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Copper supplementation amplifies the anti-tumor effect of curcumin in oral cancer cells

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ABSTRACT

Background: Oral cancer is the sixth most common cancer worldwide and 90% of oral malignancies are caused by oral squamous cell carcinoma (OSCC). Curcumin, a phytocompound derived from turmeric (Curcuma longa) was observed to have anti-cancer activity which can be developed as an alternative treatment option for OSCC. However, OSCC cells with various clinical-pathological features respond differentially to curcumin treatment.
Hypothesis: Intracellular copper levels have been reported to correlate with tumor pathogenesis and affect the sensitivity of cancer cells to cytotoxic chemotherapy. We hypothesized that intracellular copper levels may affect the sensitivity of oral cancer cells to curcumin.
Methods: We analysed the correlation between intracellular copper levels and response to curcumin treatment in a panel of OSCC cell lines derived from oral cancer patients. Exogenous copper was supplemented in curcumin insensitive cell lines to observe the effect of copper on curcumin-mediated inhibition of cell viability and migration, as well as induction of oxidative stress and apoptosis. Protein markers of cell migration and oxidative stress were also analysed using Western blotting.
Results: Concentrations of curcumin which inhibited 50% OSCC cell viability (IC_{50}) was reduced up to 5 times in the presence of 250 μM copper. Increased copper level in curcumin-treated OSCC cells was accompanied by the induction of intracellular ROS and increased level of Nrf2 which regulates oxidative stress responses in cells. Supplemental copper also inhibited migration of curcumin-treated cells with enhanced level of E-cadherin and decreased vimentin, indications of suppressed epithelial-mesenchymal transition. Early apoptosis was observed in combined treatment but not in treatment with curcumin or copper alone.
Conclusion: Supplement of copper significantly enhanced the inhibitory effect of curcumin treatment on migration and viability of oral cancer cells. Together, these findings provide molecular insight into the role of copper in overcoming insensitivity of oral cancer cells to curcumin treatment, suggesting a new strategy for cancer therapy.

Introduction

Several plant-derived phytochemicals are now known to reduce the risk of cancer development. Among them, curcumin, the yellow pigment extracted from *Curcuma longa*, has been shown to have significant anti-cancer activities both *in vitro* and *in vivo* via targeting multiple oncogenic pathways (Kunnumakkara et al. 2008). Curcumin, like several other phytoagents, is known as an antioxidant that prevents cancer via antagonizing carcinogen-triggered oxidative stress by scavenging free radicals and/or activating endogenous defence systems such as Nrf2-regulated antioxidant genes or pathways. However, recent findings have suggested that the anti-cancer

Abbreviations: DAPI, 4,6-diamidine-2-phenylindole; DCFDA, dichlorofluorescin diacetate; DMEM, dulbecco's minimal essential medium; DMSO, dimethyl sulfoxide; EMT, epithelial-to-mesenchymal transition; FITC, fluorescein isothiocyanate; NAC, N-,acetyl cysteine; NOK, normal oral keratinocyte; Nrf2, nuclear factor E2-related factor 2; PBS, phosphate-buffered saline; PI, propidium iodide; OSCC, oral squamous cell carcinoma; ROS, reactive oxygen species.

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activity of curcumin may be attributed to the induction of ROS in cancer cells (Liang et al. 2014). Intriguingly, pro-oxidation of curcumin was recently shown to be enhanced in the presence of copper (Lou et al. 2010). Copper is a redox-active metal ion that fluctuates between the oxidized (Cu^{2+}) and reduced (Cu^{+}) states and is commonly utilized by organisms living in oxygen-rich environments (Ridge et al. 2008). More than 90% of serum copper is bound by ceruloplasmin, an oxidative enzyme (Hellman and Gitlin 2002). However, high levels of free form copper is part of the radical-reactive cellular environment and its role in cancer has long been the subject of speculation (Schwartz 1975). In this regard, there is a growing body of evidence shows that levels of copper are altered on the onset and progression of malignant diseases (Gupte and Mumper 2009, Khanna et al. 2013). Recently, high serum levels of copper were observed in patients with prostate cancer and the cytotoxic action of disulfiram in cancer cells was found to occur in a copper-dependent manner (Safi et al. 2014). When excessive concentrations of free form of metal ions exist, classic antioxidants such as curcumin, catalyze the redox cycling of metal ions by reducing their oxidized form. As a result, a burst of hydroxyl free radical production ensues and the phytoagents become prooxidants (Lee et al. 2013). This mechanism potentiates a novel therapeutic approach targeting elevated copper and related oxidative stress in aggressive tumors (Gupte and Mumper 2009, Trachootham et al. 2009).

Head and neck neoplasias represent a major public health burden which accounts for 13.2% of all cancer incident among the population in Malaysia (Omar and Ibrahim Tamin 2011). One of the subtypes of head and neck cancer are the most common form of all oral malignancies (90%) arising in the oral cavity and collectively known as oral squamous cell carcinomas (OSCC) (Reis et al. 2011). It is commonly known that local recurrence, lymph node metastases and resistance to clinical drugs often cause the failure of oral cancer treatment (Rikiishi et al. 2007). Hence there is a pressing need to characterize the genetic and biochemical processes that underlie carcinogenesis and malignancy of OSCC in order to seek appropriate treatments. Pertinently, a recent study reported a progressive and elevated copper level in patients with oral OSCC when compared to the normal group, suggesting copper could be a potential biomarker for OSCC carcinogenesis (Khanna et al. 2013). Concordantly, a representative panel of OSCC cell lines which were derived from oral cancer patients with various clinicopathological characteristics was examined with different levels of endogenous copper in our laboratory. All the OSCC lines were found to possess higher copper content compared to normal oral keratinocytes (NOK). Whilst curcumin has been reported to inhibit oral cancer cell proliferation and invasion (Zhen et al. 2014), our data broadly demonstrated that the tested OSCC lines showed differential responses to curcumin treatment. In this study, we hypothesized intracellular copper levels may affect the sensitivity of cancer cells to cytotoxic chemotherapy and aimed to investigate the role of copper in regulating oral cancer cell response to a curcumin therapeutic regimen. Using a sub-set of cell lines show low copper content and insensitivity to curcumin to supplement with exogenous copper, our results revealed that copper demonstrated a remarkable effect in modulating prooxidation, anti-metastasis and cytotoxicity of curcumin in OSCC cells, potentially providing evidence for developing this natural product as a therapy option for OSCC.

Materials and methods

Herbal substance

Curcumin (mixture of curcumin, demethoxycurcumin, and bisdemethoxycurcumin), assay percentage range (98+%), Acros Organics (218580100).

Chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), curcumin, copper (II) chloride dehydrate ($CuCl_2 \cdot 2H_2O$), and N-acetyl cysteine (NAC) were purchased from Sigma-Aldrich (MO, USA). DCFDA, DMEM, DMEM, Nutrient Mixture F-12 (DMEM-F12) and Phen Green FL were from Invitrogen (CA, USA). Antibodies of Nrf2, horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse were obtained from Santa Cruz (CA, USA). Antibody of PARP, *E*-cadherin and vimentin was from Cell Signaling Technology (MA, USA). Apoalert DNA Fragmentation Kit and Annexin-V/PI Apoptosis Kit were purchased from Clontech (CA, USA) and BD Biosciences (CA, USA) respectively.

Maintenance of cells

The H-series OSCC cell lines were obtained from European Collection of Cell Cultures (ECACC) and the ORL OSCC cell lines were kindly provided by Prof Sok-Ching Cheong (Cancer Research Malaysia, Malaysia). All the OSCC cell lines were grown in DMEM-F12 supplemented with 10% fetal bovine serum, 0.5 µg/ml sodium hydrocortisone succinate, 100 units/ml of penicillin and 100 units/ml of streptomycin. Normal primary oral keratinocytes (NOK) 232 N and 337 N (by Prof Sok Ching from Cancer Research Malaysia, Malaysia) and immortalised nasopharyngeal epithelial cell lines (NP69, NP460: provided by Professor George Tsao from the Department of Anatomy, The University of Hong Kong) were cultured in keratinocyte serum free media (KSFM; GIBCO, Carlsbad, CA, USA) supplemented with 25 µg/ml bovine pituitary extract, 0.2 ng/ml epidermal growth factor, 0.031 mM calcium chloride, 100 units/ml of streptomycin and 100 units/ml of penicillin. All the cell cultures were maintained at 37 °C with 5% carbon dioxide.

Determination of cell viability

OSCC and NOK cells were cultured in 96-well plates at a density of 3000–5000 cells per well and allowed to adhere overnight, and then treated with vehicle (0.05% DMSO) and curcumin at the indicated concentrations for 24 h and 48 h Cell viability was then measured using the MTT-based colorimetric assay. Briefly the cells were incubated using 5 mg/ml of MTT reagent in PBS for 3 h and after the medium removed and replaced with 100 µl of DMSO to solubilize the crystal formazan formed in the cells, followed by measuring absorbance at 570 nm using the Tecan Infinite 200 plate reader. All cell viability assays were performed in quadruplicate. The percentage of viable cells was calculated using the formula:

Cell viability (%) = (Absorbance of treated groups/Absorbance of untreated groups) × 100%

Detection of intracellular copper

Phen Green FL method was used to detect intracellular copper levels as described previously (Lou et al. 2010). OSCC and NOK cells were cultured in 96-well plates at a density of 3000–5000 cells per well and allowed to adhere overnight. For detection of basal copper level, all cells were incubated with 5 µM of Phen Green FL in culture medium for 30 min at 37 °C. For detection of cellular copper level after treatment, OSCC cells were treated at indicated concentrations for 1 h before incubation with Phen Green FL. Then, the cells were washed three times with PBS and fluorescence measured using the Tecan Infinite 200 plate reader with excitation at 490 nm and emission at 529 nm. Binding of Phen Green FL to copper quenches its fluorescence. Therefore the fluorescence indicates free Phen Green FL which is in inversely proportional to cellular