Int J Clin Exp Pathol 2015;8(8):8857-8868
www.ijcep.com /ISSN:1936-2625/IJCEP0011896

Original Article

Down-regulation of neutrophil gelatinase-associated lipocalin in head and neck squamous cell carcinoma correlated with tumorigenesis, not with metastasis

Lei Wang[1*], Chen Chen[2*], Fen Li[2], Qingquan Hua[1], Shiming Chen[1,2], Bokui Xiao[2], Mengyuan Dai[1], Man Li[1], Anyuan Zheng[1], Di Yu[1], Zhangwei Hu[1], Zezhang Tao[1,2]

[1]Departments of Otolaryngology Head and Neck Surgery, [2]Research Institute of Otolaryngology Head and Neck Surgery, Renmin Hospital of Wuhan University, Wuhan 430060, PR China. [*]Equal contributors and co-first authors.

Received June 26, 2015; Accepted July 29 2015; Epub August 1, 2015; Published August 15, 2015

Abstract: To examine the significance of the Neutrophil gelatinase-associated lipocalin (NGAL) in diagnosing head and neck squamous cell carcinoma (HNSCC) and predicting regional metastasis. We first used GEO dataset to analyze the NGAL gene expression in HNSCC. Then, we summarized the characteristics of patients retrospectively selected in clinic. Expression of NGAL protein in human HNSCC tumor, lymph node and normal samples were analyzed using immunohistochemistry. Next, we further investigated the NGAL expression in a tissue microassay to analyze the relationship between NGAL protein expression and TNM stage. Finally, we tested the NGAL protein expression in head and neck cancer cell lines. Analysis of GEO dataset concluded that NGAL gene expression in HNSCC was lower than that in normal tissue ($P<0.01$). There was no statistically significant difference of NGAL gene expression between T-stage and N-stage ($P>0.05$). NGAL protein expression in tumor was lower than that in normal tissue ($P<0.01$). There was no statistically significant difference of NGAL protein expression between metastasis group and non-metastasis group ($P>0.05$). Expression of NGAL protein was not correlated with TNM stage of HNSCC. Aggressive HNSCC cell lines have lower NGAL protein expression. Our data demonstrated that the expression of NGAL protein was correlated with tumorigenesis of HNSCC, but not with regional metastasis. It may serve as a novel biomarker for prognostic evaluation of patients with HNSCC.

Keywords: GEO dataset, NGAL, HNSCC, biomarker, tissue microassay

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequent cancer in the world, more than 40,000 new cases are diagnosed every year in the United States [1]. Multidisciplinary treatment combining surgery, radiotherapy and chemotherapy is the conventional treatment for HNSCC. In recent years, the 5 years survival rate was optimistically improved, 54.7% in 1992-1996, and increased to 65.9% in 2002-2006 [2], suggesting the enhancement of the treatment. However, for the purpose of improving the life quality of patients, the more effective way of prediction of early stage of cancer and regional metastasis was urged to be found. Although the advanced techniques, such as CT and MRI, were widely used to detect the tumor, they were not totally reliable. Some biomarkers have been reported for diagnosis and prognosis of HNSCC, while none of them showed enough clinical efficacies.

Neutrophil gelatinase-associated lipocalin (NGAL), a member of the lipocalin protein family, also known as Lipocalin2 (LCN2), was initially discovered as constituent of human neutrophil specific granules and was function as antimicrobial by blocking the trafficking of iron in bacteria [3]. It was a well recognized innate immunity protein expressed in most normal tissues for anti-microbacteria. Innate immunity was the primary role of NGAL. In clinic, it has been reported as predictive role in early diagnosis of platin-reduced renal Injury or nephrotoxicity [4, 5]. What's interesting, NGAL has been found overexpression in many types of non-

Table 1. Details of information of GEO dataset

Series accession	Organism	Type	Platform
GSE33205	Homo sapiens	Expression profiling by array	Affymetrix Human Exon 1.0 ST Array
GSE6631	Homo sapiens	Expression profiling by array, count, 2 disease state, 22 individual sets	Affymetrix Human Genome U95 Version 2 Array
GSE39368	Homo sapiens	Expression profiling by array; Genome variation profiling by SNP array	Affymetrix Genome-Wide Human SNP 6.0 Array

microbially-associated cancers in recent years, including pancreas [6], breast [7], and ovarian carcinomas [6]. Later, it was also found overexpression in many other types of cancers, such as esophageal [8, 9], gastric [10], lung [11]. In addition, NGAL promoted tumorigenesis through promoting tumor cell survival, proliferation, and possible metastasis [12]. One study reported NGAL could form a complex with MMP-9 to prevent its degradation and increase the MMP-9 activity [13]. Moreover, NGAL bound to siderophore and participated in iron metabolism in mammalian. NGAL transported iron into epithelial cell in a transferrin-independent manner. While the NGAL way of iron transport seemed to occur in response to stimuli, such as inflammation, bacterial infection or anemia [14]. Thus iron homeostasis was speculated to be involved with NGAL in promoting cancer.

However, the characteristics of NGAL expression in HNSCC remained to be explored; The aim of this work is to study the NGAL expression in HNSCC; meanwhile, to investigate the significance of NGAL as a biomarker of HNSCC.

Materials and methods

Gene expression profiles

Microarray data set GSE33205 (Control with HNSCC tumor and uvupopalatopharyngoplasty samples), GSE6631 (Analysis of paired normal tissues and tumor samples from patients with HNSCC), GSE39368 (A total of 163 samples were considered. A total of 138 tumor arrays remained after removing low-quality arrays, duplicate arrays, and arrays from non-HNSCC samples) was down-loaded from Gene Expression Omnibus (GEO), a public functional genomics data repository. Detailed information of the dataset was showed in **Table 1**. Annotation file was also acquired. The expression values of NGAL gene were transformed to relative expression.

Patients selected and tissue microassay

One hundred and ninety paraffin sections, including 70 cases of primary tumor of HNSCC, 70 cases of lymph nodes and 50 cases of normal mucosa, were acquired from the Pathology Department of the Renmin Hospital of Wuhan University in China, collected from 2013 to 2014. Sources of the lymph node were consistent with the primary tumor. Tumor localization included larynx, pharynx and nasopharynx. None of the patients received preoperative radiotherapy. Patients selected were divided into two groups: HNSCC group and normal group. The clinical characteristic, tumor type, age, gender and differentiation grade were shown in **Table 2**. All paraffin-embedded tissue blocks were cut and dried on 4 micrometer thick paraffin slides for immunohistochemistry performance. In addition, primary tumor and lymph node were respectively divided into two groups: metastasis group and non-metastasis group. This study has been approved by the Ethical Committees of the hospital.

An independent tissue microassay (US Biomax company, USA) (Production number: HN803b) was purchased. The tissue microassay consisted of 10 normal tissues, 32 tongue carcinoma, 31 larynx carcinoma and 7 nose carcinoma, including 62 men and 18 women. The mean age was 53.4 years old (ranged from 18 to 90 years old).

Cell culture

The human normal broncial epithelial cell line 16HBE and human HNSCC cell lines CNE-1, CNE-2, FADU were cultured in RMPI1640 (Invitrogen) with 10% fetal bovine serum at 37°C, 5% CO_2. Cells in logarithmic growth phase were used for western blotting.

Immunohistochemical staining

The tissue sections and the tissue microassay were deparaffined with standard pure xylene

Table 2. Characteristics of patients selected

Characteristics	Primary tumor (n=70)	Lymph node (n=70)	Normal (n=50)
Sex			
Male	68	68	30
Female	2	2	20
Localization			
Glottis	56	56	50
Hypopharynx	6	6	0
Supraglottis	5	5	0
Nasopharynx	2	2	0
Tonsil	1	1	0
Differentiation grade			
Well	65	62	0
Moderate	4	5	0
Poor	1	3	
Samples			
Metastasis	35	35	0
Non-metastasis	35	35	0
Normal	0	0	50

for 15 minutes three times at room temperature and hydrated in graded alcohols, phosphate buffer saline (PBS) was used to wash the sections. Antigen retrieval was performed in boiling citrate buffer (PH 6.0) for 15 minutes. Sections were then cooled down to room temperature in the buffers. After washing sections in PBS for 5 minutes three times, 0.3% hydrogen peroxide phosphate-citrate buffer was used to block endogenous peroxidase activity for 10 minutes. Rinsed with PBS for 5 minutes, tissue sections were incubated with primary antibody NGAL (Sigma-Aldrich; dilution 1:100) for 12 hours at 4°C. The sections were incubated with Poly-HRP Goat anti-rabbit (Maixin. Bio, FuZhou, China) for 30 minutes. Slides were dyed with diaminobenzidine for 5 minutes. Haematoxylin was used to counterstain the nucleus, followed by dehydtation and mounted. Images of sections were taken using an Olympus BX40 microscope and CC-12 Soft-Imaging System (Olympus, Tokyo, Japan).

Evaluation of immunohistochemical staining

NGAL showed both cytoplasmic and membrane expression. All of the immunostained sections and tissue microarray were analyzed and scored for intensity (0-3) and frequency (0-4). The intensity was scored as grade (0), negative; grade (1), weak intensity; grade (2), moderate intensity; grade (3), strong intensity. The frequency scores were respectively assigned when 0-25, 26-50, 51-75 and 76-100% of the tumor cell were positive. To use statistical analysis, NGAL protein intensity and frequency were transformed into a Composite Expression Score (CES) utilizing the formula CES = Intensity × Frequency. The range of CES was from 0 to 12. The CES was scored as negative (0), weak positive (1~4), positive (5~8), strong positive (9~12).

Western blotting

The cells were rinsed twice with cold phosphate-buffered saline (PBS) and lysed in buffer containing 1% Nonidet-P40 supplemented with complete protease inhibitor 'cocktail' (Roche) and 2mM dithiothreitol. Lysates were resolved by 12% SDS-polyacrylamide gel, electrotransferred to nitrocellulose membranes and blocked in 5% nonfat dry milk in Tris-buffered saline. Membranes were immunoblotted with primary anti-NGAL monoclonal antibody (Sigma-Aldrich, USA, 1:300). Proteins were detected with enhanced chemiluminescence reagent (Thermo Scientific) after immunoblotted with secondary antibodies; all experiments were carried out for at least three times.

Statistic analysis

All values were expressed as the mean ± SD. NGAL expression in primary tumor and lymph node was analyzed with t-test. NGAL expression measurements in tissue microassay and cell lines were analyzed by one-way of ANOVA and Bonferroni's multiple comparison tests among groups. Probability values <0.05 were considered statistically significant.

Results

Analysis of GEO dataset

GEO dataset GSE33205 included the HNSCC and uvupopalatopharyngoplasty samples which were non-paired materials (**Figure 1A**). The relative expression of NGAL gene of the two groups was compared. The result showed that NGAL expression in tumor was lower than that in normal tissue ($P<0.01$). Then, data of paired samples from GEO dataset GSE6631 was analyzed, showing the same outcome (**Figure 1B**). These data indicated that NGAL gene was down-regulated in HNSCC.

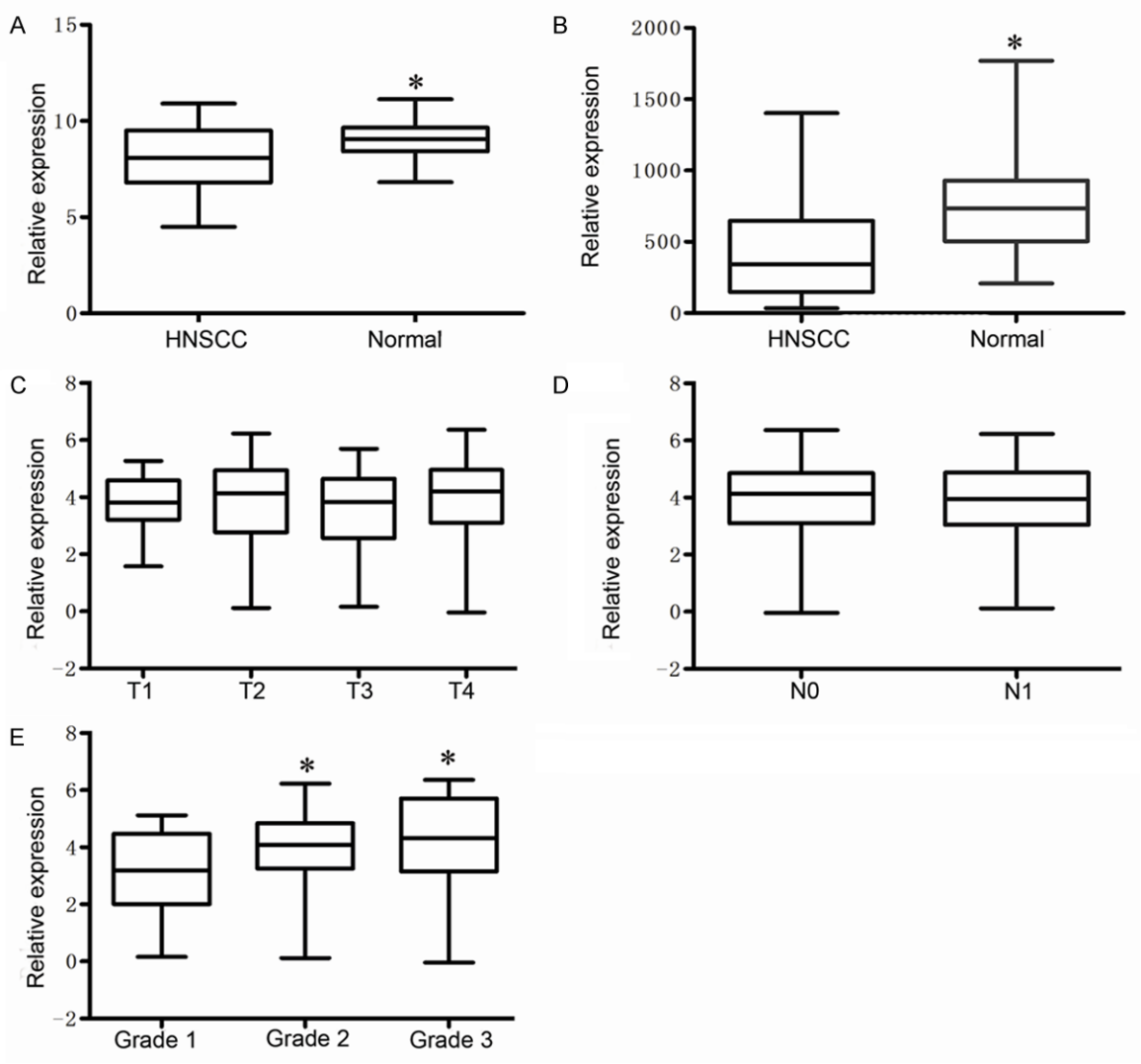

Figure 1. Analysis of gene relative expression of NAGL in HNSCC from GEO dataset. A. Analysis of the gene relative expression of unpaired HNSCC tumors and uvupopalatopharyngoplasty samples from patients with HNSCC. Normal tissue showed the higher expression of NGAL gene. B. Analysis of paired normal tissues and tumor samples from patients with HNSCC. Normal tissue showed the higher expression of NGAL gene. C. Gene expression in different T-stage of HNSCC, there was no statistically significance between different T-stages. D. Gene expression in different N-stage of HNSCC, there was no statistically significance between different N-stages. E. Gene expression in different grade of HNSCC, there was statistically significance between grade 2/3 and grade 1, while there was no statistically significance between grade 2 and grade 3.

We further investigated the NGAL expression in T-stage, N-stage and different grade in patients with HNSCC. Data from GEO dataset GSE39368 showed that there was no statistically significant difference between T-stages or N-stages ($P>0.05$) (**Figure 1C**, **1D**). While there was statistically significant difference between tumor grades; the NGAL expression in grade 1 was lower than that in grade 2/3 ($P<0.05$). But there was no statistically significance between grade 2 and grade 3 ($P>0.05$) (**Figure 1E**). These results showed that NGAL expression in HNSCC tissues was correlated with grade but had no correlation with T-stage, N-stage.

Characteristics of patients retrospectively selected and TMA

The summary of baseline characteristics of all patients from the clinic was showed in **Table 2**. For patients with HNSCC collected from our institution, male was a predominance and the

Table 3. Details of the characteristics of Tissue microassay (TMA)

Patients demographics	Total N=80
	n (%)
Age, Mean	53.4±10.5
Localization	
Tongue	42 (52.5)
Larynx	31 (38.6)
Nose	7 (8.9)
T classification	
T1	5 (8.3)
T2	30 (50.0)
T3	17 (28.3)
T4	8 (13.3)
N classification	
N0	42 (68.9)
N1	16 (26.2)
N2	3 (5.0)
Differentiation	
1	12 (16.7)
2	36 (50.0)
3	24 (33.3)
Staging	
I	5 (0.81)
II	33 (54.1)
III	14 (23.0)
IV	9 (14.8)
Metastasis	8 (10.0)
NAT	2 (2.5)
Normal	9 (11.2)

median age was 60.4 years old. Glottis was the most common place where tumor forms (80.0%). 92.8% patients showed a well differentiation.

The summary of characteristics of the TMA was shown in **Table 3**. The tissue microassay consisted of 10 normal tissues, 32 tongue carcinoma, 31 larynx carcinoma and 7 nose carcinoma, including 62 men and 18 women. The mean age was 53.4 years old (ranged from 18 to 90 years old).

Analysis of NGAL expression in tumor and normal tissue

The intensity and frequency of immunohistochemical (IHC) staining in all tissue sections was analyzed. We integrated intensity and frequency into IHC index (CES) for a single numerical measurement. NGAL located at cytoplasmic membrane. **Figure 2A** showed the representative images of NGAL expression in tumor and normal tissue. A significant decreased CES was noted in primary tumor than that in normal tissue ($P<0.01$) (**Figure 2B**). These data demonstrated that NGAL was down-regulated in HNSCC, which was consistent with what we analyzed using the GEO dataset.

Correlation between NGAL expression and regional metastasis

To analyze the correlation between NGAL expression and regional metastasis, Tissue sections from primary tumor and lymph node which both contained metastasis and non-metastasis group were used to test the NGAL protein expression. Representative images were shown in **Figure 3A**. Our result showed that there was no statistically significant difference between primary tumor or lymph node with metastasis and that without metastasis ($P>0.05$) (**Figure 3B**). These data indicated that NAGL may have no correlation with regional metastasis.

Analysis of NGAL expression in TMA of HNSCC

In addition to completing an analysis of NGAL in clinic samples of HNSCC, we used tissue microarray contained three types of head and neck cancer (tongue, larynx and nose) to have a subset analysis, normal tissues were also included in tissue microassay. T-stage, N-stage and grade were compared respectively. To better compare the results, we also use CES for measurement.

We chose the images of NGAL expression from all the T-stage sections of TMA, representative images of NGAL expression were shown in **Figure 3A**. NGAL protein expression in T-stage sections showed weak positive. There were no statistically significant difference of NGAL expression between different stage ($P>0.05$) (**Figure 3B**). These data suggested that NGAL had no correlation with T-stage of HNSCC. N-stage was also compared using the same method. Representative images were shown in **Figure 4A**. The CES showed that there was no statistically significant difference between N-stages ($P>0.05$) (**Figure 4B**). These results were also consistent with what we analyzed

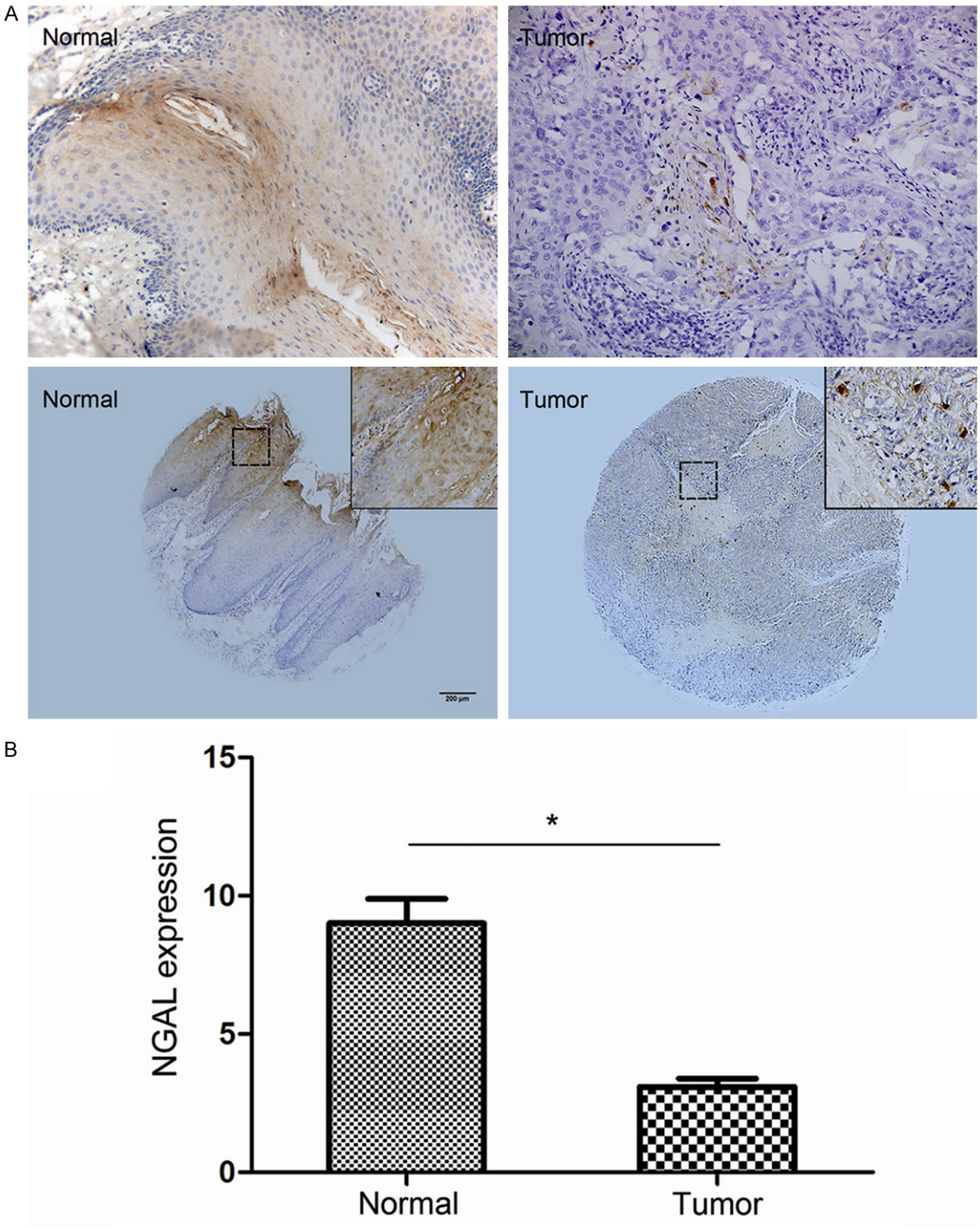

Figure 2. A. Represent images of IHC staining for NGAL protein in human tumor and normal tissue. Samples were from our institution and TMA. A zoomed in ×200 magnification of a small area of the same section in the top right corner was showed the staining details. B. IHC index (intensity × % tumor cells) was showed that NGAL expression in tumor was lower than normal tissue (P<0.01).

from the GEO dataset. As N+ represents regional metastasis of tumor, these results suggested that NGAL had no correlation with regional metastasis of HNSCC.

Finally, we compared the tumor grade. Tumor grade 1, 2, 3 represent high differentiation, moderately differentiation and poorly differentiation, respectively. There was significantly

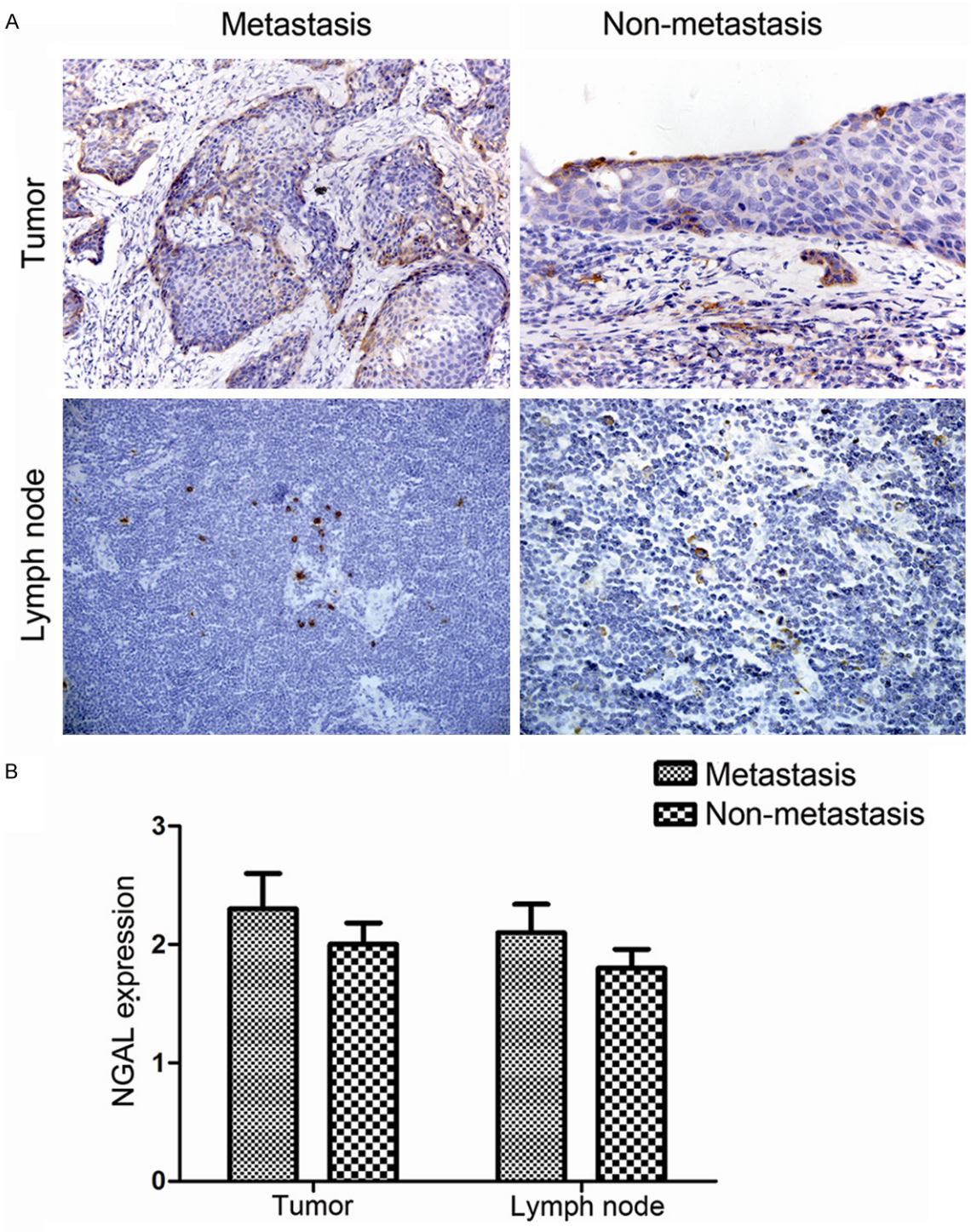

Figure 3. A. Represent images of IHC staining for NGAL protein in human primary tumor and lymph node with metastasis or without metastasis. All magnification 200×. B. IHC index (intensity × % tumor cells) was showed that there was no statistically significance between metastasis group and non-metastasis group (P<0.01).

increased CES in grade 2 compared with grade 1 (P<0.05), meanwhile, grade 3 have a markedly increased CES than grade 2 (P<0.05). Representative images and the IHC index were shown in **Figure 5A**, **5B**.

NGAL expression in HNSCC cell lines

Tissue IHC staining confirmed that low expression of NGAL was found in HNSCC than normal tissues. We then tested the NGAL expression in

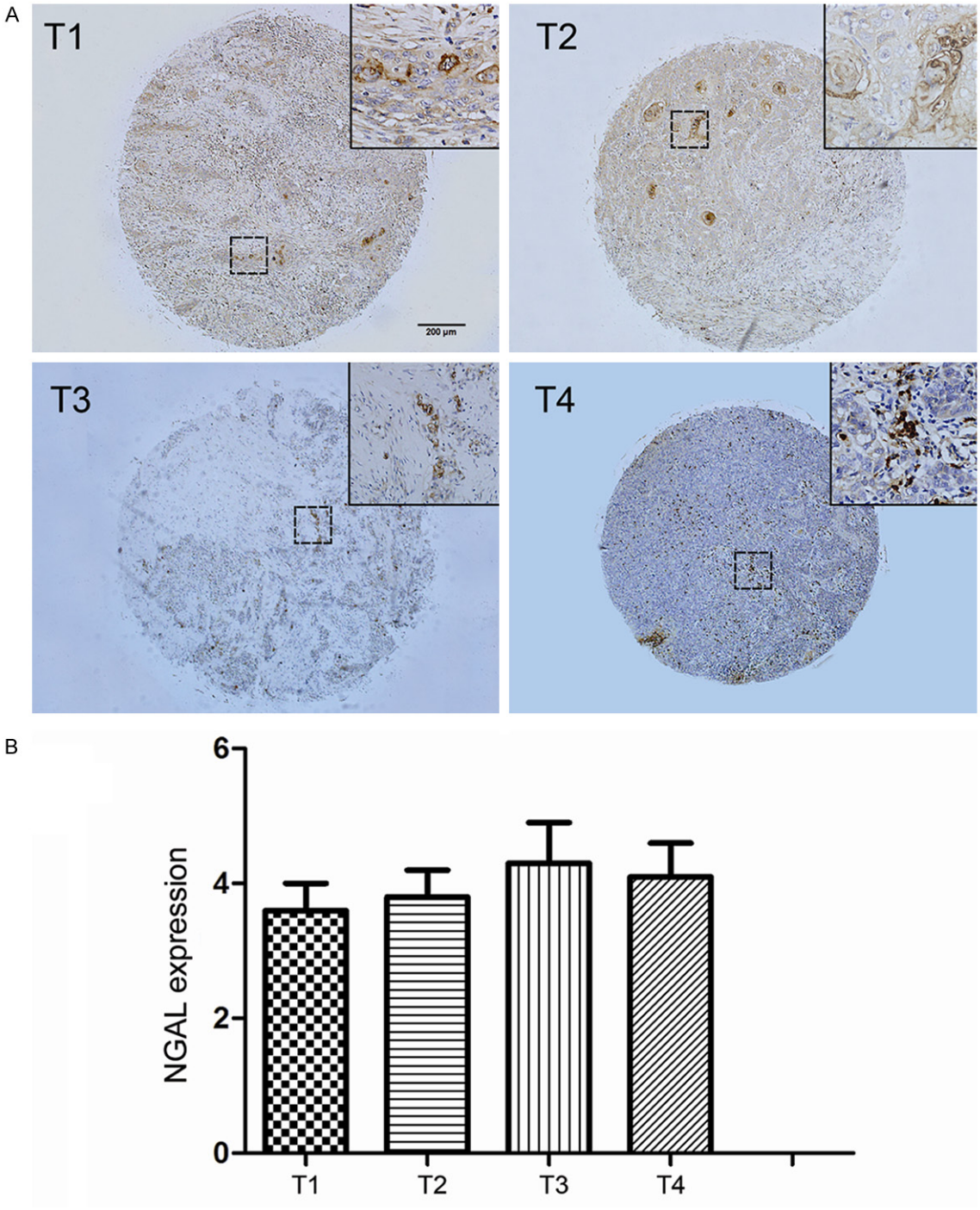

Figure 4. A. Represent images of IHC staining for NGAL protein in T-stage of TMA. All magnification 100×. A zoomed in ×200 magnification of a small area of the same section in the top right corner was showed the staining details. B. IHC index (intensity × % tumor cells) was showed that there was no statistically significance between T-stages.

four types of cell lines (16-HBE, CNE-1, CNE-2 and FADU). NGAL expression in tumor cells was lower than that in 16HBE cells ($P<0.05$). There were no statistically significant difference between different tumor cells ($P>0.05$). The results were shown in **Figure 6A**, **6B**. The data was consistent with the results of immunohistochemical staining.

Discussion

Head and neck cancer is a broad term that contains epithelial malignancies that occur in the

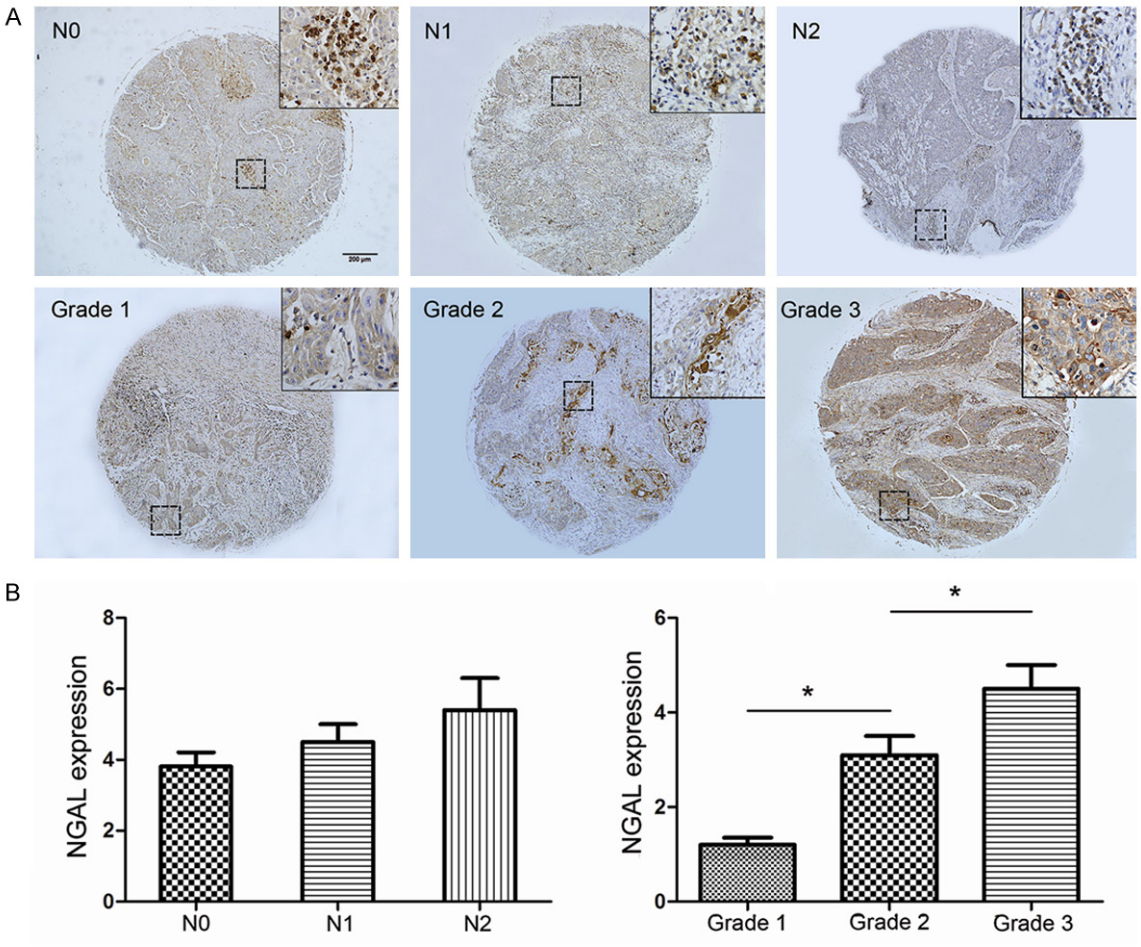

Figure 5. A. Represent images of IHC staining for NGAL protein in N-stage of TMA. All magnification 100×. A zoomed in ×200 magnification of a small area of the same section in the top right corner was showed the staining details. B. IHC index (intensity × % tumor cells) was showed that there was no statistically significance between N-stages.

paranasal sinuses, nasal cavity, oral cavity, pharynx and larynx. Most of these epithelial malignancies are squamous cell carcinoma. The complex anatomy and important physiological role of the structures where tumor involved indicate that the goals of the treatment are not only to improve the survival but also to protect organ function. Nowadays, surgical techniques, chemotherapy and radiotherapy are the major method to improve the survival. Moreover, molecularly targeted agents like the epidermal growth factor receptor inhibitors have been successfully used in clinic [15]. New molecular targeted agents should be investigated to enhance the survival of HNSCC. Biomarkers with diagnostic and prognostic value for predicting HNSCC are crucial for determining treatment method and may provide a strategy of targeted therapy of HNSCC.

NGAL was initially defined as a useful bacteriostatic agent actively against bacterial, it was found over-expressed in many types of cancers including breast, pancreatic and ovarian cancers [14]. We first analyze the data from GEO dataset, concluding that NGAL gene was down-regulated in HNSCC tumor. Next, samples from our institution and tissue microassay were used for immunohistochemical staining. We found that NGAL expression in tumor tissue was lower than that in normal tissue. The results were consistent with what we analyzed from the GEO dataset. Finally, four types of cell lines 16-HBE, CNE-1, CNE-2 and FADU were chosen in the Westen blot assay for detection of NGAL protein, and the results supported the speculation. Tumor cell contained lower NGAL expression than normal cell. These result indicated that down-regulation of NGAL in HNSCC

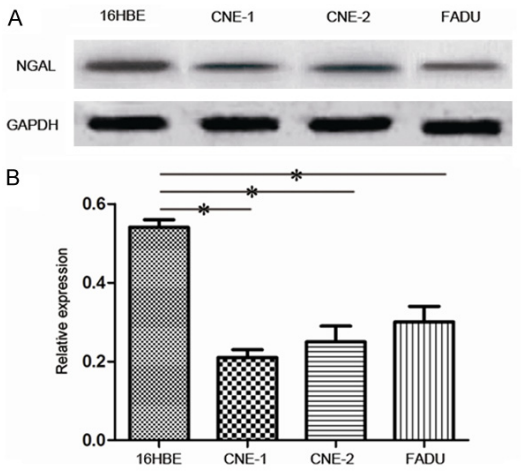

Figure 6. A. The NGAL expression in cell lines of 16HBE, CNE-1, CNE-2 and FADU. B. NGAL expression in tumor cell lines was lower than that in normal cells.

may correlate with tumorgenesis; While several studies reported that NGAL was overexpression in many cancers, the detailed mechanism was not totally understood; We suggested that larger number cases were needed to be explored to confirm the result.

NGAL was reported to be involved in tumorigenesis and played potential role in metastasis [16]. One study demonstrated the metastatic potential of anaplastic thyroid carcinoma cells was regulated by NGAL [17]. Tung reported that the growth and invasion of prostate cancer cells were suppressed when knockdown of NGAL [18]. Chalinee showed that NGAL knockdown by siRNA decreased the invasion of human cholangiocarcinoma cells [19]. Based on these researches, NGAL was speculated to be a new kind of metastasis biomarker. While there were also studies showing that NGAL had no correlation with metastasis. Several publications reported the negative correlation: there was no effect of NGAL on aggressiveness of cancer in the MMTV-PyMT/FVB/N mouse model for breast cancer [20], nodal metastasis of oral cancer seems to be not associated with NGAL [21, 22], and disruption of NGAL gene in mice suppressed primary mammary tumor formation but did not decrease lung metastasis [23].

In our study, Analysis of GEO dataset showed that there was no statistically significant difference of NGAL expression between T-stage and N-stage, suggesting that NGAL may have no correlation with regional metastasis. To further explore the relationship between NGAL protein and metastasis, we compared the NGAL expression in primary tumor and lymph node confirmed metastasis with that without metastasis. There was no statistically significant difference between the two groups. Meanwhile, TMA was used to have a subset analysis, there was no statistically significant difference between T-stage and N-stage, which was consistent with the analysis from GEO dataset. Tumor grade had correlation with NGAL expression, while analysis of NGAL expression in TMA was a little different with what we got from GEO dataset. There may several reasons for this situation. First, The mechanisms of post-transcriptional and translational which is complicated and varied are not yet sufficient well understood. RNA and protein expression level may have the poor correlations, even have the opposite expression level. There may be some other reasons like tumor microenvironment that could influence the NGAL expression. Hypoxia is one factor that could activate NGAL expression [24]. During inflammation, NGAL is also strongly up-regulated in epithelial tissues [25]. Therefore, RNA levels cannot be used as substitute for corresponding protein levels. In addition, NGAL expression in tumor cell lines was assayed, there was no statistically significant difference between tumor cells. Higher aggressive tumor cell did not showed more NGAL expression than low aggressive one.

In a word, we concluded that NGAL protein correlated with tumorigenesis, but not with regional metastasis; These result may enhance the accuracy of diagnosing HNSCC.

Acknowledgements

This work was funded by the National Natural Science Foundation of China (NO. 81372880; NO. 81172569); the doctoral program of Higher Education Research Fund (NO. 20130141120093; NO. 20110141110062); the Natural Science Foundation of Hubei province (No. 2012FFA045).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zezhang Tao, Department of Otolaryngology-Head and Neck Surgery, Renmin Hospital of Wuhan University, Wuhan 430060, PR China. Tel: +86-27-88041911-86605; Fax: +86-27-88043958; E-mail: taozezhang@hotmail.com; Dr. Chen Chen, Research Institute of Otolaryngology Head and Neck Surgery, Renmin Hospital of Wuhan University, Wuhan 430060, PR China. Tel: +86-18062134851; Fax: +86-27-88043958; E-mail: chenchen_md@whu.edu.cn

References

[1] Ota I, Okamoto N, Yane K, Takahashi A, Masui T, Hosoi H and Ohnishi T. Therapeutic strategies for head and neck cancer based on p53 status. Exp Ther Med 2012; 3: 585-591.

[2] Pulte D and Brenner H. Changes in survival in head and neck cancers in the late 20th and early 21st century: a period analysis. Oncologist 2010; 15: 994-1001.

[3] Goetz DH, Holmes MA, Borregaard N, Bluhm ME, Raymond KN and Strong RK. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. Mol Cell 2002; 10: 1033-1043.

[4] Kos FT, Sendur MAN, Aksoy S, Celik HT, Sezer S, Civelek B, Yaman S and Zengin N. Evaluation of renal function using the level of neutrophil gelatinase-associated lipocalin is not predictive of nephrotoxicity associated with cisplatin-based chemotherapy. Asian Pac J Cancer Prev 2013; 14: 1111-1114.

[5] Seker MM, Deveci K, Seker A, Sancakdar E, Yilmaz A, Turesin AK, Kacan T and Babacan NA. Predictive role of neutrophil gelatinase-associated lipocalin in early diagnosis of platin-induced renal injury. Asian Pac J Cancer Prev 2015; 16: 407-410.

[6] Lim R, Ahmed N, Borregaard N, Riley C, Wafai R, Thompson EW, Quinn MA and Rice GE. Neutrophil gelatinase-associated lipocalin (NGAL) an early-screening biomarker for ovarian cancer: NGAL is associated with epidermal growth factor-induced epithelio-mesenchymal transition. Int J Cancer 2007; 120: 2426-2434.

[7] Yang J, McNeish B, Butterfield C and Moses MA. Lipocalin 2 is a novel regulator of angiogenesis in human breast cancer. FASEB J 2013; 27: 45-50.

[8] Zhang H, Xu L, Xiao D, Xie J, Zeng H, Wang Z, Zhang X, Niu Y, Shen Z, Shen J, Wu X and Li E. Upregulation of neutrophil gelatinase-associated lipocalin in oesophageal squamous cell carcinoma: significant correlation with cell differentiation and tumour invasion. J Clin Pathol 2007; 60: 555-561.

[9] Du ZP, Wu BL, Wang SH, Shen JH, Lin XH, Zheng CP, Wu ZY, Qiu XY, Zhan XF, Xu LY and Li EM. Shortest path analyses in the protein-protein interaction network of NGAL (neutrophil gelatinase-associated lipocalin) overexpression in esophageal squamous cell carcinoma. Asian Pac J Cancer Prev 2014; 15: 6899-6904.

[10] Shimura T, Dagher A, Sachdev M, Ebi M, Yamada T, Yamada T, Joh T and Moses MA. Urinary ADAM12 and MMP-9/NGAL complex detect the presence of gastric cancer. Cancer Prev Res (Phila) 2015; 8: 240-8.

[11] Tong Z, Wu X, Ovcharenko D, Zhu J, Chen CS and Kehrer JP. Neutrophil gelatinase-associated lipocalin as a survival factor. Biochem J 2005; 391: 441-448.

[12] Leng X, Wu Y and Arlinghaus RB. Relationships of lipocalin 2 with breast tumorigenesis and metastasis. J Cell Physiol 2011; 226: 309-314.

[13] Fernández CA, Yan L, Louis G, Yang J, Kutok JL, Moses MA. The matrix metalloproteinase-9_ neutrophil gelatinase- associated lipocalin complex plays a role in breast tumor growth and is present in the urine of breast cancer patients. Clin Cancer Res 2005; 11: 5390-5395.

[14] Rodvold JJ, Mahadevan NR and Zanetti M. Lipocalin 2 in cancer: when good immunity goes bad. Cancer Lett 2012; 316: 132-138.

[15] Modjtahedi H, Cho BC, Michel MC and Solca F. A comprehensive review of the preclinical efficacy profile of the ErbB family blocker afatinib in cancer. Naunyn Schmiedebergs Arch Pharmacol 2014; 387: 505-521.

[16] Saverio Candido RM, Jerry Polesel, Alessia Catania, Francesca Maira, Santo S. Signorelli, James A. McCubrey and Massimo Libra. Roles of neutrophil gelatinase-associated lipocalin (NGAL) in human cancer. Oncotarget 2014; 5: 1576-1594.

[17] Volpe V, Raia Z, Sanguigno L, Somma D, Mastrovito P, Moscato F, Mellone S, Leonardi A and Pacifico F. NGAL controls the metastatic potential of anaplastic thyroid carcinoma cells. J Clin Endocrinol Metab 2013; 98: 228-235.

[18] Tung MC, Hsieh SC, Yang SF, Cheng CW, Tsai RT, Wang SC, Huang MH and Hsieh YH. Knockdown of lipocalin-2 suppresses the growth and invasion of prostate cancer cells. Prostate 2013; 73: 1281-1290.

[19] Nuntagowat C, Leelawat K and Tohtong R. NGAL knockdown by siRNA in human cholangiocarcinoma cells suppressed invasion by reducing NGAL/MMP-9 complex formation. Clin Exp Metastasis 2010; 27: 295-305.

[20] Cramer EP, Glenthoj A, Hager M, Juncker-Jensen A, Engelholm LH, Santoni-Rugiu E, Lund LR, Laerum OD, Cowland JB and Borregaard N. No effect of NGAL/lipocalin-2 on aggressiveness of cancer in the MMTV-PyMT/FVB/N mouse model for breast cancer. PLoS One 2012; 7: e39646.

[21] Lin CW, Tseng SW, Yang SF, Ko CP, Lin CH, Wei LH, Chien MH and Hsieh YS. Role of lipocalin 2 and its complex with matrix metalloproteinase-9 in oral cancer. Oral Dis 2012; 18: 734-740.

[22] Noorlag R, van der Groep P, Leusink FK, van Hooff SR, Frank MH, Willems SM and van Es RJ. Nodal metastasis and survival in oral cancer: Association with protein expression of SLPI, not with LCN2, TACSTD2, or THBS2. Head Neck 2014; 37: 1130-6.

[23] Berger T, Cheung CC, Elia AJ and Mak TW. Disruption of the Lcn2 gene in mice suppresses primary mammary tumor formation but does not decrease lung metastasis. Proc Natl Acad Sci U S A 2010; 107: 2995-3000.

[24] Viau A, El Karoui K, Laouari D, Burtin M, Nguyen C, Mori K, Pillebout E, Berger T, Mak TW, Knebelmann B, Friedlander G, Barasch J and Terzi F. Lipocalin 2 is essential for chronic kidney disease progression in mice and humans. J Clin Invest 2010; 120: 4065-4076.

[25] Karlsen JR, Borregaard N and Cowland JB. Induction of neutrophil gelatinase-associated lipocalin expression by co-stimulation with interleukin-17 and tumor necrosis factor-alpha is controlled by IkappaB-zeta but neither by C/EBP-beta nor C/EBP-delta. J Biol Chem 2010; 285: 14088-14100.