

Detection of MAGE and SSX Gene Expressions by RT-nested PCR Using Common Primers in Head and Neck Cancer

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Objectives. The melanoma antigen gene (MAGE) and synovial sarcoma on X chromosome (SSX) gene families are silent in most normal adult tissues, but are expressed in a variety of malignant lesions. Therefore, detection of MAGE and SSX transcription may be useful for the diagnosis of head and neck cancers. The aim of this study is to detect MAGE and SSX gene transcripts of head and neck cancers using the MAGE 1-6 assay and the SSX 1-9 assay.

Methods. The transcripts of MAGE 1-6 and SSX 1-9 genes were detected by the MAGE 1-6 assay and the SSX 1-9 assay respectively, in cancer cell lines, cancer tissue, and induced sputum specimens from head and neck cancer patients.

Results. The transcripts of MAGE 1-6 and SSX 1-9 genes were detected in 82.8% and 75.9% of head and neck cancer tissues (N=29) respectively, and 96.6% of cancer tissues expressed at least one of MAGE 1-6 or SSX 1-9 genes. In the induced sputum of head and neck cancer patients (N=18), the transcripts of MAGE 1-6 and SSX 1-9 genes were detected in 72.2% and 77.8%, respectively, and 94.4% of the sputum specimens were positive for either the MAGE 1-6 or the SSX 1-9 assay.

Conclusion. These results suggest that the combination of MAGE 1-6 and SSX 1-9 assays may be useful in the diagnosis of head and neck cancer.

Key Words. MAGE and SSX gene, Head and neck cancer, RT-PCR

INTRODUCTION

The genes of cancer/testis antigens (CTA) such as melanoma antigen gene (MAGE) and synovial sarcoma on X chromosome (SSX) are thought to be silent in normal adult tissues except testis. However, these genes are expressed at a high frequency in a large variety of cancer cells. Therefore, the corresponding transcripts represent attractive targets for cancer immunotherapy and cancer diagnosis (1-4).

The MAGE A family consists of several subtypes, including MAGE-1 to MAGE-12. During the past several years, many researchers have studied the expression of individual MAGE A genes for cancer diagnosis. However, the use of MAGE genes in the

detection of a small number of cancer cells by reverse transcription-polymerase chain reaction (RT-PCR) has been limited by the low expression frequency of individual MAGE genes in various cancer tissues. It has been reported that the probability of a cancer cell expressing at least one MAGE gene is very high (5-7). In order to improve the detection rate of MAGE genes, we have recently designed common primers that can bind simultaneously to the cDNA of MAGE-1, -2, -3, -4a, -4b, -5a, -5b and -6 (MAGE 1-6). We also developed a MAGE 1-6 assay that can simultaneously detect the transcripts of MAGE 1-6 (8).

The SSX gene family, which was originally identified as fusion partners to the SYT gene in synovial sarcomas, consists of 9 subtype genes (SSX 1-9). The known SSX family members share high homology at the protein and DNA level. Also, naturally occurring serologic responses mounted by cancer patients against one SSX family member cross-react with other members of the family (9-11). The high level of homology between the subtypes at the DNA and protein levels suggests that it may be possible to design a common primer for the SSX family. In this study, we

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designed a common SSX primer, and developed an SSX 1-9 assay that can detect cancer cells expressing at least one of the 9 SSX genes by RT-nested PCR using the common SSX primers.

MATERIALS AND METHODS

Cell culture

Thirty five cancer cell lines derived from stomach cancer (SNU 484, SNU 620, SNU 638, SNU 668), colon cancer (SNU C1, SNU C4, SNU C5, HT29, HCT 116), head and neck cancer (AMC-HN3, AMC-HN4, AMC-HN7), leukemia (U937, HL 60) cervical cancer (Caski, C4-II, ME-180, Hela, CUNC-6, SiHa), lung cancer (NCI-H292, NCI-H522, NCI-H1703, A-549), prostate cancer (PC 3, DU 145), hepatocellular carcinoma (HEPG2, SNU 182, SNU 354, SNU 387, SNU 398, SNU 423), kidney cancer (HEK 293), breast cancer (MDA 231), and osteosarcoma (SAOS 2) were studied for the expression of SSX 1-9 genes. These cancer cell lines were grown in DMEM or RPMI1640 supplemented with 10% FBS in a 5% CO₂ incubator at 37°C. Then total RNA was extracted with the trizol chloroform method.

Cancer tissue and sputum specimens

The cancer tissue samples were obtained from 29 head and neck cancer patients who were surgically treated at the Department of Otolaryngology, School of Medicine, Kosin University, Busan, Korea. The tissue samples were immediately frozen and kept at -70°C. Then total RNA was extracted with the trizol chloroform method.

Induced sputum samples were obtained from 18 patients with head and neck cancer and 22 patients with benign pulmonary diseases such as pneumonia, bronchitis, and tuberculosis. Induced sputum was collected after inhalation of 100 µg of Ventolin (GlaxoSmithKline, London, UK) and nebulization with 3% hypertonic saline solution for 10 min. The induced sputum was mixed with 10 mL of specimen protector (iC&G Co., Daegu, Korea) and stored at -20°C until RNA isolation. The messenger RNA in sputum was extracted using a sputum mRNA extraction kit (iC&G Co.) according to the manufacturer's instructions. Briefly, after thawing the sputum that had been digested with the specimen protector, the undigested debris was removed by centrifugation, and capture beads were then added to the liquefied supernatants. After mixing the liquefied sputum and beads for 30 min at room temperature, the beads were separated in a magnet rack and washed 3 times with washing buffer. Messenger RNAs attached to the beads were eluted with an elution buffer and stored at -70°C until the reverse transcription reaction.

MAGE 1-6 assay

Detection of transcripts of MAGE 1-6 gene was performed using the Cancer Hunter kit (iC&G Co.). Briefly, reverse transcription reactions were carried out in a 20 µL reaction mixture contain-

ing 7 µL of RT master mixture, 0.5 µL of RNase inhibitor, 1 µL of RTase and 11.5 µL of eluted RNA solutions. The reaction mixture was incubated at 42°C for 60 min, 95°C for 5 min and then stored at -20°C until the polymerase chain reaction (PCR).

The first PCR was carried out in a 20 µL reaction mixture containing 8 µL of PCR master mixture, 0.5 µL of outer primers, 2 µL of the RT reaction products and 9.5 µL of distilled water. The cycling parameters were as follows: denaturation was initiated at 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 45 sec and 65°C for 60 sec. The final extension incubation was performed at 65°C for 5 min. After the first PCR, 20 µL of nested PCR mixture containing 8 µL PCR master mixture, 0.5 µL inner primers and 11.5 µL DW was added to the first PCR tube. Nested PCR was carried out under the same conditions as the first PCR except for the annealing temperature, which was set at 62°C. The nested PCR products were separated on 1% agarose gels impregnated with ethidium bromide (0.5 µg/mL). The DNA sequences of MAGE primers were as follows: (MMRP1, 5'-CTGAAGGA-GAAGATCTGCC-3'; MMRP2, 5'-CTCCAGGTAGTTTCTTGCAC-3'; MMRP3, 5'-CTGAAGGAGAAGATCTGCCWGTG-3', W is A or T; MMRP4, 5'-CCAGCATTTCTGCCTTTGTGA-3').

Designing common primers for SSX 1-9 assay

The sequences of SSX 1-9 gene were obtained from National Center for Biotechnology Information (Bethesda, MD, USA), and the DNA homology of each SSX gene was analyzed using the DNAsis program (Hitachi, Tokyo, Japan). The DNA sequences that are commonly present in SSX 1-9 subtype genes that were used for designing the common primers were as follows: (outer sense [OS] primer, 5'-GTGCCATGAACGGAGAYGA-3', Y is C or T; outer antisense [OAS] primer, 5'-TCTGTGGGTCCAGGCATGT-3'; inner antisense [IAS] primer 5'-TGTYTCCCCCTTTGGGTCC-3', Y is C or T).

SSX 1-9 assay

Detection of transcripts of SSX 1-9 genes was performed using a Cancer Hunter kit (iC&G Co.) with some modifications. In the first and nested PCR, the common primers for SSX 1-9 gene were used in place of common primers for MAGE 1-6. All other procedures and temperature conditions for PCR were identical to the MAGE 1-6 assay.

RESULTS

Primer design and development of SSX 1-9 assay

In order to design common primers that bind to 9 SSX cDNAs together, DNA sequences of the SSX subtypes were compared. The SSX-1 sequence showed more than 90% DNA homology with all other SSX subtype sequences (data not shown). Using highly homologous DNA sequences, common primers that can

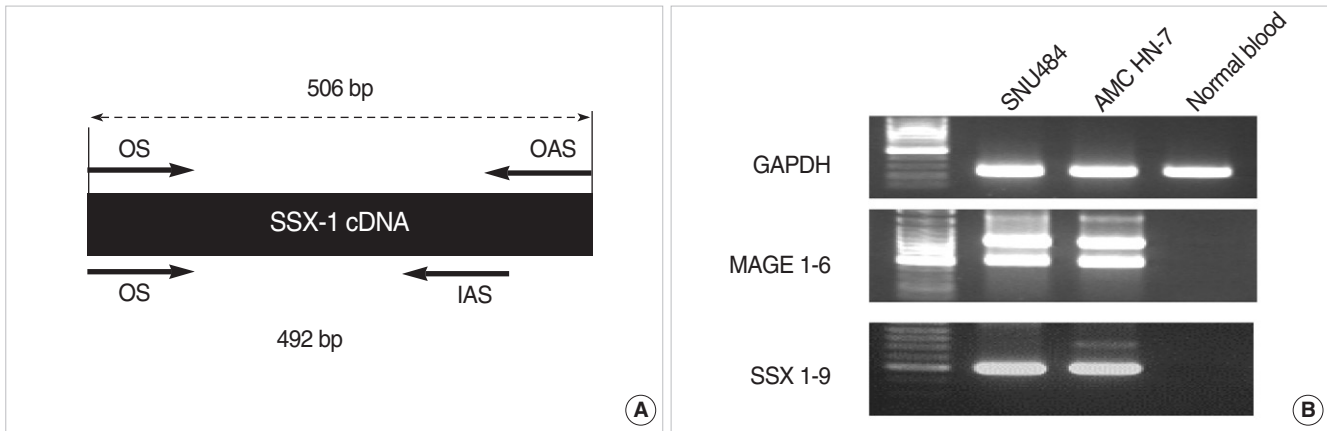


Fig. 1. The binding sites of common primers on SSX-1 cDNA (A) and PCR products amplified by MAGE 1-6 and SSX 1-9 assay (B).

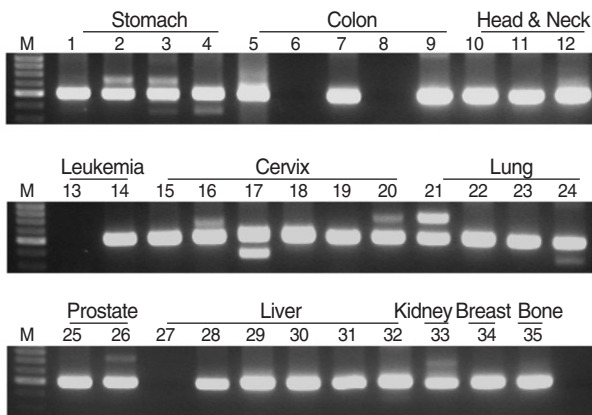


Fig. 2. Detection of SSX 1-9 transcripts of cancer cell lines by SSX 1-9 assay. M: Marker; 1: SNU 484; 2: SNU 620; 3: SNU 638; 4: SNU 668; 5: SNU C1; 6: SNU C4; 7: SNU C5; 8: HT29; 9: HCT 116; 10: AMC-HN3; 11: AMC-HN4; 12: AMC-HN7; 13: U937; 14: HL 60; 15: Caski; 16: C4-II; 17: ME-180; 18: Hela; 19: CUNC-6; 20: SiHa; 21: NCI-H292; 22: NCI-H522; 23: NCI-H1703; 24: A-549; 25: PC 3; 26: DU 145; 27: HEPG2; 28: SNU 182; 29: SNU 354; 30: SNU 387; 31: SNU 398; 32: SNU 423; 33: HEK 293; 34: MDA 231; 35: SAOS 2.

bind simultaneously to SSX 1-9 cDNAs were designed, and used for the SSX 1-9 assay. The binding site of common primers on SSX-1 gene and the products amplified by the SSX1-9 assay and the MAGE 1-6 assay are summarized in Fig. 1. All primers (OS, OAS, and IAS) had 100% homology with the sequences of the 9 SSX cDNAs, and were used for the SSX 1-9 assay. The transcripts of MAGE 1-6 genes and SSX 1-9 genes were detected in SNU484 and AMC HN-7 cell lines, but not in normal blood cells. The expected DNA sizes amplified by the SSX 1-9 assay and the MAGE 1-6 assay were 490-492 bp and 448-472 bp, respectively.

Detection of the transcripts of SSX 1-9 genes in cancer cell lines

In order to evaluate the sensitivity of the SSX 1-9 assay, mRNA was isolated from 35 cancer cell lines described previously in this

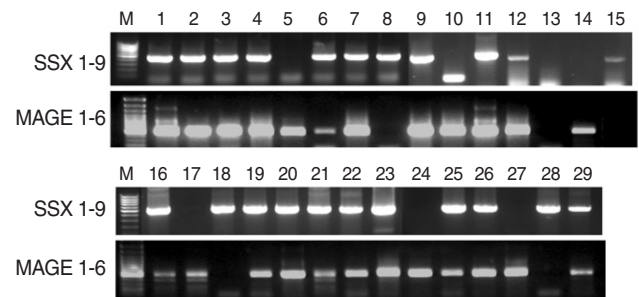


Fig. 3. Detection of MAGE 1-6 and SSX 1-9 gene expressions in cancer tissue of the patients with head and neck cancer by MAGE 1-6 assay and SSX 1-9 assay.

paper, and transcripts of SSX 1-9 genes were detected by the SSX 1-9 assay. Thirty one (88.6%) of the 35 cancer cell lines were positive (Fig. 2). All 3 head and neck cancer cell lines were positive in both gene assay (data not shown).

Detection of the transcripts of SSX 1-9 and MAGE 1-6 in cancer tissues

The transcripts of MAGE 1-6 and SSX 1-9 genes were detected in 24 (82.8%) and 22 (75.9%) of 29 fresh cancer tissues from head and neck cancer patients. Eighty six percent (18/21) and 72.7% (16/21) of squamous cell carcinoma cells expressed MAGE 1-6 genes and SSX 1-9 genes, respectively. Sixty three percent (5/8) of other pathologic types of cancers expressed both MAGE 1-6 genes and SSX 1-9 genes (Fig. 3, Table 1).

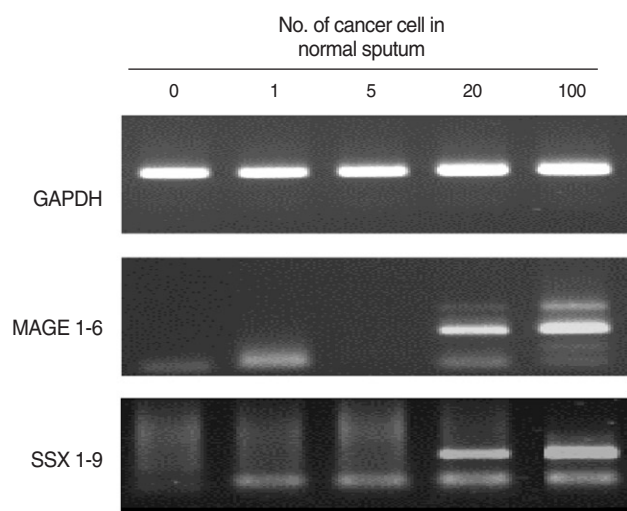
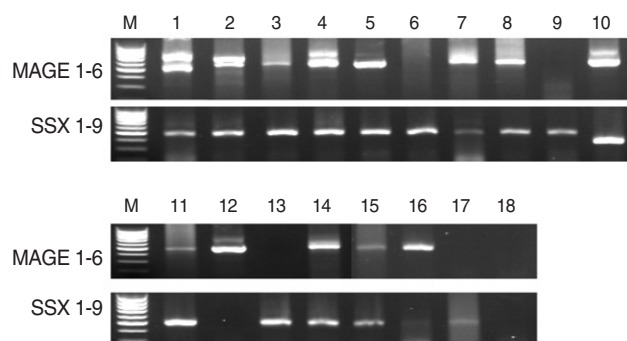
Detection of the transcripts of SSX 1-9 and MAGE 1-6 in sputum samples

In order to investigate the ability of MAGE 1-6 and SSX 1-9 assays to detect small numbers of cancer cells mixed in sputum, SNU484 (MAGE- and SSX-positive cell lines) were added to normal sputum. After digesting the sputum with specimen protector, mRNA was isolated, and MAGE 1-6 and SSX 1-9 assays were performed. Both assays detected transcripts in samples

Table 1. Analysis of the profile of MAGE and SSX gene expression in cancer tissue according to the pathology of head and neck cancer

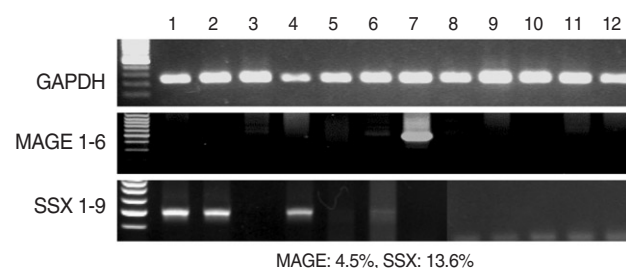
Pathology	Primary site	No. of positive reaction (%)	
		MAGE	SSX
Squamous cell carcinoma	Larynx (9)*	9 (100)	7 (77.8)
	Nasopharynx (2)	0 (0)	2 (100)
	Oropharynx (4)	3 (75)	1 (25)
	Hypopharynx (2)	2 (100)	2 (100)
	Oral cavity (4)	4 (100)	4 (100)
Verrucous carcinoma	Larynx (1)	1 (100)	1 (100)
Undifferentiated carcinoma	Nasopharynx (1)	0 (0)	1 (100)
Lymphoma	Lymphatics (2)	1 (50)	1 (50)
Thyroid ca	Thyroid (3)	3 (100)	2 (66.7)
Adenoid cystic carcinoma	Submandibular gland (1)	1 (100)	1 (100)
Total (n=29)		24 (82.8)	22 (75.9)

*No. of total cases tested. MAGE: melanoma antigen gene; SSX: synovial sarcoma on X chromosome.

**Fig. 4.** Detection of the transcripts of MAGE 1-6 and SSX 1-9 genes in sputum containing SNU484 by MAGE 1-6 assay and SSX 1-9 assay.**Fig. 5.** Detection of MAGE 1-6 and SSX 1-9 gene expressions in sputum of the patients with head and neck cancer by MAGE 1-6 assay and SSX 1-9 assay.**Table 2.** Analysis of the profile of MAGE and SSX gene expression in sputum according to the pathology of head and neck cancer

Pathology	Primary site	No. of positive reaction (%)	
		MAGE	SSX
Squamous cell carcinoma	Larynx (12)*	8 (66.7)	9 (75)
	Hypopharynx (1)	0 (0)	0 (0)
	Nasopharynx (1)	1 (100)	1 (100)
	Oropharynx (1)	1 (100)	1 (100)
Adenocarcinoma	Hypopharynx (1)	1 (100)	1 (100)
Adenoid cystic carcinoma	Oropharynx (1)	1 (100)	1 (100)
Basaloid carcinoma	Oropharynx (1)	1 (100)	1 (100)
Total (n=18)		13 (72.2)	14 (77.8)

*No. of total cases tested. MAGE: melanoma antigen gene; SSX: synovial sarcoma on X chromosome.

**Fig. 6.** Representative results of MAGE 1-6 and SSX 1-9 expression in sputum of the patients with benign lung disease (n=22) by MAGE 1-6 or SSX 1-9 assay.

containing more than 20 SNU484 cells (Fig. 4).

Eighteen samples of induced sputum from head and neck cancer patients and 22 samples of induced sputum from patients with benign lung disease were collected. After digestion of sputum with specimen protector and isolation of mRNA in liquefied sputum, MAGE 1-6 and SSX 1-9 assays were performed. The transcripts of MAGE 1-6 and SSX 1-9 genes were detected in 72.2% (13/18) and 77.8% (14/18) of fresh induced sputum samples from head and neck patients (Fig. 5, Table 2). The MAGE 1-6 assay detected transcripts in 33.3% of sputum samples from TNM stage T1 tumors, 88.9% of T2 tumors, 75% of T3 tumors and 100% of T4 tumors. The SSX 1-9 assay detected transcripts in 100% of sputum samples from T1 tumors, 77.8% of T2 tumors, 75% of T3 tumors and 100% of T4 tumors (Table 3). In order to evaluate the specificity of both assays, transcripts of the MAGE 1-6 and SSX 1-9 assays in induced sputum samples from patients with benign lung disease were detected. The MAGE 1-6 assay was positive in 4.5% (1/22) and the SSX 1-9 assay was positive in 13.6% (3/22) of the samples (Fig. 6). These results represents MAGE 1-6 assay is more specific than SSX 1-9 assay.

Combination of MAGE 1-6 and SSX 1-9 assay in the cancer tissue and induced sputa of head and neck cancer patients

In head and neck cancer patients, the MAGE 1-6 assay was posi-

Table 3. Analysis of the profile of MAGE and SSX gene expression in sputum according to the cancer stage of head and neck cancer

Cancer stage (N0)	No. of positive reaction (%)	
	MAGE	SSX
T1 (3)	1 (33.3)	3 (100)
T2 (9)	8 (88.9)	7 (77.8)
T3 (4)	9 (75)	3 (75)
T4 (1)	1 (100)	1 (100)

MAGE: melanoma antigen gene; SSX: synovial sarcoma on X chromosome.

tive in 82.8% of cancer tissues and 72.2% of induced sputa. The SSX 1-9 assay was positive in 75.9% of cancer tissues and 77.8% of induced sputa. When both MAGE 1-6 assay and SSX 1-9 assay were performed on the same specimen, 96.6% of cancer tissues and 94.4% of induced sputa were positive in either one of the two assays (Table 4).

DISCUSSION

Cancer antigens that have a high frequency of expression exclusively in cancer cells can be excellent markers for cancer diagnosis. The expression of MAGE and SSX genes are highly specific to cancer cells, but their use in the detection of a small number of cancer cells by RT-PCR has been limited by the low expression frequency of individual genes. If the transcripts of several cancer antigen genes could be detected simultaneously by RT-PCR, cancer detection rates could be increased. Two major methods have been described one is multiplex PCR, which combines several specific primers in the same reaction (12), and the other is PCR that uses common primers that simultaneously bind to multiple targets. Whereas it is difficult to amplify more than 5 targets using multiplex PCR, multiple targets can be detected with few limitations by performing PCR using common primers. In a previous study, we showed that RT-nested PCR using common primers was a very useful method for detecting MAGE-expressing cancer cells (8).

Expression analysis has demonstrated that SSX is a member of the recently described cancer/testis antigen class. It is expressed in a variety of different human neoplasms, but not in normal tissues, with the exception of testis and a weak expression in the thyroid. The SSX family of genes consists of several subtypes Tureci et al. (13) studied the expression of SSX genes in 325 specimens of human neoplasms, and found that SSX-1, -2, and -4 subtypes are expressed in 8%, 15% and 15% of the cancers, respectively, while the expression of the SSX-5 or SSX-3 subtypes were rare or absent. Expression of at least one of the SSX family members was most frequently observed in head and neck cancer (75%), followed by ovarian cancer (50%), malignant melanoma (43%), lymphoma (36%), colorectal cancer (27%) and breast cancer (23%). These results suggest that simultaneous detection of tran-

Table 4. Detection of MAGE and SSX gene expression by MAGE 1-6 or SSX 1-9 assay in patients with head and neck cancer

MAGE	SSX	No. of positive reaction (%)	
		Tissue	Sputum
+	and -	6 (20.7)	3 (16.7)
-	and +	4 (13.8)	4 (22.2)
+	and +	18 (62.1)	10 (55.6)
-	and -	1 (3.4)	1 (5.6)
+		24 (82.8)	13 (72.2)
	+	22 (75.9)	14 (77.8)
+	or +	28 (96.6)	17 (94.4)

MAGE: melanoma antigen gene; SSX: synovial sarcoma on X chromosome.

scripts of multiple SSX genes by RT-nested PCR will be valuable in the diagnosis of SSX-expressing cancer. The known SSX family members share more than 90% homology at the DNA level. In the present study, we designed new common primers and developed an SSX 1-9 assay that can detect cancer cells expressing at least one of the 9 SSX genes. Our SSX 1-9 assay successfully detected the transcripts of all SSX 1-9 genes in 31 out of 35 cancer cell lines.

In order to compare the sensitivity of MAGE 1-6 and SSX 1-9 assays, and to evaluate the value of combining both assays in diagnosis, we detected the transcripts of MAGE and SSX genes in 29 cancer tissue samples of head and neck cancer patients. The MAGE 1-6 and SSX 1-9 assays were positive in 82.8% and 75.9% of cancer tissues, and 96.6% of cancer tissues were positive for at least one of the two assays. The SSX-positive rate in head and neck cancer tissues observed in the present study is higher than the rate reported by Tureci et al. (13). The results from the present study show that the SSX genes, as well as the MAGE genes, are expressed in head and neck cancer tissue, and that combination of the MAGE 1-6 assay and the SSX 1-9 assay can detect more than 94% of head and neck cancers.

RT-PCR-based amplification of transcripts that are expressed in cancer cells, but not in normal non-neoplastic cells, is increasingly being used as a sensitive diagnostic tool for detecting rare disseminated or exfoliated cancer cells to improve the accuracy of cancer staging and to aid in developing early detection protocols. In particular, detection of transcripts of cancer markers in sputum may be very useful for cancer screening or early diagnosis of cancer of the respiratory tracts. In this study, we evaluated the sensitivities of MAGE 1-6 and SSX 1-9 assays in detecting cancer cells in induced sputum of head and neck cancer patients. It has been reported that head and neck cancer can be diagnosed by sputum cytology (14, 15). The reports suggested that cancer cells from head and neck malignancies can be expelled from cancer tissue and end up in sputum. Matsuda et al. (14) detected 29.4% of T1 lesions and 63.3% of T2 lesions of head and neck malignancies by sputum cytology. In the present study, 33.3% of T1 lesions (N=3) and 88.9% of T2 lesions (N=9) were detect-

ed by MAGE 1-6 assay, and 100% of T1 lesions (N=3) and 77.8 % of T2 lesions (N=9) were detected by the SSX 1-9 assay. All T1 and T2 lesions were detected by either one of the two assays, and there was no relationship between MAGE or SSX gene expression and the tumor stage. These results suggest that it may be possible to detect small numbers of cancer cells that express at least one of MAGE 1-6 or SSX 1-9 genes in sputum or in malignant tissue by performing the MAGE 1-6 and the SSX 1-9 assays together. Thus the combination of these assays may become very useful for screening or early diagnosis of primary or recurrent head and neck cancers.

The MAGE 1-6 and SSX 1-9 gene transcripts were detected in 4.5% and 13.6% of sputum of patients with benign lung disease. These false positive results may be due to the detection of dysplastic cells in sputum. One possible explanation for the false positive amplification of MAGE or SSX transcripts is the activation of MAGE genes by dysplastic cells during early tumorigenesis (16). Another possible explanation is the low level of transcription of MAGE or SSX genes in normal cells (17, 18). We have been following up these patients at 3-6 month intervals some of patients were negative for both MAGE and SSX assays during the follow-up period, while others consistently tested positive. If the results show continuous positive on follow up test, it is need to do the screening work-up to rule out occult head and neck cancer.

CONCLUSION

We developed an SSX 1-9 assay to detect the transcripts and of 9 SSX genes simultaneously, and investigated the usefulness of SSX 1-9 MAGE 1-6 assays in the detection of cancer cells in tissue and sputum samples of head and neck cancer patients. The results of the present investigation suggest that the combination of MAGE 1-6 and SSX 1-9 assays may be a sensitive diagnostic tool in detecting small numbers of malignant cells in the cancer tissue and induced sputum of head and neck cancer patients.

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