EMMPRIN Expression in Oral SCC Is Regulated by FYN Kinase

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Abstract. *This study shows that the expression of the extracellular matrix metalloproteinase inducer (EMMPRIN) in oral squamous cell carcinoma cells (SCC) depends upon activation of the Src Family kinaseFyn; and that EMMPRIN and β6 form a complex that requires active Fyn and the full length β6 integrin cytoplasmic domain. Fyn is also important for matrix remodeling as it regulates both matrix type 1 metalloproteinase (MT1-MMP) and tissue inhibitor of metalloproteinase-1 and -2 (TIMP1/2). The tumor promoter/suppressor caveolin-1, which associates with MT1-MMP, also requires FYN activation for expression. Lastly, EMMPRIN expression can act as a readout for the mitogen-activated protein kinase (MAPK) pathway, since when MAPK is blocked, so is the expression of EMMPRIN. In oral cancer, the activation of FYN occurs post β6 integrin ligand binding. That the activation of FYN drives EMMPRIN expression and several important pathways associated with invasive oral SCC is now demonstrated.*

Approximately 96% of all oral carcinomas are squamous cell carcinoma (SCC) (1). The extracellular matrix (ECM) regulates many aspects of cell behavior, including growth, survival and invasion. Membrane type 1 metalloprotease (MT1-MMP) is a transmembrane metalloprotease that plays a major role in ECM remodeling, directly by degrading several of its components and indirectly by activating pro-MMP2 (2). During oral SCC progression, the ECM is remodeled by matrix metalloproteinases (MMPs) many of which are activated by EMMPRIN (extracellular matrix metallo-proteinase inducer), a transmembrane molecule belonging to the immunoglobulin superfamily (3). The mitogen-activated protein kinase (MAPK/ERK1/2) pathway is one of the most thoroughly characterized and important intracellular signaling pathways involved in growth, stress and differentiation (18).

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Our previous work showed that β6 integrin/Fyn interaction phosphorylates MAPK (ERK1/2) (6). MAPK is activated by MEK (MAPK kinase) which phosphorylates key tyrosine[185] and threonine[183] residues. Our group previously noted that EMMPRIN expression was up-regulated with β6 integrin in oral cancer (4-6). Some studies have indicated that in tumor cells EMMPRIN regulates vascular endothelial growth factor (VEGF) *via* PI3K/Akt, a serine/threonine protein kinase that plays a key role in multiple cellular processes such as cell proliferation, apoptosis, transcription and cell migration (7). More recently it was shown that EMMPRIN can complex with β1 integrin (α3 and α6) and may play a role in regulating MMP production (8). Caveolin-1, a component of caveolae, is reportedly both a tumor promoter and tumor suppressor and directly interacts with integrin α1 (β1) (17). It also complexes with MT1-MMP and acts as a membrane adapter between integrin and Fyn (17). Caveolin-1 has also been shown to play a role in integrin α5β1 signaling (9). We have found caveolin-1 to be regulated by FYN activity which associates it with tumor promotion.

Materials and Methods

SCC cells. The SCC9 cell line was derived from a base of tongue lesion and was a generous gift from Dr. James Reinwald (Brigham and Woman's Hospital, Harvard School of Medicine). The SCC9β6 and SCC9CAFyn cell lines were established by stable transfection of the SCC9 cells with cDNAs for full length β6 and constitutively active FYN (CAFyn) respectively (6, 12). The SCC9β6 cells were transduced with the cDNA for a kinase-dead FYN or a truncated β6 cDNA lacking the *C*-terminal 11 AA to establish the SCC9β6KDFyn and the SCC9β6D1 lines, respectively (6, 13). The FYN cDNAs were a generous gift of Dr H. Kawakatsu (University of California, San Francisco). The cDNA for the full length β6 and the cytoplasmic deletion were generously provided by Dr. Dean Sheppard (University of California, San Francisco).

Growth conditions. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum plus penicillin (100 U/ml) and streptomycin (100 g/ml). Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

Reagents. Anti-EMMPRIN mouse monoclonal antibodies MAb 1354 (clone 7F11; Chemicon International, Temecula, CA, USA), and rabbit monoclonal antibodies (clone EPR4052; Gen Tex Inc.,

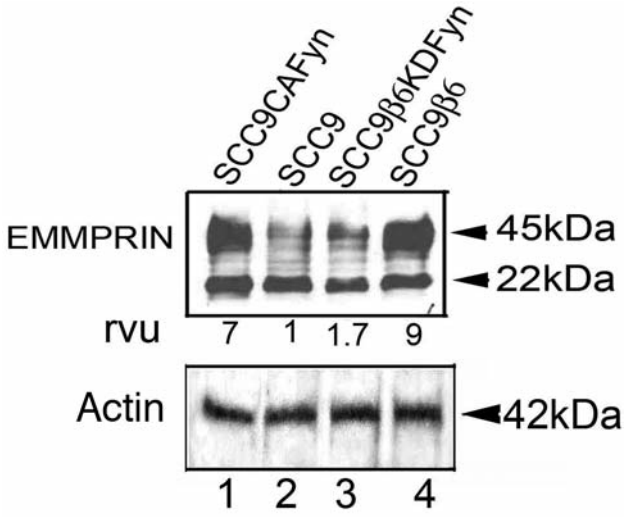

Figure 1. *EMMPRIN expression in relation to FYN. Western blot of SCC cells with anti-EMMPRIN antibodies (45 and 22 kDa). rvu, Relative value units. Antibodies to β-actin were used as a loading control.*

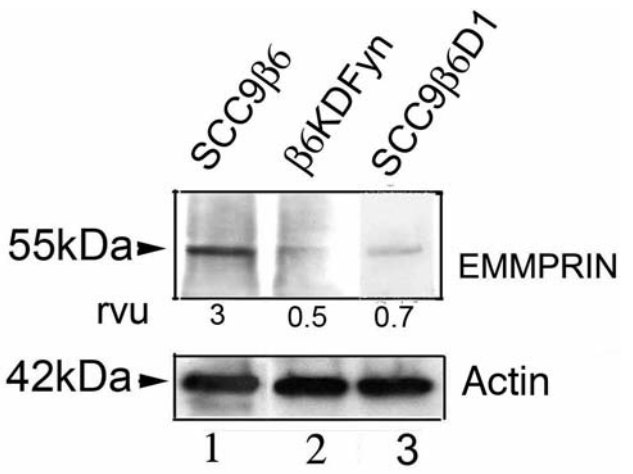

Figure 2. *EMMPRIN in relation to integrin β6. Anti-β6 antibodies immune precipitates of lysed SCC cells analyzed by Western blotting with anti-EMMPRIN antibodies. rvu, Relative value unit. Antibodies to β-actin were used as a loading control.*

Irvine, CA, USA) were used for Western blotting. Goat anti-mouse polyclonal (AF772) and goat anti-rabbit polyclonal (clone R1930) antibodies were purchased from R and D (Minneapolis, MN, USA) and Sigma Aldrich (Louis, MO, USA), respectively. Monoclonal antibodies to β-actin were purchased from Sigma-Aldrich. Monoclonal antibodies to to β6 (RG69 were provided by Dr. Dean Sheppard (University of California, San Francisco). MEK inhibitor U0126 was purchased from Calbiochem (San Diego, CA, USA). Polyclonal antibodies to caveolin-1 (#3238) were purchased from Cell Signaling (Danvers, MA, USA). Monoclonal antibodies to TIMP1 (clone 102D1) and TIMP2 (clone T2101) and rabbit polyclonal antibodies to MT1-MMP were purchased from Thermo Fisher Scientific (Fremont, CA, USA).

Immunoprecipitation (IP). The SCC cells (2×10^5/ml) were lysed by treatment with Nonidet P-40 lysis buffer and incubated with the primary anti-β6 antibodies, RG69 and Csβ6 overnight at 4˚C. The immunocomplex was captured by adding protein A-agarose for 1 h at 4˚C. The immunoprecipitate was washed with lysis buffer prior to determination of protein content and analyzed by Western blotting.

Western blotting. SCC cells were serum-starved for 24 h and then plated onto fibronectin (10 μg/ml) for 24 hours at a density of 2×10^5/ml. The cells were lysed in Nonidet P-40 lysis buffer (1.5% Nonidet P-40, 150 mM, NaCl, 0.2% SDS, 1 mM EDTA, 20 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 mM NaF). Protein concentration in the lysate was determined by BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The proteins were separated by SDS-PAGE and transferred to a membrane (Micron Separation Inc, Westborough, MA, USA) using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA, USA). The membranes were then developed using an ECL Chemiluminescence Kit (Amersham, Piscataway, NJ, USA) and assigned relative value units (rvu) using an image analysis program (NIH Image, Bethesda, MD, USA).

Results

Effect of FYN kinase on EMMPRIN expression. As shown by Western blot the SCC9CAFyn and the SCC9β6 cell lines expressed more than 5-fold more EMMPRIN than the SCC9 and SCC9β6KDFyn cell lines (Figure 1). This demonstrated that in the presence of activated FYN, the expression of EMMPRIN was increased.

EMMPRIN complexes with β6. We previously showed β6 forms a complex with FYN kinase (6). The SCC9β6, SCC9β6KDFyn and SCC9β6D1 cells grown were IP using anti-β6 antibodies before Western blot analysis. A band of 55 kDa was detected in the SCC9β6 cell lysate and was 6-fold greater than that seen in the SCC9β6KDFyn and 4-fold greater than that found in the SCC9β6D1 cell lysates (Figure 2). The residual complex formation seen in both the SCC9β6KDFyn and SCC9β6D1 was probably due to incomplete suppression by the dominant negative constructs used to establish the cell lines. These results demonstrated that EMMPRIN and β6 formed a complex which required active FYN and the full length β6.

Effect of FYN activation on MT1-MMP. The SCC cell lines (SCC9, SCC9CAFyn, SCCβ6 and SCC9β6KDFyn) were analyzed by Western blot for expression of MT1-MMP (Figure 3). The expression of MT1-MMP was 3-fold greater in the SCC9CAFyn and SCC9β6 cell lines compared to the SCC9β6KDFyn or the SCC9β6D1 cells, indicating that active FYN promoted MT1-MMP expression.

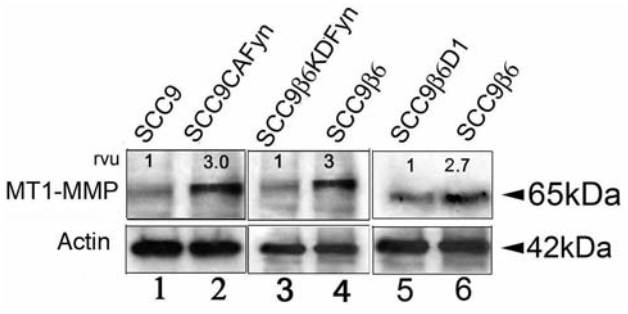

Figure 3. *Activation of FYN regulates expression of MT1-MMP. Western blot for MT1-MMPexpression using three pairs of lysates from SCC cell subtypes. rvu, Relative value unit. Anti-actin was used as a loading control.*

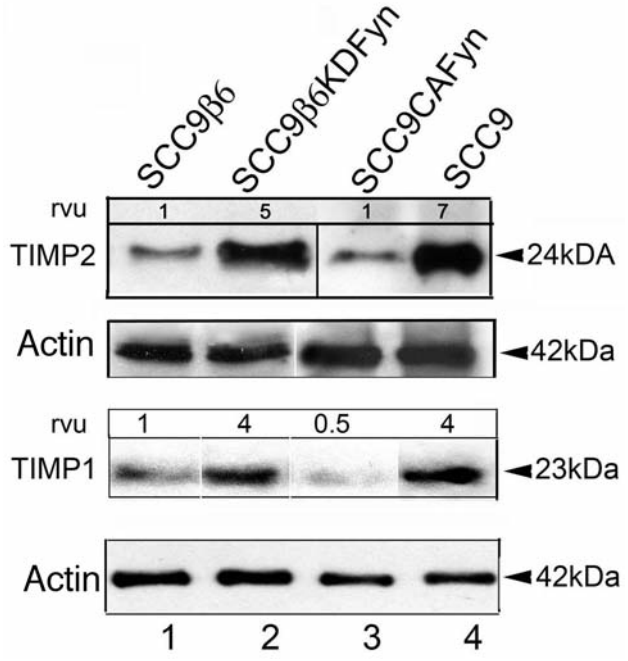

Figure 4. *Effect of FYN kinase on expression of TIMP1/TIMP2. Western blot of the expression of TIMP2 and TIMP1 in SCC cell subtypes. rvu, Relative value units. Actin was used as a loading control.*

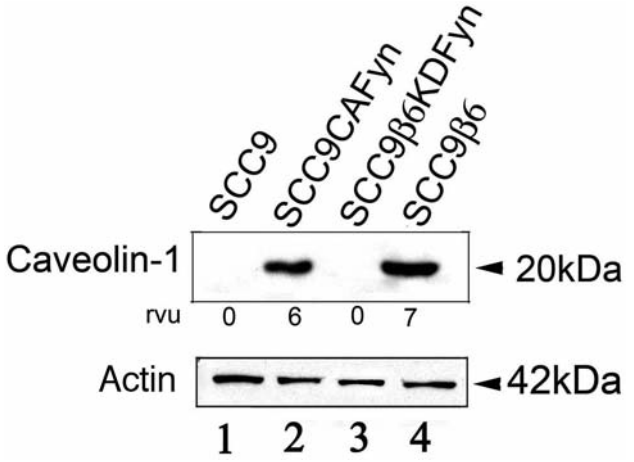

Figure 5. *Effect of FYN on expression of caveolin-1. Western blotting of SCC cell subtypes with antibodies to caveolin-1. rvu, Relative value unit. Actin was used as a loading control.*

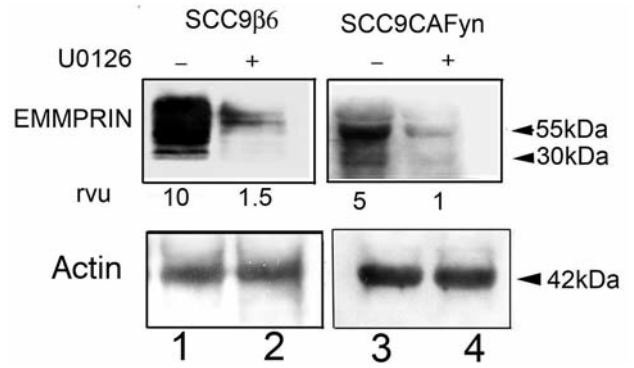

Figure 6. *EMMPRIN expression in relation to the MAPK pathway. Western blot of SCC9β6 and SCC9CAFyn cells grown serum-free on FN for 24 h in the presence (+) or absence (–) of MEK inhibitor U0126 (0.1 μM). rvu, Relative value unit U0126: (–): (lane 1; rvu: 10; lane 3; rvu 5); (+) (lane 2; rvu 1.5; lane 4, rvu 1).*

Effect of FYN kinase on TIMP1/2Western blot showed that TIMP1/2 expression was increased when FYN activation was suppressed (Figure 4). When FYN was suppressed in the SCC9β6KDFyn cell line, the expression of TIMP2 and TIMP1 was approximately 5-fold greater than in the SCC9β6 cell line. Similarly, the SCC9 cells expressed 4- to 7-fold greater levels of TIMP1 and TIMP2, respectively, compared to the SCC9CAFyn cells. These results clearly demonstrated that FYN kinase suppressed TIMP1/2 expression.

Effect of FYN on caveolin-1 expression. Western blotting Showed that caveolin-1 was not detectable when FYN activation was low (SCC9 and SCC9β6KDFyn) but was robustly expressed in the SCC9β6 and SCC9CAFyn cell lines (Figure 5), demonstrating that activation of FYN promoted caveolin-1 expression.

EMMPRIN expression in relation to MAPK. To test whether MAPK activation was required for EMMPRIN expression, the SCC9β6 cells were treated with the MEK1 inhibitor U0126 (0.1 μM) for 48 h. The cells were washed, then grown on FN for 24 h (serum-free) before Western blotting. In the presence of the MEK inhibitor the expression of EMMPRIN was reduced between by 80 and 90% (Figure 6), clearly demonstrating that EMMPRIN expression required MAPK activation.

Discussion

We showed previously that the progression from dysplasia to oral cancer is accompanied by an increase in expression of EMMPRIN coinciding with invasion by oral SCC cells (4).

EMMPRIN expression was found to be downstream of FYN kinase since the forced expression of a kinase-dead FYN, noticeably reduced EMMPRIN expression, reinforcing the importance of post β6 ligand binding events. Our previous work was shown to suppress experimental metastasis when the kinase-dead FYN construct was expressed by invasive oral SCC cells (6).

The present study now demonstrated that EMMPRIN expression was increased in the presence of a constitutively active FYN, suggesting that FYN acts as an intermediate, regulating expression of EMMPRIN through β6-ligand binding. FYN activation is central to the invasive potential of oral cancer cells and promotes changes to the organization of the ECM. EMMPRIN binds to the actin and tubulin cytoskeleton and helps regulate cell shape and promote cell motility (3).

EMMPRIN complexes with α3β1 and α6β1 laminin receptors (8), and, as now demonstrated, β6 integrin and EMMPRIN also form a complex. The exact function or purpose of these complexes is unclear at this time. The EMMPRIN/β6 complex formation required activated FYN and the full length β6 integrin and was significantly reduced with β6 11 AA truncation (β6D1) or a kinase-dead FYN. Based on previous work from our laboratory, it appears that β6/FYN interactions are essential for many phases of oral cancer progression, including the deposition of the ECM (6).

EMMPRIN–MT1-MMP interactions have also been shown to be important in the regulation of EMMPRIN activation (19) and it was suggested that releasing a 22 kDa fragment into the surrounding stroma may promote MMP activation in the tissue, thus facilitating invasion. In the present study, both MT1-MMP and EMMPRIN were positively influenced by activation of FYN. The reverse was also true for both EMMPRIN and MT1-MMP. This clearly pointed to a direct positive feedback loop between FYN activation in oral SCC and the expression of MT1-MMP.

SRC family kinases (SRC, YES, and FYN) phosphorylate caveolin-1 and induce association with MT1-MMP (14). This interaction is pivotal to MT1-MMP-mediated locomotion (14). The current study found high expression of caveolin-1 in the SCC9β6 and the SCC9CAFyn cells, both of which express high levels of active FYN, whereas without FYN activation, caveolin-1 was not detected. The pattern of matrix degradation and deposition in oral SCC is regulated to a great extent by the activation of MMPs by EMMPRIN. This is countered by TIMP activity, which regulates the degradation of the ECM by MMPs (20). A negative feedback loop was found between the activation of FYN and expression of TIMP1/2, which was appropriate, as during tumor invasion and matrix remodeling negative regulators of MMPs should be turned off. Inhibiting FYN kinase was shown by our group previously to suppress MAPK (ERK1/2) and this was important for experimental metastasis (6). Using the MEK inhibitor U0126 to block MAPK activation, the expression of EMMPRIN was clearly suppressed, demonstrating that EMMPRIN expression was optimal when MAPK was activated. This is a complex process initiated by the initial β6 integrin ligand binding. Coupled to our previous studies on EMMPRIN, we demonstrate that in a subset of oral cancer cells, the β6/FYN/EMMPRIN backbone is central to invasion. When oral cancer cells are plated onto FN substrates, the full length β6 forms a complex with FYN kinase and EMMPRIN. and disruption of this complex may serve as a target for future oral SCC therapy.

Acknowledgements

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