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

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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

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 increase 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 considered as a prognostic biomarker in patients with CRC.

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

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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. 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.

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, it is over-expressed in the NSCLC (Non-Small Cell Lung Cancer) cells and enhances NSCLC invasion and cell migration. Because 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 characteristics of the tumor in 67 samples of CRC tissues and their adjacent normal tissues.

MATERIALS AND METHODS

**CRC and adjacent normal tissues**

67 samples of colorectal tumors and their adjacent normal tissues were collected from Iranian patients with CRC who had not received 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 density 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 1 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 Livak method were used.

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 nodes status, the tumor samples were categorized in two groups consisted of lymph nodes...
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 expression 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.

### 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 involvement was associated with NEBL gene expression ($p = 0.001$).

![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.](image1)

![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.](image2)
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. NEBL (LASP2) belongs to nebulin protein family and is more closely to LIM and SH3 protein 1 (LASP1), which have two transcriptional genes. 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.

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. Also LASP1 gene expression is associated with the epithelial-mesenchymal markers in CRC.

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

NEBL is highly 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. NEBL is involved in mixed lineage leukemia (MLL) rearrangements and has an oncogenic role in leukemia.

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. 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.

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.

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 patients were in stage I and 12 patients in stage 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.

REFERENCES

1. Siegel RL, Miller KD, Fedewa SA, Ahnen DJ, Meester RGS, Barzi A, et al. Colorectal cancer statistics, 2017. CA Cancer J Clin 2017;67:177-93. doi:10.3322/caac.21395.

2. Tanaka T, Tanaka M, Tanaka T, Ishigamori R. Biomarkers for colorectal cancer. Int J Mol Sci 2010;11:3209-25. doi:10.3390/ijms11093209.

3. Lech G, Słotwiński R, Słodkowski M, Krasnodębski J. Colorectal cancer tumour markers and biomarkers: Recent therapeutic advances. World J Gastroenterol 2016;22:1745-55. doi:10.3748/wjg.v22.i5.1745.

4. Pappas CT, Bliss KT, Zieseniss A, Gregorio CC. The Nebulin family: an actin support group. Trends Cell Biol 2011;21:29-37. doi:10.1016/j.tcb.2010.09.005.

5. Sequeira V, Nijenkamp LL, Regan JA, van der Velden J. The physiological role of cardiac cytoskeleton and its alterations in heart failure. Biochim Biophys Acta 2014;1838:700-22. doi:10.1016/j.bbamem.2013.07.011.

6. Bliss KT, Chu M, Jones-Weinert CM, Gregorio CC. Investigating lasp-2 in cell adhesion: new binding partners and roles in motility. Mol Biol Cell 2013;24:995-1006. doi:10.1091/mbc.E12-10-0723.

7. Ort M, Cazes A, Butt E, Grunewald TG. An update on the LIM and SH3 domain protein 1 (LASP1): a versatile structural, signaling, and biomarker protein. Oncotarget 2015;6:26-42. doi:10.18632/oncotarget.3083.

8. Wang B, Zhang L, Zhao L, Zhou R, Ding Y, Li G, et al. LASP2 suppresses colorectal cancer progression through JNK/p38 MAPK pathway mediated epithelial-mesenchymal transition. Cell Commun Signal 2017;15:21. doi: 10.1186/s12964-017-0179-9.

9. Zhang X, Cai L, Zhou H, Liu Y, Fan C, Wang L, et al. Lasp2 enhances tumor invasion via facilitating phosphorylation of FAK and predicts poor overall survival of non-small cell lung cancer patients. Mol Carcinog 2017;56:2558-65. doi: 10.1002/mc.22700.

10. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods 2001;25:402-8. doi: 10.1006/meth.2001.1262.

11. Zieseniss A, Terasaki AG, Gregorio CC. Lasp-2 expression, localization, and ligand interactions: A new Z-disc scaffolding protein. Cell Motil Cytoskeleton 2008;65:59-72. doi:10.1002/cm.20244.

12. Zhao L, Wang H, Liu C, Liu Y, Wang X, Wang S, et al. Promotion of colorectal cancer growth and metastasis by the LIM and SH3 domain protein 1. Gut 2010;59:1226-35. doi:10.1136/gut.2009.202739.

13. Wang H, Shi J, Luo Y, Liao Q, Niu Y, Zhang F, et al. LIM and SH3 Protein 1 Induces TGFβ-Mediated Epithelial–Mesenchymal Transition in Human Colorectal Cancer by Regulating S100A4 Expression. Cancer Res Clin Oncol 2014;20:5835-47. doi:10.1158/1078-0432.CCR-14-0485.

14. Hailer A, Grunewald TG, Ort M, Reiss C, Kneitz B, Spahn M, et al. Loss of tumor suppressor mir-203 mediates overexpression of LIM and SH3 Protein 1 (LASP1) in high-risk prostate cancer thereby increasing cell proliferation and migration. Oncotarget 2014;5:4144-53. doi: 10.18632/oncotarget.1928.

15. Zheng J, Yu S, Qiao Y, Zhang H, Liang S, Wang H, et al. LASP-1 promotes tumor proliferation and metastasis and is an independent unfavorable prognostic factor in gastric cancer. J Cancer Res Clin Oncol 2014;140:1891-9. doi: 10.1007/s00432-014-1759-3.

16. Chiyomaru T, Enokida H, Katarano S, Uchida Y, Kawahara K, et al. Functional role of LASP1 in cell viability and its regulation by microRNAs in bladder cancer. Urol Oncol 2012;30:434-43. doi:10.1016/j.uroon.2010.05.008.
17. Zhao T, Ren H, Li J, Chen J, Zhang H, Xin W, et al. LASP1 is a HIF1α target gene critical for metastasis of pancreatic cancer. *Cancer Res* 2015;75:111-9. doi: 10.1158/0008-5472.CAN-14-2040.

18. Grunewald TG, Butt E. The LIM and SH3 domain protein family: structural proteins or signal transducers or both? *Molecular Cancer* 2008;7:31. doi:10.1186/1476-4598-7-31.

19. Deng XA, Norris A, Panaviene Z, Moncman CL. Ectopic expression of LIM-nebulette (LASP2) reveals roles in cell migration and spreading. *Cell Motil Cytoskeleton* 2008;65:827-40. doi:10.1002/cm.20304.

20. Emerenciano M, Kowarz E, Karl K, de Almeida Lopes B, Scholz B, Bracharz S, et al. Functional analysis of the two reciprocal fusion genes MLL-NEBL and NEBL-MLL reveal their oncogenic potential. *Cancer Lett* 2013;332:30-4. doi:10.1016/j.canlet.2012.12.023.

21. Qiu X, Feng JR, Wang F, Chen PF, Chen XX, Zhou R, et al. Profiles of differentially expressed genes and overexpression of NEBL indicates a positive prognosis in patients with colorectal cancer. *Mol Med Rep* 2018;17:3028-34. doi:10.3892/mmr.2017.8210.