RESEARCH ARTICLE

Significance of Expression of Human METCAM/MUC18 in Nasopharyngeal Carcinomas and Metastatic Lesions

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Abstract

Human METCAM/MUC18, a cell adhesion molecule (CAM) in the immunoglobulin-like gene super family, plays a dual role in the progression of several epithelium cancers; however, its role in the nasopharyngeal carcinoma (NPC) remains unclear. To initiate the study we determined human METCAM/MUC18 expression in tissue samples of normal nasopharynx (NP), NPCs, and metastatic lesions, and in two established NPC cell lines. Immunoblotting analysis was used for the determination in lysates of frozen tissues, and immunohistochemistry (IHC) for expression in formalin-fixed, paraffin-embedded tissue sections of 7 normal nasopharynx specimens, 94 NPC tissue specimens, and 3 metastatic lesions. Human METCAM/MUC18 was expressed in 100% of the normal NP, not expressed in 73% of NPC specimens (or expressed at very low levels in only about 27% of NPC specimens), and expressed again in all of the metastatic lesions. The level of human METCAM/MUC18 expression in NPC tissues was about one fifth of that in the normal NP and metastatic lesions. The low level of human METCAM/MUC18 expression in NPC specimens was confirmed by a weak signal of RT-PCR amplification of the mRNA. Low expression levels of human METCAM/MUC18 in NPC tissues were also reflected in the seven established NPC cell lines. These findings provided the first evidence that diminished expression of human METCAM/MUC18 is an indicator for the emergence of NPC, but increased expression then occurs with metastatic progression, suggesting that huMETCAM/MUC18, perhaps similar to TGF-β, may be a tumor suppressor, but a metastasis promoter for NPC.

Keywords: HuMETCAM/MUC18 - immunoblot - immunohistochemistry - RT-PCR - NPC progression

Introduction

Nasopharyngeal carcinoma (NPC) is a non-lymphormatous, sqamous-cell carcinoma that develops in the epithelial lining of the nasopharynx cavity (Raab-Traub, 1992). NPC is prevalent in several regions around the world: Southern China, Mediterranean Africa, and Eskimos inhabited areas (De The', 1982). The disease is the major cause of cancer death among Southern Chinese; especially those in the Canton province so that NPC bears a notorious name of Canton tumor (De The', 1982; Raab-Traub, 1992). Radiotherapy is an effective treatment for NPC, and more than 80% of patients with early disease are curable (Wei et al., 2005). Unfortunately, most of the NPC patients are diagnosed at later stages, where treatment is much less effective and difficult (Wei et al., 2005; Lee et al., 2012). If these patients are diagnosed earlier or if relapses can be predicted sooner, clinical management would be greatly improved.

Epidemiological studies suggest that three major etiological factors, such as genetic susceptibility, environmental factors, and infection with Epstein Barr virus (EBV), contribute to the extraordinary incidence in endemic areas (Ho, 1972; De The', 1982; Raab-Traub, 1992; Wei et al., 2005; Lee et al., 2012). Though contribution of these factors to NPC incidence provides a model system to study the interaction of multiple factors resulting in cancer, the specific contribution of each of the three factors to the tumorigenesis and metastasis of NPC remains elusive. Nevertheless, these etiological factors may induce aberrant expression of cell adhesion molecules (CAMs) in NPC, since CAMs govern the social behaviors of cells and altered expression of CAMs affects the motility and invasiveness of many tumor cells in vitro and metastasis in vivo (Cavallaro et al., 2004). For examples, up-regulation of ICAM (Yu et al., 2004) and down-regulation of E-cadherin (Huang et al., 2001; Li et al., 2004) and connexin 43 (Yi et al., 2006) correlates

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Table 1. Patient Characteristics (N = 94)

Characteristics	No. of Cases	%
Age (years)		
Range	24-74	
Median	49	
Sex		
Male	67	71.3
Female	27	28.7
Karnofsky scale		
90%	57	60.6
80%	34	36.2
70%	2	2.1
60%	1	1.1
Pathology (WHO classification)		
Type I	24	25.5
Type II	48	51.1
Type III	22	23.4
T stage (1997 AJCC)		
T1	17	18.1
T2	41	43.6
T3	17	18.1
T4	19	20.2
N stage (1997 AJCC)		
N0	13	13.8
N1	34	36.2
N2	27	28.7
N3	20	21.3
Overall stage		
II	30	31.9
III	32	34
IV	32	34
Radiotherapy		
Yes	94	100
No	0	0
Concurrent chemotherapy		
Yes	47	50
No	47	50
Adjuvant chemotherapy		
Yes	20	21.3
No	74	78.7
Failure sites		
Nasopharynx	8	8.5
Neck	1	1.1
Distant metastasis	19	20.2
Nasopharynx + Neck	0	0
Nsopharynx + Distant metastasis	3	3.2
Neck + Distant metastasis	1	1.1
Nsopharynx + Neck + Distant metastasis	1	1.1

WHO, World Health Organization; AJCC, American Joint Committee on Cancer

with the progression of NPC; however, the expression of CD44 does not (Huang et al., 2001).

The expression of human METCAM/MUC18 (huMETCAM/MUC18), a cell adhesion in the immunoglobulin gene super-family (Lehmann et al., 1989; Shih, 1999; Wu, 2005), in normal nasopharynx (NP) and NPC tissues has not been studied (Shih, 1999; Wu, 2005). Based on the following reasons, we hypothesize that the expression of human METCAM/MUC18 (huMETCAM/MUC18) is likely to play a role in the malignant progression of NPC: (a) the huMETCAM/MUC18 gene resides in the region of the chromosome11q22-23, in which hyper-methylation is associated with the NPC

development during the progression of NPC (Lung et al., 2004; Lung et al., 2005), and (b) HuMETCAM/MUC18, a cell adhesion molecule (CAM) in the immunoglobulin gene super family, which is expressed in several normal tissues, such as hair follicular cells, smooth muscle cells, endothelial cells, cerebellum, normal mammary epithelial cells, basal cells of the lung, activated T cells, and intermediate trophoblast (Shih, 1999), has been evident in playing very intriguing roles in the progression of several epithelial cancers (Shih, 1999; Wu, 2005). Over-expression of huMETCAM/MUC18 promotes the metastasis of melanoma (Xie et al., 1997; Schlag-bauer-Wadl et al., 1999; Shih, 1999; Wu, 2005; Wu et al., 2008), prostate cancer (Wu et al., 2001a; Wu et al., 2001b; Wu, 2004; Wu et al., 2004; Wu et al., 2005; Chiang et al., 2005; Wu et al., 2011), angiosarcomas (Wu, 2005), osteosarcoma (McGary et al., 2003), and breast cancer (Zeng et al., 2011; Zeng et al., 2012a; Zeng et al., 2012b). On the contrary, under-expression correlates with the malignant progression of haemangioma (Li et al., 2003) and over-expression of huMETCAM/MUC18 suppresses the tumorigenesis of one mouse melanoma cell line and human ovarian cancer cell lines in an immunodeficient mouse model (Wu, 2012), suggesting that its expression may suppress the tumorigenesis of these cancers and perhaps other cancer types (Wu, 2012).

To test the above hypothesis, we have investigated in this report the possible expression of huMETCAM/MUC18 in normal NP and NPC tissues and two established NPC cell lines in association with the progression of NPC.

Materials and Methods

Materials

Normal NP and NPC tissue specimens were from the tissue archive of Taichung Veterans General Hospital, Taichung, Taiwan. Two NPC cell lines, NPC-TW01 and NPC-TW04, were from Dr. Chin-Tarng Lin, Department of Pathology, National Taiwan University, Taipei, Taiwan (Lin et al., 1990; Lin et al., 1993). Anti-huMETCAM/MUC18 antibody was prepared from chicken immunized with the middle fragment of a recombinant huMETCAM/MUC18 protein, which was over-expressed in E. coli (Wu et al., 2001a; Wu et al., 2001b; Wu, 2004). Since this portion of the huMETCAM/MUC18 protein does not have any N-glycosylation sites, the antibody could recognize the epitopes of the huMETCAM/MUC18 protein expressed in human cancer cell lines and formalin-fixed, paraffinized tissue sections with a minimal interference from glycans. The antibody also has a high specificity and a minimal cross-reactivity with mouse METCAM/MUC18 protein (Wu et al., 2001a; Wu et al., 2001b; Yang et al., 2001; Wu, 2004).

Patient information

Ninety-four patients were randomly selected from both genders for the study (Table 1). As shown in Table 1, 71.3 % of patients were males, and an age distribution from 24 to 74 years with a median age of 49-year-old. The pathological grades of NPC, and the clinical stages and the treatment of the patients are also listed in Table 1.

Growth of cancer cell lines

The human melanoma cell line SK-Mel-28 and the prostate cancer cell line DU145 from ATCC were maintained in Eagle's MEM supplemented with sodium pyruvate and 10% fetal bovine serum (FBS). The prostate cancer cell line PC-3 from ATCC was maintained in a RPMI1640/F12K (1:1) medium with 10% FBS. The bladder cancer cell line TSU-Pr1 from Dr. John T. Isaacs was maintained in RPMI1640 medium with 10% FBS. The prostate cancer cell line LNCaP from ATCC was maintained in a modified RPMI1640 medium supplemented with 20 mM Hepes buffer, 1 mM sodium pyruvate, 2% glucose, and 10% FBS. All the above cell lines were grown in the media with penicillin and streptomycin and in 5% CO_2 in a 37 °C incubator. Two NPC cell lines, NPC-TW01 and NPC-TW04, were maintained in DMEM (4.5% glucose) supplemented with 5% fetal bovine serum and 100 μg/ml kanamycin and in 5% CO_2 in a 37 °C incubator. All media were from GIBCO/BRL, Life technology. FBS was from Cellgro/MediaTech Inc.

RT-PCR amplification of huMETCAM/MUC18 cDNA from total RNA

Total RNA from cell lines or homogenized NP or NPC tissues was prepared according to the one step acid-guanidinium thiocyanate-phenol-chloroform extraction procedure (Chomczyuski et al., 1987), checked for integrity in a formaldehye-impregnated 1.2 % agarose horizontal gel (Ausubel et al., 1987), and used for RT-PCR analysis of the abundance of huMETCAM/MUC18 mRNA (Wu et al., 2001a; Wu et al., 2001b; Yang et al., 2001; Wu, 2004; Wu et al., 2008). 0.5 to 1 μg of total RNA was used for the reverse transcription (RT) reaction by using a RT system from Promega, Madison, WI. The quality of the RT product was verified by the production of a strong positive human GAPDH cDNA signal in a PCR reaction. A typical PCR was carried out in 20 μl, which contained 2 μl of the RT product and other necessary ingredients. The full length huMETCAM/MUC18 cDNA was amplified for 30 cycles in a PCR by using a primer pair of BF-1 and ER6a (Wu et al., 2001a; Wu et al., 2001b; Wu, 2004; Wu et al., 2008). The C-terminal fragment 197 bp of the huMETCAM/MUC18 cDNA was amplified by using a primer pair of HuCD (21-mer, AAGAAGGGCAAGCTGCCGTGC) and ER6a (25-mer, TCGGGGCTAATGCCTCAGATCGATG), which were designed from the published sequence (Wu et al., 2001b). The 197 bp fragment was amplified for 30 cycles (hot start for 5 min at 94 °C, and 30 sec at 80 °C, then 29 cycles of PCR reaction for 1min at 94 °C, 2 min at 61 °C, and 3 min at 72 °C, and finally one cycle for 1min at 94 °C, 2 min at 61 °C, and 20 min at 72 °C). The PCR products were cloned to the pGEM-T or pGEM-T easy vector (Promega). Positive (white) clones were first identified by restriction enzyme analysis (New England Biolabs, Beverly, MA, or American Allied Biochemicals, Aurora, CO) and further characterized by DNA sequencing (Wu et al., 2001b).

DNA sequence analysis

DNA sequencing was carried out by using an ABI prism dye terminator cycle sequencing ready reaction kit (Perkin-Elmer) in an automated ABI 373XL sequencer (Emory DNA Sequence Core Facility).
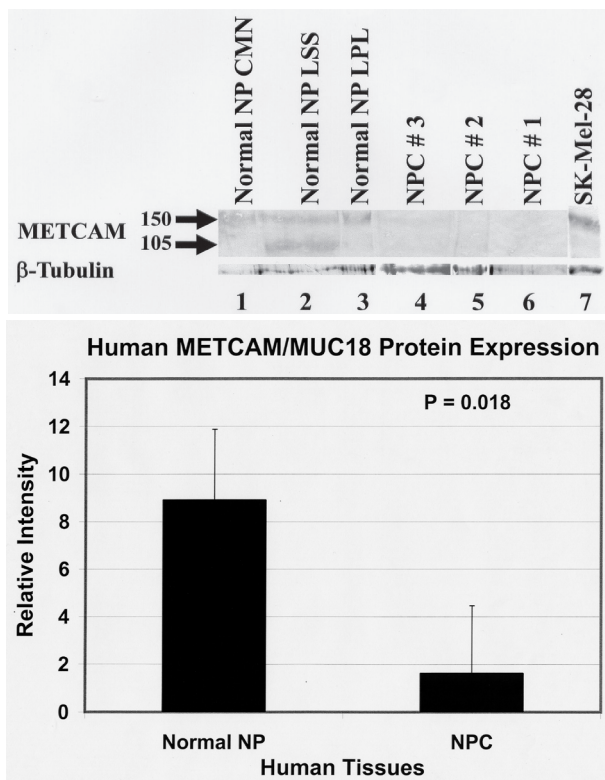
Immunoblot (Western blot) analysis

Cell extracts from various cultured cell lines were prepared by directly lysing the monolayer cells with the Western blot lysis buffer, added an anti-proteolytic cocktail that included 1 mM benzamidine, 0.5 mM PMSF, and 1 μg/ml each of antipain, leupeptin, chymostatin, and pepstatin A (Sigma Chemical Co.), boiled, and kept frozen until use (Wu et al., 2001a; Wu et al., 2001b; Yang et al., 2001; Wu, 2004; Wu et al., 2008). The extract from each frozen normal NP or NPC tissue was prepared as described (Wu et al., 2001a; Wu et al., 2001b; Wu, 2004; Wu et al., 2005; Chiang et al., 2005; Wu et al., 2008; Wu et al., 2011; Zeng et al., 2011; Zeng et al., 2012a). A portion of the homogenate was used for RNA preparation as described above. The rest of the homogenate was used for making a Western blot lysate (Wu et al., 2001a; Wu et al., 2001b; Wu, 2004; Wu et al., 2005; Chiang et al., 2005; Wu et al., 2008; Wu et al., 2011; Zeng et al., 2011; Zeng et al., 2012a). HuMETCAM/MUC18 protein expression in cellular and tissue extracts was determined by the standard procedure of Western blot analysis by using a 1/235-350 dilution of the chicken anti-recombinant huMETCAM/MUC18 protein IgY (reacting at room temperature for 2-5 hours or at the cold room for 16 hours) (Wu et al., 2001a; Wu et al., 2001b; Wu, 2004; Wu et al., 2005; Chiang et al., 2005; Wu et al., 2008; Wu et al., 2011; Zeng et al., 2011; Zeng et al., 2012a). The image of the huMETCAM/MUC18 band was scanned with an Epson Photo Scanner model 1260 and its intensity was quantitatively determined by a NIH Image J program version 1.31. The data were statistically analyzed by the Student's t test. The same membrane was subsequently used for the detection of actin and β-tubulin in the lysates; a dilution of 1/200 of the primary antibodies against human actin (SC-1615, goat polyclonal antibody from Santa Cruz Biotech, CA) or human β-tubulin (SC-9104, rabbit polyclonal antibody from Santa Cruz Biotech, CA) was used. A dilution of 1/2000 AP-conjugated secondary antibodies, rabbit anti-goat (AP106 A, Chemicon) or goat anti-rabbit (AP132A, Chemicon), was used.

Immunohistochemistry

Paraffin-embedded tissue sections were used. A human malignant melanoma tissue section was used as an external positive control for the immunohistochemistry. The human tissue sections of a few melanoma cases, 7 specimens from normal patients, and 94 specimens from NPC patients were de-paraffinized, and rehydrated, and immunohistochemically stained as described (Wu et al., 2001a; Wu et al., 2001b; Wu, 2004; Wu et al., 2005; Chiang et al., 2005; Wu et al., 2008; Wu et al., 2011; Zeng et al., 2011; Zeng et al., 2012a). The primary antibody used was the chicken anti-recombinant huMETCAM/MUC18 protein IgY (1/500 dilution) and the secondary antibody the biotinylated rabbit anti-chicken IgY (1/250 dilution) (G289A, Promega) (Wu et al., 2001a; Wu et al., 2001b; Wu, 2004; Wu et al., 2005; Chiang et al., 2005;
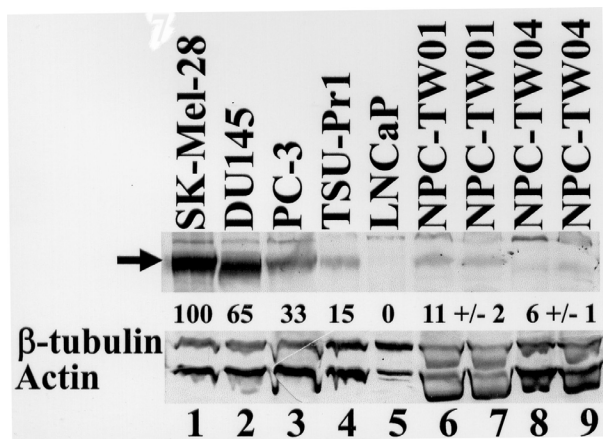
Figure 1. Expression of huMETCAM/MUC18 Protein in Normal NP and NPC Tissues. (A) shows the results of Western blot analysis of the lysates (10 μg proteins) from three normal nasopharynx (NP) tissues, LPL, LSS, and CMN, (lanes 1-3), and three NPC tissues (lanes 4-6). The lysate of a human melanoma cell line, SK-Mel 28, was used as a positive control (lane 7). The numbers show the molecular weights (kD) and the arrows the locations of the two forms of huMETCAM/MUC18. β-tubulin band is shown as the loading control. (B) shows the quantitative result of the Western blot analysis carried out in (A). The expression level of huMETCAM/MUC18 protein in a human melanoma cell line, SK-Mel-28, was assumed to be 100%. The P-values was calculated by comparing the two sets of data obtained from normal NP and NPC

Wu et al., 2008; Wu et al., 2011; Zeng et al., 2011; Zeng et al., 2012a). Classification of the NPC tissue sections was carried out by a histological staining with H & E and followed by observation with a light microscope.
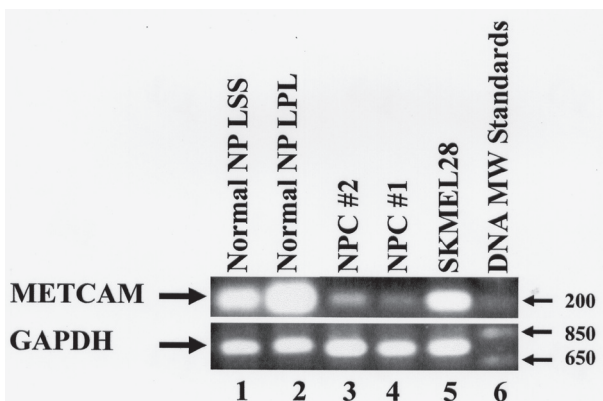
Results

Expression of HuMETCAM/MUC18 protein in the normal NP and NPC tissues, metastatic lesions, and two established NPC cell lines

Figure 1A shows that the huMETCAM/MUC18 protein was expressed in all three normal NP tissues (lanes 1-3), but weakly expressed in the three NPC tissues (lanes 4-6). The molecular weight of huMETCAM/MUC18 protein (about 150 kDa) expressed in NPC tissues was similar to that in a human melanoma cell line and prostate cancer cell lines. The lower molecular weight species (about 105 kDa) in lanes 2 and 4 was probably representing the under-glycosylated huMETCAM/MUC18 protein, which was similarly observed in angiosarcomas and endothelial cells (Lehmann et al., 1989; Wu, 2005). Figure 1B shows the quantitative result of the expression level

Figure 2. Expression of huMETCAM/MUC18 Protein In Human Npc Cell Lines in Comparison with One Melanoma Cell Line, Three Prostate Cancer Cell Lines, and One Bladder Cancer Cell Line Tsu-Pr1. 10 μg proteins from each lysate of the cell lines were loaded in each lane for the Western blot analysis. The expression of huMETCAM/MUC18 protein is shown in one human melanoma cell line, SK-Mel-28 (lane 1), three prostate cancer cell lines, DU145, PC-3, and LNCaP (lanes 2, 3 and 5, respectively), one bladder cancer cell line, TSU-Pr1 (lane 4), and the two human NPC cell lines, NPC-TW01 and NPC-TW04 (lanes 6-9). The arrow shows the location of huMETCAM/MUC18. The number (%) below each lane of the top row represents the relative expression level of huMETCAM/MUC18 in each cell line with respect to that in SK-Mel-28, assuming to be 100%. Loading controls, β-tubulin and actin, are shown in the bottom rows. Loading controls, β-tubulin and actin, are shown in the bottom rows


Figure 3. RT-PCR Verification of the Presence of huMETCAM/MUC18 mRNA in NPC Tissues. The top row shows the 197 cDNA fragment of the RT-PCR amplified huMETCAM/MUC18 mRNA in two NP and two NPC specimens. The bottom row shows the 908 cDNA fragment of the RT-PCR amplified human GAPDH mRNA as a control. The DNA molecular size markers shown on the right are the 1 kb ladder (Life Technology/GIBCO/BRL)

of huMETCAM/MUC18 protein in these tissues. The mean expression level of huMETCAM/MUC18 in NPC tissues was about 1/5 of that in the normal counterparts and metastatic lesions. Figure 2 shows the expression of huMETCAM/MUC18 protein in two established NPC cell lines, NPC-TW-01, which was established from a keratinizing squamous nasopharyngeal carcinoma (WHO type I), and NPC-TW-04, which was established from a

Table 2. Human METCAM/MUC18 Expression in the Tissues of Normal Nasopharynx, Nasopharyngeal Carcinomas, and Metastatic Lesions

| Histological/pathological classification	Number of cases expression (+5)	Number of cases expression (+1-+2)	Number of cases no expression	% Cases expression
Normal nasopharynx	7 out of 7	6 out of 7	0 out of 7	100
WHO type I (KSCC)	0/24	6 out of 24	18/24	25
WHO type II (NKSCC)	0/49	16/49	33/49	32.7
WHO type III (UDC)	0/22	5 out of 22	17/22	22.7
Metastatic lesions (bone, liver, and lung)	3 out of 3	0/3	0/3	100

WHO, World Health Organization
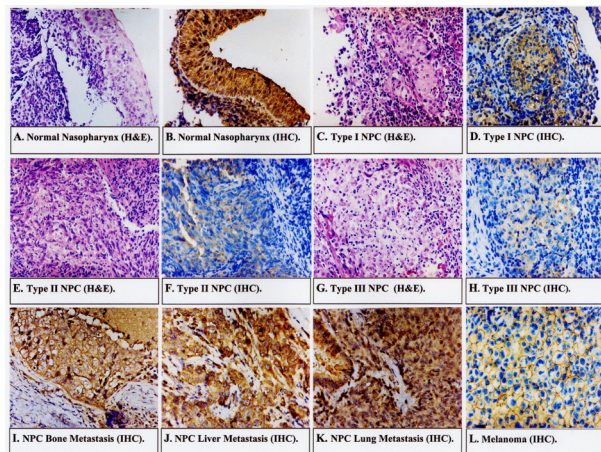
Figure 4. Histology and Immunohistochemistry of the Expression of huMETCAM/MUC18 Antigen in Normal Nasopharynx, NPC Tissue Sections, and Three Metastatic Lesions. Histology is shown in normal nasopharynx (A), type I NPC (C), type II NPC (E), and type III NPC (G). Immunohistochemistry is shown in normal nasopharynx (B), type I NPC (D), type II NPC (F), and type III NPC (H), and metastatic lesions (I-K). Immunohistochemistry of metastatic lesions in bone (I), liver (J), and lung (K) is shown. A human melanoma tissue section (L) was used as a positive control for the immunohistochemistry by using anti-huMETCAM/ MUC18 antibody

non-keratinizing squamous nasopharyngeal carcinoma (WHO type II) (Lin et al., 1990; Lin et al., 1993). As shown in Figure 2, the molecular weight of huMETCAM/ MUC18 expressed in NPC-TW01 was similar to that in other cancer cell lines, but that in the NPC-TW04 was slightly lower than that in most of human cancer cell lines. The huMETCAM/MUC18 level in NPC-TW01 was about 2 folds of that in NPC-TW-04 (Figure 2). However, the huMETCAM/MUC18 levels in the NPC-TW01 and NPC-TW04, respectively, were about 1/9 and 1/17 of that in a human malignant melanoma cell line, SK-Mel-28, about 1/6 and 1/11 of that in a metastatic human prostate cancer cell line, DU145, about 1/3 and1/5.5 of that in a metastatic human prostate cancer cell line, PC-3, and about 1/1.4 and 1/2.5 of that in a human bladder cancer cell line, TSU-Pr1 (Figure 2). Thus the low expression levels of huMETCAM/MUC18 in NPC tissues were also reflected in the two NPC cell lines as well as in five other established cell lines (data not shown).

HuMETCAM/MUC18 cDNA sequence the NPC tissues

To Confirm the expression of huMETCAM/MUC18 in NPC tissues, we amplified the cDNA of the total RNA
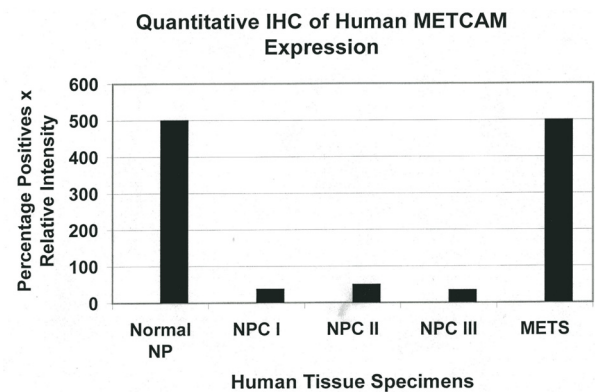
Figure 5. The Quantitative IHC of the Expression of huMETCAM/MUC18 Antigen in Normal NP, NPC and Metastatic Lesions. The results were taken from the IHC results in Figure 4

prepared from two normal NP and eight NPC tissues, and determined the huMETCAM/MUC18 cDNA sequences from these specimens. Figure 3 shows that the amplified cDNA from huMETCAM/MUC18 mRNA expressed in normal NP was higher than that in NPC tissues, in parallel to the protein level in both types of tissues. The sequences of the huMETCAM/MUC18 cDNA in most normal NP and NPC tissues were identical to that of the major form of huMETCAM/MUC1820 except that one NPC sample had two point mutations at nucleotide #1891 A -> G (corresponding to 631Lys ->Glu) and at nucleotide # 1894 A-> C (corresponding to a neutral mutation at 632Arg), which may be due to single nucleotide polymorphisms (SNPs).

HuMETCAM/MUC18 antigen is expressed in the formalin fixed, paraffinized NP and NPC tissue sections and metastatic lesions

Figure 4 shows that the results of IHC. Both a normal NP and a human melanoma specimen, as a positive control for the IHC, expressed the huMETCAM/MUC18 antigen mostly in cytoplasmic membrane and some in the cytoplasm, as expected (Figures 4B & L). The positively stained WHO type I NPC also expressed the antigen mostly in cytoplasmic membrane and some in the cytoplasm (Figure 4D). In contrast, the positively stained WHO type II and III NPCs expressed the antigen mostly in the cytoplasm and some in the cytoplasmic membrane (Figures. 4 F & H). The expression levels of huMETCAM/ MUC18 appeared to decrease from normal NP tissues to all three types of NPC tissues with the lowest level in the WHO type III NPC tissues. Surprisingly the expression

level of the protein was increased in all the three metastatic lesions (bone, liver, and lung). Table 2 summarized the result of IHC of seven normal NP, ninety-four human NPC, and three metastatic specimens. As shown in Table 2, huMETCAM/MUC18 antigens were expressed in 100% of normal nasopharynx specimens; however, they were not expressed in 73% and very weakly expressed in 27 % of the ninety-five NPC specimens. HuMETCAM/MUC18 expression was in a slightly higher percentage of patients with the WHO Type II NPC (33%) than those with both the WHO Type I NPC (25%) and WHO Type III NPC (23%). The overall expression frequency and level of huMETCAM/MUC18 in all the NPC specimens was less than 1/10 of that in the normal nasopharynx (Figure 5). In contrast, the three metastatic lesions (bone, liver, and lung) expressed a level of huMETCAM/MUC18 similar to that in the normal NPs (Figure 5).

Discussion

We have presented evidence to show that METCAM/MUC18 was expressed in all normal NP tissues, but not expressed in most (about 73%) of NPC tissues (or if expressed, 27% of NPC specimens expressed at very low levels), and expressed in all metastatic lesions of NPC. The expression of huMETCAM/MUC18 in NP and NPC tissues was confirmed by the positive presence of the mRNA in these tissues. To further support the notion that huMETCAM/MUC18 was expressed at a low level in NPC tissues, we showed that low levels of huMETCAM/MUC18 were also expressed in seven established NPC cell lines in comparison with that in a human malignant melanoma cell line, SK-Mel-28, two metastatic human prostate cancer cell lines, DU145 and PC-3, and a human bladder cancer cell line, TSU-Pr1. Taken together, we concluded that the diminishing or the loss of huMETCAM/MUC18 expression may be a new potential biomarker for emergence of NPC, whereas the re-gain of its expression may be a new potential biomarker for the progression of NPC to malignant stages, in addition to other potential markers for this cancer, such as the loss of E-cadherin and connexin 43 expression (Huang et al., 2001; Li et al., 2004; Yi et al., 2006), the gain of ICAM expression (Yu et al., 2004), and the presence of EBV-specific LMP1 (Tsai et al., 2002; Yoshizaki, 2002).

Previously we have shown that huMETCAM/MUC18, a CAM in the immunoglobulin-like super-family, is able to promote tumorigenesis and initiate metastasis in prostate cancer cells (Wu et al., 2001a; Wu et al., 2001b; Wu, 2004; Wu et al., 2004; Wu, 2005; Wu et al., 2005; Wu et al., 2011). HuMETCAM/MUC18 also enhances metastasis of melanoma cells; however it has little effect on promoting their tumorigenesis (Xie et al., 1997; Schlagbauer-Wadl et al., 1999; Wu et al., 2008). Furthermore, huMETCAM/MUC18 is able to promote tumorigenesis and metastasis of human breast cancer cells (Zeng et al., 2011; Zeng et al., 2012a; Zeng et al., 2012b). From our previous experiences with the human prostate cancer LNCaP clones/cells and human breast cancer clones/cells (Wu et al., 2004; Wu, 2005; Wu et al., 2011; Zeng et al., 2011; Zeng et al., 2012a), we showed that a huMETCAM/

MUC18-transfected LNCaP clone, which expressed a low level of huMETCAM/MUC18, were capable of promoting tumorigenesis and metastasis in nude mice (Wu et al., 2004; Wu et al., 2011). However, it is not clear if this is applicable to NPC cell lines, so that the low expression levels of huMETCAM/MUC18 in NPCs may promote tumorigenesis and metastasis. Consistent with this notion, the two NPC cell lines, which only expressed about 6-11 % of the protein in comparison with that in SK-Mel-28 cells, were previously shown that they were able to form tumor subcutaneously and to establish metastatic lesions on the surface of diaphragm and in lung parenchyma and liver, when the very high number of cells (5 x 10⁷ cells) were subcutaneously injected into Balb/c nude mice ((Lin et al., 1990; Lin et al., 1993). However, the possibility that this might be an experimental artifact of injecting a very high number of cells in immunodeficient mice can not be completely ruled out until the results of a similar animal test by using a much lower number of cells for injection are confirmed. Taken together, we suggest that enforced expression of huMETCAM/MUC18 might suppress the tumor growth, but increase metastasis of the NPC cell lines. However, this requires further testing the effect of enforced or decreased expression of huMETCAM/MUC18 in the two NPC cell lines on their abilities to induce tumorigenesis and metastasis in an immunodeficient mouse system. Our preliminary results of animal tests suggest that enforced expression of huMETCAM/MUC18 indeed suppressed the tumorigenesis of one NPC cell line (Wu et al., unpublished results).

Several plausible reasons may account for that the majority of the NPC tissues lost the expression of huMETCAM/MUC18, but some had low expression levels, whereas the metastatic lesions regained the expression of huMETCAM/MUC18: (a) environmental carcinogens may induce mutations in the regulatory region of the promoter of the huMETCAM/MUC18 gene, which alter the expression of the gene, (b) SNP variations in patients may decrease or augment the expression of the gene, (c) hypermethylation in the promoter region of the gene, which is caused by the expression of LMP-1 of EBV, may diminish or repress the expression of the gene, whereas partial or complete reversal of hyper-methylation may account for regaining the expression of the gene in metastatic lesions.

Recent evidence seems to be inline with the above possibilities. For examples, there is a positive correlation of the TAP1 gene polymorphisms in a Tunisian population with the risk of (Hassen et al., 2007) as well as the TGF-β1 gene polymorphisms in a Chinese population with the genetic susceptibility to NPC (Wei et al., 2007). The evidence of association of SNPs with cancer metastasis in many cancer types has also begun to emerge (Craefor ad Hunter, 2006). The LMP1 gene of EBV has been shown to down regulate the E-cadherin expression via the activation of DNA methytransferases (Tsai et al., 2002; Krishna et al., 2005) and also promoter methylation down-regulates the expression of connexin 43 in NPC (Yi et al., 2006). The fine mapping in the chromosome11q22-23 region, in which the huMETCAM/MUC18 gene resides, indicates that hyper-methylation of the region may abolish the

expression of the gene and is associated with the NPC development during the progression of NPC (Lung et al., 2004; Lung et al., 2005). Finally the LMP1 gene of EBV has also been shown to up-regulate the expression of matrix metalloproteinase-9, urokinase type-plasminogen activator via activation of NF-$\varkappa$B and AP-1, VEGF via cyclooxygenase-2 activation, and interleukin-8 through NF-$\varkappa$B activation, and to enhance cell motility by activation of the transcription factor ets-1 (Yoshizaki, 2002), which may up-regulate the transcription of the huMETCAM/MUC18 promoter (Wu, 2012).

In summary, since there is a direct correlation of the loss of the expression of the gene with the majority of the NPC specimens, it is tempting to suggest that huMETCAM/MUC18 may serve as a tumor suppressor in development of NPC and but as a metastasis promoter in some of the NPC patients, similar to the actions of TGF-β in many cancer types (Roberts and Wakefield, 2003).

Acknowledgements

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