RESEARCH ARTICLE

Diagnostic Relevance of Overexpressed Serine Threonine Tyrosine Kinase/Novel Oncogene with Kinase Domain (STYK1/NOK) mRNA in Colorectal Cancer

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Abstract

Background: Alterations in gene expression levels or mutations of tyrosine kinases are detected in some human cancers. In this study, we examined whether serine threonine tyrosine kinase 1 (STYK1)/novel oncogene with kinase domain (NOK) is overexpressed in patients with colorectal cancer. We also examined the clinical relevance of STYK1/NOK expression in cancer tissues. Materials and Methods: In tumor samples of patients with colorectal cancer and their matched non-cancerous samples, STYK1/NOK messenger RNA (mRNA) expression was analyzed by quantitative reverse transcriptase polymerase chain reaction. Associations between the expression levels of STYK1/NOK and clinicopathological characteristics of colorectal cancer were also assessed using Mann-Whitney U and Kruskal-Wallis tests. Results: Upregulation of STYK1/NOK was found in cancer tissues even at early stage of colorectal cancer compared to normal adjacent tissues. The optimal cutoff point of 0.198 the STYK1/NOK expression showed 0.78 sensitivity and 0.75 specificity for diagnosis. Overexpressed STYK1/NOK was correlated with tumor size but had no association with other clinicopathological characteristics of colorectal cancer. Conclusions: These results indicate that STYK1/NOK mRNA is widely expressed in the patients with colorectal cancer and suggest that inhibition of this molecule could potentially serve as a novel therapeutic target.

Keywords: Colorectal cancer - oncogene - tyrosine kinase - NOK/STYK1 - diagnosis

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Introduction

Colorectal cancer (CRC) is the fourth most common cancer in the world and the most common form of colorectal cancer presentation is primary adenocarcinoma with non-urgent symptoms with non-urgent symptoms (Safaee et al., 2010, Siegel et al., 2013). Given the effectiveness of early diagnosis and treatment in colorectal cancer and the performance gaps of current non-invasive colorectal cancer markers, the development of novel biomarkers could result in improved patient outcomes for colorectal cancer (Barrett et al., 2006, Shemirani et al., 2011). Tyrosine kinases are of the former class that their abnormalities cause them to stuck in "on" position and contribute to the uncontrolled proliferation and anti-cancer drug resistance of a vast spectrum of human cancer cells (Hubbard and Till, 2000). In the light of In the light of these evidences and reports, kinase inhibition is of the considerable interest in recent research work (Zwick et al., 2002). Despite the gradual progress in colorectal cancer prognosis, the detection of cancer in early stages remains a relatively open research field (Samanian et al., 2011, Barouni et al., 2012, Karimi et al., 2013). To increase the efficiency of cancer management programs, it is of urgent importance to identify candidate genes that their unique signature may hold promise as a novel diagnostic tool for detecting tumors at an early stage of colorectal cancer, particularly adenocarcinomas.

Receptor protein tyrosine kinases (RPTKs) are currently well-recognized for their pivotal roles in diverse cellular processes (Blume-Jensen and Hunter, 2001). Their dysregulation has been reported to result in an abnormal kinase activity and malignant transformation (Shepard et al., 2008). The structure of these subfamilies consists of a diverse ligand-specific ectodomain, a single trans-membrane domain and an intracellular tyrosine kinase domain (Fambrough et al., 1999).

Serine Threonine Tyrosine kinase (STYK1)/ Novel oncogene kinase (NOK) is a newly identified member of RPTK-like protein family that lacks extracellular domain enabling it to act in a non-specific ligand manner (Liu et al., 2004). It is reported to be an important factor in tumorigenesis and metastasis in nude mice, and it is and it is proved to lead growth factor-independent cell

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proliferation among murine, bone-marrow-derived, lymphatic BaF3 cells and surface-adhesion-independent growth and colony forming in NIH3T3 and BaF3 cells (Chen et al., 2005 , Li et al., 2009, Ding et al., 2012). Furthermore, NOK/STYK1 oncogenic potential in breast, lung, prostate and ovarian cancers has been reported (Amachika et al., 2007, Kimbro et al., 2008, Jackson et al., 2009). It is therefore an intriguing possibility that STYK1/NOK serves as a critical molecule in colorectal tumorigenesis and is thus of immediate interest as a candidate in the colorectal cancer prognosis and treatment. In this paper, we hypnotized that over-expression of STYK1/NOK may be an inevitable circumstance in cancer cells. However, expression level of this gene has not been yet quantified in colorectal cancer cells and the previous observations raised the question that to what extent the CRC tissue samples express STYK1/NOK compared to normal tissue samples.

In line with previous studies, we quantitatively analyzed the expression levels of STYK1/NOK in CRC tissues relative to their non-tumor counterparts. Moreover, the potential relationship between STYK1/NOK levels and clinicopathological and prognostic outcomes in CRC patients has been investigated.

Materials and Methods

Patients and Tissue samples

In this prospective study, a total of 40 CRC samples and normal adjacent tissues were collected following colonoscopy and sigmoidoscopy at Imam Reza Hospital (Tabriz, Iran), the first affiliated hospital of Tabriz University of Medical Sciences. The non-tumor counterparts were obtained from a section of the resected specimen at the farthest distance from tumor (>2cm from tumor). All study participants were Iranian-born individuals. The study was approved by the Research Ethics Committee of Imam Reza Hospital in accordance with institutional protocol and informed consents were obtained from all patients. Resected specimens were routinely processed for histopathological assessment. The clinicopathological factors and histological grades of cancer were classified using the TNM staging system of the American Joint Committee on Cancer (AJCC; 2010) and International Union Against Cancer (UICC), according to the standard of the World Health Organization (WHO). A number of cases were excluded from subsequent statistical analysis for the following reasons: the patients had a previous or secondary malignancy and/ or had undergone chemotherapy, radiation therapy or immunotherapy, the samples had histology other than adenocarcinomas.

Sample preparation and RNA isolation

All the tissue samples were immediately flash frozen in liquid nitrogen and stored at -80°C until RNA extraction. We applied phenol based total RNA extraction using TRIzol reagent (Takara) according to the manufacturer's instructions with some small changes. Briefly, tissue samples were homogenized quickly along with liquid nitrogen and then suspended in 1 ml TRIzol reagent and incubated at room temperature for 15 min. By adding 300µl chloroform (MERK) and then centrifugation at 12,000×g for 15 min at 4°C nucleic acids were recovered from the lysate. The aqueous phase was carefully transferred into another microcentrifuge tube. Isopropanol was used to precipitate nucleic acid and stored at 20°C overnight followed by a 13,000×g centrifuge for 10 min at 4°C, pelleting the nucleic acid. The pellet was washed with 75% ethanol, air dried and at the end RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water. The RNA concentration and purity were measured with a NanoDrop ND-1000 Spectrophotometer. The integrity of the RNAs was checked using agarose gel electrophoresis stained with an intercalating dye. The extracted RNAs were stored at -80°C until cDNA synthesis. To degrade any DNA contamination in extracted RNAs we performed a 10µl DNase I treatment reaction which contained 1µg of total RNA, 1µl 10X reaction buffer, Iµl of 1U/µl of DNase I (Takara) and DEPC-treated water and was incubated at 37°C for 15 min. To heat inactive the DNase I, 1µl of 25 nM Ethylenediaminetetraacetic acid (EDTA) was added to the previous mixture and (was) firstly incubated at 65 C for 15 min and then was replaced on ice for 1 min with the Eppendorf 5331 thermal cycler.

Reverse transcription reaction

The reverse transcription to synthetize cDNA was conducted using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) and was divided into two parts. First reaction was a 12µl mixture containing 8µl of total RNA, was procured from the DNase I treatment reaction, 1µl oligo(dt) primer, DEPC-treated water, and was incubated at 65°C for 5 min in thermal cycler. The second one was a reverse transcription reaction added to the previous nuclease-free tube of fist reaction and contained 4µl 5X reaction buffer, 1µl RiboLock RNase Inhibitor(20u/µl), 2µl of 10nM dNTP Mix, 1µl RevertAid M-MulV Reverse Transcriptase(200u/µl) and DEPC-treated water. The total 20µl mixture was firstly incubated at 37°C for 15 min, followed by incubation at 85°C for 5 min for reverse transcriptase deactivation. The first stranded synthetized tube was restored at -20°C until following procedure.

Real-time polymerase chain reaction

STYK1/NOK mRNA expression was determined by real time polymerase chain reaction by using a Power SYBR Green (Takara) with an iCycler iQ™ (Biorad). Results were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The gene-specific primers for STYK1/NOK and GAPDH were as follow:

STYK1/NOK forward primer, 5'-TCTCGGGA AATCTGAAATGATGC-3'; reverse primer: 5'-GGACT GGGACAGCAGAAGTG-3';

GAPDH forward primer, 5'-GTGAACCATG AGAAGTATGACAAC-3'; Reverse primer: 5'-CATGAGTCCTTCCACGATACC-3'.

The 20µl mixture of PCR consisted of 10µl SYBR Green suppermix, DEPC-treated water, 0.5µl forward primer, 0.5µl reverse primer and 1-3µl reverse transcribed product.

The cycling program involved preliminary denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s and elongation at 72°C for 30 s, followed by a final elongation step at 60°C for 10 min. All samples were processed in triplicate. The threshold cycle (CT) was defined as the cycle number at which the fluorescence passed the fixed threshold. A control without a template was included in each experiment. The final products of real-time PCR were confirmed by Polyacrylamide gel electrophoresis (PAGE).

Statistical analysis

The expression levels of STYK1/NOK in CRC tissues relative to matched non-tumor counterparts were analyzed using the $2^{-\Delta\Delta Ct}$ method. Briefly, the threshold cycle (Ct) of fluorescence for each sample was determined. ΔCt indicated the difference in expression levels with the Ct value between STYK1/NOK and GAPDH ($\Delta Ct=Ct_{STYK1/NOK} - Ct_{GAPDH}$), and ΔΔCt indicated the difference in the ΔCt value between cancer tissue and the matched control ($\Delta\Delta Ct=\Delta Ct_{cancer} - \Delta Ct_{control}$). The $2^{-\Delta\Delta Ct}$ value (fold value) was also calculated. When the fold value was >1, there was a high expression of STYK1/NOK in the cancer tissues compared to their non tumorous counterparts. The statistical differences in STYK1/NOK expression in cancer tissues relative to the matched adjacent non tumor tissues were analyzed by a paired t-test. Moreover, the association between STYK1/NOK expression and clinicopathological parameters was analyzed by a non parametric test (Mann Whitney U test between 2 groups and Kruskal Wallis H test for ≥3 groups). Statistical analysis was performed using the GraphPad Prism 6. Moreover, Receiver operating characteristic (ROC) curves were constructed to evaluate the specificity and sensitivity of predicting CRC and normal tissue by STYK1/NOK and the sensitivity/specificity at various cutoff values was calculated using SigmaPlot.

Results

Expression of STYK1/NOK in CRC tissues.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis of STYK1/NOK was performed in 36 pairs of CRC tissues and matched adjacent non-tumor counterparts. The results showed that STYK/NOK expression levels were significantly elevated in CRC tissues compared to normal matches. The value of ΔCt (mean±SD) was 0.037±0.030 in CRC tissues and 0.013±0.011 in their matching adjacent non-tumor tissues. The mean difference and the range of SD was 0.024±0.0051 (p value<0.01, paired t-test; Figure 1).

Capability of STYK1/NOK to function as a CRC tumor marker. Receiver operating characteristic (ROC) curves was constructed and the area under the curve (AROC) was calculated to evaluate the specificity and sensitivity of predicting CRC and normal tissue by STYK1/NOK expression levels. Based on the analysis of ROC curved STYK1/NOK mRNA showed a ROC area (AROC) of 0.79. (Figure. 2; p<0.001) The plot shows sensitivity and specificity at different cut-off points. To calculate the optimal cut-off value we performed a post-test from pre-test probability of 0.5 and cost ration of 1.00. The optimal cut-off point was 0.198 with 0.78 and 0.75 Sensitivity and specificity respectively.

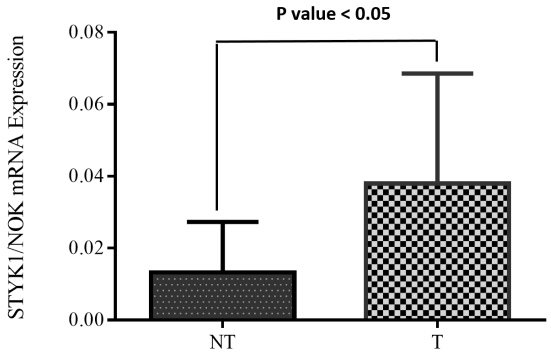

Figure 1. Expression of STYK1/NOK mRNA in Cancerous and Adjacent Non-Cancerous Tissues from CRC Patients. Expression of NOK mRNA was measured using quantitative RT-PCR and is reported relative to the amount of GAPDH mRNA. STYK1/NOK was differentially expressed between CRC tissues (T) and matched non-tumor adjacent tissues (NT). STYK1/NOK was significantly upregulated in CRC tissues compared to the matching adjacent non-tumor tissues (P<0.05; paired t-test)

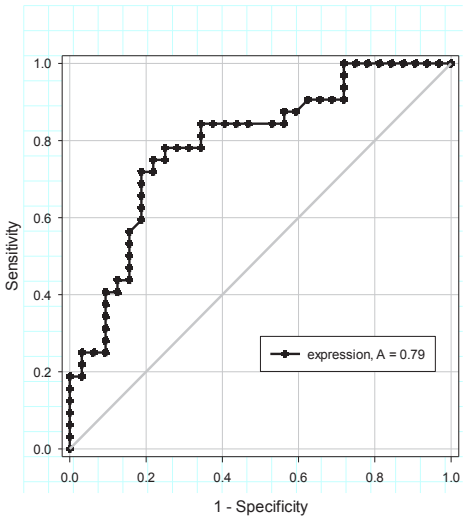

Figure 2. Receiver Operating Characteristic (ROC) Curve in Detection of CRC. The curve was automatically generated from 62 points of cutoff values set by the software SigmaPlot. The area under the ROC curve (AROC) is 0.79 out of 1

Correlation between STYK1/NOK expression levels and clinical staging in CRC patients. The relationship between STYK1/NOK expression levels and clinical stages determined by tumor size, invasion and metastasis to lymph nodes and distant organs was pursued in CRC samples. The results indicated that STYK1/NOK mRNA expression was elevated even at early stages of CRC and furthermore the median mean STYK1/NOK mRNA expression in stage I was 0.0375± 0.0356 compared with 0.0381± 0.0244 in other stages included II-IV (p value >0.05) and all was shown in Figure 3.

Association between STYK1/NOK expression levels and other clinicopathological characteristics. In total,

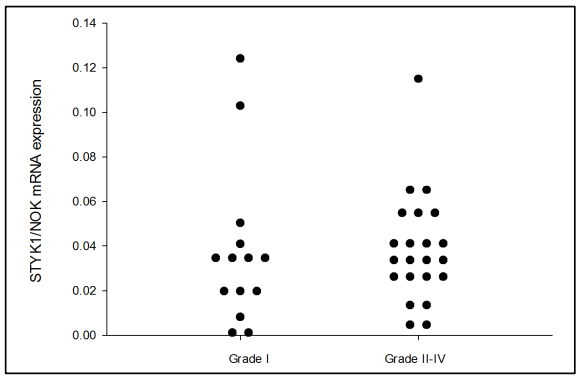

Figure 3. STYK1/NOK mRNA Expression at Different Clinical Stages of CRC. STYK1/NOK mRNA expression was measured using quantitative RT-PCR and expressed relative to the amount of GAPDH mRNA

Table 1. Relationships between NOK/STYK1 Expression Levels in Cancer Tissue Samples from Patients with CRC and Clinicopathological Features

Feature	N	miR-205, ΔCt	Statistical significance	p value
Gender				
Male	22	0.03±0.02	NS	0.77
Female	14	0.04±0.03		
Age (years)				
< 65	12	0.03± 0.1	NS	0.06
≥ 65	24	0.04±0.03		
Smoking status				
Never	11	0.07±0.01	NS	0.15
Current or ex-smoker	25	0.03±0.05		
Tumor location				
Colon	21	0.03±0.01	NS	0.79
Rectum	15	0.03±0.03		
Tumor size (cm)				
<5	16	0.02±0.03	**	0.0017
≥ 5	20	0.04±0.02		0.0017
Histological grade				
Well differentiated	17	0.03± 0.02	NS	0.93
Moderately differentiated	13	0.04±0.03		
Poorly differentiated	6	0.03±0.02		
pTNM stage				
I+II	22	0.03±0.02	NS	0.16
III+IV	14	0.04±0.03		
Invasion into lymphatic vessels				
Positive	13	0.03±0.03	NS	0.47
Negative	23	0.03±0.03		

*TNM, tumor–node–metastasis; Data presented as mean±SD; NS, not statistically significant (P ≥ 0.05); P-values obtained using Mann Whitney U test and Kruskal Wallis H test

36 patients (22 male, 14 female) were included in the study. The non-parametric test between the relative expression levels of STYK1/NOK in CRC cases and its clinicopathological characteristics have been exerted to investigate the associations with clinicopathological variables. Somewhat surprisingly, no significant associations were detected between expression of STYK1/NOK and clinicopathological variables, including age, gender, tumor stage, differentiation, localization, smoking status and lymphocyte infiltration. However, increased expression levels of STYK1/NOK in patients with CRC tended to be associated with increased tumor sizes as shown by non-parametric tests (p=0.0017; Mann-Whitney U test). Patient characteristics with respect to increased STYK1/NOK expression are shown in Table 1.

Discussion

The past decades have seen advances in the diagnosis and treatment of colorectal cancer. However, due to the asymptomatic onset of colorectal cancer most patients are in advanced or metastatic condition at the time of diagnosis, resulting in a poor prognosis. RPTKs constitute the largest family of novel oncogenes and perturbation of RPTKs expression levels has been documented frequently in promoting cellular transformation and tumorigenesis (Blume-Jensen and Hunter, 2001, Danilkovitch-Miagkova and Zbar, 2002). These findings imply their importance in the development and/ or function of tumor cell as they function in such signal pathways that control cell shape, proliferation, differentiation and migration (Hirai et al., 1987, Berclaz et al., 1996, Robinson et al., 1996, Lamorte and Park, 2001). Existing diagnostic tools and biomarkers for CRC have many inherent deficiencies. Currently FGFR family is one of the most important group of RPTKs that their aberrant expression in CRC was frequently reported (Matsuda et al., 2012). However, it has been proved that FGFR is associated just with well-differentiated histological type (Sato et al., 2009). Furthermore the lack of correlation between FGFR evaluation and genetic/ mRNA level and its protein occurrence indicated that in order to exploit FGFR as a predictive marker, the assessment of the receptor at immunohistochemical level is required (Spano et al., 2005). STYK1/NOK is a naturally occurring from an unknown protein with certain sequence homology with FGFR/PDGFR family member and resembles the structural feature of FGFR4 missing complete extracellular domain (Liu et al., 2004). Several previous studies validated its dysregulation in acute Leukemia and breast, prostate, ovarian and lung cancer (Amachika et al., 2007, Kimbro et al., 2008, Chung et al., 2009, Jackson et al., 2009, Kondoh et al., 2009).

Using a QRT-PCR we have determined for first time, to our knowledge, STYK1/NOK was dramatically upregulated in CRC tissues, suggesting that high expression of STYK1/NOK involved in colorectal carcinogenesis. Its high sensitivity and specificity in optimal cut-off point confirmed its high diagnostic and prognostic value. We also assessed the association between expression elevation of STYK1/NOK and clinicopathological features of patients. However, in our cohort, just the increased tumor size in CRC was closely correlated with the high expression of STYK1/NOK. Although the increased tumor size in CRC was not part of the staging system, previous studies have suggested that tumor size is also an important prognostic factor in CRC (Kornprat et al., 2011). Thus, collectively, STYK1/NOK might be important biological marker in carcinogenesis of CRC and the high expression of STYK1/NOK may contribute to the proliferation of CRC tissues.

Specifically STYK1/NOK expression was found to be high even at the early clinical stages of CRC, which might explain why associations with other clinical variables were absent in our tumor panel. Nevertheless it is unambiguous whether the increased expression of STYK1/NOK is one of the causes of CRC carcinogenesis or it is a consequence of normal colorectal cell transformation to tumor cells.

In conclusion, our study showed that STYK1/NOK is dramatically upregulated in colorectal cancer tissues and that it could be used as a tumor marker. Furthermore we demonstrated that the high expression of STYK1/NOK is significantly associated with tumor size. Consistent with the observations reported by Liu et al. (Liu et al., 2004), which revealed the function of overexpressed STYK1/NOK in tumorigenesis and metastasis of non-cancerous cells, and taking into account the structural differences between STYK1/NOK and RTKs, which lack extracellular domain, we speculate that over expression of STYK1/NOK might result in growth factor-independent cell proliferation, surface adhesion-independent growth and may contribute to the carcinogenesis of CRC in synergism. The Confirmation of this theory requires large-scale and long-term follow-up studies. Further insights into the functional and clinical implications of STYK1/NOK may contribute to the early diagnosis of colorectal cancer and help with CRC therapeutic approaches.

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