

mTOR Inhibitor Induces Autophagy and Reduces Tumor Vascularization to Prevent Tumor Growth of TP53 Mutant HNSCC

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ABSTRACT

Introduction: TP53 is the most frequently mutated gene in head and neck squamous cell cancer (HNSCC), comprising 80% of HPV- negative tumors. These patients have the worst clinical outcomes with 50-60% recurrence rates after definitive treatment. In a recent phase II clinical trial, adjuvant therapy with Everolimus (mTOR inhibitor) after complete response to definitive therapy was shown to significantly increase 2-year progression free survival in p53 mutated patients. Mutant p53 proteins acquire new biological properties referred to as gain-of-function (GOF) that contribute to cancer progression, treatment resistance, invasion, and metastasis. In solid malignancies, mutant p53 driven mTOR activation causes inhibition of autophagy and the cytoplasmic stabilization of Hif-1 α . Hif-1 α contributes to malignant progression by stimulating oncologic processes such as tumor angiogenesis, aerobic glycolysis and cell proliferation. Here, using cell lines and a tumor xenograft model, we investigated the effect of Everolimus on autophagy and Hif-1 α mediated tumor angiogenesis.

Methods: mutant TP53 HNSCC cell lines were grown in regular culture conditions, treated with Everolimus (100 ng/ml) for 24 hours, and protein and mRNA was isolated. Western blot, qRT-PCR, and immunofluorescence were employed to evaluate the proteins involved in autophagy, apoptosis and cell cycle. The Hif-1 α protein and its target proteins were also analyzed. In the xenograft model HN31 (p53 mutant) cells were injected in the flank of athymic nude mice. The treatment group received oral suspension of Everolimus (5mg/Kg in 1% CMC-Na), daily whereas the control group was given 1% CMC-Na only. Tumor volume and mice weight were monitored three times per week. Tumors were harvested and analyzed by western blot and immunohistochemistry to examine autophagy and tumor vascularization. Moreover, tumor lysate was analyzed by western blot to determine changes in Hif-1 α and VEGF-A.

Results: In vivo, Everolimus effectively reduced tumor volume and significantly increased survival of mice with HN31 tumors. The autophagic flux was increased by Everolimus treatment both in cell lines and in animal model. However, we did not observe induction of apoptosis when cells were treated with Everolimus. Hif-1 α protein level was effectively reduced by mTOR inhibitor treatment both in vitro and in vivo. Moreover, the level of VEGF-A was also downregulated by everolimus treatment. Immunohistochemistry of CD31 showed that microvascular density is significantly reduced by Everolimus treatment in HN31 tumor.

Conclusion: Everolimus induces autophagy both in vitro and in vivo in HN31 (p53 mutant) HNSCC. The Hif-1 α protein level was also downregulated by Everolimus both in cell lines and tumor xenograft. Tumor growth and tumor vascularization of p53 mutant HNSCC were also inhibited. Everolimus holds promise for the treatment of p53 mutant HNSCC.

BACKGROUND

Head and neck squamous cell carcinoma (HNSCC) is the 6th most common cancer worldwide.¹ In the United States, HNSCC accounts for more than 60,000 new cases and over 13,000 deaths per year.² TP53 is the most frequently mutated gene in HNSCC. Alterations in this gene play a significant role in the development, progression, and treatment resistance of HNSCC.³⁻⁶ mutTP53 HNSCCs exhibit poor overall survival and prognosis compared to wild-type TP53 (wtTP53), warranting an urgent need for effective therapy.⁷ Our phase-two clinical trial shows exciting results of HNSCC patients with mutant TP53 benefiting from Everolimus treatment. Everolimus treatment showed significant increase in the 2-year progression-free survival of TP53 mutant patients.

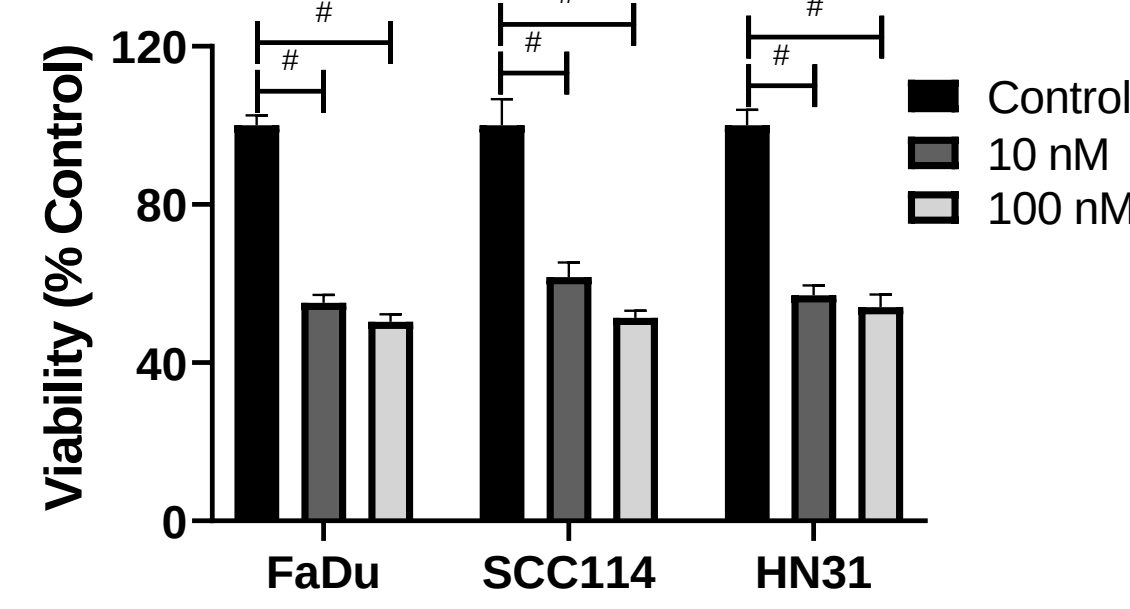
OBJECTIVES

To investigate the underlying molecular mechanisms of how Everolimus prevents tumorigenesis of HPV-negative TP53 mutant HNSCC:

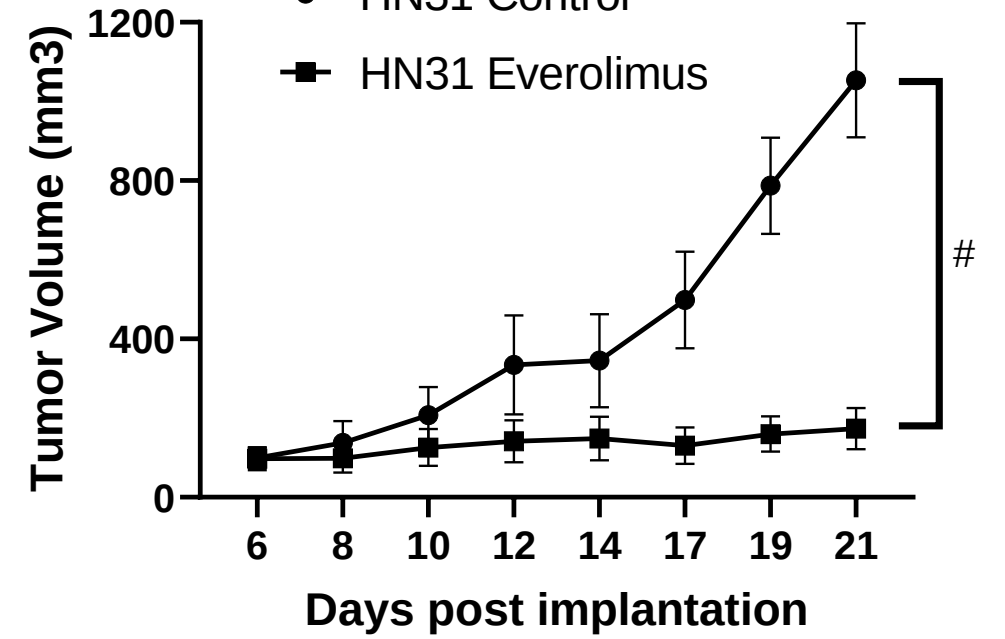
- Evaluate the effect of Everolimus on autophagy induction through activation of ULK1 to prevent tumor growth.
- Assess the effect of Everolimus on tumor angiogenesis through mTOR/pSTAT3/Hif-1 α /VEGF-A pathway.

METHODS AND RESULTS

(1A)

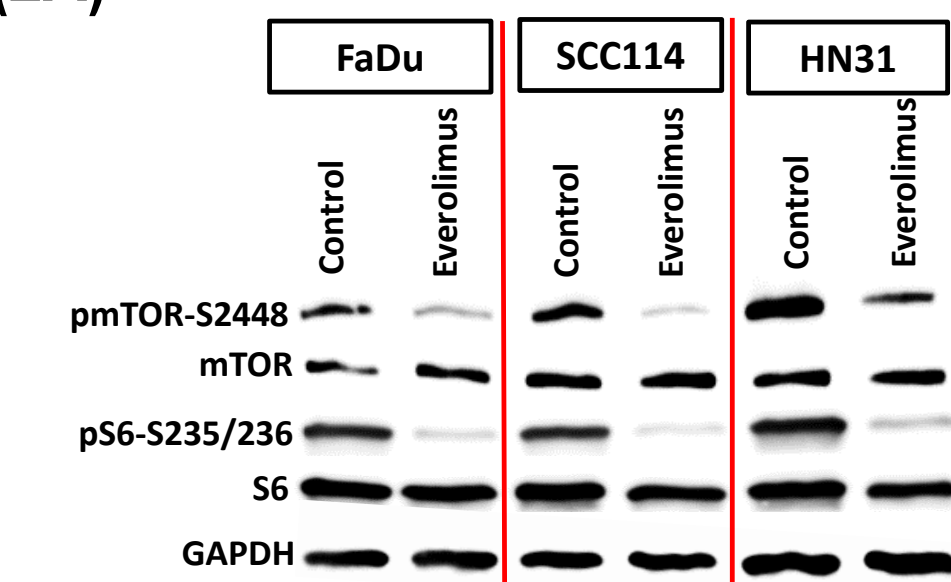


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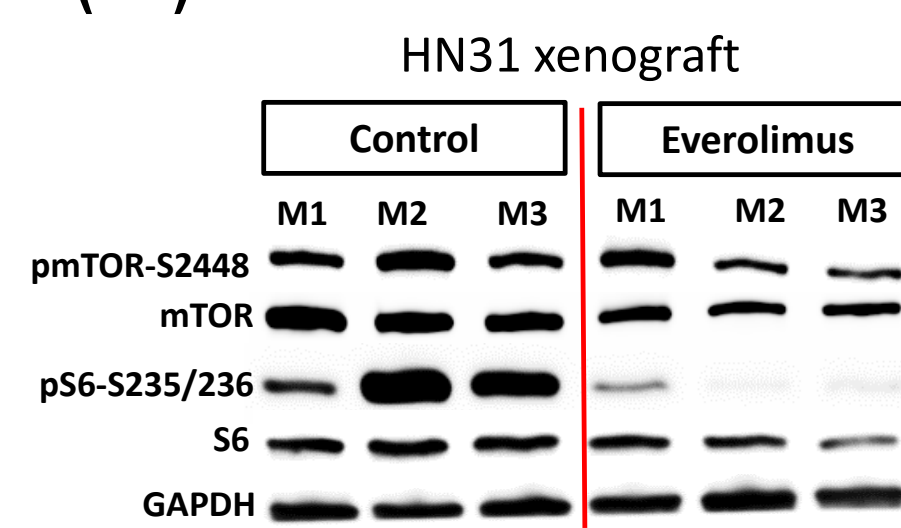


Everolimus inhibits cell growth of HPV-negative mutTP53 HNSCC. (1A) Cells were treated with 100 nM of Everolimus for 72hrs. Cell viability was measured using Cell Titer-Glo assay. **(1B)** Everolimus inhibits the tumor growth of HN31 cell line xenograft. 1x10⁶ of HN31 cells were injected into the flank of athymic nude mice. After five days, mice were treated with oral suspension of Everolimus (5mg/kg in 1% CMC-Na) for three weeks, and control mice received 1% CMC-Na.

(2A)

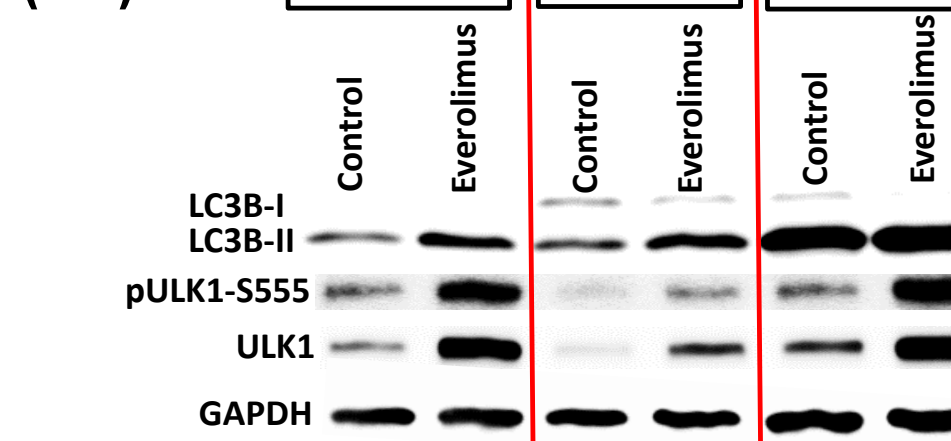


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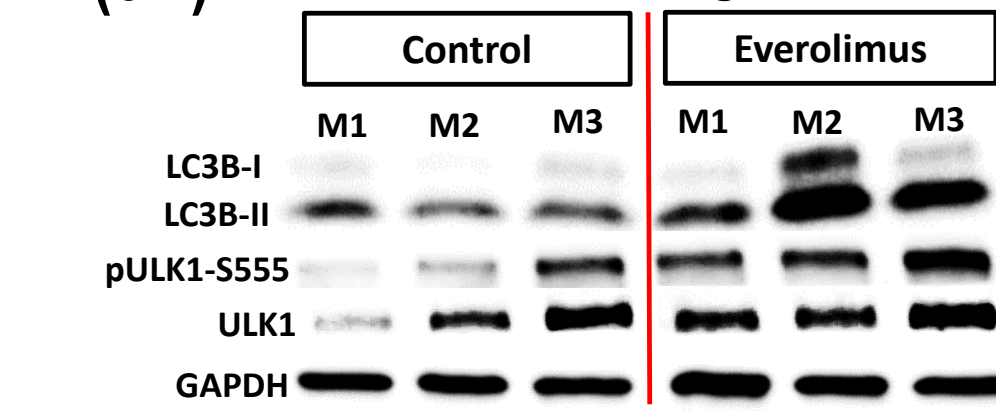


Everolimus inhibits mTORC1 signaling in HPV-negative mutTP53 cell lines and xenograft. HPV-negative mutTP53 HNSCC cell lines were treated with 100nM of Everolimus for 24 hours. **(2A)** Western blot for pmTOR S2448 and pS6 S235/236 in HPV-negative mutTP53 HNSCC cell lines. **(2B)** Western blot analysis for pmTOR S2448 and pS6 S235/236 in HN31 cell line xenograft treated with either vehicle (control) or Everolimus.

(3A)

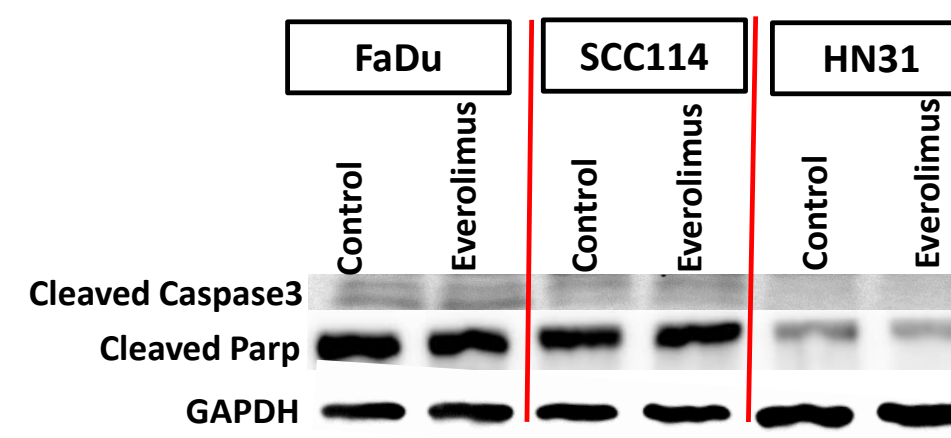


(3B)

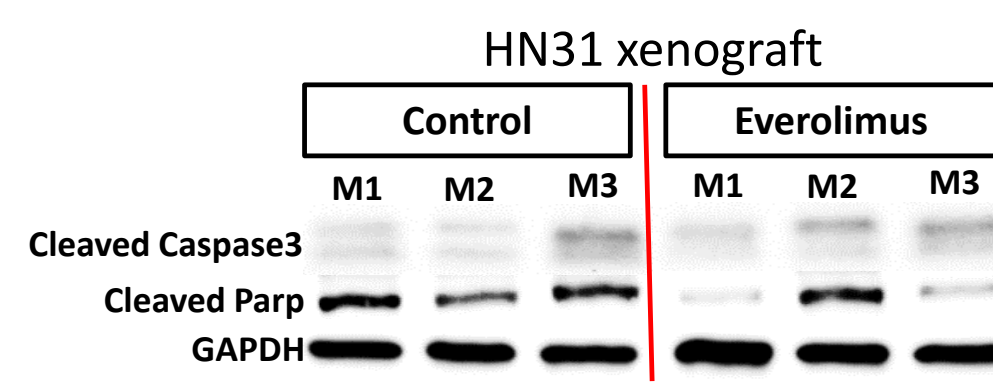


Everolimus induces autophagy in HPV-negative TP53 mutant cell lines and xenografts through activation of ULK1. (3A) Cells were treated with Everolimus (100nM) for 24hrs. Western blot of LC3B, P-ULK1 S555 and ULK1 was done. **(3B)** Western blot for LC3B, P-ULK1 S555, and ULK1 in HN31 cell line xenograft. Everolimus treatment induces autophagy, as evident from the upregulation of LC3B-II, with concomitant activation of ULK1.

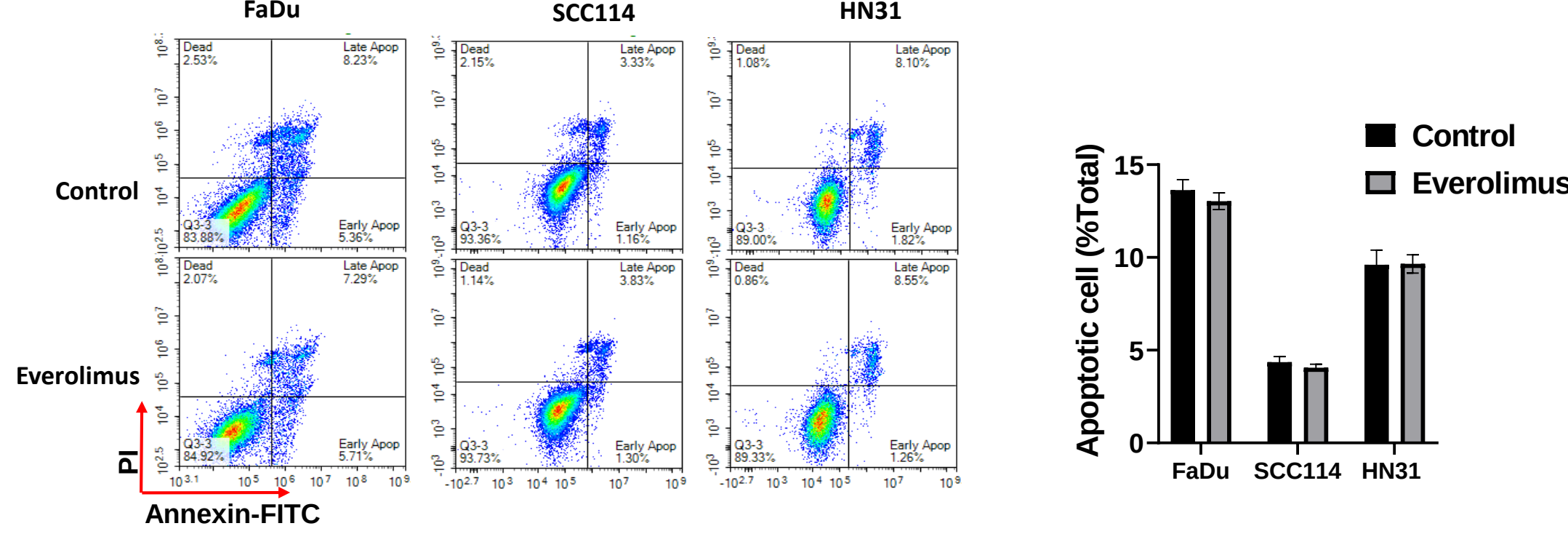
(4A)



(4B)

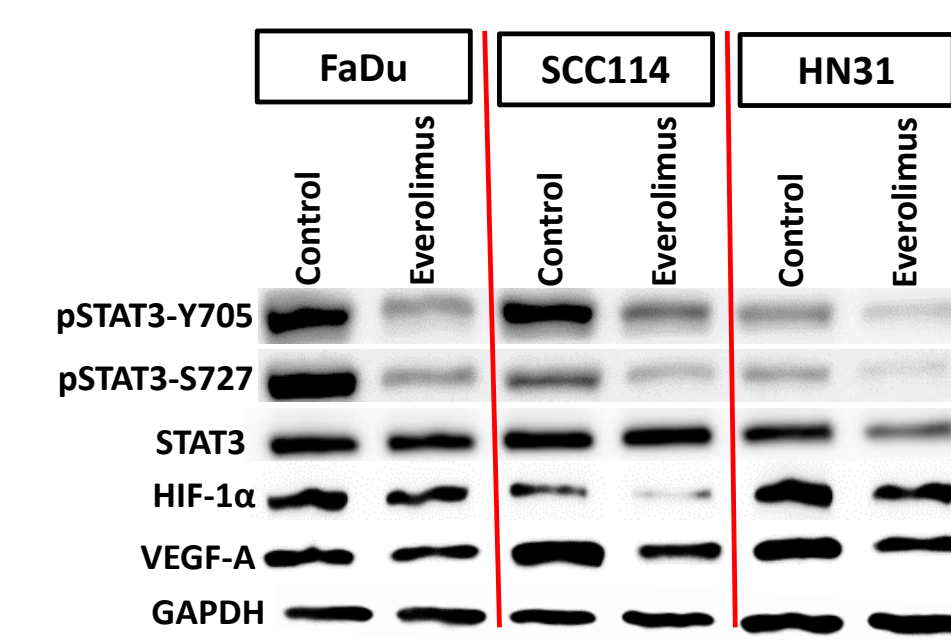


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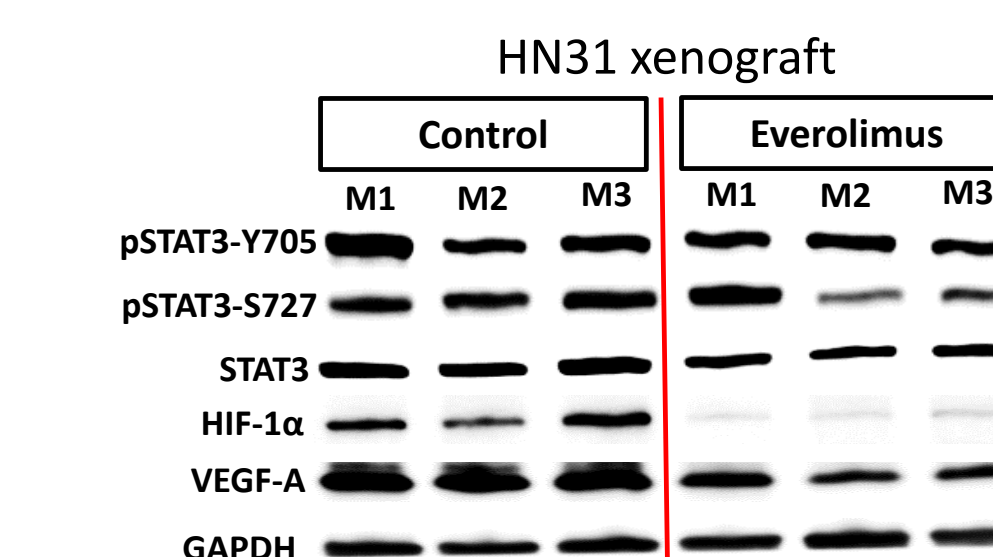


Everolimus does not induce apoptosis of HPV-negative TP53 mutant HNSCC cell lines and xenografts. (4A) Western blot of apoptotic marker proteins, cleaved Caspase-3 and cleaved PARP in FaDu, SCC114 and HN31 cell lines after the cells were treated with Everolimus (100nM) for 24 hours. **(4B)** Western blot of apoptotic marker proteins, cleaved Caspase-3 and cleaved PARP in HN31 xenograft **(4C)** Flow cytometry histogram and corresponding quantification for FaDu, SCC114, and HN31 cell lines after the cells were treated with Everolimus (100nML) for 72 hours.

(5A)

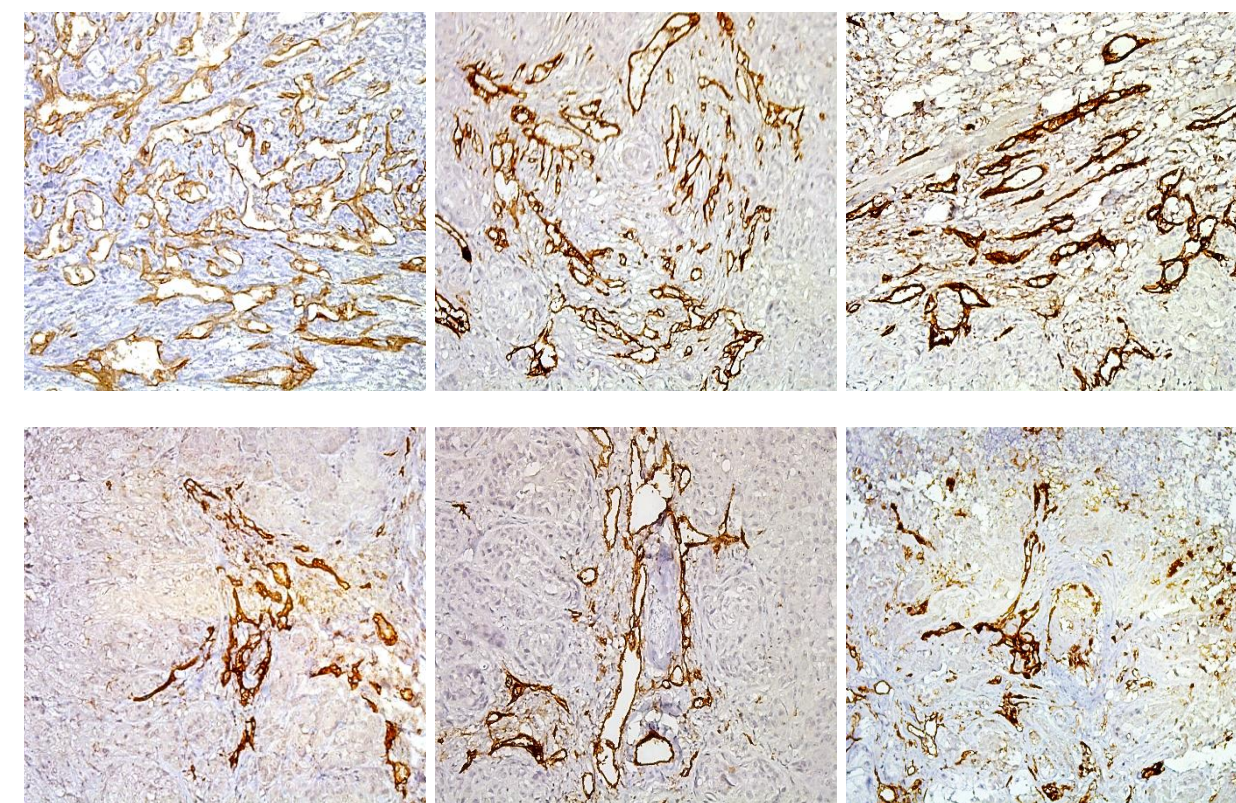


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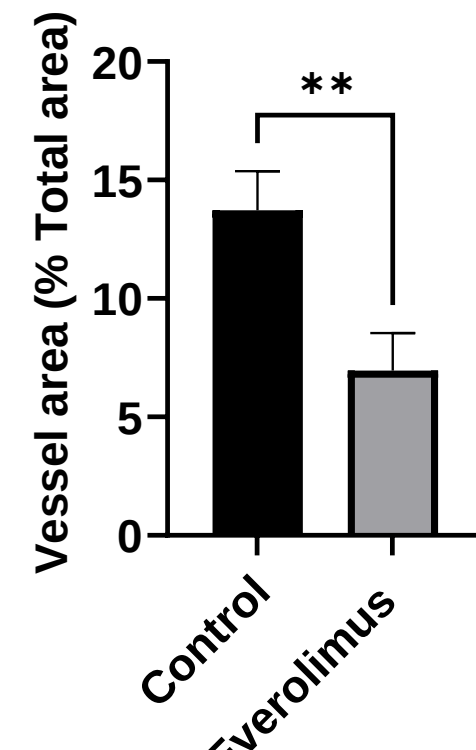


Everolimus downregulates Hif-1 α and VEGF-A levels in HPV-negative TP53 mutant cell lines and xenograft. (5A) Western blot analysis of STAT3, Hif-1 α and VEGF-A in FaDu, SCC114 and HN31 cell lines. **(5B)** Western blot of STAT3, Hif-1 α and VEGF-A in HN31 xenograft. Everolimus treatment reduces the level of pSTAT3 as well as Hif-1 α levels both in cell lines and xenograft. The level of Hif-1 α target protein, VEGF-A was also reduced by Everolimus treatment.

(6A)



(6B)



Everolimus inhibits angiogenesis of HN31 xenograft. Immunohistochemistry of CD31(200X) showing vessels in control and Everolimus-treated mice. **(6A)** The FFPE xenograft tissue was stained with anti-mouse CD31 (Cell Signalling Technology, cat#77699) **(6B)** Quantification of vascularized area as a percent of total area. Everolimus reduces the vessel (CD31 positive) area of HN31 xenograft.

CONCLUSIONS

- Everolimus effectively prevents the growth of HPV-negative TP53 mutant HNSCC both in cell culture and cell line xenografts.
- Everolimus induced autophagy through the activation of autophagy initiating protein ULK1, causing non-apoptotic autophagy-dependent cell death.
- Everolimus attenuated neovascularization through downregulation of mTOR/pSTAT3/ Hif-1 α /VEGF-A pathway.
- Our studies suggest a promising role for mTOR inhibitors in treatment of HNSCC patients exhibiting mutant p53 status.

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