Decreased Srcasm Expression in Esophageal Squamous Cell Carcinoma in a Chinese Population

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Abstract. *Backround: Src-family tyrosine kinases (SFKs) play critical roles in regulating cellular differentiation and proliferation. Src-activating and signaling molecule (Srcasm) is a novel molecule that down-regulates SFK activity and promotes cell differentiation. The aim of this study was to determine whether Srcasm expression was altered in esophageal squamous epithelial carcinoma compared with normal epithelium in a Chinese population. Materials and Methods: We examined Srcasm immunohistochemical staining in 30 cases in both normal esophageal epithelium and esophageal squamous cell carcinoma (SCC) from the same patient in formalin-fixed paraffin embedded tissue blocks. Results: Srcasm protein expression levels are decreased in esophageal SCC compared to the esophageal normal epithelium. Conclusion: This pattern of Srcasm expression suggests that it may act as a negative regulator in esophageal SCC cell signaling.*

The esophageal lining is protected by a non-keratinized stratified squamous epithelium. Esophageal squamous epithelium is self-renewing tissue that maintains a stable integument by carefully regulating epithelial cell growth and terminal differentiation. The mechanisms controlling the switch from a differentiated to a proliferative state in esophageal epithelium are the key point events to understanding esophageal epithelial disorders, especially the origin of esophageal carcinoma.

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Src-family tyrosine kinases (SFK) play critical roles in regulating cellular differentiation and proliferation (1). Three SFKs (Src), (Fyn) and (Yes) are ubiquitously expressed in the human body, whereas others such as (Lyn), (Lck), (Hck) are mainly expressed in non-adherent cells of the hematopoietic system. Elevated SFK protein expressions have been observed in some kinds of human carcinomas, such as colon carcinoma (2), breast carcinoma (3), and non-small cell lung cancer (4).

Src-activating and signaling molecule (Srcasm), a recently described molecule, is a substrate of SFKs that can down-regulate levels of activated SFKs (5). *In vitro* studies, increased Srcasm expression in human primary keratinocytes regulates the activity of p44/42 (MAP) kinases, and promotes differentiation (6). *In vivo* studies showed that K14-Fyn transgenic mice were derived and characterized; they demonstrate K14-Fyn mice demonstrate a thickened, hyperplastic, and scaly epidermis dependent on increased Fyn expression. In contrast, K14-Fyn/Srcasm transgenic mice did not manifest the hyper-proliferative phenotype. Increasing Srcasm expression suppresses Fyn-induced epidermal hyperproliferation, and promotes cellular differentiation (6). In addition, Srcasm protein expression is reduced in human skin squamous cell carcinoma (1) and other cutaneous hyperproliferative lesions (7), as determined by immunohistochemistry and Western blotting, suggesting that reduced Srcasm levels are associated with squamous epithelial carcinoma.

Given the properties of Srcasm, it was hypothesized that Srcasm expression may be altered in esophageal squamous epithelial carcinoma compared with normal epithelium. To evaluate this hypothesis, we examined Srcasm expression in formalin-fixed paraffin-embedded tissue blocks.

Materials and Methods

Immunohistochemistry. This retrospective study, which used surgically resected specimens from the archives of the Department of Thoracic Surgery at the University of Zhengzhou in Zhengzhou, Henan, China, included 30 cases of both normal esophageal

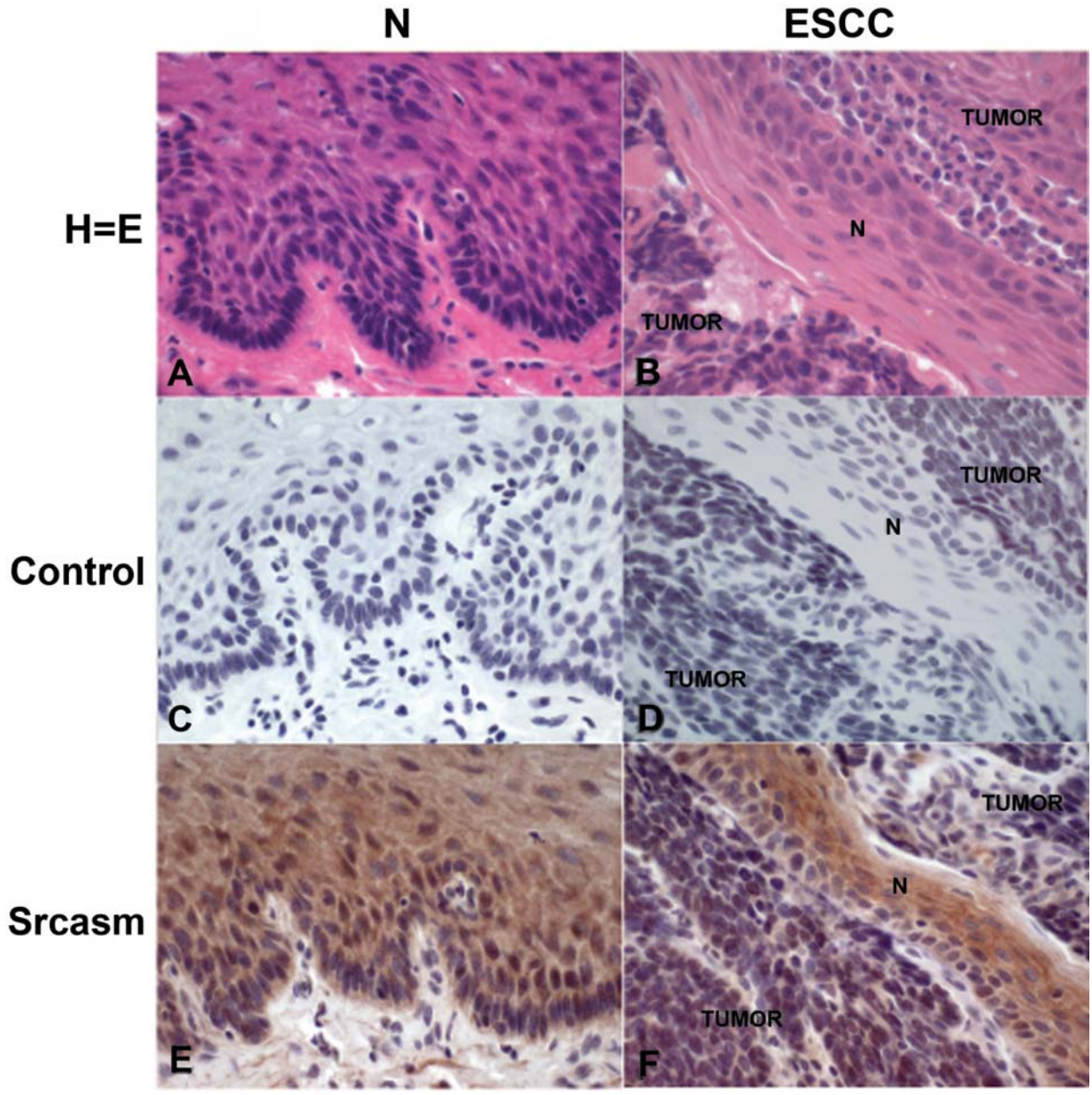

Figure 1. *Immunohistochemical staining for Src-activating and signaling molecule (Srcasm) in normal esophageal epithelium (N) and esophageal squamous cell carcinoma (ESCC). A, B: Normal epithelium and ESCC staining with hematoxylin and eosin (×400). C D: Negative control slides, where the primary Srcasm was replace by purified rabbit IgG, showed no staining of the Srcasm in the normal esophageal epithelium (N) and ESCC. E: Srcasm staining was stronger in normal esophageal mucosa layer, especially in the epithelium, than in the lamina propria layer and the muscularis mucosa layer. F: Srcasm staining was weak in ESCC. In one slide, the Srcasm staining in normal epithium was significantly stronger than in the ESCC.*

epithelium and esophageal squamous cell carcinoma from the same patient. In all cases, recut slides were stained with hematoxylin/eosin and examined to confirm the diagnosis (Figure 1 A, B). The preparation, specificity and reliability of rabbit Srcasm antibody used in the study were described previously (1). In brief, the sample slides were heated, deparaffinized, rehydrated and rinsed in distilled water. In order to expose masked epitopes, the sections were incubated in 10 mM citrate buffer (pH 6.0) at 85˚C for 20 minutes, and then kept at room temperature for 30 minutes, followed by distilled water wash. The activity of endogenous peroxidase was blocked in 3% H_2O_2 for 10 minutes, then the slides were washed three times in (PBST). The tissue sections were blocked at room temperature for 1 hour with 10% normal goat serum, then incubated with 1:50 dilution of affinity purified rabbit Srcasm antibody overnight at 4˚C. Affinity purified biotinylated goat anti-rabbit polyclonal immunoglobulin (BD Biosciences,

Pharmingen, San Diego, CA, USA) was applied to the tissue sections at a 1:100 dilution for 30 minutes. Prediluted streptavidin-horseradish peroxidase (BD Biosciences) was applied to the sections and incubated for 40 minutes, and histochemical development was performed using a liquid 3,3'-diaminobenzidine tetrahydrochloride substrate kit (Invitrogen, San Francisco, CA, USA) for 2 minutes. The sections were rinsed in PBST between the incubations. The slides were subsequently counterstained in hematoxylin, dehydrated and cleared. For negative control, purified rabbit IgG were instead of the primary antibody (Invitrogen). In all cases, there was no staining in the negative control (Figure 1 C, D).

Analysis of staining intensity. All slides were evaluated independently by two individuals for staining intensity and the extent of staining in esophageal squamous cell carcinoma (ESCC) and normal esophageal epithelium (N). The intensity of staining was graded as follows: 1, none; 2, weak; 3, moderate; and 4, strong. No lesion exhibited a zero staining intensity. The extent of staining was assessed as follows: 1, 0-25%; 2, 25-50%; 3, 50-75%; 4.75-100%. For each specimen, a staining index was determined by multiplying the intensity factor by the extent factor. The maximum index is 16. The data show the average staining index for each sample with the standard deviation (Figure 2).

Statistical analysis. The two tailed Student's *t*-test was performed to analyze the means and standard deviations of the Srcasm expression in the normal and ESCC groups, *P*-value<0.05 were considered statistical significantly.

Immunoblotting. For Western blotting, Srcasm antisera was diluted 1:1000. Three pairs of tissue samples of ESCC and normal esophageal epithelium tissue were obtained after esophagectomy and frozen at –80˚C until use. Portions of ESCC tissue and normal esophageal mucosa tissue were homogenized in a radiommune precipitation (RIPA) lysis buffer and the protein concentration was determined by standard curve methods; 30 μg of each protein lysate were subjected to SDS-PAGE analysis.

Results

A total of 30 cases of ESCC from Chinese patients were examined. In normal epithelium group, the Srcasm staining was stronger in the esophageal mucosa layer, especially in the epithelium tissue, than in the lamina propria and the muscularis mucosa (Figure 1E). On the contrary, in ESCC group, Srcasm staining was weak in the same patient (Figure 1F).

To further characterize this inverse relationship, we performed a semiquantitative assessment of staining on the two groups. The Srcasm staining index in the ESCC group was markedly lower compared with that of the normal epithelium group (Figure 2), and this difference was statistical significantly (Table I).

Protein lysates derived from the three pairs of patient samples were subjected to Western blot analysis to determine levels of Srcasm in ESCC and normal epithelium. Analysis of protein derived from normal esophageal epithelium contained greater amounts of Srcasm than equivalent amounts of protein lysate derived from ESCC (Figure 3A). A densitometric analysis of Western blot data after standardization to β-actin signals is represented graphically (Figure3B).

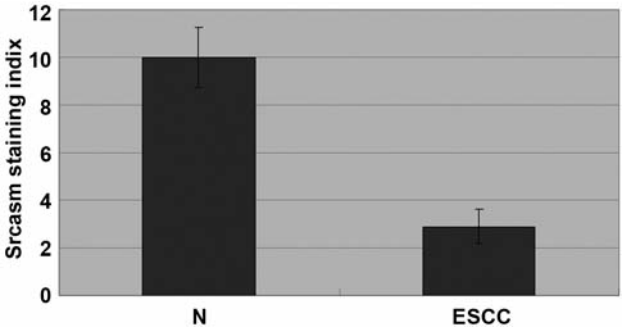

Figure 2. *Src–activating and signaling molecule (Srcasm) staining index in normal esophageal epithelium (N) and esophageal squamous cell carcinoma (ESCC). Standard deviations of mean values are indicated.*

Table I. *Student's t-test p-value for the Srcasm staining index.*

	N	Mean	Standard deviation	*P*-Value
Normal epithelium	30	10	2.51	<0.01
ESCC	30	2.88	1.45	

Discussion

To explore the role of Srcasm in human esophageal squamous cell carcinoma, we studied the expression of Srcasm protein in ESCC and normal epithelium. The data show that the Srcasm protein expression in the normal epithelium was significantly stronger than that in ESCC (Figure 1). At the same time, Western blot analysis confirmed our these findings (Figure 3). The results suggest that a decreased Srcasm level is a common feature of ESCC, and implies that Srcasm may participate in the ESCC cell signaling, promoting esophageal epithelial cell differentiation. Until now, it was not known what signaling pathway Srcasm is involved in and how Srcasm down-regulates esophageal squamous cell differentiation.

Previous studies indicated that the SFKs play important roles in regulating cellular proliferation and differentiation (1). Activation of SFK is an important mechanism for promoting epithelial cell growth. On the other hand, increased activity of SFKs is observed in several kinds of epithelial tumors. In theory, increased SFK activity in tumors could be caused by activating mutations and/or impairment of down-regulatory mechanisms. However, SFK activation mutations in these tumors are rare(8), hence impaired down-regulation of activated SFK is perhaps the main mechanism

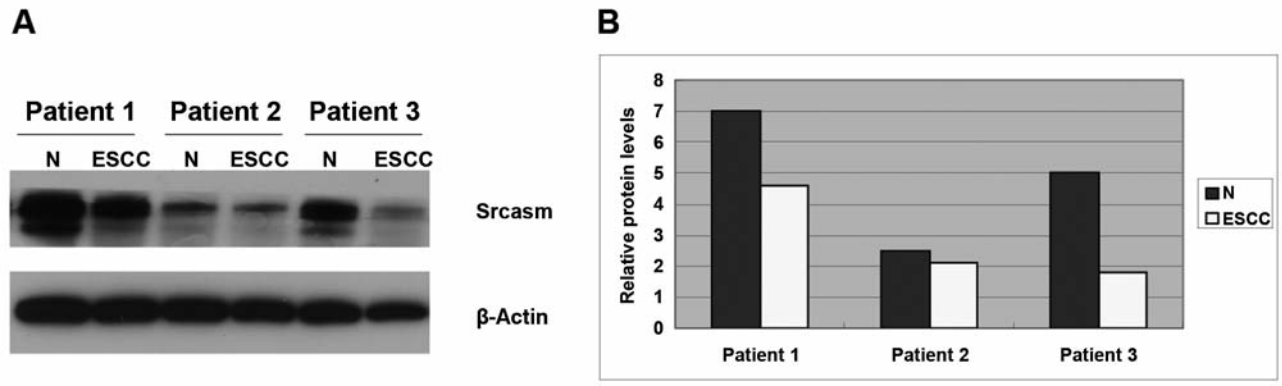

Figure 3. *Immunoblotting analysis of Srcasm in ESCC and normal epithelium. A: Three pairs tissue samples of esophageal squamous cell carcinoma (ESCC) and normal esophageal epithelium (N) were homogenized in a radiommune precipitation (RIPA) lysis buffer and protein concentration was determined by standard curve methods. Lysates were subjected to SDS-PAGE analysis. B: A densitometric analysis of Western blot data after standardization to β-actin signals is represented graphically.*

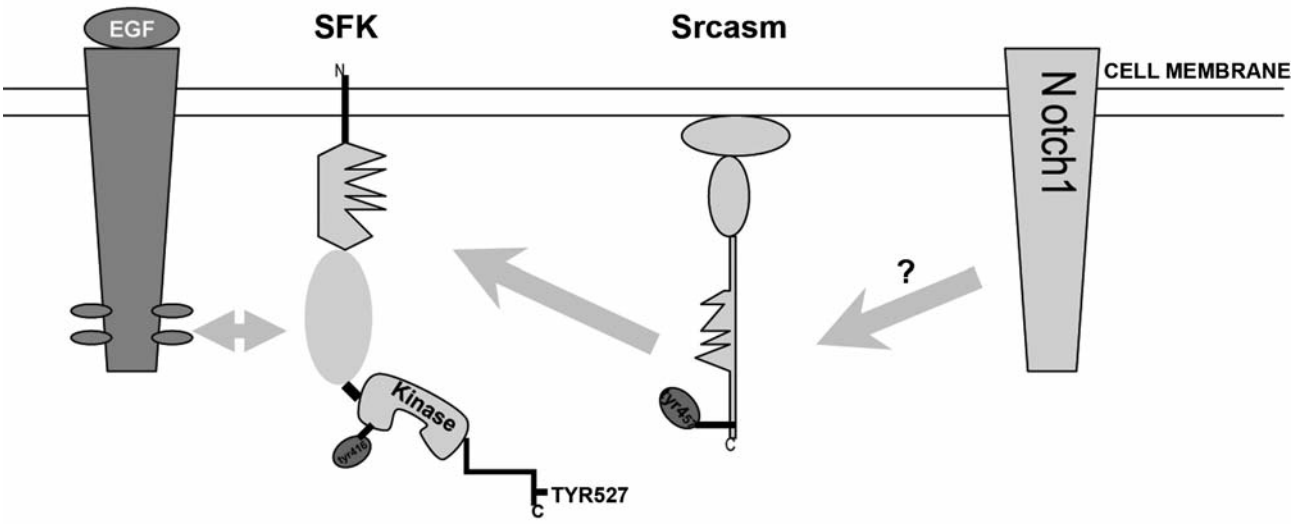

Figure 4. *Srcasm modulation hypothesis in human esophageal squamous cell carcinoma. Srcasm is a component of the EGFR and SFK-dependent signaling pathway (1), and acts as a negative regulator. What is the upstream modulating molecule of Srcasm? Our data show Srcasm protein expression levels are decreased in esophageal SCC compare to the normal esophageal epithelium. It is consistent with the reported Notch1 expression in ESCC (14). We hypothesize that Srcasm is a intermediate molecule of receptor tyrosine kinase (RTK) cell signaling and Notch1 cell signaling pathways, and serves as a conjunct downstream signaling molecule.*

to account for the high level of SFK in these kinds of tumors. In structural analysis, the SFK are characterized by four highly conserved Src homology (SH) domains termed SH_1 to SH_4, of which, SH_2 and SH_3 domains are protein to protein interaction domains interacted with proline-rich sequences and phosphotyrosine-containing motifs, respectively. The competitors of the SH_2 and SH_3 domains can alter the activity of kinases. Srcasm contains the optimal ligands for SH_2 and SH_3 domains of SFK, and can regulate the activation of SFK (9). These features suggest that Srcasm may play a role in regulating the levels of tyrosine kinase signaling and may be associated with the transition from proliferation to differentiation of cells.

Li *et al.* (1) first examined the expression of Srcasm protein. A total of 17 actinic keratoses (early dysplasia), 8 squamous cell carcinoma *in situ* (SCISs), and 12 squamous cell carcinoma (SCCs) biopsy samples were examined. The results showed that, there were readily detectable decreases in Srcasm staining in 15/17 actinic keratoses, 7/8 SCISs, and 10/12 invasive SCCs. The relative decrease of Srcasm

staining between lesional and normal epidermis was more readily detected in SCC than in SCIS and actinic keratoses. This suggests that there is an inverse correlation between Srcasm expression and keratinocyte proliferation. Meulener *et al*. (7) further analyzed 16 seborrheic keratoses (SKs), 7 basal cell carcinomas (BCCs) and 5 samples of normal epidermis with Srcasm antibody and Ki-67 antibody. The results manifested that more staining was detected in the stratum spinosum and granulosum in normal epidermis. Meanwhile, a low percentage of Ki-67-positive staining was detected in the same site. In BCCs, Srcasm expression was markedly diminished. In contrast, the Ki-67 staining in all BCCs was higher. They confirmed that the intensity of Srcasm staining correlates well with the degree of cellular differentiation. In the SK group, similar results were found. In light of these data, they hypothesized that the Srcasm levels are inversely related to keratinocyte proliferation and are directly associated with keratinocyte differentiation. We found a similar relationship between the ESCC group and the normal esophageal epithelium group.

Srcasm expression in ESCC and normal esophageal epithelium appeared to have an inverse relation to the epidermal growth factor receptor (EGFR) protein expression (10). In human keratinocytes, Srcasm is a component of the EGFR and SFK-dependent signaling pathway (1). However, the exact mechanism is not clear. Interestingly, at the same time, we notice that Notch1 signaling pathway is implicated in selfrenewal of stem cells, cell fate determination of progenitor cells, and terminal differentiation of proliferation (11). Notch1 protein was highly expressed in the basal layer of mouse esophagus (12) and human skin of epidermis (13). In esophageal cancer, Notch1 typical ligand expression in esophageal cancer is significantly lower than that in adjacent tissue (14). According to our study, Srcasm protein expression in ESCC (Figure 1 E, F) is consistent with the reported Notch1 expression.

These data can help us to hypothesize that Srcasm is an intermediate molecule of receptor tyrosine kinase (RTK) cell signaling pathway and Notch1 cell signaling pathway, and serve as a conjunct downstream signaling molecule (Figure 4). The activation and the inactivation of Srcasm may regulate the transition of cells from proliferation to differentiation.

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