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Research Article
Comparative Study on the Role of Nagalase Expression in Different Cancer Cell Lines

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Abstract

Background and Objective: The α-N-acetylgalactosaminidase (nagalase) is an enzyme related to immune-suppression in cancer patients. Nagalase aids the migration and invasion of cancer cells. This study aims to investigate the relative expression of nagalase as blocker of Gc protein-derived Macrophage Activating Factor (GC-MAF) in six different cell lines known to have different aggressiveness and metastatic rates. **Materials and Methods:** In this study, HeLa (Human cervical cell line), SW460 and SW480 (Human colon cancer cell lines) MCF-7 and MDA-MB-231 and human mammary carcinoma cell line and A549 (human lung carcinoma cell line) were used. The relative levels of nagalase protein were evaluated by western blotting. Cells were homogenized and centrifuged. The blocked membranes were probed with NAGA mouse monoclonal antibody and HRP anti β-actin primary antibodies and scanned. **Results:** Among the studied cell lines, HeLa cells recorded the higher level of nagalase, followed by colon cancer cell lines, breast cancer cell lines and lung cancer cells as cells with the lowest level of nagalase. The obtained results were normalized to β-actin, serving as an internal loading control and were then presented as a percentage of the control. The relationship between nagalase and the aggressiveness of the studied cell lines was discussed in detail. **Conclusion:** Nagalase may have an important role in the invasion of cancer cells and can be considered as a candidate for further studies.

Key words: Cancer, α-N-acetylgalactosaminidase (nagalase), human cervical cell line, human colon cancer cell lines, human mammary carcinoma cell line, human lung carcinoma cell line, immune-suppression

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancer stands as a leading contributor to global mortality, posing a significant obstacle to advancing life expectancy worldwide. According to the World Health Organization (WHO) estimates in 2019, cancer ranks as the primary or secondary cause of death before the age of 70 in 112 out of 183 countries and in an additional 23 countries, it is classified as the third or fourth leading cause[1].

In the context of the COVID-19 pandemic, it is crucial to underscore that various factors heighten the susceptibility of cancer patients to COVID-19. The elevated risk of SARS-CoV-2 infection in individuals with cancer can be attributed to factors such as malnutrition, comorbidities, anti-cancer treatments and notably, the immunosuppressive nature of cancer itself[2-4].

Concerning the immunosuppressive character of cancer, it is interesting to highlight the crucial role of activated macrophages in the fight against different types of cancers. It is well known that vitamin D binding protein (Gc globulin), after being modified by sialidase and galactosidase, as two enzymes secreted by T and B lymphocytes, gains the capability to stimulate macrophages. This protein with N-acetylgalactosamine as the remaining sugar moiety is a precursor of GcMAF-mediated macrophage activation cascade (group specific component macrophage activating factor)[5,6].

The liver secretes GC-MAF, which enhances the superoxide-generating capability of macrophages[6]. It also boosts both general and antibody-dependent phagocytic functions and induces the translocation of FcgR1 and FcgR2 receptors[7]. The GC-MAF has been demonstrated to influence host susceptibility to various diseases, including multiple types of cancer such as melanoma[8], squamous cell carcinoma[9] and oral cancer[10].

Macrophages activated by GcMAF undergo significant receptor variations that enable them to identify abnormalities on malignant cell surfaces, rendering them highly effective against tumors[11]. In many instances, the deactivation of GcMAF is facilitated by an enzyme known as α-N-acetylgalactosaminidase (nagalase).

It is well documented that nagalase accumulates in the serum of viral-infected patients and cancer patients. It catalysis the deglycosylation of GcMAF, finally leading to immunosuppression as a common character in progressive cancer patients. It is well known that cancer cells release nagalase in such a magnitude that its level reflects tumor burden, aggressiveness and metastasis of the disease[12,13].

A few studies have examined the level of nagalase in cancer cell lines and their results specify that levels are greatly varied in different cell lines.

While human embryonic lung fibroblasts (HEL299) and human gingival fibroblasts (HGF) exhibit normal levels, elevated levels of nagalase were observed in cell lines associated with human salivary gland adenocarcinoma (HSG) and squamous cell carcinoma (SCCTM)[14,15]. In western blotting, the normalization process is a crucial step to ensure the reproducibility and accuracy of results. While, under ideal conditions, normalization might not be deemed necessary, practical considerations such as sample loading and transfer proficiency make this step indispensable for obtaining meaningful and consistent data.

At present, there is a notable absence of diverse clinical management options for various cancer types that can distinguish between aggressive tumors and those with a more indolent nature. Recently established tissue-based molecular markers have enhanced the ability to assess the risk associated with the disease. The early detection of nagalase as a biomarker for cancer aggressiveness holds potential advantages, such as an increased probability of recovery, a decrease in the need for aggressive treatment approaches and a minimized progression of the disease to advanced or metastatic stages. This information initiates our interest to assay the normalize relative nagalase protein expression by western blotting in six different cell lines representing cervical (HeLa), colon (SW480 and SW620), breast (MCF-7 and MDA 231) and lung (A 549) cancers. This might help to clarify the relationship between nagalase and cancer burden, aggressiveness and progression, provide a non-invasive diagnostic tool for the early detection of cancer development and intervention to neutralize the effects of nagalase in cancer patients.

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MATERIALS AND METHODS

Study area: The study was conducted at Central Research Laboratory, Center for Female Scientific and Medical Colleges, King Saud University, Riyadh, Saudi Arabia from February, 2022 to June, 2022.

Cancer breast cell lines (MCF-7 and MDA-MB-231), cancer colon cell lines (SW620 and SW480), cancer cervix cell lines

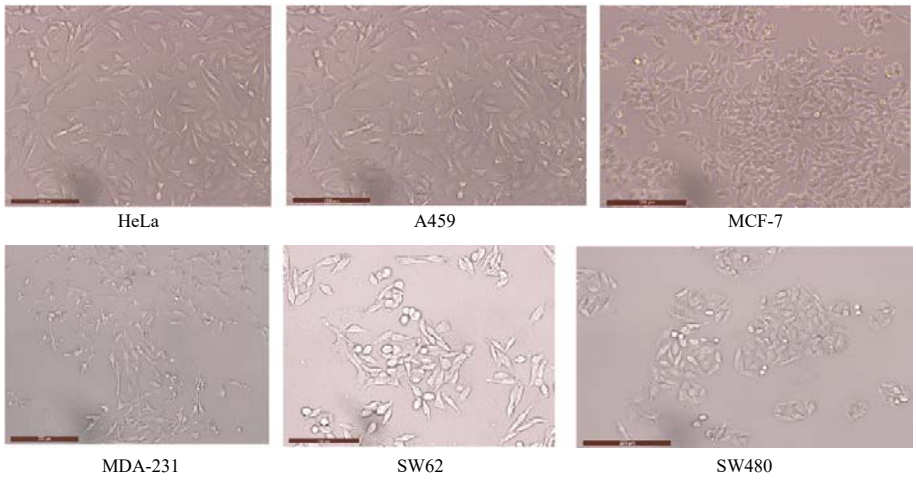

Fig. 1: Morphology of the six studied cell lines

Table 1: Description of the cell lines employed in the present study

Name	Species	Tissue	Morphology	Disease	Media
HeLa	*Homo sapiens*, human	Uterus, cervix	Epithelial	Adenocarcinoma	Eagle's Minimum Essential Medium (EMEM)
SW480	*Homo sapiens*, human	Large intestine, colon	Epithelial	Colorectal; Dukes' type B	Leibovitz's L-15 Medium
SW620	*Homo sapiens*, human	Large intestine, colon	Epithelial	Colorectal; Dukes' type C	Leibovitz's L-15 Medium
MDA-231	*Homo sapiens*, human	Breast	Epithelial	Cancer	DMEM
MCF-7	*Homo sapiens*, human	Breast	Epithelial	Cancer	DMEM
A549	Human	Lung	Epithelial	Cancer	DMEM

DMEM: Dulbecco's modified Eagle's medium

(HeLa) and cancer lung cell lines (A549) are characterized as adherent and continuous cells when grown in the optimized cell culture. The MDME media (sigma-D8437) containing L-glutamine,15 mM HEPES buffer and sodium bicarbonate (Sigma Aldrich, UK) 10% fetal bovine serum (FBS) (Sigma Aldrich, UK) and 1% antibiotic anti-mitotic (penicillin 10.000 units/mL-streptomycin 10.000 µg mL^{-1}) (Sigma Aldrich, UK). The cell lines used in the current study were listed in Table 1.

The cells were cultured at optimal growth conditions of 5% CO_2 in air atmosphere and 37°C. Cells were regularly checked using an inverted microscope (Leica DMI 6000).

Cell culture and growth: cancer breast cell lines (MCF-7 and MDA-MB-231), cancer colon cell lines (SW620 and SW480), cancer cervix cell lines (HeLa) and cancer lung cell lines (A549) were cultured in a 10 mL Sterile flask (Nunc™, Denmark) containing optimal cell medium and optimum atmosphere to about 90% confluent. The adherent cells were then trypsinised by incubation of cancer cells for 1-3 min with a diluted 0.5% trypsin-EDTA solution (Sigma Aldrich, UK). The morphology of all the six studied cell lines was shown in Fig. 1. At the end of incubation, the trypsin was neutralized with serum-containing medium before centrifuging cell suspension at 3000 rpm to remove trypsin. The dissociated cells were washed two times with 1X PBS as individual cells. Finally, cells were collected as a pellet to begin protein extraction.

Sample collection: The cells were washed with PBS and ice homogenized in (RIPA buffer plus protease inhibitor cocktail) (10 volume/weight). The supernatant was clarified by centrifuging the homogenate at 14000 rpm for 30 min, the supernatant containing protein was collected which was then used for western blot.

Measurement of nagalase protein using western blot: The total protein concentration in the different cancer cell lines was measured by Nanodrop spectrophotometer. About 20 µg protein was separated on SDS-PAGE, electro-transferred to PVDF membranes and blocked in 5% bovine serum albumin (BSA) in BPS Tween 20. The blocked membranes were probed with NAGA Mouse Monoclonal Antibody and HRP anti-β-actin primary antibodies (OriGene Technologies, Inc., Abcam, respectively). Following the washing step, the membranes underwent incubation with secondary antibodies and were developed using an enhanced chemiluminescence kit from Bio-Rad, USA. Subsequently, the blots were scanned and the intensities of the pertinent bands were quantified utilizing

Image Lab software. The obtained results were normalized to β-actin, serving as an internal loading control and were then presented as a percentage of the control[16].

Normalization of the experimentally observed nagalase signals using β-actin: Each lane normalization factor was determined to be used to normalize the experimental nagalase intensity values. For accurate normalization of β-actin protein bands on the blot, it is essential to identify and utilize the highest detected signal for β-actin. To establish the lane normalization factor, the observed signal value for the β-actin protein in each lane should be divided by the highest observed β-actin signal on the blot. In the current study, as lane 6 exhibited the highest β-actin signal, the signal values for β-actin in all other lanes were divided by the signal value of β-actin in lane 6 (Eq. 1)[16]:

$$\text{Lane normalization factor} = \frac{\text{Observed signal of β-actin protein for each lane}}{\text{Highest observed β-actin protein on the blot}} \quad (1)$$

The subsequent step involves dividing the normalized signal of each experimental nagalase band and the observed signal intensities of each experimental target band by the lane normalization factor (Eq. 2)[16]:

$$\text{Nagalase protein normalized signal} = \frac{\text{Observed nagalase signal}}{\text{Lane normalization factor}} \quad (2)$$

RESULTS

The results demonstrated the expression levels of nagalase protein (55 KD) and β-actin (as internal control) in the HeLa, SW480, SW620, MDA-231, MCF7 and A549. HeLa cells recorded the highest level of nagalase, followed by SW480 and SW620 representing colon cell lines, MDA-231, MCF7 as breast cancer cell line, with lost level in A459, a lung cancer cell line as shown in Fig. 2. The relative nagalase expression blot in the six different cancer cell lines were presented in Fig. 3. Table 2 presented the lane normalization factor using the lane normalization factor Eq. 1 and Table 3 presented the normalized signals of nagalase bands.

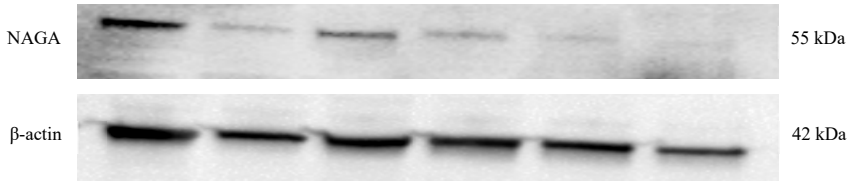

Fig. 2: Relative nagalase protein expression assay by western blot in the six different cancer cell lines

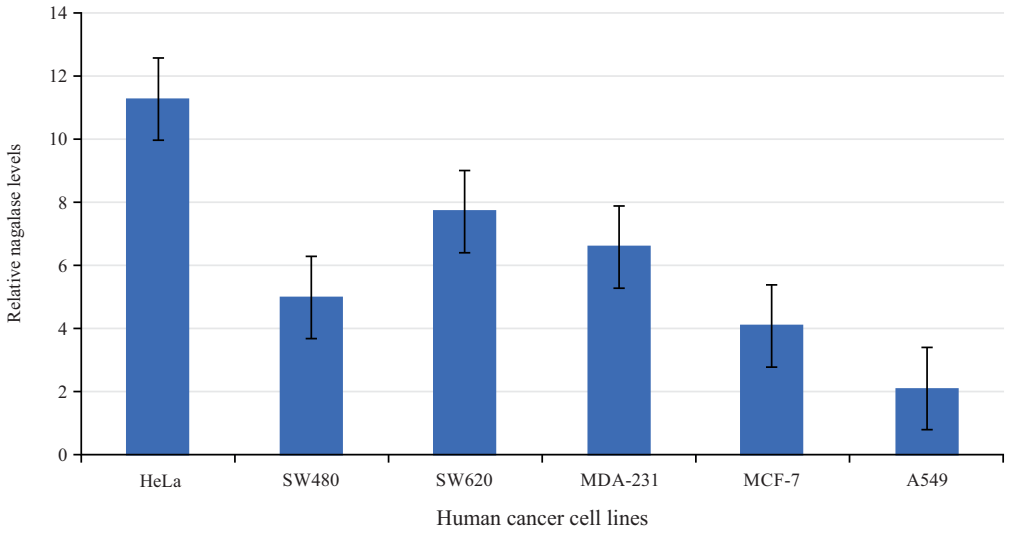

Fig. 3: Histogram presents relative intensities of the relevant bands±SD, quantified using the Image Lab software
Results were normalized to β-actin

Table 2: Determination of lane normalization factor, normalization factors were determined using the lane normalization factor Eq. 1

Lane	Normal β-actin	Lane normalization factor
1	10388466	0.179
2	25678905	0.443
3	11468921	0.198
4	24535451	0.4236
5	31392151	0.542
6	57911671	0.1

Table 3: Determination of normalized signals of nagalase bands

Lane	Observed nagalase signals	Lane normalization factor	Normalized nagalase signals
1	10388466	0.179	58036122
2	6220584	0.443	14041950
3	8362710	0.198	42041950
4	7525980	0.4236	17766713
5	6999588	0.542	12914369
6	4498065	0.1	8299012

DISCUSSION

Based on the fact that cancerous cells and viral entities that make nagalase are not normally present, so appearance of this enzyme in the plasma of cancer patients is a serious issue from a diagnostic perspective. When nagalase shows up, even in very small quantities, it is an early sign of a new cancer or viral infection. Nagalase appears in the circulation when a nascent cancer is just a small cluster of abnormal cells, long before conventional diagnostic methods can detect it.

Figure 1 demonstrated that HeLa cervical cells show the highest level of nagalase between the six studied cell lines. This can be supported by the previous record of Lyapun *et al.*[17] which reported that HeLa cells come from a particularly aggressive human cervical tumor. In contrast to a normal population of human cells that divide from 40 to 50 times before they die, HeLa cells can divide indefinitely. These cells tend to overgrow other cell cultures grown in the same incubator. The HeLa cell line usually proliferated rapidly and was even more stable than other cancer cells[18].

Next to HeLa cell line, colon cancer (SW460 and SW480) recorded a remarkably higher protein level of nagalase. The association between colon cell line nagalase level and the metastatic activity of this type of cancer could be proved by considering the previous work of Yamamoto *et al.*[11]. In this work, they proved that administration of GcMAF therapy significantly increased the MAF precursor activities of all patients and conversely decreased serum nagalase activities. This confirmed that serum nagalase is proportional to tumor burden and can be used as a prognostic index for time course analysis of GcMAF treatment. Interestingly, after 32-50 weekly administrations of 100 ng GcMAF, all colorectal cancer patients showed normal levels of serum nagalase, indicating abolition of metastatic cancer cells. Seven years post the completion of GcMAF treatment, their serum nagalase level did not increase, indicating any recurrence of cancer. Moreover, the remarkably observed higher level of nagalase in colon cancer cell lines (Fig. 1) can be supported by the work of Bakunina *et al.*[19] in which expression of colon cancer nagalase was significantly reduced upon the treatment of human colon carcinoma DLD-1 cells with the sulfated polysaccharides fucoidan, extracted from the brown alga *Fucus evanescence*.

Breast cancer cell lines (MCF-7 and MDA231) recorded much lower levels of nagalase compared to HeLA cervical cell lines and SW460 and SW480 colon cancer cell lines. Despite the lower level of nagalase in the two studied breast cancer cell lines, the observed nagalase protein level could find support in the work of Yamamoto *et al.*[11] in which they reported that normalization of serum nagalase activity in breast carcinoma patients may represent an index of tumor eradication which again support the relationship between nagalase level and breast cancer severity[11].

Interestingly, in most breast cancer cases, the expression level of Estrogen Receptors α (ERα) is directly proportional to tumor growth[20]. It is well known that estrogen receptor-negative breast cancer cells such as (MDA-MB-231) are significantly more aggressive and invasive and known to be resistant to several anti-cancer agents when compared to ERα positive cells (e.g. MCF-7)[21-23]. This level of aggressiveness is going parallel with the western blot recorded expression of nagalase in MCF-7 and (MDA-MB-231) cell lines with the latter having remarkably higher nagalase compared to MCF-7 as presented in Fig. 1. The reported lower nagalase in the MCF-7 breast cancer cell line could explain the previously recorded effectiveness of Gc-MAF in stimulating macrophage angiogenesis, proliferation, migration and inhibiting metastasis of MCF-7 human breast cancer cell line[12,24].

The lowest level of nagalase was observed in the lung cancer cell line (A590) (Fig. 1). This could be consistent with the fact that while many types of cancer are targets for Gc-MAF treatment, it has not been used as a clinical therapy for lung and brain cancer[25].

An earlier report of Korbelik *et al.*[26] and current data could support nagalase measurement as a diagnostic and prognostic index that might allow oncologists to design the dosage or nature of the treatment.

Despite its special function as a biomarker of various aggressiveness of cancer cell lines. Strong, prospective comparative data on tissue biopsies of various tumour types and stages are desperately needed to further our understanding of nagalase's role in clinical practice as a cancer biomarker.

CONCLUSION

The remarkable differences of western blot relative nagalase protein expression in cervical, colon, breast and lung cancer cell lines together with the discussed relationship between these levels and the response to GC-MAF treatment. This could help to suggest an assessment of serum nagalase as a reliable biomarker for the early detection of cancer, checking progression and response to different treatment strategies. The GcMAF-based immunotherapy should be encouraged as an effective intervention for reducing serum nagalase levels and helping to reach successful clinical outcomes in patients with various stages and numerous types of cancer. Screening for effective and safe natural products with high potency to reduce the expression of nagalase as an aggressive enzyme could be of great help in treating cancer patients. However, further *in vitro* and *in vivo* studies are needed to explore the molecular mechanism of inhibitory activity on alpha-nagalase expression.

SIGNIFICANCE STATEMENT

Currently, there is a lack of clinical management alternatives for different types of cancer that can differentiate between aggressive tumours and those that are indolent. Tissue-based molecular indicators that were recently developed have improved the risk assessment for the disease. Early identification of nagalase as a biomarker of cancer aggressiveness could provide several benefits, including a higher likelihood of recovery, fewer aggressive treatment options and a reduction in the disease's development to advanced or metastatic stages.

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