Deficient Expression of $O^6$-Methylguanine-DNA Methyltransferase Combined With Mismatch-Repair Proteins hMLH1 and hMSH2 Is Related to Poor Prognosis in Human Biliary Tract Carcinoma

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Background: $O^6$-Methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme that transfers methyl groups from $O^6$-methylguanine to itself. Alkylation of DNA at the $O^6$ position of guanine is an important step in the induction of mutations in the organism by alkylating agents. The $O^6$-methyl G:T mismatch is recognized by the mismatch-repair (MMR) pathway. The biliary duct is highly exposed to alkylating agents because of its anatomical location.

Methods: We examined 39 surgically resected gallbladder carcinomas and 35 extrahepatic bile duct carcinomas and evaluated the expression of MGMT and MMR protein (hMLH1 and hMSH2) by immunohistochemical staining.

Results: MGMT-negative staining was detected in 59.0% of gallbladder carcinoma specimens and 60.0% of extrahepatic bile duct carcinoma specimens. In gallbladder carcinoma, hMLH1- and hMSH2-negative staining was observed in 51.3% and 59.0%, respectively, whereas in extrahepatic bile duct carcinoma, the respective values were 57.1% and 65.7%. MGMT-negative staining correlated with hepatic invasion in gallbladder carcinoma and with poor prognosis in both types of tumor. Furthermore, a combined MGMT and MMR status was shown to be a more significant prognostic biomarker in both tumor types.

Conclusions: Combined MGMT and MMR is a possible prognostic marker that probably reflects an accumulation of genetic mutations.

Key Words: Biliary tract carcinoma—MGMT—hMLH1—hMSH2.

Alkylation of DNA at the $O^6$ position of guanine is an important step in the induction by alkylating agents of mutations in the organism. $O^6$-methylguanine is capable of pairing with both thymine and cytosine during DNA replication, resulting in a G:C to A:T transition mutation in DNA. $O^6$-Methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme that transfers methyl groups from $O^6$-methylguanine to itself to repair the premutagenic base in DNA. The level of MGMT varies among different organs and tissues, whereas most tumor cells express high levels of MGMT; approximately 20% of human tumor cell lines are deficient in MGMT expression. MGMT-lacking cell lines are hypersensitive to alkylating agents, and these cell lines, termed Mer− or Mex−, show little or no methyltransferase activity. The mechanisms responsible for regulating MGMT expression remain unknown. Abnormal MGMT expression may induce either human oncogene activation or tumor suppressor gene inactivation, thereby contributing to carcinogenesis and tumor progression. Therefore, abnormal MGMT expression may correlate with the malignant grade of cancer and the prognosis of patients with neoplastic disease.

$O^6$-Methylguanine on its own is not deleterious to cells, and it does not inhibit DNA enzymatic processes,
such as replication and transcription. However, the preferred base pairing during DNA synthesis results in incorporation of thymine opposite \( O^6 \)-methylguanine rather than cytosine, and this in turn results in a G:C to A:T transition mutation if not repaired. The \( O^6 \)-methyl G:T mismatch is recognized by the mismatch-repair (MMR) pathway of the cell,\(^{10}\) which subsequently excises the errant thymine residue in the daughter strand. However, unless the \( O^6 \)-methylguanine is repaired before the resynthesis step in MMR, there is a high likelihood that thymine will be reinserted opposite the lesion. It is believed that the ensuing repetitive cycle of futile MMR results in the generation of chronic strand breaks, which in turn elicits an apoptotic response.\(^{11}\) Of five or more distinct proteins that are involved in the MMR pathway,\(^{12}\) two—MLH-1 and MSH-2—have frequently been found to be suppressed in tumor cells.\(^{13}\)

Biliary tract carcinoma is a relatively rare tumor that is characterized by a poor prognosis. Mortality rates are highest among American Indian women from the southwestern United States and in Chilean and Japanese women.\(^{14}\) Alkylating agents are metabolized and activated in hepatocytes and are released from the bile duct and stored in the gallbladder. The epithelium of the gallbladder and extrahepatic bile duct is highly exposed to alkylating agents because of its physiological function and anatomical location.

Previous studies have observed that genetic abnormalities in biliary tract carcinoma are focused mainly on dominant oncogene \( K-ras \) mutations\(^{15-18}\) and on abnormalities of the tumor suppressor genes \( p53 \)\(^{16-19}\) and \( p16{^{NK4}} \).\(^{20}\) Abnormalities of these factors are considered to be early changes during carcinogenesis and are not correlated with pathologic factors or patient prognosis.\(^{21}\)

The aim of this study was to analyze the association of deficient or reduced MGMT and MMR enzyme (hMLH1 and hMSH2) expression with clinicopathologic factors and prognosis in human biliary tract carcinomas.

**MATERIALS AND METHODS**

**Patients**

Thirty-nine gallbladder carcinoma specimens and 35 extrahepatic bile duct carcinoma specimens were obtained from patients who underwent surgical resection at the Department of Surgery, Saga Medical School, from April 1989 to February 2000.

**Antibody Preparation**

Polyclonal rabbit antibodies against human MGMT protein were prepared by using tryptophan operon of *Escherichia coli* (TrpE) fusion protein, as previously described.\(^{22}\) *E. coli* BL21 (DE3) carrying pET3d:TrpE-hMGMT-1, which encodes the TrpE polypeptide fused to a part of MGMT (residues 1–45) at its C terminus, was used to produce each fusion protein,\(^{23}\) and polyclonal antibodies against the fusion protein were raised in rabbits. First, the serum was applied to the TrpE-hMGMT-1-coupled column, and bound materials were eluted at pH 2.3 and dialyzed against 10 mM of Tris-HCl (pH 7.4) and 150 mM of NaCl. To recover the antibody fraction at a much higher specificity, the eluted fraction was applied to an affinity column with TrpE-mMGMT-1, in which a corresponding part of mouse MGMT (residues 1–58) was fused to TrpE\(^{24}\) as a ligand, and the bound fraction was eluted and dialyzed. This fraction was used as the anti-MGMT antibodies.

**Determination of Anti-MGMT Antibody**

We determined the specificity of this MGMT antibody by Western blot analysis by using cell-line lysate. HeLa S3 is an MGMT-proficient (Mer\(^+\)) cell line\(^{25,26}\) of cervical carcinoma. HeLa MR is an MGMT-deficient (Mer\(^-\)) cell line,\(^{25,26}\) and HeLa MRV-11 is a transfected vector only. HeLa MR5-2 is an MGMT overexpressor that is transfected with human MGMT expression vector. We performed semiquantitative analysis with these cell-line lysates, and the specificity of this antibody was checked.\(^{27}\)

**Immunohistochemical Analysis**

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections of all 39 gallbladder carcinoma and 35 extrahepatic bile duct carcinoma specimens. The primary antibodies used in this study were rabbit polyclonal antibody against MGMT and monoclonal antibody against hMLH1 (clone G168-728; PharMingen, San Diego, CA) and hMSH2 (clone G219-1129; PharMingen). Sections (5 \( \mu \)m) were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. The sections were placed in .01 M of citrate buffer (pH 6.0) and exposed to microwaves for 15 minutes. Next, .3% hydrogen peroxidase was applied for 10 minutes to block endogenous peroxidase activity, and the tissues were then incubated in 10% normal goat serum for 10 minutes to abolish nonspecific protein binding. The sections were then incubated with a primary antibody at 4°C overnight. The MGMT antibody was used at a dilution of 1/200. The hMLH1 and hMSH2 antibodies were used at a dilution of 1/50. Immunostaining was performed by the streptavidin-biotin-peroxidase complex method by using a Histofine SAB-PO kit (Nichirei Co., Tokyo, Japan). Staining was visualized...