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.
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 hypothesized 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 patients has been investigated.

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 12,000xg 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 13,000xg 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, 1μl 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 synthesize 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 first 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 synthesized 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 AATCTGAATGATGC-3’; reverse primer: 5’-GGACT GGGACACGCAAGTG-3’;

GAPDH forward primer, 5’-GTGAACCATG AGAAGTATGACAAC-3’; Reverse primer: 5’-CATGAGTCCTTCCAGCATACC-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.

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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-ΔΔCt 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 (ΔCt = Ct_STYK1/NOK - Ct_GAPDH), and ΔΔCt indicated the difference in the ΔCt value between cancer tissue and the matched control (ΔΔCt = ΔCt_cancer - ΔCt_control). The 2-ΔΔCt 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 STYK1/NOK expression levels were significantly elevated in CRC tissues compared to normal tissues. 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 curve STYK1/NOK mRNA showed a AROC 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-

![Figure 1. Expression of STYK1/NOK mRNA in Cancerous and Adjacent Non-Cancerous Tissues from CRC Patients.](image)

Expressed NOK mRNA was calculated using the GraphPad Prism 6. The area under the ROC curve (AROC) was calculated to evaluate the specificity and sensitivity of predicting CRC and normal tissue by STYK1/NOK mRNA showed a ROC area (AROC) of 0.79. (Figure. 2; p<0.001) The plot shows sensitivity and specificity at various cutoff values was calculated using SigmaPlot.
Table 1. Relationships between NOK/STYK1 Expression Levels in Cancer Tissue Samples from Patients with CRC and Clinicopathological Features

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

*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 (Komprat 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.
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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.

Acknowledgements

Our gratitude goes to Tabriz Imam Reza Hospital nurses and staff at the endoscopy department and patients for their helpful collaboration. We would also like to thank doctors Kamal Boostani and Saleh Azadbakht for their helpful collaboration. We would also like to thank doctors Kamal Boostani and Saleh Azadbakht for their helpful collaboration. We would also like to thank doctors Kamal Boostani and Saleh Azadbakht for their helpful collaboration. We would also like to thank doctors Kamal Boostani and Saleh Azadbakht for their helpful collaboration. We would also like to thank doctors Kamal Boostani and Saleh Azadbakht for their helpful collaboration.

References