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Original paper

# Possible role of α-mannosidase and β-galactosidase in larynx cancer

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**Background:** Lysosomal exoglycosidases, such as α-mannosidases (MAN) and β-galactosidases (GAL), are found in different glycoside hydrolase sequence-based families. Considerable research has proved plays the role of MAN, which play a key role in the modification and diversification of hybrid *N*-glycans, processes with strong cellular links to cancer. Therefore the study aim was to investigate the activities of MAN and GAL in larynx cancer compared to controls.

**Material and methods:** Larynx cancer ($n$ = 21) and normal healthy tissue ($n$ = 21) were collected from patients during total laryngectomy. A biopsy of macroscopically healthy tissue in the area of the lower 1/3 of omohyoid muscle was taken for frozen sections in each case and these served as controls. The release of p-nitrophenol from p-nitrophenol derivatives of MAN and GAL was used.

**Results:** In all specimens we observed significantly higher activity of investigated enzymes in larynx cancer compared with controls. The mean release of MAN from activated cells was 3.702 ±1.3245 nkat/g wet tissue compared to controls (1.614 ±0.8220 nkat/g wet tissue). The mean release of GAL from the activated cells was 3.383 ±2.1980 nkat/g wet tissue compared to controls (2.137 ±1.3685 nkat/g wet tissue). Differences in observed activity were statistically significant.

**Conclusion:** The present data indicate that MAN and GAL are significantly and consistently elevated in larynx cancer growth. It also means that catabolic reactions involving glycoproteins, glycolipids and proteoglycans may play a role in larynx cancer. Further research should also evaluate the relative importance of these particular exoglycosidases in indicating the progress of the disease in considering the spectrum of identified marker mediators.

**Key words:** α-mannosidase, β-galactosidase, laryngeal cancer, exoglycosidase activity.

## Background

Larynx cancer is one of the most common cancers in the head and neck region. The growing number of patients with head and neck cancer is a reason to search for new markers and treatment strategies. The discussion on possible markers is still open. A group of enzymes called lysosomal exoglycosidases are markers for several types of cancer such as renal cancer and salivary gland cancer [1, 2]. Therefore it was the reason for us to make an attempt to evaluate the activity of selected lysosomal glycosidases, i.e. α-mannosidase (MAN) and β-galactosidase (GAL), in squamous cell larynx carcinoma. Results of treatment for locally advanced squamous cell head and neck cancer with surgery and/or radiotherapy are still unsatisfactory. In Europe, head and neck cancer accounted for approximately 143 000 new cancer cases in 2007 and were responsible for more than 68 000 deaths [3]. They have broadly varying rates of incidence and mortality around the world. In North America and Europe, tumours usually arise from the oral cavity, oropharynx and larynx. Numerous studies have highlighted the increasing role of the human papillomavirus (HPV) in the occurrence of head and neck cancers. Most HPV positive cancers occur in the oropharynx [4]. HPV positive rates for larynx ranged from 5 to 16% [5-7]. However, other authors have reported that HPV was identified in 35.5% of patients with squamous cell carcinoma of the larynx [8]. Head and neck cancers are strongly associated with environmental and lifestyle risk factors. The largest risk factor for the disease is the use of tobacco [9]. Alcohol consumption is also frequently associated with tobacco use as a co-factor in oncogenic risk in larynx cancer [10]. Treatment of head and neck cancer depends on the initial localization of the tumour, on patient's comorbidity and potential side effects of treatment. Surgical resection, radiotherapy, radiochemotherapy, induction chemotherapy, and radiobiotherapy (with anti-epidermal growth factor receptor (EGFR) therapies such as cetuximab and other anti-EGFR) are the therapeutic methods in locally advanced cases, the most frequent mode of presentation of head and neck cancers [10].

Selective cell to cell adhesion of laryngeal cancer cells may be mediated by oligosaccharide chains of glycoproteins present on the extracellular membrane. These oligosaccharide chains are recognized by cadherins on the cellular membranes of the neighbouring cells. The adhesion of the cellular membranes of epithelial cells to components of the extracellular matrix is mediated by integrins and integral membrane proteoglycans. Exoglycosidases which degrade sugar chains of glycoconjugates (glycoproteins, glycolipids and proteoglycans) may participate in reducing adhesion to the neighbouring cells and creating channels in the extracellular matrix.

Lysosomal exoglycosidases, such as mannosidases and galactosidases, are found in different glycoside hydrolase sequence-based families. The research has proved the importance of α-mannosidases, which play a key role in the

modification and diversification of hybrid *N*-glycans – processes with strong cellular links to cancer [11].

Lysosomal α-mannosidase (α-MAN, EC 3.2.1.24) is an exoglycosidase which cleaves α-linked mannose residues from the non-reducing end during the ordered degradation of *N*-linked glycoproteins [12]. Therefore its activity may be associated with the intensity of catabolism of *N*-linked oligosaccharide chains of glycoproteins. MAN is extractable from human liver, fibroblasts and other tissues [13].

β-galactosidase (EC 3.2.2.23, GAL) is a glycoside hydrolase localized in the lysosome [14, 15]. It catalyses the hydrolysis of terminal β-glycosidic bonds present in oligosaccharide chains of glycolipids, glycoproteins and glycosaminoglycans [16] and therefore its activity may reflect the intensity of catabolism of oligosaccharide chains containing galactose, i.e. glycosaminoglycans, glycoproteins and glycolipids. β-galactosidase has been taken as an indicator for lysosomal enzyme release [17]. The enzyme is capable of degrading extracellular matrix components, mainly glycosaminoglycans and glycoproteins. The activity of the enzyme is maximal at acid pH 3-5 [15, 18].

Although exoglycosidases have been shown to manifest tissue degradation in different diseases, their activity levels in larynx cancer are hardly known. In our previous paper we presented the activity of *N*-acetyl-β-glucosaminidase (hexosaminidase) in laryngeal cancer and cholesteatoma [19-21] and now in the present investigation, for the first time, we investigated the activities of α-mannosidase and β-galactosidase in larynx cancer, performing identical, blinded assays in normal tissue biopsies taken from macroscopically healthy tissue during the same total laryngectomy. We also demonstrated the correlation between the activities of investigated enzymes in larynx cancer and healthy tissue specimens.

## Material and methods

All procedures were approved by the Medical University Investigational Review Board and patients were consented for matched-healthy tissue biopsies in addition to the planned laryngological procedure. Larynx cancer specimens ($n$ = 21) and healthy tissue from a region deemed to be macroscopically without cancer and located in the area of the lower 1/3 of the omohyoid muscle ($n$ = 21) were harvested and immediately frozen at –80°C from the same patients during total laryngectomy due to larynx cancer. A biopsy of macroscopically healthy tissue was taken for frozen sections in each case. The histopathological evaluation of them was received during the surgery.

The age of patients ranged between 50 and 74 years (mean age: 59.83). There were 17 males and 4 females included in the study. Among the 21 cancers of the larynx 12 were localized in the vocal folds, anterior commissure and sinus of Morgagni and 9 in the subglottic area. The history of laryngeal cancer ranged from 3 months to 11 months. Histopathological evaluation of the tumour revealed squamous cell carcinoma in all cases. TNM staging was estimated according to the American Joint Committee on Cancer to define laryngeal cancer in all investigated cases [22]. In 16 cases the staging assessment revealed T3N2aM0, in 3 cases T3N2bM0 and in 2 cases T4N2aM0. Due to cervical lymph node metastasis 17 patients underwent post-operative radiotherapy.

### Preparation of homogenate

Larynx cancer and healthy tissue specimens were thawed out and weighed. Specimens were washed with 0.9% NaCl to remove leukocytes and fragments of mucosa. Specimens were suspended in 0.05 M citrate-phosphate buffer at pH 4.3 at 1:9 ratio (w/v) and homogenized for 2 minutes using a homogenizer. Homogenates were then centrifuged for 30 minutes (12 000 × g) at 4°C. Supernatant was stored at –70°C for further studies.

### Reagents

The reagents p-nitrophenyl-α-mannoside and p-nitrophenyl-β-galactoside were from Sigma, St. Louis, MO, USA, and other reagents were from Polish Chemical Reagents, Gliwice, Poland.

### α-Mannosidase and β-galactosidase release and assay

Activity of MAN and GAL in laryngeal cancer and healthy tissue homogenates was determined by the mean p-nitrophenol release from p-nitrophenol derivatives (p-nitrophenol-α-mannopyranoside and p-nitrophenol-β-D-galactopyranoside). These methods offer a robust method of quantifying activity of exoglycosidase activity levels in nkatals per gram wet tissue. The wavelength used to measure the absorbance was 410 nm.

Statistical comparisons of enzyme activity within larynx cancer and healthy tissue samples were compared with analyses conducted using STATISTICA StatSoft program. Comparisons were made with the Wilcoxon matched pairs test: differences at the $p < 0.05$ level were considered significant.

## Results

In all larynx cancer specimens we observed higher activity of MAN compared with that in normal tissue specimens. The mean activity of MAN was 3.702 ±1.3245 nkat/g wet tissue as compared to controls (1.614 ±0.8220 nkat/g wet tissue). We observed the statistical difference in 18 per 21 cases respectively. As data have nonparametric measurements, we used a Wilcoxon signed rank test.

The descriptive statistics of the exoglycosidase activity are shown in Fig. 1.

The correlation of two variables, MAN activity in larynx cancer and MAN activity in healthy tissue specimens, is shown in Fig. 2. Pearson's coefficient $r$ = 0.11902, proving that the correlation is weakly positive.

In 17 of 21 specimens we observed significantly higher activity of GAL in larynx cancer compared with that in normal healthy tissue specimens.

The mean activity of GAL from the activated cells was 3.383 ±2.1980 nkat/g wet tissue as compared to controls (2.137 ±1.3685 nkat/g wet tissue). In five larynx cancer specimens, the activity of GAL was 1.94 to 3.05 fold higher than in the healthy tissue.

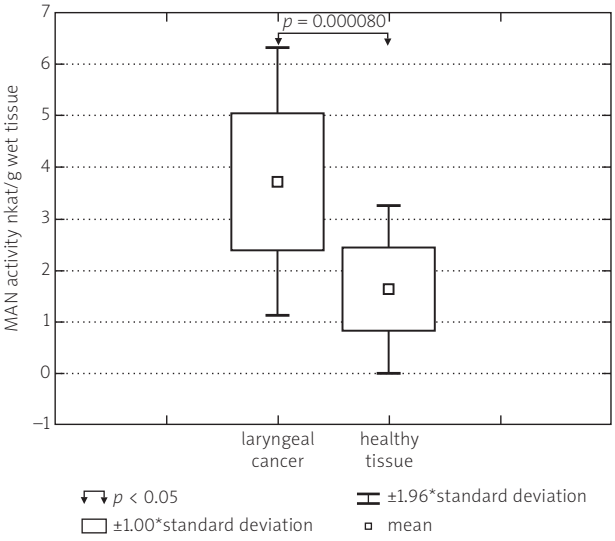


**Fig. 1.** Activity of α-mannosidase (MAN) in laryngeal squamous cancer specimens (statistical differences)

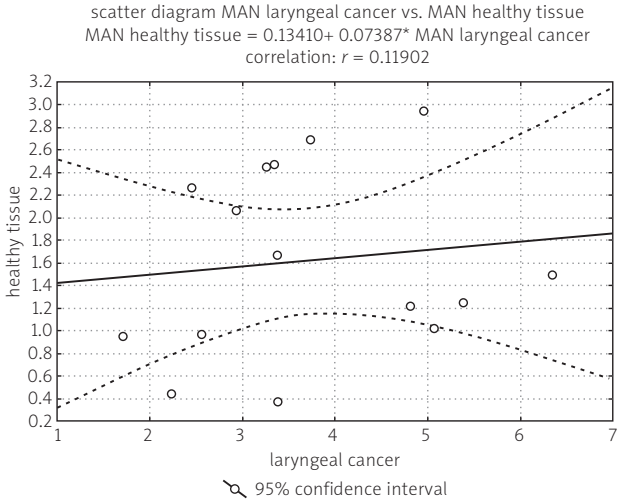


**Fig. 2.** Distributions of α-mannosidase activity levels in larynx cancer and healthy tissue serving as controls. Wilcoxon matched pairs test. Pearson's coefficient is weakly positive ($r$ = 0.11902)

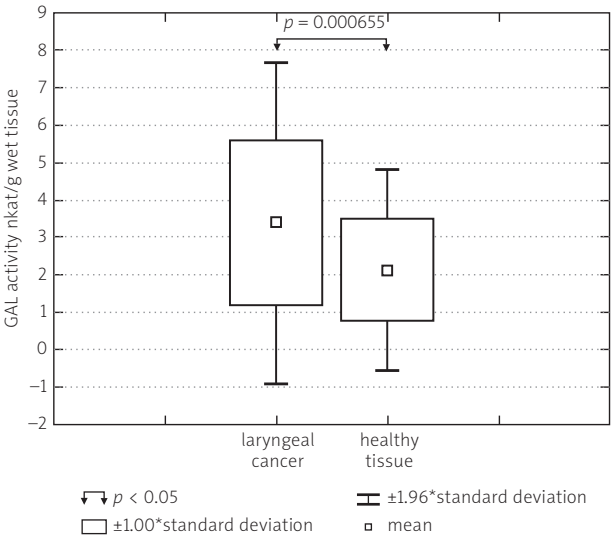


**Fig. 3.** Activity of β-galactosidase (GAL) in laryngeal squamous cancer specimens (statistical differences)

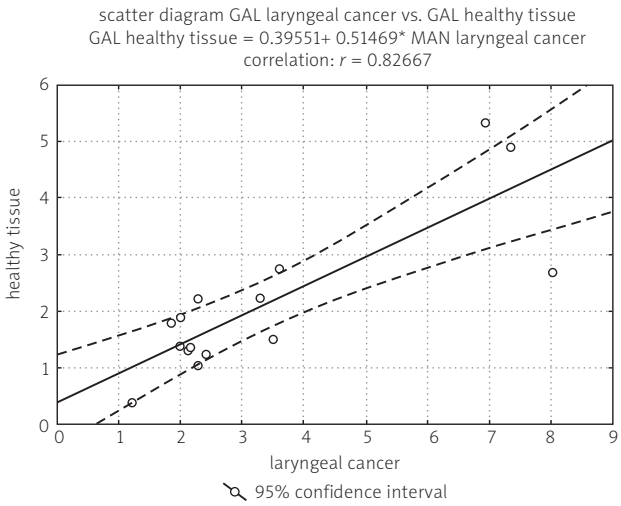


**Fig. 4.** Distributions of α-galactosidase activity levels in larynx cancer and healthy tissue specimen. Wilcoxon matched pairs test. Pearson's coefficient is strongly positive ($r$ = 0.82667)

In eight larynx cancer specimens the activity of GAL revealed small decrements relative to control tissue (1.05-1.61 fold higher in larynx cancer compared to the healthy tissue).

The descriptive statistics of laryngeal cancer are shown in Fig. 3.

We did not find any correlation between TNM staging of the disease and the level of MAN and GAL activity.

Statistical differences in GAL levels using Wilcoxon matched pairs test are shown in Fig. 4, which describes the correlation of the two variables GAL activity in larynx cancer and GAL activity in healthy tissue specimens. Pearson's coefficient $r$ = 0.82667, proving that the correlation is strongly positive.

## Discussion

The family of exoglycosidases has been only marginally considered in head and neck cancer, especially laryngeal cancer. It is undoubtedly true that exoglycosidases are important in tissue destruction and in providing access to proteases for highly glycosylated proteins. Our study is the first one addressing the question of exoglycosidase activity in larynx cancer. The lack of interest in catabolism of glycoproteins is surprising in light of the fact that most bone matrix macromolecules are glycosylated. High levels of carbohydrates have been demonstrated and they may significantly affect the proteolytic cleavage of the extracellular matrix. The process of

cancer development in the larynx induces molecular and cellular defects. Those defects are manifested in the form of invasion, migration, hyperproliferation and aggressiveness. Polypeptide chains of glycocalyx and extracellular matrix are degraded by proteases, while oligo- and heteropolysaccharide chains of glycoconjugates are degraded by endo- and exoglycosidases. During the course of catalysis, an oxonium ion-like transition state is thought to be generated, which is stabilized by a deprotonated carboxyl group from the enzyme [23]. In the literature we can find only one publication considering the level of mannosidase in the aspect of gene expression in papillomatosis [24]. However, Oktem *et al*. measured urinary *N*-acetyl-β-D-glucosaminidase (U-NAG) level in larynx cancer patients. The enzyme demonstrates the highest activity in different diseases, such as renal cancer, cholesteatoma, rheumatoid arthritis, juvenile arthritis, etc. *N*-acetyl-β-D-glucosaminidase belongs to a group of lysosomal exoglycosidases. Oktem *et al.* suggest that the level of U-NAG might be used in the diagnosis of laryngeal cancer and the early detection of recurrence during follow-up evaluation [25]. Due to the lack of publications concerning the role of lysosomal exoglycosidases in larynx cancer but taking into consideration the small number of papers we made an attempt to evaluate the activity of MAN and GAL in larynx cancer as a preliminary report for further investigations. Zhang H *et al*. searched for the expression of metalloproteinase-14 (MMP-14) and proved that MMP-14 was significantly increased in the T3 supraglottic cancer and neck nodal metastasis groups compared with the T1-2 group and the group without nodal metastasis [26]. Expression of MMP-14 mRNA and protein was also higher in tumours of patients with stage III-IV disease compared to patients with clinical stage I-II tumours [26]. The authors suggest that MMP-14 may play an important role in the progression of supraglottic carcinoma and be a novel prognostic factor for patients with supraglottic carcinoma.

The enzymatic activity of selected exoglycosidases such as MAN and GAL suggests that its presence in laryngeal squamous cell carcinomas could have diagnostic value. However, it is important to emphasize that the causes of laryngeal cancer are multifactorial and complex. The exoglycosidases may play a role in allowing the cancer to develop and seem to correlate with the change from healthy to diseased tissue. This is probably a result of the switch to cancer rather than a cause.

In our previous study we demonstrated the increased activity of one exoglycosidase, i.e. *N*-acetyl-β-D hexosaminidase (HEX), in larynx cancer and chronic otitis media with cholesteatoma [19-21]. The increase in HEX activity was observed in several inflammatory diseases, such as rheumatoid arthritis, idiopathic juvenile arthritis, osteoarthritis and chronic glomerulonephritis, as well as tumours, e.g. renal cancer [27-30]. Owing to the essential role of HEX in different cancers it may be assumed that the significance of MAN and GAL as catabolic enzymes is also crucial in the pathogenesis of laryngeal squamous cancer. MAN may indicate the catabolism of *N*-linked glycoproteins, glycolipids and proteoglycans. We demonstrated, for the first time, that MAN and GAL are present in the larynx cancer specimens. The revealed levels of MAN and GAL were also found to be significantly increased as compared to those in healthy tissue.

In conclusion: the present data indicate that lysosomal exoglycosidases MAN and GAL are significantly and consistently elevated in larynx cancer. This raises the need to further assess correlations between levels of MAN and GAL and larynx cancer growth. It also means that catabolic reactions involving glycoproteins, glycolipids and proteoglycans may play a role in larynx cancer. Further research should also evaluate the relative importance of these particular exoglycosidases in indicating the progress of the disease when considering the spectrum of identified marker mediators.

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