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ATOX1 gene silencing increases susceptibility to anticancer therapy based on copper ionophores or chelating drugs

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ABSTRACT

Copper is a catalytic cofactor required for the normal function of many enzymes involved in fundamental biological processes but highly cytotoxic when in excess. Therefore its homeostasis and distribution is strictly regulated by a network of transporters and intracellular chaperones. ATOX1 (antioxidant protein 1) is a copper chaperone that plays a role in copper homeostasis by binding and transporting cytosolic copper to ATPase proteins in the *trans*-Golgi network. In the present study the Caco-2 cell line, a colon carcinoma cell line, was used as an in vitro model to evaluate if ATOX1 deficiency could affect sensitivity to experimentally induced copper dyshomeostasis. Silencing of ATOX1 increased toxicity of a short treatment with a high concentration of Cu^{2+}. Copper ionophores, such as 5-chloro-8-hydroxyquinoline, induced a copper-dependent cell toxicity which was significantly potentiated after ATOX1 silencing. The copper chelator TPEN (N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylenediamine) produced a form of cell toxicity that was reversed by the addition of Cu^{2+}. ATOX1 silencing increased Caco-2 cell sensitivity to TPEN toxicity. Our results suggest the possibility of a therapy with copper-chelating or ionophore drugs in subtypes of tumors showing specific alterations in ATOX1 expression.

1. Introduction

Copper assumes a critical role in many biological processes mainly as a catalytic cofactor of enzymes, such as multi-copper oxidase (ceruloplasmin/hephaestin) and lysyl oxidase, and its homeostasis is guaranteed by the activity of metal transporters and intracellular chaperones [1,2]. In humans, disruption of this tightly regulated cellular copper homeostasis affects normal tissue development and a growing number of reports indicates an alteration of essential metal ion homeostasis (metallostasis) in tumors [3,4].

Several small molecules able to bind metals, such as copper and zinc, have been proposed as anticancer agents [5,6]. It has been suggested that such molecules can be distinguished in two classes: *metal chelators* able to remove metals from a biologically active site, such as TPEN (N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylenediamine), and *metal ionophores* capable of transferring multiple metal ions across biological membranes such as hydroxyquinolines [5,6]. The effects of "metal chelators" can be reversed by an increase in metal concentration while the ionophore effect is potentiated in a metal-dependent manner. Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline, CQ) is a member of the metal-ionophore class and is known to exhibit a variety of biological activities, such as antibacterial [7] and anticancer [8] activities. CQ displays an anticancer effect in *in vitro* [9,10] and *in vivo* [11] preclinical models, inducing apoptosis in breast cancer cells through a caspase-dependent apoptotic pathway [12,13]. Moreover, CQ induces apoptosis in leukemia and myeloma cells by inhibiting histone deacetylase activity [14]. In addition, Zhai and collaborators [15] demonstrated that both 8-hydroxyquinoline (8-OHQ) and CQ/copper complexes are able to inhibit the proteasome activity, resulting in proliferation suppression and apoptosis in cultured breast cancer cells. Recently a new mechanism, called "parapoptotis", was described in HeLa cells treated with the CQ–copper complex at 14 μM [16,17]. This mechanism is characterized by a massive cytoplasmic vacuolization due to accumulation of unfolded proteins in the endoplasmic reticulum [16]. Conversely, the activation of the caspase-3 during the apoptotic process was observed in HeLa cells treated with CQ at the highest concentration (50 μM) [17].

On the contrary, TPEN is a cell permeable, high-affinity zinc and copper chelator. Different studies demonstrated that TPEN, at a concentration higher than 1 μM induces apoptosis. In particular, exposure of PC3 and DU145 prostate cancer cells to TPEN (2–20 μM) activated caspase 3/7 activity [18]. Similar results were obtained in cultured hippocampal neurons through inhibition of the ERK signaling pathway and activation of caspase-3 [19]. Although TPEN can chelate all the endogenous transition metals such as zinc, iron, and copper, TPEN-induced apoptosis is most likely caused by chelating and thus depleting intracellular zinc, and possibly copper. This conclusion was deduced

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0162-0134/

Table 1
Sequence-specific oligonucleotide primers and probes for Quantitative Real Time PCR (qRT-PCR).

CCND1F-Exon3	5′-AGAGGCGGAGGAGAACAAAC-3′
CCND1 PROBE	5′-FAM-AGATCATCCGCAAACACGCGCAGA-TAMRA-3′
CCND1R-Exon4	5′-AGGGCGGATTGGAAATGAAC-3′
ATOX1F-Exon2	5′-TGTGCTGAAGCTGTCTCTC-3′
ATOX1 PROBE	5′-FAM-AGTTAAGTATGACATTGACCTGCCCAACAAGA-TAMRA-3′
ATOX1R-Exon3	5′-GCTCAGATTCAATGCAGACC-3′
GAPDHF-Exon1	5′-TCTATAAATTGAGCCCGCAGCC-3′
GAPDH PROBE	5′-FAM-CCTCCTGTTCGACAGTCAGCCGCATCTTCTTT-TAMRA-3′
GAPDHR-Exon2	5′-TTGACTCCGACCTTCACCTTCC-3′

from the demonstration that addition of equimolar copper or zinc, but not iron, blocked TPEN-induced apoptosis, and from the affinities of metals with TPEN (copper > zinc > iron) [20].

In the present study we aimed to investigate if the expression of components of the copper homeostasis functional network [2] can modify the sensitivity to the cytotoxicity of copper and copper-binding compounds. Indeed, a better knowledge of the determinants of such sensitivity can be exploited in the selection of cancer types to be treated and may provide further pharmacological targets to potentiate the cytotoxic effect.

ATOX1 is a copper chaperone that plays a role in copper homeostasis by binding and transporting cytosolic copper to ATPase proteins in the *trans*-Golgi network [21]. It has been reported that ATOX1-deficient cells display a marked increase in intracellular copper content secondary to impaired copper efflux [22,23] and an abnormal distribution of intracellular copper [24]. Since the ATOX1 chaperone function is crucial in the proper management and transport of intracellular copper, we evaluated if ATOX1 gene silencing in colorectal cancer cell cultures could increase sensitivity to cytotoxic drugs acting with a copper-dependent mechanism (copper ionophore or chelating drugs).

2. Experimental

2.1. Chemicals and reagents

5-Chloro-8-hydroxyquinoline (ClHQ) and the copper chelator (*N,N,N′N′,-tetrakis* (*2-pyridylmethyl*) *ethylenediamine* (TPEN) were obtained respectively from Sigma Aldrich Co., St. Louis, MO 63103, USA and Santa Cruz Biotechnology, CA. Compounds were dissolved in dimethyl sulfoxide (DMSO) (D5879, Sigma Aldrich Co., St. Louis, MO 63103, USA).

2.2. Cell culture

Human colon carcinoma cell line Caco-2 (ATCC number: HTB-37, 2.5–3.0 × 103 cells/0.33 cm^2), was grown in DMEM medium (Dulbecco's modified Eagle medium 1×; GIBCO, cat. No. 31965-023 containing 4.5 g/l of D-glucose), supplemented with 10% FBS (fetal bovine serum) and 100 U/ml of penicillin–streptomycin. The cell culture was incubated at 37 °C in humidified atmosphere with 5% of CO_2 and 95% of air. The medium was changed twice in the week.

2.3. High-resolution DNA copy number analysis

Genomic DNA (gDNA) was extracted from a Caco-2 cell line using the QIAamp DNA Mini Kit according to the manufacturer's instructions. DNA concentration and quality was determined using a ND-1000 spectrophotometer (NanoDrop, Thermo Scientific, USA).

High-resolution genome-wide DNA copy number and SNP (single nucleotide polymorphism) genotyping analysis were performed as previously described in Barresi et al. [25,26], using Affymetrix SNP 6.0 arrays (Affymetrix, Inc., Santa Clara, CA, USA) that interrogates 906,600 SNPs and 945,826 copy number probes (SNP/CNV array). Array scanning and data analysis were performed using Affymetrix® "GeneChip Operating Software" (GCOS) and "Genotyping Console™ (GTC) version 3.0.1". The following algorithms were used: 1) SNP 6.0 Birdseed v2 algorithm for genotyping, 2) BRLMM-P-Plus algorithm and Hidden Markov Model with regional GC correction for copy number analysis, and 3) the LOH (loss of heterozygosity) algorithm. As a quality control of the genotyping and copy number results "Contrast QC value" and "Median Absolute Pairwise Difference" (MAPD) were calculated as implemented in the GTC 3.0.1 software. The log2ratio between signal for each marker in each sample and the corresponding median value in a reference group (270 HapMap individuals) were calculated. The log2ratio has been smoothed using a Gaussian kernel, to improve per marker signal to noise ratio, and was calibrated so that the numerical values provided a direct estimate of copy number.

2.4. Gene expression analysis

Total RNA was extracted from tissue using RNeasy Mini Kit according to the manufacturer's instructions. RNA concentration and quality was determined using a ND-1000 spectrophotometer (NanoDrop, Thermo Scientific, USA).

Whole transcript (WT) expression analysis was performed using 100 ng of total RNA to produce amplified targets labeled in sense orientation for hybridization to the "GeneChip Human Transcriptome Array 2.0" according to the protocol supplied by the manufacturer (Cat. No. 902310, Cat. No. 900720, Affymetrix, Inc., Santa Clara, CA, USA). Human Transcriptome Array 2.0 contains >6.0 million distinct probes for analyzing simultaneously 44,699 coding transcripts and 22,829 non-coding transcripts. Array scanning and data analysis were performed using Affymetrix® Expression Console™ software (v.1.4) the application of which provides signal estimation and QC functionality for the GeneChip® Expression Arrays and the "Transcriptome Analysis Console (TAC)" which performs statistical analysis to obtain a list of differentially expressed genes. Expression level analysis was performed using the normalization method based on the following processing algorithm: Robust Multi-array Average (RMA) [27].

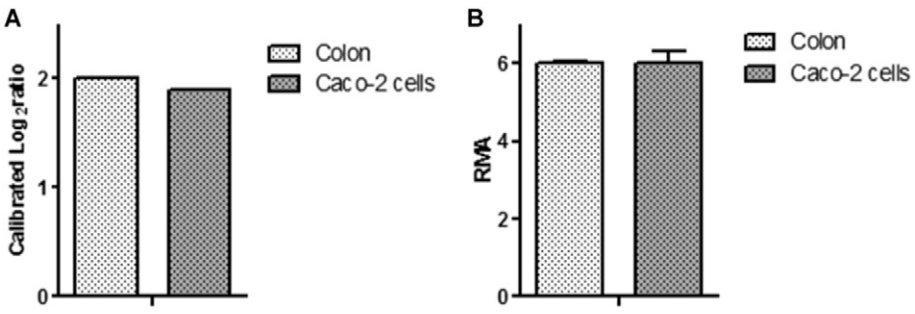

Fig. 1. A) ATOX1 gene copy number values are reported in Caco-2 cells (1.89) and in colon mucosa (2.0). The smoothed log2ratio is calibrated to copy number and anti-logged. B) Expression levels of the ATOX1 in Caco-2 cells and in colon mucosa show the same amounts of ATOX1 transcripts, in both.