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Araştırma Makalesi / Research Article

Seramid Metabolizmasının HNSCC Hücrelerinde Primer Silia Uzunluğu ve Tümör Metastazına Etkisi

Impact of the Ceramide Metabolism on Primary Cilia Length and Tumor Metastasis in HNSCC Cells

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Öz- Son on yılda, belirli sfingolipitler, hücre zarı fonksiyonlarına katılımları ve çok çeşitli hücresel davranış sistemini düzenleyen sinyalizasyon işlemleriyle önemli hale gelmiştir. Seramid, sfingolipid metabolizmasının ana molekülüdür. Hücre farklılaşması, hücreden hücreye iletişim, apoptoz ve proliferasyon gibi temel hücresel fonksiyonlara aracılık etmede hayati rol oynar. Son zamanlarda yapılan çalışmalar seramid metabolizmasının ve metabolik enzimlerinin hücre göçü ve hücre mobilitesi sürecinde önemli rol oynadığını göstermiştir. Bununla birlikte, söz konusu sfingolipidlerin moleküler mekanizması bilinmemektedir. Mekanik olarak sistemi anlamaya çalışan bu çalışmadaki verilerimiz bu bilinmeyene ışık tutacaktır. Bu çalışmada, özellikle, seramid sentezlerinin UM-SCC-22A baş ve boyun skuamöz karsinom hücrelerinin göç kabiliyeti üzerindeki etkisine baktık. Kısaca, biyoaktif seramidleri azaltan, farmakolojik inhibitör fumonisin B1 ile seramid metabolizmasındaki değişikliklerin, TGF-ß reseptör tip I ve II (TßRI / II) hücre yüzeyine lokalizasyonunun aktifleşmesine ve hücre göçünün / yayılımının artmasına yol açtığını tespit ettik. Öte yandan, UM-SCC-22A hücrelerinde primer silia uzunluğu, seramid miktarında azalma ile değişmedi. Bu nedenle, bu veriler seramid sentezlerinin / seramidin, TßRI / II'yi primer siliuma taşınımını düzenleyen, hücre göçünü ve kanser metastazını kontrol eden yeni bir faktör olduğunu ortaya koymaktadır.

Anahtar Kelimeler: Seramid, Metastaz, Primer Silia, Baş ve Boyun Skuamöz Karsinomu

A*bstract-* **In the last decade, particular sphingolipids have become renowned for their participation in cell membrane functions and signaling proceedings that regulate a wide array of cellular manners. Ceramide is a major molecule of sphingolipids metabolism. It plays vital roles in mediating the major cellular functions such as cell differentiation, cell-to-cell communication, apoptosis, and proliferation. Recent studies have shown that ceramide metabolism and their metabolic enzymes take important role during migration and cell mobility process. However, the molecular mechanism of sphingolipids involved is unknown. Our efforts in this study, which are serving mechanistically the understanding of ceramide, will be shade some light on this unknown. In this work, particularly, we looked at the effect of ceramide syntheses on migration ability of UM-SCC-22A head and neck squamous carcinoma cells. Briefly, we identified that alterations in ceramide metabolism by pharmacological inhibitor fumonisin B1, reducing bioactive ceramides, results in activation of the TGF-ß receptor type I and II (TßRI/II) cell surface localization and, leading to increased cell migration/invasion in UM-SCC-22A head and neck squamous carcinoma cells. On the other hand, primary cilia length in UM-SCC-22A cells did not changed by decreased ceramide amount. Thus, these data reveal that ceramide synthases/ceramide is a novel factor that regulates TßRI/II to the primary cilium, controlling cell migration and cancer metastasis.**

***Keywords*- Ceramide, Metastasis, Primary Cilia, Head and Neck Squamous Carcinoma**

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I. INTRODUCTION

Head and neck cancers originates from the malignancies in nasopharynx, oropharynx, larynx, hypopharynx, oral cavity, nasal cavity, paranasal sinuses, cervical lymph nodes, and salivary glands [1]. Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cause of cancer related morbidity and mortality worldwide [2]. Nearly 600,000 new cases diagnosed with HNSCC each year [3]. The most common risk factors of HNSCC is human papillomavirus (HPV) and carcinogens like alcohol and tobacco. HNSCC can be segregate into two depending on HPV status. HPV negative tumors are associated with tobacco and alcohol consumption. HPV positive tumors are associated with HPV infection and harbored fewer mutations compared to HPV negative tumors. The mutations in HPV positive tumors are driven by E6 and E7 viral proteins on the other hand HPV negative tumors have TP53 mutations [3, 4]. HNSCC patients suffer from poor prognosis with almost (40-50 %) survival rate over 5 years in most cases. From a molecular point of view, there are variety of genetic mutations drive the initiation and the progression of HNSCC. The genetic variations had been identified in both tumor suppressors and oncogenes. There are multiple signaling pathways taking role in formation of the mutations including EGFR, TP53, and NOTCH signaling [2]. One of the most common genetic mutation that cause it; is TGF-β/SMAD genetic mutations [5, 6]. It is important to find new alternative therapeutic strategies to cure head and neck squamous cell carcinoma.

Ceramide, the central molecule of sphingolipids, is an important tumor suppressor also act as a regulator of differentiation, proliferation and apoptotic processes. Regulation of ceramide metabolism plays a key role in the anticancer activities [7, 8]. Ceramide-mediated processes are differentially regulated dependently localization of ceramide, cell and tissue type [9].

Metastasis is the common term used for the process of spreading cancer cells from where they first formed (primary tumor) to surrounding tissues and distant organs. Ceramide directly or indirectly has an important role on invasion and metastasis [8]. For example, matrix metalloproteinases (MMPs) are enzymes that can induce metastasis with its proteolytic activation on extracellular matrix. It has been shown that MMPs, especially MMP-2 and MMP-9, are over expressed in cancer cells compared to normal cells. Ceramide inhibits MMP-2 and MMP-9's expression leading to reduced metastatic activities [10]. Recent studies show that TGF-β signaling also has a connection with primary cilia and has an important role in cell migration. Activation of TβRI/II signaling in the primary cilium leads to the induction of Sonic Hedgehog signaling and activation of Smo. Eventually increasing cell migration and metastasis [6, 8, 10].

Ceramide is excessively regulated with metabolic enzymes and their activity has crucial roles in promoting cancer cell survival or death. There are six mammalian ceramide synthase enzymes, CerS 1-6. CerS's control salvage pathway and de novo pathway of ceramide metabolism [11]. Ceramide synthase enzyme is responsible of generating ceramide with different fatty acyl chain lengths as a consequence has an important role in cancer cell signaling. For example, C18-Ceramide is generated by CerS1, it has 18 carbon-long fatty acid chain. CerS2 generates very long chain ceramides such as C22-, C24 and C26-Ceramides. CerS4 mainly generates C18-Ceramide and C20-Ceramide. Different fatty acyl chain long ceramides have different biological functions. C18-Ceramide induces cancer cell death and suppression of head and neck tumor growth but on the other hand, C16-Ceramide induce head and neck cancer proliferation [10, 12, 13].

The primary cilia is an organelle that serves as an antenna for signaling factors and sensing both biophysical and biochemical changes in the extracellular environment. [14] It occurs singly on numerous cells such as neurons, epithelial, stem and muscle cells. It has been reported that the primary cilia play key role on signaling pathways, cell polarity and in tissue homeostasis such as the liver and kidney. In addition, primary cilia is essential for important biological processes like differentiation, proliferation and cell migration. Defects on primary cilia or ciliopathies can result in various disorders including polycystic kidney disease, skeletal malformations, obesity, von Hippel–Lindau syndrome and retinopathies. [15, 16]. Ciliogenesis, formation of primary cilia, can occur via two different mechanisms, intracellular pathway and extracellular pathway [17]. Ciliogenesis is directly associated with many factors such as starvation, injury or bioactive sphingolipids, ceramides. It has been shown that the inhibition of ceramide results primary cilia loss and C16-, C24-Ceramide is important for formation of the cilia [15, 18]. Similarly, various factors effect primary cilia elongation. The importance of cilia's length is known yet not fully understood. A well-known example of the length control is via intracellular cAMP level. Decrease intracellular cAMP level results shortening of primary cilia [19]. In addition, kinesins and dyneins play an important role in ciliary length regulation. Decreasing the expression of dynein leads to shortening cilia. [20, 21]. Primary cilia is essential for various signaling pathways like PDGFRα, Wnt and Sonic hedgehog. These signaling pathways have crucial roles such as cell survival, growth, differentiation and migration. In addition, the length of primary cilia is also dynamically changed in the cells, as

a result and/or cause, during the progression of various diseases including cancer and neurological disorders [22].

Studies that are more recent have presented bioactive sphingolipids and their metabolic enzymes show important role during migration and cell mobility process. However, the molecular mechanism of sphingolipids involved is unknown. Our efforts in this study, which is serving mechanistically the understanding of bioactive lipids, will be shade some light on this unknown. In this work, particularly, we looked at the effect of ceramide syntheses on migration by focusing on primary cilia length and TGF-beta signal pathway, in head and neck squamous cell carcinoma (HNSCC). Therefore, here we are investigating how this mechanism lead to the abrogation of metastasis and invasion of UM-SCC-22A cells.

II. EXPERIMENTAL STUDY

A. Cell Culture

UM-SCC-22A cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Cellgro) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1% penicillin and streptomycin (Cellgro). UM-SCC-22A cells were provided from Medical University of South Carolina by Prof. Dr. Besim Ogretmen.

B. Fumonisin B1

Fumonisin B1 (FB1) was purchased from Sigma (St. Louis, MO, USA). A stock solution of FB1 for cellular assays was set in phosphate-buffered saline (PBS) and then diluted in the optimal medium (≤10 µl/ml). UM-SCC-22A cells were treated with 50 µM FB1 for 24h [23, 24].

C. Total RNA Isolation and RT-PCR

Total RNA were isolated from cells to detect changes in the expression levels of the ceramide synthase 1-6 genes (Qiagen, RNeasy Mini Kit).500 ng of total RNA were translated into complementary DNA (cDNA) by using the Reverse Transcriptase enzyme kit. Q RT-PCR was used to quantitatively determine the real-time changes in the expression levels of the CerS 1-6 genes. Beta-actin was used as an internal positive control. The PCR reaction was performed in an automatic thermocycler programmed as shown in Table 1. Primers used in this study are described in Table 2.

Table 1: Polymerase chain reaction steps

PCR step	**Temperature (in °C)**	**Time**
Pre-denaturation	95°C	5 minutes
Denaturation	95°C	20 seconds
Annealing	56-60 °C	30 seconds
Extension	72°C	45 seconds
Final Extension	72°C	5 minutes

Table 2: Primers Sequence

Gene	Primers	Orientation Sequence
Actin	Forward	5 ′- ATTGG AATGAGCGGTTCC -3 ′
	Reverse	5 ′- GGTAGTTT GTGGATGCCACA -3 ′
CerS1	Forward	5 ′- ACGCTACGCTATACATGGACAC -3 ′
	Reverse	5 ′- AGGAGGAGACGATGAGGATGAG -3 ′
CerS2	Forward	5 ′- CCGATTACCTG TGGAGTCAG -3 ′
	Reverse	5 ′- GGCGAAGACGAGAAGATGTTG -3 ′
CerS3	Forward	5 ′- ACATTCCACAAGGCAACCATTG -3 ′
	Reverse	5 ′- CTCTTGATTCCGCCGACTCC -3 ′
CerS4	Forward	5 ′- CTTCGTGGCGGTCATCCTG -3 ′
	Reverse	5 ′- TGTAACAGCAGCACCAGAGAG -3 ′
CerS5	Forward	5 ′- TGTAACAGCAGCACCAGAGAG -3 ′
	Reverse	5 ′- GCCAGCACTGTCGGATGTC -3 ′
CerS6	Forward	5 ′- GGGATCTTAGCCTGGTTCTGG -3 ′
	Reverse	5′- GCCTCCTCCGTGTTCTTCAG -3 ′

F. Migration Assay

For the migration assay, the cells were seeded into 6 well plates. Plates were incubated for about 24 hours at 37°C, allowing cells to adhere and spread on the substrate completely. After 24 hours, cells seated in the wells were scratched with a p1000 pipette tip. Cell debris was removed, and the edge of the scratch was smoothened by washing the cells once with 1 ml of the growth medium. In order to obtain the same area during image acquisition, markings were made close to the drawn region. The determined reference points were imaged at 0 and 24 hours under a microscope (Olympus CKX41). Images were analyzed with Wimasis image analysis software.

G. Flow Cytometry

Cell were suspended in 0.5–1 ml 1X PBS and added formaldehyde to obtain a final concentration of 4%. After fixing for 15 minutes at 25°C the cells washed by centrifugation with excess 1X PBS. Supernatant was discarded and cells were resuspended in 0.5-1 ml 1X PBS. To permeabilize the cells ice-cold 100% methanol was added slowly to pre-chilled cells to a final concentration of 90% methanol. Cells were incubated 30 minutes on ice. Desired number of cells were added into tubes. To remove methanol, cells washed by centrifugation in excess 1X PBS and supernatant was discarded. Cells were resuspended in 100 µl of diluted primary antibody and incubated for 1 hour at 25°C. Cells washed by centrifugation in incubation buffer and supernatant was discarded. Then, cells were resuspended in 100 µl of diluted fluorochrome-conjugated secondary antibody and incubated for 30 minutes at 25°C. Cells washed by centrifugation in incubation buffer supernatant was discarded. Lastly, cells were resuspended in 1X PBS and analyzed on flow cytometry.

H. Confocal Microscopy

Following immunohistochemistry, sections were initially checked for labelling quality using the fluorescence settings on an Olympus AX70 light microscope, equipped with epi-fluorescence capabilities. All subsequent imaging work was carried out using a Zeiss LSM 510 upright laser scanning confocal microscope with a 100X oil immersion objective at the Medipol University Microscopy. Detector gain and amplification settings were adjusted to reduce the signal-to-noise ratio, and at least ten alveoli, from all four quarters of each section were imaged. Cilia were imaged as a series of Z slices obtained using immunofluorescence specific laser excitation lines 488 nm and 543 nm, according to the staining protocol employed. Nuclei were imaged from the midsection of the selected Z-slice using standard fluorescence excitation. In cases where digital zoom was used to enhance the view of individual cilia, the corresponding field view was recorded to maintain information of ciliary orientation in relation to lumina. All LSM image files were processed in ImageJ to create composite micrographs. Images from each of the red and green detector channels were stacked to create a single image.

Where necessary, minor adjustments to colour threshold levels were made to optimise signal-to-noise ratio. The single-colour images were then merged to create single composite RBG micrographs, and scale bars added.

İ. Ceramide analysis by HPLC/MS

UM-SCC-22A cells were harvested with a scraper into 10 ml of ice-cold PBS, than they were pelleted. Total ceramide were measured by the Lipidomics Core facility at the Medical University of South Carolina, using liquid chromatograpy and mass spectrometry as described previously [25]. Molecular species of ceramide were semisynthetically prepared as described in Materials and Methods. Each species was reacted with 1-anthroyl cyanide, and the resultant anthroyl derivative was purified by silicic acid column chromatography. Seventeen species of purified anthroyl derivatives were mixed and separated by HPLC on a reversed-phase column (Merck LiChrosorb RP-18, 4 mm x 250 mm) with the mobile phase of acetonitrile–methanol–ethyl acetate 12:1:7 (by vol) at a flow rate 1.2 ml/min. Each peak was detected using a spectrofluorometer with excitation at 365 nm and emission at 412 nm [25].

J. Statistical Analysis

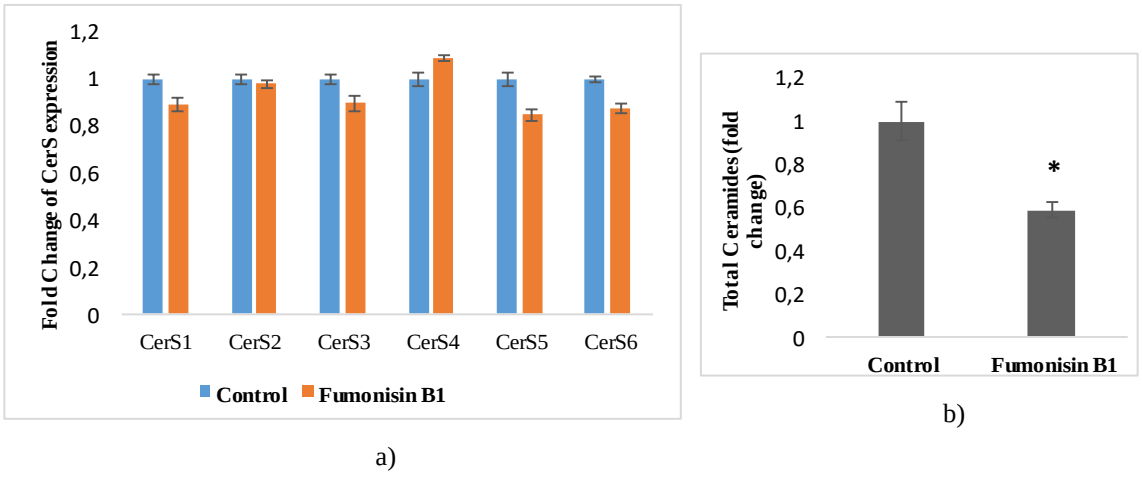
Experiments were performed in three sets independent of each other and the results were expressed as mean ± standard error. Statistical comparisons were determined according to Student's t-test assuming equal variance. Differences were considered as statistically significant at $p \leq 0.05$. Data are expressed as ±SEM.

III. RESULTS AND DISCUSSION

Ceramide is an important bioactive sphingolipid that acts as a tumor suppressor and a regulator of differentiation, proliferation and apoptosis processes. Regulation of ceramide metabolism plays a key role in the anticancer activities. Recent studies have shown that ceramide and primary cilia collaborate in some instances. Primary cilia known as the new organelle that has multiple functions about cell physiology and pathology such as biological processes like differentiation, proliferation and metastasis [14-16]. Metastasis is the common term used for the process of spreading cancer cells from where they first formed to surrounding tissues and distant organs. Ceramide and primary cilia directly or indirectly have an important role on invasion and metastasis. Thus, lipid metabolism and primary cilia overlaps at this point. In our study, we wanted to illuminate their effects on tumor metastasis by targeting primary cilia via ceramide.

It has been demonstrated that increased amount of ceramide induces cancer cell apoptosis. To see ceramide's anticancer activities, we used a clinical inhibitor named fumonisin B1. Fumonisin B1, mycotoxin produced by Fusarium verticillioides, is a specific inhibitor of ceramide synthase [11]. We tested whether ceramide synthase (CerS) inhibition by fumonisin B1 alters the cell migration in head and neck cancer cell line 22A.

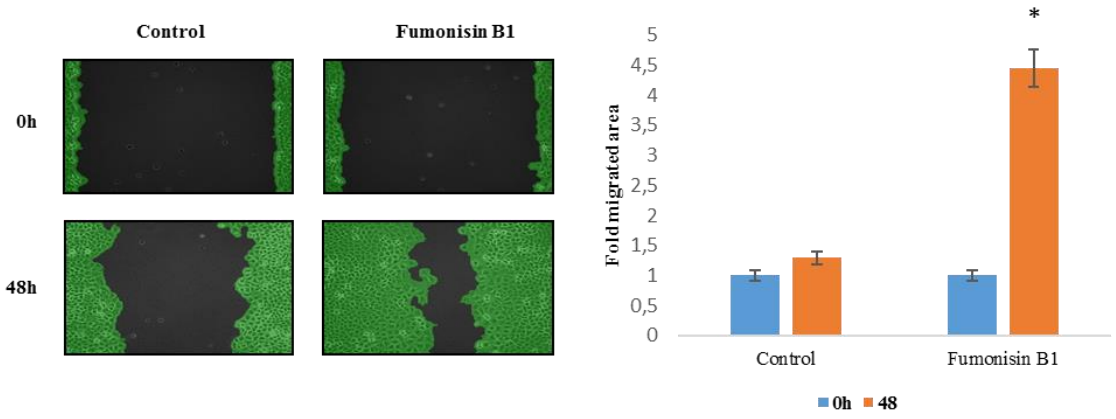
UM-SCC-22A cells were treated with 50 μM FB1, right amonut of solution in view of the fact that its toxicity, for 24h. Afterwards we used PCR to see the change in CerS expressions. As a result, it has seen that CerS(1-6) mRNA expression levels did not changed by treatment of 50 μM FB1 (Figure 1a). However, the total ceramide level was decreased by fumonisin B1 treatment almost 50% percentage compared to untreated control. Thus, resulting in decreased intracellular ceramide level (Figure 1b).



a) b)

Figure 1. a) PCR results after treating cells with 50 µM FB1 for 24h. Orange bars are fumonisin B1 treated cells, blue bars are untreated controls. **b)** Ceramide analysis by HPLC/MS.

In order to see the metastatic change we performed wound healing scratch assay. When the cells are confluent enough, a straight line scraped through the cell monolayer with a pipet tip. Cell debris was removed, and the edge of the scratch was smoothened by washing the cells once with 1 ml of the growth medium. We checked the cells every 6-12 hours. The pictures shows the tumor metastasis of the control and fumonisin B1 treated cells. After 48 hours, fumonisin B1 treated cells have showed more metastatic activity then control.



a) b)

Figure 2. a) Wound healing assay images in UM-SCC-22A cells after 48 hours. **b)** Fold change of the fumonisin B1 treated cells and controls after 48 hours.

The images were analisyed by Wimasis program and quantitative results were obtained. While control cells hve showed a fold change of 1,25 after 48h, fumonisin B1 treated cells have showed a fold change of 4,20 (Figure 2). This results has also proved that ceramide might has a key role in metastasis and decreased level of ceramide induces tumor metastasis.

Various signal pathways involve in the biological process called metastasis but the molecular mechanism is not fully understood. In order to understand this mechanism we focused on the very known TGF-β pathway. It has been demonstrated that TGF- β pathway is one of the most crucial pathways on tumor metastasis [10]. Thus, we examine the to TβRI/II internalization via flow cytometry. The results showed that fumonisin B1 treated cells had more than two folds TβRI/II localization on the membrane (Figure 3). In other

words, decreased number of ceramide leads to the localization of TβRI/II on the membrane and induce tumor metastasis.

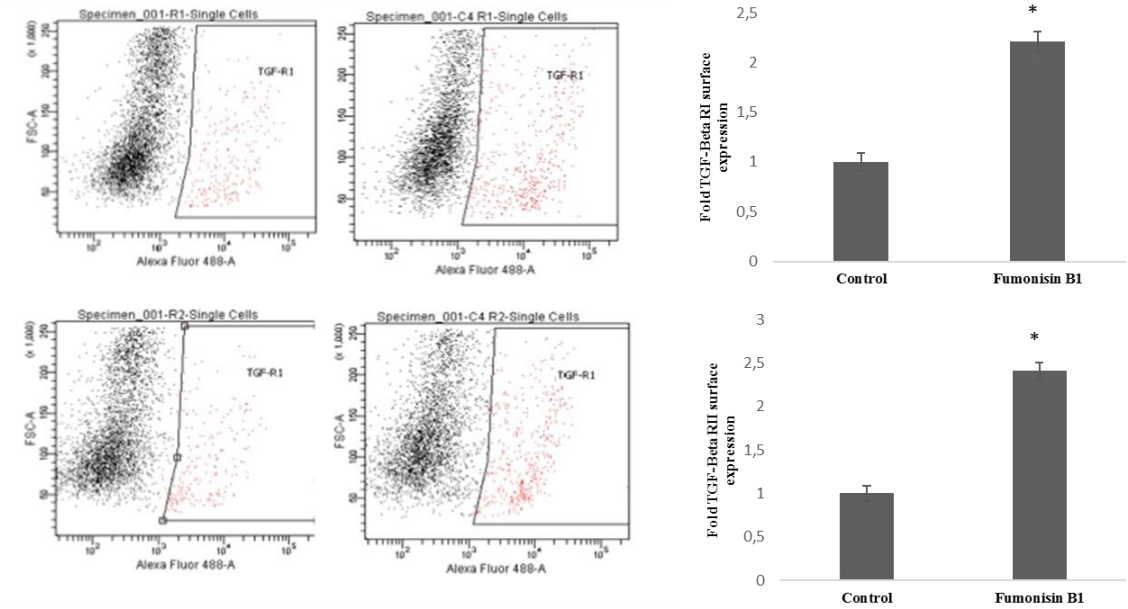
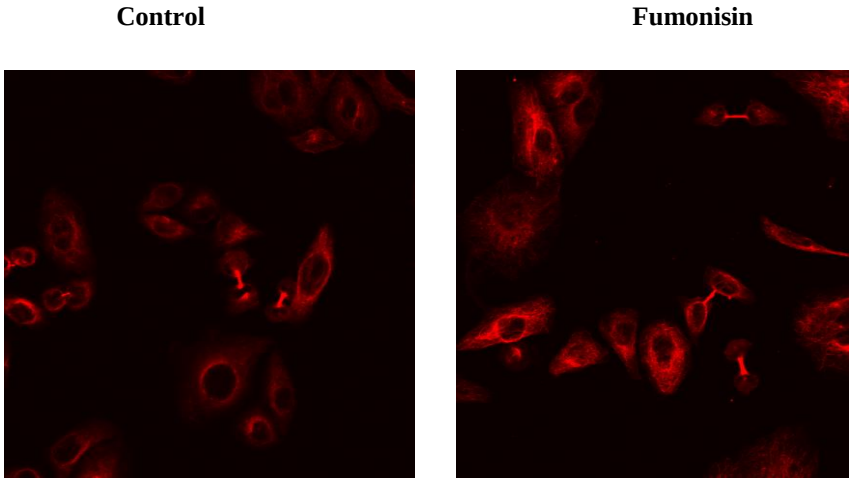


Figure 3. TβRI and TβRII surface 2expression levels was measured by flow cytometry.

Recent studies shows that TGF-β also localize on the primary cilia [20-22]. To investigate whether ceramide metabolism has an important effect on the primary cilia lenght, we used confocal microscopy to indicate primary cilia by using acetylated alpha-tubulin antibody. Results show that there were not particular differences such as primary cilia length and number between untreated control cells and fumonisin B1 treated cells (Figure 4).



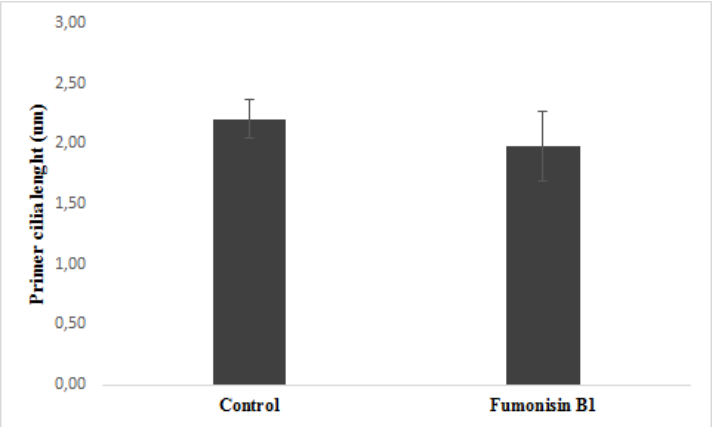


Figure 4. Confocal microscopy images of primary cilia in UM-SCC-22A cells. To image of primary cilia was used antibodies against acetylated alpha-tubulin (labeling primary cilia, red). (Magnification 63X, Scale bar 5µm).

IV. CONCLUSION

Our goal was to see whether ceramide metabolism has an impact on primary cilia length and tumor metastasis in head and neck squamous carcinoma cells. In order to investigate this aim, we first inhibit the expressions of ceramide synthases (CerS). Therefore, we used a clinical inhibitor named Fumonisin B1 and did RT-PCR to quantify the mRNA expression levels of CerS. As a result of the study, CerS's expressions did not changed in UM-SCC-22A cells after treatment of Fumonisin B1 but total ceramide level decresed significantly. We than did wound healing assay to see whether ceramide levels affect the UM-SCC-22A cell migration. The results have shown that fumonisin B1 tereated cells show significantly increased migration ratio compare to the untreated controls. Herewith fumonisin B1 treated cells have become more aggressive and metastatic cells. Also, we showed that decreased ceramide level leads to the localization of TβRI/II on the membrane surface and induce tumor metastasis as well. We used confocal microscopy to see the changes in primary cilia length but there were not significantly changes between unterated control and fumonisin B1 treated cells. The overall goal of these results are to define the mechanisms by which ceramide metabolism regulates TGF-ß receptor type I/II (TßRI/II)-signaling at the primary cilia to limit cancer cell migration/ invasion, and use this information for the development of mechanism-based strategies to inhibit tumor metastasis.

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