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RESEARCH

# Serine Protease Inhibitor Kazal Type 1 (SPINK1) Promotes Proliferation of Colorectal Cancer Through the Epidermal Growth Factor as a Prognostic Marker

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**Abstract** Serine protease inhibitor Kazal type-1 (SPINK1), a trypsin kinase inhibitor, is involved in inflammation, cell proliferation and carcinogenesis. The role and association between SPINK1, EGFR and Ki-67 in colorectal adenoma (CRA) and colorectal cancer (CRC) are still unknown. In this study, we used immunohistochemical stain to evaluate expression of SPINK1, EGFR and Ki-67 proteins in 30 CRA and 53 CRC patients semiquantitatively, and then analyzed their correlation with clinicopathologic parameters. Our results revealed that SPINK1 expression was noted in the upper and basal parts of the crypts in CRA and was more intensely related with cellular atypia. EGFR expression was found in 13 out of 30 adenomas, including 9 out of 15 adenomas with dysplasia or synchronous CRC (60 %), and 4 out of 15 adenomas without dysplasia (26.7 %). In CRC, high SPINK1 expression was significantly associated with males ($p$=0.041) and advanced disease stage ($p$=0.015). EGFR positivity was significantly correlated with higher T stage ($p$=0.004) and disease stage (stage I-IV, $p$=0.017; early vs. late, $p$=0.015). Pearson's correlation showed positive correlation between the SPINK1 intensity and EGFR immunoreactivity ($p$=0.011), and Ki-67 and SPINK1 intensity or percentage ($p$=0.017 and $p$=0.039 respectively). In Kaplan-Meier analyses, patients with high SPINK1 intensity tended to have shorter overall survival ($p$=0.03). Concomitant expression of high SPINK1 intensity and EGFR was also identified as being associated with poor prognosis ($p$=0.015). In conclusion, high SPINK1 expression is associated with advanced stage and poor prognosis. There is positive correlation between high SPINK1 expression, EGFR immunoreactivity, and high Ki-67 labeling index. The SPINK1 protein seems to play a role in tumor proliferation and malignant transformation through the EGFR pathway. SPINK1 may serve as a prognostic biomarker in therapeutic targeting in the future.

**Keywords** Colorectal cancer · Epidermal growth factor receptor (EGFR) · Serine protease inhibitor Kazal type-1 (SPINK1) · Tumor-associated trypsin inhibitor (TATI)

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## Introduction

14.1 million new cancer cases occurred worldwide in 2012, of which an estimated 1.36 million were colorectal cancer cases (CRC). CRC is the third most common neoplasm in men and the second in women [1]. Although several possible pathogenesis pathways have been found, many patients have died due to this aggressive disease even under current treatment modalities. Some patients had colon cancer due to congenital gene disorder, but others developed sporadically. They might

have had previous colonic adenoma and then experienced malignant transformation into adenocarcinoma during long-term follow-up, especially in old age [2]. Clinicopathological parameters, such as age, tumor differentiation, margin, lymphovascular invasion, perineural invasion, tumor-node-metastasis (TNM) status, and stage, were established to predict outcome of the disease, but these are insufficient to provide a definitive prognosis and further guidelines of treatment. Therefore, it is both important and urgently necessary to identify major specific disease-related biomarkers for personalized medicine.

Serine protease inhibitor Kazal-type 1 (SPINK1) is a trypsin kinase inhibitor, also called pancreatic secretory trypsin inhibitor (TATI) or pancreatic secretory trypsin inhibitor (PSTI), which was initially found to prevent autodigestion or proteolysis of pancreatic glands in pancreatic acinar cells [3]. It is known to be involved in inflammation, cell proliferation and cancer pathogenesis. Mutation in this gene is associated with chronic or hereditary pancreatitis [4]. Moreover, altered expression levels of SPINK1 related to disease prognosis have also been identified in numerous cancers [5] and even used as a target therapy in prostatic cancer [6].

Epidermal growth factor receptor (EGFR) is the cell-surface receptor of the ErbB tyrosine kinase family of extracellular protein ligands. Once activated, it can regulate intracellular message transduction [7]. EGFR overexpression is related to advanced stage and poor prognosis in variable neoplasm [8]. Therefore, tyrosine kinase inhibitor has been used as a treatment to inhibit tumor growth and increase sensitivity to chemotherapy of tumor cells [9].

In a previous study, SPINK1 was identified as having a half-similar structure to epidermal growth factor (EGF) [10], which suggested the ability of SPINK1 to act as a growth factor. SPINK1 and EGF bind to the EGFR, and SPINK1 stimulates cell proliferation through the mitogen-activated protein kinase (MAPK) cascade in pancreatic cancer cell lines [11]. Co-expression of SPINK1 and EGFR was found in pancreatic tubular adenocarcinoma, intraductal papillary mucinous neoplasm, and pancreatic intraepithelial neoplasia [12]. In prostate cancer, Ateeq et al. also demonstrated that SPINK1 mediated its oncogenic effects through EGFR in vitro and in vivo, even as a potential therapeutic target by decreasing tumor size [6]. However, the role and association between SPINK1 and EGFR in colon adenoma and cancer are still unknown.

Ki-67 is also a nuclear protein which is associated with and possibly necessary for cellular proliferation [13]. Ki-67 proliferative index has been used as a prognostic and predictive marker in variable cancers.

The aim of this study was to evaluate the prognosis and predictive significance of the expressions of SPINK1, EGFR and Ki-67 and their corrections with clinicopathologic factors to identify the possible pathogenesis pathway in CRC. To our knowledge, this study is the first literature to use SPINK1, EGFR and Ki-67 concomitantly in CRC to find the prognostic values by immunohistochemical staining (IHC).

## Materials and Methods

### Patients Population and Clinical Data Collection

The study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital (KMUH-IRB-20130096). We analyzed specimens of 53 CRC patients treated with surgical intervention between 2007 and 2009 in Kaohsiung Medical University Hospital. No patient received preoperative chemotherapy or radiotherapy. The clinical information was collected by chart review. An additional 30 cases of colorectal adenoma (CRA) (11 tubular adenoma and 19 tubulovillous adenoma) with or without high-grade dysplasia or synchronous CRC under colofibroscopic biopsy were compared. Eight adenomas (3 tubular adenomas and 5 tubulovillous adenomas) with synchronous CRC were included.

### Pathologic Evaluation

All specimens were processed according to standard pathologic procedures. The hematoxylin and eosin (H&E) slides were reviewed to confirm the diagnosis and pathologic features, such as the status of tumor grade, lymphovascular and perineural invasion, and disease stage. The tumor stage were assigned according to the American Joint Committee on cancer staging manual system published in 2010 [14].

### Immuohistochemistry and Scoring

Formalin-fixed, paraffin-embedded blocks of CRA and CRC were collected. All 4 μm sections were dried, deparaffinized, rehydrated, and heat-mediated antigen retrieval was carried out by boiling under pressure in Target Retrieval Buffer (Leica, pH 6.0; abcam, pH 9.0 and DAKO, pH 9.0, respectively) for 8 min. Three percent hydrogen peroxide was also used for 5 min to block endogenous peroxidase activity at room temperature, and the slides were washed with Tris buffer solution. IHC was performed using anti-EGFR (1:50, Leica, UK), anti-SPINK1 (1:800, abcam, USA) and Ki-67 (1:75, DAKO, Denmark) as the primary antibodies. Positive and negative control sections were included in each run of quality control. Semi-quantitative analysis of expression of EGFR and SPINK1 were independently evaluated by two pathologists. If there was any discrepancy, the pathologists re-analyzed the IHC slides together and came to made a consensus on the final score.

Status of SPINK1 expression was evaluated according to the percentage and intensity of tumor cells cytoplasmic staining by IHC published previously [15–17]. Cytoplasmic immunoreactivity of percentage in tumor cells was scored in the following manner: 0, nonreactivity; 1, 1–10 %; 2, 11–50 %; and 3, >50 %. Intensity was assessed by a score of 0 to 3. The highest values of cancer cells were analyzed. Specimens with more than 50 % or high intensity (score 3) of cytoplasmic immunoreactivity in cancer cells were considered as high SPINK1 expression [15, 17].

Extent of EGFR expression was recorded as the percentage of tumor cells membranously stained by IHC. Status of EGFR staining was assigned in a score of 0 (negative), 1 (weak and incomplete staining more than 10 %), 2 (moderate and complete staining more than 10 %) and 3 (more than 10 % strong and complete staining). EGFR-positive staining was defined as any IHC of more than 10 % tumor cells whether it was complete or incomplete circumferential membrane stain [18].

Ki-67 score was evaluated by the percentage of total number of tumor cells with positive nuclear staining. High Ki-67 labeling index was defined as more than 40 % positive cells [19–21].

### Statistical Analysis

All statistical analyses were performed with the SPSS 18.0 software. Chi-square test was applied for correlation analysis between protein expression in IHC and clinicopathological parameters. Pearson's correlation coefficient was used to analyze the correlation between SPINK1, EGFR and Ki-67. Cancer-specific survival probabilities were estimated using the Kaplan-Meier method. Overall survival (OS) was measured from the date of surgery to the date of death. The observations were censored at the end of the study period. All tests were 2-sided and a p value less than 0.05 was regarded as statistically significant.

## Results

### SPINK1 Expression in Normal Colonic Mucosa and CRA

In normal colonic mucosa, SPINK1 expression was noted in the goblet cells in the basal parts of the crypts. In adenomas, the upper part of the polyp also showed SPINK1 expression, and the higher staining intensity was related to the higher-grade cellular atypia. High cytoplasmic immunoexpression of SPINK1 was identified in 8 (100 %) adenomas with synchronous CRC.

EGFR expression was found in 13 of 30 adenomas, including 3 of 11 (27.3 %) tubular adenomas and 10 of 19 tubulovillous adenomas (52.6 %). Nine of 15 adenomas with high-grade dysplasia or synchronous CRC (60 %) had more EGFR immunoreactivity than 4 of 15 adenomas without dysplasia (26.7 %). Adenomas with synchronous CRC had 62.5 % EGFR positivity.

### Association of SPINK1, EGFR and Ki-67 Expression and Clinicopathologic Features

The expression of SPINK1 in relation to colorectal cancer and patient characteristics is listed in Table 1.

High expression of SPINK1 in percentage and staining intensity was found in 36 (52.8 %) and 7 (13.2 %) of 53 tumors respectively. High SPINK1 expression (intensity score 3) was significantly associated with male gender ($p$=0.041) and advanced disease stage (stages I-II v.s stages III-IV) ($p$=0.015) (Fig. 1). All patients with high SPINK1 expression were advanced stage, compared with half of the patients with low SPINK1 expression. None of the female patients had high SPINK1 immunoreactivity.

EGFR membrane immunostaining was positive (score 1–3) in 35 (66.0 %) of 53 CRC patients. Immunoreactivity of EGFR was significantly correlated with higher T stage ($p$=0.004) and disease stage (stage I-IV, $p$=0.017; early vs. late, $p$=0.015). More than half the patients with positive EGFR expression had higher T stage, disease stage and late stage. Status of Ki-67 expression showed no significant association with clinical and pathological characteristics.

### Simultaneous Immunostaining and Correlations of SPIN K1, EGFR and Ki-67 Labeling Index

The combination SPINK1>50 %/EGFR+ was present in 12 (22.6 %), SPINK1>50 %/EGFR− in 5 (9.4 %), SPINK1 ≦50 %/EGFR+ in 23 (43.4 %), and SPINK1 ≦50 % /EGFR − in 13 (24.5 %) patients. Co-expression of SPINK1>50 %/ EGFR+ was correlated with advanced disease stage ($p$=0.048). The combination SPINK1 intensity 3/EGFR+ was noted in 5 (9.4 %), SPINK1 intensity 3/EGFR− in 3 (5.7 %), SPINK1 intensity ≦2/EGFR+ in 26 (49.1 %), and SPINK1 intensity ≦2 /EGFR− in 19 (35.8 %) patients.

The combination SPINK1>50 %/Ki-67>40 % was observed in 14 (26.4 %), SPINK1 intensity >50 %/Ki-67≦40 % in 3 (5.7 %), SPINK1 ≦50 %/ Ki-67≦40 % in 19 (35.8 %), and SPINK1 ≦50 %/Ki-67≦40 % in 17 (32.1 %) patients. The combined SPINK1 intensity 3/Ki-67>40 % was present in 6 (11.3 %), SPINK1 intensity 3/Ki-67 ≦40 % in 1 (1.9 %), SPINK1 intensity ≦2/Ki-67>40 % in 27 (50.9 %), and SPINK1 intensity ≦2/Ki-67≦40 % in 19 (35.8 %) patients. Concomitant expression of SPINK1 intensity 3/Ki-67>40 % was found to be associated with advanced disease stage ($p$=0.030) and higher N stage ($p$=0.049).

A Pearson correlation coefficient was computed to assess the relationships between variables of SPINK1, EGFR and Ki-67. There was a positive correlation between the SPINK1

**Table 1** Correlation of SPINK1 expression percentage and intensity with clinicopathological parameters in CRC patients by Chi-square test

| Variable | All | SPINK1 expression percentage | | | SPINK1 expression intensity | | |
|---|---|---|---|---|---|---|---|
| | | ≦50 % | >50 % | *p* value | ≦2 | 3 | *p* value |
| All | 53 | 17 (47.2 %) | 36 (52.8 %) | | 46 (86.8 %) | 7 (13.2 %) | |
| Age (y/o) | | | | 1.000 | | | 0.588 |
| ≦75 | 44 (83.0 %) | 14 (31.8 %) | 30 (68.2 %) | | 39 (88.6 %) | 5 (11.4 %) | |
| >75 | 9 (17.0 %) | 3 (33.3 %) | 6 (66.7 %) | | 7 (77.8 %) | 2 (22.2 %) | |
| Gender | | | | 0.555 | | | 0.041* |
| Male | 34 (64.2 %) | 12 (35.3 %) | 22 (64.7 %) | | 27 (79.4 %) | 7 (20.6 %) | |
| Female | 19 (35.8 %) | 5 (26.3 %) | 14 (73.7 %) | | 19 (100 %) | 0 (0 %) | |
| Tumor size (cm) | | | | 0520 | | | 1.000 |
| ≦5 | 38 (71.7 %) | 11 (28.9 %) | 27 (71.1 %) | | 33 (86.8 %) | 5 (13.2 %) | |
| >5 | 15 (28.3 %) | 6 (40 %) | 9 (60 %) | | 13 (86.7 %) | 2 (13.3 %) | |
| Grade | | | | 0.295 | | | 0.438 |
| I | 3 (5.7 %) | 0 (0 %) | 3 (100 %) | | 3 (100 %) | 0 (0 %) | |
| II | 44 (83.0 %) | 16 (36.4 %) | 28 (63.6 %) | | 37 (84.1 %) | 7 (15.9 %) | |
| III | 6 (11.3 %) | 1 (16.7 %) | 5 (83.3 %) | | 6 (100 %) | 0 (0 %) | |
| Site | | | | 0.167 | | | 0.650 |
| Colon | 41 (77.4 %) | 11 (26.8 %) | 30 (73.2 %) | | 36 (87.8 %) | 5 (12.2 %) | |
| Rectum | 12 (22.6 %) | 6 (50 %) | 6 (50 %) | | 10 (83.3 %) | 2 (16.7 %) | |
| Lymph-vascular invasion | | | | 0.760 | | | 0.408 |
| Positive | 17 (32.1 %) | 6 (35.3 %) | 11 (64.7 %) | | 16 (94.1 %) | 1 (5.9 %) | |
| Negative | 36 (67.9 %) | 11 (30.6 %) | 25 (69.4 %) | | 30 (83.3 %) | 6 (16.7 %) | |
| Perineural invasion | | | | 0.511 | | | 1.000 |
| Positive | 13 (24.5 %) | 3 (23.1 %) | 10 (76.9 %) | | 11 (84.6 %) | 2 (15.4 %) | |
| Negative | 40 (75.5 %) | 14 (35 %) | 26 (65 %) | | 35 (87.5 %) | 5 (12.5 %) | |
| Tumor stage (T) | | | | 0.188 | | | 0.199 |
| 1 | 7 (13.2 %) | 2 (28.6 %) | 5 (71.4 %) | | 7 (100 %) | 0 (0 %) | |
| 2 | 11 (20.8 %) | 1 (9.1 %) | 10 (90.9 %) | | 11 (100 %) | 0 (0 %) | |
| 3 | 27 (50.9 %) | 12 (44.4 %) | 15 (55.6 %) | | 21 (77.8 %) | 6 (22.2 %) | |
| 4 | 8 (15.1 %) | 2 (25 %) | 6 (75 %) | | 7 (87.5 %) | 1 (12.5 %) | |
| Lymph node stage (N) | | | | 0.904 | | | 0.139 |
| 0 | 28 (52.8 %) | 9 (32.1 %) | 19 (67.9 %) | | 26 (92.9 %) | 2 (7.1 %) | |
| 1 | 14 (26.4 %) | 5 (35.7 %) | 9 (64.3 %) | | 10 (71.4 %) | 4 (28.6 %) | |
| 2 | 11 (20.8 %) | 3 (27.3 %) | 8 (72.7 %) | | 10 (90.9 %) | 1 (9.1 %) | |
| Metastasis status | | | | 0.520 | | | 1.000 |
| Yes | 15 (28.3 %) | 6 (40 %) | 9 (60 %) | | 13 (86.7 %) | 2 (13.3 %) | |
| No | 38 (71.7 %) | 11 (28.9 %) | 27 (71.1 %) | | 33 (86.8 %) | 5 (13.4 %) | |
| Disease stage | | | | | | | |
| I | 15 (28.3 %) | 3 (20 %) | 12 (80 %) | 0.622 | 15 (100 %) | 0 (0 %) | 0.103 |
| II | 8 (15.1 %) | 3 (37.5 %) | 5 (62.5 %) | | 8 (100 %) | 0 (0 %) | |
| III | 13 (24.5 %) | 4 (30.8 %) | 9 (69.2 %) | | 10 (76.9 %) | 3 (23.1 %) | |
| IV | 17 (32.1 %) | 7 (41.2 %) | 10 (58.8 %) | | 13 (76.5 %) | 4 (23.5 %) | |
| Early (I-II) | 23 (43.4 %) | 6 (26.1 %) | 17 (73.9 %) | 0.555 | 23 (100 %) | 0 (0 %) | 0.015* |
| Late (III-IV) | 30 (56.6 %) | 11 (36.7 %) | 19 (63.3 %) | | 23 (76.7 %) | 7 (23.3 %) | |

* $p<0.05$

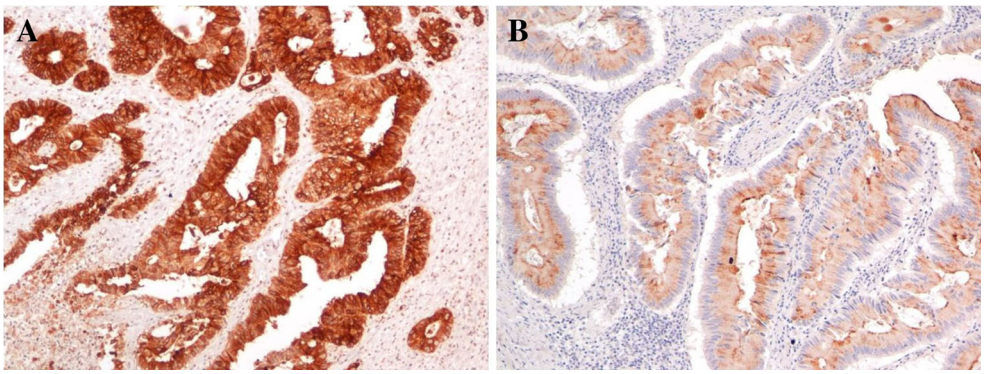


**Fig. 1** Representaitve immunohistochemical staining pattern of SPINK1 in colorectal cancer. Higher SPINK1 expression was identified in colorectal cancer with advanced disease stage (**a**) compared with the colorectal cancer in early stage (**b**)

intensity and EGFR ($r$=0.348, $n$=53, $p$=0.011). Overall, increased SPINK1 intensity expression was correlated with positive EGFR expression. There was also positive correlation between Ki-67 and SPINK1 intensity or percentage ($r$=0.326, $n$=53, $p$=0.017 and $r$=0.285, $n$=53, $p$=0.039, respectively). High SPINK1 expression was associated with high Ki-67 labeling index.

### The Relationships of SPINK1, EGFR and Ki-67 in Overall Survival

In Kaplan-Meier analyses, patients with high SPINK1 intensity cells tended to have shorter OS ($p$=0.03). Conversely, high SPINK1 friction, EGFR or Ki-67 expression had no significant correlation with clinical outcome (data not shown).

Combined with these three variables, concomitant expression of high SPINK1 intensity and positive EGFR was also identified to be correlated with adverse OS ($p$=0.015) (Fig. 2). There was a trend between co-expression of high SPINK1 intensity with high Ki-67 and shorter OS, but the difference was not significant ($p$=0.058). Other combinations showed no significant association with cancer-specific survival (data not shown).

## Discussion

SPINK1, encoded by *SPINK* gene, is a trypsin inhibitor, which is secreted from pancreatic acinar cells into pancreatic juice. It presents trypsin-catalyzed premature activation of zymogens within the pancreas and the pancreatic duct [3, 5]. It is also known to play an important role in inflammation, cell proliferation and cancer pathogenesis. Increased expression of SPINK1 in tissue or serum which was related with poor prognosis was also found in other epithelial cancers, including pancreatic, gastric, renal, hepatic, breast, ovarian, esophageal and prostatic cancers [5, 22, 23]. Conversely, loss of SPINK1 expression was associated with inferior outcomes in bladder cancer [15]. These differences might be explained by differences in tissue histology and cancer biology.

In the present study, we identify that SPINK1 expression is not only in the goblet cells of normal colorectal mucosa but also in CRAs, which is of increased intensity related with dysplastic grade [24]. The role of SPINK1 has been shown to be associated with tumor growth in tissue culture and with increased tumor size in CRC patients [6, 25]. We demonstrate that high SPINK1 expression is associated with aggressive tumor behavior in CRC patients, such as advanced pathologic stage and shorter survival. The results are in concordance with previous studies [17, 26]. SPINK1, as a weak inhibitor of serine proteinase, is hypothesized to play an important mediating role in cancer invasion, metastasis and progression due to disrupted balance of cell-matrix interactions [5, 27]. In vitro, Gouyer V et al. reported that SPINK1 was involved in autocrine induction of invasion and metastasis in human colon cancer cell line [27], which is similar in bladder cancer [28]. Previous studies have presented the correlation of high expression of SPINK1 in tissue with liver metastasis [17] and high serum SPINK1 level with impaired overall survival in

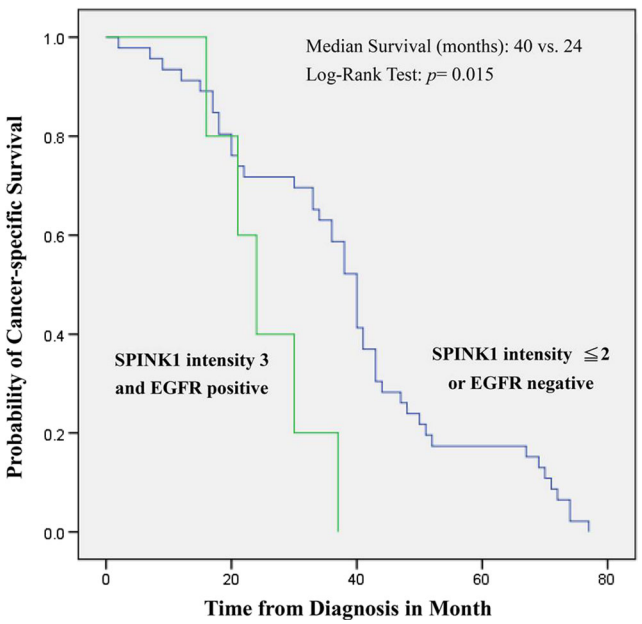


**Fig. 2** Kaplan-Meier plots of cancer-specific survival estimates in 53 CRC patients by combined SPINK1 intensity score and EGFR status

CRC patients [26, 29]. These data suggest that SPINK1 plays a role in CRC associated with tumor development. The discrepancy between our results and those of another study [16] might be due to tissue processing, selected antibodies and different interpretations of scoring in immunoreactivity.

EGFR is a tyrosine kinase receptor which is frequently identified in epithelial cancer. [8]. Elevated EGFR expression is known to be involved in carcinogenesis, including cell proliferation, apoptosis, angiogenesis and metastasis, which is associated with poor prognosis [30]. Overexpression of EGFR is found in 70–80 % of CRC patients, which is proposed as a target therapy [31]. We presented that EGFR overexpression was associated not only with advanced CRAs but also higher T stage and disease stage in CRC. We also identified more positive EGFR immunoreactivity in tubulovillous adenoma, high-grade dysplasia, and adenoma with synchronous CRC. These results were in accordance with previous findings [32]. It suggests that overexpression of EGFR progressively increases with malignant transformation, and that it is an independent adverse prognostic factor [30, 32–34].

In a previous study, SPINK1 was shown to bind to the surface receptors and to stimulate DNA synthesis in fibroblasts [35]. Structurally SPINK1 includes a fifty percent sequence homology and the presence of 3 intrachain disulfide bridges similarities with epidermal growth factor as demonstrated by radioreceptor assay [10], which indicates the ability of SPINK1 to act as a growth factor. SPINK1 can induce dimerization and phosphorylation of EGFR and promote signal transduction and cell proliferation by MAPK pathway [6, 11]. Co-expression of SPINK1 and EGFR was observed in pancreatic cancer and precancerous lesions [12]. We also found that co-high expression and correlation of SPINK1 and EGFR are associated with poor prognosis. This demonstrates SPINK1 is a new ligand for EGFR and develops autocrine stimulation in EGFR-positive cells to induce cell transformation. The potential of SPINK1 as a therapeutic target due to its similarities with EGF may be considered. However, the results in prognostic expressions of combined SPINK1 and EGFR in our study differ from a previous study [36]. The contradictory results may be attributed to several factors, such as IHC techniques, scoring methods, inter-observer interpretation, and different populations.

The downstream cascades of EGFR in PI3K/AKT, JAK/STAT and MAPK pathways are all related to DNA synthesis and cell proliferation [6, 34]. Ki-67 protein is also a proliferative index marker indicating high activity in cells, which is correlated with high SPINK1 status in our study. In vivo, Ateeq et al. observed a significant decrease in Ki-67 labeling index in the prostate cancer tissue by inhibition of SPINK1 [6]. This indicates that SPINK1 plays an important role in cellular proliferation. Although the definite mechanism for SPINK1 in the tumor aggressiveness is still unknown, there is much evidence to show protease inhibitors may influence the tumor invasion-promoting effects. For example, plasminogen activator inhibitor 1 (PAI-1) and tissue inhibitor of metalloproteinases 1 (TIMP-1) were identified to be associated with poor prognosis in breast cancer [37, 38]. The complexity of the interactions of proteases and their inhibitors or other proliferative associated receptors in human cancer warrants further exploration.

Our study has several limitations. First, it is a retrospective design lacking data regarding surgical techniques, surgeons' preferences, experience, chemotherapy regiments, and duration. However, all specimens were collected at a single academic medical center, which strengthens our results. The second limitation is the duration of follow-up. Long-term follow-up for stage I CRC is needed to identify defined OS. To reduce this bias, we re-classified the cases into early and advanced groups and found better statistically significant differences. Another possible limitation is the reliability of IHC techniques. The results of IHC may be influenced by tissue fixation techniques, choice of antibody, antibody concentration, and interpretation criteria. A prospective study with standardized inclusion or exclusion criteria will be able to present the value of SPINK1 with EGFR or Ki-67 as a predictive and prognostic biomarker in CRC, and even in target therapy.

In conclusion, we found that high expression of SPINK1 was associated with advanced stage and poor prognosis. The expression of SPINK1 was correlated with positive EGFR status and high Ki-67 labeling index. The SPINK1 may be considered as a proliferative tumor protein through the EGFR pathway. Further studies on the interactions and molecular mechanisms in CRC are needed. Although SPINK1 expression revealed no independent value in outcomes, it might serve as a biomarker in clinical decision-making and in personalized therapeutic targeting in the future.

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