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Original Article

Targeting COPZ1 non-oncogene addiction counteracts the viability of thyroid tumor cells☆

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ABSTRACT

Thyroid carcinoma is generally associated with good prognosis, but no effective treatments are currently available for aggressive forms not cured by standard therapy. To find novel therapeutic targets for this tumor type, we had previously performed a siRNA-based functional screening to identify genes essential for sustaining the oncogenic phenotype of thyroid tumor cells, but not required to the same extent for the viability of normal cells (non-oncogene addiction paradigm). Among those, we found the coatomer protein complex ζ1 (COPZ1) gene, which is involved in intracellular traffic, autophagy and lipid homeostasis. In this paper, we investigated the mechanisms through which COPZ1 depletion leads to thyroid tumor cell death. We showed that siRNA-mediated COPZ1 depletion causes abortive autophagy, endoplasmic reticulum stress, unfolded protein response and apoptosis. Interestingly, we observed that mouse tumor xenografts, locally treated with siRNA targeting COPZ1, showed a significant reduction of tumor growth. On the whole, we demonstrated for the first time the crucial role of COPZ1 in the viability of thyroid tumor cells, suggesting that it may be considered an attractive target for novel therapeutic approaches for thyroid cancer.

Introduction

Cancer cells often depend on non mutated genes and pathways to support their unchecked growth. The activity of these genes is essential for tumor cells, but not required to the same extent by normal cells; this concept is known as “non-oncogene addiction” (NOA) paradigm [1]. NOA genes represent tumor vulnerabilities and their inhibition results in the failure of the oncogenic phenotype. Nowadays, large-scale siRNA-based functional screening of cancer cell lines represent a powerful strategy for the identification of NOA genes that can be investigated as therapeutic targets for many types of tumors [2]. We used this approach to unveil nodal points for therapeutic intervention for thyroid carcinoma (TC), which represents the most frequent endocrine cancer, with a rapidly increasing incidence [3].

The majority of TC originates from epithelial cells and includes well-differentiated papillary (PTC) and follicular (FTC) carcinomas, poorly differentiated (PDTC) and undifferentiated anaplastic carcinomas (ATC). Most TCs are effectively treated by standard therapy, involving surgery, thyroid stimulating hormone and radioiodine. Nevertheless, a fraction of patients cannot be cured due to local recurrence (in up to 20% of patients), distant metastasis (in approximately 10% at 10 years [4]) and/or radioresistant disease.

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Furthermore, patients with PDTC and ATC have a very poor prognosis, with survival of few months in the case of ATC [5,6]. Even though recent molecular findings allowed the development of several therapies designed to specifically target thyroid oncoproteins or their downstream pathways, this strategy showed modest results and only partial response [7,8]. Few or no therapeutic options are currently available for patients with aggressive and iodine-refractory thyroid tumors. Thus, there is a need to better understand the mechanisms of thyroid carcinogenesis and to improve the treatment of the most aggressive tumor forms not curable by standard therapy.

We have recently discovered several thyroid tumor cell vulnerabilities. By screening a siRNA library, we identified a set of genes whose silencing inhibited the growth of a panel of thyroid tumor cells, but not of normal immortalized thyrocytes. The COPZ1 gene was found among the top ranked genes [9]. COPZ1 (coatomer protein complex ζ1) belongs to the coatomer protein complex I (COPI), an heptameric complex which is involved in: assembly of coated vesicles on Golgi membranes, retrograde transport of luminal and membrane proteins in the ER-Golgi secretory pathway, endosome maturation, autophagy [10,11] and lipid homeostasis [12]. It has been demonstrated that COPZ1 knockdown causes cell death in both proliferating and nondividing tumor cells. Unlike tumor cells, normal cells are not sensitive to COPZ1 inhibition [13]. We identified COPZ1 as a thyroid tumor cell specific survival gene, as its inhibition induced cell death in tumor cell lines but not in immortalized thyrocytes. This evidence, together with a not significant variation of expression and absence of mutations in PTC, allowed us to classify COPZ1 as an example of "non-oncogene addiction" for thyroid cancer cells [9].

Here we investigated the mechanisms through which COPZ1 depletion leads to thyroid tumor cell death and explored its potential use as therapeutic target for thyroid carcinoma. We found that cell death induced by COPZ1 depletion is associated with abortive autophagy, ER stress, UPR and apoptosis. Interestingly, local treatment with siCOPZ1 oligos reduced tumor growth in an *in vivo* model of thyroid carcinogenesis.

Materials and methods

Cell lines

Thyroid tumor cell lines, primary thyrocytes and mammary epithelial h-TERT-HME1 cells were cultured as previously described [9,14,15]. Source and culture conditions are reported in Supplementary. GFP-LC3 stable cell lines were generated by transfecting 2 μg of pEGFP-LC3 plasmid (from Tamotsu Yoshimori's laboratory [16], using the Lipofectamine 3000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) for TPC-1, and Lipofectamine LTX (Invitrogen Life Technologies) for 8505C cells, according to manufacturer's instruction. Transfected cells were selected and propagated in medium containing G418 (500 μg/ml, Invitrogen Life Technologies).

Detection of LC3 puncta

Cells growing on glass coverslips were transfected as described below and 24 h, 48 h, 72 h later fixed for 20 min with 4% paraformaldehyde. After washing with PBS, cells were treated with the ProLong Diamond Antifade mountant with DAPI (P36966, Molecular Probes, Invitrogen Life Technologies) and imaged with immunofluorescence microscopy (Eclipse E1000; Nikon Instruments, Inc. NY, USA). The percentage was calculated as the ratio between cells displaying LC3 puncta and total cells (at least 150), considering six randomly selected 60× fields.

siRNA reagents and transfection

siRNA transfection was performed using 20 nM of siRNA oligos (see list in Supplementary) and the Lipofectamine RNAiMAX reagent (Invitrogen Life Technologies), according to manufacturer's instruction.

Colony formation assay

Cells were plated in 100 mm dishes and transfected the following day with 20 nM of siRNAs. One day later, cells were collected and plated as follows: 2×10^4 cells in 24 well-plates for colony formation assay; 1.5×10^5 cells in 60 mm dishes for western blot analysis. For the colony formation assay, cells were fixed five days later with formaldehyde 3.7% v/v solution for 30 min, washed with PBS and stained for 20 min with 0.1% crystal violet (w/v). Pictures were taken using an Epson image scanner.

Western blot analysis

WB analysis was performed as previously described [17]. The list of antibodies is provided in Supplementary.

RNA extraction and real time PCR

RNA extraction and Real time PCR were performed as previously described [17]. The following TaqMan gene expression assays (Applied Biosystem, Foster City, CA) were used: Hs01023197_m1 for COPZ1 and Hs00358796_g1 for CHOP expression; Hs02800695_m1 for HPRT1, used as housekeeping gene for normalization among samples.

Apoptosis assay

Cells were transfected with 20 nM of siRNA oligos in the presence of 2 μM of the fluorogenic substrate for activated caspase-3/7 (CellEvent™ Caspase-3/7 Green Detection Reagent, Invitrogen Life Technologies). Live-cell fluorescence images were taken 72 h later with microscope (Eclipse TE2000-S; Nikon Instruments).

In vivo studies

Female CD-1 nu/nu mice (5-weeks old) (Charles River, Calco, Italy) were injected subcutaneously into the left flank with 8505C cells (10×10^6). When xenograft tumors reached the mean volume of 0.085 mm^3 (range 0.040–0.144), they were randomly divided into three groups and then locally treated with 20 μg of siCOPZ1 oligos (4457308, Ambion® ID s22427) or non-targeting oligos (4457289, Ambion® *In Vivo* Negative Control #1 siRNA) mixed in 50 μl of MaxSuppressor™ In Vivo RNA-LANCEr II reagent (Bioo Scientific, Austin, TX, USA), or with MaxSuppressor™ only. Treatments were carried out at day 17, 20, 23, 27, 30 after cell injections. Tumor growth was followed for 34 days and assessed by monitoring tumor weight (TW), as previously described [18]. Animal studies were reviewed and approved by the Ethics Committee for Animal Experimentation of the hosting institution and are in accordance with the guidelines of the UK Coordinating Committee for Cancer Research [19].

Immunohistochemistry

Serial sections from paraffin-embedded tumor xenografts (2-μm thick) were stained with hematoxylin and eosin and evaluated under a light microscope to assess the pattern of tumor growth. Antigen retrieval was performed using 1 mM citrate buffer (pH 6), then sections were immunostained with a primary rat polyclonal antibody anti-human COPZ1 (1:400 SAB4500896 Sigma-Aldrich St Louis, Mo, USA), anti-human Ki-67 (Mib1) (1:200 M7240 Dako, Agilent Technologies, CA, USA). Immunostains were performed using standard immunoperoxidase protocol followed by diaminobenzidine chromogen reaction (Dako REAL™ EnVision™ Detection System, K5007 Dako). The intensity of staining for COPZ1 was scored as low (1+), medium (2+) or high (3+). The percentage of cells immunostained for Ki67 and necrotic cells was estimated counting one 10× field, randomly selected. One sample of each group of tumor explants was excluded from the IHC analysis due to technical problems.

Gene/miRNA expression analysis

Gene expression data of *COPZ2* gene (log scale) from 58 normal-thyroid tissue samples compared to 31 ATC, 72 PTC and 17 PDTC samples, were obtained combining GSE3467 [20], GSE6004 [21], GSE33630 [22,23] and GSE76039 [24] publicly available datasets. Raw intensity expression values on U133 Plus 2.0 array (Affymetrix, Santa Clara, CA) were processed by Robust Multi-array Average procedure [25], with the re-annotated Chip Definition Files from BrainArray libraries version 20.0.0 [26], available at http://brainarray.mbni.med.umich.edu. Batch effects were removed by using sva R/Bioconductor package [27]. The analysis of the thyroid TCGA dataset from 58 paired normal/PTC samples was performed as previously described [9]. Normalized reads per million miRNA mapped (RPM) data were obtained for the same 58 tumor samples from Illumina HiSeq Level 3 isoform quantification files (TCGA Data Portal website), by summing up the reads aligned to each 3p or 5p miRBase v16 mature strands [28,29]. Pearson's correlation method was applied to evaluate the correlation between RNAseq normalized gene and miRNA read counts (log scale) in 58 tumor samples.

Results

COPZ1 depletion affects thyroid tumor cell viability

The issue that COPZ1 represents vulnerability for thyroid tumor cell lines but not for normal thyrocytes [9] is further documented