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2016 Gastric Cancer: Global view

Update on a tumor-associated NADH oxidase in gastric cancer cell growth

Hsiao-Ling Cheng, Yi-Hui Lee, Tein-Ming Yuan, Shi-Wen Chen, Pin-Ju Chueh

Hsiao-Ling Cheng, Yi-Hui Lee, Pin-Ju Chueh, Institute of Biomedical Sciences, National Chung Hsing University, Taichung 40227, Taiwan

Tein-Ming Yuan, Shi-Wen Chen, Department of Surgery, Feng-Yuan Hospital, Ministry of Health and Welfare, Taichung 42055, Taiwan

Pin-Ju Chueh, Graduate Institute of Basic Medicine, China Medical University, Taichung 40402, Taiwan

Pin-Ju Chueh, Department of Medical Research, China Medical University Hospital, Taichung 40402, Taiwan

Pin-Ju Chueh, Department of Biotechnology, Asia University, Taichung 41354, Taiwan

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Correspondence to: Pin-Ju Chueh, PhD, Institute of Biomedical Sciences, National Chung Hsing University, Taichung 40227, Taiwan. pjchueh@dragon.nchu.edu.tw
Telephone: +886-4-22840896
Fax: +886-4-22853469

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Abstract

Gastric cancer is one of the most common human malignancies, and its prevalence has been shown to be well-correlated with cancer-related deaths worldwide. Regrettably, the poor prognosis of this disease is mainly due to its late diagnosis at advanced stages after the cancer has already metastasized. Recent research has emphasized the identification of cancer biomarkers in the hope of diagnosing cancer early and designing targeted therapies to reverse cancer progression. One member of a family of growth-related nicotinamide adenine dinucleotide (NADH or hydroquinone) oxidases is tumor-associated NADH oxidase (tNOX; ENOX2). Unlike its counterpart CNOX (ENOX1), identified in normal rat liver plasma membranes and shown to be stimulated by growth factors and hormones, tNOX activity purified from rat hepatoma cells is constitutively active. Its activity is detectable in the sera of cancer patients but not in those of healthy volunteers, suggesting its clinical relevance. Interestingly, tNOX expression was shown to be present in an array of cancer cell lines. More importantly, inhibition of tNOX was well correlated with reduced cancer cell growth and induction of apoptosis. RNA interference targeting tNOX expression in cancer cells effectively restored non-cancerous phenotypes, further supporting the vital role of tNOX in cancer cells. Here, we review the regulatory role of tNOX in gastric cancer cell growth.

Key words: Apoptosis; Capsaicin; Gastric cancer cells; Protein expression; Tumor-associated NADH oxidase

Core tip: Gastric cancer is one of the most common human malignancies, and its prevalence has been shown to be well-correlated with cancer-related deaths worldwide. Here, we review the role of tumor-associated NADH oxidase tNOX (ENOX2) in cancer, in particular its regulation of gastric cancer cell growth. The most common inhibitors of tNOX and the phenotypes associated with tNOX depletion are also discussed.

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INTRODUCTION

According to Global Cancer Statistics, nearly 1 million new gastric cancer cases and more than 700000 gastric cancer-associated death were reported in 2012[1]. Unfortunately, despite progression in the diagnosis and treatment of advanced gastric cancer, many patients suffer from metastasis and later recurrence of this disease. Thus, their poor prognosis reflects the fact that gastric cancers are often diagnosed at an advanced stage. Current research efforts have focused on available diagnostic and prognostic biomarkers of gastric cancer. For example, carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA19-9) have been used as standard biomarkers for gastric cancer diagnosis[2]. A method for detecting circulating tumor cells in gastric cancer patients, which could provide important targets for treatment and critical surrogate markers, is also being developed[3]. Moreover, polymorphisms of certain genes, such as epidermal growth factor receptor (EGFR), have been used as risk markers for gastric cancer[4]. Serum level of interleukin-18 (IL-18), which plays a role in the pathogenesis of malignancies and is a determinant of clinical outcome in gastric cancer patients, is another risk marker[5]. Recent studies have revealed that several microRNAs, when abnormally expressed, are potential biomarkers of gastric cancer[6]. Although intensive efforts to identify biomarkers in this field are ongoing, universal biomarkers for gastric cancer are scarce because of the heterogeneous properties of this cancer[2]. Thus, new and specific gastric cancer markers for diagnoses and therapeutic purpose are urgently needed.

We have previously described tumor-associated NADH oxidase (tNOX, also known as ENOX2), a member of a family of growth-related NADH (or hydroquinone) oxidases[7-10]. Unlike its counterpart CNOX (ENOX1), identified in normal rat liver plasma membranes and shown to be stimulated by growth factors and hormones, tNOX activity purified from rat hepatoma cells is unresponsive to growth stimuli and is instead constitutively active[7]. Subsequent studies have confirmed that tNOX is present in an array of cancer cell lines, including those derived from breast, cervix, colon, and lung cancer as well as leukemias[11-14]. Its activity is also observed in the sera of cancer patients but not in those of healthy volunteers, suggesting its clinical relevance[15-17]. tNOX cDNA was subsequently cloned from a HeLa cell cDNA library[9], and functional motifs of tNOX protein have been identified. The important role of tNOX in cell growth regulation is supported by a study that showed that the growth rate of mouse embryo fibroblasts (MEFs) from tNOX-overexpressing transgenic mice is approximately twice that of wild-type cells[18]. Given its expression in an array of cancer cell lines and presence in sera of cancer patients, taken together with other characteristics discussed below, tNOX possesses potential as a biomarker. Here, we briefly review the regulatory role of tNOX in gastric cancer cell growth.

EXPRESSIONS OF tNOX PROTEIN IN GASTRIC CANCER CELLS

Although tNOX expression has been demonstrated in an array of cancer cell lines, there is relatively less information regarding tNOX protein expression in gastric cancer cells. To demonstrate the expression of tNOX protein in gastric cancer cells, we utilized cell lines derived from human stomach cancers, including AGS (gastric adenocarcinoma), TMC-1 (derived from the lymph node of a moderately differentiated stomach adenocarcinoma), MKN45 (from the lymph node of a moderately differentiated stomach adenocarcinoma), SNU-1 (from a poorly differentiated primary carcinoma of the stomach), TMK-1 (from a poorly differentiated adenocarcinoma), and SCM (human gastric carcinoma) cells. Western blot analyses showed that tNOX protein was expressed to varying degrees in all gastric cancer lines (Figure 1). tNOX expression was higher in TMC-1, SCM, and TMK-1 cells than in SNU-1, MKN45, and AGS cells. Antisera used in western blot analyses were raised against bacterial tNOX, as described previously[12], and they recognized the same tNOX protein band as that identified by a commercially available anti-tNOX polyclonal antibody[14] (Protein Tech Group, Inc. Chicago, IL, United States).

We also continuously monitored cell growth dynamics, measured as cell impedance and displayed in the form of cell index (CI) values[19-22]. In this application of the cell impedance assay, the existence of cells on top of the electrodes creates an increase in electrode impedance,

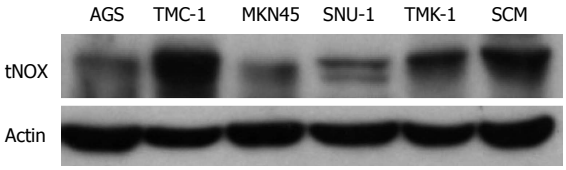

Figure 1 Tumor-associated NADH oxidase protein expressions in different human gastric cancer lines. Aliquots of cell lysates were separated by SDS-PAGE and analyzed for tNOX expression by western blot analysis. β-Actin was used as an internal control to monitor for equal loading. Gastric cancer cell lines including, AGS (gastric adenocarcinoma), TMC-1 (derived from the lymph node of a moderately differentiated stomach adenocarcinoma), MKN45 (from the lymph node of a moderately differentiated stomach adenocarcinoma), SNU-1 (from a poorly differentiated primary carcinoma of the stomach), TMK-1 (from a poorly differentiated adenocarcinoma), and SCM (human gastric carcinoma), were used to study the expression of tNOX protein. SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; tNOX: Tumor-associated NADH oxidase.

the degree of which is determined by cell numbers and the level of cell adhesion. By comparing the results from cell-impedance measurements with those obtained with various commonly used cytotoxicity assays, we confirmed that this novel measurement of electrical impedance provides an effective, continuous-monitoring method for examining cellular processes in a dye-free setting[23,24]. Using this approach, we found that both SCM and AGS cell grew much faster than MKN45 and TMC-1 cells (Figure 2). Surprisingly, the growth rate of TMC-1 cells, as determined using this method, was slower, notwithstanding the fact that they were derived from patients with metastatic stomach cancer. It has been reported that TMC-1 cells grown *in vitro* exist as a mixture of attached and suspended cells[25]. Thus, since the degree of cell impedance is determined by both cell numbers and the level of cell adhesions, the cell impedance method used here may yield a CI value that underestimates the number of TMC-1 cells.

INHIBITION OF tNOX PROTEIN

Numerous chemopreventive and anti-cancer drugs have been shown to reduce tNOX activity accompanied by a decrease in cell growth. These agents include capsaicin, the major component of chili pepper[11], (-)-epigallocatechin-3-gallate (EGCg)[26], phenoxodiol[27], and doxorubicin (trade name, Adriamycin)[28,29]. Interesting, these chemopreventive agents, often used to reverse cancer progression, preferentially inhibit tNOX activity in cancer cells, reducing cancer cell growth but having little effect on non-cancerous cells[11,26]. This is important, since the dietary pattern and availability of fresh fruits and vegetables as well as gastric cancer incidence differ greatly among countries. Statistical data bear this out, showing that South Korea, Mongolia, Japan, and China in Eastern Asia have the highest incidence rates of gastric cancer, whereas Northern America and most parts of Africa have the lowest rate[1]. Recent progress has focused on the chemopreventive effects of capsaicin, reflecting its anti-growth activity against various human cancer cell systems, including prostate[30-32], colon[33,34], hepatoma[35,36], breast[11,37] cancer, as well as leukemic[38-40]. However, there are few studies available reporting on the cytotoxicity of capsaicin in gastric cancer cells[41-43].

We have investigated the effects of capsaicin on different gastric cancer cell lines, including SCM, SNU-1, and TMC-1. In these studies, which measured metabolic activity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assays, we verified that capsaicin exerted a concentration-dependent inhibitory effect on SCM cell proliferation. After 24 h exposure, 100 μmol/L and 200 μmol/L capsaicin decreased SCM cell viability to less than 70% and 50% of control groups, respectively. Cell proliferation, measured by counting SCM cell number, was also significantly decreased in a concentration and time-dependent manner by capsaicin exposure[44]. We studied the anticancer activity of capsaicin on the proliferation of SNU-1 cells, which were derived from a poorly differentiated human gastric carcinoma, and TMC-1 cells, a metastatic gastric carcinoma line[45]. These assays showed that capsaicin induced significant cytotoxicity in SNU-1 cells at 100 μmol/L, diminishing cell numbers to smaller than 40% and 30% of control groups after 48 and 72 h exposure, respectively. To our surprise, 100 μmol/L capsaicin did not decrease the number of TMC-1 cells, even after a 72 h exposure[45]. These results imply that capsaicin exerted differential cytotoxic effects on gastric cancer lines derived from different stages of cancer progression.

DUEL EFFECTS OF CAPSAICIN ON CELL GROWTH AND tNOX IN TWO GASTRIC CANCER LINES

To further investigate whether the differential inhibitory effects of capsaicin on cell growth inhibition involves cell death, specifically apoptosis, we analyzed cells for apoptotic subpopulations using flow cytometry. Interestingly, capsaicin provoked cytotoxicity in SCM cells concurrently with caspase 3-mediated poly (ADP-ribose) polymerase (PARP) cleavage and apoptosis induction[44]. Using the pan-caspase inhibitor Z-VAD-FMK, we confirmed that capsaicin-induced apoptosis in these cells was dependent on caspase activity[44]. Consistent with results obtained in other gastric cancer cell lines, capsaicin was found to induce cytotoxicity and apoptosis in SNU-1 cells, possibly through up-regulation of p53[42]. We also demonstrated that 100 and 200 μmol/L capsaicin triggered apoptosis in 16.1% and 26.2% of SNU-1 cells, respectively[45]. In contrast, TMC-1 cells were fundamentally unresponsive to the apoptotic effect of capsaicin; exhibiting very little apoptosis in response to capsaicin exposure. The greater cytotoxicity of capsaicin toward SCM and SNU-1 cells was also reflected in the apoptotic activity

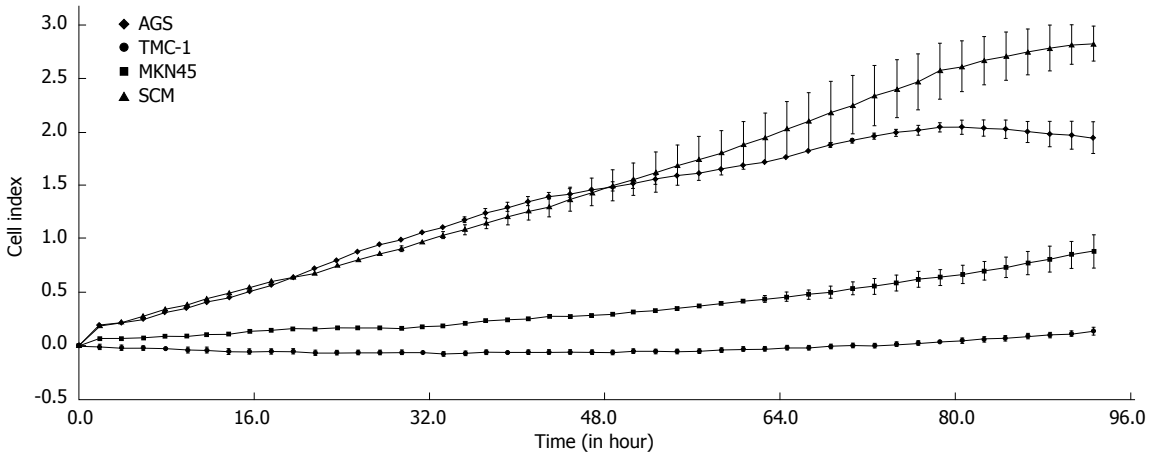

Figure 2 Cell growth monitored by cell impedance technology. For continuous monitoring of dynamic changes in cell proliferation, cells (10^4 cells/well) were seeded onto E-plates and incubated for 30 min at room temperature, after which E-plates were positioned onto the Real-Time Cell Analysis station (Roche, Mannhein, Germany). Cells were grown, and impedance was determined every hour, as previously described[21,22]. Cell impedance is defined by the cell index (CI) = (Z_i - Z_0) [Ohm]/15[Ohm], where Z_0 is background resistance and Z_i is the resistance at an individual time point. A normalized cell index was determined as the cell index at a certain time point (CI_{ti}) divided by the cell index at the normalization time point (CI_{nml_time}). Normalized cell index values measured over 92 h are shown. Gastric cancer cell lines, including AGS (gastric adenocarcinoma), TMC-1 (derived from the lymph node of a moderately differentiated stomach adenocarcinoma), MKN45 (from the lymph node of a moderately differentiated stomach adenocarcinoma), and SCM (human gastric carcinoma), were used to study cell growth pattern using cell impedance measurements.

of capsaicin in these two lines, whereas capsaicin induced neither cytotoxicity nor apoptosis in TMC-1 cells.

To identify molecular mechanisms underlying the differential cytotoxicity of capsaicin in gastric cancer cells, we assessed mitochondrial function, by measuring 3,3'-dihexyloxacarbocyanine iodide [DiOC6(3)] retention. We showed that capsaicin instigated changes in mitochondrial membrane potential in SNU-1 cells but not in TMC-1 cells[45]. Further protein analyses confirmed that capsaicin up-regulated pro-apoptotic Bak protein and down-regulated anti-apoptosis Bcl-2 protein in SNU-1 cells, whereas these changes were less prominent in TMC-1 cells[45].

Given that capsaicin-induced cytotoxicity and inhibition of tNOX activity was previously established in cancer/transformed cells[11], we next tested the possibility that tNOX protein is involved in the differential effects of capsaicin in these two gastric cancer lines. Interestingly, we found that capsaicin enhanced tNOX down-regulation in SCM cells concurrently with capsaicin-induced apoptosis[44]. Additionally, in SNU-1 cells, capsaicin-induced tNOX down-regulation was accompanied by a concentration-dependent increase in caspase 3-mediated PARP cleavage and apoptosis[45]. In contrast, capsaicin treatments promoted very slight changes in tNOX expression or caspase 3-directed PARP cleavage in TMC-1 cells, resulting in limited apoptosis. These various lines of evidences indicate that tNOX is a potential molecular target for capsaicin in gastric cancer cells that is important for gastric cancer survival.

PHENOTYPES OF tNOX-DEPLETED CELLS

To examine whether tNOX expression level is crucial for the survival of gastric cancer cells, we knocked down tNOX expression in TMC-1 cells using small interfering (hairpin) RNA (shRNA). These tNOX-depleted TMC-1 cells were more responsive to capsaicin treatments, as evidenced by enhanced caspase 3-mediated PARP cleavage, greater loss of mitochondrial membrane potential, and higher intracellular oxidative stress level compared with control groups[45]. Thus, decreased tNOX expression in TMC-1 cells resulted in greater sensitivity to the effects of capsaicin. Interestingly, reduced expression of tNOX appeared to affect cell-cycle progression such that capsaicin-induced G1 accumulation was enhanced and proliferation was reduced in tNOX-depleted TMC-1 cells[45], supporting a significant role for tNOX in TMC-1 cell growth.

We also examined the possibility that decreasing tNOX protein levels in cancer cells is sufficient to diminish tumor growth in animals. To this end, mice were injected with parental (wild type) HCT116 human colon cancer and either control (scrambled RNAi) HCT116 cells or tNOX-knockdown HCT116 cells, and xenograft tumors were examined after 60 d. We found that the growth of HCT116 xenograft tumors was significantly reduced in tNOX-knockdown groups[46], indicating that tNOX depletion in cancer cells reduces their capacity to form tumors *in vivo*.

The regulatory role of tNOX in cell growth is not limited to gastric cancer cells. Utilizing a loss-of-function approach, Chueh *et al*[47] reduced tNOX expression in HeLa cervical cancer cells using antisense

oligonucleotides and found that tNOX deficiency reduced cell proliferation, as determined by colony-formation assays. A subsequent study utilizing shRNA to specifically and effectively inhibit tNOX expression in HeLa cells showed that tNOX knockdown attenuated cell proliferation and migration by interfering with the Rac pathway[14]. In another study, tNOX-knockdown was shown to sensitize cells to stress-induced apoptosis in human HEK293 cells derived from human embryonic tissues[13], which share properties with cancer. Notably, we found that tNOX is abundantly expressed in these embryonic cells. Conversely, a gain-of-function approach showed that tNOX over-expression in non-cancerous MCF-10A cells gave rise to enhanced invasiveness, an aggressive feature of cancer cells, confirming a vital role for tNOX in cancer progression[47]. Moreover, transient up-regulation of tNOX in HCT116 cells augmented cell proliferation and migration *in vitro* and *in vivo*[46]. These phenomena were also observed in A549 human lung cancer cells, demonstrating that an epithelial-to-mesenchymal transition mechanism may be involved in the enhanced cell migration associated with tNOX up-regulation[22].

CONCLUSION

In this review, we summarized the recent literature on the biological function of tNOX in gastric cancer cells. We also considered the paradoxical effect of capsaicin on cancer growth and tNOX expression, which results in differential cellular outcomes. Collectively, these various lines of evidence establish a significant regulatory role for tNOX in cancer cell proliferation, survival, and migration. This information may provide a reasonable framework for the future development of tNOX-targeting agents as a new class of anti-tumor therapeutics.

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