Glucose-regulated protein 78 regulates multiple malignant phenotypes in head and neck cancer and may serve as a molecular target of therapeutic intervention

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Abstract
Glucose-regulated protein 78 (Grp78) is an endoplasmic reticulum chaperone protein and is overexpressed in various cancers. However, it is unclear how significance of this molecule play an active role contributing to the oncogenic effect of head and neck cancer (HNC). To investigate the potential function of Grp78, six HNC cell lines were used. We found that Grp78 is highly expressed in all six cell lines and many of the proteins were localized in the periphery regions, implying other function of this molecule aside from endoplasmic reticulum stress response. Knockdown of Grp78 by small interfering RNA significantly reduced cell growth and colony formation to 53% to 12% compared with that of controls in all six HNC cell lines. Using in vitro wound healing and Matrigel invasion assays, we found that cell migration and invasive ability were also inhibited to 23% to 2% in all these cell lines tested. In vivo xenograft studies showed that administration of Grp78-small interfering RNA plasmid into HNC xenografts significantly inhibited both tumor growth in situ (>60% inhibition at day 34) and liver metastasis (>90% inhibition at day 20). Our study showed that Grp78 actively regulates multiple malignant phenotypes, including cell growth, migration, and invasion. Because knockdown Grp78 expression succeeds in the reduction of tumor growth and metastatic potential, this molecule may serve as a molecular target of therapeutic intervention for HNC. [Mol Cancer Ther 2008; 7(9):2788–97]

Introduction
Stress proteins represent a class of molecular chaperones that, when expressed at high levels, can protect host cells from death by modulating the activity of proteins involved in regulating the cell cycle and in apoptosis (1). The glucose-regulated proteins (Grp) are a set of endoplasmic reticulum (ER) stress proteins originally discovered by inducing glucose starvation (2). Grp78, a 78-kDa protein, is a well-characterized ER chaperone, belonging to the heat shock protein 70 family (and therefore also named Hsp70-5 or HspA5; ref. 3). It has also been referred to as immunoglobulin heavy chain-binding protein (BiP), as it is involved in the folding and assembly of newly synthesized proteins in the ER (3). In cancer patients, depletion of glucose and oxygen induces a state of physiologic stress in the tumor’s microenvironment. To adapt to these conditions, stress proteins including Grp78 are induced in tumor cells. These proteins participate in, among other processes, the unfolded protein response, which is activated to help cells survive (3, 4). Grp78 targets misfolded proteins for proteasome degradation, regulates calcium homeostasis, and serves as a sensor of ER stress (5). Therefore, it appears to play cytoprotective and antiapoptotic functions. Recently, Grp78 expression was found elevated in many tumors and cancer cell lines, including lung (6, 7), breast (8, 9), stomach (10, 11), prostate (12, 13), colon (14), and liver (15, 16) cells. However, it is unclear whether and how of this molecule play roles associated with oncogenesis.

Grp78 association with head and neck cancer (HNC), a common cancer with an estimated ≥500,000 new cases diagnosed annually around the world (17), has not been well studied. The overall 5-year survival rate for patients with HNC is among the lowest of the major cancers and has not changed over the past two decades. The standard treatment is surgery or radiation or a combination of the two (18, 19), which provide acceptable local control and survival rates for early-stage tumors but are limited to offer patients with advanced disease. Even with a good response to treatment, patients with advanced disease often suffer from substantial functional and cosmetic morbidity. A better understanding of the molecular mechanisms underlying HNC may lead to improve therapy.

Received 2/18/08; revised 3/20/08; accepted 4/7/08.

Grant support: Ministry of Education, Taiwan grant EMRPD150221 and National Science Research Grant of Taiwan grant NSC 95-2745-B-182-004-URD.

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Although Grp78 has been reported overexpressed in many tumors, it is unclear whether Grp78 elevation is a consequence of tumor stress or it also plays an active role contributing to malignant phenotype. For that reason, we investigated how Grp78 regulates homeostasis of HNC and whether this molecule serves as molecular target of therapeutic intervention.

Materials and Methods

Patients, Cell Lines, and Culture

Six HNC cell lines were used, including nasopharyngeal cancers [BM1 (20), BM2, and 076 (21)], oral cancer [OECM1 (22)], and pharyngolaryngeal cancers [Fadu (ATCC HTB-43) and Detroit 562 (ATCC CCL-138)]. BM2 cell line is derived from a bone marrow metastatic lesion of a 40-year-old male patient with nasopharyngeal carcinoma (Chang Gung Memorial Hospital). Biopsy of the primary tumor of this patient showed a WHO type II, nonkeratinized carcinoma, with histopathologic stage of T4N3M0. The BM1, BM2, and OECM1 cells were grown in RPMI 1640, 076 cells in DMEM, and Fadu and Detroit cells in MEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. Cells were grown at 37°C in a humidified incubator containing 5% CO2. Cell viability was determined by staining with 0.25% trypan blue.

Five consecutive patients with HNC seen at the Head-Neck Surgery clinics at Chang Gung Memorial Hospital were recruited for study, with approval from the Institutional Review Board of Chang Gung Memorial Hospital. Written informed consent was obtained from all patients. Biopsies of the tumor sample and grossly normal mucosa cells were obtained from each subject before chemotherapy or radiotherapy.

Protein Extraction and Western Blot Analysis

Tissue samples (~50 mg) or cell pellets were homogenized in 300 μl lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1.5 mmol/L MgCl2, 1% Triton X-100] and incubated on ice for 30 min. After centrifugation at 14,000 x g for 30 min at 4°C, the supernatant containing the protein extracts was collected. For Western blot analysis, 20 μg of the proteins were separated by 8% SDS-PAGE on anitrocellulose membrane. The membrane was hybridized with anti-Grp78 monoclonal antibody (clone N-20; Santa Cruz Biotechnology) and then incubated with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology). The protein images were developed by using a Renaissance Western Blot Chemiluminescence Reagent kit (NEN Life Science Products) following autoradiography. To define the relative expression of Grp78 in clinical samples, the band density of each tumor sample was compared with that of the normal tissue sample from the same patient after normalization with an internal control (actin protein expression). Grp78 expression in tumor tissue greater than 2-fold of the normal counterpart was defined as overexpressed.

Immunofluorescent Staining and Confocal Microscopy

Glass coverslips were coated with 1% poly-l-lysine (Sigma) for 10 min at room temperature and then air dried, after which 5 x 10^5 cells were seeded onto a coverslip and incubated at 37°C overnight. After washing with PBS, the cells were fixed in 3.7% formaldehyde in PBS buffer for 10 min and then permeabilized with a permeation buffer (0.05% Triton-X-100 in PBS) at 4°C for 10 min and blocked with 1% FBS in PBS at 37°C for 30 min. The coverslips were incubated with anti-Grp78 antibody, rinsed, and stained with FITC-conjugated secondary antibody. The coverslips were then mounted with mounting medium containing 4',6-diamidino-2-phenylindole fluorescence (Vector Laboratories) and the fluorescence was visualized with a confocal laser microscope (Leica TCS Sp2 MP).

Cloning of Small Interfering RNA Plasmid

The pTOPO-U6 vector was used to construct a Grp78-small interfering RNA (siRNA) and Grp78-scramble plasmid as described previously (22, 23) in which a 22-nucleotide sense and antisense hairpin oligonucleotide was generated complementary to Grp78 mRNA. This hairpin oligonucleotide included two restriction enzyme cleavage sites corresponding to the blunt and overhanging ends matching EcoRV- and BbsI-digested pTOPO-U6. The sequence of the Grp78-siRNA oligonucleotide was 5’-AAGGATGTTATGATGCTGAGAATCTCAGTACACAGTATACC-3’. The Grp78-scramble plasmid contained the same nucleotides as the Grp78-siRNA plasmid but in a random sequence (5’-AAGGATATTGATGCTGAGAACTTCAATCTCAGTACATATT-3’). Ligation of the hairpin oligonucleotides and pTOPO-U6 at the cloning sites produced the Grp78-siRNA and Grp78-scramble plasmids (Fig. 2A).

Plasmid Transfection and Cell Growth Assay

For plasmid transfection, cells were seeded at a density of 5 x 10^5 in a 100-mm dish and cultured for 16 h. When 50% confluence was reached, the cells were transfected with 6 μg Grp78-siRNA or the vector plasmids using LipofectAMINE 2000 reagent (Invitrogen) in Opti-MEM medium (Invitrogen) for 16 h, after which the medium was replaced with fresh complete medium. The transfection efficiencies were all >70% in each of the HNC cell lines. Changes in the cellular phenotype were determined. To determine cell growth, cell numbers were counted daily with a hemocytometer.

Colony Formation Assay

A total of 1,000 to 3,000 cells, transfected with either Grp78-siRNA or vector plasmids, were seeded onto six-well plates and allowed to grow without moving for 7 days in complete culture medium containing 20% FBS. The plates were then stained with 5% crystal violet for 15 min, after which the cell colonies were counted.

Cell Migration Assay

Cell migration was evaluated by an in vitro wound healing assay. After transfection with either Grp78-siRNA or vector plasmid, 2 x 10^5 transfectants were seeded in each
well of a six-well fibronectin-coated plate (Corning) and incubated for 6 h to allow monolayer cell formation. The cell layer was wounded with a micropipette tip and then incubated in the presence of 1% FBS culture medium for 48 h. Cell migration toward the wounded area was observed and photographed.

**Cell Invasion Assay**

A cell invasion assay was done by using a BioCoat Matrigel (Becton Dickinson Biosciences) and Millicell invasion chamber (Millipore). The Matrigel, 100 μL diluted at 5 mg/mL, was coated onto the membrane of the Millicell upper chamber with a pore size of 8 μm in a 24-well plate for 12 h at 37°C followed by washing with PBS and blocking with 1% BSA for 1 h at 37°C. Cells (1 × 10⁵) transfected with either Grp78-siRNA or vector plasmids in 1% FBS medium were seeded into the upper chamber. The lower chamber contained complete culture medium, which included 10% FBS to trap invading cells. Cells in the lower chamber, which have passed through the Matrigel-coated membrane, were counted daily for 4 days.

**Mice and Xenograft Tumors**

To investigate the effects of Grp78-siRNA treatment on tumor growth in vivo, we established a xenograft Fadu and Detroit tumors in BALB/c nude mice. The 5-week old female BALB/c-null mice were used and cared for according to institutional guidelines. For xenografting in situ study, the mice were injected s.c. in the upper portion of the hind limb with 5 × 10⁶ Fadu or Detroit cells. Three days after tumor cell xenografting, the mice were randomly divided into two groups of seven mice each. The experimental group was injected i.v. with 50 μg Grp78-siRNA plasmid in 50 μL PBS followed by a booster of 25 μg of the plasmid in 25 μL PBS twice a week for 3 weeks. The two control groups were injected on the same schedule as the experimental group but with either vector or scramble plasmids.

For in vivo imaging system examination, luciferin substrate (100 μL of 30 mg/mL in PBS; Promega) was injected s.c. After 10 min, mice were anesthetized with an isoflurane-oxygen mixture. Photoemissions from the luciferin-luciferase reaction were detected with a sensitive CCD camera. The imaging system first produced a photographic image in the chamber under dim illumination followed by luminescent image acquisition. The overlay of the pseudocolor images represents the spatial distribution of photo counts produced by active luciferase. Living Image software (Xenogen) was used to integrate the bioluminescence signals and measure photo flux obtained from the mice. The mice were then sacrificed and the livers were examined to evaluate the metastatic potential of the tumor xenografts.

**Results**

**Grp78 Is Overexpressed and Localized in the Periphery Region of Cancer Cells**

To understand subcellular localization of Grp78 in normal and cancer cells, confocal microscopic examination was done using immunofluorescent staining with anti-Grp78 antibody on four normal oral keratinocytes and six HNC cells (BM1, BM2, 076, OECM1, Fadu, and Detroit), whereas staining with 4,6-diamidino-2-phenylindole for nucleus serves as controls. As shown in Fig. 1A, Grp78 presented as basal levels of expression in normal keratinocytes (Fig. 1A, in contrast to high levels of expressions in all the HNC cells (Fig. 1B). In those cancer cells, many of the Grp78 proteins were localized in the periphery regions, implying other functions aside from ER stress response in cancer cells.

To examine the clinical significance of Grp78, paired normal and cancerous tissues from five patients with HNC were obtained for study. As shown in Fig. 1C, all of the cancer tissues showed higher Grp78 protein expression than the normal counterparts. These results indicate that Grp78 play an important role in the carcinogenesis of HNC.
Figure 1. Differential expression of Grp78 between normal keratinocytes and HNC cells. A and B, immunofluorescent staining and confocal microscopy examination for Grp78 localization in four cell lines of human normal oral keratinocytes (NHOK1-NHOK4; A) and six HNC cell lines (BM1, BM2, 076, OECM1, Fadu, and Detroit; B). Grp78 was detected using a Grp78-specific primary antibody with a FITC-conjugated secondary antibody (green). 4',6-Diamidino-2-phenylindole staining was done for nuclear localization (blue). Merge imaging was shown to indicate subcellular localization of Grp78 in a cell. C, overexpression of Grp78 in tumor (T) tissues compared with paired grossly normal mucosa (N) from patients with HNC. Grp78 protein expression was determined by Western blot. Expression of actin protein served as an internal control.
Grp78 Knockdown Inhibits Cell Growth and Colony Formation

To validate whether Grp78 functions in cell growth regulation, six different HNC cell lines were used to examine changes in cellular phenotypes after Grp78 knockdown by siRNA. After 2 days of transfection with Grp78-siRNA plasmids, scramble plasmids, or vector, the cells were harvested for Western blot analysis. Compared with controls, Grp78 was significantly knocked down (>90% inhibition) by the Grp78-siRNA plasmid, indicating the effectiveness of this siRNA design (Fig. 2).

The cellular effects of Grp78-siRNA were examined. Treatment with Grp78-siRNA resulted in a gradual but significant decrease in cell growth in all six cell lines tested (Fig. 3A). At day 5, compared with the vector treatment controls, cell growth was reduced to 18%, 39%, 40%, 53%, 12%, and 48% in BM1, BM2, OECM1, Fadu, and Detroit cells, respectively. Consistently, Grp78-siRNA significantly suppressed cell colony formation. Colony number, size, and cell density were all lower than with the vector or scramble controls (Fig. 3B). Compared with the vector treatment controls, the colony density of Grp78 knockdown cells were reduced to 41%, 36%, 27%, 26%, 31%, and 25% in BM1, BM2, OECM1, Fadu, and Detroit cells, respectively.

Grp78 Knockdown Suppresses Cell Migration and Invasion

We examined whether Grp78 also participates in the malignant phenotypes of cell migration and invasion. Cell migration and invasion were determined using in vitro wound healing and Matrigel Transwell invasion assays. Monolayer cultures of HNC cells, transfected with either Grp78-siRNA or control vector, were wounded by a micropipette tip. Cell migration toward the wounded area was observed. As shown in Fig. 4, although different lines of cells migrated at different rates, all the Grp78-siRNA transfectants in each line migrated slower toward the wounded area compared with controls. At 24 h, the wounded area was almost completely covered by BM1, BM2, OECM1, Fadu, and Detroit cells, whereas the siRNA-transfected cells were still moving toward into the area. The migrations in Fadu and Detroit cells were slower; however, significant differences were also found at 48 h. Knockdown of Grp78 thus reduces migration, suggesting a positive regulation of this protein in cell migration.

Figure 2. Knockdown of Grp78 expression by siRNA. A, two plasmids clones were designed: Grp78-siRNA and Grp78-scramble, with their sense and antisense hairpin structures illustrated. B, suppressing Grp78 expression by Grp78-siRNA plasmids in six HNC cell lines (BM1, BM2, OECM1, Fadu, and Detroit). For each experiment, $5 \times 10^5$ cells were seeded in a 10-mm dish plate and transfected with Grp78-siRNA, Grp78-scramble, or vector plasmids. Cells were further cultured for 2 d and the cellular proteins were extracted for determination of Grp78 expression by Western blot analysis. Actin protein level was determined as internal control.

Suppression of Grp78 expression thus apparently reduces cell growth and the colony-forming ability of HNC cell lines.
For the invasion assay, Grp78-siRNA-transfected cells were seeded in the upper chamber of Matrigel-coated Millicell. The number of cells invading the lower chamber was determined daily. As shown in Fig. 5, significant reduction of Grp78-siRNA-transfected cells were found invaded to the lower chamber compared with controls. By day 2, more than 50% of reductions were found in all six cell lines. By day 4, the invading cells were reduced to 23%, 14%, 15%, 1%, 17%, and 2% in BM1, BM2, 076, OECM1, Fadu, and Detroit cells, respectively. Apparently, knockdown of Grp78 expression suppresses the invasion ability of HNC cells.

**GRP78 Knockdown Inhibits Tumor Growth In vivo**

To investigate the effects of Grp78-siRNA treatment on tumor growth in vivo, we established a xenograft Fadu

**Figure 3.** Grp78 knockdown inhibits cell growth (A) and colony formation (B) in six HNC cell lines: BM1, BM2, 076, OECM1, Fadu, and Detroit. A, for each experiment, 5 × 10^5 cells were seeded in a 10-mm dish plate, transfected with Grp78-siRNA, Grp78-scramble, or vector plasmids, and then cultured for up to 5 d. Cell numbers were determined every 24 h. Experiments were done in triplicate. B, after transfection with Grp78-siRNA, Grp78-scramble, or vector plasmids, 1,000 cells were seeded in a six-well plate and incubated for 7 d to allow colony formation. Cell colonies were visualized by 5% crystal violet staining. The relative number of colonies was quantitatively determined by spectrometry measurement of the absorbance at 560 nm in each plate after elution of crystal violet solution. After normalization with a vector control, the average results were indicated at the bottom of each plate. Experiments were done in duplicate.
and Detroit tumor in BALB/c nude mice. After injection of the tumor cells, siRNA or vector plasmids were administered i.v. into seven animals per treatment group and continuously monitored for total of 40 days. Figure 6A shows the average tumor volume between these two groups. In both xenografts, tumors in mice given siRNA had sustained, significant growth arrest compared with the controls. On average, Grp78-siRNA treatment resulted in 44% less tumor growth than controls at day 18 ($P = 0.03$) and 69% less at day 34 ($P = 0.004$) for Fadu tumors (Fig. 6A-1). The corresponding results for Detroit tumors were 60% less at day 18 ($P = 0.017$) and 66% less at day 39 ($P = 0.016$; Fig. 6A-2).

To examine whether the suppression of tumor growth is associated with the effect of injected Grp78-siRNA, the xenograft tumors were dissected to examine Grp78 expression by immunohistochemical method. As shown in Fig. 6B, the Grp78 proteins were knockdown in the siRNA-injected xenograft tumors in both Fadu (Fig. 6B-1) and Detroit (Fig. 6B-2) cells compared with the higher levels in the vector-injected tumors, whereas the expressions of Grp78 in hair follicles of the two samples were used as internal controls. These results indicate that the inhibition of tumor growth induced by Grp78-siRNA is associated with decreased expression of this molecule.

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**Figure 4.** Grp78 knockdown reduces cell migration in six HNC cell lines (BM1, BM2, 076, OECM1, Fadu, and Detroit). After transfection with Grp78-siRNA, Grp78-scramble, or the vector plasmids, $2 \times 10^5$ transfectants were seeded per well in a six-well fibronectin-coated plate and incubated for 6 h to allow monolayer cell formation. Cell layers were wounded with a micropipette tip and then incubated in the presence of 1% FBS culture medium for up to 48 h. Cell migration toward the wounded area was observed and photographed.
Having shown that Grp78-siRNA knockdown suppressed cell migration and invasion in vitro (Figs. 4 and 5), we investigated the same question in vivo by injecting mice with luciferase-transfected Fadu cells followed by administration of Grp78-siRNA plasmids, scramble plasmids, or vector plasmids. As shown in Fig. 6C, the luciferin flux in the group of siRNA-treated mice was significantly lower than in scramble-plasmid-treated controls. Quantitation for the luciferin flux showed average reductions to 8.8% at day 11 and 7.0% at day 20 (Fig. 6C-1). Examination on the livers of the siRNA-treated mice also showed that Grp78 knockdown suppressed tumor metastasized to the organs (Fig. 6C-2). Six of the seven mice in scramble-treated groups had one or more liver tumor mass, whereas none of tumor was found in the livers of siRNA-treated group.

**Discussion**

Previously, the study of the Grp78 emphasizing on the cytoprotective and antiapoptotic functions in response to ER stress, leading to the modulation of chemosensitivity. For example, overexpression of Grp78 suppresses apoptosis of human gastric cancer cells induced by celecoxib, whereas knockdown of Grp78 drastically enhances apoptosis (24). Likewise, down-regulation of Grp78 by transfection of antisense cDNA increases cisplatin-induced cell death (25). Grp78 is also thought to play a protective role against ultraviolet radiation- and selenium-induced cell death (26–28). It has been reported to form complexes in the outer ER membrane with procaspases, such as caspase-7 and mouse caspase-12, and to suppress their activity (29, 30). Because caspase-7 is a downstream executioner protein activated by a variety of chemotherapeutic agents, suppression of this protein by Grp78 may confer chemo-resistance in a variety of human cancers.

In this study, we further show that Grp78 also plays an active role contributing to multiple malignant phenotypes. Previously, Grp78 has been implicated in the regulation of tumorigenesis. It has been shown that spontaneous activation of a reporter gene driven by the Grp78 promoter was found to be highly expressed in xenograft tumors in mice (31). Inhibition of Grp78 in xenografted fibrosarcoma suppressed tumor growth in mice presumably due to a cytotoxic T-cell response (32). In agreement with these studies, we further showed that Grp78 actively regulates several malignant phenotypes, including cell growth (Fig. 3), cell migration (Fig. 4), and invasion (Fig. 5). Moreover, we found that Grp78 is highly expressed in the periphery regions of cancer cells (Fig. 1B). These results are consistent with previous findings in the rhabdomyosarcoma and prostate cancer cells (33, 34), implying a function or interaction with molecules located in the membrane compartment. Because membrane compartment of cancer cells contains many receptors and regulatory molecules involved in cell growth, migration, and invasion, it may be worthwhile to investigate whether Grp78 contributing to malignant phenotype is associated with certain membrane molecules.

Understanding the mechanisms underlying oncogenesis has wide-ranging implications for choosing cancer therapy.

**GRP78 Knockdown Inhibits Tumor Metastatic Potential In vivo**

Having shown that Grp78-siRNA knockdown suppressed cell migration and invasion in vitro (Figs. 4 and 5), we investigated the same question in vivo by injecting mice with luciferase-transfected Fadu cells followed by administration of Grp78-siRNA plasmids, scramble plasmids, or vector plasmids. As shown in Fig. 6C, the luciferin flux in the group of siRNA-treated mice was significantly lower than in scramble-plasmid-treated controls. Quantitation for the luciferin flux showed average reductions to 8.8% at day 11 and 7.0% at day 20 (Fig. 6C-1). Examination on the livers of the siRNA-treated mice also showed that Grp78 knockdown suppressed tumor metastasized to the organs (Fig. 6C-2). Six of the seven mice in scramble-treated groups had one or more liver tumor mass, whereas none of tumor was found in the livers of siRNA-treated group.
treatment targets. Directing treatment at molecules such as Grp78 that are overexpressed in tumor tissues ought to minimize cytotoxic effects on normal cells. It has been reported that a chimeric peptide with a Grp78-binding motif fused to an apoptosis-inducing sequence suppressed tumorgrowth inmurineprostateandbreastcancermodels (34). In the present study, we found that direct knockdown Grp78 expression by siRNA delivery renders potent antitumor effects, with significant inhibition of xenografted

tumor growth in situ (Fig. 6A) and suppression of the metastatic potential (Fig. 6C). These findings suggest that Grp78 is a potential molecular target in the development of adjuvant therapy for HNC.

Our study thus adds to the literature on the direct association of Grp78 with the aggressiveness of HNC, particularly on its functions of positive regulations in cell growth, migration, and invasion. Knockdown of the gene expression succeeds in the reduction of malignant
phenotypes in both cell lines and tumor xenografts in mice. The growing body of evidence on the effects of Grp78 and its manipulation serves as a foundation for further application of this molecule as a molecular target of therapeutic intervention for HNC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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