommendations. Aminoethyl carbozole was used as the chromogen. After immunostaining, sections were counterstained with hematoxylin.

A semiquantitative scoring scheme was designed to describe the immunohistochemical staining observed. Cell nuclei were positive or negative for Mcm3. The percentage of positively stained tumor cells was scored and they were divided into four categories as follows: 1 (0 to 25% cells stained), 2 (26 to 50% cells stained), 3 (51 to 75% cells stained), and 4 (76 to 100% cells stained).

Tumorigenicity. The in vivo studies were approved by the ethics committee of our institution. Mice were given filtered tap water ad libitum in accordance with Guide for the Care and Use of Laboratory Animals (NIH). To assess in vivo tumorigenicity, HEK 293 cells (5 × 106 cells) stably transfected with the MCM3 gene were injected s.c. into the posterior lateral aspect of the trunk of 5-week-old athymic nu/nu female BALB/c mice.

![Image](http://clincancerres.aacrjournals.org)
Mice that received injections of HEK 293 cells stably transfected with the MCM3 gene were killed when the tumors reached 1.5 to 2.5 cm in diameter approximately after 6 weeks. Mice that received injections of vector-transfected cells were killed after 6 weeks. The size of the tumors was measured every 3 to 4 days. Tumors were fixed in 10% formalin for histologic examination.

RESULTS

Cloning of a MCM3 Overexpressed in Cervical Cancer.

To discover genes involved in cervical carcinogenesis, we applied a differential display reverse transcription-PCR. Total RNAs were isolated from the normal cervical tissue, the cervical cancer tissue, metastatic lymph node tissue, and CUMC-6 cervical cancer cells but was not expressed in normal cervical tissue (Fig. 1). Using the partial cDNA CG262 as a probe, we next screened normal human lung cDNA library to isolate the full-length cDNA. One clone with a size of 3091 bp named HCC-5 for human cervical cancer proto-oncogene 5 (GenBank accession number AY032603) was isolated. Se-

292-bp partial cDNA fragment CG262 that was overexpressed in cervical cancer tissue, metastatic lymph node tissue, and CUMC-6 cervical cancer cells but was not expressed in normal cervical tissue (Fig. 1). Using the partial cDNA CG262 as a probe, we next screened normal human lung cDNA library to isolate the full-length cDNA. One clone with a size of 3091 bp named HCC-5 for human cervical cancer proto-oncogene 5 (GenBank accession number AY032603) was isolated. Se-

Table 1

<table>
<thead>
<tr>
<th>Tissue origins</th>
<th>No. of normal tissues</th>
<th>No. of tumor tissues</th>
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<td>4</td>
</tr>
<tr>
<td>Breast</td>
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<td>10</td>
</tr>
<tr>
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<td>3</td>
</tr>
<tr>
<td>Bone</td>
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<td>2</td>
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<tr>
<td>Soft tissue</td>
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<td>1</td>
</tr>
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<td>Lung</td>
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<tr>
<td>Spleen</td>
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<td></td>
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<tr>
<td>Tonsil</td>
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Fig. 2 MCM3 gene expressions in human tissues and cell lines. Northern blot analyses were done to determine the expression pattern in different human tissues and cell lines. A. Normal 12-lane multiple tissue Northern blot purchased from Clontech was probed with a radioactively labeled CG262 partial cDNA. Human β-actin cDNA control probe provided by Clontech was used as a loading control. B. Human cancer cell line multiple Northern blot was probed with a CG262 partial cDNA. C, comparison of MCM3 mRNA expressions in fresh human cancer tissues and their corresponding normal counterparts. Total RNAs were extracted from fresh normal (N) and cancer (C) tissues, and Northern blotting was done with CG262 as a radioactive probe. Human β-actin cDNA control probe was used as a loading control.

Mice that received injections of HEK 293 cells stably transfected with the MCM3 gene were killed when the tumors reached 1.5 to 2.5 cm in diameter approximately after 6 weeks. Mice that received injections of vector-transfected cells were killed after 6 weeks. The size of the tumors was measured every 3 to 4 days. Tumors were fixed in 10% formalin for histologic examination.

Fig. 3 MCM3, Ki-67, and PCNA protein expression levels in human tissues. A. Whole protein lysates were assessed for expression of MCM3 by Western blot analysis with polyclonal antibody. Comparison of MCM3 protein expression in human cancer tissues (C) and their corresponding normal counterparts (N). Three different blots containing the same samples were probed with antibodies against PCNA (B), Ki-67 (C), and β-actin (D), respectively.
sequence analysis showed that this cDNA encodes MCM3 gene (GenBank accession number NM_002388) with a molecular mass of 100 kDa.

**MCM3 Gene Expressions in Human Cancers.** Northern hybridization was done on a human normal multiple tissue mRNA blot and a human cancer cell line mRNA blot to analyze MCM3 expressions in various normal tissues and cancer cell lines. The MCM3 mRNA (~3 kb) was present in low levels in some normal tissues, including the small intestine, spleen, and colon (Fig. 2A). Contrary to normal tissue samples, the MCM3 transcript was strongly detected in cancer cell lines, including lymphoblastic leukemia cell line MOLT-4, Burkitt’s lymphoma cell line Raji, and cervical cancer cell line HeLa. Overexpressions of MCM3 were also detected in promyelocytic leukemia cell line HL-60, chronic myelogenous leukemia cell line K-562, and colon cancer cell line SW480, whereas it was expressed at a moderate level in melanoma cell line G361 and A549, a lung cancer cell line (Fig. 2B). Thus, Northern blot analyses show that MCM3 mRNA exists abundantly in cancer cell lines compared with its little or no expression in most normal tissues. To additionally examine MCM3 expressions in fresh human cancer tissues, Northern hybridization was carried out to blots containing total RNAs from tissues such as colon, lung, cervix, stomach, kidney, and breast cancers and their corresponding normal tissues. Each pair on the blot consists of normal and cancerous tissues obtained from the same patient. The result revealed that MCM3 is expressed much higher in primary cancer tissues, including carcinomas of colon, cervix, stomach, kidney, and breast than in their normal counterparts, except lung cancer tissue (Fig. 2C). Although lung cancer sample didn’t show the high expression on this particular blot, we could also detect the overexpression of MCM3 transcripts in other lung cancer samples (data not shown). This finding reassures the general requirement of MCM3 function in several types of human cancers.

**MCM3, Ki-67, and PCNA Protein Expressions in Human Cancers.** To detect the MCM3 protein, we raised polyclonal antibody against MCM3 expressed and purified in a prokaryotic system. We applied this polyclonal antibody to tissue Western blot. We could observe the cancer-specific expression of MCM3 protein corresponding to a molecular mass of ~100 kDa in multiple cancer tissues, including carcinomas of colon, lung, cervix, stomach, kidney, and breast, although the amount of proteins expressed varies in each cancer sample (Fig. 3A). Although this result is consistent with the mRNA expres-
sion data (Fig. 2C), the heterogeneous level of MCM3 protein contrasts to the relatively constant levels of mRNA among different cancer samples. We also observed the smaller protein band with a faster electrophoretic mobility in lung, kidney, and cervix cancer tissues, respectively (Fig. 3A). However, given the fact that MCM3 protein shows the specific proteolytic cleavage during apoptosis (38), it can probably be a cleaved form of Mcm3p or a nonspecific band. We then applied antibodies against PCNA and Ki-67, known as conventional proliferation markers to the same blots (39–41). PCNA showed the cancer-specific expressions only in colon and breast cancer tissues. Although the PCNA was overexpressed in lung, cervix, stomach, and kidney cancer tissues, their corresponding normal tissues also showed faint or weak PCNA expressions. In contrast, Ki-67 was detected only in stomach and colon cancer tissues, which was observed in a multiple band pattern because of its modifications such as phosphorylations and glycosylations. Moreover, only stomach cancer strongly expressed Ki-67, whereas colon cancer was very weakly positive for Ki-67. According to immunoblotting experiments, MCM3 was most specific for cancer, whereas PCNA was relatively less effective and Ki-67 failed to detect several human cancers.

**Anti-MCM3 Antibody Detects Malignant Cells in Tissue Sections.** Our data described above suggest that anti-MCM3 antibody might serve as a marker for malignant cells derived from multiple anatomic sites. To examine this, we tested affinity-purified polyclonal antibody on paraffin-embedded sections consisting of various human cancer tissues. A total of 110 tumor tissues was included in this immunohistochemical analysis as shown in Table 1. Positive immunostaining was observed in the nuclei of cancer cells derived from multiple tissues with unlabeled nuclei appearing pale with the hematoxylin counter stain (Fig. 4). Levels of Mcm3 expression were variable among tumor specimens studied. Ninety-three of 110 cancer samples analyzed showed the moderate or high levels of expressions, whereas the remaining samples showed cytoplasmic or no expressions, corresponding to >80% of a diagnostic efficiency for human cancer. To additionally confirm that anti-Mcm3 antibodies efficiently detect malignant cells, we also stained the normal tissue specimens on paraffin-embedded sections (Fig. 5). We included several normal tissues in this analysis (see Table 1). As expected, most of normal tissues were negative or weakly positive in staining. We observed that some normal tissues had the reactivity with anti-MCM3 antibodies, although the staining was strictly restricted to the proliferating fractions (data not shown here). For example, in testes, MCM3 expression was high in spermatogonia in which sperms are actively formed and growing. The MCM3 expression in normal tonsil was also confined to germinal centers having active proliferating activities. Likewise, MCM3 expression is tightly associated to the proliferating activities of tumor and normal tissues. Because the proliferative fraction is associated to the degree of malignancy, the identification of proliferating fraction within the tumors is being used in a prognosis and diagnosis of cancers. Therefore, the use of anti-MCM3 antibodies will be proven valuable as a marker for detecting abnormal malignant cells, although its usage is not separable from that of a proliferative marker.

**The Delayed Down-Regulation of MCM3 in Cancer Cells.** Next, we were interested to investigate the down-regulation of MCM3 protein to see if it is differently regulated in...
normal and cancer cells, respectively. We first looked for MCM3 protein expression pattern under the serum starvation and could see the gradual decrease of MCM3 labeling in normal BJ fibroblast cells during the time course of serum starvation (Fig. 6A). The clear nuclear staining was observed in most of cells at 0 hour and disappeared from a majority of cells at 48-hour time point. It was then completely lost in all BJ cells up to 96 hours after serum starvation. In contrast, MCF-7 showed the persistent expression of MCM3 even at 96 hours after serum starvation. This delayed down-regulation in MCF-7 implicates that MCM3 protein level is aberrantly regulated in cancer cells. To determine whether this delayed down-regulation in general in cancer cells, we analyzed the MCM3 protein levels under the same condition as above by including several normal and cancer cell lines (Fig. 6B). All cancer cells (MCF-7 breast cancer, A549 lung cancer, and HeLa cervical cancer cells) tested showed no degradation even at 96 hours after serum starvation, whereas all normal cell lines (BJ fibroblasts, IMR-90 fibroblasts, and MCF-12F mammary cells) had their own characteristic degradation kinetics (Fig. 6B). It is possible that the degradation kinetics might reflect on the proliferation potential of each cell type. According to this notion, IMR-90 seems to most rapidly lose its growth ability and enter into the quiescent state. To compare with other proliferation markers, we applied anti-PCNA (Fig. 6C) and anti-Ki67 antibodies (Fig. 6D) onto the same blot. Notably, Ki-67 antigen was positive only in MCF-12F, A549, and HeLa but negative in BJ, IMR-90, and MCF-7 (Fig. 6D). This shows that the level of Ki-67 is not always related to the proliferation potential. Moreover, its level decreases during the time course of serum starvation even in cancer cells such as A549 and HeLa, as well as in MCF-12F mammary cells. However, PCNA behaved similarly to MCM3 in that it was decreas-
ing in normal cells, except BJ. It was highly stable in all cancer cells (Fig. 6C) It is interesting that it was detected at the highest level in IMR-90 and highly stable in BJ, which differs from MCM3 in those cell lines. In this regard, MCM3 seems most directly associated to the growth ability of normal cells. It also seems to fit into the definition of a tumor marker because it can detect only cancer cells but not normal cells under the prolonged nutritional deprivation.

**Ectopic Expression of MCM3 Induces Tumorigenicity.**

We have shown that MCM3 is overexpressed in almost all human cancers examined thus far. It implies that MCM3 might facilitate the tumorigenesis by playing a role in the malignant transformation of cells. To determine whether the ectopic expression of MCM3 can induce the tumorigenicity in human cells, we introduced MCM3 into HEK 293 cells, and stable cell lines were selected in medium containing 0.6 mg/mL G418 by culturing for 21 days. Western blot analysis revealed that 128 kDa product were expressed in these MCM3-transfected cells (Fig. 7A). We next examined whether MCM3 can contribute to the tumorigenic transformation of HEK 293 cells with in vivo nude mouse tumor assay. Cells were harvested from the culture dish in media containing G418. We then injected 5 × 10^6 cells into each 10 nude mice. These cells exhibited tumorigenic potentials as shown by their development as tumors in 9 of 10 nude mice (Fig. 7B), whereas cells with vector alone failed to grow as tumors. We then analyzed tumors derived from nude mice for their origins. Tumors from nude mice injected with HEK 293 cells stably transfected with MCM3 displayed characteristics of epithelial carcinoma (Fig. 7C). For morphologic comparison between MCM3-derived tumor cells and epithelial cells, we determined whether MCM3-derived tumor cells express epithelial cell marker, keratin. Tumor-derived cells exhibited the positive staining for keratin, indicating they are originated from the epithelial cells (Fig. 7D).

**DISCUSSION**

Although genetic characterization of tumor tissues shows that mutation of the p53 is the most common genetic alteration in human cancers, the mutation rate of the p53 in cervical cancer is relatively low (42, 43). It implies that there might be other factors involved in cervical carcinogenesis.

In our study, we applied the differential display reverse transcription-PCR method to explore genes related to the tumorigenesis of human cervical tissue and identified MCM3, a component of the MCM2–7 complex. We have showed here that the majority of human tumors overexpress MCM3, and as a result, anti-MCM3 antibody is potentially valuable as a tumor marker. The high level of MCM3 expression generally observed in human cancer might contribute to the growth of cancer cells by facilitating overall replication activities. It has been reported that the overexpression of MCM proteins might contribute to cell growth by increasing the transcription rate (44) or through interaction with the retinoblastoma protein (45). Indeed, we observed the ability of HEK 293 cells stably transfected with MCM3 to induce the tumor formation in nude mouse, implying the role of MCM3 in tumorigenesis.

The rapid proliferation rate is one of the features common to most neoplasms. Identification of a proliferating fraction within the tumor cell population has been useful in a diagnosis or prognosis in a range of human cancers, including prostate and breast cancer, and lymphoma (46). Biological markers indicative of cell cycle state has proven useful for determining growth fractions because previous methods mostly focused on S-phase
Overexpression of MCM3 in Human Cancers

labeling and resulted in missing a considerable fraction of inter-mitotic cells that are proliferating but not in S-phase. PCNA has a major limitation as a proliferation marker because of its redundant role in DNA repair. This is in accordance with our observations (Figs. 3B and 6C), which shows its considerable expression in normal human tissues and cell lines without proliferating activities. Meanwhile, the expression of Ki-67 antigen is restricted to proliferating fractions but disappears rapidly when cells enter a resting state. The MCM proteins are key regulators of eukaryotic DNA replication. Their expression is stable throughout the cell cycle and, thereby, is an excellent indicator of cell cycle state. In particular, they are distinct from other proliferation markers in that they can also identify cells that are resting but still replication competent (47).

Previous studies on application of anti-MCM antibodies to the diagnostic tumor pathology were mostly done with those against MCM2 and MCM5 (48–51). We additionally extended this by analyzing the expression patterns of MCM3 in multiple human cancers in both mRNA and protein levels. Immunostaining on paraffin sections was positive for various human cancers (Fig. 4). Although this should be done in a larger clinical scale, we can conclude that our affinity-purified anti-MCM3 antibodies identify malignant cells with high sensitivity and specificity in a broad range of human cancers.

One of the interesting observations that we made is that the amount of MCM3 proteins among primary tumors shows the sample-to-sample variations (Fig. 3). This is one of the reasons why we saw the heterogeneous staining among tumor sections in immunohistochemical analysis (Fig. 4). This suggests that there might be a cascade of regulations to control its expression levels during the process of mRNA translation in addition to the control mechanism from its promoter sequences. It is likely that the stability or translation rate of mRNA might be crucial factors for variable amount of proteins in each tumor. This notion is supported by our pulse-chase immunoprecipitation experiments, showing that the turnover rate of MCM3 protein was nearly identical between normal and cancer cells.4 It indicates that regulation activity to determine the protein level is exercised not at the protein level but possibly at the mRNA level. Thus, their levels might be differently regulated possibly depending on several factors characteristic of each tumor, which await to be determined. Regardless of this, it will be of interest to uncover this regulation mechanism together with its promoter activity control.

We measured and compared the down-regulation of MCM3 proteins under the nutrient deprivation to that of PCNA and Ki-67 (Fig. 6). The prolonged expression of both MCM3 and PCNA under this unfavorable environment makes them more attractive as tumor markers because there is a possibility of a nutritional deprivation operating regionally in solid tumors. In a case like this, a tumor marker can be defined separately from a proliferation marker because MCM3 was detectable only in cancer cells but not in normal cells after long serum starvation. In addition, their delayed down-regulation also indicates that antibodies against MCM3 and PCNA can also detect cells that stopped proliferating but still retain the replicative ability. This agrees with the previous study (47). However, there still remain problems in PCNA as shown in its overexpression in IMR-90 and in its high stability in BJ. It suggests that the PCNA marker must have a higher chance of detecting normal cells as well as cancer cells.

In conclusion, this study shows the aberrant expressions of MCM3 in cancer cells and its potential usefulness as a tumor marker was also assessed. Additional studies on the control mechanisms for MCM3 expression will provide new insights on how these mechanisms are deregulated in human cancers and how significant they are in maintaining normal tissues.

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Cancer-Associated Expression of Minichromosome Maintenance 3 Gene in Several Human Cancers and Its Involvement in Tumorigenesis

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