Expression patterns of LC3A and LC3B in normal brain and glioblastoma cell lines, and the effect of the inhibition of autophagy on cell radio-sensitization.

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Introduction

Autophagy is a significant intracellular pathway that contributes to the recycling of damaged proteins and organelles via a process of engulfing them in autophagosomes and fusing with lysosomes[1]. Moreover, autophagy could play a key role on cancer cell resistance to radiotherapy. In this study, we investigated the expression pattern of two of the most significant autophagic proteins, LC3A and LC3B, in two glioblastoma cell lines, T98G and U87MG compared with normal brain[2]. Furthermore, the expression of these proteins were suppressed using siRNA and the cells radio-resistance was studied. Finally, after suppression of these proteins with specific LC3A and LC3B siRNA interference we noticed a radiosensitizing effect, following establishment of dose-response curves using the Alamar Blue® viability assay.

Materials & Methods

Western Blotting

Forty micrograms of total protein derived from whole fraction of T98G and U87MG cells and normal brain were separated on 12.5% discontinuous SDS gels. Then, proteins transferred on PVDF-PSQ membranes (0.2 μm pore size, Millipore Corp., ISEQ00010) and membranes blocked with 5% non-fat dry milk in TBS- Tween 20 at room temperature for 2 hours while they were hybridized overnight at 4°C with rabbit polyclonal antibody to MAP1LC3A (1:1.000; Abcam, ab62720) and mouse monoclonal antibody to MAP1LC3B (1.1.000, Nanotools, 5F10), respectively. Finally, membranes were hybridized for 2 h at 37ºC with the secondary antibody, goat anti- rabbit IgG (1:3.000, Biorad) or with goat anti-mouse IgG (1:3.000, DAKO) conjugated to HRP. The bands of these proteins were detected using Chemidoc MP, Imaging System, Biorad.

Silencing of LC3A and LC3B

A pool from four specific siRNAs for MAP1LC3A and from three specific siRNAs for MAP1LC3B were used in 50nM in order to suppress the expression of these two proteins in U87MG and T98G cell lines. 150000 cells were seeded in six-well plates and were incubated for 24h with transfection complexes which were formed after a co-incubation of HiPerfect®, Qiagen, Germany and siRNA, GenePharma, China for 10 minutes at room temperature. Then, the transfection mix were removed and cells were cultured in DMEM for 24h more. Thus, after a 48h incubation a whole fraction of proteins was collected for each of these samples in order for silencing to be confirmed using Western Blot analysis. After this confirmation, the protocol for siRNA in 96-well plates of Qiagen was used in order to test the changes of cells sensitization to radiotherapy[3].

Alamar Blue® assay

Cells were plated in 96-well plates. Fluorescence was measured with Fluo-star (OMEGA) and irradiation of the plates was performed using the 6MV beam of a linear Accelerator PRECISE (ELEKTA) endowed with Multileaf Collimator[3]. The Alamar Blue® assay used to assess cell viability. Alamar blue® in 10% v/v concentration was added and seven hours later fluorescence was measured. Analysis is based on both:

$$_{i/in}\text{RFU-ratio} = \frac{\text{RFU irradiated} - \text{RFU negative controls}}{\text{RFU non-irradiated} - \text{RFU negative controls}}$$

$$\%\text{ABr} = \frac{\text{RFU irradiated} - \text{RFU negative controls}}{\text{RFU fully reduced} - \text{RFU negative controls}}$$

RFU : the relative fluorescence units

RFU ratio : the ratio of RFU recorded from a well divided by the RFU recorded from an other well of reference

%ABr :absolute % Alamar Blue reduction

Results

Expression pattern of LC3A & LC3B

In this research study we investigate the expression levels of LC3A and LC3B in two glioblastoma cell lines, U87MG and T98G, and normal brain (Figure 1).

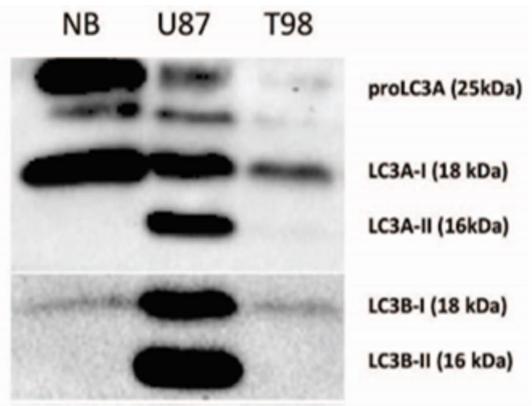


Figure 1: Expression patterns of LC3A and LC3B in normal brain, U87MG and T98G.

A strong over-expression of proLC3A and LC3A-I is observed in normal brain, contrary to LC3A-II and LC3B that is weakly expressed. LC3A–II and LC3B are highly expressed in U87MG, but not inT98G.

Confirmation of LC3A & LC3B suppression

Western Blot was used in order to confirm the suppression of LC3A and LC3B. It is observed that there is 95% suppression of LC3A and LC3B in T98G cell line while there is approximately 80% suppression of LC3A and 90% suppression of LC3B in U87MG cell line (Figure 2).

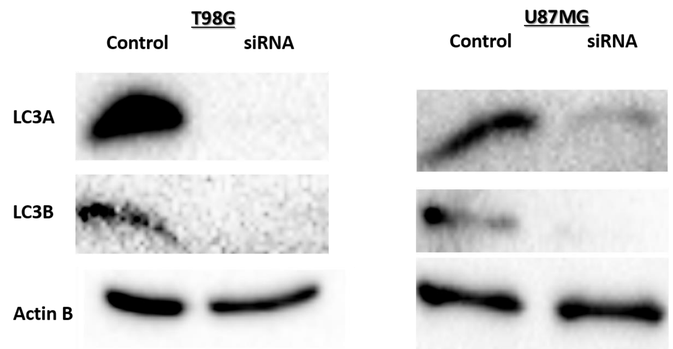


Figure 2: Confirmation of LC3A and LC3B suppression in U87MG and T98G, using Western Blot.

Study of T98G and U87MG radio-sensitization after LC3A & LC3B suppression

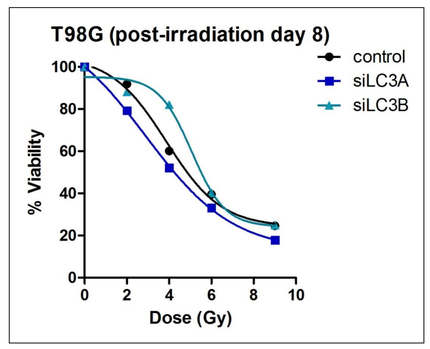
Protocol for suppression of LC3A and LC3B was used for T98G and U87MG cells followed by treatment with multi-doses of radiation[3]. Cells viability was measured seven days after radiation treatment using Alamar blue® assay.

Cell viability curve for T98G shows that 3Gy is the IC50 value for cells which were treated with siLC3A while the untreated cells exhibit an IC50 value near to 4Gy. On the other hand, suppression of LC3B does not affect on sensitization of T98G to radiotherapy (Figure 3a).

In U87MG cell line, it is observed that both of two suppressions influence the cell resistance on radiation. Analytically, siLC3A reduces IC50 by 1.5Gy while siLC3B 1Gy, approximately (Figure 3b).

Results

a)



b)

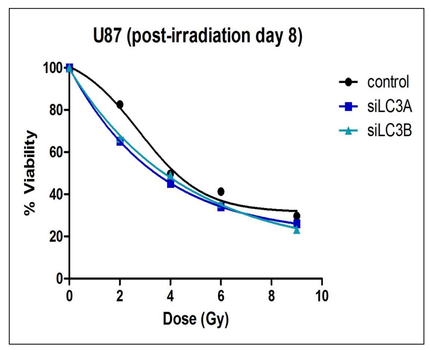


Figure 3: Survival curves of a) T98G and b) U87MG cell line after radiation and treatment with siRNA

Conclusions

- Normal brain expressed high levels of proLC3A and LC3A-I
- The U87MG cell line had high expression levels of LC3A-I, LC3A-II and LC3B-I and LC3B-II
- In the T98G cell line there was a weak expression of both LC3A and B
- Suppression of LC3A and LC3B sensitizes U87MG cells to radiation.
- Suppression of LC3A, but nor of LC3B, sensitizes T98G to radiation. This phenomenon could be explained by low levels of LC3B autophagic flux in T98G

References

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2. Kabeya Y, Mizushima N, Ueno T *et al.* LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J*. 2000;19, 5720-5728.
3. Abatzoglou I., Zois C.E., Pouliliou S., Koukourakis M.I., Establishment and validation of a method for multi-dose irradiation of cells in 96-well microplates. *Biochem. Biophys. Res. Commun.* 2013 Feb;15;431(3):456-9.