Abstract. Aim/Purpose: We investigated the expression of melanoma-associated antigen gene (MAGE), human synovial sarcoma on X chromosome (SSX) and their clinical implications in sporadic colon cancer. Materials and Methods: Fresh tissue samples from 37 patients with colorectal adenocarcinomas were analyzed for MAGE and SSX mRNA by reverse transcription-polymerase chain reaction (RT-PCR) and their paraffin-embedded tissues were used for immunohistochemistry for cyclooxygenase 2 (COX2), vascular endothelial growth factor (VEGF), and survivin. Results: Expression of MAGE and SSX was not detected in normal tissues. Colon cancer expressed SSX in 32.4% and MAGE in 51.4% of cases. Co-expression of MAGE and SSX was directly correlated with liver metastasis (p=0.024) and also correlated with nuclear expression of survivin (p=0.016), yet did not correlate with expression of COX2 and VEGF. Nuclear survivin expression (83.3%) was found in the cancer tissues exclusively. No significant relationships between the expression of COX2 (73.9%) and VEGF (72.4%) and other clinicopathologic variables were found. Conclusion: Our results suggest that nuclear expression of survivin, lymph node metastasis, vascular and perineural invasion, and co-expression of MAGE and SSX may be associated with the metastasis of colorectal cancer to the liver.

Cancer-testis antigen (CTAs) were the first human tumor-shared specific antigens to be characterized at the molecular level. CTAs are expressed in normal testis and in a wide array of human cancer types, yet are expressed at very low levels, or not at all, in most other health tissue (1). CTA gene products are often immunogenic in cancer patients, and make ideal targets for cancer immunotherapy. It is important to note that no individual CTA is specific to any cancer type, and CTAs are therefore not expected to be useful for molecular diagnosis.

The biological functions of CTAs are largely unknown (1). Melanoma-associated antigen gene (MAGE), GAGE, and New York esophageal-1 (NY-ESO-1) have been classified as CTA genes (1). Human synovial sarcoma on X chromosome (SSX) gene was also recently classified as a CTA gene (1). The gene was first identified as being involved in the t(X;18) translocation in synovial sarcoma (2-4). SSX is actually a multigene family comprising nine genes on Xp11 (5). The SSX family members show strong sequence homology with each other, with nucleotide homologies range from 88% to 95%, and amino acid homologies range from 77% to 91% (6). Although the SSX proteins lack a DNA-binding domain and they appear to function as transcriptional co-repressors, the true function of the SSX genes remains unclear (7).

The survivin gene, located in chromosome 17q25, is a new member of the inhibitor of the apoptosis family expressed predominantly in fetal tissue, but is also found to be expressed in many common types of human cancer (8). There are some reports that survivin expression correlates with poor survival of cancer patients, including those with non-small cell lung cancer (9), breast carcinoma (10), esophageal cancer (11), and gastric carcinoma (12). Therefore, survivin expression is considered an important prognostic marker in cancer. However, the clinical significance of survivin expression and correlation with the biological aggressiveness of cancer remain unclear in colon cancer.

Angiogenesis is essential for development, growth, and advancement of solid tumors. COX2 and vascular endothelial growth factor (VEGF) are recognized as angiogenic factors in various tumor types (13, 14). One report suggests that COX2 is involved in the course of tumor angiogenesis of colorectal cancer, acting through VEGF (13) or inducible nitric oxide synthase (14).

Over 14% of patients with colorectal cancer have synchronous liver metastases of colorectal cancer (15). The aim of this our study was to elucidate the factors involved in
hepatic metastasis of colorectal cancer by assessing expression of the CTAs SSX and MAGE, the angiogenic factors COX2 and VEGF, and the anti-apoptotic factor survivin.

Materials and Methods

Patients and clinical samples. Surgical specimens were obtained from 37 consecutive patients diagnosed with various stages of colorectal carcinoma who had undergone resection between October, 2002 and August, 2003. The mean age was 59.22 (±18.635) years. Pathologies were confirmed by analysis of frozen sections of resected specimen. RNA was isolated from fresh frozen primary colorectal carcinoma samples and non-neoplastic, normal-looking colorectal mucosa taken from an area more than 2 cm from the tumor mass.

Histology and RNA isolation. Fresh frozen colorectal cancer and corresponding non-neoplastic colorectal tissue blocks were cut into 4 μm sections using cryostat microtome at –20˚C. The first and last sections were immediately stained with methylene blue and examined under a microscope to confirm histologically normal tissues without tumor cell infiltration and tumor tissue consisting of at least 80% tumor cells. Total RNA was isolated from each of the 37 samples via lysis in guanidinium isothiocyanate and phenol extraction using a commercial kit (Trizol; Invitrogen Laboratories, San Diego, CA, USA).

Reverse-transcription polymerase chain reaction (RT-PCR). cDNA was synthesized from 4 μg of total RNA in a 25-μl reaction mixture containing 6 μl of 5× reverse transcriptase reaction buffer, 1 μl of oligo(dT)(100 pmol/μl), 4 μl of 10 mM dNTP, 40 unit/μl of RNAsin, 0.5 μl of 200 units/l Moloney leukemia virus reverse transcriptase. The mixture was incubated at 4˚C for 60 minutes, heated to 94˚C for 3 minutes, and then chilled on ice. In order to verify the integrity of the cDNA, GADPH was amplified in each sample.

MAGE primer design. In order to simultaneously detect the expression of MAGE A-1 to 6 and to avoid amplification of genomic MAGE DNA, the followings MAGE common primer sets were used: sense 5’-CTGAAGGAGAAGATCTGCC and antisense 5’-CTCCAGGTAGTTTTCCTGCAC for first round PCR, and sense 5’-CTGAAGGAGAAGATCTGCCWGTG and antisense 5’-CCAGCA TTCTGCGCTTGTGA for the second round PCR. These primer sets were designed such that the 5’ (sense) and 3’ (antisense) primers span at least one intron in the genomic DNA. Therefore, each of the sense and antisense primers were complementary to two exon sequences at either side of an intervening intron to prevent

Figure 1. The electrophoretic analysis of nested RT-PCR amplification products with common SSX primers (495 bp) in the first panel and MAGE primers (490 bp) in the second panel from healthy colorectal mucosa (N) and colorectal cancer (T); GAPDH is shown in the third panel (450 bp).
hybridization of the genomic DNA. These common MAGE primer pairs were used for the nested PCR of the reverse-transcribed cDNAs. All oligonucleotide primers were synthesized by the Bioneer Company (Bioneer, Taejung, South Korea). Oligonucleotide primers were dissolved in Tris-EDTA buffer to 100 pmol/μl, aliquoted, and stored at –75˚C. Each aliquot was diluted to 10 pmol/μl before use.

**SSX primer design.** In order to simultaneously detect the expression of SSX1 through SSX9, the common primers for SSX based on the homology of each subtype were used: first round PCR: sense 5’-GTGCCATGAACGGAGACGA, antisense 5’-GTCTGTGGGTCCAGGCATGT; second round PCR: sense 5’-GTGCCATGAACGGAGACGA, antisense 5’-TGTTTCCCCCTTTTGGGTCC. Each of the sense and antisense primers were complementary to two exon sequences at either side of an intervening intron, which prevented hybridization of the genomic DNA. These common primers were used for the nested PCR of the reverse-transcribed cDNAs. All oligonucleotide primers were synthesized by Bioneer Company. Oligonucleotide primers were dissolved in Tris-EDTA buffer at 100 pmol/μl. Aliquots were diluted to 10 pmol/μl before use.

**Direct DNA sequencing of PCR products.** Wizard Plus SV Minipreps Kit (Promega, Fitchburg, WI, USA) was used to prepare the template DNA for sequencing after subcloning of the RT-PCR products. An automatic DNA sequencer (ABI sequencer 3700; Macrozen, Seoul, Korea) was used for sequencing, and sequence data were analyzed by the NCBI Blast search program (NIH, USA).

**Immunohistochemistry.** The expression of survivin, COX2, and VEGF in colorectal carcinomas and adjacent healthy colorectal tissue samples were evaluated by immunohistochemistry of the paraffin embedded tissue. For the nuclear and cytoplasmic survivin assessment, the intensity (I) and distribution (D) of the immunostaining were scored on a scale of 1 to 4. An ID score (ID) ≤4 was interpreted as low-level expression, while ID >4 was interpreted as being highly positively expression.

**Statistical analysis.** Statistical analysis was performed using SPSS (version 14.1; Stanford, CA, USA). The significance level was set at p<0.05.

**Results**

SSX and MAGE mRNA was not observed in normal colon tissue (Figure 1), whereas colorectal cancer tissues expressed SSX mRNA in 32.4% (12/37) of cases and MAGE mRNA in 51.4% (19/37) of cases (Table I). Liver metastasis was positively correlated with regional lymph node metastasis (p=0.006), vascular invasion (p=0.001), perineural invasion (p<0.001), and co-expression of SSX and MAGE (p=0.024) (Tables I-III). Seventy-five percent of primary tumors in colorectal cancer patients with liver metastasis expressed both MAGE and SSX (Table III).
Clinicopathologic variables were detected (Table III). Between the expression of these genes and other relationship between VEGF expression and COX2 expression VEGF (Table II). However, there was a significant survivin (survivin expression, and their association with liver metastasis. Their genes have several common characteristics: they frequently map to chromosome X; they exist as multigene families; they are immunogenic in cancer patients, and they are ideal targets for cancer immuno-therapy (16-18). In this study, 32.4% of colorectal tumors expressed SSX mRNA and 51.4% of the expressed MAGE mRNA. The RT-PCR based analysis of SSX family expression in 325 human neoplasia specimens of Tureci et al. showed that at least one SSX family member was expressed in 27% of colorectal carcinomas, an incidence lower than that detected in our study (20). This discrepancy could be attributed to a higher efficiency with one set of common primers to detect SSX expression. We found MAGE A1-6 expression in 51.4% of tumors analyzed. Previous studies have reported rates of MAGE gene family expression in colorectal carcinoma ranging from 30% to 88% by RT-PCR (21-23). Our data fall between these two extremes. These inconsistencies could result from the use of different primer sets or be due to physiological variations in the different clinical samples. Our data suggest that patients with co-expression of MAGE and SSX demonstrated a higher frequency of the primary tumors with liver metastasis than those with non-expression. Some reports suggest that MAGE expression correlates with a poor prognosis(21-23), which partially supports our results. However, because our study is the first to report co-expression of MAGE and SSX in metastatic colorectal cancer, these data should be verified in future large-scale studies.

Survivin was localized to the nucleus in 83.3% of cancer tissues that expressed survivin, and nuclear expression of survivin was exclusive to cancer tissue (Table II and Figure 2). COX2 and VEGF were expressed in 73.9% and 72.4% of cancer cases, respectively.

MAGE and SSX correlated with nuclear expression of survivin (p=0.016), but not with the expression of COX2 and VEGF (Table II). However, there was a significant relationship between VEGF expression and COX2 expression (p=0.023) (Table II). No other significant relationships between the expression of these genes and other clinicopathologic variables were detected (Table III).

Discussion

In this study, we evaluated the clinical implications of MAGE and SSX expression, their correlation with COX2, VEGF, and survivin expression, and their association with liver metastasis in patients with colorectal cancer.

CTAs, including MAGE, SSX, GAGE, and NY-ESO-1, are expressed in normal testis and in a wide array of human cancer types, yet have little or no expression in most other normal tissues (16-19). Their genes have several common characteristics: they frequently map to chromosome X; they
in our report as compared with these previous studies could result from using different target epitopes for immunohistochemical analysis, or could be due to the small number of cases studied. Further larger-scale studies are required to verify our results.

**Conclusion**

Co-expression of *MAGE* and *SSX*, as well as nuclear expression of survivin, may predict colorectal cancer-derived liver metastasis, and these cancer antigen genes may be targets for anti-metastasis therapy for patients with colorectal cancer. Significant prognostic value of survivin, COX2, and VEGF in colorectal cancer were not demonstrated here.

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**References**


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