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Dual Roles of Metcam in the Progression of Nasopharyngeal Carcinomas

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Published Date: May 15, 2016

ABSTRACT

METCAM/MUC18, an integral membrane Cell Adhesion Molecule **(CAM)** in the I_g-like gene super-family, is capable of performing typical functions of CAMs, such as mediating cell-cell and cell-extracellular interactions, crosstalk with intracellular signaling pathways, and modulating social behaviors of cells. METCAM/MUC18 is expressed in about nine normal cells/tissues. Aberrant expression of METCAM/MUC18 has been associated with the progression of several epithelial tumors. Since METCAM/MUC18 has been implicated in the progression of nasopharyngeal carcinomas, we initiated the study of the possible roles of METCAM/MUC18 in the malignant progression of NPC. We showed that METCAM/MUC18 was expressed in all

of the normal nasopharynx, but weakly expressed in only 27% of the NPC tissues, suggesting that METCAM/MUC18 may function as a tumor suppressor in the development of NPC during the progression of the disease. To test the hypothesis, we investigated the effect of METCAM/MUC18 over-expression on *in-vitro* cellular behavior and *in-vivo* tumorigenesis of two NPC cell lines in athymic nude mice. Indeed, METCAM/MUC18 over-expression suppressed the tumor growth of NPC-TW01 cells, which were established from type I NPC. Surprisingly, METCAM/MUC18 over-expression promoted the tumor growth of NPC-TW04 cells, which were established from type II NPC. We suggested that METCAM/MUC18 plays a tumor suppressor role in the type I NPC, however, a tumor promoter role in the type II NPC. The dual role played by METCAM/MUC18 in the progression of two different types of NPC's may be modulated by different intrinsic factors and also in different stromal microenvironment. Furthermore, radio-sensitivity of tumors induced from both cell lines was increased by increased expression of METCAM/MUC18. Thus ectopically increased expression of this protein may be used for clinical treatment. This may serve as a model for understanding the contribution of three etiological factors to triggering the malignant progression of NPC and for translational applications.

INTRODUCTION

Nasopharyngeal Carcinoma (NPC)

Nasopharyngeal Carcinoma **(NPC)** is a malignant head and neck cancer; 90% of that develops in the non-lymphomatous, squamous epithelial lining of posterior nasopharynx [1]. NPC takes one of the three patterns (or three subtypes): keratinizing squamous cell carcinomas (WHO type I), non-keratinizing squamous cell carcinomas (WHO type II), and undifferentiated carcinomas (WHO type III) [2].

NPC is prevalent in several regions around the world: Southern China, Mediterranean Africa, and Eskimos inhabited areas [3]. 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 [1,3]. Radiotherapy is an effective treatment for NPC, and more than 80% of patients with early disease are curable [4]. Unfortunately, most of the NPC patients are diagnosed at later stages, where treatment is much less effective and difficult [4,5]. 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 [1-6]. 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* [7]. For examples, up-regulation of ICAM [8] and down-regulation of E-cadherin [9,10] and connexin 43[11] correlates with the progression of NPC; however, the expression of CD44 does not [9]. Though it is not known how the three major etiological factors contribute to the extraordinary incidence in endemic areas. But from their induction of aberrant expression of Cell Adhesion Molecules **(CAMs),** which has been proven evident in NPC, perhaps the mechanism can be deduced.

METCAM/MUC18

Human METCAM/MUC18 (or MCAM, Mel-CAM, S-endo1, CD146, or A32), an integral membrane Cell Adhesion Molecule **(CAM)** in the immunoglobulin (I_g)-like gene super family, has an N-terminal extra-cellular domain of 558 amino acids, a transmembrane domain, and a short intra-cellular cytoplasmic domain (64 amino acids) at the C-terminus, as shown in the following Figure 1[12-13].

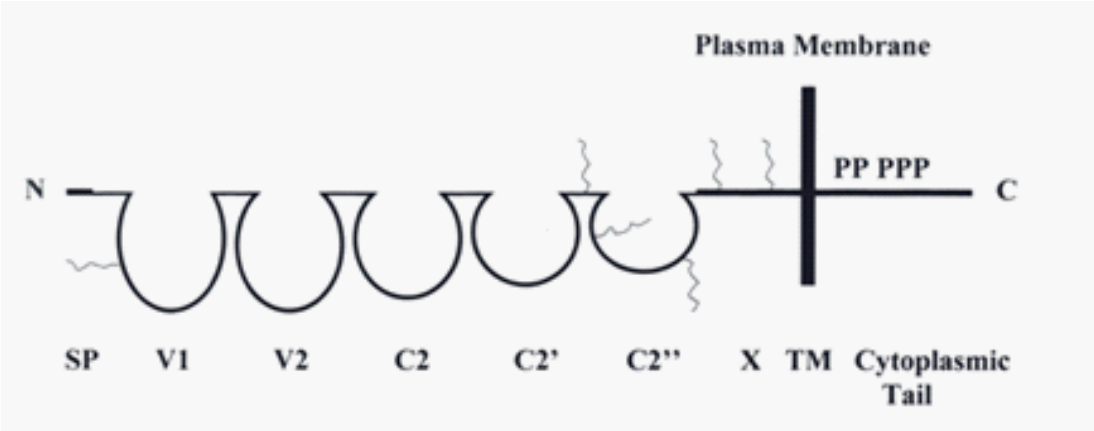

Figure 1: HuMETCAM protein structure: SP stands for signal peptide sequence, V1, V2, C2, C2', C2'' for five Ig-like domains (each held by a disulfide bond) and X for one domain (without any disulfide bond) in the extracellular region, and TM for transmembrane domain. P stands for five potential phosphorylation sites (one for PKA, three for PKC, and one for CK2) in the cytoplasmic tail. The six conserved N-glycosylation sites are shown as wiggled lines in the extracellular domains of V1, between C2' and C2'', C2'', and X.

As shown in the above Figure 1, the extra-cellular domain of the protein comprises a signal peptide sequence and five immunoglobulin-like domains and one X domain [12,13]. The cytoplasmic domain contains five consensus sequences potentially to be phosphorylated by PKA, PKC, and CK2 [12,13]. Thus human METCAM/MUC18 is capable of performing typical functions of CAMs, such as governing the social behaviors by affecting the adhesion status of cells and modulating cell signaling. Therefore, an altered expression of METCAM/MUC18 may affect motility and invasiveness of many tumor cells *in-vitro* and tumorigenesis and metastasis *in-vivo* [13].

Human METCAM/MUC18 is only expressed in several normal tissues, such as hair follicular cells, smooth muscle cells, endothelial cells, cerebellum, basal cells of the lung, activated T cells, intermediate trophoblasts [14], normal breast epithelium [15], ovarian epithelium [16], and nasopharyngeal epithelium [17]. Human METCAM/MUC18 is also expressed in several epithelial tumors, such as melanoma, prostate cancer, osteosarcoma, breast carcinoma, and intermediate trophoblast tumors [13,14]. METCAM/MUC18 has dual effects on different tumors: either it has no effect or a positive effect on tumorigenesis of some cancers, or a negative effect on tumorigenesis of some other cancers. It may also manifest a positive or a negative effect on metastasis of different cancer cells. For examples, over-expression of METCAM/MUC18 promotes the tumorigenesis of prostate cancer [18] and breast carcinoma [19,20], but it has a minimal effect on the tumorigenesis of melanoma [21]. Over-expression of METCAM/MUC18 also initiates the metastasis of prostate cancer [22] and promotes the metastasis of melanoma [21] and breast carcinoma [23].

On the contrary, the possibility that the over-expression of METCAM/MUC18 might play a tumor suppressor role was first suggested by Shih et al. [15], who found that METCAM/MUC18 expression suppressed tumorigenesis of a breast cancer cell line MCF-7 in SCID mice. However, this notion was contradicted by recently published evidence, which supported the positive role of METCAM/MUC18 in the progression of breast cancer cells [19,20,23], similar to its role in the progression of melanoma and prostate cancer cells [13].

The role of METCAM/MUC18 in the progression of NPC has not been studied. The following section describes the recent findings for the role of METCAM/MUC18 in this aspect.

IMPLICATION OF METCAM/MUC18 IN THE PROGRESSION OF NPC

The expression of HuMETCAM/MUC18 in normal Nasopharynx **(NP)** and NPC tissues has not been studied [13,14]. 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 [24,25]. (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, intermediate trophoblast and ovarian epithelial cells [14,16], has been evident in playing very intriguing roles in the progression of several epithelial cancers [13,26]. Over-expression of HuMETCAM/MUC18 promotes the metastasis of melanoma [21,27,28], prostate cancer [18,22,29,30], angiosarcomas [13], osteosarcoma [31] and breast cancer [19,20,23]. On the contrary, under-expression correlates with the malignant progression of haemangioma [32] and over-expression of HuMETCAM/MUC18 suppresses the tumorigenesis of one mouse melanoma cell line [33] and two human ovarian cancer cell lines [34] in an immune-deficient mouse model, suggesting that its expression may suppress the tumorigenesis of these cancers and perhaps other cancer types [26].

We initiated the study of possible role of HuMETCAM/MUC18 in the malignant progression of NPC by investigating the possible expression of HuMETCAM/MUC18 in normal NP and NPC tissues and two established NPC cell lines. We found that all of the normal nasopharynx specimens expressed a good level of HuMETCAM/MUC18; however 73% of NPC clinical tumor specimens did not express and 27% weakly expressed the protein in all the three subtypes of NPC [17]. Down regulation and loss of the expression of the METCAM/MUC18 gene suggests that METCAM/MUC18 may serve as a tumor suppressor, but as a metastasis promoter during the progression of NPC [17].

METCAM/MUC18 PLAYS A TUMOR SUPPRESSOR ROLE IN THE PROGRESSION OF TYPE I NPC

To test the above hypothesis, we transfected the NPC-TW01 cell line with the HuMETCAM/MUC18 cDNA gene and selecting high expression clones and using these clones to subcutaneously inject these cells into female and male nude mice. We found that METCAM/MUC18 expression suppressed tumorigenesis of NPC-TW-01 [35].

Cell morphology of NPC-TW01 cells

NPC-TW01 cells, which were established from Type 1 NPC [36,37], can be easily cultured *in-vitro* in a humidified 37o C incubator without CO_2. The following Figure 2 shows that most of the cells are mesenchymal-like and only a small percentage is epithelial-like, suggesting that they are highly tumorigenic and highly malignant.

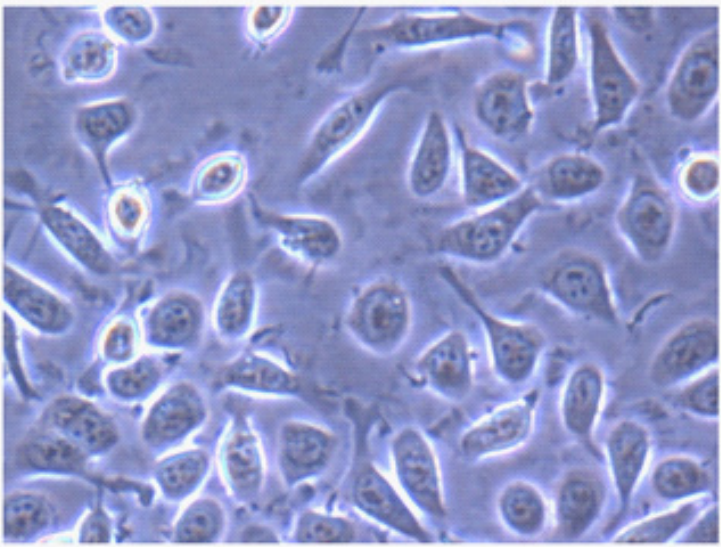

Figure 2: *In-vitro* Cell morphology of NPC-TW01 cells. NPC-TW01 cells were maintained in DMEM medium supplemented with 5% fetal bovine serum and 100 μg/ml kanamycin in a humidified incubator with or without CO_2.

Expression of HuMETCAM/MUC18 protein in the clones of NPC-TW01 cell line

To investigate the effect of METCAM/MUC18 expression on *in-vitro* behaviors and *in-vivo* tumorigenesis, it is necessary to obtain high-expressing clones from NPC-TW01 cells since they weakly expressed METCAM/MUC18 [17]. This can be achieved by transfecting with HuMETCAM/MUC18 cDNA by using lipofecting reagents. We had tried several kinds of lipofecting agents, such as DAMRIE-C (Life Technology), Lipofectamine (Invitrogen), FuGene HD (Roche), and Lipofectamine 2000 (Invitrogen). Lipofectamine 2000 had been found to be most effective to obtain high-expressing clones: 2.9% high-expressing clones, 5.7% middle-expressing clones, 60% low-expressing clones, and 31.4% very-low expressing clones. Figure 3 shows three typical clones (clones #92, #45, and #85) with various expression levels of METCAM/MUC18.

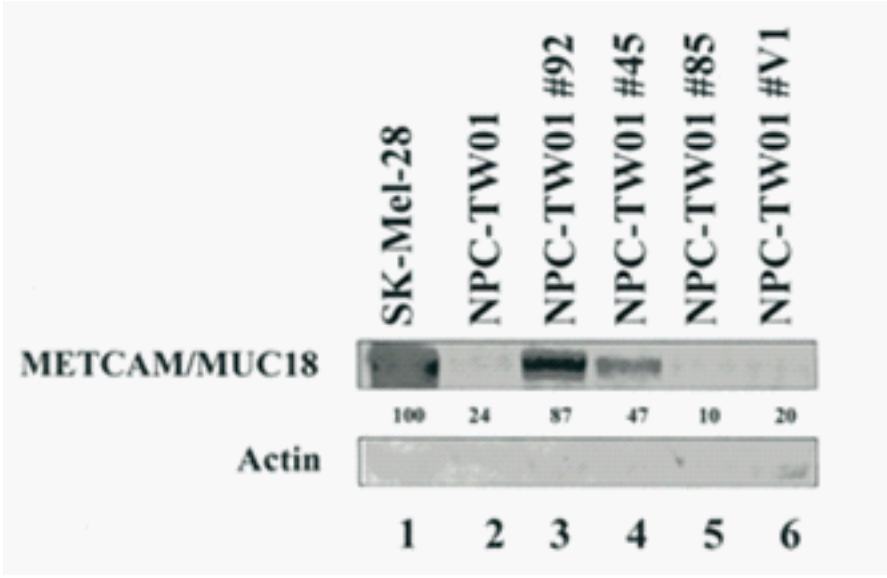

Figure 3: Expression levels of HuMETCAM/MUC18 in NPC-TW01 clones. Western blot was used to determine the expression levels of HuMETCAM/MUC18 in each cell line/clones. Lane 1 shows the expression level of the protein in human melanoma cell line, SK-Mel-28, as a positive control (100%). Lanes 2-5 show the expression levels of the protein in the parental cells and three clones #92, #45, and #85, and lane 6 shows that of the vector control clone V1. The number under each lane was the relative expression level of each clone. Actin was the loading control.

Over expression of HuMETCAM/MUC18 decreases the migration and invasiveness of NPC-TW01 cells

To determine the effect of HuMETCAM/MUC18 expression on the *in-vitro* behaviors of NPC-TW01 cells, migration and invasiveness of clones #92, #45, and V1 were determined. As shown in Figure 4, expression of HuMETCAM/MUC18 decreased the *in-vitro* motility and invasiveness

of NPC-TW01 clones. As also shown in Fig. 4, the anti-HuMETCAM/MUC18 antibody either had no effect on the motility and invasiveness of the cells except the vector control clone V1 which increased both behaviors. We conclude that HuMETCAM/MUC18 decreased the Epithelial-To-Mesenchymal Transition (EMT) of NPC-TW01 cells.

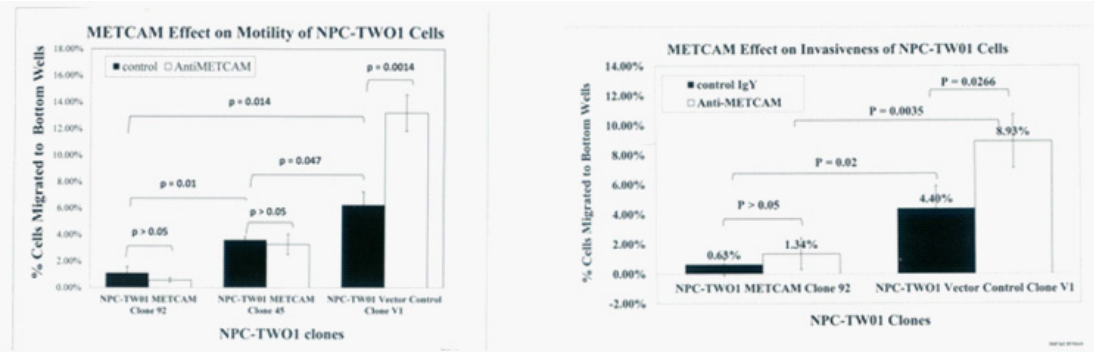

Figure 4: Effects of HuMETCAM/MUC18 expression on *in-vitro* migration and invasiveness of NPC-TW01 cells. Boyden type transwell system was used to determine the effects [38]. Percentages of cells in each clone migrated to the bottom wells were determined after 20 hours. Filled columns showed the percentages of cells migrated to bottom wells in the absence of an anti-huMETCAM/MUC18 antibody and empty columns that in the presence of the antibody.

Over-expression of HuMETCAM/MUC18 altered the growth in 3D basement membrane assay

We had difficulties to demonstrate formation of anchorage-independent colony in soft agar (*in-vitro* tumorigenesis) from both clones of NPC-TW01 cell line. Alternatively, we could demonstrate a limited (less aggressive) growth of the clone #92, which formed spheroid growths in the 3D basement membrane culture assay [39] than the vector control clone V1, which formed a more aggressive (extended) tubular-like growth, as shown in Figure 5. The limited (less aggressive) growth of clone #92 could be reversed in the presence of anti-METCAM/MUC18 antibodies, as also shown in Figure 5. Taken together, we conclude that over-expression of METCAM/MUC18 could promote a limited (less aggressive) growth of NPC-TW01 cells in the 3D basement membrane culture assay and it was due to the direct effect of METCAM/MUC18.

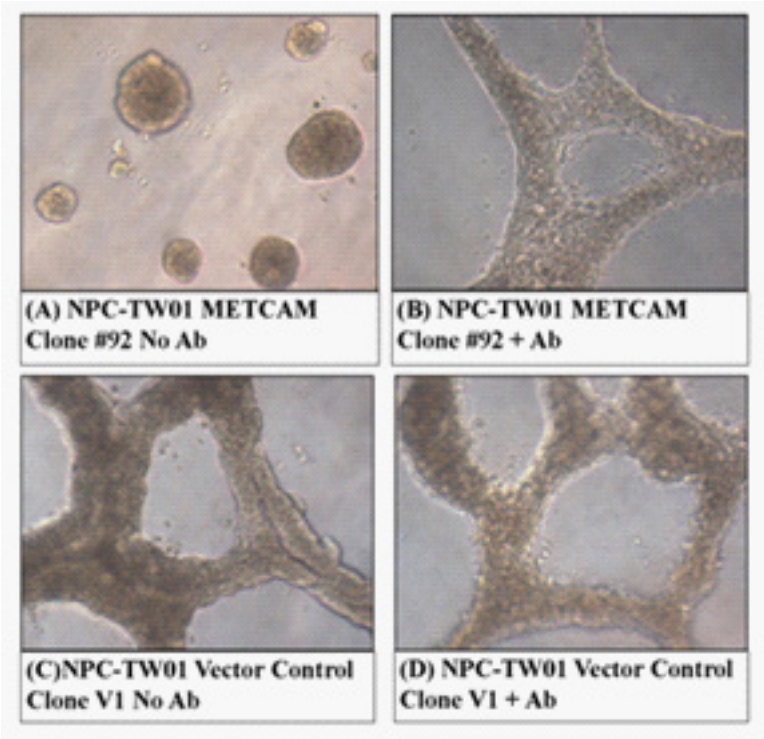

Figure 5: Effects of HuMETCAM/MUC18 expression on the growth of NPC-TW01 cells in 3D basement membrane culture assay in the presence or absence of an anti-huMETCAM/MUC18 antibody. The method of 3D basement membrane culture assay was used [39].

Over expression of HuMETCAM/MUC18 decreases the tumorigenesi

Previously NPC-TW01 cells could yield tumors via Subcutaneous (SC) injection of the cells (non-orthotopic injection route) in a Balb/C athymic nude mouse model [36,37]. However, an unusually high number of cells, about 10^7 cells, were used for the injection [36,37]. To eliminate the possibility that injection of an extremely high number of cells may yield experimental artifacts, we used a much lower cell number for the injection [40]. In the presence of Matrigel, we found when 1. 5 x 10^6 cells, about 1/10 of that had previously been used, were able to induce tumor formation in Balb/C athymic nude mice. We also found that an even lower number of cells (1.5 x 10^5) were also able to induce tumor at the SC site (data not shown). Tumorigenicity appeared to be similar between male and female Balb/C nude mice. When1. 5 x 10^6 cells were used for injection, tumor formation by the V1 clone was much more efficient than the clone #92, as shown in Figure 6 (top figures). Figure 6 shows that tumor formation by the clone V1 was clearly visible at 7 days after injection, more prominent at 28 days after, and 16 times larger at 40 days. At the end point (40 days after injection) when the mice were terminated, the final mean tumor weight of the V1 clone was 18 times larger than that of the clone #92, as shown in Figure 6 (top figures). When 1.5 x 10^5 cells were used for injection, tumor formation by the V1 clone was also much more

efficient than the clone #92, which could not induce any visible tumor in nude mice (data not shown). METCAM/MUC18 expression suppressed tumorigenesis of NPC-TW-01 at both a low (1.5 x 10^5) and a high number (2 x 10^6) of cells. From these results, we concluded that over-expression of METCAM/MUC18 suppressed the tumorigenicity and decreased the final tumor weight of NPC-TW01 clones/cells in an athymic nude mouse model. In addition, tumor suppression appeared to be proportional to the dosages of HuMETCAM/MUC18 expressed, as shown in Figure 6 (bottom figures).

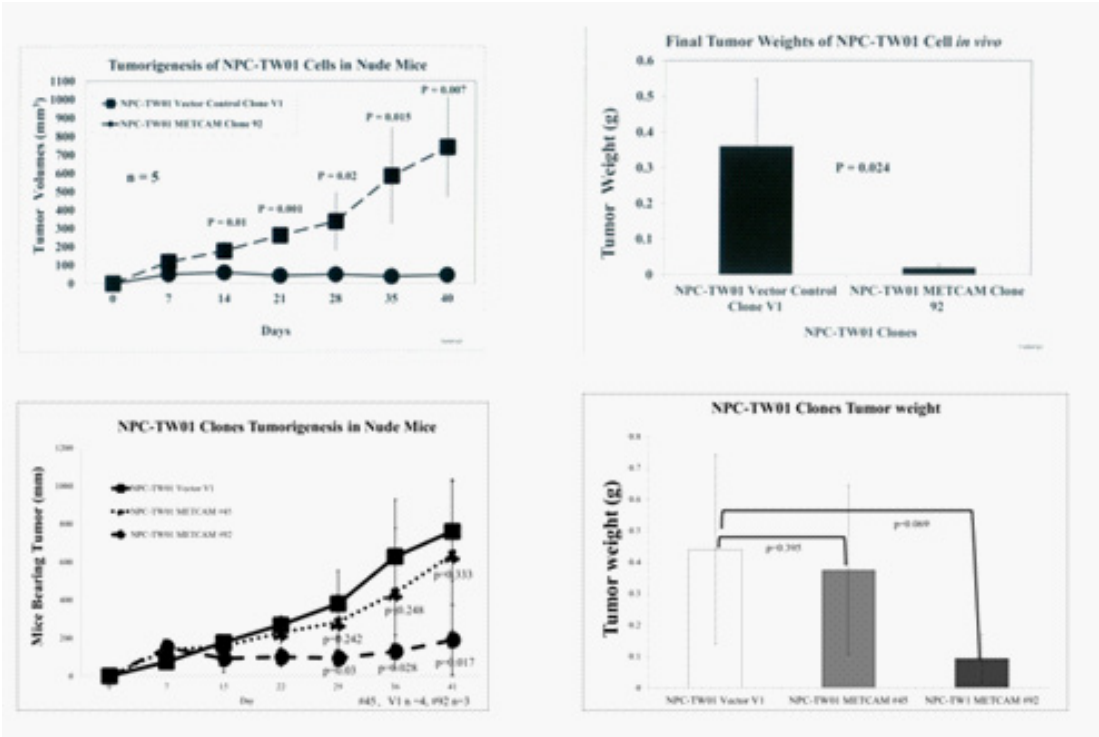

Figure 6: Effects of HuMETCAM/MUC18 expression on the tumorigenesis of NPC-TW01 cells in athymic nude mice. The method of SC injection of cells (1.5 x 10^6 cells/mouse) with matrigel in Balb/C athymic nude mice was used to investigate effects of METCAM/MUC18 over-expression on tumorigenesis of NCP-TW01 cells. The tumors were weekly measured by an electronic caliper. Tumor volume versus time was plotted. At the end of the experiment, tumors were excised and weighed.

MECHANISMS OF HUMETCAM/MUC18-INDUCED TUMOR SUPPRESSION

The mechanism by which METCAM/MUC18 expression affects tumorigenesis of NPC cells has not been studied. By deducing knowledge which we learned from tumorigenesis of other tumor cell lines (such as, melanoma and cancers in breast, ovary, and prostate) induced by METCAM/MUC18, METCAM/MUC18 may affect tumorigenesis by cross-talk with many signaling pathways

that regulate proliferation, survival, apoptosis, metabolism, and angiogenesis of tumor cells [26]. We therefore predicted that enforced expression of METCAM/MUC18 may affect tumorigenesis by altering expressions of its downstream effectors, such as indexes of apoptosis/anti-apoptosis, proliferation, survival, aerobic glycolysis, and angiogenesis, in the tumor cells. For this purpose, we determined the expression of levels of Bcl2, Bax, PCNA, the ratio of phospho-AKT/AKT, LDH-A, VEGF, and VEGFR2 in tumor lysates. Figure 7 shows that Bax was increased in tumor lysates of clone #92, but PCNA, ratio of phospho-AKT/AKT, LDH-A, and VEGF, and VEGFR2 were decreased in tumor lysates of clone #92. But no difference was found for Bcl2.

Taken together, we concluded that enforced expression of METCAM/MUC18 suppressed tumorigenesis of NPC-TW-01 cells by increased an apoptosis index (Bax), and decreased a proliferation index (PCNA), a signal for survival and proliferation pathway (ratio of phospho-AKT/AKT), aerobic glycolysis (LDH-A), and angiogenesis indexes (VEGF and VEGFR2).

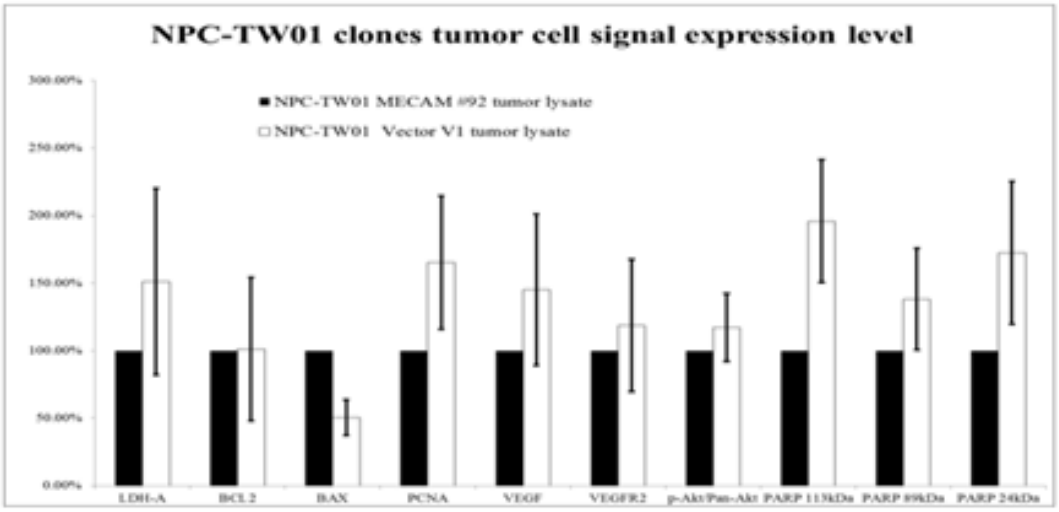

Figure 7: Expression levels of various key effectors down-stream of HuMETCAM/MUC18 in tumors. Western blot was used to determine the expression levels of various down-stream effectors (such as, BCl2, Bax, PCNA, LDH-A, Phospho-AKT, pan-AKT, VEGF, and VEGFR2) in tumor lysates. Relative levels of various downstream effectors expressed in tumors induced by clones #92 and V1 are plotted.

REVERSAL EFFECT OF SHRNA ON THE ROLE OF METCAM/MUC18 ON NPC-TW01 CELLS [wu et al. unpublished results]

The effect of shRNA on reversal the effects of METCAM/MUC18 on the ability of NPC-TW01 cells in *in-vitro* and *in-vivo* tumorigenesis in athymic nude mice was tested [30]. Locations of three HuMETCAM/MUC18-specific shRNAs are shown in Table 1. To ensure stable expression of the shRNAs, they were cloned into a lentivirus vector, pGIPZ (Thermo Scientific) and used for

transfection into NPC-TW01 cells, and stable clones enriched in the presence of puromycin were pooled. The efficiency of knockdown expression of NPC-TW01 shRNA clones was determined by Western blot. As shown in Figure 8 and summarized in Table 2, three different shRNAs had different efficiencies in knocking down the expression of METCAM/MUC18 in comparison with the control shRNA, #46.

Table 1: METCAM/MUC18-specifc shRNA inserts in the lentivirus vector pGIPZ.

Short Name	Catalogue number of Thermo Sci	Sequence of sense strand	Location in METCAM cDNA
46	RHS4346 (non-silence control)	5'-ATCTCGCTTGGGCGAGAGTAAG- 3'	none
72	RHS4430-200292372 (shRNA 392652)	5'-TGGTCTTGTTCACTTGCCG-3'	1435-1453
24	RHS4430-200288324 (shRNA 392649)	5'-TCAACTACAAGTTCGCTCT-3'	1843-1861
27	RHS4430-200295227 (shRNA 382648)	5'-TTGTCTTCTTTAACCAGCT-3'	661-679

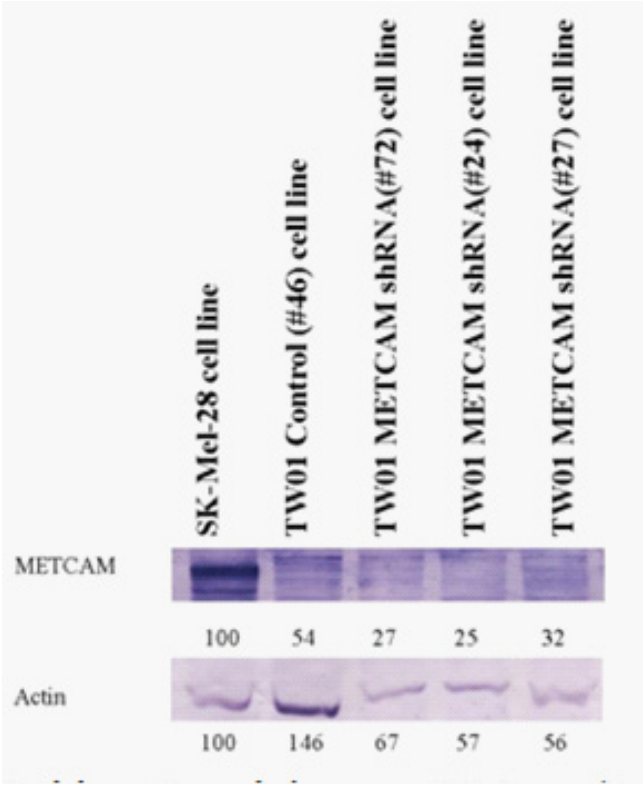

Figure 8: Expression levels of HuMETCAM/MUC18 in NPC-TW01 clones after transfection with pGIPZ with shRNA -coding genes. Western blot was used to determine the expression levels of HuMETCAM/MUC18 in each cell line/clones. Lane 1 shows the expression level of the protein in human melanoma cell line, SK-Mel-28, as a positive control (100%). Lanes 2-5 show the expression levels of the protein in the four clones.

Table 2: Knockdown METCAM/MUC18 expression in each of the NPC-TW01 shRNA clones.

ShRNA-knockdown clones	METCAM/MUC18 expression level	Number of mice injected
TW01-shRNA #46 (p58->p59)	100%	5 mice
TW01-shRNA #72 (p58->p59)	54%	5 mice
TW01-shRNA #24 (p58->p59)	77%	5 mice
TW01-shRNA #27 (p60->p61)	33%	5 mice

The tumorigenesis of four NPC-TW01 shRNA knockdown clones was investigated after SC injection of these cells in athymic nude mice. Figure 9 showed the results that shRNA #72 was more efficient than shRNAs #24 and #27 in reversal of the tumor-suppressor effects of METCAM/MUC18-overexpression on NPC-TW01 cells.

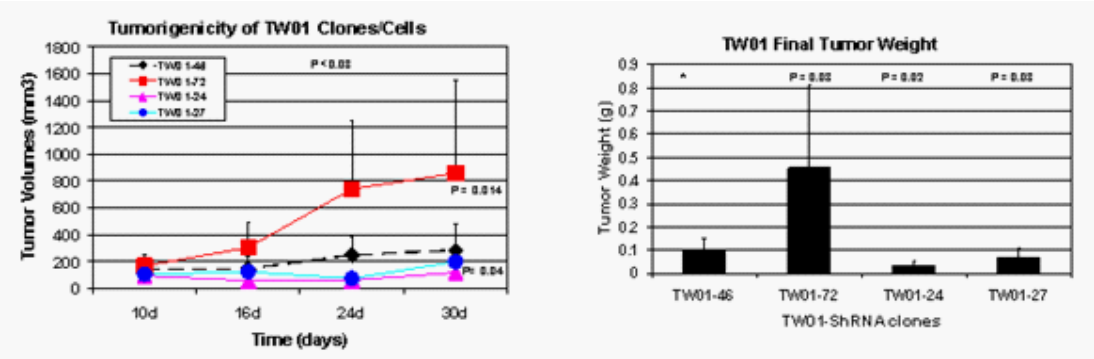

Figure 9: Tumorigenesis and final tumor weights of tumors induced by four NPC-TW01 shRNA knockdown clones. 2 x 10^6 cells were SC injected in each mouse. After 30 days mice were euthanatized and tumors were excised and weighed.

Taken together, we conclude that shRNAs had some effects on reversing the effect of METCAM/MUC18. However, the residual low level expression of METCAM/MUC18 may make the results not as clear-cut as predicted.

MTCAM/MUC18 PLAYS A TUMOR PROMOTER ROLE IN THE PROGRESSION OF TYPE II NPC

To test the above hypothesis, we also transfected the NPC-TW04 cell line with the HuMETCAM/MUC18 cDNA gene and selecting high expression clones and using these clones to subcutaneously inject these cells into female and male nude mice. We found that METCAM/MUC18 expression promoted the tumorigenesis of NPC-TW-04 cells [35].

Cell morphology of NPC-TW04 cells

NPC-TW04 cells, which were established from Type II NPC [36,37], can also be easily cultured *in-vitro* in a humidified 37°C incubator without CO_2. The following Figure 10 shows that most of the cells are mesenchymal-like and only a small percentage is epithelial-like, suggesting that they are highly tumorigenic and highly malignant.

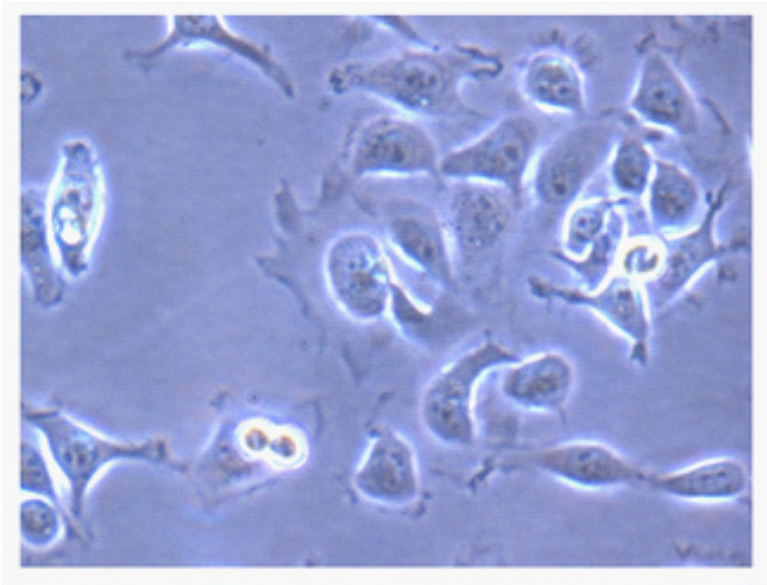

Figure 10: In-vitro Cell morphology of NPC-TW04 cells. NPC-TW04 cells were maintained in DMEM medium supplemented with 5% fetal bovine serum and 100 μg/ml kanamycin in a humidified incubator with or without CO_2.

Expression of huMETCAM/MUC18 protein in the clones of NPC-TW04 cell line

To investigate the effect of METCAM/MUC18 expression on *in-vitro* behaviors and *in-vivo* tumorigenesis, it is necessary to obtain high-expressing clones from NPC-TW04 cells since they also weakly expressed METCAM/MUC18 [17]. This can be achieved by transfecting the cells with HuMETCAM/MUC18 cDNA by using various lipofecting reagents. Lipofectamine 2000 had been found to be most effective to obtain high-expressing clones: 2.8% high-expressing clones, 0% middle-expressing clones, 38.9% low-expressing clones, and 58.3% very-low expressing clones. Figure 11 shows that two typical clones (clones #76 and #72) expressed with various levels of METCAM/MUC18.

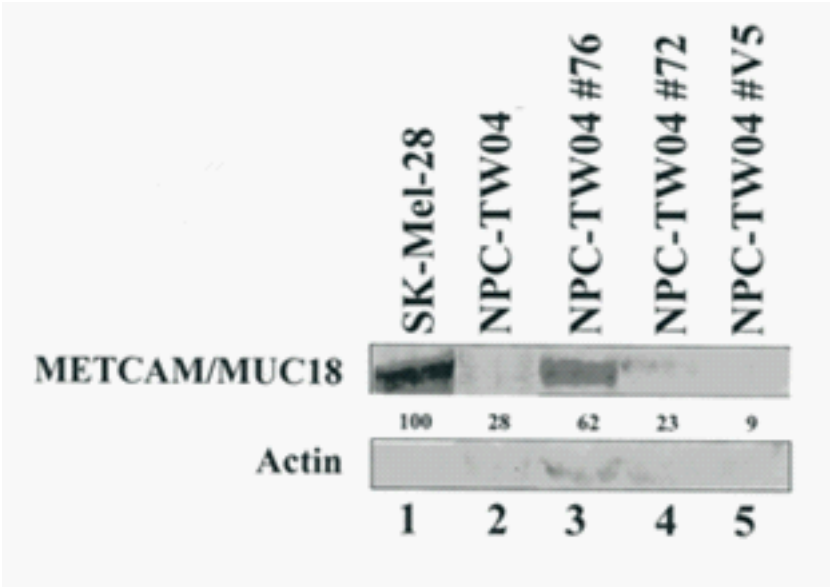

Figure 11: Expression levels of HuMETCAM/MUC18 in NPC-TW01 clones. Western blot was used to determine the expression levels of HuMETCAM/MUC18 in each cell line/clones. Lane 1 shows the expression level of the protein in human melanoma cell line, SK-Mel-28, as a positive control (100%). Lanes 2-4 show the expression levels of the protein in the parental cells and two clones #76 and #72, and lane 5 shows that of the vector control clone V5. The number under each lane was the relative expression level of each clone. Actin was the loading control.

Over-expression of HuMETCAM/MUC18 increases the migration and invasiveness of NPC-TW04 cells

To determine the effect of HuMETCAM/MUC18 expression on the *in-vitro* behaviors of NPC-TW04 cells, migration and invasiveness of clones #76 and V5 were determined. As shown in Figure 12, expression of huMETCAM/MUC18 increased the *in-vitro* motility and invasiveness of NPC-TW01 clones. As also shown in Figure 12, the anti-huMETCAM/MUC18 antibody somewhat decreased the motility and invasiveness of the clone #76 except the vector control clone V5. We conclude that HuMETCAM/MUC18 increased the Epithelial-To-Mesenchymal Transition (EMT) of NPC-TW04 cells.

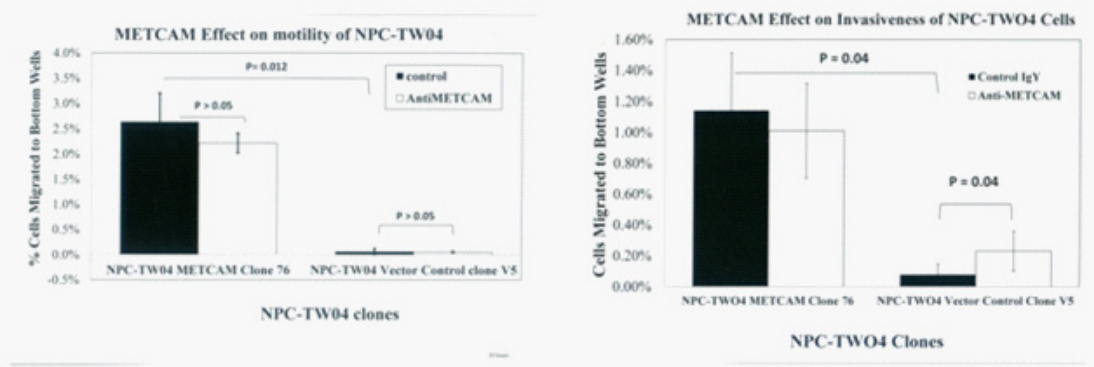

Figure 12: Effects of HuMETCAM/MUC18 expression on in vitro migration and invasiveness of NPC-TW04 cells. Boyden type transwell system was used to determine the effects [38]. Percentages of cells in each clone migrated to the bottom wells were determined after 20 hours. Filled columns showed the percentages of cells migrated to bottom wells in the absence of an anti-huMETCAM/MUC18 antibody and empty columns that in the presence of the antibody.

Over-expression of HuMETCAM/MUC18 altered the growth in 3D basement membrane assay

Similar to NPC-TW01 cell line, we had difficulties to demonstrate formation of anchorage-independent colony in soft agar (*in-vitro* tumorigenesis) from both clones of NPC-TW04 cell line. Alternatively, we could demonstrate a more aggressive growth of the clone #76, which formed tubular-like growths in the 3D basement membrane culture assay than the vector control clone V5, which formed spheroid growths, as shown in Figure 13. The aggressive growth of #76 could somewhat be reversed in the presence of anti-METCAM/MUC18 antibodies, as also shown in Figure 13. Taken together, we conclude that over-expression of METCAM/MUC18 could promote a more aggressive growth of NPC-TW04 cells in the 3D basement membrane culture assay and it was due to the direct effect of METCAM/MUC18.

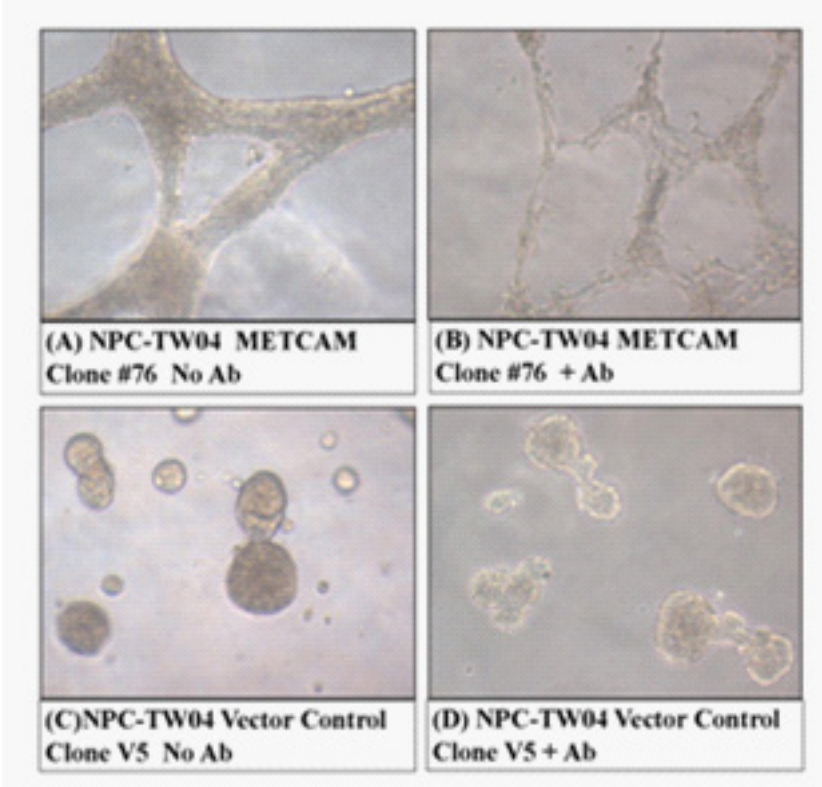

Figure 13: Effects of HuMETCAM/MUC18 expression on the growth of NPC-TW04 cells in 3D basement membrane culture assay in the presence or absence of an anti-huMETCAM/MUC18 antibody. The method of 3D basement membrane culture assay was used [39].

Over expression of HuMETCAM/MUC18 increases the tumorigenesis

Similar to the *in-vivo* tumorigenesis test was carried out for the NPC-TW04 clones. As shown in Figure 14, METCAM/MUC18 expression promoted tumorigenesis of NPC-TW-04 at a low number ($1x10^5$ or $2x10^6$) of cells [40], but only statistically insignificantly slightly promoted tumorigenesis of NPC-TW04 at a high cell number (4 x106).

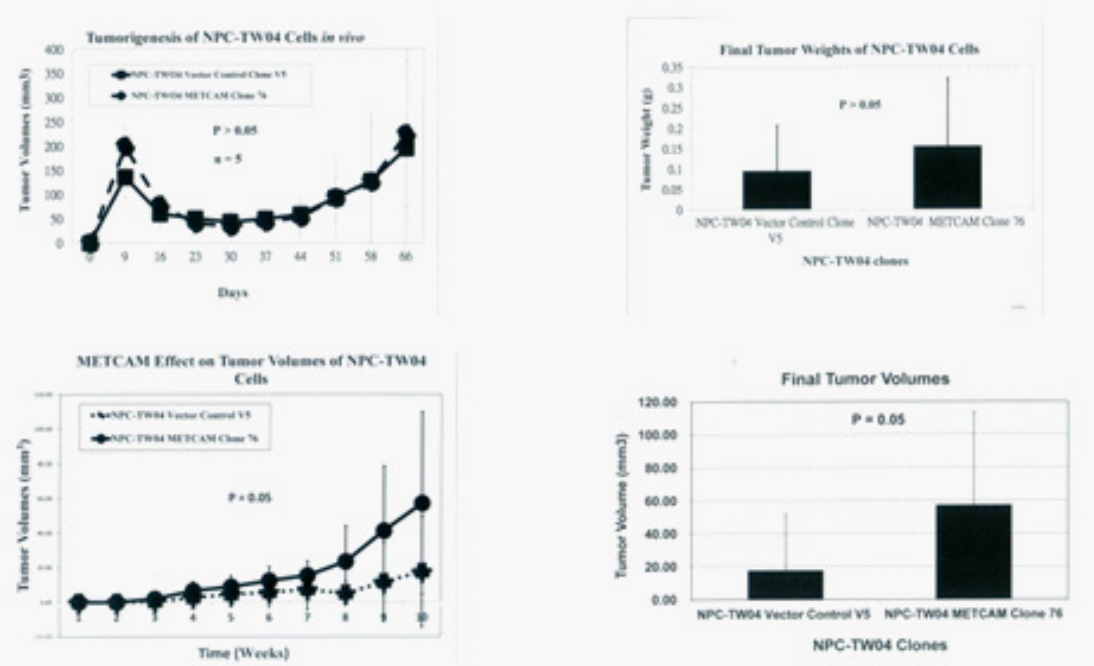

Figure 14: Effects of HuMETCAM/MUC18 expression on the tumorigenesis of NPC-TW01 cells in athymic nude mice. The method of SC injection of cells (4 x 10^6 cells/mouse in the top figures and 1.5 x 10^5 cells/mouse in the bottom figures) with matrigel in Balb/C athymic nude mice was used to determine effects of huMETCAM/MUC18 over-expression on tumorigenesis of NPC-TW04 cell line. The tumors were weekly measured by an electronic caliper. Tumor volume versus time was plotted. At the end of the experiment, tumors were excised and weighed.

MECHANISMS OF HUMETCAM/MUC18-INDUCED TUMOR PROMOTION

To understand the mechanism by which METCAM/MUC18 expression affects tumorigenesis of NPC cells, we determined the expression of levels of Bcl2, Bax, PARP, PCNA, the ratio of phospho-AKT/AKT, LDH-A, VEGF, and VEGFR2 in tumor lysates. Figure 15 shows that BCl2, Bax, PCNA ratio of phospho-AKT/AKT, LDH-A, VEGF and VEGFR2 were increased in tumor lysates of clone #76 in comparison with those in the vector clone V5. But no difference was found for Bcl2. Taken together, we concluded that enforced expression of METCAM/MUC18 promoted tumorigenesis of NPC-TW-04 cells by increased anti-apoptosis index (Bcl2), a proliferation index (PCNA), a signal for survival pathway (ratio of phospho-AKT/AKT), aerobic glycolysis (LDH-A), and angiogenesis indexes (VEGF and VEGFR2).

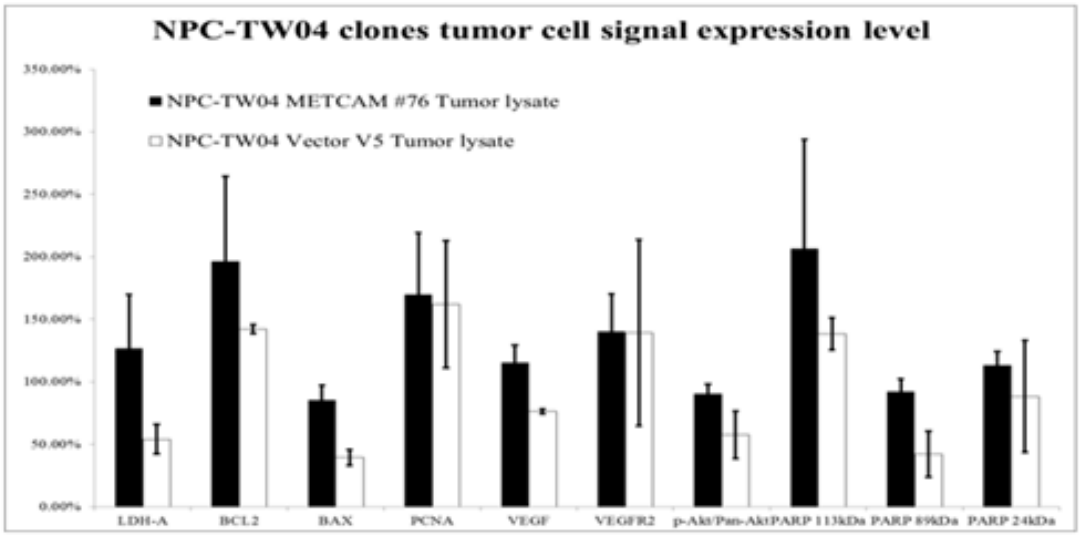

Figure 15: Expression levels of various key effectors down-stream of HuMETCAM/MUC18 in tumors. Western blot was used to determine the expression levels of various down-stream effectors in tumor lysates. Relative levels expressed in tumors induced by clones #76 and V5 are plotted.

REVERSAL EFFECT OF SHRNA ON THE ROLE OF METCAM/MUC18 IN NPC-TW04 CELLS [Wu et al. unpublished results]

The effect of shRNA on reversal the effects of METCAM/MUC18 on the ability of NPC-TW-04 cells in *in-vitro* and *in-vivo* tumorigenesis and metastasis in athymic nude mic was tested [30].

The four shRNA knock down clones of NPC-TW04 were obtained, similar to NPC-TW01 cells. The efficiency of knockdown expression of NPC-TW04 shRNA clones was also determined by Western blot. As shown in Figure 16 and summarized in Table 3, three different shRNAs had different efficiencies in knocking down the expression of METCAM/MUC18 in comparison with the control shRNA, #46 and knock down expression of METCAM/MUC18 was most efficient by the shRNA #27.

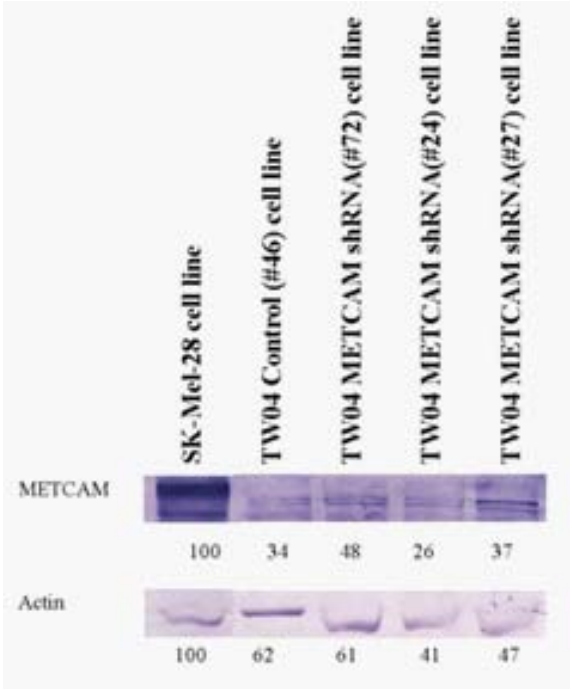

Figure 16: Expression levels of HuMETCAM/MUC18 in NPC-TW04 clones after transfection with pGIPZ with shRNA -coding genes. Western blot was used to determine the expression levels of HuMETCAM/MUC18 in each cell line/clones. Lane 1 shows the expression level of the protein in human melanoma cell line, SK-Mel-28, as a positive control (100%). Lanes 2-5 show the expression levels of the protein in the four clones. Actin was the loading control.

Table 3: METCAM/MUC18 expression in each of the shRNA knock-down of NPC-TW04 clones/ cell lines.

ShRNA knockdown clones	METCAM/MUC18 expression level	Number of mice injected
TW04-shRNA #46 (p66->p67)	100%	5 mice
TW04-shRNA #72 (p66->p67)	89%	5 mice
TW04-shRNA #24 (p66->p67)	68%	5 mice
TW04-shRNA #27 (p66->p67)	21%	5 mice

Similar to NPC-TW01 clones, the tumorigenesis of four NPC-TW04 shRNA knockdown clones was investigated after SC injection of these cells in athymic nude mice. As shown in Figure 17, shRNAs #27 was more effective than shRNA #72 and #24 in reversal of the tumor-augmentation effects of METCAM/MUC18-overexpression on NPC-TW04 cells.

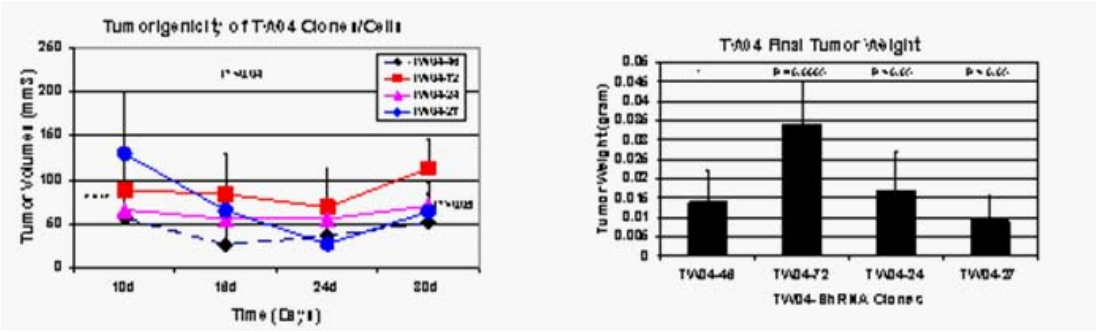

Figure 17: Tumorigenesis and final tumor weights of tumors induced by four NPC-TW04 knockdown clones were determined similar to NPC-TW01 clones. 2 x 10^6 cells were SC injected in each mouse. After 30 days mice were euthanatized and tumors were excised and weighed. Taken together, we conclude that shRNAs had some effects on reversing the effect of METCAM/MUC18. However, similar to NPC-TW01 cells the residual low level expression of METCAM/MUC18 may make the results not as clear-cut as predicted.

METCAM/MUC18 EXPRESSION IN NPC CELL LINES ALTERS THEIR RADIO-SENSITIVITY [Wu et al. unpublished results]

In clinical practice, Radiation Therapy (RT) is the main treatment modality for NPC. The major reasons include the surgically unapproachable anatomy and the favorable sensitivity to RT. However, once the tumor clones develop radiation resistance, the treatment outcome declines from fair to poor prognosis. Yet, the intrinsic factors modulating radio-sensitivity of NPC cells remain unclear. Since METCAM/MUC18 may suppress tumorigenesis of NPC, we tested the possibility that its enforced or knock-down expression may render NPC more sensitive to RT [41] (Figure 18). We tested if METCAM/MUC18 expression might alter the intrinsic radio-sensitivity of tumors induced by these clones. The following figures show that increased expression of HuMETCAM/MUC18 in both NPC-TW01 and NPC-TW04 cell lines increased the radio-sensitivity of tumors induced by both NPC-TW01 clone #92 and NPC-TW04-clone #76, when in comparison to that by the vector-control clones (NPC-TW01 clone V1 and MPC-TW04 clone V5, respectively).

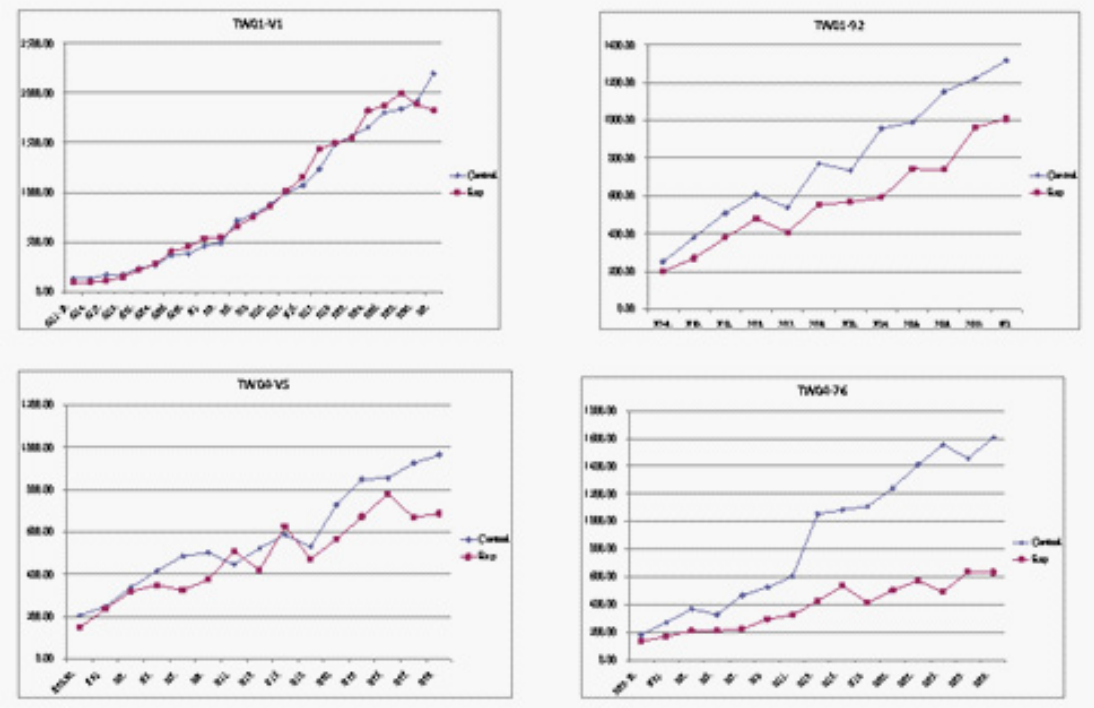

Figure 18: Radiation treatment of tumors induced by NPC-TW01 clone #92 and V1 (top figures) and NPC-TW04 clone #76 and V5 (bottom figures). Tumorigenesis and final tumor weights of tumors induced by knockdown clones of both NPC-TW01 and NPC-TW04 were determined in athymic nude mice.

PERSPECTIVES AND POSSIBLE CLINICAL APPLICATIONS

The results of this research allowed us to further understanding the possible role of METCAM/MUC18 in modulating the development of NPC. Interestingly, METCAM/MUC18 plays a dual role in the tumorigenesis of NPC, with a suppressor role in type I and with a promoter role in type II NPC. Our results suggest that METCAM/MUC18 may also be a therapeutic target for the treatment of clinical NPC by using peptides derived from METCAM/MUC18 to block the tumor angiogenesis and hematogenous metastasis. Ectopically increasing the expression of METCAM/MUC18 in NPC tumors may be used for enhancing the sensitivity of NPC to radiation therapy and thus the possible translational application to clinical treatment of NPC.

ACKNOWLEDGEMENT

This work was supported by research grants from Georgia Cancer Coalition, USA, National Science Council (NSC101-2320-B-033-001 & 003), Taiwan, and the McKay Memorial Hospital-Chung Yuan Inter-campus collaborative fund (MMH-CY-10102&10202), Taiwan.

References

1. Raab-Traub N. Epstein-Barr virus and nasopharyngeal carcinoma. Semin Cancer Biol. 1992; 3: 297-307.
2. Shanmugaratnam K, Sobin LH. "The World Health Organization histological classification of tumors of the upper respiratory tract and ear. A commentary on the second edition." Cancer. 1993; 71: 2689-2697.
3. De the' G. Epidemiology of Epstein-Barr virus and associated diseases. In: Roizman B, editor. The Herpesviruses. New York. Plenum Press. 1982; 1: 25-87.
4. Wei WI, Sham JS. Nasopharyngeal carcinoma. Lancet. 2005; 365: 2041-2054.
5. Lee AW, Ng WT, Chan YH, Sze H, Chan C, Lam TH. The battle against nasopharyngeal cancer. Radiother Oncol. 2012; 104: 272-278.
6. Ho JHC. Current knowledge of the epidemiology of nasopharyngeal carcinoma (NPC). In: Biggs P, de the' G, Payne L, editors. Oncogenesis and Herpesviruses. Lyon: IARC; 1972; 357-366.
7. Cavallaro U, Christofori G. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. Nat Rev Cancer. 2004; 4: 118-132.
8. Yu Y, Dong W, Zhou X, Li S. The significance of serum soluble intercellular adhesion molecule 1 and transforming growth factor alpha in patients with nasopharyngeal carcinoma. Arch Otolaryngol Head Neck Surg. 2004; 130: 1205-1208.
9. Huang GW, Mo WN, Kuang GQ, Nong HT, Wei MY, et al. Expression of p16, nm23-H1, E-cadherin, and CD44 gene products and their significance in nasopharyngeal carcinoma. Laryngoscope. 2001; 111: 1465-1471.
10. Li Z, Ren Y, Lin SX, Liang YJ, Liang HZ. Association of E-cadherin and beta-catenin with metastasis in nasopharyngeal carcinoma. Chin Med J (Engl). 2004; 117: 1232-1239.
11. Yi ZC, Wang H, Zhang GY, Xia B. Downregulation of connexin 43 in nasopharyngeal carcinoma cells is related to promoter methylation. Oral Oncol. 2007; 43: 898-904.
12. Lehmann JM, Riethmüller G, Johnson JP. MUC18, a marker of tumor progression in human melanoma, shows sequence similarity to the neural cell adhesion molecules of the immunoglobulin superfamily. Proc Natl Acad Sci USA. 1989; 86: 9891-9895.
13. Wu GJ. METCAM/MUC18 expression and cancer metastasis. Curr Genomics. 2005; 6: 333-349.
14. Shih IM. The role of CD146 (Mel-CAM) in biology and pathology. J Pathol. 1999; 189: 4-11.
15. Shih LM, Hsu MY, Palazzo JP, Herlyn M. The cell-cell adhesion receptor Mel-CAM acts as a tumor suppressor in breast carcinoma. Am J Pathol. 1997; 151: 745-751.
16. Wu GJ, Dickerson EB. Frequent and increased expression of human METCAM/MUC18 in cancer tissues and metastatic lesions associates with the clinical progression of human ovarian carcinoma. Taiwanese J of Obstetrics and Gynecology. 2014; 53: 509-517.
17. Lin JC, Chiang CF, Wang SW, Wang WY, Kwan PC, Wu GJ. Significance of expression of human MECAM/MUC18 in nasopharyngeal carcinomas and metastatic lesions. Asian Pacific J Cancer Prevention. 2014; 15: 245-252.
18. Wu GJ, Wu MW, Wang C, Liu Y. Enforced expression of METCAM/MUC18 increases tumorigenesis of human prostate cancer LNCaP cells in nude mice. J Urol. 2011; 185: 1504-1512.
19. Zeng GF, Cai SX, Wu GJ. Up-regulation of METCAM/MUC18 promotes motility, invasion, and tumorigenesis of human breast cancer cells. BMC Cancer. 2011; 11: 113.
20. Zeng G, Cai S, Liu Y, Wu GJ. METCAM/MUC18 augments migration, invasion, and tumorigenicity of human breast cancer SK-BR-3 cells. Gene. 2012; 492: 229-238.
21. Wu GJ, Fu P, Wang SW, Wu MW. Enforced expression of MCAM/MUC18 increases in vitro motility and invasiveness and *in vivo* metastasis of two mouse melanoma K1735 sublines in a syngeneic mouse model. Mol Cancer Res. 2008; 6: 1666-1677.
22. Wu GJ, Peng Q, Fu P, Wang SW, Chiang CF, et al. Ectopical expression of human MUC18 increases metastasis of human prostate cancer cells. Gene. 2004; 327: 201-213.
23. Zeng Q, Li W, Lu D, Wu Z, Duan H, et al. CD146, an epithelial-mesenchymal transition inducer, is associated with triple-negative breast cancer. Proc Natl Acad Sci USA. 2012; 109: 1127-1132.
24. Lung HL, Cheng Y, Kumaran MK, Liu ET, Murakami Y, et al. Fine mapping of the 11q22-23 tumor suppressive region and involvement of TSLC1 in nasopharyngeal carcinoma. Int J Cancer. 2004; 112: 628-635.
25. Lung HL, Bangarusamy DK, Xie D, Cheung AK, Cheng Y, et al. THY1 is a candidate tumor suppressor gene with decreased expression in metastatic nasopharyngeal carcinoma. Oncogene. 2005; 24: 6525-6532.
26. Wu GJ. Dual Roles of METCAM in the Progression of Different Cancers. J Oncol. 2012; 2012: 853797.

27. Xie S, Luca M, Huang S, Gutman M, Reich R, et al. Expression of MCAM/MUC18 by human melanoma cells leads to increased tumor growth and metastasis. Cancer Res. 1997; 57: 2295-2303.

28. Schlagbauer-Wad H, Jansen B, Müller M, Polterauer P, Wolff K, et al. Influence of MUC18/MCAM/CD146 expression on human melanoma growth and metastasis in SCID mice. Int J Cancer. 1999; 81: 951-955.

29. Wu GJ, Fu P, Chiang CF, Huss WJ, Greenberg NM, et al. Increased expression of MUC18 correlates with the metastatic progression of mouse prostate adenocarcinoma in the TRAMP model. J Urology. 2005; 173: 1778- 1783.

30. Wu GJ. Human METCAM/MUC18 as a novel biomarker to drive and its specific SiRNAs to block the malignant progression of prostate cancer. J Cell Sci Therapy. 2015; 6.

31. McGary EC, Heimberger A, Mills L, Weber K, Thomas GW, et al. A fully human antimelanoma cellular adhesion molecule/MUC18 antibody inhibits spontaneous pulmonary metastasis of osteosarcoma cells in vivo. Clin Cancer Res. 2003; 9: 6560-6566.

32. Li Q, Yu Y, Bischoff J, Mulliken JB, Olsen BR. Differential expression of CD146 in tissues and endothelial cells derived from infantile haemangioma and normal human skin. J Pathology. 2003; 201: 296-302.

33. Wu GJ. Over-expression of METCAM/MUC18 suppresses tumorigenesis and metastasis of mouse melanoma K1735-9 cells in syngeneic mice. 2016.

34. Wu GJ, Zeng GF. METCAM/MUC18 is a novel tumor and metastasis suppressor for the human ovarian cancer SKOV3 cells. BMC Cancer. 2016; 16: 136.

35. Liu YC. The putative role of human METCAM/MUC18 in modulating the development and progression of nasopharyngeal carcinoma. 2014.

36. Lin CT, Wong CI, Chan WY, Tzung KW, Ho JK, et al. Establishment and characterization of two nasopharyngeal carcinoma cell lines. Lab Invest. 1990; 62: 713-724.

37. Lin CT, Chan WY, Chen W, Huang HM, Wu HC, et al. Characterization of seven newly established nasopharyngeal carcinoma cell lines. Lab Invest. 1993; 68: 716-727.

38. Wu GJ. The role of MUC18 in prostate carcinoma. In: Hayat MA, editor. Immunohistochemistry and in situ hybridization of human carcinoma. Molecular pathology, lung carcinoma, breast carcinoma, and prostate carcinoma. Amsterdam. Elsevier Science/ Academic Press. 2004; 2: 347-358.

39. Lee GY, Kenny PA, Lee EH, Bissell MJ. Three-dimensional culture models of normal and malignant breast epithelial cells. Nat Methods. 2007; 4: 359-365.

40. Huang CY, Wu GJ. Mechanism of METCAM/MUC18-promoted progression of human breast cancer cells: METCAM/MUC18 promoted tumorigenesis of human breast cancer SK-BR-3 cells in a dosage-dependent manner. Taiwanese J Obstetrics & Gynecology. 2016; 55.

41. Chen YJ, Liao HF, Tsai TH, Wang SY, Shiao MS. Caffeic acid phenethyl ester preferentially sensitizes CT26 colorectal adenocarcinoma to ionizing radiation without affecting bone marrow radio response. Int J Radiat Oncol Biol Phys. 2005; 63: 1252-1261.