

# Interrelationship of Autophagy and Oxidative Stress in Malignant Transformation of Oral Submucous Fibrosis

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## ABSTRACT

Oral submucous fibrosis (OSMF) is a potentially malignant disorder. Malignant transformation is a major concern related with OSMF. Despite array of studies discussed, pathogenic factors responsible for carcinogenesis in OSMF is still a field of research. Autophagy is a degradation of unwanted cytoplasmic material to maintain cellular biosynthesis when cell is under metabolic stress. Tissue microenvironment consists of diverse mechanisms. It is found that the autophagy has a major impact on these mechanisms to maintain homeostasis. However, dysregulated autophagy has been involved in disruption of the homeostasis and leads to formation of various diseases including cancer. Altered autophagy enhances oxidative stress in the cell which is a critical cause of genetic instability and oncogenesis. Plethora of studies supports that autophagy is implicated in carcinogenesis process and are interlinked with each other. In OSMF, areca nut causes increase in production of reactive oxygen species and formation of oxidative stress which is being wielded by autophagy. Furthermore, modulated autophagy has an impact on senescence and immunity which can lead to cancer. There is an explicit cause and effect relationship present between autophagy and oncogenesis in OSMF. It is our sincere efforts to elucidate this relationship in OSMF so that it can be incorporated in therapeutic purpose at an early stage.

**Keywords:** Autophagy, Carcinogenesis, Oral submucous fibrosis, Oxidative stress.

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## INTRODUCTION

Oral submucous fibrosis (OSMF) is a potentially malignant disorder. It is mostly seen in South Asian countries but recently has been recognized in Europe and North American countries.<sup>1,2</sup> It has the highest rate of prevalence (8.09%) among all the potentially malignant disorders.<sup>3</sup> Epidemiological data suggested that there is persistent increase in the number of cases in the recent time.<sup>2</sup> Oral submucous fibrosis confirms the significant cause of mor-

bidity to the patient. However, more concern is associated with high chance of malignant transformation. Long-term follow-up studies over a period of 17 years showed that the transformation rate of OSMF was 7.6%<sup>4</sup> and develop in 14% of OSMF lesions.<sup>5</sup>

Undoubtedly, areca nut is one of the major factors responsible for the malignant transformation.<sup>6</sup> It has been proposed that oral cancer arising in OSMF is clinicopathologically separate entity. It is believed that malignancy in OSMF originated from differential mechanisms of carcinogenesis.<sup>7</sup>

Recently, autophagy mechanism has gained an immense attention globally because of its implication in cancer formation. Autophagy is a self-degradative and highly modulated catabolic process.<sup>8</sup> It acts as a "housekeeping agent" by removing unwanted material.<sup>8</sup> It functions as a "cell survival mechanism" when cell is under stress, such as starvation, hypoxia, and chemo/radiotherapy. However, dysfunctional autophagy is implicated in a range of diseases, such as neurodegenerative disease, muscular disorder, diabetes, and cancer. Plethora of research has proved its role in oncogenesis in various types of cancers.<sup>9-13</sup> Alteration in autophagic mechanism leads to initiation and progression of cancer.<sup>9</sup> Many studies support the fact that oxidative stress and autophagy are interdependent on each other. Interaction of both the factors results in carcinogenesis.<sup>10</sup> Oral submucous fibrosis is the condition in which cells are continuously under oxidative stress, because areca nut induces reactive oxygen species (ROS).<sup>11</sup> Although pathogenesis of OSMF has been elaborated for many times, mechanism of malignant transformation is still unrevealed to some extent. Therefore, aim of this paper is to reveal the correlation between the effect of modulated autophagy and carcinogenesis process in OSMF, thus proposed a concept of cause and effect relationship between the same in OSMF.

## AUTOPHAGY PROCESS AND AUTOPHAGIC GENE

Autophagy is a Greek word and literally means "self-eating."<sup>8</sup> Autophagy is a bulk nonspecific degradation of cytoplasmic material which is transferred to lysosomes for degradation process. It is critically important for almost all the tissues.<sup>12</sup> It consists of formation and expansion of isolation membrane (phagophore) which is followed by fusion of the edges of phagophore to form autophagosomes.

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These autophagosomes then finally fuse with lysosome for degradation.<sup>8</sup> Some molecules act as initiators for autophagy, such as adenosine monophosphate-activated protein kinase, UNC51-like kinase 1, and mammalian target of rapamycin (mTOR) 1 complexes.<sup>13</sup> It is an adaptive catabolic process when the cell is under different form of cytotoxic stress, such as nutrient deprivation, growth factor depletion, and hypoxia.<sup>14</sup> When unwanted material starts to accumulate inside the cell, autophagy triggers and helps in discarding accumulated redundant material. It aids in removal of damaged organelles, aggregated protein, reduces ROS, and mitochondrial abnormality.<sup>15</sup> By doing this, it maintains quality control of macromolecules and energy homeostasis within the cell.<sup>8-12</sup> It enables the starved and dying cell to survive by fueling nutrient supply to the cell.<sup>9</sup>

There are more than 20 genes (called as Atg) which govern the execution of autophagy TOR kinase is the master negative regulator of autophagy.<sup>9</sup> It stimulates cascade of genes for induction of autophagy process.<sup>16</sup> Genetic alteration in autophagy gene may be hereditary and predisposes individual to various diseases, such as autoimmune and autoinflammatory diseases.<sup>17</sup>

Various experiments demonstrated that autophagy has a role in healthy as well as diseased conditions.<sup>8-12</sup> Studies supported its action in initiation, promotion, and progression of cancer.<sup>9-17</sup> Sannigrahi et al<sup>18</sup> showed that how modulated autophagy involved in formation of "apoptosis-refractory tumor" of head and neck cancer. It was showed that dysregulated autophagy can no longer be responsible for execution of accumulated unwanted protein results in stressed mitochondria, increase oxidative stress and thus forced the cell to enter into carcinogenesis process.<sup>15</sup>

Selective autophagy is a mechanism in which selective elimination of unwanted components, such as aberrant protein aggregate, lipid droplets, and dysfunctional organelles occurs.<sup>19</sup>

Selective autophagy is responsible for binding of one end of an ubiquitinated protein with molecular adaptor protein (p62, NBR1, NDP52, VCP, optineurin) and other end binds with autophagosome-specific proteins, such as the members of the LC3/GABARAP/Gate16 family. Selective autophagy usually functions in recognition of ubiquitinated protein for degradation.<sup>20</sup> Receptors like p62 (sequestosome 1/SQSTM1) are actively involved in the selective autophagy process.<sup>19</sup>

The p62 is a ubiquitously present cellular protein found in cellular inclusion bodies along with polyubiquitinated proteins.<sup>8,12,19-21</sup> LC3 is microtubule-associated protein light chain 3 which is encoded by the mammalian homolog of Atg8. It is expressed in most of the cell types as a full length cytosolic protein.<sup>8</sup> The p62 directly interacts with the LC3 through LC3 interacting regions and

subsequently integrates into autophagosomes for final degradation. The p62 is responsible for aggregate formation which is subsequently removed by autophagy.<sup>20</sup>

Beclin-1, the mammalian ortholog of yeast Atg6 (autophagic), is required for double-membrane autophagosome formation. It is associated with regulation of autophagic process.<sup>22</sup> It promotes the initial stages of autophagy via binding with autophagic protein, such as Bcl-2, p150, Vps34, and Atg14L.<sup>13</sup> Hama et al<sup>9</sup> found out that heterozygous deletion of Beclin-1 is associated with spontaneous formation of tumorigenesis. Therefore, reduced levels of Beclin-1 protein is related with increase in formation of epithelial malignancy.<sup>20,22</sup> Monoallelic loss of Beclin-1 results in ROS formation, unrepaired DNA damage, and genomic instability which culminate in tumor formation.<sup>9</sup>

Moreover, it was found that an initial event in carcinogenesis process causes inhibition of autophagic process. These events consist of loss of heterozygosity of Beclin-1 and over expression of antiapoptotic proteins (Bcl-2 family).<sup>17</sup>

Other autophagic (ATG) genes are related with different types of cancer, for example, ATG5 is associated with gastric, colorectal, and hepatocellular cancer. ATG2B, ATG5, and ATG9B (alone or in combination) are mutated in gastric and colorectal cancer.<sup>23</sup>

### Interrelationship of Autophagy and Various Factors

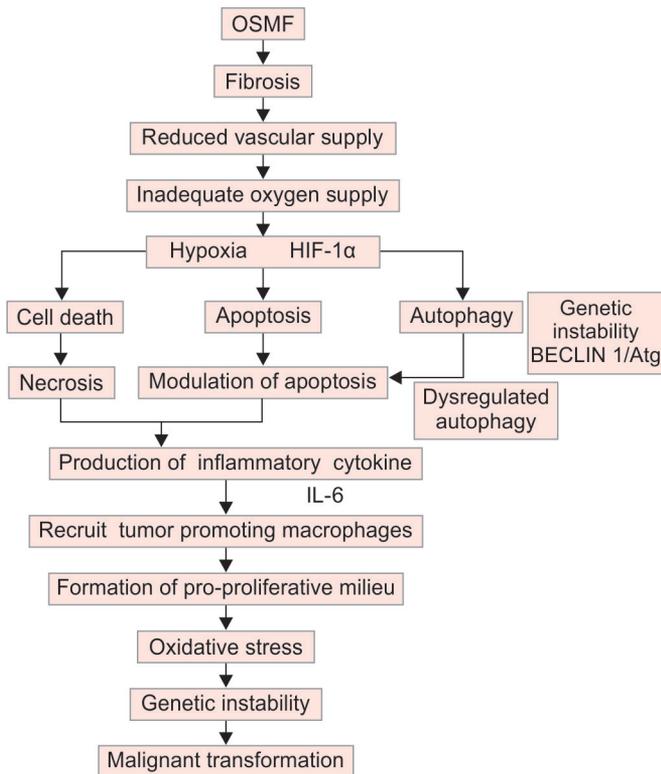
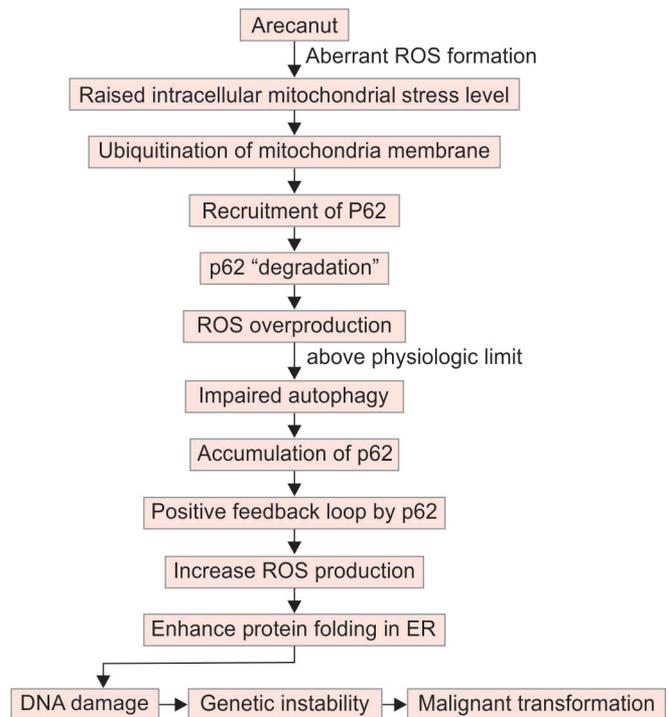
Oral submucous fibrosis is a precancerous condition associated with increase in fibrosis and inflammation.<sup>6</sup> It is potentially malignant disorder with significantly enhanced risk of cancer.<sup>4</sup>

In tissue, interaction and cross-talk occur between the cells and various mechanisms for maintaining microenvironmental homeostasis. Many modulating factors are present in the tissue microenvironment which perturb normal mechanism and make the cell more prone for cancer formation.

### Effect of Interrelation of Autophagy-Hypoxia in OSMF

In OSMF, production of collagen fibers increases while degradation of collagen decreases by 75% (Flow Chart 1).<sup>7</sup> Formation of bundles of collagen results in fibrosis. Compression of blood vessels leads to inadequate oxygen supply to microenvironment. Consequently, cell becomes hypoxic.<sup>6</sup>

Although hypoxia either stimulates cell to die or induce apoptosis, Zhang et al<sup>24</sup> demonstrated that hypoxic condition generates HIF-1 alpha-factor which initiates autophagy in oxygen-deprived cell through the expression of BNIP3 (a member of BH3 subfamily).

**Flow Chart 1:** Interrelationship of autophagy and hypoxia in OSMF**Flow Chart 2:** Interrelationship of selective autophagy and oxidative stress in OSMF

The study showed that when areca nut extract treated with fibroblast, leads to the release of cytokines, such as IL-6 and IL-8 which are responsible for the formation of ROS in the cell.<sup>6</sup> In hypoxic condition, autophagy triggers and removes accumulated oxidized material formed by ROS. It was found that genetic alteration in autophagy is inherited and can be inactivated by the changes occurring in somatic cells, for example, oxidative stress beyond physiologic limit. Inactivated autophagy disturbs the tissue homeostasis and supports the condition for cancer formation.<sup>17</sup>

Furthermore, when cell is under oxidative stress, impaired autophagy and apoptosis induced changes in the cell result in production of inflammatory cytokines and recruit tumor promoting macrophages at the site of lesion.<sup>25</sup> Consequently, it forms a pro-proliferative milieu for the cells by increasing oxidative stress which results in genetic instability and makes the cell prone to cancer formation.<sup>20</sup>

### Effect of Interrelationship of Selective Autophagy and Oxidative Stress in OSMF

Selective autophagy critically removes selected proteins from the cell. Areca nut induces aberrant ROS formation and accumulates oxidized material which leads to increase the stress level in oral keratinocytes (Flow Chart 2).<sup>11,26</sup>

In high oxidative stress, selective autophagy activates. Oxidative stress causes increase in intracellular mitochondrial stress level, ubiquitination of mitochondrial

membrane, and recruitment of p62 in the cell membrane. p62 delivers oxidized material to autophagosomes for degradation. As ROS production increases above its physiologic limit, impairment of autophagic mechanism occurs.<sup>20</sup> Study done on autophagy-deficient cells showed that recruitment of p62 will occur, but because of the absence of autophagy, cells are unable to clear ubiquitinated proteins and recruit p62. So, p62 starts accumulating. Increased accumulated p62 stimulates a positive feedback loop by which p62 itself leads to increased ROS production, enhanced protein-folding mechanism in ER responsible for increase in ER stress.<sup>27</sup> Unfolded protein starts to accumulate. If proteins persist, they are equivalent to noninherited mutation and responsible for the increased oxidative stress and genomic instability which are beneficial conditions for oncogenesis.<sup>28</sup>

Studies showed that cytoskeletal proteins (keratin family) present in the cell wall get degraded along with p62.<sup>29,30</sup> Nonkeratinized buccal mucosa expresses a range of cytokeratins like K4, K14 and keratinized palatal mucosa expresses K1, K10 type cytokeratin.<sup>5</sup> It is found that defect in autophagy results in aberrant intracellular accumulation of these proteins and can lead to increase in oxidative stress and genomic instability.<sup>28-30</sup>

Another mechanism for carcinogenesis by selective autophagy is by increase in expression of cancer forming cytokines. Impaired selective autophagy causes accumulation of misfolded proteins which cause prolonged expression of p62, as it is not able to remove aggregate. Thus, overall results in induction of aberrant growth

factors signaling [nuclear factor-kappaB (NF- $\kappa$ B)] thereby initiate molecular pathway for inflammation.<sup>20,31</sup> Concurrently, cell loses its control over cell growth and apoptotic process. It leads to inhibition of apoptosis, increase in cell survival which can alter the cell to transformed from benign to malignant one.<sup>20,31</sup>

### Effect of Interrelationship of Autophagy—Senescence in OSMF

Senescence acts as a preventive phenomenon in tumor formation as cells stop to divide at G1/S cell cycle phase.<sup>26</sup> Senescence is an early barrier for oncogenesis.<sup>17</sup> Premature senescence occurs because of multiple stresses, such as DNA damage and aberrant mitogenic signaling. One study reports that when human oral keratinocytes were treated with areca nut extract results in activation of p38MAPK, p16, p21, and NF- $\kappa$ B response which lead to formation of senescence-associated phenotypes (SAP) in more than 40% of treated cells.<sup>26</sup> A study revealed that senescence results in stimulation of autophagy and alters SAP into “senescence-associated secretory phenotype.” Above mechanism varies the expression of inflammatory cytokines, such as IL-6, IL-8, and attract immune system for immunosurveillance.<sup>20,32-34</sup>

It was found that defect in autophagy impedes the quality and efficacy of senescence. It delayed senescence by inhibiting expression of IL-6 and IL-8.<sup>33,34</sup> Moreover, impaired autophagy causes alteration in the execution of excess senescence cells and stressed cells, thus causes persistence of unwanted cells with enhanced oxidative stress. It overall results in increased pro-proliferative environment which has a positive impact on survival of these stressed cells to become malignant.<sup>20,35</sup>

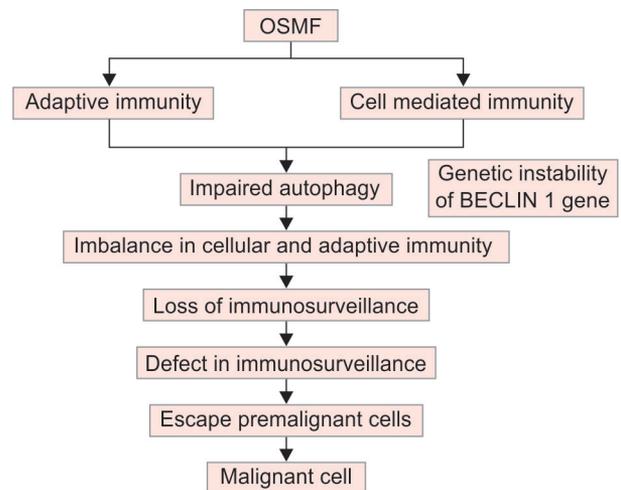
### Effect of Interrelationship of Autophagy—Immunity in OSMF

Autophagy represents a physiologic process of cross-presentation of antigens derived from either tumor cells or pathogens (Flow Chart 3).<sup>36</sup> It amended the antigenic properties of immune cells and varies their susceptibility to recognize antigenic cells.<sup>17</sup>

Study demonstrated that OSMF is associated with cell-mediated immunity. Evidence showed that OSMF was related with increase in the number of T cells and CD4+ cells.<sup>37</sup> Guruprasad<sup>38</sup> revealed that OSMF exhibits enhanced “adaptive type of immunity.” Nicotine induces increase in mucosal permeability and enhances salivary IgA levels which are thought to be a preventive and defensive mechanism of oral mucosa to toxins.

A study done by Li et al<sup>36</sup> showed that defective autophagy severely reduces the ability of presentation of endogenous tumor antigens in human embryonic kidney

**Flow Chart 3:** Interrelationship of autophagy and immunity in OSMF



cells and melanoma cells. Impaired autophagy minimizes intrinsic cellular immunity by autophagy-mediated degradation of intracellular pathogens, diminishes innate immunity by reducing activation of the innate immune cells, and restrains adaptive immunity by decreasing efficient cross-presentation.

Finally, it results in imbalance in adaptive immunity and cellular type of immunity.<sup>36</sup> This makes favorable condition for evasion of premalignant cells from immunosurveillance and pushes the cell in cancer formation.<sup>17</sup>

### Effect of Interrelation of Autophagy-p53 in OSMF

p53 plays a dual role in relation with autophagy. It acts a pro-autophagic nuclear factor and antiautophagic cytoplasmic factor. When damage to DNA occurs, p53 senses the damage results from instability of cell homeostasis and activates autophagy. It alleviates cell stress and prevents further DNA damage.<sup>9</sup> p53 try to prevent the alleviation of cellular stress by inducing autophagy. However, continuous oxidative stress and DNA damage suppress the p53 activation. However, continuous activated autophagy helps the cancer forming (DNA damaged) cell to proliferate. It was demonstrated that autophagy induces mammary tumor growth in DNA damaged cells by suppressing p53 activation.<sup>39</sup> Kavitha<sup>40</sup> demonstrated overexpression of p53 gene in OSMF. Increase in expression of p53 stimulates continuous autophagy, at one point above basal cell level, suppression of p53 occurs while activated autophagy would help the premalignant cells to survive.<sup>39</sup>

### Effect of Interrelationship of Autophagy-MicroRNA in OSMF

MicroRNA (miRNA) has been associated with regulation of autophagy process. Autophagy plays an important role in miRNA homeostasis. When cells faced stress condition, they reprogrammed their gene expression through

“miRNA involved signaling pathways.” It was found that miRNA was upregulated in autophagic condition through dysregulation of mTOR pathway.<sup>41</sup> According to research done, miRNA was found to be critical regulator of pathogenesis and malignant transformation of OSMF. Increase in miRNA expression was associated with malignant transformation of premalignant condition.<sup>41</sup> In OSMF, when hypoxic condition increases above the cellular level, autophagy activated and enhances above basal level, miRNA increases through dysregulation of mTOR pathway, and subsequently leads to tumorigenic condition.

## CLINICAL IMPLICATIONS

Exploiting autophagic process provides a potential approach for prevention and treatment in cancer therapy. Pathways which involve autophagic process and malignant transformation of OSMF have to be targeted. As autophagy is more beneficial cell death as compared to necrosis, autophagy targeted anticancer therapy is used as potential approach to overcome carcinogenesis.<sup>42</sup>

Autophagy-mediated stress tolerance helps in facilitation of cell survival by sustaining energy production which results in tumor growth and therapeutic resistance. In such conditions, inhibition of autophagy by utilizing its antitumorigenic property will aid cessation of cancer. Furthermore, it was demonstrated that autophagy inhibition refurbishes chemosensitivity and enhances anticancer potential of various drugs.<sup>43</sup>

Another important mechanism by which exploitation of autophagy process can be achieved is by activation of autophagic process. Experiments proved that autophagy directly targets and kills the “converted cancer cells” which are more apoptosis resistant.<sup>43</sup>

Some autophagy modulator drugs are available, for example, gefitinib acts as a tyrosine kinase inhibitor (nasopharyngeal carcinoma); YC-1, AEW541 are hypoxia modulator (pancreatic cancer), which were utilized as an anticancer therapy.<sup>9</sup> While some anticancer approaches are in the form of “gene target therapy.” Autophagy in the form of drugs can be implemented as an autophagy modulator; for example, ATG12 protein helps in maintenance of mitochondrial homeostasis and apoptosis.<sup>15</sup> More substantial and profound genetic sequencing of autophagic genes are required to help in assessing its role in carcinogenesis, so that mutational scenario of related genes is more specifically defined.

To inhibit or enhance the process of autophagy completely depends on the tissue microenvironment mechanisms.<sup>43</sup> It will be beneficial to assess the process of autophagy to evaluate the condition of affected cell which will help in cancer prevention at an early stage.

## CONCLUSION

Oral submucous fibrosis is associated with the high risk of cancer. Initiation and promotion of cancer is dependent on dynamic surrounding microenvironmental mechanism. Lots of evidence-based researches support the fact that autophagy is “potent modulator” of factors and responsible for carcinogenesis in various cells. It is very important to emphasize that, as many processes are present in the cellular microenvironment, autophagy found to govern the control of progression of most of the mechanisms and are mutually interdependent. However, research work on autophagy in oral lesions is at a very preliminary stage. It is evident from the research that a definite cause and effect relationship is present between altered autophagy and carcinogenesis in OSMF. It bears a mutual interrelationship for oncogenesis. Hence, we explored and highlighted the connection between autophagy and its consequence in cancer formation in OSMF. More research work is needed in this particular field so as to establish the above fact and redefine the modulatory factors for cancer formation in OSMF. It will help in generating more precise targeted therapies at an early stage of cancer formation in OSMF.

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