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Genomic Database Analysis for Head and Neck Cancer Prevention Targets: MTOR Signal Transduction Pathway

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Abstract. *Background: Type II diabetes agents have anticancer effects on head and neck squamous cell carcinoma (HNSCC). The mechanistic target of rapamycin (MTOR) pathway represents a putative target. Materials and Methods: We interrogated an Affymetrix HNSCC dataset for MTOR-related gene expression. Results: MTOR expression itself was unchanged, but various related genes demonstrated differential expression. Pathway promoters ras homolog (RHEB), MTOR-associated protein (MLST8), and ribosomal protein S6 kinase B1 (RPS6KB1) were up-regulated. Expression of growth suppressors tuberous sclerosis complex 2 (TSC2), programmed cell death 4 (PDCD4), and BCL2 apoptosis regulator-associated agonist of cell death (BAD) were reduced in HNSCC. Upstream, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), AKT serine/threonine kinase 1 (AKT1), and phosphatase and tensin homolog (PTEN) were up-regulated in cancer. Conclusion: Several MTOR pathway promoters and tumor suppressors were found to be differentially expressed, favoring MTOR pathway up-regulation in HNSCC. Genomic databases can be interrogated to identify intervention targets and endpoints in HNSCC trials.*

Squamous cell carcinoma of the head and neck (HNSCC) is a devastating disease which affects approximately 53,000 patients in the United States each year, and results in over 10,000 deaths annually (1). A better molecular understanding of head and neck carcinogenesis would allow for the development of improved chemotherapy and chemoprevention agents. Our Molecular Oncology Program investigates carcinogenesis mechanisms to identify agents appropriate for intercepting the development of invasive cancer.

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We have pursued head and neck cancer prevention by repurposing Food and Drug Administration-approved agents for type II diabetes, specifically pioglitazone and metformin, alone and in combination. Previous studies demonstrated at least a 35% reduction in both head and neck, and lung cancer incidence in diabetics treated with thiazolidinediones (2). Our group has demonstrated anticancer effects of these agents in preclinical studies as well as National Institutes of Health-sponsored head and neck chemoprevention trials. Metformin acts to antagonize mechanistic target of rapamycin (MTOR) pathway-associated head and neck carcinogenesis, and has demonstrated cancer prevention trends in patients with type II diabetes who are prescribed metformin (3). This effect may have utility in cancer chemoprevention.

MTOR is a cellular pathway involved in the interpretation of environmental cues and regulation of cell-cycle proliferation, growth, and metabolism, and may provide druggable targets for chemoprevention and cancer treatment in head and neck cancer. Previous work by Gutkind *et al.* uncovered up-regulation of the phosphoinositide 3-kinase (PI3-K), MTOR signaling pathway in HNSCC, and clinical trials of the MTOR inhibitor, rapamycin, yielded promising initial results (4). However, the emergence of acquired resistance to MTOR-specific targeting presents an opportunity to identify alternative *MTOR* pathway genes which may be viable pharmacotherapy targets.

Databases such as the Cancer Genome Atlas can be analyzed for the purpose of identifying treatment targets and disease biomarkers, based on mRNA expression in cancer specimens compared with controls. We examined the MTOR and associated signal transduction pathways in a cancer genomic database we developed using Affymetrix microarray data (5). We found we were indeed able to interrogate this database to identify expression levels of genes of interest in signal transduction pathways which appear important for chemoprevention. We conclude this type of analysis can be melded with other translational and basic science and epidemiology to have a better understanding of the pathophysiology of head and neck cancer for new agent development and treatment biomarkers.

Materials and Methods

Gene expression was compared between 41 HNSCC specimens and 13 mucosal controls using microarray analysis. Mucosal specimens were taken from healthy volunteers. Data were interrogated and analyzed from our previously published dataset (5), which contains all details of acquisition and analysis, human subject consent, experimental methods, and bioinformatic analytical techniques.

Expression of 19 genes of interest was analyzed in 41 HNSCC and 13 normal oral mucosal samples. *MTOR* and related pathway genes were identified in the original Affymetrix dataset and copied into a new Microsoft Excel spreadsheet (Microsoft Excel 2016; Microsoft Corporation, Redmond, WA, USA) to generate a specific database. A two-sided *t*-test was employed to compare HNSCC tumor gene expression to control gene expression for each expressed sequence tag (EST) of interest. Welch's correction was used, with a value of $p<0.05$ indicating significant difference. For genes with multiple ESTs, low-intensity ESTs were discarded if they were less than 20% expression of the maximum EST intensity for that gene. All statistical analysis was performed in Microsoft Excel 2016.

Table I. *Clinical features of patients with head and neck cancer (HNSCC) and normal controls (5).*

	HNSCC		Normal controls	
	n	%	n	%
Sample size	41		13	
Gender, n (%)				
Male	29	71%	6	46%
Age, years				
Median (range)	64		60	
Tobacco use[1], n (%)				
Yes	37	90%	5	38%
Alcohol use[2], n (%)				
Yes	14	32%	0	
Anatomical location, n (%)				
Oral cavity	18	44%		
Oropharynx	4	10%		
Hypopharynx	1	2%		
Larynx	15	37%		
Sinus	3	7%		
Clinical stage, n (%)				
Stage I-II	13	32%		
Stage III-IV	28	68%		
Tumor differentiation, n (%)				
Well	6	15%		
Moderate	20	48%		
Poor[3]	15	37%		

[1]Any past or present use. [2]≥4 Alcoholic drinks/day. [3]Including samples with mixed moderately/poorly differentiated histology.

Results

To assess the gene-expression patterns of genes involved in the *MTOR* cellular signaling pathway, we re-interrogated a microarray dataset of 41 patients with HNSCC, compared with 13 mucosal controls obtained from the buccal mucosa of healthy individuals. The clinical characteristics of the 41 patients and 13 controls are displayed in Table I.

Mean expression levels of *MTOR* and related pathway genes of interest were compared between the tumor tissue from patients with HNSCC and buccal mucosa tissue from controls (6). Table II displays the change in expression of each gene assessed and the associated *p*-value. Gene-expression analysis of *MTOR* itself did not demonstrate differential expression in HNSCC specimens when compared with the controls ($p=0.97$). Pathway promoter ras homolog (RHEB) demonstrated up-regulation in the HNSCC samples in two ESTs ($p<0.0001$). MTOR associated protein (MLST8) also demonstrated up-regulation in cancer ($p=0.045$). The pathway suppressor tuberous sclerosis 1 (TSC1) did not demonstrate differential expression in HNSCC, however tuberous sclerosis 2 (TSC2) expression was reduced in the HNSCC samples ($p=0.01$), suggesting a potential mechanism for indirect up-regulation of MTOR activity.

MTOR targets eukaryotic translation initiation factor 4B (EIF4B) and EIF4B-binding protein (EIF4EBP1) were analyzed. Both *EIF4B* and *EIF4BP* demonstrated no change in expression ($p>0.05$). MTOR target eukaryotic translation initiation factor 4E (EIF4E) was down-regulated in one ($p=0.04$) of two ESTs. The ribosomal protein S6 kinases (RPS6KB1 and RPS6KB2) are also targets of mTOR. When analyzed, *RPS6KB1* was up-regulated in one ($p=0.037$) of two ESTs and *RPS6KB2* demonstrated down-regulation in HNSCC ($p<0.0001$). Analysis of one of the targets of RPS6KB, programmed cell death 4 (*PDCD4*), yielded down-regulation ($p<0.0001$) in HNSCC. *PDCD4* targets MDM2 proto-oncogene (*MDM2*) and BCL2 apoptosis regulator-associated agonist of death (BAD) were also analyzed. HNSCC samples demonstrate no change in *MDM2* expression ($p>0.05$) and reduced expression of *BAD* ($p<0.01$).

We also assessed upstream effectors of the MTOR pathway, specifically the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT pathway. Analysis of PI3K regulatory subunit 1 (*PIK3R1*) expression in HNSCC demonstrated up-regulation in two ESTs ($p<0.044$). Catalytic subunits *PIK3CA* ($p=0.0012$) and *PIK3CD* ($p<0.0001$) were up-regulated and *PIK3CB* was down-regulated ($p=0.0018$), however, in *PIK3CD*, one of the two queried ESTs had no change in expression ($p>0.05$). Downstream of PI3K, AKT serine/threonine kinase (*AKT1*) revealed up-regulation in the cancerous specimens ($p=0.0021$). PI3K pathway tumor suppressor phosphatase and tensin homolog (*PTEN*) was also up-regulated in HNSCC samples relative to the control in one ($p=0.027$) of two ESTs.

Table II. *Changes in gene expression of mechanistic target of rapamycin (MTOR) pathway genes in head and neck squamous cell carcinoma (HNSCC) relative to the control specimens, as determined by Affymetrix microarray cDNA expression data.*

Gene	Encoded protein	Change in HNSCC	Fold change	*p*-Value
Pathway promoters				
MTOR	Serine/threonine-protein kinase mTOR	No change	1.00	0.97
RHEB	GTP-binding protein Rheb	Up-regulated	1.22	**4×10^{-8}**
			1.51	**6×10^{-4}**
MLST8	Target of rapamycin complex subunit LST8	Up-regulated	1.25	**0.045**
EIF4B	Eukaryotic translation initiation factor 4B	No change	0.98	0.86
EIF4E	Eukaryotic translation initiation factor 4E	Down-regulated (1/2 ESTs)	0.92	**0.037**
			0.80	0.41
EIF4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1	No change	1.00	0.98
RPS6KB1	Ribosomal protein S6 kinase beta-1	Up-regulated (1/2 ESTs)	0.91	**3.5×10^{-4}**
			1.76	0.40
RPS6KB2	Ribosomal protein S6 kinase beta-2	Down-regulated	0.41	**7.90×10^{-6}**
PIK3R1	Phosphatidylinositol 4,5-bisphosphate 3-kinase regulatory subunit alpha	Up-regulated	1.31	**0.044**
			1.47	**0.017**
PIK3CA	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform	Up-regulated	1.51	**0.0012**
PIK3CB	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta isoform	Down-regulated	0.74	**0.0018**
PIK3CD	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit delta isoform	Up-regulated (1/2 ESTs)	2.52	**4×10^{-7}**
			0.83	0.14
AKT1	RAC-alpha serine/threonine-protein kinase	Up-regulated	1.23	**0.0021**
Pathway suppressors				
TSC1	Hamartin	No change	1.00	0.94
TSC2	Tuberin	Down-regulated	0.77	**0.011**
PTEN	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase	Up-regulated (1/2 ESTs)	1.24	**0.027**
			0.92	0.35
PDCD4	Programmed cell death protein 4	Down-regulated	0.38	**7×10^{-5}**
MDM2	E3 ubiquitin-protein ligase MDM2	No change	0.90	0.57
			1.3	0.32
BAD	BCL2 apoptosis regulator (BCL2)-associated agonist of cell death	Down-regulated	0.75	**2.7×10^{-4}**
			0.81	**0.014**

Statistically significant *p*-values are shown in bold.

Discussion

MTOR is a serine/threonine kinase which functions as the catalytic subunit of MTORC1 and MTORC2, promoting cellular proliferation and growth (6). In this analysis, we found a number of significant gene-expression changes in pathway elements of MTOR associated with head and neck squamous tumors compared to buccal mucosa from healthy controls. *MTOR* itself is similarly expressed in tumor and normal mucosa, however, differential expression upstream and downstream of *MTOR* may result in increased cellular proliferation.

The gene products of *RHEB* and *MLST8* act on MTOR to increase phosphotransferase activity. Gene products of *TSC1* and *TSC2* act together to convert RHEB into its inactive form (6), resulting in overall suppression of the MTOR pathway (Figure 1). RHEB and MLST8 up-regulation, as well as TSC2 down-regulation, are putative mechanisms to increase MTOR activity in head and neck carcinogenesis, despite no change in MTOR gene expression itself.

MTOR targets RPS6KB1 and 2 are involved the overall promotion of translation *via* the phosphorylation of ribosomal protein S6 (6). We discovered up-regulation of *RPS6KB1* which may contribute to cellular proliferation in cancer. Increased S6 phosphorylation has been well-demonstrated in cancerous tissue (7), and our findings are in accordance with this. RPS6KB1 is also involved in cellular proliferation by its degradative effects on PDCD4 which promotes apoptosis when active (6). *PDCD4* acts to suppress proliferation *via* *MDM2* and *BAD* (8). In addition to the potential effects that up-regulation of *RPS6KB1* would have on deactivating apoptosis *via* PDCD4 degradation, we found *PDCD4* and *BAD* to be down-regulated in HNSCC, which would also contribute to increased cellular survival.

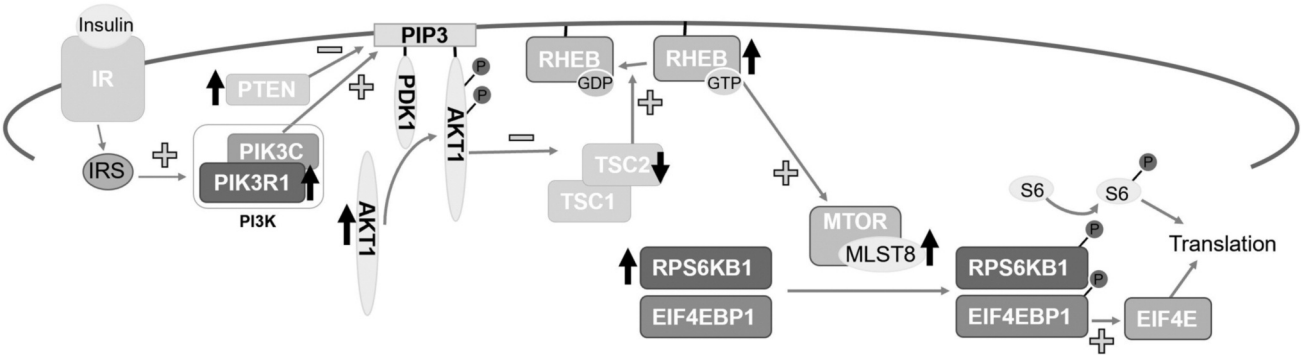

Figure 1. *Mechanistic target of rapamycin (MTOR) pathway gene components, and their genetic changes in cancer. The + and – signs indicate typical physiological effects of each gene product on the pathway. Vertical arrows indicate change in gene expression in cancerous compared with normal tissue. IR: Insulin receptor; IRS: insulin receptor substrate 1; PIK3R1: phosphoinositide-3-kinase regulatory subunit 1; PIK3C: phosphatidylinositol 3-kinase subunits; PTEN: phosphatase and tensin homolog; AKT1: AKT serine/threonine kinase 1; TSC: tuberous sclerosis; PDK1: 3-phosphoinositide dependent protein kinase 1; RHEB: Ras homolog; MLST8: MTOR-associated protein; EIF4E: eukaryotic translation initiation factor 4E; EIF4EBP1: EIF4E binding protein 1; RPS6KB1: ribosomal protein S6 kinase B1.*

Upstream of MTOR, the PI3K/AKT pathway promotes cellular growth in the presence of environmental growth factors. PI3K is a heterodimer made up of PI3KR1 and PI3KC subunits. PI3K converts phosphatidylinositol (3,4)-*bis*phosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3), recruiting and activating AKT (Figure 1) (6). The activation of the PI3K/AKT pathway ultimately increases MTORC1 activity (9). PTEN is a tumor suppressor which acts on the PI3K pathway by dephosphorylating PI3K (10). We observed up-regulation of *PIK3R1, PIK3CA, PIK3CD*, and *PTEN* in the cancer specimens. Up-regulation of *PIK3R1* and *PIK3C* pathway genes may favor increased expression of PIP3, thus promoting the tumorigenic phenotype. Finally, we demonstrated up-regulation of *AKT1* in cancerous specimens, a molecular finding that is well-demonstrated in studies of carcinogenesis (11), and specifically seen in head and neck cancer (4).

In summary, up-regulation of the MTOR complex with ultimate phosphorylation and activation of S6 can be promoted by alterations of expression of *PIK3R1, TSC2, RHEB* and *RPS6KB1;* the only inconsistent (but perhaps important) finding for pathway elements was the up-regulation of tumor suppressor *PTEN*.

The purpose of this project was to examine MTOR signal transduction, an important pathway in oral carcinogenesis progression (12). We have been investigating pioglitazone/metformin combination agent (Actoplus MET) (clinicaltrials.gov NCT02917629) in a window-of-opportunity trial and are collaborators for a recently completed metformin leukoplakia trial (clinicaltrials.gov NCT02581137). Some of the MTOR pathway elements we examined here are surrogate endpoints in these clinical trials. We identified differentially expressed genes that can be used for clinical trial endpoints, as well as potential therapeutic targets. In the future, it will be important to meld these genomic findings with other basic science technologies. One caveat of this initial study is that we used a low stringency for fold change. This way we captured the greatest number of targets for further analysis. In such a study, it would be quite useful to determine inositol triphosphate levels, which would give insight into the hyperfunctionality of MTOR. Ultimately, this analysis will help guide clinical trial biomarker modulation for current and future studies employing MTOR pathway inhibitors, such as rapamycin or metformin. Importantly, we feel the analysis of gene-expression databases with the goal of identifying pathways for therapy and prevention is feasible.

Conflicts of Interest

The Authors declare they have no conflicts of interest regarding the study.

Authors' Contributions

FO and CK designed the study. CK and FO participated in data analysis, and final article preparation. As the principal investigator, FO supervised the study. All Authors read and approved the final article.

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References

1 Siegel R, Naishadham D and Jemal A: Cancer statistics. CA Cancer J Clin *70*: 7-30, 2013. PMID: 23335087. DOI: 10.3322/caac.21166

2 Govindarajan R and Siegel ER: The effect of exposure to thiazolidinediones on the development of head-and-neck cancer in patients with diabetes mellitus. Transl Res Oral Oncol *2*: 2057178X1773980, 2017. DOI: 10.1177/2057178x17739809

3 Seabloom DE, Galbraith AR, Haynes AM, Antonides JD, Wuertz BR, Miller WA, Miller KA, Steele VE, Miller MS, Clapper ML, O'sullivan MG and Ondrey FG: Fixed-Dose combinations of pioglitazone & metformin for lung cancer prevention. Cancer Prev Res *10*: 116-123, 2017. PMID: 28052934. DOI: 10.1158/1940-6207.CAPR-16-0232

4 Molinolo AA, Amornphimoltham P, Squarize CH, Castilho RM, Patel V and Gutkind JS: Dysregulated molecular networks in head and neck carcinogenesis. Oral Oncol *45*: 324-334, 2009. PMID: 1000000221. DOI: 10.1038/jid.2014.371

5 Ginos MA, Page GP, Michalowicz BS, Patel KJ, Volker SE, Pambuccian SE, Ondrey FG, Adams GL and Gaffney PM: Identification of a gene expression signature associated with recurrent disease in squamous cell carcinoma of the head and neck. Cancer Res *64*: 55-63, 2004. PMID: 14729608. DOI: 10.1158/0008-5472.CAN-03-2144

6 Ma XM and Blenis J: Molecular mechanisms of mTOR-mediated translational control. Nat Rev Mol Cell Biol *10*: 307-318, 2009. PMID: 19339977. DOI: 10.1038/nrm2672

7 García-Carracedo D, Ángeles Villaronga M, Álvarez-Teijeiro S, Hermida-Prado F, Santamaría I, Allonca E, Suárez-Fernández L, Victoria Gonzalez M, Milagros Balbín M, Astudillo A, Martínez-Camblor P, Su GH, Rodrigo JP and García-Pedrero JM: Impact of PI3K/AKT/mTOR pathway activation on the prognosis of patients with head and neck squamous cell carcinomas. Oncotarget *7*: 29780-29793, 2016. PMID: 27119232. DOI: 10.18632/oncotarget.8957

8 Sridharan S and Basu A: Distinct roles of mTOR targets S6K1 and S6K2 in breast cancer. Int J Mol Sci *21*: 1-17, 2020. PMID: 32054043. DOI: 10.3390/ijms21041199

9 King D, Yeomanson D and Bryant HE: PI3King the Lock: Targeting the PI3K/AKT/mTOR pathway as a novel therapeutic strategy in neuroblastoma. J Pediatr Hematol Oncol *37*: 245-251, 2015. PMID: 25811750. DOI: 10.1097/MPH.0000000000000329

10 PTEN phosphatase and tensin homolog. Natl Libr Med (US), Natl Cent Biotechnol Inf, 2004. Available at: https://www.ncbi.nlm.nih.gov/gene/5728 [Last accessed April 15, 2020]

11 Brazil DP, Yang ZZ and Hemmings BA: Advances in protein kinase B signalling: AKTion on multiple fronts. Trends Biochem Sci *29*: 233-242, 2004. DOI: 10.1016/j.tibs.2004.03.006.

12 Molinolo AA, Marsh C, El Dinali M, Gangane N, Jennison K, Hewitt S, Patel V, Seiwert TY and Gutkind JS: mTOR as a molecular target in HPV-associated oral and cervical squamous carcinomas. Clin Cancer Res *18*: 2558-2568, 2012. PMID: 22409888. DOI: 10.1158/1078-0432.CCR-11-2824

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