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AUTOPHAGY IN LUNG CANCER

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Abstract

Lung cancer is the leading cause of cancer-related deaths worldwide. The relatively poor cure rate in lung cancer patients has been associated with a resistance to chemotherapy and radiation that is at least in part related to defects in cellular apoptotic machinery. Exploitation of another form of cell death, autophagy, has the capacity to improve the therapeutic gain of current therapies. In an effort to develop novel treatment strategies to enhance the therapeutic ratio for lung cancer, we wish to better understand the role of autophagic cell death for the sensitization of lung cancer. This text reviews the most up to date protocols and techniques for the study of autophagic cell death in lung cancer models. Others may use these techniques as a framework for study within their experimental models.

1. INTRODUCTION

Lung cancer is the most prevalent cancer worldwide. Despite improvements in multimodality therapy, it continues to be the leading cause of cancer-related death in the United States, with an estimated 161,840 deaths in 2008 (Jemal *et al.*, 2008). The majority of lung cancer patients present with advanced disease, and standard therapeutic regimens include radiation, platinum-based chemotherapy, and, rarely, surgery. Though there have been advances in radiation delivery, enhanced platinum pharmacological profiles, introduction of targeted biological agents and optimization of treatment schedules, there is still significant room for improvement in both the prognosis and side-effect profiles for this patient population (Rigas and Kelly, 2007).

A major barrier to curative therapy in lung cancer is the dysregulation of cell death signaling (Abend, 2003; Fesik, 2005; Melet *et al.*, 2008; Moretti *et al.*, 2007). Of the cellular death processes, apoptosis has been the best studied. Multiple investigators have demonstrated that deficits in apoptotic machinery can lead not only to abnormal proliferation but also to insensitivity to cytotoxic therapy. In fact, there have been efforts over the past decade to enhance apoptosis utilizing various activating antibodies, peptides and small molecules to restore the apoptotic machinery of tumor cells for the purpose of triggering cell death or rendering tumor cells more sensitive to chemotherapy and radiation (Fesik, 2005).

Autophagic (type II or macroautophagic) cell death was described as early as the 1970s, as a cell death mechanism that can occur independent of apoptosis (Clarke, 1990). It is an evolutionarily conserved process, which morphologically involves the formation of double-membrane-bound autophagic vacuoles, called autophagosomes (Baehrecke, 2002; Reggiori *et al.*, 2004a,b). These autophagosomes degrade and recycle proteins and cellular organelles by fusing with lysosomes to form autolysosomes (Levine and Klionsky, 2004). The role of autophagy in mammalian cell death is suggested by a study that implicates the autophagic genes, *ATG7* and *beclin 1* Shimizu *et al.*, 2004; Yu, 2004). These studies not only demonstrate an antagonistic interplay between apoptosis and autophagic cell death but also suggest that they are nonexclusive events that can at times be observed in the same

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senescent cell (Gonzalez-Polo *et al.*, 2005; Shimizu *et al.*, 2004; Yu, 2004). The role of autophagy in cell death is still controversial, however, and a recent study finds that *ATG7* and *beclin 1* act in a protective manner in the same system (Wu *et al.*, 2008).

Autophagy has multiple roles in the promotion of carcinogenesis. It ensures survival in nutrient-poor conditions through lysosomal recycling of intracellular nutrients, which is proposed to allow time for the development of adaptive changes in gene expression and metabolic activity (Ogier-Denis and Codogno, 2003). Additionally, it may promote evasion of chemotherapy and radiation-induced apoptosis through removal of damaged organelles (Boya *et al.*, 2005; Lum, 2005; Ogier-Denis and Codogno, 2003; Paglin *et al.*, 2001). Although the autophagic response to starvation is less pronounced in cancer cells, it is still up-regulated in many tumor types suggesting it is an important survival mechanism (Gozuacik and Kimchi, 2004; Ogier-Denis and Codogno, 2003).

Suppression of carcinogenesis has also been proposed to be an important feature of autophagy. Cancer cells that are unable to undergo apoptosis secondary to genetic mutations are still susceptible to autophagic cell death (Paglin *et al.*, 2001). Additionally, some cancer cells are dependent on a blockade of autophagy for maintenance of their malignant phenotype (Liang *et al.*, 1999). This hypothesis was tested in several experimental models of hepatic carcinogenesis, where preneoplastic nodules and frank hepatocellular carcinomas demonstrate decreased autophagic capacity as compared to normal liver cells (Canuto *et al.*, 1993; Schwartz *et al.*, 1993; Schwarze and Seglen, 1985). It is proposed that this decreased autophagic activity associated with malignant cells may be related to the prevention of excessive protein loss upon starvation of tumor cells (Canuto *et al.*, 1993; Schwarze and Seglen, 1985).

Three are a number of cancer therapies that currently induce autophagy (Table 14.1). One of the most notable of these is temozolomide, which is a DNA alkylator that induces autophagy in malignant glioma cells (Kanzawa *et al.*, 2004). This is the first chemotherapeutic agent that has demonstrated efficacy in this difficult population. In addition to temozolomide, there are multiple therapies that are known to induce autophagy. These include the class of histone deacetylase inhibitors (Shao *et al.*, 2004), arsenic trioxide (Kanzawa *et al.*, 2003, 2005), tamoxifen (Bursch *et al.*, 1996, 2000), rapamycin (Takeuchi *et al.*, 2005), and irradiation (Ito *et al.*, 2005; Paglin *et al.*, 2001; Yao *et al.*, 2003). These drugs may actually cause autophagic cell death. For example, 3-methyladenine (3-MA) is an inhibitor of autophagosome formation and prevents tamoxifen-induced cell death in breast cancer cells (Paglin *et al.*, 2001). However induction of autophagy does not necessarily signify a causal therapeutic benefit. For example, 3-MA treatment results in radiosensitization with increased cell death observed in irradiated malignant glioma cells (Ito *et al.*, 2005). In the case of irradiation, induction of autophagy may represent a survival mechanism, or the balance of cellular decisions is tipped toward apoptotic death.

The rapid evolution in the study of autophagy has prompted a recent publication to establish guidelines to more clearly establish a basic foundation for the understanding of autophagic processes.(Klionsky *et al.*, 2008) The authors outline the current tools that at a minimum should be used by investigators to determine whether full autophagy (autophagic flux) has occurred, rather than accumulation of markers of autophagy, such as the autophagosome. Our text will outline these protocols with a special emphasis on the study of lung cancer models.

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2. METHODS

2.1. Cell culture

We utilize multiple cell lines in the study of cell death in lung cancer tumors. Primary mouse embryonic fibroblasts (MEFs) are a useful tool in the study of cell death mechanisms within various genetic backgrounds. We use MEFs with deficiencies in various key apoptotic proteins (e.g., caspases, Bcl-2 family members) to characterize cell death in response to irradiation with and without other cytotoxic agents (Kim *et al.*, 2008a,b). To complement these studies, we also evaluated well-characterized human lung cancer cell lines (e.g., H460) *in vitro* and *in vivo*. As neoangiogenesis also plays an important part in tumor control, we perform experiments with human umbilical vein endothelial cells (HUVECs) as a surrogate for tumor blood vessel response (Albert *et al.*, 2006; Cao *et al.*, 2005; Kim *et al.*, 2008a,b).

1. MEFs are derived from wild-type (WT), caspase-$3^{-/-}/7^{-/-}$ double knockout (DKO) mice and Bax-Bak DKO mice. They are then immortalized by transfection with a plasmid containing SV40-T-antigen.
   a. The MEFs are cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (Invitrogen, Cat. No. 10313-039) and 0.5 μmol/L 2-mercaptoethanol at 37 °C.
2. NCI-H460 (H460) lung cancer cells are obtained from ATCC (Cat. No. HTB-177).
   a. The H460 cells are cultured in RPMI 1640 (Invitrogen, Cat. No. 11875-119) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C and humidified 5% CO_2.
3. Human Umbilical Vein Endothelial Cells (HUVECs) are obtained from Clonetics (now Lonza, Cat. No. CC-2519).
   a. HUVECs are maintained in EBM-2 medium (Clonetics (now Lonza), Cat. No. CC-3156) supplemented with EGM-2 MV (Bio-whittaker (now Lonza), Cat. No. CC-4147) single aliquots at 37 °C.

2.2.Drugs in autophagy analysis

We utilize various drugs in our studies and these agents serve various purposes (Table 14.1). The lysosomal protease inhibitors help verify the presence of autophagic flux. By blocking lysosomal degradation, we can evaluate the time-dependent accumulation of autophagosomes and LC3-II (Klionsky *et al.*, 2008). This allows for better differentiation of the presence of autophagosome accumulation versus changes in the rates of autophagic degradation (Klionsky *et al.*, 2008).

Inhibitors of autophagy (e.g., 3-methyladenine) are necessary in the validation of autophagy. They are most often used to determine a change in autophagosome production, as determined by GFP-LC3 puncta formation in response to therapy (Shintani, 2004). They can also be used to monitor and estimate the turnover rates of autophagic organelles (e.g., autophagosomes, autolysosomes) (Kirkegaard *et al.*, 2004; Shintani, 2004). There is also a complex interplay between apoptosis and autophagy (Jaboin *et al.*, 2007). We use inhibitors of apoptosis (e.g., caspase inhibitors) to better study this relationship.

The number of inducers of autophagy has been growing steadily over the past decade. These agents vary in their specificity and abilities to induce cell death, but some may prove extremely useful for the improvement of therapeutic ratio in a host of tumors. Listed in

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Table 14.1 are a few of the better characterized agents that either stimulate or inhibit autophagy (Lefranc *et al.*, 2007).

2.3. Clonogenic assay

The clonogenic assay is an *in vitro* cell survival assay that has been the standard for evaluating reproductive cell death following irradiation for many years (Franken *et al.*, 2006). It has the advantage of analysis of cell death without significant confounding factors of changes in cellular proliferation or metabolic changes. This tool is useful in the study of various cellular models, and multiple cytotoxic agents.

1. H460 cells and MEFs are treated with DMSO or drug (e.g. M867) at varying concentrations (1.4, 5, and 10 n*M* in H460 cells; 5 and 10 n*M* in MEFs) for a period 24 h at 37 °C in 60-mm tissue culture dishes.
2. Cells are subsequently treated with siRNAs against empty vector, *caspase-3*, *caspase-7*, *beclin 1*, and/or *ATG5*.
3. Cells are then irradiated with 0–6 Gy at a dose rate of 1.8 Gy/min using a ^{137}Cs irradiator (J.L. Shepherd and Associates, Glendale, CA).
4. After irradiation, cells are incubated at 37 °C to allow for at least 6 cell divisions, which typically is a period of 6–14 days.
5. The medium is removed, and cells are then fixed for 15 min with 3:1 methanol: acetic acid and stained for 15 min with 0.5% crystal violet (Sigma-Aldrich, Cat. No. C0775) in methanol.
6. Following staining, colonies are counted. A colony is defined as containing a minimum of 50 viable cells.
7. Surviving fraction is calculated as (mean colony counts)/(cells inoculated) × (plating efficiency (PE)), where PE is defined as (mean colony counts)/(cells inoculated for nonirradiated controls).
8. Dose enhancement ratio (DER) is calculated as the dose (Gy) for radiation alone divided by the dose (Gy) for radiation plus drug (normalized for drug toxicity) necessary for a surviving fraction of 0.25. Experiments are conducted in triplicate and mean, standard deviation, and P values are calculated.

2.4. Endothelial cell morphogenesis assay: tubule formation

Angiogenesis is an important facet of cancer therapy, as it is critical in maintaining nourishment after tumors reach a critical size (Folkman, 2007). As such, *in vitro* studies of novel drugs and combination therapies would not be complete without an analysis of tumor endothelial cells. Most of our studies have been performed utilizing human umbilical vein endothelial cells, as a surrogate for tumor endothelial cells (Albert *et al.*, 2007; Kim *et al.*, 2008a).

1. HUVECs are grown to ~70% confluency, and treated with DMSO, Z-DEVD (50 μ*M* for 24 h), RAD001 (10 n*M* for 2 h) or combined Z-DEVD with RAD001 (at the same doses for a period of 24 h), and then cells are treated with 5 Gy.
2. The media is removed from the culture dish and washed once with PBS. The PBS is aspirated, and 1 ml of trypsin-EDTA is added to the plate. The plate is rocked to ensure that the entire surface is covered. Incubate the dish at room temperature for 1–3 min, and observe under a microscope. When the cells are completely round, gently dislodge the cells from the surface of the dish by rapping.

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3. They are seeded at 48,000 cells per well on 24-well plates coated with 300 μl of Matrigel (BD Biosciences, Cat. No. 354234).
4. The cells undergo differentiation into capillary-like tube structures, and are periodically observed by microscopy.
5. Twenty-four h later, cells are stained with hematoxylin and eosin and photographs are taken via microscopy.
6. The average number of tubes is calculated from examination of three separate microscope fields (100X) and representative photographs are taken.

2.5. Analysis of autophagosomes (Immunofluorescence Microscopy)

Detection of fluorescence-tagged GFP is a useful tool for the microscopy detection of autophagosome production. LC3 is tagged with GFP at the amino terminus. The cells are subsequently transiently transfected to overexpress the GFP-LC3 proteins, and treated in various cellular conditions. At various time points, the cells are analyzed by confocal microscopy with images recorded for analysis of punctate GFP-LC3 as an indication of autophagosome production.

There are limitations to this strategy. A stable transfection may result in fewer artifacts, and decreased background over transient transfection (Klionsky *et al.*, 2008). Analysis would also be easier, because nearly every cell would express the GFP-LC3. Generally we prefer the transient transfection method, as the effect of LC3 overexpression is detected shortly after transfection, which theoretically reduces alterations of the cellular machinery over the time it would take to generate stable transfectants.

1. H460 cell and MEFs are seeded at a density of 2×10^5 cells into 6-well tissue culture plates.
2. After 16 h, the cells are transfected with 2.5 μg of GFP-LC3 expression plasmid using the Lipofectamine 2000 reagent (Invitrogen Life Technologies, Cat. No. 12566-014) per the manufacturer's protocols.
3. Twenty-four h later, the cells are treated with 5 Gy of radiation, with or without drug.
4. After 24 and 48 h time points, the fluorescence of GFP-LC3 is observed using confocal microscopy.

2.6. Immunoblotting

Western immunoblotting has been useful for the evaluation of autophagic proteins in our models. In mammalian systems, determinations of the total levels of LC3 is not sufficient, as it does not account for the variations in conversion and degradation of LC3-I and LC3-II (Klionsky *et al.*, 2008). Thus, Western analysis of LC3-I and LC3-II, and determination of a LC3-II/LC3-I ratio has been considered a good marker for autophagy.

There are limitations to this approach. Changes in LC3-I and LC3-II levels vary with cell type and conditions, which can present significant challenges for heterogeneous populations (Mizushima *et al.*, 2004). Also there are reported variations in the sensitivity of various antibodies to LC3-I with less lability associated with LC3-II (Klionsky *et al.*, 2008). That combined with the fact that LC3-II is degraded by autophagy can confound assessments. Finally, LC3-II and the LC3-II/LC3-I ratio can represent autophagosome accumulation at a given point in time. It is better to also assess LC3-II in the presence and absence of lysosomal protease inhibitors to determine the amount of LC3-II delivered to lysosomes, which is a better determination of autophagic flux (Mizushima and Yoshimori, 2007).

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1. H460 cells (0.5×10^6) are treated with varying doses of radiation and drugs.
2. They are subsequently collected at multiple time points, and washed with ice-cold PBS twice before the addition of lysis buffer (20 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 20 m*M* EDTA, 1% NP40, 50 m*M* NaF, 1 m*M* Na_3Vo_4, 1 m*M* $NaMO_4$ and protease cocktail inhibitor (Sigma-Aldrich, Cat. No. P8340)).
3. Protein concentration is quantified using the Bio-Rad protein assay kit (Bio-Rad, Cat. No. 500-0001).
4. An equal amount of 2X SDS-polyacrylamide gel electrophoresis sample loading buffer is added to each sample, and the samples are heated at 100 °C for 5 min.
5. Equal amounts of protein are loaded into each well, and resolved on a 12.5% SDS-PAGE gel.
6. The blots are transferred to a polyvinylidene difluoride (PVDF) membrane at 300 mA for 3 h at 4 °C.
7. The PVDF membranes are blocked using 5% nonfat dry milk in TBS-T (Tris-buffered saline (TBS) and 0.1% Tween-20 with 5% nonfat dry milk) for 1 h at room temperature.
8. The blots are then incubated with various primary antibodies in TBS with 5% nonfat milk for 1 h at room temperature or overnight at 4 °C.
   - a. Rabbit anti-LC3 polyclonal antibody: Medical & Biological Laboratories ITL, Cat. No. PD012
     - i. LC3-I: 16 kDa
     - ii. LC3-II: 18 kDa
   - b. Rabbit anti-caspase-3 polyclonal antibody: Cell Signaling, Cat. No. 9662
     - i. Full length caspase-3: 35 kDa
     - ii. Cleaved caspase-3: 17 kDa
   - c. Rabbit anti-caspase-7 polyclonal antibody: Cell Signaling, Cat. No. 9492
     - i. Full length caspase-7: 35 kDa
     - ii. Cleaved caspase-7: 20 kDa
   - d. Rabbit anti-Akt polyclonal antibody: Cell Signaling, Cat. No. 4685
     - i. Akt: 60 kDa
   - e. Rabbit anti-phospho-Akt (Ser-473) monoclonal antibody: Cell Signaling, Cat. No. 4058
     - i. Phospho-Akt: 60 kDa
   - f. Rabbit anti-S6 ribosomal protein monoclonal antibody: Cell Signaling, Cat. No. 2217
     - i. S6 ribosomal protein: 32 kDa
   - g. Rabbit anti-phospho-S6 ribosomal protein (Ser-240/244) monoclonal antibody: Cell Signaling, Cat. No. 4838
     - i. Phospho-S6 ribosomal protein: 32 kDa

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h. Rabbit anti-Actin polyclonal antibody: Santa Cruz Biotech, Cat. No. sc-10731

   i. Actin: 45 kDa

9. The membranes are washed with TBS-T 3 times for 10 min.
10. Then, the membranes are incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase for 45 min at room temperature.
11. Immunoblots are developed by using the chemiluminescence detection system (PerkinElmer) according to the manufacturer's protocol and autoradiography.

2.7. Quantification of autophagic flux

To distinguish between autophagic induction and autophagic flux, we assay for the levels of LC3-II by Western analysis following treatment with either 3-MA or lysosomal protease inhibitors.

1. H460 cells are seeded in 6-well tissue culture plates at a density of 2×10^5 cells.
2. Sixteen h later, the cells are treated with Z-DEVD (50 μ*M* for 24 h).
3. Then 23 h later, if indicated, the cells are treated with RAD001 (10 n*M* for 1 h).
4. One h later, the cells are irradiated at 5 Gy, and then treated with either 200 μ*M* 3-MA or a combination of pepstatin A (10 μg/ml) and E64d (10 μg/ml) for a period of 2 h.
5. Cells are then collected at 2 time points (24 and 48 h), and washed with ice-cold PBS twice before lysis and protein concentration determination (as described previously).
6. The lysate is prepared as described previously, and then probed with anti-LC3 antibody in milk for a period of 1 h.
7. The blots are washed 3 times with TBS-T for 10 min.
8. Then the blots are reprobed with antiactin antibody for standardization of LC3-II levels to actin.

In addition, the LC3-II/LC3-I ratio can be determined.

2.8. Analysis of apoptosis

There are multiple methods to detect and quantify apoptosis which include, but are not limited to, end labeling of DNA, detection of phosphatidylserine changes, DNA laddering, and Western analysis of apoptosis signaling proteins. In our studies, we find that utilizing flow cytometric analysis of phosphatidylserine changes provides reliable and reproducible quantitative analysis of early apoptosis change in response to our various cytotoxic conditions.

1. H460 cells (2.5×10^5) are plated into 10-mm dishes for each data point.
2. After 24 h of incubation at 37 °C, the cells are treated with drug and immediately irradiated with 3, 5, 10, or 20 Gy.
3. Twenty-four h later, the cells are treated with 1 ml of the cell detachment medium Accutase (Millipore, Cat. No. SCR005) for 4 min and then cell counts are recorded for each sample.

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4. Cells are centrifuged at 2000 rpm at 4 °C for 15 min, and resuspended in 1X Annexin V Binding Buffer (BD Biosciences, Cat. No. 51-66121E) at a concentration of 1×10^6 cells/ml.
5. One hundred microliters of each solution (1×10^5 cells) is transferred into 12 × 75-mm ml FACS tubes (BD Biosciences, Cat. No. 340265) to which is added 1.2 μl of Annexin V-FITC (BD Biosciences, Cat. No. 556570) and 1.2 μl of propidium iodide.
6. After 30 min incubation at room temperature in the dark, 400 μl of 1x binding buffer is added to each tube.
7. The rate of apoptosis (as determined by external membrane translocation of phosphatidylserine) is measured using the Annexin V-Fluorescein Isothiocyanate Apoptosis Detection Kit II (BD Biosciences, Cat. No. 556570) with flow cytometry per the manufacturer's protocols.

2.9. Gene knockdown utilizing siRNA transfection

Genetic manipulation utilizing siRNA can be a relatively simple way to associate a given agent to an effect. The limitations of these experiments are similar to those discussed with transient versus stable transfections of other proteins. However in the study of autophagy, where causal relationships are difficult to establish, determining the mediators of a given effect is particularly important.

1. We use siRNAs against various proteins:
   - **a.** siRNA mouse caspase-3; Santa Cruz Biotechnology, Cat. No. sc-29927
   - **b.** siRNA mouse caspase-7; Santa Cruz Biotechnology, Cat. No. sc-29928
   - **c.** siRNA mouse Beclin 1; Santa Cruz Biotechnology, Cat. No. sc-29798
   - **d.** siRNA mouse ATG5
   - **e.** 5′-AACUUGCUUUACUCUCUCAUCAUU-39 (Sense)
   - **f.** 3′-UUUUGAACGAAAUGAGAGAUAGU-59 (Antisense)
   - **g.** Control siRNA; Santa Cruz Biotechnology, Cat. No. sc-37007
2. Cells are transfected with 25 n*M* of siRNAs using Lipofectamine 2000 (Invitrogen Life Technologies, Cat. No. 12566-014).
3. The transfected cells are used for experiments 24 h later.

2.10. Human lung cancer xenograft

1. Human H460 lung cancer cells are used in a xenograft model in female athymic nude mice (nu/nu), 5–6 weeks old.
2. A suspension of 1×10^6 cells in 50 μL volume is injected subcutaneously into the left posterior flank of mice using a 27½-gauge needle.
3. Tumors are grown for 6–8 days until the average tumor volume reaches 0.25 cm^3.
4. Treatment groups consist of vehicle control (DMSO), drug, vehicle plus radiation, and drug plus radiation. Each treatment group contains 5 mice.
5. DMSO or drug is given daily by intraperitoneal (i.p.) injection at doses of 2 mg/kg for 7 consecutive days.

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6. In the case of combination treatment, drug or vehicle is given for 2 days prior to the first dose of irradiation.
7. Mice in radiation groups are irradiated 1 h after drug or vehicle treatment with daily 2 Gy fractions given over 5 consecutive days.
8. Tumors on the flanks of the mice are irradiated using an X-ray irradiator (Therapax, AGFA NDT).
9. The non-tumor-bearing parts of the mice are shielded by lead blocks.
10. Tumors are measured 2–3 times weekly in 3 perpendicular dimensions using a Vernier caliper and the volume is calculated using the modified ellipse volume formula (volume = (height × width × depth)/2).
11. Growth delay is calculated for treatment groups relative to control tumors.

2.11. Immunohistochemistry

1. Mice are implanted with H460 cells and treated as described previously in the tumor volume studies.
2. After 7 days of daily treatments, the mice are sacrificed and tumors are paraffin fixed.
3. Slides from each treatment group are then stained for von Willebrand factor (vWF) using anti-vWF polyclonal antibody (Millipore, Cat No. AB7356).
4. Blood vessels are quantified by randomly selecting 400X fields and counting the number of blood vessels per field.
5. This is done in triplicate and the average of the three counts is calculated. Ki67 and terminal deoxynucleotidyl transferase(TdT)-mediated dUTP nick end labeling (TUNEL) staining are performed in the Vanderbilt University pathology core laboratory according to the following protocols.

2.11.1. Protocol: TUNEL staining

2.11.1.1. Solutions

TdT Buffer Stock Solution:	
Tris-HCl (MW: 157.6)	1.97 g
Sodium cacodylate, trihydrate (MW: 214.0)	21.4 g
Bovine serum albumin (BSA)	0.125 g
Distilled water	100 ml
Adjust pH to 6.6, and store aliquots at −20 °C.	
Cobalt Chloride Stock Solution:	
Cobalt chloride, hexahydrate (MW: 237.9)	0.6 g
Distilled water	100 ml
Mix to dissolve, and store aliquots at −20 °C.	
TdT Reaction Buffer:	
TdT buffer stock solution	40 µl
Cobalt chloride stock solution	8 µl
Distilled water	160 µl
Mix well. Store at −20 °C.	

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TdT Storage Buffer:	
Potassium phosphate (K_2HPO_4; MW: 174.18)	1.05 g
KCl (FW: 74.55)	1.12 g
Distilled water	50 ml

Stir to dissolve and adjust pH to 7.2 using concentrated HCl. Add 50 ml of glycerin (100% glycerol), 0.5 ml of Triton X-100, and 8 μl of 2-mercaptoethanol (99% solution. FW: 78.13). Store at −20 °C.

2.11.1.2. Reagents

PBS:	
Sodium phosphate, dibasic (Na_2HPO_4)	1.44 g
Sodium chloride	8 g
Potassium phosphate (KH_2PO_4)	0.24 g
Potassium chloride	0.2 g
Distilled water	800 ml
Adjust pH to 7.4, and adjust to a final volume of 1 liter with additional distilled water.	
PBS-T (PBS/Tween Solution):	
0.1% volume of Tween 20 prepared in PBS	
Triton X-100 (octylphenolpoly(ethyleneglycolether):	
Roche Diagnostics, Cat. No. 11332481001.	
Enzyme Reagent:	
TdT (Roche Diag., Cat. No. 03333574001)	4 μl
TdT storage buffer	100 μl
Mix well and store at −20 °C.	
Label Reagent:	
Biotin-16-dUTP (Roche Diag., Cat. No. 11093070910)	4 μl
TdT reaction buffer	1 ml
Mix well and store at −20 °C.	
TdT Reaction Mixture:	
Enzyme reagent	100 μl
Label reagent	900 μl
Mix just before use. Use the remaining 100 μl of label solution as a negative control.	
Stop Wash Buffer:	
NaCl (MW: 58.44)	1.75 g
Sodium citrate, trihydrate (MW: 294.11)	0.88 g
Distilled water	100 μl
Mix to dissolve and store at room temperature.	

2.11.1.3. Protocol

1. Deparaffinize sections in 2 changes of xylene for 5 min each in labeled 2.0 ml microcentrifuge tubes

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2. Hydrate with 2 changes of 100% ethanol for 3 min each, followed by 95% ethanol for 1 min.
3. Rinse in distilled water.
4. Add 800 μl of lysis buffer, and add 9 μl of proteinase K (20 mg/ml; Promega, Cat. No. V302B).
5. Vortex samples for 15 s, and incubate at 55 °C until tissue is completely lysed (may need to be overnight). Vortex samples occasionally.
6. Add 180 μL of 5 *M* NaCl and vortex well. The solution will become frothy.
7. Spin tubes at 13,000 rpm for 5 min, and the salted out debris will pellet.
8. Transfer the supernatant fractions to cryotubes (screw cap).
9. Add 420 μL of ice-cold isopropanol (2-propanol) to the supernatant fractions.
10. Mix slowly by inversion 5–10 times. CAUTION: Do not vortex.
11. DNA fibers may be seen at this time.
12. Centrifuge the tube at 13,000 rpm for 10 min.
13. The DNA pellet should be visible
14. Pour out the supernatant to discard.
15. Add 400 μL of 70% ethanol to wash the DNA pellet.
16. Wash for 20 min on a cell rotator at room temp.
17. Centrifuge the tubes at 13,000 rpm for 5 min and pour out the ethanol carefully! Note that the pellet may be loose. If the pellet is loose, pipette the ethanol out, being careful to not disturb the pellet.
18. Dry the DNA pellet in a speed vac on high for 10 min.
19. Resuspend the pellet in distilled H_2O.
    a. If a small pellet add approximately 50 μL
    b. If a large pellet add approximately 100 μL
20. Let the tubes stand at room temp overnight.
21. Perform 2 washes of 2 min each with PBS-T.
22. Incubate the sections in TdT Reaction Buffer for 10 min.
23. Incubate the sections in TdT Reaction Mixture for 1–2 h at 37 °C in a humidified chamber.
24. Rinse the sections in stop wash buffer for 10 min.
25. Perform 3 washes of 2 min each with PBS-T.
26. Counterstain with propidium iodide or DAPI for 20 min.
27. Rinse in PBS for 5 min.
28. Mount the sections with antifading mounting medium.

The number of positive cells per field are scored and graphed by averaging 3 repeated assessments.

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3. CONCLUSION

Techniques for the analysis of autophagy have been rapidly evolving over the past decade. As a result, there has been a concerted effort by the leaders in this field to maintain strict guidelines regarding the interpretation and appropriate methods for analysis of autophagy (Klionsky *et al.*, 2008). Though there have been relatively few studies into autophagy and its role in lung cancer, we hope that this chapter will serve as the basis for further investigations. As these techniques continue to evolve, it will be important to continue investigating this process to develop optimal therapeutic combinations for the improvement of therapeutic ratio in lung cancer.

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Table 14.1

Characteristics of agents targeting autophagy in lung cancer

CLASS	AGENT	MECHANISM OF ACTION
Lysosomal Protease Inhibitors	E64d	Lysosomal Inhibitor (proteases)
	Leupeptin	
	Pepstatin A	
	Vinca alkaloids	Microtubule Inhibitors
	Bafilomycin A_1	Increases Lysosomal pH
Autophagy Sequestration Inhibitors	3-methyladenine (3-MA)	Class I/III PI3K Inhibition
	LY294002	
	Wortmannin	
	Cycloheximide	Not established
Caspase Inhibitors	M687 (Kim *et al.*, 2008b); Merck	Not established
	Z-DEVD	Not established
Inducers of Autophagy	Arsenic Trioxide	Inhibition of mTOR (Kanzawa *et al.*, 2005)
	Butyrate & suberoylanilide hydroxamic acid	Histone Deacetylase Inhibitor (Shao *et al.*, 2004)
	Ceramide	Increased Beclin 1 (Daido *et al.*, 2004)
	Rapamycin, RAD001 (Everolimus) (Lefranc *et al.*, 2007)	Inhibition of mTOR (Albert *et al.*, 2006; Cao *et al.*, 2006; Kamada *et al.*, 2000; Kim *et al.*, 2006)
	Resveratrol (Opipari *et al.*, 2004)	Not established
	Tamoxifen (Bursch *et al.*, 1996, 2000)	Increased Beclin 1 expression (Scarlatti *et al.*, 2004)
	Temozolomide	DNA Alkylation (Kanzawa *et al.*, 2004)
	Endostatin (Chau *et al.*, 2003)	Not established