Published in final edited form as:
Laryngoscope. 2011 January ; 121(1): 167–170. doi:10.1002/lary.21164.

Expression of Mucin (*MUC*) Genes in Mucoepidermoid Carcinoma

Nima Shemirani, MD[2], **Vladimir Osipov, MD**[3], **Alex Kolker, PhD**[3], **Pawjai Khampang, MS**[2], and **Joseph E Kerschner, MD**[1,2]

[1]Division of Pediatric Otolaryngology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

[2]Department of Otolaryngology and Communication Sciences, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

[3]Department of Pathology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

Introduction

Salivary gland carcinomas make up approximately 3% of all head and neck carcinoma[1]. The most common subtype is mucoepidermoid carcinoma (MEC). MEC consists of both epithelial and mucin type cells, and is further characterized by histopathologic examination. It can be classified as low, intermediate and high grade based on cytologic features, invasion patterns and cellular type. Low-grade tumors typically have more mucin containing cells and those of high grade tend to have more epithelial cells[1,2]. Also, criteria such as mitotic figures per high power field, presence of anaplasia and perineural invasion are utilized in determining the grade of MEC[1,2]. Prior studies have examined factors associated with clinical course in MEC. Overall decreased 5-year survival has been linked to: 1) age greater than 40 years 2) T stage of T3 or T4 3) presence of metastatic lymph node disease and 4) high tumor grade[3]. Furthermore, when analyzed independently, age over 40 (RR=3.8) and T3/T4 (RR=3.1) has slightly worse prognoses than those of high tumor grade (RR=2.6). Additional studies have also linked decreased survival to more severe disease as staged clinically[2]. More recently, mucin gene expression has been investigated in MEC and linked to prognosis. Immunohistochemistry (IHC) has been utilized to assess expression of mucin gene 1 (*MUC1*) protein and the presence of *MUC1* expression in MEC was associated with decreased progression-free survival compared to *MUC1* negative tumors[4]. The differences in these tumor characteristics were relatively dramatic with none of the patients with *MUC1* negative tumors experiencing recurrence or death at five years. Utilizing another technique of IHC, the association of greater expression of *MUC1* with a higher tumor grade, lower disease free survival, and higher rate of recurrence and metastasis was confirmed[4]. Presence of mucin gene 4 (*MUC 4*) protein was inversely proportional to the presence of *MUC1* and

Corresponding Author: Joseph E. Kerschner, M.D., Children's Hospital of Wisconsin, 9000 W. Wisconsin Avenue, Milwaukee, Wisconsin 53226, Phone: (414) 266 6476, Fax: (414) 266 2693, kersch@mcw.edu.

Portions of this manuscript presented at the Triological Society Middle Section Meeting, January 18th, 2008.

The authors have no other funding, financial relationships, or conflicts of interest to disclose.

patients with MEC and a higher *MUC4* expression had a more favorable prognosis[5]. These previous studies looking at mucin genes and MEC prognosis also investigated other the glycoprotein products of other mucin genes utilizing IHC; including mucin gene 2 (*MUC2*) mucin gene 5AC (*MUC5AC*) mucin gene 6 (*MUC6*) and mucin gene 7 (*MUC7*). Molecular techniques using quantitative real time PCR provide a different and quantitative means of further assessing the relationship between mucin gene expression and mucoepidermoid salivary gland carcinoma.

This current study also sought to evaluate MEC for expression of a number of more recently identified mucin genes not previously examined in MEC including: mucin gene 12 (*MUC12*), mucin gene 13 (*MUC13*), mucin gene 17 (*MUC17*), mucin gene 18 (*MUC18*) and mucin gene 19 (*MUC19*) and to see if their expression could be correlated to tumor characteristics or clinical behavior. Quantitative reverse-transcriptase polymerase chain reaction (qPCR) techniques were also utilized to assess these mucin genes. Molecular techniques such as qPCR provide a quantitative measure to assess gene expression and also a different means of assessing tumor biology compared with IHC. Utilization of this technique to assess a series of patients with MEC examining the broad spectrum of mucin genes was the primary aim of this investigation.

Methods

Approval for this study was obtained from the Medical College of Wisconsin (MCW) Institutional Review Board. A retrospective chart review was performed utilizing the clinical database in the Department of Pathology at the MCW over a ten-year period (1996 – 2006) to identify all patients with a diagnosis of mucoepidermoid. Patients were excluded if a sample of their tumor was not available for analysis. Clinical information including tumor grade, stage at presentation, location of tumor, length of follow-up, death, presence of metastasis and recurrence were recorded on a Microsoft Excel worksheet.

All pathologic slides were reviewed by a single pathologist (VO), and the diagnosis of MEC and tumor grade were confirmed. Areas of tumor and normal surrounding salivary gland were noted on the slide. The corresponding areas were also demarcated on the paraffin embedded tissue blocks. Tissue was then isolated utilizing a 2-mm punch biopsy in both tumor and normal tissue from the paraffin embedded blocks. In 4 specimens, the tumor tissue sample available for analysis in embedded paraffin block did not contain any meaningful normal salivary gland tissue adjacent to the MEC. In 1 of these 4 patients only a small normal specimen could be obtained and this was utilized for qPCR. In the remaining 3 patients no normal tissue could be identified for utilization in the comparative studies. RNA was isolated from each of the tissues using a commercial kit (Qiagen FFPE, Alameda California). qPCR was performed utilizing standard techniques previously published in our laboratory[6]. However, briefly cDNA was created from the isolated RNA utilizing reverse transcriptase. Subsequently, for PCR, a 20 μL sample was mixed with Taqman Platinum Blue Mastermix (Invitrogen, Carlsbad, CA). PCR reactions were performed with 18.6 μL of the mix, 0.2 μL of each forward and reverse primer and 1 μL of RT+ cDNA, RT- cDNA, or water as a control. The samples underwent PCR for a total of forty cycles.

For analysis of *MUC12, MUC13, MUC17, MUC18* and MUC*19* primers previously created in our laboratory were utilized. These primers were used in control specimens that were known to express these MUC genes (small intestine and trachea). The primers for these genes, and housekeeping gene, β-actin are displayed in Table 1. Tissue samples underwent PCR and were consequently run on a 2% agarose gel with GelStar (Cambrex Rockland, ME) and a 50bp ladder. The bands were then isolated and sequenced to confirm specificity for the mucin gene being investigated. Mucin genes were compared to the housekeeping gene, β-actin, whose expression has been shown in a previous study to be unaffected in mucoepidermoid carcinoma[7]. β-actin was utilized to ensure the integrity of the samples and as a comparative value as the differential expression of mucin genes was assessed between tumor and normal tissue.

In performing qPCR, cDNA samples were combined with Ambion TaqMan Master Mix™ and commercial primers (Applied Biosystems (Foster City, CA) with bound fluorophores utilizing the iCycler® PCR machine. The emitted fluorescence from both tumor and normal tissue was compared with the emittance from the housekeeping gene. The difference in expression between the two samples was calculated as a fold difference. The critical threshold (Ct) of the mucin gene of interest (GOI) was compared to the housekeeping gene, β-actin utilizing the formula ΔC=CtGOI-Ctβ-actin. The fold difference between tumor and normal tissue was $2^{-\Delta C}$ for the tumor tissue divided by $2^{-\Delta C}$ for the normal tissue as outlined in the manufacturer's guidelines[8]. Fischer exact test was used to determine statistical significance. This portion of the experiment provides a quantitative level of expression, which may not be visible on qualitative PCR, therefore there were 2 more normal specimens that were analyzed compared to qualitative PCR.

Results

RT-PCR mucin gene expression

There were 23 tumors, of which, 19 also had enough normal salivary gland tissue available for analysis of both mucin gene expression and quantitative analysis. Table 2 demonstrates the clinical stage, location of tumor, and grade of tumor. The majority of patients (57%) in this cohort presented with stage IV disease. Sixty-one percent of samples were located in major salivary glands (parotid and submandibular) and 39% presented in minor salivary glands. Finally, there was preponderance for high-grade tumors at 44%.

RT-PCR performed on these specimens revealed that 65% (15/23) of tumors expressed *MUC 19*, whereas only 26% (5/19) of the adjacent normal salivary gland tissue expressed *MUC 19* (p=0.02). Additionally, 13% (3/23) tumors expressed *MUC 13*, whereas no expression was found in normal salivary gland tissue (Table 3). *MUC 12* and *MUC 17* were not expressed in either tissue samples and *MUC 18* was expressed with equal frequency. No conclusions could be made regarding MUC gene expression and clinical survival or presence of metastasis. Normal salivary gland tissue was available for only one of four patients with either death or metastasis therefore meaningful statistical comparison for these clinical outcomes was not possible.

qPRC

There were 21 pairs of normal and tumor tissue available from this portion of the study. This portion of the experiment provided a quantitative level of expression, which may have not been visible on qualitative PCR, therefore there were 2 more normal specimens which were analyzed compared to qualitative PCR. Figure 1 demonstrates the MEC clinical stage compared to expression of *MUC1* and *MUC4* utilizing qPCR. *MUC1* was expressed 4.2 fold higher for stage I disease compared to stage IV disease (p=0.008). *MUC4* was expressed 21 fold higher in stage I disease compared to stage IV (p=0.05). There was no difference in expression of *MUC18* between normal and tumor tissue. *MUC19* was only expressed in 33% (7/21) of normal tissue specimens, making statistical analysis impractical due to the large majority of patient samples not having a normal comparative value.

Discussion

There currently exist nineteen unique human mucin genes that have been identified. Much of the work examining the association of mucin genes and carcinomas has been done with malignancies of the breast, lungs, prostate and skin (melanoma) [9,10,11,12]. There exists very little data on mucin gene expression and head and neck cancer. The previous studies examining mucin gene expression and MEC of the salivary glands utilized IHC to assess mucin gene expression and focused on the membrane-bound mucins of *MUC1* and *MUC4*. These studies did also examine a variety of other mucin genes known at the time but were limited in that a variety of mucin genes had not yet been characterized at the time of their completion. In a study by Handra-Luca and colleagues the expression of *MUC4* was correlated with a better histologic grade and improved prognosis. This study found no significance with *MUC1* expression and prognosis. Finally, they concluded that expression of *MU5AC* was found more often in tumor tissue compared to normal tissue. In a second study, by Alos and colleagues, expression of *MUC1* in greater than 50% of tumor cells was associated with higher histologic grades, recurrence rates, metastasis rates, and shorter disease-free survival[5]. The results from Alos confirmed that increased *MUC4* was protective in MEC. These patients demonstrated longer disease free survival, lower grades, and lower recurrence rates. In this current study, qPCR results were different than those described by Alos and Handra-Luca with respect to *MUC1*. Patients with a greater expression of *MUC1* compared with normal salivary tissue actually demonstrated a lower clinical staging which would be expected to correlate with less aggressive disease and increased survival. Similar to the previous 2 studies, *MUC4* expression did demonstrate a protective effect in our patient series. Greater *MUC4* expression in relation to normal gland tissue was associated with a lower clinical stage, as well as likely less aggressive behavior. No conclusions could be made regarding survival and metastasis as there were not enough normal salivary gland tissue samples from the aggressive and fatal forms of MEC in our patient population.

Of the more recently identified mucin genes investigated in this study, MUC 19 was found more often in tumor tissue when compared to normal. This may serve as a marker for carcinoma, an indicator of prognosis, and a possible immune target for future chemotherapy. There are scant data regarding *MUC19* and this study may aid in future developments for cancer research. *MUC13* was found in 13% of our tumor tissue population and in none of

the normal salivary gland tissue. This finding could also be utilized in future pathologic studies but requires additional confirmation from additional patient specimens. *MUC18* has been found to be expressed in more aggressive melanomas and prostate carcinomas [9,10,12]. However, *MUC18* was not expressed in a higher percentage of tumor specimens and was not upregulated in comparing tumor tissue to normal salivary gland tissue. This finding suggests that *MUC18* over-expression may be specific in certain carcinomas but does not appear play a significant role in MEC.

This investigation further solidifies the utility of mucin gene assessment in patients with certain malignancies. With respect to MEC, *MUC4* analysis appears to be a particularly useful gene marker to incorporate into routine tissue assessment. Given the routine ability to perform qPCR in clinical laboratories adding this assessment to patients with MEC is feasible and broader, prospective studies with long-term clinical follow-up appear warranted based on the results of this study and others. The utility of qPCR in assessing these patients is particularly interesting given that it allows for quantitative reporting of results. This will allow for objective measurements and comparison with clinical staging and outcomes. This may allow for utilization of this gene expression to guide clinical decision making pathways including adjunctive therapy and the role of lymph node assessment. With respect to *MUC1* and the conflicting results now reported in several studies it appears that more investigation is needed. In particular, it would be helpful to study *MUC1* in a prospective fashion to ensure that tissue is selected as far distant from the malignancy as possible. This would be true of the investigations with respect to *MUC 19* expression as well. The primary rationale for conducting these studies is that PCR is an extremely sensitive test of the molecular activity of cells. There is a possibility that, although histologically normal in appearance, that the “normal” salivary gland tissue surrounding the MEC has already undergone some changes in its gene expression profile.

Conclusions

Utilization of specific genetic markers in the assessment of malignancies has become increasingly common. Development of quantitative molecular tools such as qPRC allow for sensitive and quantitative measures of gene expression. In the case of MEC, it appears that qPCR measurements of *MUC 4* expression can be utilized to predict favorable prognosis and *MUC 1* and *MUC 19* to predict less favorable prognosis. Further research in these areas is certainly warranted.

Acknowledgments

This work was funded by the National Institutes of Health grants NIDCD: DC007903 (JEK) and the Department of Otolaryngology and Communication Sciences, Medical College of Wisconsin.

References

1. Bailey, BJ.; Healy, GB.; Johnson, JT., et al. Head and Neck Surgery-Otolaryngology: Salivary Gland Neoplasms. Philadelphia, PA: Lippincott Williams & Wilkins; 2006. p. 230-232.
2. Boahene DK, Olsen KD, Lewis JE, Pinheiro AD, PAnkratz VS, Bagniewski SM. Mucoepidermoid carcinoma of the parotid gland: the Mayo clinic experience. Arch Otolaryngol Head Neck Surg. 2004 Jul; 130(7):849–56. [PubMed: 15262762]

3. Pires FR, de Almeida OP, de Araújo VC, Kowalski LP. Prognostic factors in head and neck mucoepidermoid carcinoma. Arch Otolaryngol Head Neck Surg. 2004 Feb; 130(2):174–80. [PubMed: 14967746]
4. Handra-Luca A, Lamas G, Bertrand JC, Fouret P. MUC1, MUC2, MUC4, and MUC5AC expression in salivary gland mucoepidermoid carcinoma: diagnostic and prognostic implications. Am J Surg Pathol. 2005 Jul; 29(7):881–9. [PubMed: 15958852]
5. Alos L, Lujan B, Castillo M, Nadal A, Carreras M, Caballero M, de Bolos C, Cardesa A. Expression of membrane-bound mucins (MUC1 and MUC4) and secreted mucins (MUC2, MUC5AC, MUC5B, MUC6, and MUC7) in mucoepidermoid carcinomas of salivary glands. Am J Surg Pathol. 2005 Jun; 29(6):806–13. [PubMed: 15897748]
6. Kerschner JE, Meyer TK, Burrows A. Chinchilla middle ear epithelial mucin gene expression in response to inflammatory cytokines. Arch Otolaryngol Head Neck Surg. 2004 Oct; 130(10):1163–7. [PubMed: 15492162]
7. Nagel H, Laskawi R, Eiffert H, Schlott T. Analysis of the tumour suppressor genes, FHIT and WT-1, and the tumour rejection genes, BAGE, GAGE-1/2, HAGE, MAGE-1, and MAGE-3, in benign and malignant neoplasms of the salivary glands. Mol Pathol. 2003 Aug; 56(4):226–31. [PubMed: 12890744]
8. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec; 25(4):402–8. [PubMed: 11846609]
9. McGary EC, Heimberger A, Mills L, Weber K, Thomas GW, Shtivelband M, Lev DC, Bar-Eli M. A fully human antimelanoma cellular adhesion molecule/MUC18 antibody inhibits spontaneous pulmonary metastasis of osteosarcoma cells in vivo. Clin Cancer Res. 2003 Dec 15; 9(17):6560–6. [PubMed: 14695161]
10. Wu GJ, Peng Q, Fu P, Wang SW, Chiang CF, Dillehay DL, Wu MW. Ectopical expression of human MUC18 increases metastasis of human prostate cancer cells. Gene. 2004 Mar 3; 327(2): 201–13. [PubMed: 14980717]
11. Chen Y, Zhao YH, Kalaslavadi TB, Hamati E, Nehrke K, Le AD, Ann DK, Wu R. Genome-wide search and identification of a novel gel-forming mucin MUC19/Muc19 in glandular tissues. Am J Respir Cell Mol Biol. 2004 Feb; 30(2):155–65. Epub 2003 Jul 25. [PubMed: 12882755]
12. Ostmeier H, Fuchs B, Otto F, Mawick R, Lippold A, Krieg V, Suter L. Prognostic immunohistochemical markers of primary human melanomas. Br J Dermatol. 2001 Aug; 145(2): 203–9. [PubMed: 11531780]

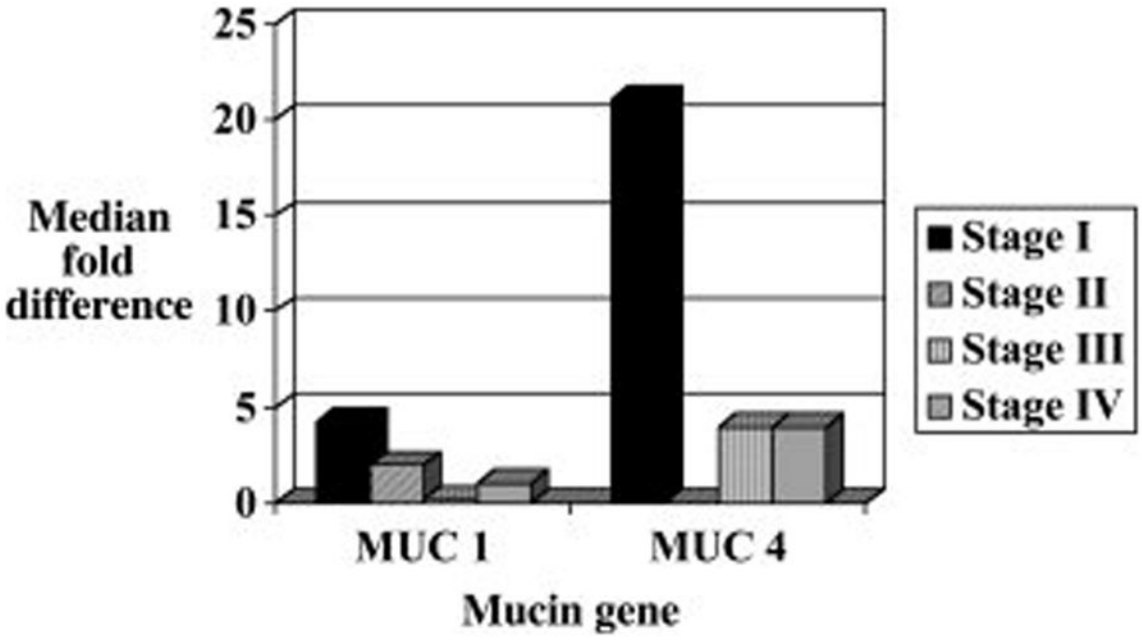

Figure 1.

TABLE I

Primer Pairs Utilized for PCR

Gene	Direction	Primer Sequence	Base Pair Length
MUC12	Forward (sense)	5’-TGTGTCTACTGAAAGCCTGG-3’	510
	Reverse (anti-sense)	5’-ACCTAAAGTGGCGTTGAGTT-3’	
MUC13	Forward (sense)	5’-ACAATGGTTCCTTCTGAAAC-3’	214
	Reverse (anti-sense)	5’-ACCCTTCTAAACACAGGCAA-3’	
MUC17	Forward (sense)	5’-CTCCTCTTGACACAAGCACA-3’	154
	Reverse (anti-sense)	5’-TCAGTGGAAGTTATCACAGG-3’	
MUC18	Forward (sense)	5’-GCCATGTCGACTGGTTTTCT-3’	241
	Reverse (anti-sense)	5’-TCCTCCGGAGCTTTGTAGAC-3’	
MUC19	Forward (sense)	5’-GAGTTCAGATGGCAAAATGCA-3’	144
	Reverse (anti-sense)	5’-TGCCATCAGGACAGTCAAGTA-3’	
β-actin	Forward (sense)	5’-CTACAATGAGCTGCGTGTGGC-3’	271
	Reverse (anti-sense)	5’-CAGGTCCAGACGCAGGATGGC-3’	

TABLE II

Tumor Stage, Grade and Location.

Stage at Presentation	Percentage of Patients (n=23)
I	4/23 (17%)
II	4/23 (17%)
III	2/23 (9%)
IV	13/23 (57%)
Location of tumor	
Major salivary gland	14/23 (61%)
Minor salivary gland	9/23 (39%)
Tumor grade	
Low	7/23 (30%)
Intermediate	6/23 (26%)
High	10/23 (44%)

TABLE III

Mucin Gene Expression in Tumor and Control Specimens

Gene	Tumore (n=23)	Normal (n=19)	p value
MUC12	0%	0%	NS
MUC13	1 (13%)	0 (0%)	p< 0.001
MUC17	0 (0%)	0 (0%)	NS
MUC18	18 (78%)	15 (79%)	NS
MUC19	15 (65%)	5 (26%)	p = 0.02