

# The Expression of MAGE and SSX, and Correlation of COX2, VEGF, and Survivin in Colorectal Cancer

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**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

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*Key Words:* Colorectal cancer, SSX, MAGE, COX2, VEGF, liver metastasis.

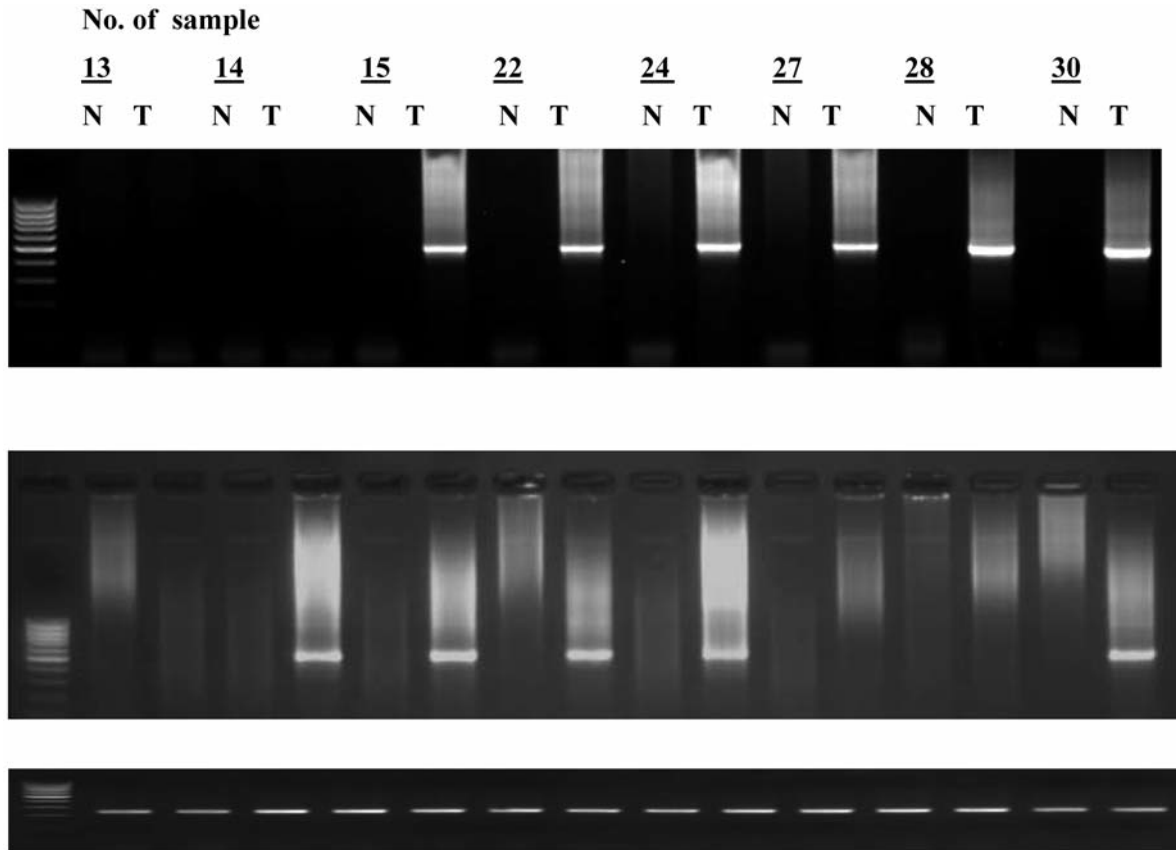


Figure 1. The electrophoretic analysis of nested RT-PCR amplification products with common SSX primers (495 bp) in the first panel and MAGE primers (490 bp) in the second panel from healthy colorectal mucosa (N) and colorectal cancer (T); GAPDH is shown in the third panel (450 bp).

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 ( $\pm 18.635$ ) 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  $\mu$ m sections using cryostat microtome at  $-20^{\circ}\text{C}$ . 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  $\mu$ g of total RNA in a 25- $\mu$ l reaction mixture containing 6  $\mu$ l of 5 $\times$  reverse transcriptase reaction buffer, 1  $\mu$ l of oligo(dT)(100 pmol/ $\mu$ l), 4  $\mu$ l of 10 mM dNTP, 40 unit/ $\mu$ l of RNAsin, 0.5  $\mu$ l of 200 units/l Moloney leukemia virus reverse transcriptase. The mixture was incubated at  $4^{\circ}\text{C}$  for 60 minutes, heated to  $94^{\circ}\text{C}$  for 3 minutes, and then chilled on ice. In order to verify the integrity of the cDNA, GAPDH 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'-CTCCAGGTAGTTTTCTGCAC for first round PCR, and sense 5'-CTGAAGGAGAAGATCTGCCWGTG and antisense 5'-CCAGCA TTTCTGCCTTTGTGA 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

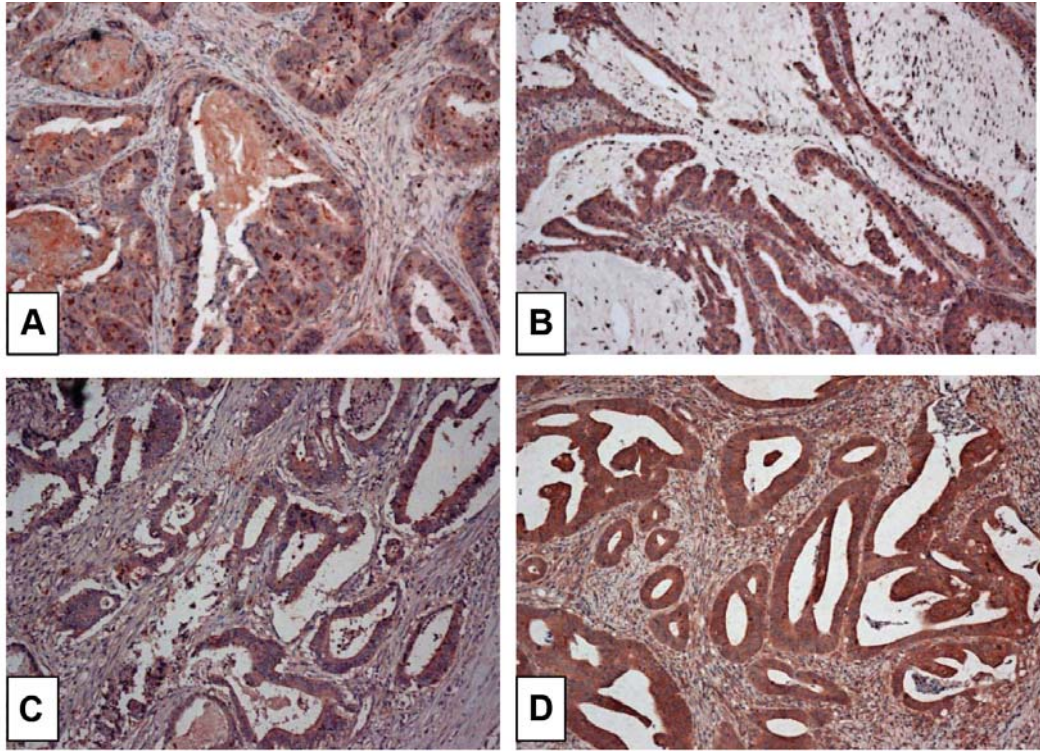


Figure 2. Immunohistochemical analysis of colorectal adenocarcinomas (ABC,  $\times 200$ ). The staining of nuclear survivin (A) and cytoplasmic VEGF (B). Cytoplasmic immunostaining of survivin (C) and COX2 (D) in colorectal carcinoma.

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, Taejeon, South Korea). Oligonucleotide primers were dissolved in Tris-EDTA buffer to 100 pmol/ $\mu$ l, aliquoted, and stored at  $-75^{\circ}\text{C}$ . Each aliquot was diluted to 10 pmol/ $\mu$ l before use.

**SSX primer design.** In order to simultaneously detect the expression of SSX1 through 9, 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/ $\mu$ l. Aliquots were diluted to 10 pmol/ $\mu$ l 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)  $\leq 4$  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).



Table I. The association of *MAGE* and *SSX* expression with clinical and pathological parameters for 37 patients with colorectal cancer.

Characteristic	No. of patients (n=37)	<i>MAGE</i>		<i>SSX</i>	
		+	-	+	-
Total number (%)	37	18 (48.6)	19 (51.4)	25 (67.6)	12 (32.4)
Gender					
Male	19 (55.9%)	9	9	14	5
Female	18 (48.6%)	9	9	11	7
Depth of tumor invasion (pT)					
1	1 (2.7%)	1	0	1	0
2	6 (16.2%)	4	2	5	1
3	14 (37.8%)	12	2	14	0
4	16 (43.2%)	1	15	1	11
Lymph node metastasis					
Negative	23 (62.2%)	15	8	16	7
Positive	14 (37.8%)	3	11	9	5
Vessel invasion					
Negative	26 (70.3%)	13	13	21	5
Positive	11(29.7%)	5	6	5	6
Perineural invasion					
Negative	28 (75.7%)	17	11	21	7
Positive	9 (24.3%)	1	8	4	5
Liver metastasis					
Negative	33 (89.2%)	18	15	24	9
Positive	4 (10.8%)	0	4	1	3

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

exist as multigene families; they are immunogenic in cancer patients, and they are ideal targets for cancer immunotherapy (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 expression is inversely correlated with apoptosis during tumorigenesis, and inhibition of apoptosis by survivin is positively correlated with proliferation and angiogenesis, predicting a poor prognosis and shorter survival time for various types of human cancer (6-12). Here, we analyzed survivin mRNA expression in normal and colorectal cancer tissues. We detected survivin expression in 94.6% of cancer samples and 51.4% of normal colon tissue by RT-PCR. However, it has been reported that survivin expression is not detectable in normal adult tissues by immunohistochemical methods (12, 22). In this study, we detected nuclear localization of survivin in 83.3% of cancer tissue samples, and only in the cancer samples.

COX2 and VEGF are recognized as being potent angiogenic factors in tumors (13, 14). In some reports, COX2 has been shown to be associated with tumor metastasis, and inhibiting COX2 inhibits liver metastasis of colon cancer (24). In this study, COX2 and VEGF expression rates were 73.9% and 72.4% in colorectal tumors, with no distinct correlation to liver metastasis. Our incidence data agree with other previous reported incidences of COX2 expression, which range from 86.7% (25) and to 67.3% (26). It is reported that COX2 may be involved in the course of tumor angiogenesis of colorectal cancer *via* VEGF (13) or iNOS (14). Other reports suggest that expression of *VEGF* mRNA isoforms is correlated with liver metastasis, M stage, and poor prognosis in colon cancer (27) The different results

Table II. Nuclear and cytoplasmic expression of survivin, and expression of *VEGF* and *COX2* in patients with colorectal cancers.

Characteristic	Positive cases [n (%)]			
	Survivin		VEGF	COX2
	Nuclear	Cytoplasmic		
Positive rate	25/30 (83.3)	25/30 (83.3)	21/29 (72.4)	17/23 (73.9)
Depth of tumor invasion (pT)				
I	0 (0)	0 (0)	0 (0)	0 (0)
II	3/5 (60.0)	4/5 (80.0)	3/5 (60.0)	5/5 (100.0)
III	1/1 (100.0)	0/1 (0.0)	1/1 (100.0)	1/1 (100.0)
IV	21/14 (87.5)	21/24 (87.5)	17/23 (73.9)	11/17 (64.7)
Lymph node metastasis				
Negative	15/18 (83.3)	14/18 (77.7)	11/17 (64.7)	11/13 (84.6)
Positive	10/12 (83.3)	11/12 (91.7)	10/12 (83.3)	6/10 (60.0)
Vessel invasion				
Negative	17/21(80.9)	18/21 (85.7)	15/20 (75.0)	13/16 (81.2)
Positive	8/9 (88.9)	7/9 (77.8)	6/9 (66.7)	4/7 (57.1)
Perineural invasion				
Negative	19/23 (82.6)	19/23 (82.6)	15/22 (68.2)	12/17 (70.6)
Positive	6/7 (85.7)	6/7 (85.7)	6/7 (85.7)	5/6 (83.3)
Liver metastasis				
Negative	5/27 (18.5)	4/27 (14.8)	18/26 (69.2)	14/20 (70.0)
Positive	3/3 (100.0)	2/3 (66.7)	3/3 (100.0)	3/3 (100.0)

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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Table III. Co-expression of *MAGE* and *SSX* and the incidence of liver metastasis.

Liver metastasis	Co-expression of <i>MAGE</i> and <i>SSX</i> [n (%)]	
	Negative (n=27)	Positive (n=10)
Yes	1 (25.0)	3 (75.0)
No	26 (77.8)	7 (22.2)

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