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Anti-Metastatic Effect of Maghemite Nanoparticles on CRL-1739 Adenogastric Carcinoma Cell

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ABSTRACT

Over the past four decades, the spreading of gastric adenocarcinoma has been increased statically world-wide. Even though cancer treatment is really challengeable as metastasis still shows critical stage for treatment in cancer patients. However, most of drugs have cytotoxic effect on cancer cells but failed at metastatic state. Maghemite nanoparticles encompass massive biomedical applications inducing cytotoxic effects. In this study, we focussed to evaluate the anti-metastatic and cell regulatory consequence of maghemite nanoparticles on gastric adenocarcinoma (AGS) cell. The anti metastatic strength was questioned by monitoring the production of metastatic factor nitric oxide (NO) which shows statistically considerable reduction while compared to untreated AGS cells. Clonogenic assay revealed low cloning efficiency and proliferation of these cells. Failing wound closer and cell migration, defeat of cell adhesion to extracellular matrix owing to decreased expression of cell adhesion factors in nanoparticle treated cells were noted from scratch assay analysis and cell adhesion assay respectively. Semi-quantitative PCR for cell cycle regulative genes illustrated that maghemite nanoparticles intention to arrest AGS cells at the G2/M phase transition and leads to progression of mitotic catastrophe. Further, analysis of proteins via two dimensional gel electrophoresis and LC-MS/MS endorsed the down regulation of proteins. The identified proteins are mainly concerned in the regulation of cell survival, proliferation, cell morphology and structure maintenance, drug resistance, tumour genesis and metastasis of AGS cells. From the results obtained here, it was concluded that maghemite nanoparticles have potent anti-metastatic activity and persuade cell death via mitotic catastrophe in gastric cancer cells.

Keywords: Gastric adenocarcinoma; Maghemite nanoparticles; Anti-metastatic; G2/M phase arrest; Mitotic catastrophe

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INTRODUCTION

Tumour metastasis in addition to invasion is perceived as problematical and multi step cellular process [1]. Conflict of the drugs antagonist to neoplast is the vast underlying principle for failure of therapy that responsible disease recurrence and metastasis. The genetic root of resistance to cancer therapy is generally involving complex multiple processes like DNA repair and drug metabolism [2]. In gastric cancer patients at the advanced stage most of approved drugs get refused to accept in chemotherapy together with cisplatin, 5-fluorourasil. After treatment the gastric cancer cell lines SGC7901 and AGS are acquired the resistant to 5-fluorourasil, emerge as cancer stem cell character with resistance to multiple chemotherapeutic drugs, self renewable ability and enhanced tumorigenic potential. The gastric cancer patients treated with 5-fluorourasil will become resistant within the period of 8 months [3]. Ramucirumab is a monoclonal antibody used to prevent gastric cancer spreading (metastasis) in the advanced

stomach cancer, which have shown severe side effects. Radiation therapy has further side effects such as fatigue, diarrhea, skin problems and low blood cell counts. Side effects were often more when given along with chemotherapy. Surgery is limited as only 23% for gastric cancer patients at stage IV which extents survival to one year [4]. There is also a lack of effective anti- metastatic therapeutic agent for gastric cancer without or mild side effects in better-quality of therapy to improve patient's survival. However, unique physic-chemical properties nanoparticles have been used in range of application including diagnostics, therapeutics and technologies in medicine [5]. Maghemite nanoparticle shows cellular uptake, bio distribution, metabolism and toxicity depends on the property and route administration of maghemite nanoparticles. Iron oxide nanoparticles have shown to be improve the effectiveness of anticancer drugs, used as carrier in targeted therapy and withdraw the multi drug resistance in cells [6]. Several research group shown the proliferative efficiency of cells decreased with intracellular concentration of iron oxide nanoparticles [7]. In our recent study, we showed the anti-proliferative and apoptotic potential on AGS cells in addition to null effect in normal intestinal epithelial cell (IEC-6) in response to maghemite nanoparticles. Though, the anti metastatic efficiency and a lack of facts of molecular/ cellular events by which mechanism; maghemite nanoparticle tempts apoptosis in gastric cancer further need to be elucidated for betterment of gastric cancer therapy. Proteomic will be the fundamental to study the time lapse of expression of protein, interlinked network pathways of molecular systems and those together to control cell, tissues, organs and organisms. From proteomic analysis the understanding of human biology at molecular level improves applications in diagnostic, therapeutic and medical [8]. Identification and quantification of protein and their post-translational modification in biological systems is made simple with proteomics [9]. At any given point of time in the physiological/ pathological state proteins were the dynamic and representing protein equivalents of transcriptome. Hence, our current study aimed to look into the cellular events that maghemite nanoparticle bring apoptosis, anti metastatic activity in AGS cell by means of analysing cell metastatic, cell migration, cell adhesion and cell cycle regulatory factors.

MATERIALS AND METHODS

Cell Culture

The human gastric adenocarcinoma (AGS) cell line (ATCC CRL-1739) was obtained from the National Centre for Cell Science, Pune (NCCS). The cells were grown in F12-K medium supplemented with 2 mM L-glutamine, 1% of penicillin/streptomycin and 10% of foetal bovine serum (FBS). The cultures were maintained at 37°C with 5% CO_2 and sub cultured periodically.

Preparation of Ion Oxide Nanoparticle

Maghemite nanoparticles (γ-$Fe_2O_{3)}$ was obtained from Sigma- Aldrich (size $\leq$ 50 nm), prepared as 5 mg/ml concentration stock in F12-K basal medium and sonicated for 9' pulse 9' interval for 5 minutes at 4°C and filtered to get sterile stock solution.

Griess Nitrite Assay (Level of Nitric Oxide)

Nitric oxide is important in cell angiogenesis and was measured colorimetrically. AGS cells were treated with varying concentrations maghemite nanoparticles (12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml) and without nanoparticles for 24 h and cell lysates were collected in phosphate buffered saline (PBS). To the cell lysate 0.3 ml of sodium nitroprusside (100 mM) was added and incubated for 3 h. To this 0.5ml of Griess reagent containing 1% of sulphanilamide, 2% of phosphoric acid, 0.1% of *N* -(1-naphthyl)ethylenediamine was added then measured spectrophotometrically at 516 nm.

Clonogenic Assay

Assay was performed by plating before treatment. AGS cells were treated with varying concentrations maghemite nanoparticles (12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml) and without nanoparticles. After 24 h of treatment cells were allowed to form a colony. A single colony contains minimum of 50 cells and above. Then cells were washed with PBS and fixed with 6.0% glutaraldehyde followed by stained with 0.5% crystal violet and photographed [10].

Scratch Assay

AGS cells were grown to confluence in 6 wells plate. Before 80% of confluence each plate were scraped with a 20-200 µl tip to form scratch wound. Then cells were treated with and without nanoparticles for 24 h. The wound areas at various points were photographed and wound distance was measured using NIS-Element software.

Cell Adhesion Assay

The cell adhesion assay commonly analysis ability of cell attachment to extra cellular matrix (ECM). Type Colloge-I coated 96 well plate was blocked with bovine serum albumin (BSA). After 24 h treatment cells had trypsinized and were seeded in BSA blocked wells. Then cells were allowed to attach for specific time and non attached cells were washed with PBS. Cells were fixed with 4% formaldehyde and stained with 0.1% crystal violet. The images of the fibroblasts were captured using a Nickon camera. In another one setup of experiment the stained crystal violet was solubilized with 0.2% TritanX -100 and measured colorimetrically at 570 nm.

Semi Quantitative PCR

After 24 h of maghemite nanoparticles treatment with various concentrations, total RNA was isolated from AGS cells using TRIZOL reagent. For DNAase treatment 3 µg of RNA was incubated with DNAse buffer (1X) and DNAse enzyme (3 units) in 30 µl of volume for 15 minutes at room temperature. Reaction was stopped by adding EDTA 3 µl (1 mM/L) and heat inactivated at 65°C for 10 min. After DNAse treatment, total RNA (1 µg) was reverse transcribed (RT) using M-MuLV RT enzyme and 0.5 µg of Oligod Tprimers in a 20 µl reaction volume. Amplified cDNA were subjected to PCR and amplified products resolved in 2% agarose gel electrophoresis. The digital image of gel was captured by using gel documentation system and then dentiometric analysis was performed with image lab platform 2.1 software. The targeted gene expressions were normalized to GAPDH as an internal control. The details of primers used in this study were described below in the Table 1.

Table 1: The following targeted genes were analysed by semi quantitative PCR

Genes	Forward Primer (5'-3')	Reverse Primer (5' – 3')	T_a	Amplicon size
Cyclin A	TGAAGATGCCCTGGCTTTTA	AGAAGATGAAAAGCCAGTGAGTG	55˚C	150 bp
CDK4	CCGAAGTTCTTCTGCAGTCC	GTTGGGCAAAATCTTTGACC	55˚C	137 bp
CDK6	TGGAGACCTTCGAGCACC	CACTCCAGGCTCTGGAACTT	57˚C	149 bp
GAPDH	CCATCACCATCTTCCAGGAG	CCTGCTTCACCACCTTCTTG	50ºC	576bp

Sample Preparation for Proteomic Study

After 24 h of treatment the control and maghemite nanoparticles treated AGS cells (50 µg/ml, IC50 value calculated from MTT cell proliferation assay) in 100 mm petri dishes were washed with ice cold Tris (pH=7.4) for 3 times. Then 100 µl of ice cold UTCD sample Buffer (urea 7 M, thiourea 2 M, CHAPS 4%, DTT 40 mM, IPG Buffer 0.5% and protease inhibitor cocktail) was added per petri dish. Collected in microfuge tube and centrifuged for 14000 rpm at 4°C for 15 minutes. Collected supernatant was aliquoted, and stored at -80°C until use. Protein was quantified with GE 2D Quant Kit.

Isoelectric Focussing

The isoelectric focussing was performed using 13cm immobilized precast pH gradient (IPG) strips with a linear pH range between 4-7. The strips were active rehydrated with 250 µl of rehydration buffer containing destreak solution (from GE Health Care), 0.5% IPG buffer (pH 3-7) for overnight in the strip holder. Then 250 µg of protein sample was cup loaded at cathode end. IEF was carried out at 20°C with the maximum current of 50 µA/strip by using the following programme in GE- Ettan IPGphor 3 IEF System: Step-1: 80 V, 8 h; Step-2: 300 V, 1 h; Grad-3: 1000 V, 4 h; Grad-4: 5000 V, 2 h; Grad-5: 8000 V, 1 h; Step-6: 8000 V, 5 h for the total Vh of 55640. After completion of the first dimension, the IPG strip was removed from the IEF equipment and place it on the channels of IPG box tray facing the gel side up. Added 0.5 ml of 1% of DTT equilibration buffer on the entire length of IPG strip and incubated for 15 minutes. Then placed strip on the new channel of the tray. Added 0.5 ml of 1% of iodoacetamide on strip and allowed incubation for 15 minutes. Then spots were separated with 10% SDS polyacrylamide gel electrophoresis with protein marker. The gel was stained with coomassie brilliant blue-250 (G250) and spots were image analysed by using the software Image Master Platinum 7.0.

LC-MS/MS Analysis and Protein Identification

More than two fold differentially expressed protein spots were subjected to tryptic in-gel digestion and peptide extracts were analysed by Nano LC-MS/MS. Zip tip purified peptides were analysed using nano−RPLC (Thermo Scientific, USA) coupled with an Orbitrap Elite Mass spectrometer (Thermo Scientific, USA). Peptide mixtures were dissolved in 2% ACN with 0.1% of formic acid and loaded onto a guard column. Purified peptides were released into a C18 capillary column (100 µm × 10 cm) and separated using a linear gradient solvent system (5-100% ACN) for 80 min at a flow rate of 300 nl/min. Peptides were ionized by positive mode electrospray with an

ion spray voltage of 1.9 kV. The MS data were acquired in positive ion mode over mass range m/z 350- 4000 Da using Xcalibur software.

Statistics

The statistical significance was represented as mean ±SEM. The variation between groups was examined by one way analysis of variance. Post hoc testing was worked out for comparisons using Tukey's multiple comparisons test. The difference with P-values was considered to be statistically significant using Graphpadprism 6.0 software. The P values represent mean ± SEM for n=3 and statistically significant at **** P<0.0001, ***P<0.001, **P<0.01, *P<0.05.

RESULTS

Effect of Maghemite Nanoparticle in Angiogenicity and Clonogenicity of AGS

Here, we revealed the anti-metastatic prospective of maghemite nanoparticles on AGS cells. Nitric oxide is a potent cell type reliant metastatic signalling factor which was analysed, in results from Figure 1a drop in NO production intensity significantly from 12.5 µg/ml and high level of significance showed from 25 µg/ml compared to untreated control AGS cells. Commencing clonogenic assay the proliferation efficiency of AGS cells were decreased with the effect of nanoparticles shown in Figure 1b. The number and size of the clones were reduced (decreased clonogenicity) during nanoparticle treated AGS cells with dosage dependent manner when compared to untreated control AGS cells. Very few numbers of colonies with small size were noted from 50 µg/ml of nanoparticle concentration condition.

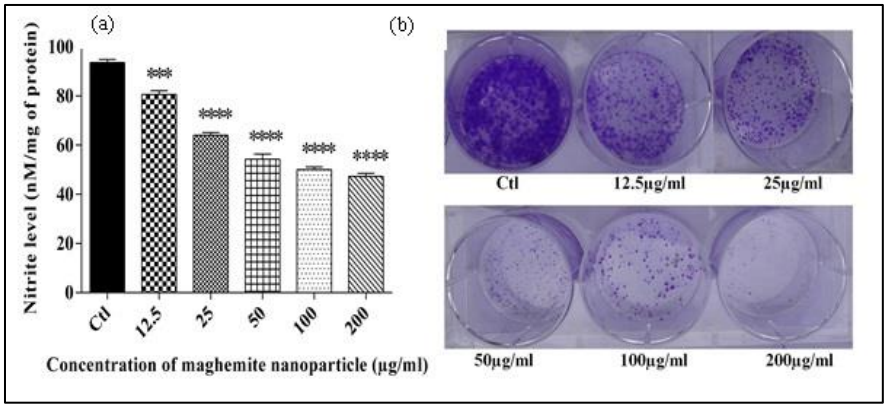

Figure 1: (a) Nitric oxide as an important metastatic signalling factor analyzed colorimetrically after treatment for 24 h, (b) Proliferative efficiency of AGS cells after 24 h treatment with maghemite nanoparticle was analyzed by clonogenic assay. The P values represent mean ± SEM for n=3 and statistically significant at **** P<0.0001, ***P<0.001, **P<0.01, *P<0.05

Effect of Maghemite Nanoparticle on AGS cell Migration and Adhesion

Migratory behaviour of AGS cells were distinguished from (Figure 2a) microscopic analysis and its corresponding graphical (Figure 2b) representation. In the graphical representation, wound distance was measured in nanoparticle treated AGS cells against the wound distance in untreated control). From both the analysis, increasing nanoparticle concentration increases the wound distance (reduces wound healing) and through which slowed up of AGS cell migration, but cell migration and wound closer event was noted with very less wound distance in untreated AGS control. The metastatic cells are well interact with extracellular matrix such as collagen, fibrin, laminin. We studied the AGS cells interaction with the collagen treated plates *in vitro*. The cell adhesion ability of AGS cells with nanoparticle were carried out both microscopically (Figure 2c) and colorimetrically (Figure 2d). From colorimetric analysis it was proved that AGS cell adhesion to the collagen was decreased significantly with dose dependently from 50 µg/ml and high significance at 200 µg/ml of nanoparticle concentration compared to untreated control AGS cells. From microscopic analysis it was conformed further that AGS cell adhesion to the collagen surface was reduced upon nanoparticle treatment.

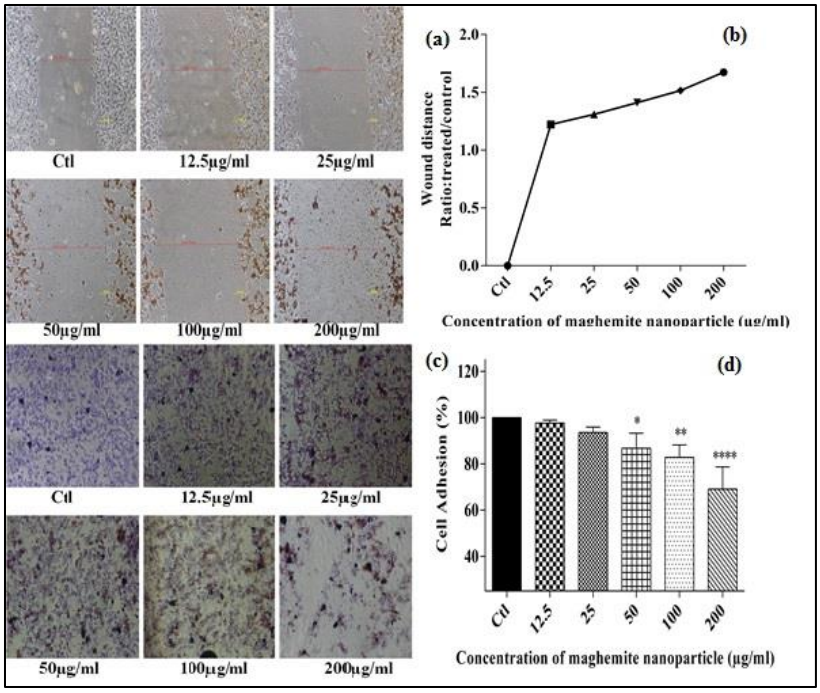

Figure 2: Cell migration and cell adhesion was analysed by (a) scratch assay and (c) adhesion assay respectively after 24 h of maghemite nanoparticle treatment and cells were photographed with 10 X magnification, (b) the wound distance formed was graphed against the control, (d) cell adhesion was also analysed by calorimetrically and graphed. The P values represent mean ± SEM for n=3 and statistically significant at **** P<0.0001, ***P<0.001, **P<0.01, *P<0.05

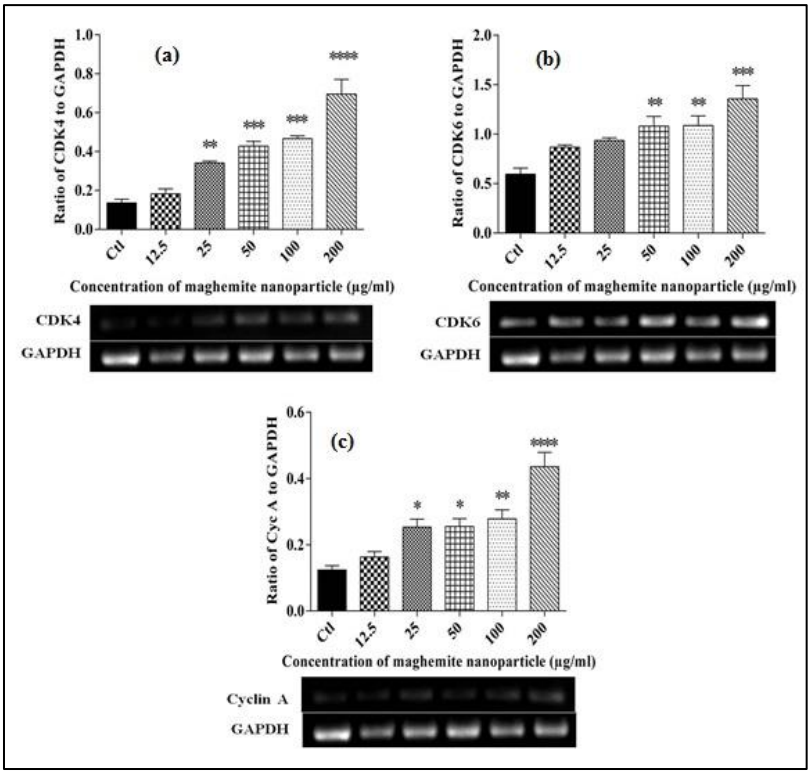

Figure 3: Semi quantitative PCR analysis of cell cycle regulator genes (a) CDK4, (b) CDK6 and (c) Cyclin A transcriptional gene expression after treatment with maghemite nanoparticle in AGS cells for 24 h. The P values represent mean ± SEM for n=3 and statistically significant at **** P<0.0001, ***P<0.001, **P<0.01, *P<0.05

Transcriptional Analysis of Cell Cyclin Genes with Nanoparticles Effect on AGS

The transcriptional expression of cell cycle regulatory gene factors cyclin dependent protein kinases- CDK4 and CDK6 was analysed by semi quantitative PCR. The PCR analysis illustrated considerable increases in CDK4 gene expression from 25 µg/ml (Figure 3a) but CDK6 expression increases from 50 µg/ml (Figure 3b) with the $P<0.01$ and shows higher significance at 200 µg/ml but no significant expression at 12.5 µg/ml of nanoparticle concentration compared to untreated control. We also examined transcriptional expression of cell cycle regulatory factors Cyclin A by semi quantitative PCR. The PCR analysis showed significant increases in Cyclin A gene expression from 25 µg/ml (Figure 3c) with the $P<0.05$ and shows higher significance at 200 µg/ml but no significant expression at 12.5 µg/ml of nanaoparticle concentration compared to untreated control.

Proteomic Regulation of Maghemite Nanoparticles in AGS Cell

We carried out a proteomic ananysis to find out the proteins that regulate AGS cell survival and cell death moreover the impact of maghemite nanoparticle on AGS cell survival and cell death treated with IC50 value (50 µg/ml) concentration. From Figure 4, we observed aproximately 500 proein spots with coomassie staining method after IEF in IPG strip (pH 4-7, 13 cm) and SDS PAGE separation of 250 µg of protein. To study the proteomic profile of 2D gels image, the Image Master Platinum 7.0 analysis was performed and it was noted that most of the spots were upregulated and some of few spots were downregulated in AGS control and nanoparticles treated cells respectively. Some of protein spots distinguished only in AGS untreated control gel which was missed in nanoparticles treated AGS gel. Among them more than 2-fold differentially/altered expressed 5 protein spots were selected and subjected to tryptic digestion followed by LC-MS/MS analysis.

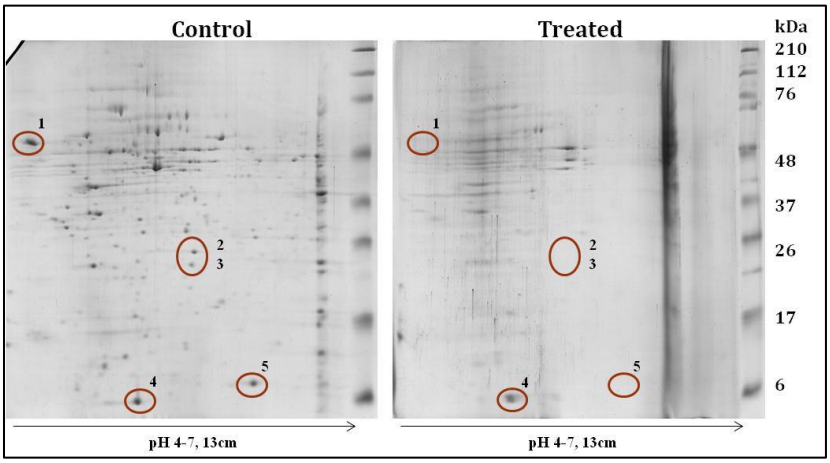

Figure 4: Two dimensional gel electrophoresis profiles

Two Dimensional Gel Electrophoresis Profiles

AGS cell control (untreated) and treated with IC50 value 50 µg/ml of maghemite nanoparticles for 24 h. Then 250 µg of protein of each control and treated sample was separated and spots were stained by coomassie brilliant blue. After Image Master Platinum 7.0 analysis of 2DE image, more than two fold differentially expressed protein spots (5) were selected

Table 2: Identification of differentially expressed proteins by LC-MS/MS and Xcalibur software analysis

Spot No.	Accession	Protein Description	Score	Coverage	MW (kDa)	Calc. PI
1	Q53G71	Calreticulin	1596.39	66.5	46.9	4.45
2	Q5VU66	Tropomyosin alpha 3 chain	803.97	79.84	28.9	4.75
3	P09211	Glutathione S transferase	1335.25	70	23.3	5.64
4	P31949	Protein S100A11	1348.4	59.05	11.7	7.12
5	P06703	Protein S100A6	291.03	80	10.2	5.48

The proteins were identified with Xcalibur software. Table 2 describes the accession number, score value, coverage value, molecular weight and calculated PI (Isoelectric Point) value of identified proteins. The protein involved in Ca^{2+} homeostasis as calreticulin (CRT), actin stabilizing protein tropomyocin (TRP3), cellular oxidative stress regulating protein glutathione s transferase (GST) and Ca^{2+} binding proteins S100-A11 and S100-A06 were identified and downregulated in AGS cells when treated with 50µg/ml (IC50 value) of maghemite nanoparticles

compared to untreated AGS control cells. All of these down regulated proteins were involved in the survival, invasion, migration and metastasis of AGS cell.

DISCUSSION

Anti-Metastatic Activity of Maghemite Nanoparticle in AGS Cells

Cancer progression and metastasis are associated with elevated levels of nitric oxide [11]. Our study showed that cell progression and the metastatic nature of AGS cell was reduced remarkably by increasing nanoparticle concentration with decreased NO level and it denotes nanoparticle as a potent anti metastatic factor. Clonogenic potential is as an important character in the progression of metastatic cells. However, the clonogenic efficiency/ proliferation efficiency of the AGS after nanoparticle treatment was not success. The metastatic character from an AGS cell was not transferred to the upcoming clone. Still, the clonogenicity of AGS cells were decreased with increasing nanoparticles treatment. Each and every step in the metastatic cascade, cell migration is required [12]. In our study, it was noted from both microscopic and graphical representation of scratch assay where AGS cell migration was significantly arrested and reduced with maghemite nanoparticle administration and as a result, invasiveness of AGS cells get impaired. The previous study described that nanoparticle produces ROS in human microvascular endothelial cells and changes the actin dynamics such as cell migration, locomotion and invasion [13]. From our cell adhesion assays analysis, it was evident that in AGS cell, metastatic properties like adhesion and migration were reduced along with cell adhesion towards collagen coated surface and cell – cell contact between AGS cells was decreased upon treatment with increasing nanoparticle concentration.

Maghemite Nanoparticle Transcriptionaly Arrest AGS Cells at G2/M Phase

Polymerization and depolymerization of microtubule is an important at specific time lapse for cell structure maintanence, signalling and mitotic cell division. Maghemite nanoparticle induces depolymerization of microtubules, decreases its polymerization ability and change the microtubules steady state equilibrium [14]. Maghemite nanoparticle reduces/affects the actin and microtubule cytoskeleton organization (also required in mitotic phase) and leads to the blockade of cell cycle progression. Elevated expression of CDK4/6 is required to arrest cells at G2/M phase and provide protection against proliferating cells that bypass G1 checkpoint [15]. From our pervious study, cell cycle was analysed by fluorescence assisted cell sorting (FACS) and results showed halt in G2/M phase transition when treated with 50 µg/ml of maghemite nanoparticles. With respect to above phenomenon, it was confirmed that maghemite nanoparticle induced the expression of CDK4/6 and Cyclin A in mRNA level drastically and arrests AGS cells at G2/M phase.

Maghemite Nanoparticle Reduces AGS Cell Survival, Tumourigenecity and Metastasis

Spot-1:

In the stromal compartment of malignant tissues CRT is upregulated compared to than in non-malignant tissues. In CRT knockdown cells, 992 genes are downregulated (63%) while a total of 1579 genes are upregulated in presence of CRT gene expression [16]. In our two dimensional gel electrophoresis of proteomic analysis most of protein spots were downregulated when the pH 4 - 7 strip range used in nanoparticles treated AGS cell. This reduction in total protein expression is associated with the down regulation of CRT expression when compared to untreated AGS cell. In most of the gastric cancer patients CRT over expression upregulates the proangiogenic factors PIGF, VEGF which increases cell proliferation and migration. Gastric cancer patients with CRT negativity shows increased survival rate [17]. Angiogenesis is promoted with activation of nitric oxide signalling pathway with amplified CRT expression [18]. In most of cancer, CRT over expression is upregulate the phosphorylation of AKT pathway [19] which enhances tumour invasiveness and metastasis [20]. In this study we already determined the reduced NO level in nanoparticle treated AGS cells. Therefore down regulation of CRT expression in nanaoprticle treated AGS cell may reduces its downstream proangiogenic factors like NO, AKT phosphorylation and VEGF mediated signalling pathway which leading AGS cell less tumourigenic and metastatic nature. However in the untreated AGS cell where CRT was over expressed and responsible for cell angiogenesis and metastasis. The inductive effect of p53 gene is modulated by CRT expression. Upon CRT knockdown, the three p53 target genes (TP53I11, TP53TG5 and SERPINB5) are upregulated in the direction to inhibit invasive and migratory potential of breast cancer cells [16]. Ischemia-Reperfusion Injury induced apoptosis in neuronal cell was inhibited by binding of calreticulin with Fas ligand and downregulates its downstream effectors caspase-8 and caspase-3 during the early stage of ischemic stroke [21]. In our earlier study results p53 gene expression was upregulated at transcriptional point considerably when treated with nanoparticles in AGS cells and bring about cell death. Since this proteomic analysis, the CRT down

regulation might permit p53 and its downstream targets to be active and responsible for AGS cell less proliferative, G2/M phase arrest and apoptotic cell death. However it was revealed that over expressed CRT in AGS cell down regulates the p53, this phenomenon was accountable for cell survival and continued proliferation.

Spot-2:
In the N0, N1 and N2 stages of breast cancer, tropomyosin alpha 3 chain is upregulated at the significance level 69%, 69% and 75% respectively [22]. Tropomyosin alpha 3 chain is involved in the regulation of cancer cell transformation [23]. In non-muscle cells, tropomyosin functions to stabilize and modulate the actin filaments and involved in cell signalling, migration and invasion [24]. Down regulated tropomyosin alpha 3 chain was also affect AGS cell less metastatic, less migratory and less invasive when treated with nanoparticles. In vitamin C treated AGS cell tropomyosin expression is drop off which might be related with morphological and migratory changes [25]. In our earlier study results from morphological analysis it was shown to be round, irregular and small size of AGS cells when treated with different concentration of nanoparticles.

Spot-3:
Physiologically aerobic cells produces reactive oxygen species (ROS) which is essential to mediate intracellular signalling pathways for cell survival, but in the condition of cell injury/damage and death the level of ROS increased. To avoid this cell irreversible process, antioxidant system is upregulated by increased ROS as an adaptive response system to restore the redox homeostasis [26]. Over expression of Glutathione s transferase is responsible for *de novo* resistance to chemotherapy and acquired resistance in chemotherapy as multidrug resistant in human breast cancer cells [27]. Reduced glutathione (GSH) involved in the detoxification of some endogenous compounds and xenobiotics which are catalysed by GST. GSH is able to interact with drugs, ROS and involved in the DNA repair process or preventing DNA /proteins from damages. Depletion in GSH and GGT in melanoma cells increases the cytotoxicity via oxidative stress [28]. ROS causes G2/M phase arrest in human prostate cancer cells by diallyl tri-sulfide [29]. The prior study stated that G2/M phase arrest come about by means of p53 dependent and p53 independent pathways [30]. Over two fold down regulation of GST expression in AGS cells when treated with 50µg/ml of maghemite nanoparticles conformed our preceding study where maghemite nanoparticle induces production of ROS which damages DNA, cell membrane, G2/M arrest of AGS cells and activates p53 mediated apoptosis. However the cytotoxicity was not observed on IEC-6 normal cells. GST encompass the novel nonenzymatic role in addition to its transferase enzymatic activity and carry out protein-protein interactions with MAPK pathway members including JNK1 (c-Jun N-terminal kinase 1), ASK1 (apoptosis signal-regulating kinase 1, which involved in apoptosis) followed by affecting the interaction with their downstream targets and responsible for inhibition of apoptosis and drug resistant phenotype [31]. There was decreased GST protein expression observed in nanoparticle treated AGS cell. This relates our previous colorimetric examination of GST activity and GSH level where both was decreasing with increasing nanoparticle concentration in AGS cells significantly compared to untreated AGS cells. This reduction of GST expression and activity causes AGS more prone to oxidative stress which induces damages to cell membrane, DNA, other cellular components and stress related signalling pathways; reduces resistance to drugs; and may activates MAPK pathway those which are cumulatively reduces AGS cell tumourigenicity and leads to cell death pathway.

Spot-4:
In most cancer types S100 family proteins S100A6, S100A8, S100A9 and S100A11 are expressed [32]. S100A6 furthermore regulates the cellular Ca^{2+} homeostasis, cell cytoskeleton dynamics, proliferation and differentiation [33]. CacyBP/SIP play an important role such as inhibition of cancer cell proliferation, tumorigenesis, cytoskeleton rearrangements and cell differentiation or degeneration by interacting with Siah-1, Skp1, tubulin, ERK1/2 kinases and to be involved in transcriptional activation of Tcf/LEF and degradation of β-catenin by its ubiquitination. The activity of CacyBP/SIP is negatively regulated by S100A6 in gastric cancer cells [34]. S100A6 enhances tumuorigenesis *in vivo* and cell proliferation and migration *in vitro* of human hepatocellular carcinoma. Overexpression of intracellular S100A6 in liver cancer resulted in down-regulation of N-cadherin and E-cadherin, phophorylation of AKT of PI3/AKT pathway in addition to expression and nuclear accumulation of β-catenin [35]. After nuclear translocation cyclin D, u-PAR and C-myc are the downstream target genes for β-catenin in the nucleus and increases the proliferation and invasion of human hepatocellular carcinoma [36]. The reduced proteomic expression of S100A6 in nanoparticle treated AGS cells leads cell less tumourigenic and proliferation. In AGS cell the S100A6's negative regulation of activity of CacyBP/SIP may reduced upon nanoparticle treatment which leads to reduction in β-catenin and AKT mediated signalling pathway. In our previews study analysis, one of the β-catenin

downstream target gene C-myc transcriptional expressions was decreased significantly upon nanoparticle treatment and which is responsible AGS cell diminished tumourigenic.

Spot-5:
S100A11 is overexpressed in both mRNA and protein level in laryngeal cancer tissue then in the noncancerous tissue [37]. The S100A11 is translocated in nucleus of normal tissues but exported into the cytoplasm of most of the cancer cells. This cytoplasmic tranlocation of S100A11 protein decreases the transcriptional expression of $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ which are negative regulator for cell proliferation and growth in cancer cells [32]. The S100A11 protein was downregulated in AGS cell after nanoparticle administration which may increases the transcriptional expression of CKI (Cyclin dependent protein kinases - $p21^{CIP1/WAF1}$ and $p16^{INK4a}$) as tumour suppressors and expose AGS to apoptotic pathway.

Heterodimer effect of S100B with S100A6 and S100A11:
S100A6 and S100A11 are the specific targets for S100B [38]. S100B was elevated in melanoma patients and responsible for metastatic growth and malignancy of melanoma particularly in the stage IV. The survival of melanoma patients were significantly increased in reduced S100B expression compared to those with elevated expression levels [39]. S100B downregulates p53 expression by feedback loop mechanism as binding with p53. S100B also downregulates intracellular p53 level by blocking its covalent modification and by polyubiquitylated degradation. This phenomenon allows cell proliferation and growth [40]. Both S100B and S100A6 co-expressed and are able to interact with distinct RAGE and trigger different cellular signaling pathway to modulate cell survival differentially [41]. There are possible cumulative mechanism for S100 family proteins (S100A6, S100A11 which both were over expressed in untreated control but down regulated upon maghemite treatment) that S100A6/S100AB down regulate proapoptotic p53 and upregulates oncogene C-myc further more S100A11/S100B down regulates the tumour suppressor genes and responsible for AGS cell apoptotic resistance and survival.

Maghemite nanoparticle induces cell death in AGS cells via mitotic catastrophe followed by apoptosis:
In cancer therapy G2/M checkpoint is considered to be a potential target. International Nomenclature Committee on Cell Death, 2012 defined the name mitotic catastrophe for a cell that undergoes an irreversible antiproliferative fate of death or senescence upon mitotic failure was sensed by oncosuppressive mechanism [42]. The rate of chromosome missegregation decides whether aneuploidy suppress or promote tumour progression. Low and high rate of chromosome missegregation determines tumourigenesis and cell death respectively [43]. Mitotic catastrophe from where, the cell definitely follows cell death. Mitotic catastrophe happens upon defective in G2 checkpoint associated with DNA damage. G2/M checkpoint was the last chance to halt cell upon impaired DNA damage repair which have escaped from the checkpoints G1 and S phase [44]. G2 cell cycle arrest, polyploidy, mitotic catastrophe and apoptosis was identified with cyclin A upregulation which is a downstream player of p53 dependent regulation in TOV-21G ovarian, H1299 non- small lung and renal carcinoma cells [45]. A mitotic catastrophe cell contains number of micronuclei with uncondensed chromosomes and cells are flat and large in morphology. From our previews study, the semi quantitative PCR analysis, the results supported that the nanoparticle increases in the expression of tumour suppressor gene p53 in AGS cells which increases the expression of its downstream player of cyclin genes cyclin A, CDK4/6 and down regulates oncogene C-myc in AGS cells. Cell death via mitotic catastrophe does not form DNA laddering pattern in DNA fragmentation assay [46]. This was correlated with our previous study results, from the DNA fragmentation assay analysis; there was no formation of ladder pattern in nanoparticle treated AGS cells but the presence of DNA smear as DNA damage. These portrays our results, where the nanoparticle induces the following sequential events such as induction of oxidative stress, deregulation of mitotic organization, G2/M phase cell cycle arrest, polyploidy and mitotic catastrophe by p53 dependent pathway in AGS cells. Mitotic catastrophe usually followed by apoptosis but mitotic catastrophe does not follow apoptosis [47]. It was summarized that the maghemite nanoparticle treatment in AGS cells causes apoptosis mediated by either ROS/p53 dependent pathway or both dependent pathways through mitotic catastrophe (Figure 5).

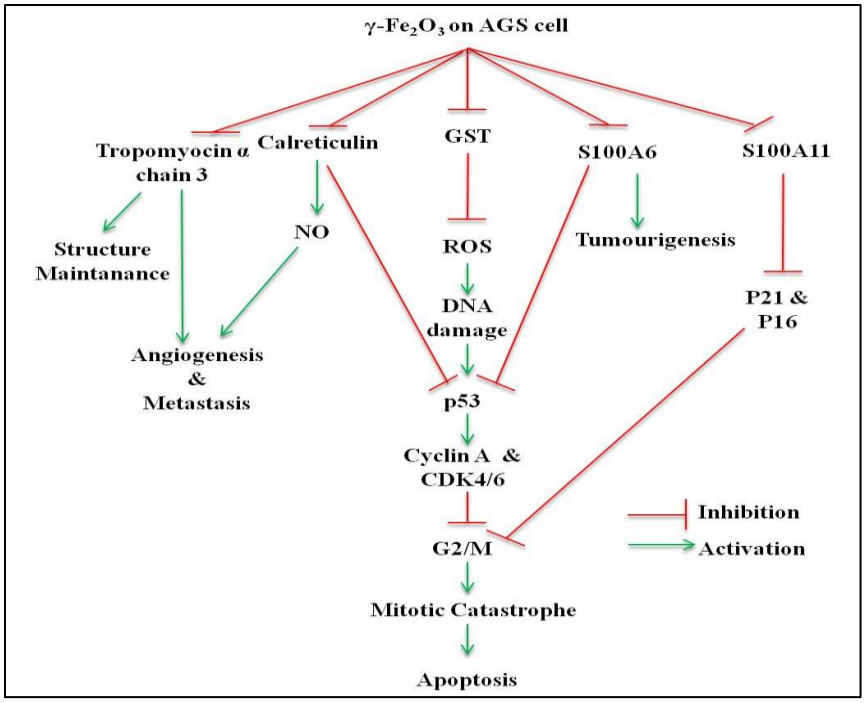

Figure 5: From this summary picture it was clarified that maghemite nanoparticle reduces tumourigenesis potential and increases tumour suppressive and oxidative stress mediated gene expression which leading mitotic catastrophe mediated apoptotic cell death in AGS cells

CONCLUSION

Maghemite nanoparticle reduces the clonogenic character of AGS cells. The dwindling level of metastatic factor NO, decreases AGS cell migration and invasion behaviour and decreased cell adhesion to collagen surface showed anti metastatic activity of maghemite nanoparticle. Upon nanoparticle treatment tumour suppressor p53 expression and its intra cellular concentration was increased with rising oxidative stress due to down regulation of both activity and expression of GST. S100A6 down regulation reduces polyubiquitinated degradation of p53 and decreases expression of oncogene C-myc. Reduction in S10011, allows CKI like p21 and p16 to be active in response to cellular damage. CRT downregulation allows p53 to be active. The activated p53 increases expression of its downstream target genes like cyclin A, CDK4/6, TP53I11, TP53TG5 and SERPINB5. This phenomenon leads AGS cell arrest at G2/M phase. G2/M phase arrest with increased Cyclin A involved in the apoptosis mediated via the formation of mitotic catastrophe. This study concluded the maghemite nanoparticle inducesd apoptosis in addition to anti- metastatic activity in AGS cells as a beneficial effect to treat gastric cancer patients in prospect.

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Conflict of Interest

Conflict of interest declared none**.**

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