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Research Article

ABERRANT N-GLYCOSYLATION REGULATES INVASION OF MG-63 CELLS THROUGH EXTRACELLULAR MATRIX REMODELING

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ABSTRACT

Objective: Despite advances in multimodal therapy, osteosarcoma (OS) still imposes big challenge due to its high rate of metastasis. The previous studies reported that aberrant glycosylation in the cells mediates the invasion of several cancers including OS. However, its mechanism, particularly *N*-glycosylation in OS progression, is still poorly understood. Thus, this study aims to investigate the effect of glycosylation inhibitions toward OS cells invasiveness.

Materials and Methods: Both 1-deoxynojirimycin (DNJ) and 1-deoxymannojirimycin (1-DMJ) were used to inhibit the activities of alpha-glucosidase-I/II and alpha-1,2-mannosidase, respectively. Invasion assay and real-time polymerase chain reaction (PCR) (quantitative PCR [qPCR]) analysis of extracellular matrix-related genes were performed at post 24 h of treatment with the inhibitors, 0.5 mM 1-DNJ and 0.5 mM 1-DMJ, respectively, on the OS cell line, MG-63.

Results: Results showed that the inhibition of N-glycosylation with 1-DNJ decreases the invasion rate of MG-63 cells while the inhibition of N-glycosylation by 1-DMJ caused the invasion rate of MG-63 cells to increase. qPCR analysis showed downregulated expression of matrix metalloproteinase (*MMP2*) gene in both types of treatments while the expression of its inhibitor, tissue inhibitor of metalloproteinase (TIMP2) was upregulated in both types of treatments. In this study, *MMP9* genes were not detected in both samples; however, the expression of its inhibitor, TIMP1 was downregulated in MG-63 cells treated with 1-DNJ but upregulated in 1-DMJ treated cells.

Conclusion: It is concluded that 1-DNJ reduced the invasion rate in MG-63 cells through downregulation of *MMP2* gene which subsequently reduced degradation of collagen type IV. However, the contrasting effect showed by 1-DMJ requires further investigation to elucidate its underlying mechanism.

Keywords: Extracellular matrix genes, Glycosylation inhibitors, *N*-glycosylation, Osteosarcoma.

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INTRODUCTION

Osteosarcoma (OS) is a primary malignant bone tumor that has bimodal age distribution with the first peak of invasion occurs on children and adolescence between the ages of 10 and 20 years old, and the second peak of invasion occurs after 65 years of age [1]. Although OS incidences are rare, OS has been reported as the most common primary malignant bone tumor (36%) followed by chondrosarcoma (20-25%) and Ewing sarcoma (16%) [2,3] and it has been pre-dominated by male with the ratio of 1.6–1.7:1 [4]. To date, the current therapeutic strategies are still unable to provide promising long-term OS prognosis, whereby over 40% of patients experience relapsed and 20% of patients were diagnosed with metastatic OS [5]. Thus, it is important to explore the OS metastasis mechanism for better understanding of the disease progression.

Cancer invasion starts with activation of signalling pathways that regulate the cytoskeletal formation together with the cell-extracellular matrix (ECM) and cell–cell interactions which results in variation of adhesion strength. Subsequently, tumor cells will start to migrate and formed angiogenesis [6,7]. Integrin, a receptor for fibronectin is the primary receptor involved in the cell–cell and cell-ECM interaction [8,9]. Furthermore, integrin has been shown to be involved in cancer cells through malignancy. The binding of integrin to the ECM proteins is largely influenced by the glycosylation of the integrin itself, whereby most integrin are highly glycosylated and were found to be overexpressed in cancer cells, thus suggested as a cause for metastasis [9]. Moreover, aberrant integrin glycosylation possibly leads to alteration of the adhesion properties of integrin to the ECM, resulting in increased capacity of tumor cells to invade nearby cells [9]. To date, the most described glycosylation alterations are the accumulation of high mannose [10] and premature termination of glycan processing, synthesis of highly branched glycans [11], decreased in bisecting glycans, and increased of terminal modification such as sialylation and fucosylation [12,13].

While aberrant glycosylation is responsible for cancer progression, uncontrolled ECM remodeling also contributes to increased malignancy of cancer cells [14]. The degradation of ECM during remodeling process is associated with the upregulation of matrix metalloproteinases (MMPs) expression as found in most metastasis cases [15,16]. The expression level of MMPs by normal and cancer cells is different, suggesting its effect on the environment subsequently regulating cancer cell progression [17]. Both MMP2 and MMP9 are gelatinase that cleaves collagen type IV, the main component of ECM and the activity of both MMP2 and MMP9 are regulated by the coexpression of their respective tissue inhibitor of metalloproteinases (TIMPs) [14-17].

Although associations of cells and ECM are well documented in many cancer cells progression, there is still lack of study on OS metastasis. Therefore, this study aims to investigate the role of *N*-glycosylation in the OS cells through ECM dysregulation.

MATERIALS AND METHODS

Cell culture

The MG-63 human OS cell was purchased from the American Type Culture Collection (ATCC, USA) and was maintained in 1:1 mixture of Ham's 12 Medium Dulbecco's Modified Eagle's Medium, with 2.5 mM L-glutamine (Nacalai Tesque, JPN). The medium was supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, CA), 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of amphotericin B (Gibco, Life Technologies, CA) at 37°C in a humidified incubator with the presence of 5% CO_2.

Glycosylation inhibition assay

The 1-deoxynojirimycin (1-DNJ) and 1-deoxymannojirimycin (1-DMJ) were purchased from Cayman Chemical (Cayman Chemical, USA). The *in vitro* cytotoxicity analysis was carried out on the glycosylation inhibitors to determine the optimum concentration and incubation time to MG-63 cells since these compounds were dissolved in dimethyl sulfoxide (1 mg/ml). Briefly, the cells were seeded at a density of 1×10^5 cells/well in a 96-well plate and incubated for 24 h at 37°C in a humidified incubator with the presence of 5% CO_2. At post 24 h incubation, the media were discarded and replaced with 100 µL of treatment media containing six different concentrations (0.25, 0.5, 1, 2, and 3 mM) of 1-DNJ and 1-DMJ. The cells were further incubated for 6, 12, 18, 24, and 48 h at 37°C in a humidified incubator with the presence of 5% CO_2. The non-treated cells were assigned as the control group.

The cell viability was determined using the CellTiter 96® AQueous One Solution Cell Proliferation (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium [MTS]) colorimetric assay (Promega, USA) according to the manufacturer protocol. The effects of both 1-DMJ and 1-DNJ on the growth of MG-63 cells were measured by adding 20 µl of MTS solution to each well without discarding the treatment media. Then, the plate was incubated at 37°C in a humidified incubator with the presence of 5% CO_2 for 1 h. The colorimetric changes were measured by reading the absorbance at 490 nm using VICTOR multilabel plate reader (PerkinElmer, USA).

Cell invasion assay

The cell invasion assay was performed using the Chemicon QCM 96-well Cell Invasion Assay Kit (Millipore, USA) according to the manufacturer protocol. Briefly, a cell density of 1×10^6 cells/well was prepared before invasion assay. The cells were incubated with 1-DNJ and 1-DMJ at 0.5 mM without the presence of serum for 24 h at 37°C in a humidified incubator with the presence of 5% CO_2. At post 24 h of incubation, the cells were detached using 2 mM EDTA/PBS solution and resuspended in the serum-free culture media containing 5% of bovine serum albumin. Then, 1×10^5 cell/well was added to the invasion chamber and allowed to invade the ECMatrix® coated membrane for 24 h. Invaded cells attached to the bottom of the membrane were dissociated by incubation with 150 µl/well of cell detachment solution (supplied by the kit) for 30 min at 37°C in a humidified incubator with the presence of 5% CO_2. Next, 50 µl/well of lysis buffer/CyQuant GR Dye solution (1:75) was added for another incubation at room temperature. Finally, 150 µl of the cell mixture was transferred to a new black 96-well plate for fluorescence measurement. The fluorescent value was detected by Infinite 200 PRO NanoQuant Microplate Reader (Tecan, Switzerland) using 480/520 nm filter set and gained setting of 65.

Extraction of total cellular RNA

Total cellular RNA was extracted from MG-63 cells (control and post-treated) using the RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer protocol. Approximately 1×10^6 cells were lysed using 350 µl of lysis buffer. Then, the cells were homogenized using QIAshredder (QIAGEN, USA) and centrifuged at 14,500 rpm for 2 min. Subsequently, one volume of 70% ice-cold ethanol was added into the lysate and mix well. Next, 700 µl of all the lysate were transferred to the spin column and centrifuged 15 s at 12000 rpm. After the centrifugation, the flow through was discarded and the cells were washed with 700 µl of washing buffer, centrifuged at 12,000 rpm for 15 s. After the centrifugation, again the flow through was discarded and the spin column was added with 500 µl of mild washing buffer (with the addition of absolute ethanol) and centrifuged at 12,000 rpm for 15 s. Next, as the flow through was discarded, 500 µl of the same washing buffer was added again into the column and centrifuged at 12,000 rpm for 2 min. The spin column was dried by centrifugation at 14,500 rpm for 1 min. Finally, 30 µl of RNase-free water were added directly to the membrane of spin column and centrifuged at 12,000 rpm for 1 min to elute the RNA. The RNA was stored at −20°C until further used. The purity and concentration of the extracted RNA were determined by RNA gel electrophoresis and NanoDrop ND-100 spectrometer (data not shown).

cDNA synthesis and quantitative real-time polymerase chain reaction (PCR)

Approximately 5 µg of the extracted RNA was reverse transcribed using QuantiNova Reverse Transcription (RT) Kit (QIAGEN, Germany) according to manufacturer protocol. The RT reaction mixture was consisted of gDNA removal mix (0.1 µl/1 µl reaction), RT enzyme (0.05 µl/1 µl reaction), RT mix (0.2 µl/1 µl reaction), RNase-free water, and RNA templates. The cDNA synthesis was carried out in the Eppendorf Mastercycler® Pro Thermal Cycler (Eppendorf, Germany) with the program of 25°C for 3 min, 45°C for 10 min, and 85°C for 5 min. The cDNA template was diluted 1:10 with RNase-free water for real-time PCR application.

The amplification of cDNA for each target genes (*MMP2*, *MMP9*, *TIMP2*, and *TIMP1*) and reference genes (*GAPDH*, *18SRNA*, and *β-actin*) was done using the QuantiNova SYBR-Green PCR Kit (QIAGEN, USA) using their specific primers (Table 1). The amplification profile involved PCR heat activation step at 95°C for 2 min and 40 cycles of denaturation at 95°C for 5 s, and two-step of annealing and extension at 60°C for 10 s. The amplification was carried out in the CFX96z® real-time PCR Detection System (Bio-Rad, USA).

Table 1 lists all the primers used in this study specific to each type of gene. The primer was designed using National Center for Biotechnology Information primer design tool and has been checked for specificity using Primer-BLAST.

Table 1: List of primers and its forward and reverse sequence

Genes	Primer sequence (5'-3')	
	Forward	**Reverse**
MMP-2	ACATTGACCTTGGCACCG	AGTCCGCCAAATGAACCG
MMP-9	AGTCCACCCTTGTGCTCT	TCGACTCTCCACGCATCT
TIMP-2	CCCTCTGTGACTTCATCGTG	GATGTAGCACGGGATCATGG
TIMP-1	GTGGCACTCATTGCTTGTGG	TCAGCCTATCTGGGACCGCA
GAPDH	ATCACCATCTTCCAGGAGCG	TGGACTCCACGACGTACTCA
18sRNA	GCTTAATTTGACTCAACACGGGA	AGCTATCAATCTGTCAATCCTGTC
β-actin	ACCGCGAGAAGATGACCCAG	GGATAGCACAGCCTGGATAGCAA

MMP: Matrix metalloproteinase, *TIMP:* Tissue inhibitor of metalloproteinase

Statistical analysis

Glycosylation inhibition assay was repeated 3 times and was done in triplicate for each time. The significance of the data was evaluated by one-way ANOVA test using the SPSS statistical software. Invasion assay and RT-quantitative PCR (qPCR) were repeated 3 times and were done in triplicate for each time. The significance of the data was evaluated by t-test Statistical Package for the Social Sciences (SPSS) statistical software (version 23.0; SPSS, Inc., Chicago, USA). $p<0.05$ indicated a statistically significant differences across the data.

RESULTS

Glycosylation inhibition assay

The optimum concentration of inhibitors in MG-63 cells was determined before the cytotoxicity analysis of the compound. The cell viability plot against different incubation times for both glycosylation inhibitors, 1-DNJ (Fig. 1a) and 1-DMJ (Fig. 1b) revealed that the optimum concentration for both inhibitors was 0.5 mM with the optimal incubation time of 24 h. Thus, this dose and incubation time was used throughout this study.

Cell invasion assay

Results showed that inhibition with 0.5 mM of 1-DNJ for 24 h decreased the invasion rate in MG-63 cells by 0.62-fold while inhibition with 0.5 mM 1-DMJ for 24 h increased the invasion rate of MG-63 cells by 3.87-fold (Fig. 2).

Expression of *MMP2, MMP9, TIMP2,* and *TIMP1* genes

The RT-qPCR analysis showed that the expression level of *MMP2* gene was decreased in 1-DNJ and 1-DMJ by 0.8-fold and 0.1-fold, respectively, while the expression of its suppressor gene, TIMP2 was increased in 1-DNJ and 1-DMJ by 1.22-fold and 1.43-fold, respectively. In this study, *MMP9* gene was not detected at all in both treatments; however, the expression of its inhibitor, TIMP1 was decreased in 1-DNJ by 0.24-fold and increased in 1-DMJ by 1-fold (Fig. 3).

DISCUSSION

The endoplasmic reticulum (ER) serves as the maturation site for one-third of the total proteins synthesized in the eukaryotic cells [18]. The fate of a newly synthesized polypeptide is defined by its folding degree in the ER and this process requires assistances to ensure the folding efficiency [19]. The *N*-glycosylation process; the removal of terminal glucose and mannose residues from protein-bound oligosaccharides and the addition of specific glucose residues controls the sequential events of the ER quality controls [20,21].

In this study, the effect of aberrant glycosylation was demonstrated on cell invasion assay and analysis of matrix in responsible for ECM degradation. This was done by inhibiting the glycosylation process with 1-DNJ and 1-DMJ; a specific inhibitor of α-glucosidase-I and II and Class I α-1,2-mannosidase, respectively.

The *N*-glycan has a core structure of $Glc_3Man_9GlcNAc_2$. On entering the ER lumen for processing, the sequential events of α-glucosidase-I and II will hydrolyze a total of three glucose residues during the *N*-glycan processing [19].

As the cells were treated with α-glucosidase-I and II inhibitor (1-DNJ), the trimming process will be halted, thus releasing the tagged proteins from the calnexin (CNX) and calreticulin (CRT) which are the lectin chaperons. As the protein was not targeted either for degradation or folding enhancement, this misfolded protein will be released to the Golgi and said to have undergone aberrant glycosylation. However, a process catalyzes by uridine diphosphate glucuronosyltransferase 1 may allow the rerecognition of *N*-glycans by CNX/CRT by adding one glucose residue to the core unit, thus giving the opportunity to the protein to fold correctly and leave the ER [21]. Thus, the effect of 1-DNJ in the invasion rate of OS cells showed a correlation with the inhibition effect of 1-DNJ in the glycosylation process. Similar inhibitor was used in the previous study by Wang *et al.*; 1-DNJ has been shown to have antimetastatic effects on melanoma B16F10 cells. Incubation of B16F10 cells with 1-DNJ decreased the cells adhesion, migration, and invasion [22].

Class 1 α-1,2-mannosidase specifically hydrolyzes mannose residues with α-1,2 linkage from the core structure in the ER generating $Man_5GlcNAc_2$ [23]. The mannose trimming process in the ER has been suggested to be as regulatory factor timer that determines the folding and degradation of a protein [23]. Inhibition of Class 1 α-1,2-mannosidase by 1-DMJ will disrupt the mannose trimming process, thus cause an accumulation of *N*-glycans with high-mannose content which is one of the most alteration found in cancer cells [11,24]. This inhibition mechanism was in line with the invasion assay results of 1-DMJ that showed increase invasion rate of OS cells.

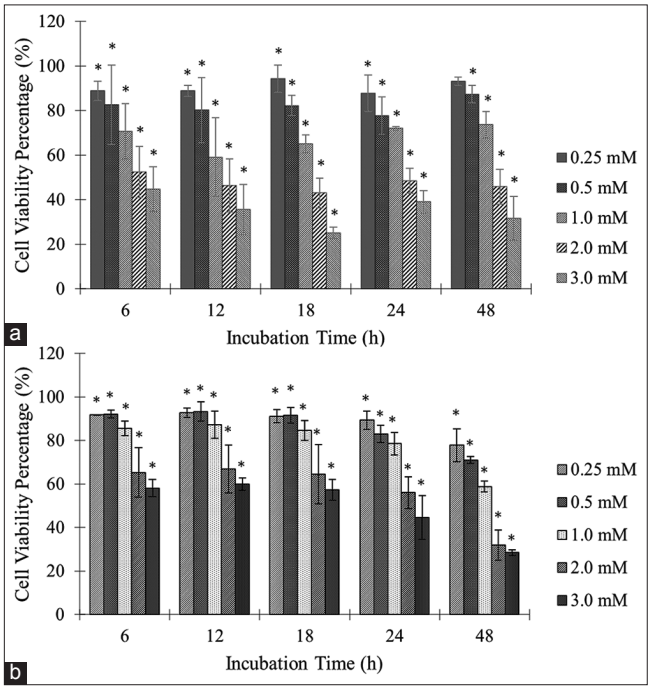

Fig. 1: Screening for optimum concentration of 1-deoxynojirimycin (DNJ) and 1-deoxymannojirimycin (DMJ) for treatment in MG-63 cells. (a) The chosen concentration for 1-DNJ was 0.5 mM due to high viability percentage of 77.7–87.4% throughout all incubation time. (b) The chosen concentration for 1-DMJ was 0.5 mM due to high viability percentage of 71–93.2% throughout all incubation time (*p<0.05)

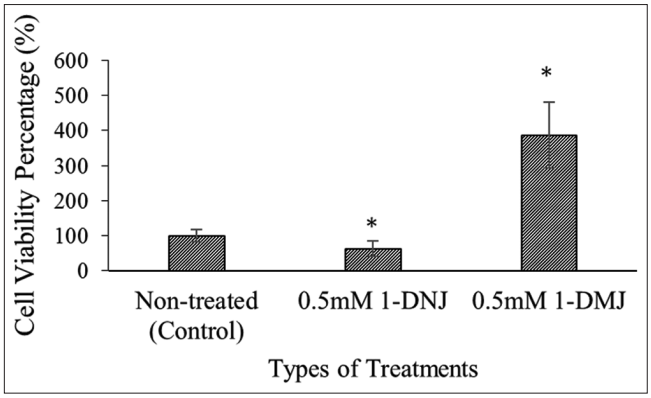

Fig. 2: Rate of invasion in MG-63 cells treated with 1-deoxynojirimycin (DNJ) and 1-deoxymannojirimycin (DMJ). The cells were harvested for invasion assay at post 24 h of treatment. 1-DNJ decreased the rate of invasion by 0.62-fold while 1-DMJ showed an increase of 3.87-fold in relative to control (*p<0.05)

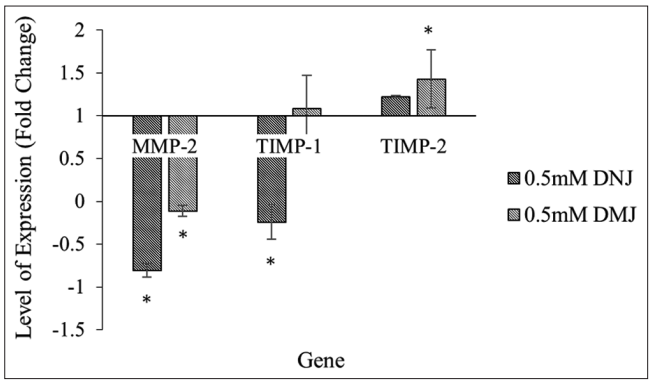

Fig. 3: Expression level of matrix metalloproteinase (*MMP2*), tissue inhibitor of metalloproteinase (*TIMP1*), and *TIMP2* genes in 1-deoxynojirimycin (DNJ) and 1-deoxymannojirimycin (DMJ)-treated cells. The expression level of *MMP2*, TIMP1, and *TIMP2* genes was detected using reverse transcription-quantitative PCR using specific primers for each gene. 1-DNJ and 1-DMJ showed decrease in MMP2 expression by and 0.8-fold and 0.1-fold, respectively, and increase in TIMP2 expression by 1.22-fold and 1.43-fold, respectively. However, 1-DNJ and 1-DMJ showed a contrasting effect on TIMP1 expression where the expression of TIMP1 was decreased in 1-DNJ by 0.24-fold and increased in 1-DMJ by 1-fold (*p<0.05)

MMP2 and MMP9 are the gelatinase that is responsible for the degradation of collagen type IV. The activity of MMPs may be regulated either by proenzyme activation or the inhibitory effects of their natural inhibitors which provide negative control on the MMPs activity [15,16]. TIMP2 has been reported to inhibit the activity off MMP2 while TIMP1 may inhibit the activity of MMP9 [17]. To investigate the association between *N*-glycosylation inhibition in OS cells and ECM regulation, the expression of MMP2, MMP9, TIMP1, and TIMP2 was analyzed.

In this study, the downregulation of MMP2 and the upregulation of *TIMP2* genes in 1-DNJ treated cells correlate with the decreased invasion rate of OS cells. This is attributable to the upregulation of *TIMP2* genes, which is a suppressor gene for MMP2. This result is in line with the results obtained previously on the correlation between MMPs activity and invasion rate. The study reported that the inhibition of the migration and invasion of hepatocellular carcinoma cells (HCC) observed with the downregulation of MMP2 expression [25]. Another study revealed that the inhibition of MMP2 activities decreases the migration and angiogenesis of retinoblastoma cells [26].

However, contrast result was obtained between *MMP2* and *TIMP2* gene expression level in MG-63 cells treated with 1-DMJ. Instead of upregulation of *MMP2* and *MMP9* genes in correlation with the increase invasion rate in MG-63 treated with 1-DMJ, the expression for MMP2 was downregulated and no signals of MMP9 were obtained from the RT-qPCR of both genes. The increase of invasion rate in MG-63 treated with 1-DMJ could be due to 1-DMJ activity that gives direct effect to TIMPs that later regulating the activity of both MMPs. This is because TIMP1 and TIMP2 have been reported to exhibit antiapoptotic activity, thus causing the cells to proliferate more [27].

Moreover, since MMP2 and MMP9 are both secretory enzymes, the expression of total RNA may not demonstrate the actual activities of the proteins. For qPCR analysis of ECM-related genes, only 1-DNJ showed correlation between MMP2/TIMP2 expression and decrease invasion of OS cells. Thus, further study will be performed to elucidate the mechanism of action underlying the glycosylation inhibitory effects of 1-DNJ and 1-DMJ on cancer metastasis.

CONCLUSION

It is concluded that 1-DNJ reduced the invasion rate in MG-63 cells through downregulation of *MMP2* gene which subsequently reduced degradation of collagen type IV. However, the contrasting effect showed by 1-DMJ requires further investigation to elucidate its underlying mechanism.

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AUTHORS' CONTRIBUTIONS

All authors contributed equally to the completion of this paper. Sarmila Hanim Mustafa conceives the idea, collected sources of information, initiated, and contributed to manuscript writing. Shareniza ab. Rahim and Mudiana Muhamad contributed to conceiving the idea, manuscript analysis, and review as well as manuscript writing.

CONFLICTS OF INTEREST

All authors have none to declare.

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