Upregulation of NETO2 gene in colorectal cancer

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From Belyaev Conference
Novosibirsk, Russia. 07-10 August 2017

Abstract

Background: Neuropilin and tolloid-like 2 (NETO2) is a single-pass transmembrane protein that has been shown primarily implicated in neuron-specific processes. Upregulation of NETO2 gene was also detected in several cancer types. In colorectal cancer (CRC), it was associated with tumor progression, invasion, and metastasis, and seems to be involved in epithelial-mesenchymal transition (EMT). However, the mechanism of NETO2 action is still poorly understood.

Results: We have revealed significant increase in the expression of NETO2 gene and deregulation of eight EMT-related genes in CRC. Four of them were upregulated (TWIST1, SNAIL1, LEF1, and FOXA2); the mRNA levels of other genes (FOXA1, BMP2, BMP5, and SMAD7) were decreased. Expression of NETO2 gene was weakly correlated with that of genes involved in the EMT process.

Conclusions: We found considerable NETO2 upregulation, but no significant correlation between the expression of NETO2 and EMT-related genes in CRC. Thus, NETO2 may be involved in CRC progression, but is not directly associated with EMT.

Keywords: Colorectal cancer, NETO2, Epithelial-mesenchymal transition, Gene expression, QPCR

Background

Colorectal cancer (CRC) is the third most common malignancy in developed countries, and furthermore, its incidence rate has continuously increased over the past few decades [1]. While early-stage CRC can be effectively treated with radical surgery, approximately 20% of CRC patients present with advanced-stage disease at the time of initial diagnosis. These patients frequently have metastases that result in increased risk of death even after radical surgery [2]. CRC is characterized by multiple genetic and epigenetic changes that affect metabolic and signaling pathways [3–6]. For instance, cancer cells have a higher glycolytic rate than normal ones [7–9], and, as a consequence, the terminal glycolytic metabolite lactate is exported to the extracellular matrix contributing the extracellular acidosis [10]. The acidic extracellular pH (pHe), in turn, can induce epithelial-mesenchymal transition (EMT) in carcinoma models and is closely associated with tumor metastasis [11, 12]. Thus, in addition to improving the current understanding of the mechanisms underlying CRC metastasis, it is important to identify novel components of EMT process that may be the potential biomarkers of the disease progression and can further contribute to both the selection of optimal treatment options and effective treatment monitoring for patients with CRC.

NETO2 gene is localized on chromosome 16 and encodes a transmembrane glycoprotein of unknown...
function. It has been shown that the abundant expression of NETO2 protein in neurons is essential for proper neurological function [13, 14]. Initially, NETO2 was believed to be a brain-specific protein [15, 16]; however, recent studies described overexpression of NETO2 in several types of cancer, including renal, lung, colon, and cervical carcinomas [17]. Accordingly, Hu et al. recently suggested high expression of NETO2 as a potential biomarker of both advanced tumor progression and poor prognosis in patients with CRC [18].

In the present study, we hypothesized that the association of NETO2 overexpression with tumor progression, invasion, and metastasis may be indicative of its involvement in the epithelial-mesenchymal transition in CRC. To investigate the validity of this hypothesis, we evaluated whether NETO2 expression was correlated with that of genes established to mediate the EMT process.

Methods

Tissue samples
A total of 44 CRC and matched morphologically normal tissue samples, which were obtained after surgical resection, but prior to patient treatment with radiation and/or chemotherapy, were frozen and stored in liquid nitrogen until use. All CRC samples were classified according to the American Joint Committee on Cancer (AJCC) staging system [19], and only those samples comprising 70% or more tumor cells were selected for analysis. Written informed consent was obtained from all patients for participation in the present study, which was approved by Herzen Moscow Cancer Research Institute - branch of National Medical Research Radiological Center, Ministry of Health of Russia Federation (Moscow, Russia), and conducted in strict accordance with the principles outlined in the Declaration of Helsinki (1964). Clinicopathologic characteristics of the CRC patients are shown in Table 1.

RNA isolation and cDNA synthesis
Total RNA was isolated from the frozen tissue samples using RNeasy Mini kit (Qiagen, Germany) according to manufacturer’s instructions. RNA quality was measured via the RNA Integrity Number (RIN) method using an Agilent RNA Bioanalyzer 2100 (Agilent Technologies, USA). RNA quantification was performed using a NanoDrop 1000 instrument (NanoDrop Technologies, USA). cDNA was synthesized from the isolated RNA using M-MLV Reverse Transcriptase (Thermo Fisher Scientific, USA) and random hexamers.

Quantitative PCR (qPCR)
Quantitative polymerase chain reaction was performed using TaqMan Assay (Thermo Fisher Scientific) primers and probes for target genes (NETO2: Hs00983152_m1, TWIST1: Hs00361186_m1, SNAIL1: Hs00195591_m1, SNAIL2: Hs0161904_m1, ZEB1: Hs01566408_m1, ZEB2: Hs00207691_m1, LEF1: Hs01547250_m1, FOXA2: Hs00232764_m1, FOXA1: Hs04187555_m1, CDH1: Hs01023895_m1, STAT1: Hs00374280_m1, BMP2: Hs00154192_m1, BMP5: Hs00234930_m1, VIM: Hs00958111_m1, SMAD2: Hs00998187_m1, SMAD3: Hs00969210_m1, SMAD4: Hs00929647_m1, SMAD7: Hs00998193_m1). Primers and probes for reference genes, GUSB and RPN1, were previously described [20, 21]. All qPCRs were carried out in triplicate in total reaction volume of 20 μL using an AB 7500 Real-Time PCR System (Thermo Fisher Scientific) to achieve cycling conditions comprising 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 60 s, and 72 °C for 30 s.

QPCR data were analyzed using Relative Quantitation (Thermo Fisher Scientific) software and ATG program taking into account the efficiency of the PCR amplification [22, 23]. The expression levels of target genes were normalized to those of the reference genes. Finally, relative (T/N) expression level of target genes was calculated using the ΔΔCt method [24]. Since the relative inner variability between the calculated mRNA levels of the reference genes was found to be less than two-fold, a variation in the expression of the target genes of two-fold or greater was considered to be significant.

Statistical analysis
Inter- and intra-group comparisons were performed using non-parametric Wilcoxon/Mann-Whitney and Kruskal-Wallis tests. Spearman’s rank correlation coefficient (r_s) was used for revealing correlations between

| Characteristic          | Total, n |
|------------------------|----------|
| Gender                 |          |
| Male                   | 23       |
| Female                 | 21       |
| Age (years)            |          |
| ≤ 60                   | 14       |
| > 60                   | 30       |
| Clinical stage         |          |
| I                      | 2        |
| II                     | 11       |
| III                    | 15       |
| IV                     | 16       |
| Distant metastases (Stage IV) | |
NETO2 and EMT-related gene expression. All statistical analyses were performed using PASW Statistics 18 (SPSS Inc., USA) software. A p-value < 0.05 was considered to indicate statistical significance.

Results

Upregulation of NETO2 gene in CRC
QPCR analysis of the relative NETO2 mRNA level across the 44 CRC samples revealed that NETO2 expression was increased by a factor of 2–50 in 41% (18/44) of cases (Fig. 1). In contrast, NETO2 expression was decreased by a factor of 2–25 in 14% (6/44) of CRC samples. These results demonstrating a significant increase in the expression of NETO2 in the analyzed CRC samples are consistent with those of the previous study by Oparina et al. [17].

Deregulation of EMT-related genes in CRC
We performed an analysis of the relevant literature and selected 17 genes related to EMT process in CRC (Table 2). Using qPCR, mRNA levels of these genes were analyzed in 44 CRC samples (Table 3).

TWIST1 gene
Up to 26-fold increase in the expression of TWIST1 gene was revealed in the majority (68%, 30/44) of CRC samples compared to matched normal tissues. In contrast, two CRC samples exhibited decreased TWIST1 expression from 4- to 6-fold. The mean value of relative mRNA level of TWIST1 gene was 2.8.

SNAIL1 and SNAIL2 genes
Quantitative analysis of SNAIL1 expression showed it to be significantly increased in 80% (35/44) of CRC cases. mRNA level of SNAIL1 gene was decreased by a factor of 6 only in one sample. The expression of SNAIL2 was found to be decreased by a factor of 2–25 in 20% (9/44) of CRC samples, and increased by a factor of 2–3 in 11% (5/44) of ones. The mean value of relative mRNA levels of SNAIL1 and SNAIL2 genes were 3.3 and 1.2, respectively.

ZEB1 and ZEB2 genes
Analysis of ZEB1 gene expression revealed it to be decreased by a factor of 2–48 in 36% (16/44) and increased in 9% (4/44) of CRC samples. The expression of ZEB2 gene was decreased in 45% (19/44) and increased in 7% (3/44) of CRC cases. The mean value of relative mRNA levels of ZEB1 and ZEB2 genes were 1.5 and 1.7, respectively.

LEF1 gene
LEF1 gene expression was increased by a factor of 2–52 in 75% (33/44) of CRC cases, and slightly decreased by a factor of two only in one sample. The mean value of relative mRNA level of LEF1 gene was 3.9.

FOXA1 and FOXA2 genes
The analysis of FOXA1 and FOXA2 gene expression showed that while FOXA1 expression was decreased by a factor of 2–79 in 52% (23/44) of CRC samples, FOXA2 expression was increased from 2- to 23-fold in 59% (26/44) of cases. Up to 4-fold increase in the expression of FOXA1 gene was detected in 75% (33/44) of examined samples. FOXA2 gene was downregulated by a factor of 2–70 in 5% (2/44) of CRC cases. The mean value of relative mRNA levels of FOXA1 and FOXA2 genes were 2.1 and 2.5, respectively.

CDH1 gene
The analysis of CDH1 gene expression showed it to be decreased by a factor of 2–86 in 16% (7/44) of CRC samples, and increased by a factor of two in 5% (2/44) of cases. The mean value of relative mRNA level of CDH1 gene was 1.3.

STAT1 gene
Quantification of STAT1 gene expression revealed it to be increased by a factor of 2–4 in 25% (11/44) of cases, and decreased by a factor of 3 in 5% (2/44) of CRC samples. The mean value of relative mRNA level of STAT1 gene was 1.4.
**Table 2** Genes involved in the epithelial-mesenchymal transition in CRC

| Gene   | Description                                                                 | References |
|--------|------------------------------------------------------------------------------|------------|
| TWIST1 | TWIST1 is a highly conserved basic helix-loop-helix (bHLH) transcription factor that regulates the EMT required for neural crest migration during vertebrate embryonic development. TWIST1 expression is positively associated with patient survival after curative CRC resection, and thus is a promising candidate biomarker of the disease progression. | (33)       |
| SNAIL1 | SNAIL1 is a transcriptional regulator of E-cadherin, which suppression is critical to facilitate the EMT process. SNAIL1 mRNA level is not detectable in the normal colon mucosa, but is upregulated in 60–70% of CRC. Importantly, aberrant SNAIL1 expression in CRC has been shown to be associated not only with poor patient prognosis, but also with a reduced relapse-free survival time. | (36)       |
| SNAIL2 | SNAIL2 has been implicated as an anti-apoptotic factor, and is thought to mediate the EMT process by repressing E-cadherin transcription. Accordingly, SNAIL2 expression in human CRC cell lines has been shown to be correlated with critical EMT properties, including the loss of E-cadherin expression and an increase in both cell migration and invasion. | (40)       |
| ZEB1   | ZEB1 mediates the EMT pathway, and in fact has been shown to be not only sufficient to induce the EMT, but also necessary for maintaining the adapted mesenchymal phenotype. ZEB1 contains zinc finger clusters in both its N- and C-terminal regions, and a homeodomain in the central region. In CRC cells, ZEB1 has been shown to critically mediate the EMT, and thus may be an important regulator of CRC metastasis. | (43)       |
| ZEB2   | ZEB2 is a member of the Zfh1 family of two-handed zinc-finger transcription factors. It is frequently expressed in colon cancer, and has been shown by several previous studies to induce the EMT, and to facilitate cancer-cell metastasis, possibly via the repression and upregulation of E-cadherin and vimentin respectively. | (46)       |
| LEF1   | LEF1 is critical for tumor-cell adhesion and/or migration, and thus, also for tumor invasion and metastasis. In addition, it plays a pivotal role in carcinogenesis and CRC progression, partly via its function in the LEF1/B-catenin complex, which is a crucial effector of the Wnt signaling pathway. Increased LEF1 expression has been shown to be correlated with node and distant metastasis, and with an advanced tumor stage. Furthermore, LEF1 was shown to be involved in CRC invasion and metastasis. | (49)       |
| FOXA1 and FOXA2 | Forkhead box (FOX) protein A1 (FOXA1) is a transcription factor belonging to the FOX gene superfamily that mediates fundamental developmental and differentiation processes. Specifically, it modulates transcriptional programs in a tissue-dependent manner by inducing nucleosomal rearrangement, and by alterting chromatin accessibility to the transcriptional machinery. FOXA1 has been shown to be overexpressed in CRC, and furthermore, to be positively associated with poor clinicopathological features. This suggests that its expression may be a promising candidate prognostic biomarker for patients with CRC. FOXA2 is a known key regulator of CRC metastasis to the liver. | (52)       |
| CDH1   | CDH1 gene encodes a classical cadherin. The E-cadherin-mediated cell adhesion system is required for both the EMT, and for cellular invasion, angiogenesis, and metastatic/tumor progression in many cancers, including CRC. | (56)       |
| STAT1  | STAT1 is a signal mediator that controls cell-death functions in the context of both pro-apoptotic and anti-proliferative interferon-dependent signaling. It appears to exhibit tumor suppressive functions, and its activity has been shown to be associated with a favorable patient prognosis in some cancers. | (57)       |
| BMP2 and BMP5 | Bone morphogenetic proteins (BMPs) are the secreted ligands of the proteins belonging to the transforming growth factor beta superfamily (TGFβ), and are important regulators of body-axis patterning during embryogenesis. In adult tissues, they regulate cell growth, apoptosis, and differentiation. The biological effects of BMPs have been predominantly studied in mesoderm-derived cells and tissues, and to a lesser degree, in epithelial cells and tissues. In general, BMPs are involved in the regulation of cancer progression and metastasis possibly through TGF-β-induced SMAD3-dependent EMT. Inactivation of BMP signaling increases the tumorigenicity of normal colon stem cells. | (59)       |
| VIM    | VIM is a Wnt-targeted gene that is expressed in normal mesenchymal cells, and that encodes the intermediate filament protein, vimentin. Previous studies have shown that vimentin mediates both cellular structure and integrity. Furthermore, vimentin has also been demonstrated to mediate cell shape and motility during the EMT process, which is required for cancer-cell metastasis. | (62)       |
| SMAD2, SMAD3, SMAD4, and SMAD7 | The SMADs are a family of structurally related signaling proteins that can be divided into three subgroups according to their respective functions in TGFβ signaling. Specifically, the receptor-activated SMADs, including SMAD2 and SMAD3, are serine-phosphorylated following TGF-receptor complex formation. The unique SMAD4 co-SMAD (which is common to both TGFβ and BMP signaling), then interacts with the phosphorylated SMAD2/SMAD3. The resulting heteropolymer migrates to the nucleus and complexes with tissue-specific transcription factors, thereby inducing the transcription of TGFβ target genes, including SMAD7. Finally, SMAD7, which is the only TGFβ-specific anti-SMAD, prevents SMAD2/5 activation, thereby providing a transient TGFβ response in the form of a negative feedback loop. Immunohistochemical analysis has revealed the expression of SMADs during EMT process in CRC. | (66)       |

**BMP2 and BMP5 genes**

The expression of both BMP2 and BMP5 genes was suppressed in 75% (33/44) and 84% (37/44) of examined CRC samples, respectively. Increase in the BMP2 gene expression was shown in only one sample (2%), while that of BMP5 gene was detected in 7% (3/44) of CRC cases. The mean value of relative mRNA levels of BMP2 and BMP5 genes were 3.2 and 7.6, respectively.
The analysis of VIM expression showed it to be increased by a factor of 2–6 in 18% (8/44) of CRC samples, and decreased by a factor of 2–4 in 7% (3/44) of cases. The mean value of relative mRNA level of VIM gene was 1.3.

SMAD2, SMAD3, SMAD4, and SMAD7 genes
QPCR analysis showed SMAD2, SMAD3, SMAD4, and SMAD7 mRNA levels to be decreased by a factor of 2–10 in 11–43% of the examined CRC samples. The mean value of relative mRNA levels of SMAD2, SMAD3, SMAD4, and SMAD7 genes were 1.4, 1.2, 1.5, and 1.8, respectively.

Table 3

| Gene | Frequency of mRNA level changes, % | Median of mRNA level changes, n-fold |
|------|-----------------------------------|-------------------------------------|
| TWIST1 | † increased expression | 68 (30/44) 5 (2/44) 2.8 † | |
| SNAIL1 | † increased expression | 80 (35/44) 2 (1/44) 3.3 † | |
| SNAIL2 | † decreased expression | 11 (5/44) 20 (9/44) 1.2 ‡ | |
| ZEB1 | † decreased expression | 9 (4/44) 36 (16/44) 1.5 ‡ | |
| ZEB2 | † increased expression | 7 (3/44) 45 (20/44) 1.7 † | |
| LEF1 | † decreased expression | 75 (33/44) 2 (1/44) 3.9 ‡ | |
| FOXA1 | † increased expression | 7 (3/44) 52 (23/44) 2.1 ‡ | |
| FOXA2 | † increased expression | 59 (26/44) 5 (2/44) 2.5 † | |
| CDH1 | † increased expression | 5 (2/44) 16 (7/44) 1.3 ‡ | |
| STAT1 | † increased expression | 25 (11/44) 5 (2/44) 1.4 † | |
| BMP2 | † increased expression | 2 (1/44) 75 (33/44) 3.2 ‡ | |
| BMP5 | † increased expression | 7 (3/44) 84 (37/44) 7.6 ‡ | |
| VIM | † increased expression | 18 (8/44) 7 (3/44) 1.3 † | |
| SMAD0 | † increased expression | 0 (0/44) 11 (5/44) 1.4 † | |
| SMAD3 | † increased expression | 0 (0/44) 11 (5/44) 1.2 † | |
| SMAD4 | † increased expression | 0 (0/44) 23 (10/44) 1.5 † | |
| SMAD7 | † increased expression | 0 (0/44) 43 (19/44) 1.8 † | |

Note: Significant frequencies (p < 0.05) are marked in bold

mRNA level of NETO2 is not correlated with that of EMT-related genes in CRC
We used the Spearman’s correlation coefficient to test the proposed hypothesis that NETO2 mRNA level in CRC correlates with that of the EMT-related genes. The results of this analysis showed that across the 44 analyzed CRC samples, 17 association pairs were identified between NETO2 and various genes involved in EMT, all of which exhibited weak relationship (Table 4). The most significant correlations were determined between NETO2 and SMAD7 expression ($r_s = 0.25$, $p < 0.05$) and between NETO2 and TWIST1 expression ($r_s = -0.24$, $p < 0.05$). These results indicate that the expression of NETO2 in CRC is only weakly correlated with that of EMT-related genes.

Discussion
The NETO2 gene encodes a transmembrane protein that is predominantly expressed in normal brain and retinal tissues. Thus, previous studies have primarily focused on NETO2 function in the context of neurobiology; in vitro analyses have revealed that NETO2 interacts with the GluK2 and GluK5 subunits of kainate receptors to significantly enhance kainate receptor-mediated signaling [25]. Recently, NETO2 has been shown to be involved in carcinogenesis. In a mutant cell line overexpressing metastasis-suppressor gene NM23-H1, which can reduce the metastatic potential of various types of cancer cells, NETO2 was amongst the nine genes identified to exhibit increased mRNA level [26]. NETO2 expression was reported to be upregulated in proliferating pediatric hemangiomas [27]. Notably, we previously demonstrated that NETO2 mRNA level is frequently overexpressed in kidney and lung cancers, and resultantly suggested it as a potential marker to early diagnosis of these diseases [17]. Hu and co-authors suggested both the potential significance of NETO2 expression in CRC carcinogenesis and its clinical relevance to the disease progression, invasion, and metastasis [18].

The EMT process is well established to be required not only for embryonic development, but also for cancer

Table 4

| Gene | Spearman’s correlation coefficient, $r_s$ |
|------|-------------------------------------|
| TWIST1 | -0.24 |
| SNAIL1 | -0.12 |
| SNAIL2 | -0.07 |
| ZEB1 | -0.05 |
| ZEB2 | 0.03 |
| LEF1 | 0.06 |
| FOXA1 | 0.06 |
| FOXA2 | 0.06 |
| CDH1 | 0.11 |
| STAT1 | 0.12 |
| BMP2 | 0.12 |
| BMP5 | 0.14 |
| VIM | 0.18 |
| SMAD0 | 0.21 |
| SMAD3 | 0.23 |
| SMAD4 | 0.24 |
| SMAD7 | 0.25 |
progression and metastasis, since it facilitates the acquisition of invasive properties that allow cancer cells to enter the surrounding stroma and thereby generate a favorable tumor microenvironment [28–30]. Moreover, EMT process is known to be closely associated with cancer recurrence and chemoresistance. Nevertheless, the mechanisms underlying the involvement of EMT process in these events seem to vary significantly between cancer types.

To date, NETO2 is known to be associated with poor prognosis and metastasis in CRC, but not with the occurrence of EMT in this context. Thus, the present study investigated whether NETO2 expression in CRC was correlated with that of key genes involved in the EMT, including TWIST1, SNAI1, SNAI2, ZEB1, ZEB2, LEF1, FOXA2, FOXA1, CDH1, STAT1, BMP2, BMP5, VIM, SMAD2, SMAD3, SMAD4, and SMAD7. The results obtained in the work confirmed that NETO2 is overexpressed in CRC. It has also been demonstrated that several genes involved in the EMT process were upregulated in CRC compared to matched normal tissues, including TWIST1, SNAI1, LEF1, and FOXA2, which mRNA levels were increased by an average factor of 2.8, 3.3, 3.9, and 2.5 (median) respectively. Conversely, the mRNA levels of FOXA1, BMP2, BMP5, and SMAD7 genes were found to be decreased by a factor of 2.1, 3.2, 7.6, and 1.8 (median) respectively that is again in accordance with the results of recently studies [31, 32].

Notably, we found no significant correlation between the expression of NETO2 gene and that of the analyzed EMT-related genes in CRC. Thus, it is likely that NETO2 is involved in CRC progression, but is not directly associated with EMT.

Conclusions
NETO2 expression was found to be considerably increased, but not significantly correlated with the mRNA levels of EMT-related genes in CRC. Thus, while NETO2 overexpression may be indicative of poor clinical prognosis and metastasis, this is unlikely to be a direct result of alterations in the EMT process. Certainly, the molecular basis for and biological relevance of NETO2 upregulation in CRC requires further investigation.

Acknowledgments
Authors thank National Medical Research Center of Radiology for supplying tumor samples and clinicopathologic data; Vavilov Institute of General Genetics for the help with data analysis; Initium-Pharm, LTD for providing computational resources. This work was performed using the equipment of EIMB RAS “Genome” center (http://www.eimb.ru/rus/clp/ccu_genome_c.php).

Funding
This work and publication costs were funded by the Russian Science Foundation, grant 14–15-01083.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

About this supplement
This article has been published as part of BMC Genetics Volume 18 Supplement 1, 2017: Selected articles from Belyaev Conference 2017: genetics. The full contents of the supplement are available online at https://bmcgenet.biomedcentral.com/articles/supplements/volume-18-supplement-1.

Authors’ contributions
MSF, AVS, and AVK conceived and designed the work; MSF, AVS, ISA, AVL, SLK, AFS, KVN, MMb, ENS, MAC, and DVS performed the experiments; MSF, AVS, EAP, KMK, NVM, AAD, AVK, and MVK analyzed the data; MSF, EAP, AAD, AVK, ADK, and BYA wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The study was approved by The Ethics committee of Herzen Moscow Cancer Research Institute - branch of National Medical Research Radiological Center, Ministry of Health of Russia Federation. The study was done in accordance with the principles outlined in the Declaration of Helsinki (1964).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Published: 28 December 2017

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