NME2 Reduces Proliferation, Migration and Invasion of Gastric Cancer Cells to Limit Metastasis

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Abstract

Gastric cancer is one of the most common malignancies and has a high rate of metastasis. We hypothesize that NME2 (Nucleoside Diphosphate Kinase 2), which has previously been considered as an anti-metastatic gene, plays a role in the invasiveness of gastric cancer cells. Using a tissue chip technology and immunohistochemistry, we demonstrated that NME2 expression was associated with levels of differentiation of gastric cancer cells and their metastasis into the lymph nodes. When the NME2 gene product was over-expressed by \textit{in vitro} stable transfection, cells from BGC823 and MKN45 gastric cancer cell lines had reduced rates of proliferation, migration, and invasion through the collagen matrix, suggesting an inhibitory activity of NME2 in the propagation and invasion of gastric cancer. NME2 could, therefore, be used as a risk marker for gastric cancer invasiveness and a potential new target for gene therapy to enhance or induce NME2 expression.

Introduction

Cancer remains as a leading cause of death, accounting for 14.1 million new cases and 8.2 million deaths in 2012 [1]. The numbers of new cases are expected to increase 70% worldwide to 22 million within the next two decades [2]. Cancers in the lungs, stomach, liver, colon and breasts have the highest mortality [3]. Gastric cancer cells can directly spread to adjacent organs (local invasion) such as the pancreas, the transverse colon, the liver and the spleen as well as to remote lymph nodes, the lungs, and bone tissue. While being two different pathological processes, local invasion and remote metastasis are interconnected where the former often promotes and propagates the latter. Genetic mutations and their aberrant products are key hallmarks and enablers of cancer cells for proliferation, resistance to apoptosis, local invasion, metastasis, immune evasion, angiogenesis, and response to DNA damage. NME (Nucleoside
Diphosphate Kinase 2 or Non-Metastatic Cells) represents a group of cancer-associated and/or cancer-regulating genes.

_NME_ consists of a family of 10 genes that are also known as the NM23 genes [4] and has been associated with suppressing cancer metastasis and invasion to local tissue [5]. Among the members of this gene family, _NME1_ and _NME2_ have been extensively studied for their cancer-suppressing activities. _NME2_, which is mapped on chromosome 17q21 [6,7], encodes the B subunit of the nucleoside diphosphate (NDP) kinase [4]. Its product has been reported to inhibit the metastasis of breast cancer and melanoma cells; and a decrease in its expression was correlated with a greater metastatic potential of these cancers [8,9]. However, _NME2_ overexpression was also associated with poor prognosis for neuroblastoma and osteosarcoma [10,11]. Moreover, the _NME2_ gene was not correlated with the metastasis of endometrial hepatocellular and thyroid carcinoma [12–14]. These apparently conflicting data suggest that _NME2_ may have differential effects on different types of cancer cells and their ability to locally invade surrounding tissues or metastasize to remote organs.

Because of these diverse _NME2_ activities on different cancer types, we analyzed tissues surgically removed from patients with gastric cancer and associated the _NME2_ expression in these tissues with their pathological characteristics. This pathology study was complemented by _in vitro_ experiments, where we examined rates of proliferation, migration and invasion of gastric cancer cells that have been stably transfected with a human _NME2_ cDNA to overexpress its product. These _in vitro_ experiments are designed to understand the invasiveness of cancer cells that express different levels of _NME2_.

### Materials and Methods

This study was approved by the ethic committee on conducting human research of Lanzhou University, School of Medicine. Patients or their guardians provided their written informed consent before being recruited into the study.

#### Tissue immunohistochemistry

We analyzed _NME2_ expression in gastric cancer and adjacent normal tissues surgically removed from 139 patients admitted to the Army Regional Hospital in the city of Lanzhou from 2011 to 2012. No patients received chemotherapy or radiation therapy before surgery. These surgically removed cancer tissues were processed for hematoxylin and eosin (HE) staining. Briefly, a wax module was punched 42 aperture holes (6×7 holes/chip) of 2 mm each. Tissue blocks were embedded in these holes (one sample/hole). This wax module was then sectioned so that tissues from 40 patients could be examined simultaneously on a single slide to ensure stain uniformity. For control, first and last holes were embedded with tissue from the non-cancerous liver and kidney, respectively. _NME2_ expression was detected using a commercial immunohistochemistry kit (Beijing ZSGB Biotechnology Co, Ltd.) with a rabbit anti-human _NME2_ antibody (1:300 dilution, Beijing Biosynthesis Biotechnology Co, Ltd.) according to the manufacturer’s instructions. Levels of _NME2_ expression were numerically scored as shown in Table 1 by a pathologist who was blinded to the clinical information of these patients.

#### Cell culture and cDNA transfection

Cells from the BGC823 and MKN45 gastric cancer cell lines were purchased from the Chinese Academy of Science (Shanghai, China). Cells in the BGC823 line were originally from a 62-year-old man with poorly differentiated adenocarcinoma of the stomach and those in the MKN45 line derived from a metastatic mass of the liver from a 62-year-old Japanese woman with an undifferentiated adenocarcinoma of the stomach. Cells from both lines had residual...
levels of NME2 expression and are, therefore, ideal for studying the impact of overexpressing this gene product on gastric cancer cells in vitro.

The cells were cultured in a DMEM medium (Sigma, St. Louis, MO, US) containing 10% of fetal calf serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China) at 37°C with 5% CO2 and 95% air. At 70–80% of confluence, these cells were transfected with a cDNA for the human NME2 gene that was cloned into a pcDNA3.1 vector (Shanghai GenePharma Co., Ltd., Shanghai, China) using lipofectamine 2000 as the DNA carrier (Invitrogen, Grand Island, NY, US). Cells stably expressing NME2 (designated as NME2) were selected by growing the transfected cells in the DMEM medium containing 1 mg/ml of G418. Control cells were transfected with the pcDNA vector without the NME2 cDNA insert (designated as mock) and underwent the same selection. Un-transfected parental cells were also examined as baseline controls. An overexpression of NME2 was determined by RT-PCR to quantify the level of NME2 mRNA and by immunoblots of transfected cell lysates using a polyclonal NME2 antibody (1:100 dilution, Beijing Biosynthesis Biotechnology Co., Ltd.)

RNA isolation and RT–PCR

Total RNA was isolated from transfected and non-transfected cells using a commercial RNA isolation kit (TaKaRa Biotechnology, Dalian, Liaoning, China) according to the manufacturer’s instructions. RNA (2 μg) from each sample was reverse-transcribed using a PrimeScript RT Kit following the manufacturer’s protocol. The complementary DNA reversely transcribed from the RNA extracted from these cells was amplified by a Taq polymerase using the following primers for the NME2 cDNA: 5’-AAGCAGCACTACATTGACCTGAAA-3’ (forward) and 5’-GGTCTCCTCCAAGCATCACTC-3’ (reverse). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also amplified as control using the following primers: 5’-GCACCGTCAAGGCTGAGAAC-3’ (forward) and 5’-TGGTGAAGACGCCAGTGGA-3’ (reverse). The amplification reaction was initiated by denaturing DNA at 95°C for 5 min, followed by 30 cycles of template denaturing at 94°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 1 min. The NME2 overexpression was quantitatively defined as an increase in NME2 mRNA by two fold or greater from the baseline set by sham transfected cells.

Immunofluorescence staining of the NME2 product

Cells stably transfected with the NME2 cDNA and a vehicle vector were plated on glass cover-slips and allowed to grow until confluence. They were rinsed twice with ice-cold phosphate-
buffered saline (PBS), fixed in 4% of paraformaldehyde in PBS for 15min, permeabilized with 0.5% of Triton X-100, and incubated with 1% of bovine serum albumin (BSA) for 30 min to block non-specific binding. The cells were then incubated with a rabbit antibody against human NME2 (1:100, Beijing Biosynthesis Biotechnology) for 24 hr at 4°C followed by incubation with a FITC-conjugated secondary antibody (1:100, Beijing Biosynthesis Biotechnology) for 1 hr. All antibodies were diluted in PBS containing 1% BSA. Nuclei were counterstained with DAPI (1:100 in PBS, 10 min at room temperature).

**Cell cycle analysis**

Cells stably expressing NME2 and control cells in culture were collected and fixed with 70% of ice-cold ethanol at 4°C for 24 hr, washed twice with ice-cold PBS, and then treated with RNase A (20 μg/ml) for 30 min at 37°C. They were incubated with propidium iodide (10 μg/ml of final concentration) in the dark for 30 min at room temperature before analysis by flow cytometry for DNA ploidy.

**Colony-formation assay**

Transfected and control cells were cultured at an initial density of 1,000 cells per 100-mm culture dish in a complete DMEM medium for up to 4 weeks. They were then stained with methyl violet. All cell colonies that were larger than 2 mm in diameter (contained ≥50 cells) were counted in three separate dishes and expressed as mean ± SEM. For the purpose of data validation, we also counted viable cells that had been cultured for up to 96 hrs. Briefly, parental BCG823 and MKN45 cells and cells that were stably transfected with either a human NME2 cDNA or the vehicle vector (pc-DNA 3.1) were plated at a density of 2 x 10^4 cells/ml. They were detached 48–96 hrs after initial seeding with 1% trypsin and centrifuged at 1600 x g. Cell pellets were re-suspended in PBS and incubated with 0.04% of trypan blue for 3 min. Surviving cells were counted on a hemocytometer.

**Assays for cell migration**

Two complementary assays were performed to measure the impact of overexpressing NME2 on the rate of cell migration. First was a wound-healing assay, where cells were plated in 6 well plates and allowed to grow to ≥ 95% confluent. After washing cells twice with PBS, a sterile pipette tip was used to scratch the cell monolayer (4–5 parallel scratches/plate). Cells were washed again with PBS, photographed to mark scratched tracks, and incubated with 2.5 ml of serum-free DMEM medium. At a baseline rate of cell migration, a scratched area was partially covered by cells migrated into the injured area 24–48 hr after injury, resulting in a smaller cell-free area. Because this wound-healing assay is semi-quantitative, the results were further validated by a Transwell cell migration assay. Briefly, 6 x 10^5 cells suspended in 500 μl of serum-free medium containing 0.1% of BSA were plated in a Transwell (BD Biosciences) that was placed in a 24 well plate. The bottom chamber contained 500 μl of complete DMEM medium. Cells were allowed to grow in the top chamber for 24 hr to facilitate cell migration from the top chamber to the bottom chamber. In the end of this incubation period, the membrane in the top chamber was fixed with 4% of paraformaldehyde for 30 min, washed with distilled water, and air dried. Cells on the opposite side of the chamber (transmigrated) were stained with 0.1% of methyl violet (Dade-Behring, Newark, DE) for 30 min, washed, and viewed under an up-right microscope (x 400, NIKON). Cell migration was defined as numbers of cells on the membrane in 5 random review fields.

**Assay for cell invasion**

Transwell chambers were coated with rat tail type I fibrillar collagen [15] for 30 min at 37°C. Excess fluid was removed and coated chambers were air dried for 1 hr in a sterile environment. Cells were
suspended in 500 μl of serum-free DMEM medium containing 0.1% of BSA to a final density of 6×10^5 and plated on the collagen matrix in a transwell that was placed in a 24 well plate. The bottom chamber contained 500 μl of complete DMEM medium. Cells remained on the membrane surface were gently removed 24 hr after cells were seeded and the membrane was fixed and stained for cells that had transmigrated through the collagen matrix to the opposite side of the transwell membrane.

**Statistical analyses**

All data were analyzed using the SPSS 20.0 statistical program. Quantitative values were expressed as mean and SEM. The following statistical analyses were used in the study: Fisher’s exact test or chi-square test for defining the relationship between NME2 expression and pathological characteristics of cancer tissue; one way ANOVA for group comparison among parental cells and cells transfected with either a vehicle vector or a human NME2 cDNA; and Pearson analysis for correlation between NME2 expression and differentiation and metastasis of cancer cells.

**Results**

**Association of NME2 expression with histological characteristics of gastric cancer**

We collected surgical specimen from 139 patients with gastric adenocarcinoma. Table 2 lists the demographic information of these patients and histological characteristics of cancer tissue from these patients. NME2 expression was weak or not detected in poorly differentiated tissue, but was intensive in well differentiated tissue at a level comparable to adjacent normal tissue (Fig. 1). A high level of NME2 expression was associated with a significantly reduced rate of metastasis to the lymph nodes (p = 0.038), but not with the size of primary tumor and depth of cancer invasion. A Pearson correlation analysis showed that NME2 expression was independently associated with rates of cell differentiation (r = 0.436, p = 0.000) and spread to the local lymph nodes (r = -0.281, p = 0.001, Table 1).

**Effect of NME2 on proliferation of gastric cancer cells in culture**

To further examine the association of NME2 expression with pathological characteristics of gastric cancer cells, we studied cells from the BGC823 and MKN45 gastric cancer lines, which expressed NME2 weakly at a level compared to poorly differentiated cancer tissue (Fig. 1). These cells were transfected with a human NME2 cDNA to overexpress NME2 as quantitative measured by an increase in the NME2 mRNA (quantitative RT-PCR) and NME2 protein product (immunofluorescence, Fig. 2).

We then investigated how overexpressing NME2 regulated cell growth by measuring the DNA ploidy and colony formation of these cells. We detected no difference in the DNA ploidy between cells overexpressing NME2 and those non-transfected and mock-transfected cells from both lines (Table 3 and S1 Fig.), suggesting that an increase in NME2 expression had no effect on cell cycles. However, NME2-overexpressing cells had a significantly reduced capacity to form colonies in culture as compared to sham transfected and non-transfected cells (Fig. 3). This observation was validated by results from a cell-counting method (Fig. 3C).

**Effect of NME2 on cell migration and invasion**

We next evaluated the ability of NME2-overexpressing cells to migrate because a directional migration is an important prerequisite for the invasion of cancer cells to surrounding tissue. This directional migration was examined using a wound-healing assay, which measures the rate of cells migration into the area of injury created by a sharp object. As shown in Fig. 4A and...
4B, the width of an injured area after 48 hrs in cultures was greater for NME2-transfected cells than for non-transfected and mock-transfected cells. The slow migration was found in cells from both cell lines, suggesting that cells overexpressing NME2 migrated much slower. Because this wound healing assay is semi-quantitative, we also quantitatively measure the rate of cell migration using a Transwell assay. Consistent with the wound-healing assay, NME2 overexpressing cells migrated at \( \times 50\% \) of non-transfected or mock-transfected cells (Fig. 4).

A decrease in cell migration could potentially reduce the ability of these cells to invade surrounding tissue. To investigate this possibility, we measure the invasion of cancer cells to the collagen matrix in a well-established transwell chamber assay. BGC823 cells that overexpressed NME2 significantly reduced their ability to invade a collagen matrix to 60% of non-transfected and mock-transfected cells (Fig. 5). The rate of invasion was similarly reduced in MKN45 cells transfected with the NME2 cDNA as compared to parental cells and those transfected with a vehicle vector (Fig. 5).

**Discussion**

We have investigated the relationship between the NME2 expression and characteristics of gastric cancer in tissues surgically removed from patients and in cultured cells from two known gastric cancer lines. The study is necessary because NME2 was initially identified as the first metastasis suppressor gene, but its ability to block cancer local invasion and remote metastasis appears to be cell type specific among cancers that have so far been studied [8–14]. For
example, an analysis of 35 patients with thyroid papillary carcinoma and 11 of metastatic lymph nodes showed a significantly reduced level of the \textit{NME1} mRNA in metastatic lymph nodes, whereas \textit{NME2} mRNA was not changed in the original tumor and lymph nodes [14],
suggesting differential roles of NME1 and NME2 in cancer invasion and metastasis. A meta-analysis of 278 patients with colon cancer, 177 with breast cancer, 137 with ovarian cancer and 77 with lung cancer also found a reduced level of NME2 expression in metastatic cancer as compared with non-metastatic cancer [17]. However, despite a negative association between NME1/NME2 expression and cancer invasion to the local lymph nodes and endometrial infiltration, NME expression was not associated with TNM scores and the differentiation of intrauterine membrane carcinoma and cervical cancer [16]. Here, we provide several lines of evidence that NME2 limits the proliferation, migration and invasion to the extracellular matrix of gastric cancer cells.

First, a higher level of NME2 expression is found in well-differentiated and less invasive tissue from gastric cancer surgically removed before chemotherapy and radiotherapy in a large...
patient cohort (Table 1 & Fig. 1). The metastasis of cancer to local lymph nodes was more frequently found in the cancer tissue that had a low level of NME2 expression and this correlation between NME2 expression and differentiation/metastasis in these patient tissues remains after the data were stratified for age, gender, the size of primary tumor and depth of invasion (Table 1 & 2). These findings are consistent with a recent report that NME2 binds the telomere repeat binding factor 2 in the nucleus to reduce telomerase activity [37], suggesting a mechanistic link between NME2 expression and survival of cancer cells.

Second, we examined effects of over-expressing NME2 on rates of proliferation, migration, and invasion to the extracellular matrix in vitro of cells from the two well-studied gastric cancer cell lines BGC823 and MKN45 [18–21]. The transfection with a human NME2 cDNA resulted in a two-fold or greater level of NME2 expression in these cells as compared to mock transfected cells (Fig. 2). This NME2 overexpression did not alter DNA ploidy (Table 3), but significantly slowed the colony formation of these cells (Fig. 3), suggesting that overexpressing

| Cell          | G0/G1     | S         | G2/M      | S+G2      |
|---------------|-----------|-----------|-----------|-----------|
| BGC823        | 66.0±2.0  | 27.9±1.6  | 6.4±0.3   | 34.3±1.5  |
| BGC-mock      | 66.4±1.1  | 27.5±1.2  | 5.4±0.6   | 32.9±1.3  |
| BGC-NME2      | 65.0±0.5  | 27.6±0.9  | 6.7±0.6   | 33.8±1.4  |
| MKN45         | 58.2±0.9  | 36.1±0.4  | 6.3±0.3   | 42.4±0.8  |
| MKN-mock      | 58.9±0.1  | 34.8±0.8  | 5.6±0.4   | 40.3±0.5  |
| MKN-NME2      | 58.9±0.8  | 35.0±1.3  | 6.5±0.4   | 41.3±1.6  |

*ANOVA group analyses found no difference among untransfected cells and those transfected with either a vehicle vector or a human NME2 cDNA.

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**Fig 3. Effect of NME2 overexpression on cell proliferation.** MKN45 (A) and BGC823 (B) cells transfected with a human NME2 cDNA or a vehicle vector (mock), and untransfected cells (BL) were cultured for 14 days and then quantitatively analyzed for numbers of colonies formed (ANOVA, n = 3, *p <.05 compared to parental cells [BL]). (C) Numbers of viable BCG823 cells in culture of these three types of cells for up to 96 hrs (ANOVA, n = 3/cell type at each time point, *p <.05 compared to parental cells).

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NME2 delays a transition from DNA replication to mitosis. This result is consistent with previous reports that NME2 does not affect the growth of cultured cancer cells and is not associated with the size of primary cancer implanted into animals [22–27]. However, it is inconsistent with reports that NME2 promotes [28,29] or reduces [30] the proliferation of other types of cancer cells. Exact reasons for the discrepancy remain to be further investigated, but these data suggest cell type-specific effects of the NME2 gene on the pathogenesis of cancers.

Third, in contrast to a minimal effect on the proliferation of gastric cancer cells, the overexpression of NME2 results in a significant reduction in cell migration and invasion through the collagen matrix as demonstrated in three distinct, but complementary assays (Figs. 4 & 5). Our data support previous findings of similar inhibitory effects on breast cancer [8], oral squamous cell cancer [28] and melanoma cells [9], although exceptions were again noted in hepatocellular [13,29] and colorectal carcinomas cells [31]. It remains to be determined as whether reduced

Fig 4. Effect of NME2 overexpression on cells migration. Parental BGC823 (A) and MKN45 (B) cells and cells that were stably transfected with either a vehicle vector (mock) or a human NME2 cDNA migrate towards the scratch areas (defined by dot lines) 48hrs after injury (panels are representative images from 3 separate sets of experiments). C & D. The migration of these cells were also quantitatively measured in a transwell assay (ANOVA, n = 3/group, *p < 0.05 compared to parental cells [BL]).

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migration and invasion of NME2-overexpressing cells are resulted from a decreased capability of these cells to proliferation.

Our results identified gastric cancer cells as sensitive to over-expressed NME2, but a critical question remains as what regulates these diverse NME2 activities among different types of cancer cells. One potential mechanism is that a NME2 activity depends on up- and downstream molecules that interact with NME2 in a given cell type. Multiple molecules have previously been identified to interact or regulate NME2, such as EGFR, c-erbB-2, c-erbB-3 and sex steroid receptor in ovarian carcinomas [32]. Plakoglobin was reported to interact with NME2 to promote its expression in cells from human tongue squamous cell carcinoma [33]. NME2 was also found to interact with MDM2 (Mouse double minute 2 homolog) to reduce the motility of renal carcinoma cells [34]. MDM2 is an E3 ubiquitin-protein ligase and serves as a negative regulator of the p53 tumor suppressor. The relationship between NME2 gene and genes of myc family appears to be more complicated. Products of the NME1 and NME2 genes have been suggested to be the downstream of the c-myc regulatory pathway [7] and involved in the down-regulation of cdc42 function in neuroblastoma [35]. NME2 is also reported to interact with the G-quadruplex DNA in the nuclease hypersensitive element of the c-myc promoter to induce c-myc expression [36]. A system biology approach to examine these specific pathways instead of individual molecules may be required to dissect roles of the NME2 gene and its product in different types of cancers.

In summary, we have demonstrated that an overexpression of NME2 reduces the migration and invasion of gastric cancer cells to the cellular matrix in vitro. As a result, NME2 expression is associated with the well differentiated and less invasive histology of gastric cancer. These results suggest that the NME2 gene and its product may serve as a potential marker for predicting the invasiveness of gastric cancer and also as a therapeutic target that can be up-regulated through gene therapy.
Supporting Information

S1 Fig. Effect of NME2 overexpression on gastric cancer cells cell cycle. The DNA ploidy of cells transfected with NME2 cDNA was analyzed by flow cytometry using a commercial kit according to the manufacturer’s instructions. The panels A-C were for parental BCG823 cells and those transfected with the NME2 cDNA or vector. The panels D-F were for MKN45 cells with the same treatments.

Author Contributions
Conceived and designed the experiments: YFL AJY JFD ML. Performed the experiments: YFL AJY WL CYW MW LHZ DCW. Analyzed the data: YFL AJY. Contributed reagents/materials/analysis tools: JFD ML. Wrote the paper: YFL AJY.

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