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Manipulation of autophagy by *MIR375* generates antitumor effects in liver cancer

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The exploration into the roles of autophagy in tumorigenesis, either as tumor suppressor or tumor promoter, has led to a great increase in the knowledge of cancer development, progression and treatment. However, there is currently no consensus on how to manipulate autophagy to improve antitumor effects. In this study, we investigated the role of autophagy in established liver cancer cells in response to hypoxia. Hypoxia not only is the most pervasive microenvironmental stress in solid tumors but is also a canonical stimulus for autophagy. The involvement of dysregulated microRNAs in hypoxia-induced autophagy and their therapeutic potential in advanced liver cancer were examined.

Keywords: autophagy, microRNA, hepatocellular carcinoma

Abbreviations: miRNA, microRNA; MIR375, microRNA 375; HCC, hepatocellular carcinoma; mRNA, messenger RNA; GFP, green fluorescent protein; LC3, microtubule-associated protein 1 light chain 3; ATG, autophagy-related; UTR, untranslated region; CASP3, caspase 3

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Although autophagy was first described more than 40 years ago, understanding its function in tumorigenesis mainly comes from investigations over the last decade. The roles of autophagy in cancer are not static and unchangeable but dynamic and highly complex. Generally, autophagy acts as a tumor suppressor during the initial stage of tumorigenesis by eliminating unwanted cellular components and thus maintaining genomic stability. However, once a tumor is established, autophagy becomes a mechanism for cell survival in response to various stresses and in turn promotes tumor growth. Hypoxia represents one of the most pervasive microenvironmental stresses in solid tumors due to overactive growth and inadequate blood supply. It is also a canonical stimulus for autophagy activation. MicroRNAs (miRNAs) are endogenous ~22-nucleotide RNAs that suppress gene expression by messenger (m)RNA cleavage and/or translational repression. It is of interest that tumor cells usually contain abnormal miRNA expression patterns. The purpose of our study was to explore whether dysregulated miRNAs in primary liver cancer [hepatocellular carcinoma (HCC)] are involved in regulation of autophagy under hypoxic conditions and to determine which miRNAs could modulate autophagy as a potential therapeutic strategy.

We first confirmed that autophagy facilitated liver cancer cell survival under hypoxia. In contrast, inhibition of autophagy decreased survival of these cells, suggesting that a therapeutic advantage may be derived from miRNAs which impair autophagy activity in liver cancer cells, under hypoxic conditions. We found multiple dysregulated miRNAs with greater than 20-fold changes in expression in liver cancer cells, compared with normal hepatocytes. One downregulated miRNA in HCC, microRNA375 (*MIR375*), showed a clear inhibitory role in autophagy activation under hypoxic conditions. As indicated by a primary screening, overexpression of *MIR375* showed potential effects on autophagy modulation, evidenced by changes in microtubule-associated protein 1 light chain 3 (LC3)-II production under hypoxia. We found that *MIR375* inhibited autophagosome formation, as indicated by the decreased number of green fluorescent protein (GFP)-LC3 puncta. In addition, both LC3-I and LC3-II detection and bafilomycin A_1 assays supported that *MIR375* blocked the conversion of

LC3-I to LC3-II, which is required for the elongation of the phagopore and in turn, the formation of the autophagosome. Furthermore, the results from transmission electron microscopy provided a visual representation of the inhibitory role of *MIR375* in autophagy.

Together, these findings indicated that *MIR375* blocked autophagy in liver cancer cells under hypoxia at the start of the autophagy pathway, i.e., at the conversion of LC3-I to LC3-II. Our interests then turned toward exploring the specific mechanism by which *MIR375* inhibited autophagy. To this end, we assessed the effects of *MIR375* on multiple important autophagy-related genes. We found that *MIR375* markedly suppressed the expression of autophagy-related (ATG) 7 at both the mRNA and protein levels. Prediction of *MIR375* targets indicated that there was indeed a binding site for *MIR375* at the 3'-untranslated region (UTR) of *ATG7* (miRanda database), and a putative miRNA binding site test supported that *MIR375* suppressed *ATG7* in a direct manner by binding its 3'-UTR. Furthermore, the increased expression of ATG7 correlated well with the downregulation of *MIR375* in human liver cancer tissues compared with their background counterparts. These results supported that *ATG7* is a key autophagy related target regulated by *MIR375*. ATG7 is required for the critical steps of autophagy activation by participating in two ubiquitin-like conjugation systems: The conjugation of ATG10 to ATG12 and the conjugation of ATG3 to LC3. Both are necessary for the conversion of LC3-I to LC3-II and for the expansion of the autophagosome. The suppression of *ATG7* by *MIR375* elucidated how *MIR375* is able to block the conversion of LC3-I to LC3-II and thus to inhibit autophagy.

We and others have found that restoration of *MIR375* function impairs tumor cell viability. We next investigated whether the suppression of ATG7 contributed to the antitumor effects of *MIR375* by inhibiting hypoxia-induced autophagy in liver cancer cells. We showed that knockdown of *ATG7* suppressed mitophagy, i.e., mitochondrial autophagy, which is predominantly activated under hypoxic conditions and serves as a mechanism for the turnover of damaged mitochondria induced by hypoxia. Subsequently, the proapoptosis protein cytoplasmic cytochrome c, released from damaged mitochondria, was increased after *ATG7* knockdown and this was accompanied by increased cleaved caspase 3 (CASP3), an apoptosis indicator. These in vitro data supported that under hypoxic conditions, autophagy acts as an adaptive mechanism and protects tumor cells from metabolic stresses.

Finally, to explore the role of *MIR375* in autophagy modulation in vivo and to evaluate its therapeutic potential, we used a *MIR375*-encoded Lentivirus to stably restore the expression of *MIR375* in liver cancer cells, and established xenograft tumors using those cells in nude mice. We found that tumor tissues harboring rescued expression of *MIR375* showed less expression of ATG7, inhibition of autophagy, and retarded tumor growth, consistent with the results in vitro. Thus, our in vitro and in vivo studies highlighted the role of *MIR375* in autophagy modulation, implying a therapeutic potential for *MIR375* in antitumor effects through the manipulation of autophagy.

Our research supported that although autophagy may have a dual role in tumorigenesis, autophagy can play a protective role in established liver cancer cell survival under hypoxic stresses. As recent studies show that blocking autophagy can enhance the efficacy of chemo-drug treatment for liver cancer, inhibition of autophagy may be a novel therapeutic avenue to pursue in advanced liver cancer. Restoring *MIR375* expression may also serve as a promising combination modality with other chemo-drugs, such as Sorafenib, which induces autophagy and thus may lose its anticancer drug efficacy due to this effect. In summary, our work highlighted the role of miRNAs in autophagy modulation in advanced HCC under hypoxic stress. Further studies will be required to develop a more efficient and safer delivery approach for using microRNA therapy in patients with malignancies.

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