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Nebulette Expression Is Associated with Lymph Node Metastasis in Patients with Colorectal Cancer

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ABSTRACT

BACKGROUND

Colorectal cancer (CRC) is one of the most common cancers among men and women worldwide. Cancer metastasis is the main cause of death in patients with cancer. NEBL (nebulette, Gene ID: 10529) protein interacts with thin filaments in the cell and may functionally destabilize focal adhesion composition. There are some studies on NEBL gene expression alteration in cancer. In the presented study we aimed to analyze NEBL gene expression in patients with colorectal cancer to explore possible association of this gene with clinicopathological features in CRC.

METHODS

Sixty-seven fresh samples of colorectal tumors and adjacent normal tissues were collected from Iranian patients with CRC. Real time polymerase chain reaction was performed to measure the level of NEBL gene expression and its association with clinico-pathological features.

RESULTS

A significant overexpression with 3 fold increse was seen in NEBL mRNA level in tumoral tissues compared with the adjacent normal tissues. In addition there was a significant association between NEBL gene expression with lymph node metastasis in patients with CRC.

CONCLUSION

The overexpression of NEBL has the capacity to be considred as a prognostic biomarker in patients with CRC.

KEYWORDS:
Nebullete, LASP2, Colorectal cancer, Lymph node metastasis, Prognostic marker

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer related mortalities worldwide.[1] Although many biomarkers have been detected in colorectal cancer, but there is an urgent need to investigate more about novel cancer biomarkers to decipher prognostic biomarkers in the cancer. Prognostic biomarkers are useful in determining the risk of clinical outcomes such as cancer recurrence or disease progression.[2,3]

Nebulin is a protein that plays an important role in cell adhesion and actin filament architecture in the cell.[4] NEBL (nebulette) or LASP2 (LIM and SH3 protein 2) gene is located on chromosome 10p12.31 and transcribed into three mRNA variants and encodes the nebulin like protein, which is abundantly expressed in cardiac muscles; however is detected in many other tissues.[5] The

Table 1: Sequences of NEBL and GAPDH primers. The size of the each primer pairs and the relative PCR amplicons size are presented in the table

Gene		Sequence	Length	Tm	Product
NEBL	F	5'- CACCAAATCTAAGGACCTACCG-3'	22	58.2	176 bp
	R	5'- CTCAATGTAATTCGCTGGGAGC-3'	22	59.7	
GAPDH	F	5'-GCAGGGGGGAGCCAAAAGGGT-3'	21	67.1	219 bp
	R	5'-TGGGTGGCAGTGATGGCATGG-3'	21	65.3	

encoded protein interacts with thin filaments and also interaction of NEBL with vinculin and paxillin may functionally destabilize focal adhesion composition then the overexpression of NEBL gene in metastatic cancer cells increases cell migration.[6] The LIM and SH3 domain protein LASP1 (previously named MLN50) is another member of nebulin proteins, which is more similar to NEBL and is involved in metastasis in some cancers.[7]

There are some studies indicating the role of NEBL as both oncogene and tumor suppressor in cancer. Although NEBL is down-regulated in CRC and suppresses CRC cell growth and migration,[8] it is over-expressed in the NSCLC (Non-Small Cell Lung Cancer) cells and enhances NSCLC invasion and cell migration.[9]

Bacuae of the controversial results on NEBL gene expression in cancer, we aimed to analyze the level of this gene expression in Iranian patients with CRC and highlight its relation with clinico-pathological charecteristics of the tumor in 67 samples of CRC tumors and their adjacent normal tissues.

MATERIALS AND METHODS

CRC and adjacent normal tissues

67 samples of colorectal tumors and their adjacent normal tissues were colected from Iranian patients with CRC who had not recieved chemotherapy referred to Hazrate-Rasoul Hospital, Tehran, Iran during the years 2014-2016. After surgery and resection of tumor, fresh tissues consisted of the tumor and adjacent normal tissue were collected in separate sterile tubes. The cancerous and adjacent normal tissues were confirmed by authorized pathologists. Then the tissue samples were frozen and stored at -70°C. All the patients' clinico-pathological information were obtained from the Pathology Department of Hazrate-Rasoul Hospital.

RNA extraction and cDNA synthesis

RNA was isolated from 100 mg tissue by using Tripure Isolation Reagent (Roche Applied Sciences, Indianapolis, USA). The quality and optical dencity of the RNA samples were checked by nanodrop (ThermoScientific, USA) and agarose gel electrophoresis. cDNAs were synthesized according to the manufacturer's protocol based on the optical density of RNAs (Thermo scientific kit).

Real-time polymerase chain reaction

To evaluate the level of NEBL gene expression, we used GAPDH as internal control gene. Real-time polymerase chain reaction (RT-PCR) was performed by a Rotor gene 6000 system (Corbett Research Pty. Ltd., Australia) with Fast-Start DNA Master SYBR-Green I kit (YTA, Iran). The PCR reaction for all the samples was contained 2000ng/µL cDNA. Samples for no-template were also included in each test to detect any contamination. For calculating the gene expression level Linreg program and Livakmethod were used.[10]

Primers were designed specifically for three mRNA transcribed variants of NEBL gene on the basis of exon–exon junction pattern. The sequences of the primers are shown in table 1.

Statistical analysis

The data were analyzed using the Statistical Package for Social Sciences (SPSS) software version 22. To identify the type of data distribution, K-S test was used. Non-parametric Mann Withney U test was used for comparison of means between the two groups and Kruskal Wallis test was used for comparison of means among more than two groups.

RESULTS

The features of the tumor samples

The features of the patients and tumors were gathered from the Pathology Department of Hazrate-Rasoul Hospital. According to lymph nods status, the tumor samples were categorized in two groups consisted of lymph nodes

Table 2: The characteristics of the patients and tumors that used for NEBL gene analyzing in this study

Tumor features	Groups	Number and percent of the patients	P value
Sex	Male	34 (50.7%)	> 0.05
	Female	33 (49.3%)	
Tumor size (cm)	< 5	24 (35.8%)	> 0.05
	5 - 8	22 (32.8%)	
	8 - 10	14 (20.9%)	
	> 10	7 (10.5%)	
Lymph node status	Positive	32 (47.8%)	0.001*
	Negative	35 (52.2%)	
Tumor stage	I	9 (13.5%)	> 0.05
	II	23 (34.3%)	
	III	23 (34.3%)	
	IV	12 (17.9%)	
Histological grade	Low	26 (38.8%)	> 0.05#
	High	22 (32.8%)	
	UnKnown	19 (28.4%)	

Histological grade comparison was performed between the two groups with low and high grades.

* Only lymph node involvment was associated with NEBL gene expresion (p = 0.001).

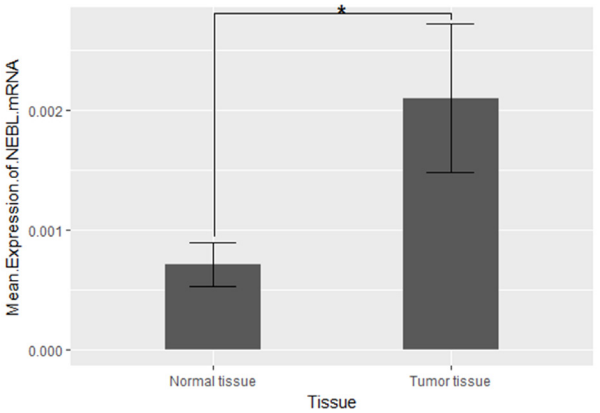

Fig.1: Comparison of NEBL mRNA mean expression level between tumoral samples and adjacent normal tissues show a 3 fold increase in CRC samples compared with the adjacent normal tissues. The relative quantification of NEBL gene expression was evaluated using GAPDH as a house keeping gene

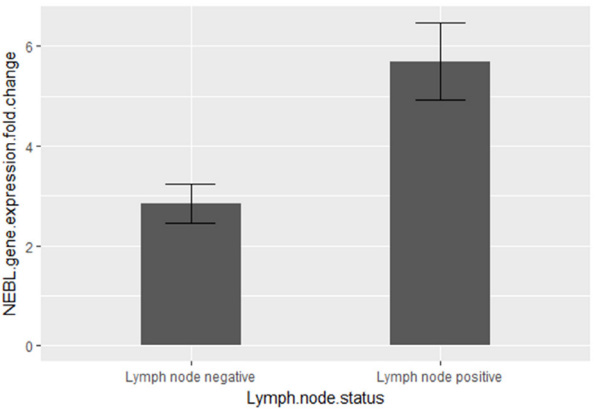

Fig.2: Comparison of NEBL fold change in CRC lymph node status. NEBL gene expression fold change is highly significant (P = 0.001) and increased in CRC lymph node positive compared with lymph node negative patients.

positive and lymph nodes negative. All colorectal tumor stages consisted of stage I, II, III, and IV existed in our patients. Also we categorized the patients' information into two sexes and four groups of tumor sizes. The information of the patients and tumor features are shown in table 2.

NEBL gene expression level

Our results indicated an overexpression in NEBL mRNA expression level in CRC compared with the adjacent normal tissue. We found 3 fold increase in NEBL mRNA expression in tumoral samples compared with the adjacent normal tissues. Figure 1 shows NEBL gene expression in tumor samples and adjacent normal tissue.

Association of NEBL expression with patients' lymph node status

Comparison of NEBL gene expression fold change indicated an association between NEBL gene expression fold change and lymph node status in patients with CRC (p = 0.001). Figure 2 shows a comparison NEBL gene

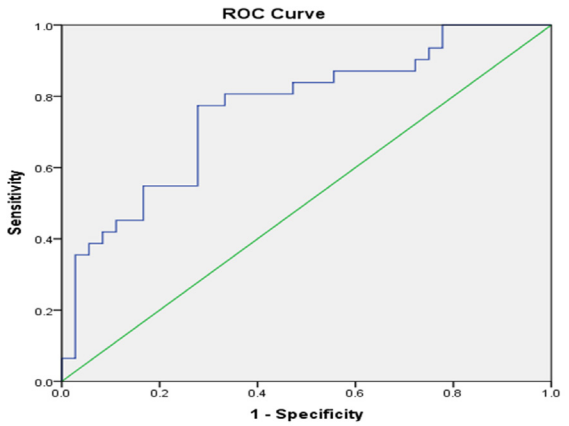

Fig.3: ROC curve for lymph node positive and lymph node negative patients suggest that NEBL gene expression is moderate useful for prognosis of lymph node status in patients with CRC. AUC was calculated as 0.763 (Youden index J = 0.4964, the best cut-off = 2.81, sensitivity = 77.42, specificity = 72.22).

expression fold change between patients with lymph node positive status and those with lymph node negative status. To evaluate NEBL gene expression fold change, each tumor sample was normalized with the adjacent normal tissue.

NEBL potential for lymph node status prognosis

To manifest and highlight NEBL gene as a prognostic marker for patients with CRC and positive lymph node, ROC curve depicted in figure 3 and area under curve (AUC) was calculated as 0.763.

DISCUSSION

Nebulette is a protein that stabilizes actin filament and allows the filament to reach its mature length.[4] NEBL (LASP2) belongs to nebulin protein family and is more closely to LIM and SH3 protein 1 (LASP1), which have two transcriptional genes.[11] LASP1 is a focal adhesion protein, which induce epithelial-mesenchymal transition and contributed in CRC metastasis and also it is more likely that NEBL as a member of the same protein family participate in cancer metastasis as LASP1.[8]

LASP1 was identified from the cDNA library of patients with breast cancer and lymph nodes metastasis. LASP1 gene expression increase in metastatic CRC tissues and is associated with overall survival of patients with CRC.[12] Also LASP1 gene expression is associated with the epithelial-mesenchymal markers in CRC.[13] Additionally LASP1 gene expression is increased in many other cancers such as prostate cancer,[14] gastric cancer,[15] bladder carcinoma,[16] and pancreatic cancer.[17]

NEBL (LASP2) is a protein involved in focal adhesion that increases the attachment of fibroblast cells on fibronectin coated surface.[18] In non-motile cells, NEBL is recruited from the cortical cytoskeleton to focal adhesion at the leading edge of cells.[19]

NEBL is highely expressed in lung cancer compared with non-cancerous tissues. In addition NEBL gene expression is increased in the non-small cell lung cancer cell lines and localized in the cell cytoplasm. NEBL promotes NSCLC migration and invasion. Also expression of the gene is significantly correlated with histological type, TNM staging, lymph node metastasis, and predicted poor survival of patients with NSCLC.[9] NEBL is involved in mixed lineage leukemia (MLL) rearrangements and has an oncogenic role in leukemia.[20]

Although NEBL is highly expressed in SW620, which is a metastatic CRC cell line, it is lowly expressed in SW48, which is the primary tumor cell line. In a study by Wang and colleagues, NEBL gene expression was down-regulated in CRC tissues.[8] In a recently published study the association between NEBL gene expression and survival rate of patients with CRC was shown. Although this gene is over-expressed in CRC, but the over-expression of NEBL was associated with longer overall survival of the patients.[21]

In the presented study we aimed to analyze NEBL mRNA expression in 67 Iranian patients with CRC. Relative quantification of NEBL gene expression was evaluated in CRC tumors and the adjacent normal tissues. We detected 3 fold increases in NEBL mRNA level in CRC tumors compared with the adjacent normal tissues. In addition, we found a significant association between the gene expression with metastasis to lymph nodes. It was shown that NEBL gene over-expression in metastatic cancer cell increases the cell migration because of destabilization of focal adhesion composition.[6]

As shown in figure 2 NEBL fold change is significantly increased in CRC lymph node positive samples. We also depicted ROC curve in figure 3 to illucidate NEBL gene potential as a prognostic marker for patients with CRC and lymph nodes metastasis. AUC was calculated as 0.763, which suggested moderate usefulness of NEBL as

a prognostic marker for patients with lymph nodes metastasis in CRC.

We also found no significant association between NEBL gene expression in patients with CRC and sex, tumor stage, tumor size, and histological grade in our patients. One of the limitations of this study was that only nine pateints were in satge I and 12 patients in satge IV.

CONCLUSION

Our results indicate an increase in NEBL gene expression in CRC tumor and its importance in invasion and metastasis of the cancer to lymph nodes. Therefore, we believe that there is a capability for NEBL gene to be a prognostic biomarker in patients with CRC.

Compliance with Ethical Standards

All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent was obtained from all participants included in the study.

The ethical standard number of this study is IR.NIGEB.EC.11.10.D

ETHICAL APPROVAL

There is nothing to be declared.

CONFLICT OF INTEREST

The authors declare no conflict of interest related to this work.

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