

## Roles of Serine Protease Inhibitor Kazal type 1 (SPINK1) in Prostate Cancer

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### Abstract

Altered genes that play a driving role in cancer development can often serve as specific diagnostic markers, criteria of molecular classification and therefore potential therapeutic targets. Serine protease inhibitor Kazal type 1 (SPINK1), also known as pancreatic secretory trypsin inhibitor or tumor-associated trypsin inhibitor, encodes a 56 amino acid secreted peptide, and its normal function is thought to be the inhibition of serine proteases such as trypsin. Recent studies have indicated marked overexpression of SPINK1 defines an aggressive molecular subtype of ETS (erythroblastosis virus E26 transformation-specific) fusion-negative prostate cancer (PCa) patients. SPINK1 may act as an autocrine growth factor and promotes PCa growth and invasion. Most recently, we suggested that SPINK1 induces epithelial-mesenchymal transition (EMT) through EGFR signaling pathway in PCa. The association between SPINK1 overexpression and poor prognosis in PCa has been reported. Notably, SPINK1 might be a novel extracellular therapeutic target in a subset of high-grade PCa patients. In this review, we will summarize the current understanding of SPINK1 involving its role in PCa biology, association with prognosis as well as perspective in therapy from the pathologist's point of view.

**Keywords:** Serine protease inhibitor kazal type 1; Prostate cancer; ETS; Prognosis; Target

Prostate cancer (PCa) is the most commonly diagnosed non-skin cancer in American men. It is estimated that 233,000 Americans will be diagnosed with PCa in 2014 and over 29,000 men will die from the disease [1]. PCa is a common heterogeneous disease with only a small fraction of patients will progress rapidly and require immediate treatment [2]. Consequently, two major clinical challenges were posed by the current inability to readily distinguish indolent from aggressive tumors in PCa patients and development of novel therapeutic strategies targeting aggressive tumors.

Altered genes that play a driving role in cancer development can often serve as specific diagnostic markers, criteria of molecular classification and therefore potential therapeutic targets [3]. The recent finding of overexpression of serine protease inhibitor Kazal type 1 (SPINK1) may represent a novel therapeutic target in selected PCa patients. In this review, we will summarize the structure of SPINK1 protein, current understanding of its role in PCa biology, association with prognosis as well as perspective in therapy from the pathologist's point of view.

### An overview of SPINK1

SPINK1, also known as pancreatic secretory trypsin inhibitor or tumor-associated trypsin inhibitor, encodes a 56 amino acid secreted peptide, which contains three disulfide bonds and a trypsin-specific binding site formed by Lys-Ile [4,5]. SPINK1 is usually with a signal peptide of approximately 20 amino acids at N-terminal. Analysis of the human SPINK1 gene reveals that it is located on 5q32 containing approximately 7.5 kb and four exons. A 40 bp DNA fragment located between kb -3.84 and -3.80 carries the element responsible for both transcriptional activity and IL-6-induced gene expression [4]. SPINK1 was originally isolated from the pancreas and its primary function is inhibition of serine proteases, such as trypsin, in the pancreas and small intestines [6]. Multiple variants of SPINK1 have been previously shown to be a strong causal factor for chronic pancreatitis possibly due to inactivation of trypsin [7,8]. Additionally, inactivation of SPINK1 in the pancreas results in autophagy of exocrine pancreatic cells, suggesting that SPINK1 may be important in cell survival. In liver cells, expression of SPINK1 can be induced by Hepatitis B virus and protects liver cells from serine protease dependent cellular apoptosis [4]. Collectively,

SPINK1 appears to play an important role in both cell survival and prevention of apoptosis by several different pathways in normal tissues.

Overexpression of SPINK1 has been observed in multiple human malignancies, including cancers of the colon, breast, liver and urinary bladder [9-12]. Notably, increased SPINK1 expression was associated with liver metastasis and was an independent predictor of poor prognosis in cancer of the colon and the breast [10,12]. Lee et al. concluded that SPINK1 overexpression contributed to cell growth advantage and enhanced the metastatic potential of tumors and suggested using SPINK1, AFP and osteopontin as combined markers for the prediction of early tumor recurrence of hepatocellular carcinoma [13].

### Expression and the Biological Role of SPINK1 in Prostate Cancer

In 2007, Paju et al. first reported SPINK1 can be overexpressed in PCa tissues and cell line 22RV1 [14]. (In 2008, using a Cancer Outlier Profile Analysis (COPA) strategy, Tomlins et al identified SPINK1 outlier expression exclusively in ETS (Erythroblastosis virus E26 transformation-specific) rearrangement negative PCa patients (~10% of total cases) [15]. Gene fusions involving ETS family transcription factors are present in approximately 50% of prostate-specific antigen (PSA)-screened localized PCas and in 15-30% of population-based cohorts [16]. Recent work has demonstrated that prostate tumors can be stratified by dominant genetic alterations, such as chromosomal rearrangements involving ETS family transcription factors [17]. In surgically resected PCa patients, Lippolis et al. [18] and Bismar et al. [19] confirmed exclusivity of SPINK1 overexpression and ETS gene fusions, but that is not the case in endocrine-treated patients [20]. In line with

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these studies, we also suggested SPINK1 is mutually exclusive with ERG rearrangement status in Chinese PCa patients and the prevalence of SPINK1 overexpression is comparable with that of western populations [21]. In contrast, some previous reports did not find such mutual exclusivity, although the majority of which suggested the incidence rate was very low for concomitance of SPINK1 and ETS fusions [20,22]. In a most recent follow-up study including 879 PCa patients treated by radical prostatectomy, Flavin et al. [23] reported that SPINK1 protein expression in 8% (74/879) of cases. Additionally, they showed SPINK1 over-expression was seen in 47 of 427 (11%) ERG negative samples and in 19 of 427 (4%) ERG positive cases. In an attempt of characterizing SPINK1 expression in uncommon histologic variant, we showed SPINK1 was expressed in 6% of ETS negative histologic variants and specifically in ductal adenocarcinoma [24]. The discrepancy might be partially explained by cohort design, patients' race, antibody selection and criteria for SPINK1 IHC positivity.

The molecular mechanism underlying SPINK1 overexpression remains unclear. Using FISH analysis, Tomlins et al. demonstrated no evidence of gene amplification or genetic arrangement, respectively, in samples with SPINK1 overexpression [15]. Thus, SPINK1 may be activated by increased transcription activity. Alternatively, SPINK1 may be activated by a unique upstream genetic event [15]. So far, research data regarding this field is still lacking.

Currently, in vitro and in vivo data suggested that SPINK1 is oncogenic in PCa [15,25]. Tomlins et al. demonstrated that SPINK1 knockdown in 22RV1 cells attenuates invasion [15]. In addition, Ateeq et al. suggested that recombinant SPINK1 protein (rSPINK1) stimulates cell proliferation in benign RWPE as well as cancerous prostate cells [25]. Of note, RWPE cells treated with either rSPINK1 or conditioned medium from 22RV1 PCa cells (SPINK1+/ETS-) significantly increased cell invasion when compared with untreated cells. In contrast, knockdown of SPINK1 in 22RV1 cells inhibited cell proliferation, cell invasion, and tumor growth in xenograft assays [25].

It remains unknown how SPINK1 contributes to PCa carcinogenesis and tumor progression. The expression of SPINK1 is invariably associated with expression of tumor-associated trypsin, which activates several matrix metalloproteinases. Several reports have suggested that the association between SPINK1 expression and adverse prognosis in cancer has been ascribed to the expression of trypsin by the tumors [6,26]. However, oncogenic effect of SPINK1 seemed to be independent of trypsin activity in PCa [25]. Indeed, recent studies have focused attention on the interaction of SPINK1 and epidermal growth factor receptor (EGFR) [11,25,27]. SPINK1 and EGF, the EGFR ligand, share 50% amino acid homology and several structural similarities [28]. Similar to EGF, Ozaki et al. [29] have showed that SPINK1 is able to phosphorylate EGFR and its downstream molecules including STAT3, Akt and ERK in pancreatic cancer cells. Ateeq et al. [25] proposed that SPINK1 functions at least in part to stimulate EGFR signaling in an autocrine loop. Clinically, the notion of SPINK1-EGFR axis was evidenced by the significant co-overexpression of SPINK1 and EGFR in PCa patients cohort [21]. Most recently, we reported that SPINK1 induces epithelial mesenchymal transition (EMT) in PCa via activating EGFR, leading to increased migratory and invasive capacity in vitro [21]. EGFR/MAPK pathway was mainly involved in this process. Connective tissue growth factor might be a main downstream molecule of this pathway. Of note, EMT is an early embryonic development program and plays a critical role in cancer progression and metastasis [30]. Thus, our data may partially explain the mechanism by which SPINK1 promotes PCa progression.

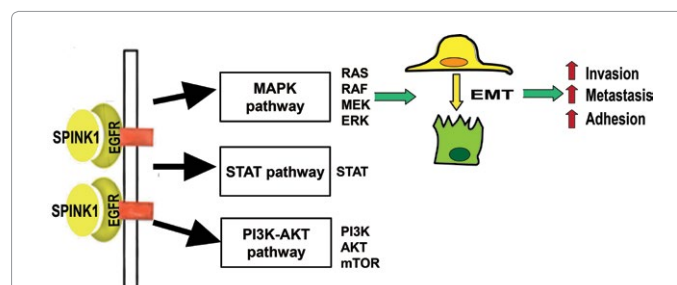
## Prognostic Association of SPINK1 Overexpression

Initial work in ETS rearrangement negative PCas indicated overexpression of SPINK1 defines a distinct molecular subtype with an aggressive phenotype [15]. Compared with other patients, SPINK1-positive PCa patients may have a worse Gleason score and a higher risk of disease recurrence, and poor prognosis. Later, Leinonen et al. [20] reported that SPINK1 expression is associated with aggressive form of the disease and could serve as a biomarker in endocrine-treated PCa patients. In consistent with these findings, our results revealed that SPINK1 overexpression is an unfavorable prognostic factor in Chinese PCa patients [21].

Conversely, there are several studies that have reported an absence of such a clinical correlation between the SPINK1 overexpression and outcome. In the study performed by Lippolis et al. [18], SPINK1 was weakly associated with pathological tumor stage, but not associated with biochemical recurrence or development of metastatic disease. Grupp et al. [31] found no association between SPINK1 status and clinico-pathologic factors or biochemical recurrence in a large cohort of surgically treated PCa patients. Similarly, Flavin et al. [23] found no positive association between SPINK1 status and prostate cancer-specific survival. Taken together, prognostic significance of SPINK1 overexpression in PCa merits further investigation.

## SPINK1: A Potential Therapeutic Target for Prostate Cancer

The goal of target therapy is to treat patients individually by targeting the pathways that are specifically present or activated in that specific tumor. Monoclonal antibody treatments have been effective against proteins that are presented outside of the cell, such as trastuzumab in breast cancers and rituximab for B cell lymphomas that express CD20 [32]. Accordingly, small-molecule inhibitors have been used to interfere with signaling networks inside of cells (Figure 1). For instance, Gleevec is a small-molecule inhibitor that can be used to treat a subset of chronic myelogenous leukemia, which is driven by BCR-ABL [33]. So far, several lines of evidence have suggested that SPINK1 may be a potential novel target for PCa treatment. First, SPINK1 is an excellent "druggable" target. Although recurrent gene fusions involving ETS family transcription factors have been identified in ~50% of the PCa patients in western countries, their products are considered poor therapeutic targets by conventional pharmaceutical approaches, owing to lack of enzymatic activity, inaccessibility in the nucleus and requirement of interaction with other proteins to achieve specificity. SPINK1 protein is a protease inhibitor, which can be



**Figure 1:** SPINK1 promotes epithelial-mesenchymal transition (EMT) through EGFR signaling pathway in PCa as a growth factor. SPINK1 binds to EGFR as an autocrine growth factor. There are at least three signaling pathways downstream of EGFR, including the PI3K/AKT, JAK/STAT, and MAPK/MEK/ERK signaling pathway. All three downstream targets are phosphorylated by SPINK1 and EGF. However, the MAPK/MEK/ERK pathway is actually mainly involved in SPINK1-induced epithelial-mesenchymal transition (EMT).

secreted into the extracellular matrix [34]. Of note, SPINK1 may be secreted by the same cell that it stimulates, and thus it can be classified as an autocrine growth factor [25]. In vivo experiments have confirmed it to be druggable [25], and because of the effect of antibodies against SPINK1 on prostate, colon, and breast cancer xenografts, it has been suggested that humanized monoclonal antibodies to SPINK1 should be developed for cancer therapy [11]. Secondly, in vivo data showed that administration of a monoclonal antibody to SPINK1 reduced cellular proliferation and the invasion and growth of SPINK1-producing 22RV1 tumor xenograft [25]. Interestingly, this inhibitory effect was enhanced by co-administration of EGFR antibody (Cetuximab) [25].

In recent years, EGFR was proposed as a promising candidate target since about 18% to 40% of advanced PCa patients showed high EGFR expression [35]. However, in a multi-center clinical trial, the use of cetuximab and other EGFR antagonists in the treatment of advanced PCa patients did not achieve the desired effect [36]. Interestingly, a response has been observed in only about 10% of the advanced PCa cases with treatment of Cetuximab and other EGFR antagonists. Of note, this is the proportion of PCas that show increased SPINK1 production; however, it is not known whether the response is associated with SPINK1 expression and whether combined use of EGFR inhibitors and SPINK1 antibodies would improve the response.

It is likely that humanized SPINK1 antibodies to be used for treatment of PCa are under development [11]. Patients suitable for such treatment need to be selected on the basis of SPINK1 expression.

## Challenges and Perspectives

Although humanized monoclonal antibodies are emerging as a new therapeutic strategy for various malignancies, the development of therapeutic antibodies requires a deep understanding of cancer serology, protein-engineering techniques, mechanisms of action and resistance, the interplay between the immune system and cancer cells, and presence of potential adverse side effects [37]. In PCa, SPINK1 has potential applications in the prognosis and therapeutic target selection on cancer patients. However, there are several issues to be solved in the forefront. Firstly, why SPINK1 is overexpressed in a subset of ETS fusion negative PCa cases? It is hypothesized that an unknown molecular lesion that initially drives ETS fusion negative tumors may predispose to activation of SPINK1 overexpression [25]. Secondly, what is the exact link between SPINK1 overexpression and PCa invasion and progression? Do any other novel molecular mechanisms exist besides SPINK1-EGFR axis? Thirdly, and most importantly, is SPINK1-targeted therapy effective to the subset of high grade PCa cases with SPINK1 overexpression? How to design the molecules against SPINK1 protein and what is the best delivery strategy? We believe the answers to above questions would pave the way for better diagnostic and/or therapeutic modalities of SPINK1+ advanced PCa patients.

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