The Possible Role of Human Papillomavirus Infection in the prognosis of Oral Squamous Cell Carcinoma in a Northern Israel Population

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ABSTRACT: **Background:** Several types of human papillomavirus (HPV) have been found to be associated with oral squamous cell carcinoma (OSCC). Still, the significance of HPV infection and its relationship to patient prognosis remains an important matter of debate.

Objectives: To investigate the incidence of HPV infection in OSCC patients in northern Israel populations to determine its role in the etiology and prognosis of OSCC.

Methods: OSCC tissues were gathered from the pathology departments at Rambam and Padeh medical centers in northern Israel. HPV DNA typing and immunohistochemistry for p16INK4A antibodies were conducted to evaluate their incidence in OSCC tissues. Statistical analysis regarding its expression in the different sub-populations (Jews, Arabs, Druze) was conducted using chi-square and Fisher's exact tests.

Results: The study included 82 patients: 53 men and 29 woman; median age 62.1 years; 54 Jews, 25 Arabs, and 3 Druze. The overall incidence of HPV expression was 45% (n=37). The median age of HPV-positive patients was 53 years vs. 65.8 in the negative group ($P < 0.001$). The 5 year overall survival of HPV-positive patients was not significantly higher than HPV-negative patients. A significant association was found between *P*16 expression and overall survival (log-rank $P = 0.001$).

Conclusions: HPV infection in OSCC was not found to be significant in this study; however, *P*16 expression in the tumor tissue was found to be a positive prognostic factor for better survival.

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KEY WORDS: oral squamous cell carcinoma (OSCC), human papillomavirus (HPV), immunocytochemistry, DNA typing

The oral cavity is build up with many different kind of tissues and may be the source of different and variable tumors. Tumors of the oral cavity may be either epithelial, mesenchymal, or hematolymphoid.

The development of oral tumors, ignoring the origin of the tissue, begins with the accumulation of disruptions in several normal cellular activities that can eventually transform normal cells into tumor cells. These disruptions upset the normal balance between cell proliferation and death, allowing cells to acquire certain capabilities essential to malignant growth and spread. This process is a multi-stage process, which is initiated and proceeded by specific carcinogens, leading to the loss of cell cycle control and selected cell growth [1].

More than 90% of oral cancers are categorized as squamous cell carcinoma (SCC). Despite the progress in treatment and diagnosis, the overall survival rate is only 40–50% [2]. The most important risk factor of head and neck carcinogenesis worldwide is smoking, with alcohol consumption coming second. The risk is dose-dependent and the simultaneous use of both increases the risk many-fold, explaining some 75–85% of the cases [3].

Recently, several types of HPV (type 16, in particular) have been found to be associated with oral squamous cell carcinoma (OSCC) [4]. Still, the significance of HPV infection and its relationship to patient prognosis remains an important matter of debate, especially when considering the contradictory results presented in different published studies [5-7].

P16 protein, which is frequently related to HPV infection, especially in oropharyngeal carcinoma, contains some 156 amino acids and acts as a tumor suppressor protein by inhibiting the catalytic activity of the cyclin-dependent kinase 4-6 complex that is required for the phosphorylation of retinoblastoma protein [8,9].

The objective of this study is to investigate the incidence of HPV infection in patients with OSCC in northern Israel populations. HPV DNA typing was examined and *P16* immunohistochemistry analysis was performed to determine the role of HPV in the etiology and prognosis of OSCC.

PATIENTS AND METHODS

PATIENTS

This study was approved by the institutional review boards of the participating medical centers and followed the principles of the Declaration of Helsinki.

STUDY DESIGN

OSCC tissues, spanning the period from October 2000 until October 2014, were gathered from paraffin block archives at the pathology departments in Rambam Medical Center, and Padeh Poriya Medical center in northern Israel. Inclusion criteria included: histologically proven OSCC, no prior history of head and neck of chemotherapy or radiation therapy, adequate clinico-pathological data, and availability of sufficient paraffin-embedded tumor material presence. Patients with less than 24 months follow-up were excluded from the study. All the patients underwent identical "intent to treat" protocols, with the primary objective of achieving a disease-free patient. Tumors were treated by surgical resection with oncological free margins (> 7 mm), neck dissection (level I–IV), and adjuvant radiotherapy when needed. Adjuvant radiotherapy was given according to the cervical lymph node status, or margins of less than 4 mm. Radiotherapy was initiated within 4 to 8 weeks after surgery. A total dose of 60 to 70 Gys was delivered in 2 Gys doses.

HPV DNA TYPING

Following surgical resection, the tumor tissues were fixed in 4% neutral-buffered formalin, embedded in paraffin blocks, and archived according to institution protocol.

A 5-micron section was cut from each paraffin-embedded block and deparaffinized in xylene, washed several times in ethanol, and digested overnight with proteinase K at 56°C (Qiagen GmbH, Germany). The following day, DNA was isolated with TRI reagent according to the manufacturer's instructions (Sigma, St. Louis, MO, USA). Briefly, 1 ml of TRI reagent was added, and then homogenized and incubated at room temperature for 5 minutes. Chloroform (200 µl) was added to each biopsy and centrifuged for 15 minutes. The DNA interphase was isolated and placed in 300 µl 100% ethanol. A sodium citrate solution (0.1 M; Sigma, Rehovot, Israel) and 1 ml 75% ethanol was used for DNA wash. After two cycles of washes the pellet was dried and dissolved in 200 µl of sterile water (deuterium depleted water). The quality of the DNA was then measured with NanoDrop (Thermo Scientific NanoDrop, IL, USA). DNA amplification was performed in 50 µl reaction tubes. Each microtube contained 2 µl extracted genomic DNA (50 ng/L), 12.5 µL PCR Taq Mix (Promega Corporation, Israel). Next, HPV typing was performed using the HPV INNO-LiPA Amp kit (Promega Corporation, Israel). The polymerase chain reaction products were denatured and hybridized to type specific HPV probes immobilized as an array strip. Positive hybridization was detected by color precipitation at the probe site and the type was determined by matching with a reference overlay.

IMMUNOHISTOCHEMISTRY

Tissue MicroArrays® (tissue MicroArrays is a pathological method in which several separate tissues tissue cores are assembled in array fashion to allow multiplexhistological analysis) was used to enable a fast, accurate, and standardized method for screening the tumor tissues. Staining of the formalin fixed, paraffin-embedded 5 micron tissue array section was performed. First, slides were deparaffinized with xylene and rehydrated. Next, endogenous peroxidase was quenched by 3% hydrogen peroxide in methanol. Slides were then subjected to antigen retrieval by boiling in citrate buffer, pH 6, blocked with 10% normal goat serum, and incubated with the primary antibody, and p16INK4A antibody (Santa Cruz BioTech, USA). Following additional washes, color was developed using the AEC reagent (Sigma, St. Louis, MO, USA), sections counterstained with hematoxylin, and mounted. Slides were scored by two independent oral pathologists as 0 = no staining, 1 = mild staining of the epithelial tissue (not including the basal cells line), 2 = moderate staining (including the basal cells line), and 3 = strong staining.

STATISTICAL ANALYSIS

Chi-square and Fisher's exact tests were used to investigate the relationship between HPV expression and clinical data (including age and gender), population (Arabs, Jews, Druze), tumor-related data (such as tumor primary location, histological differentiation, and pathological N staging), and habits such as smoking and alcohol consumption [Table 1]. Survival analysis was performed using the Kaplan–Meier curves regarding HPV and *P16* positive patients versus HPV and *P16* negative patients. *P* value < 0.05 was considered statistically significant. Statistical

Table 1. Clinical and disease related data of the study cohort

Characteristic	Number (%)	Characteristic	Number (%)
Total	82	**Histological grading**	
Jews	54 (66)	Well differentiated	50 (61)
Arabs	25 (30)	Moderate differentiated	27 (33)
Druze	3 (4)	Poorly differentiated	5 (6)
Male / Female	53/29	**P16 IHC**	32 (39)
		Strong staining (3)	7 (9)
		Moderate staining (2)	11 (13)
		Mild staining (1)	14 (17)
Median age (std.)	64.15 yrs. (17.30)	**Pathological N classification**	
		N0	62 (75)
		N1	10 (12)
		N2	8 (10)
		N3	2 (3)
Smoking habits		**Treatment**	
Yes	43 (48)	Primary resection	82 (100)
No	39 (52)	Radiotherapy	30 (36)
		Radio-chemotherapy	2 (2)
Alcohol consumption	6 (7)	**Survival**	50 (61)
Site of primary tumor			
Base of tongue	11 (13)		
Lateral tongue	14 (17)		
Buccal mucosa	23 (28)		
Floor of the mouth	14 (17)		
Gum	1 (1)		
Lower alveolus	5 (6)		
Upper alveolus	14 (17)		

analyses were performed using IBM Statistical Package for the Social Sciences statistics software, version 22 (SPSS, IBM Corp, Armonk, NY, USA).

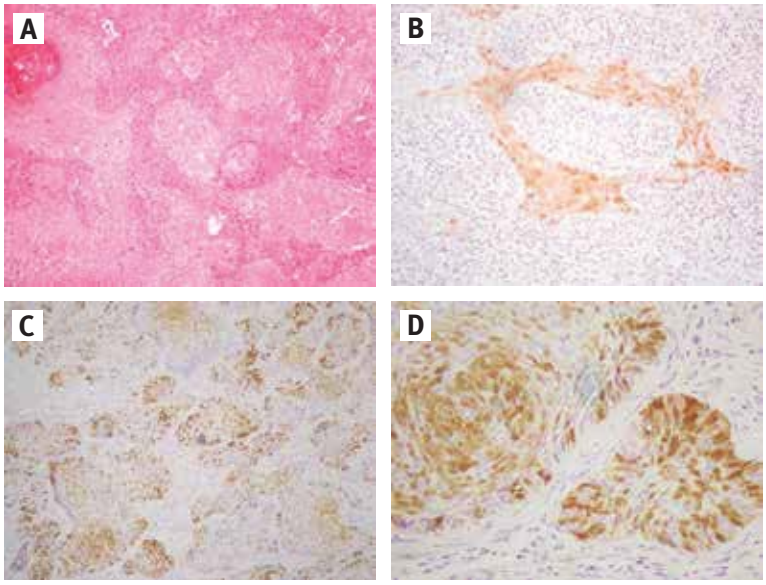
RESULTS

The study was comprised of 82 patients who met the inclusion criteria; 53 men and 29 woman, median age 62.1 years (range 26–94). Of these, 54 patients were Jews, 25 Arabs, and 3 patients were Druze. A significant portion of the patients reported smoking habits (n=43), while only six patients reported alcohol consumption. The most common site of OSCC in the study cohort was the tongue, followed by the buccal mucosa and floor of the mouth (30, 28, 17, respectively). Most of the tumors presented histological differentiation patterns (n=50). All the patients underwent intent-to-treat protocols. The treatment of choice in all the patients was surgical resection of the primary treatment with an objective of achieving a disease-free state (oncological free margins > 7 mm, neck dissection level I–IV, and adjuvant radiotherapy as indicated.

Adjuvant radiotherapy was given for positive cervical lymph nodes or negative margins of less than 4 mm (n=30). Radiotherapy was initiated within 4 to 8 weeks after surgery with a total dose of 60-70 Gy (delivered in 2 Gy fractions). One patient developed a second primary tumor of the upper aerodigestive tract (lung), 17 patients developed local recurrences, and 1 patient developed distal metastasis (to the lung). Among the study group, 31 patients died, 27 of them from oral cancer. The overall estimated survival time was 152 months. Demographic and disease-related data of the study group is presented in Table 1. In all, 32 cases were *P16* positive (39%); 14, 11, and 7 had mild, moderate, and strong patterns, respectively [Figure 1]. Most of those results were tumors located on the tongue (14/32).

The overall incidence of HPV expression (considering *P16* IHC and HPV DNA typing) was 45% (n=37). HPV DNA was detected in 23 cases (28%), and 19 of those were also positive for *P16* protein expression ($P < 0.001$). Of the 23 cases, 18 belong to HPV16, 3 cases were positive for HPV18, and 2 cases for HPV32. The median age of HPV-positive patients was 53 vs. 65.8 in the negative group ($P < 0.001$). When patients were divided by age into < 50 years and > 50 years, 63% of the < 50 aged patients were HPV positive. A significant relationship was also found between histological differentiation, and HPV-positive patients. HPV-positive patients presented a higher histological differentiation ($P = 0.045$). However, analyzing *P16* protein expression related to clinical and histological parameters, two factors showed a significant correlation: the tumor staging and histological grading ($P = 0.001$, $P = 0.04$ respectively). Cases with positive *P16* expression showed a better histological differentiation (well and well-moderate differentiation) and lower staging.

Figure 1. [A] Well differentiated squamous cell carcinoma (hematoxylineosin), original magnification ×100). **[B]** Positive moderate P16 nuclear expression (original magnification ×200). **[C,D]** Example case of well-differentiated squamous cell carcinoma and perineural invasion (hematoxylin-eosin, original magnification ×100). **[C]** Positive P16 nuclear expression (original magnification ×100). **[D]** strong positive staining for *P16* nuclear and cytoplasmic (original magnification ×200)



The N status was not significantly different in the two groups. Still, 20 out of 23 patients in the HPV-positive group were pathologically N0. No significant relationship was found between HPV detection and smoking, alcohol habits, gender, and the different subpopulations [Table 2], although a tendency was found between HPV presence and Jewish patients (15/39). The 5 year overall survival of HPV-positive patients was 73.9%, vs. 55.9% in HPV-negative patients; however, no statistically significant difference was found between those two variables (P value = 0.1). A significant association was found between *P16* expression and overall survival (log-rank $P = 0.001$). The probability of surviving 5 years in the *P16* positive group was 0.66, (81%) compared to 37.9% in the negative group [Figure 2].

DISCUSSION

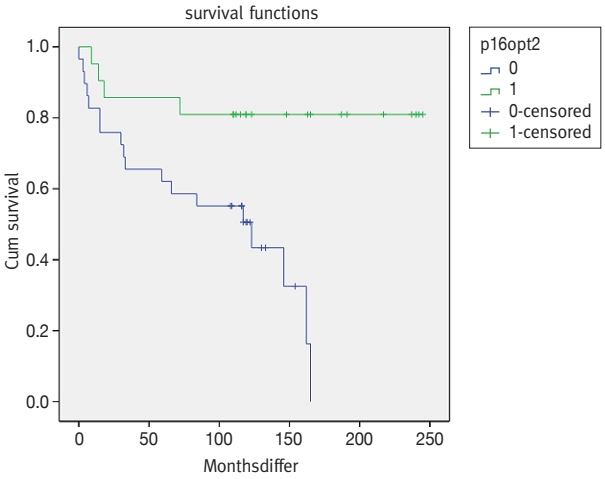
Carcinogenesis, the development of cancer, begins with the accumulation of disruptions in several normal cellular activities that can eventually transform normal cells into cancer cells. These disruptions upset the normal balance between cell proliferation and death, allowing cells to acquire certain capabilities essential to malignant growth and spread. These general hallmarks of cancer include self-sufficient growth, insensitivity to anti-growth signaling, evasion of apoptosis, limitless replication, tissue invasion/metastasis, and angiogenesis [10]. The progres-

Table 2. Clinical and histological data related to HPV DNA expression

Characteristic	HPV positive	HPV negative	*P* value
Gender			0.086
Male	18	35	
Female	5	24	
Smoking habits			0.68
Yes	11	38	
No	12	27	
Alcohol consumption			0.21
Yes	3	3	
No	20	56	
Median age	57.20	65.2	> 0.001
Population			0.56
Jews	15	39	
Non-jews	8	20	
P16 IHC staining	21	10	> 0.001
Histological differentiation			0.045
Well differentiation	38	12	
Moderate differentiation	22	5	
Poorly differentiated	2	3	
Pathological N staging			0.180
Positive N	15	3	
Negative N	20	44	
Survivals	23	59	0.349
Total: 23 (28%)			

HPV = human papillomavirus

Figure 2. Kaplan–Meier curves showing overall survival probabilities in patients with *P16*-positive (green) and *P16*-negative (blue) oral squamous cell carcinoma, $P < 0.05$



sion stage of carcinogenesis occurs when cells acquire a combination of these abilities, which allows conversion of a normal cell into a cancer cell. Similar to environmental and host-related oncogenic events, human tumor-associated viruses can lead to malignancies by providing viral mechanisms that promote one or more general hallmark of cancer. It is furthermore recognized that chronic inflammation and immunosuppression provide a microenvironment more conducive to the progression of carcinogenesis [11]. DNA tumor virus infections lead to immortalization of the infected cell through deregulation of multiple cellular pathways via expression of many potent oncoproteins. Research on various viral oncoproteins has revealed many of their novel cellular targets that are directly associated with cellular signaling, cell-cycle control, and the host's defense system.. Tumor viruses reprogram the host quiescent, G0 cell into the S phase of the cell cycle, allowing viral access to the nucleotide pools and cellular machinery that are required for replication and transmission [12].

The host cellular innate immune responses respond to viral infection by activating tumor suppressor proteins, pRB1 and p53, to induce cell death. However, the tumor viruses have evolved the means to inactivate these signaling pathways for their own benefit. Herpesvirus family members, EBV- and KSHV-encoded oncoproteins, have been shown to manipulate p53 and pRb functional activity to block apoptosis. Other DNA tumor virus-encoded oncoproteins also target tumor suppressor proteins. HPV-encoded E6 protein has been shown to bind and degrade p53 through the ubiquitin-proteasome pathway. In addition, HPV E7 oncoprotein bypasses cell cycle arrest through binding to the hypophosphorylated form of pRb, thereby inducing the degradation of pRb through a proteasome-mediated pathway [13,14]. In the present study we attempted to investigate the incidence and the possible role of HPV viral infection in oral squamous cell carcinoma in northern Israeli populations. In 1983, Syrjänen [15] proposed evidence of human papillomavirus (HPV) as an etiological factor in oral squamous cell cancer by analyzing the presence of HPV antigens in 40 oral carcinomas using immunohistochemistry. Of the 40 lesions, 16 (40%) showed HPV-suggestive changes on light microscopy; and of those, 8 expressed HPV structural proteins [15]. The latest meta-analyses of the epidemiological studies as well as the multi-center case-control studies have confirmed that HPV as an independent risk factor for head and neck cancer, with a range of odds ratios (OR) between 3.7 and 5.4 (16). Molecular and epidemiologic data associate high-risk HPV infection with cancers that arise in the oral cavity in almost 40% of the cases. Compatible with this data, the present study revealed 45% incidence of HPV (when considering either HPVDNA detection, and P16 IHC) infection in OSCC. The overall updated incidence of OSCC in Israel in 2012 was 6.48 (all rates are per 100,000; standardization to the Segi World standard population): 5.14 in Jews (0.76%) and 1.34 in Non-Jews (0.2%). This prevalence is fivefold less than the estimated prevalence in Europe (1%). Israel is a heterogenic country regarding ethnic and subpopulation groups, which differ mainly in cultural habits, socioeconomic status, and exposure to environmental carcinogens [17]. This heterogeneity is also demonstrated in OSCC prevalence and trends. In the present study, the incidence of OSCC in Jews

was 66%, compared with 34% in non-Jews. This different may be attributed to the different genetic backgrounds and environmental habits, such as alcohol consumption and smoking. Surprisingly, no significant different was found between HPV16 presences and the sub-groups, despite the difference in the sexual behavior between those two sub-population. Regarding gender distribution of OSCC, the results of the current study is compatible with world-wide epidemiological studies, which showed a significant higher male ratio (almost twofold). In addition, in the present study, a significant age difference was found between HPV-positive patients and HPV-negative patients (53 vs. 65.8 years), which may indicate a different and unique nature of HPV-related OSCC: a younger population, a significantly higher P16 protein expression, and better histological differentiation. P16, or more specifically P16InK4a, is a tumor suppressor gene that is involved in several cell pathways and eventually regulates the development of the mammalian's cells. Its inactivation is one of the main causes of cancer progression in several cancers, including head and neck cancer, esophagus, biliary duct, leukemia, and lymphoma [18].

In the current study, 32 cases were positive (39%) for P16, compatible with similar studies in oropahryngeal SCC. Positive P16 patients expressed better prognosis and survival rates. Apparently, positive P16 OSCC has a favorable molecular phenotype due to the ability of P16 to regulate the cell cycle; thus, preventing DNA damage and stopping the proliferation of mutated tumor cells [19]. P16 is a negative regulator for cell proliferation, which is a main factor in averting tumor cell proliferation and progression.

Several molecular studies have suggested that the expression of *P*16 is a positive prognostic factor in malignant tumors since it regulates the cell cycle progression by inhibiting it from preceding to the S phase. By a molecular pathway, *P*16 inhibit phosphorylation of the Rb family protein membranes and thus leads to cell cycle G1 arrest [20]. This molecular-biology interactions may explain the higher level of histological differentiation and grading in OSCC tumors, which presented positive IHC staining for *P*16 protein ($P = 0.001$, $P = 0.04$). The role of P16 in cellular senescence is well known. Its over-expression in human aging tissue and in response to oxidative stress and DNA damage have been reported in several studies [21]. This mechanism is reinforced even more by the survival results of the present study. SCC patients who were positive for P16 protein expression had a higher 2 year survival rate ($P = 0.002$) and overall survival (log-rank $P = 0.001$). Overexpression of P16 in malignant tissue is linked to stopping uncontrolled cell proliferation and cancer progression and DNA damage, thus it is used as a prognostic marker to predict a better prognosis. P16 expression in OSCC tumor tissues protect the cells from hyperproliferative signals and from various forms of stress since it acts as an activator for cellular senescence mechanism. Its expression in malignant tissue is intended to stop uncontrolled cell proliferation and cancer progression as well as DNA damage; thus, it is used as a prognostic marker to predict a better prognosis. Apparently, positive *P*16 OSCC has a favorable molecular phenotype due to the ability of P16 to regulate the cell cycle, prevent DNA damage, and stop the proliferation of mutated tumor cells.

CONCLUSIONS

The incidence of HPV DNA in northern Israel populations was compatible with its incidence in OSCC worldwide, while no significant difference was found between the different sub-groups. However, its proposed positive prognostic value was not shown since no significant difference was found between the overall survival between positive and negative HPV patients.

P16 protein expression in the tumor tissue presents a strong positive prognostic factor, indicating its important role in the etiology and progression of OSCC. Positive P16 OSCC has a favorable molecular phenotype due to the ability of P16 protein to regulate the cell cycle, prevent DNA damage, and stop the proliferation of mutated tumor cells.

We conclude that, HPV infection does not play a significant role in the etiology or prognosis of OSCC. In addition, the presence of P16 protein does not indicate previous HPV infection.

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Capsule

Commensals direct wound healing

Human skin is coated with microorganisms, some of which can cause serious infection when the skin barrier is wounded, but not always. Microorganisms on all barrier surfaces are continually monitored by the host's various immune responses. **Linehan** and co-authors found that the skin-dwelling organism *Staphylococcus epidermidis* specifically prompts an ancient arm of the immune system (the major histocompatibility complex class 1b molecule H2-M3) to respond in a way that avoids inflammation, which is distinct from responses to pathogens. H2-M3 processes and presents *S. epidermidis*-derived *N*-formyl methionine peptides to CD8+ T cells. These cells express immunoregulatory and tissue-repair gene signatures and accelerate skin wound healing. Hence, hosts and microbiota can interact in highly beneficial ways that may hold promise for therapeutic interventions.

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Eitan Israeli

Capsule

Context for immune responses

Immune cells function by recognizing pathogens and initiating a complex cellular response to mount a defense. People can show a wide range of genetically driven variation in responses to infection. **Alasoo** and colleagues investigated how the cellular environment drives change in non-coding regions associated with transcription in immune cells. Chromatin accessibility and gene expression change in pluripotent stem cell lines exposed to signals simulating bacterial infections. Genetic variants among these cell lines affected the timing of gene expression, depending on what they were exposed to. Interestingly, disease-risk variants associated with immune dysfunction, such as rheumatoid arthritis and inflammatory bowel disease, emerged from the analyses.

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Eitan Israeli

Capsule

How endothelial cells change identity

The development of healthy heart valves during mammalian embryogenesis requires that endothelial cells morph into a distinct cell type. When this identity change, called endothelial-to-mesenchymal transition (EndoMT), occurs inappropriately in adults, it can lead to disorders such as atherosclerosis, organ fibrosis, and pulmonary hypertension. To investigate the mechanisms regulating EndoMT, **Xiong** et al. studied cultured endothelial cells and mice deficient in a certain metabolic enzyme. They discovered that loss of endothelial fatty acid oxidation promotes EndoMT, most likely through changes in intracellular acetyl coenzyme A levels. These results suggest that therapies aimed at increasing fatty acid oxidation, including several drugs that already exist for other purposes, could potentially be used to treat disorders caused by aberrant EndoMT.

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Eitan Israeli