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Increased expression of transcription factor EB (TFEB) is associated with autophagy, migratory phenotype and poor prognosis in non-small cell lung cancer

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ABSTRACT

Objectives: We investigated the role of lysosomal biogenesis and hydrolase activity in the clinical behavior and postoperative outcome of lung cancer.
Materials and methods: Using immunohistochemistry we investigated the expression of the transcription factor EB (TFEB) which orchestrates lysosomal biogenesis, the lysosome membrane protein LAMP2a and of the lysosomal hydrolase cathepsin D in a series of 98 non-small cell lung carcinomas (NSCLC) treated with surgery alone. In vitro experiments with the A549 and H1299 lung cancer cell lines were also performed.
Results: Overexpression of TFEB, LAMP2a and Cathepsin D was noted in 47/98 (47.9%), 43/98 (43.9%) and 39/98 (39.8%) cases, respectively, and were significantly correlated with each other and with adenocarcinomas. High LAMP2a was related to high histology grade. Linear regression analysis confirmed significant association of TFEB with BNIP3 ($p = 0.0003$, $r = 0.35$) and LC3A with LAMP2a expression ($p = 0.0002$, $r = 0.37$). An inverse association of Cathepsin D expression with stone-like structures (SLS) was recorded ($p = 0.02$, $r = 0.22$). On univariate analysis all three lyososomal variables were associated with poor prognosis ($p = 0.05$, 0.04 and 0.01, for TFEB, Cathepsin D and LAMP2a, respectively). Multivariate analysis showed that the SLS number ($p = 0.0001$, HR5.37), Cathepsin D expression ($p = 0.01$, HR = 2.2) and stage ($p = 0.01$, HR = 1.5) were independent prognostic variables. Silencing of TFEB with siRNAs in the A549 and H1299 lung cancer cell lines did not affect proliferation but resulted in reduced migration ability.
Conclusion: Lysosomal biogenesis is linked to autophagosomal protein expression in NSCLC and characterizes subgroups of high risk patients after complete surgical lung tumor resection.

1. Introduction

Lysosomes are acidic organelles responsible for the catabolism of damaged cell organelles and macromolecules following their fusion with autophagosomes. Digestion of the phago-lysosomal content occurs by over 50 lysosomal hydrolases [1,2]. Lysosomal membrane proteins are highly glycosylated proteins decorating the luminal surface of lysosomes [3]. The most abundant of these proteins are the lysosomal membrane associated proteins LAMP1 and LAMP2 that, among other functions, are important for the auto(phago)-lysosomal fusion during the autophagic process [4]. Lysosomal biogenesis is transcriptionally regulated by Transcription factor EB (TFEB) that regulates expression of multiple genes encoding lysosomal enzymes and membrane proteins [5–7].

The role of lysosomes and lysosomal biogenesis in cancer biology and clinical behavior is obscure. Malignant transformation is accompanied by increased lysosomal biogenesis [8,9]. Overexpression of cathepsins (powerful hydrolases) in cancer cells has been linked with enhanced tumor growth and invasion, a result attributed to extra cellular release and proteolysis of the matrix [10].

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Table 1
Patient and disease characteristics ($n = 98$).

Age	
Median	67
Range	28–81
Sex	
Male	86
Female	12
Histology	
Squamous cell carcinoma	58
Adenocarcinoma	22
Large cell carcinoma	18
Grade	
Squamous cell carcinoma	5
High	8
Medium	45
Low	
Adenocarcinoma	3
High	12
Medium	7
Low	
Stage	
I	47
II	22
IIIA	23
IIIB	3
IV	3
Necrosis	
Yes	82
No	16
Fibroblastic reaction	
High	45
Medium	24
Low	29

In the current study we investigated the expression of *TFEB* and of lysosomal markers, using antibodies recognizing the lysosome membrane protein LAMP2a and of the lysosomal hydrolase cathepsin D, in a series of non-small cell lung carcinomas in an attempt to investigate correlations with histopathological variables and post-operative outcome. To investigate the role of TFEB in cell proliferation and migration, in vitro studies were also performed.

2. Materials and methods

Formalin-fixed, paraffin embedded material from 98 patients treated with curative surgery for non-small cell lung cancer were collected from the Department of Pathology, Democritus University of Thrace, Medical School, Alexandroupolis, Greece. The selected cases were serial according to the archive number. Patient and disease characteristics are shown in Table 1. Postoperative radio-chemotherapy was administered to 3 patients with stage IIIB disease, while 3 patients with stage 4 were treated with chemotherapy. Upon relapse, patients were treated with chemotherapy and/or radiotherapy as appropriate. Stage IV cases were excluded from the survival analsysis. The median overall survival was 32 months (range, 2–102). The study has been approved by the local Ethics and Research Committees. Patients were classified according to the pathologic TNM International Union Against Cancer staging system [11].

2.1. Immunohistochemistry

For the detection of lysosomal markers we used the following primary antibodies: (i) the anti-TFEB, rabbit polyclonal antibody (cat. no A303-673A; Bethyl Laboratories Inc, USA), was used a dilution of 1:500 and overnight incubation, (ii) the mouse monoclonal anti-cathepsin D, (CTD-19; ABCAM, UK) antibody was used at a dilution 1:1000, at 30 min incubation, and (iii) the rabbit polyclonal anti-LAMP2a antibody (ab18528; ABCAM, UK) was used at a dilution or 1:100, at 30 min incubation, (iv) the rabbit polyclonal antibody anti-MAP1LC3A (AP1805a, Abgent, San Diego, CA) was used at 1:20 dilution, overnight. (v) The mouse anti-MAP1LC3B monoclonal antibody was used at 1:100 overnight (LC3B 5F10 Nanotools). (vi) The rabbit anti-Beclin 1 monoclonal antibody (ab51031, Abcam, Cambridge, UK) was used at a dilution 1:50, overnight. (vii) The rabbit anti-BNIP3 polyclonal antibody (AP1321a; Abgent, San Diego, CA) was used at a dilution of 1:30 and overnight incubation. Immunohistochemical methods have been previously reported [12–15].

Both the staining intensity and the percentage of expressing cells was taken into account for the scoring of tissue samples. The proportion of cancer cells with strong cytoplasmic expression of the proteins or with nuclear expression (for TFEB) was recorded in all ×400 optical fields and the mean value was used to score each case. The median value was used to group cases as of low vs. high reactivity. Cases scored as 'negative' were grouped in the low reactivity category, as protein could be expressed at levels lower than the ones detectable with the described technique and antibody concentrations. The patterns of LC3A "stone-like" structure (SLS) expression pattern has been previously published [13]. SLS are readily recognized as large, rounded, densely stained material, amorphous or laminated, typically enclosed within an LC3 positive cytoplasmic vacuole. The number of stone-like structures (SLS) were counted in all available fields of a section at 400× magnification and expressed as the mean of all counts.

2.2. Immunoblotting

A549 and the H1299 human lung carcinoma cells (ATCC) were maintained and cultured in DMEM—Low Glucose medium, supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) at standard conditions, 37 °C, 5% CO_2 in humidified atmosphere.

Cells were washed with PBS twice and lysed in sucrose-based lysis buffer (0.25 M sucrose, 25 mM Tris–HCl, pH 7.4) containing protease inhibitors (complete mini protease inhibitor cocktail, Roche Diagnostics GmbH) and phosphatase inhibitors (phosphatase inhibitor cocktail, Cell Signaling Technology). Whole protein lysates were homogenized and protein quantification was performed according to Pierce™ BCA Protein Assay Kit (#23225, Thermo Scientific). Following blocking with TBS (pH 7.6) containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat dried milk for 1 h at room temperature, membranes were hybridized at 4 °C overnight with the abovementioned primary antibodies in the following concentrations: anti-TFEB (1:500), anti-Cathepsin-D (1:500), anti-LAMP2a (1:500), anti-MAP1LC3B (1:500) anti-MAP1LC3A (ab62720; Abcam, UK) (1/500), Anti-SQSTM1 / p62 (ab56416; Abcam, UK) (1:1000). Then, the membranes were hybridized with the appropriate secondary antibodies (1:5000, Goat Anti-Rabbit IgG (H + L)-HRP Conjugate, #170-6515, Biorad and 1:5000 bovine anti-mouse IgG-HRP, P0447, DAKO) and developed in Amersham ECL Western blotting detection reagents and analysis system (RPN2209, GE Healthcare). Each of these blots was then stripped and reprobing of the membranes with a primary anti-beta actin antibody (1:5000, ab75186, Abcam) followed. The images of the blots were captured utilizing Chemidoc™ MP imaging system (Biorad). Additional western blot analysis was performed in subset of frozen tissues from normal lungs adjacent carcinomas and from carcinomas.

2.3. TFEB silencing and proliferation/migration experiments

Pool TFEB siRNAs (5′-CAGGCUGUCAUGCAUUACATT-3′), (5′-GACGAAGGUUCAACAUCAATT-3′), (5′-GCGGCAGAAGAAAGACAA-UTT- 3′), (5′-CCGAGACCUAUGGGAACAATT-3′) were custom synthesized by GenePharma. These were used at 50 nM to transfect

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