Fibroblasts in Head Neck Squamous Cell Carcinoma Associated with Perineural Invasion Have High Level Nuclear YAP Expression

Running Title: Fibroblasts YAP expression in head neck carcinoma

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Abstract: We retrospectively studied the expression of YAP using immunohistochemical staining in 10 cases of head neck squamous cell carcinoma with associated peri-neural invasion. We find that fibroblasts in areas associated with peri-neural invasion show higher levels of nuclear YAP (nYAP), compared to fibroblasts in the stroma of normal mucosa, with a median cell count of 35.4 per high power field in the former and 3.9 in the latter. No differences were observed between the expression of YAP phosphorylated at Ser127 (pYAP) in the tumoral stroma compared to that in the normal mucosa, with a median cell count expression of 4.9 in the former versus 5.0 in the latter. Therefore, a strong and increased nYAP expression in fibroblasts associated with peri-neural invasion in head and neck squamous cell carcinoma suggests that YAP-mediated transcription programs in these fibroblasts may contribute to peri-neural invasion.

Key Words: Squamous cell carcinoma; perineural invasion; YAP expression; fibroblast
Introduction:

The tumor microenvironment plays an important role in the growth and invasive properties of tumors [1], recently reviewed factors contributing to squamous cell carcinoma invasion in the head and neck including cancer associated fibroblasts (CAFs). Fibroblasts within a tumor, CAFs, are an important component of the tumor stroma, contributing to both the extracellular matrix and the release of growth factors that promote tumor growth and invasion. [2-6] YAP is a transcriptional coactivator, known to partner with TEAD and other transcription factors to regulate gene expression. In response to Hippo signaling YAP is phosphorylated at Ser 127 (pYAP) and retained in the cytoplasm by 14-3-3-dependent cytoplasmic sequestration, preventing the activation of YAP-mediated transcription programs that regulate cell proliferation, cell death and cell-fate decisions [6, 9]. Additionally, ECM rigidity, cell tension and changes in cell geometry activate a YAP-dependent mechanoresponse independent of Hippo signaling [7, 8].
Previous studies have shown elevated YAP expression in tumor cells, e.g. YAP is strongly expressed in tumor cell islands in basal cell carcinoma [9, 10], but YAP is also expressed in fibroblasts, such as peripheral nerve fibroblasts [13] and YAP has been implicated in lung fibroblast activation and fibrosis. [11] Both total and nuclear YAP (nYAP) increased expression are associated with poor patient survival in numerous cancers, suggesting that YAP expression may have prognostic value. [8] Recent evidence from fibroblasts within mouse mammary tumors at different stages of progression that show increased nYAP suggests that YAP may have a tumor-promoting role in fibroblasts within a tumor, in addition to its more established role in epithelial cells. [12]

In the present study, we investigated the expression of YAP and pYAP in fibroblasts at the sites of peri-neural invasion in head and neck squamous cell carcinoma compared to fibroblasts in the stroma of normal mucosa and previous biopsy sites. We find that fibroblasts in areas associated with peri-neural invasion show higher levels of nYAP, compared to fibroblasts in the stroma of normal
mucosa, suggesting that YAP-mediated transcription programs in these fibroblasts may contribute to peri-neural invasion.

Materials and Method:

Tissue samples:

For this initial case series study, we selected 10 cases of head and neck squamous cell carcinoma with peri-neural invasion from our head and neck cancer database of over 500 cases. The patients were mostly elderly males with a mean age of 71.1 years and a male to female ratio of 9 to 1. Paraffin blocks were retrieved from the files of the Department of Pathology at Montefiore Medical Center, Albert Einstein College of Medicine, Bronx, NY. The Institutional Review Board of Montefiore Medical Center, Bronx, NY granted us the permission to use clinical information and tissue samples for research purposes.

All tissues were routinely fixed in 10% buffered formalin and embedded in paraffin. The YAP (catalog number 4912) and phospho-YAP(Ser127 catalog
number 4911) antibodies are from Cell Signalling, while the secondary antibody, Dako Envision+ system-HRP Labelled Polymer, anti-rabbit and chromogen DAB Substrate kit, 3,3'-diaminobenzidine are from Dako Co. YAP and pYAP expression was assessed in stroma from areas of previous biopsy site reaction and tumoral stroma.

**Immunohistochemical stain:**

The paraffin sections were cut at 5-um thickness and were placed on positively charged slides. Slides were placed in a 60°C oven for an hour, then deparaffinized and rehydrated through a series of xylene and graded alcohols. Endogenous peroxidase was quenched in 3% H2O2 for 10 minutes. Antigen retrieval was performed by placing the slides in an Oyster vegetable steamer with Cell Marque, Trilogy solution for YAP and Dako Target Retrieval Solution for phospho-YAP (Ser127). The staining procedure was performed in an automatic slide stainer, Dako Autostainer Plus. The primary rabbit polyclonal antibodies, YAP and phospho-YAP(Ser127) were applied in a dilution of 1:50 for 30 minutes
at room temperature followed by a secondary antibody for 30 minutes and DAB
Substrate, 3,3'-diaminobenzidine as chromogen for 10 minutes. Slides were
counterstained with Surgipath Hematoxylin, dehydrated through graded alcohols,
cleared in xylene, and cover slipped with cytoseal 60 from Richard-Allen
Scientific.

The staining patterns were classified as cytoplasmic or nuclear and the staining
intensity was divided into 4 categories as: 0 no staining, 1+ weakly stained, 2+
moderately stained and 3+ strongly stained. Only cells staining as 2+ or 3+ were
considered positive. The number of positive fibroblasts at each site, normal
stroma and tumoral stroma were determined by counting the total number of
positive cells in 5 high power fields and calculating the average number of cells
per high power field. The median for the average counts at each site for YAP and
pYAP are presented in Table 1. Two pathologists performed the assessment of
YAP and pYAP expression in benign and tumoral stroma. There was good
concordance between the pathologists; results from one pathologist are reported in Table 1.

**Results:**

Since YAP has been suggested as a prognostic marker in numerous cancers, YAP expression was examined with a particular focus on cancer associated fibroblasts (CAFs) in tumoral stroma and areas associated with peri-neural invasion using both an antibody that detects total YAP protein as well as an antibody that specifically detects YAP phosphorylated at Ser127.

Analysis of the 10 cases demonstrated CAFs at sites of peri-neural invasion and in cancer stroma had a higher number of fibroblasts displaying strong nYAP expression compared to fibroblasts in areas of benign mucosa. Overall, the median fibroblast count with nYAP expression was 35.4 cells / HPF in tumoral stroma, which was nearly 10 times higher than that found in the benign stroma (3.9 cells/HPF) (Table 1). Some pYAP staining was also observed in the CAFs at
sites of peri-neural invasion and cancer stroma, but the levels were lower than that of nYAP. In benign squamous stroma associated with previous biopsy site reaction, there were low levels of fibroblasts with YAP and pYAP staining.

Overall, YAP expression level was higher in both CAFs and tumor cells, especially the nuclear YAP (nYAP). The pYAP was also seen in both CAFs and tumor cells, but mainly in the cytoplasmic location. Expression of YAP and pYAP in CAFs at sites of peri-neural invasion as well as fibroblasts in tumoral stroma, benign mucosal stroma are shown (Figures 1-2).
Discussion:

We studied the expression of YAP and pYAP in tumoral stroma CAF, CAF associated with peri-neural invasion, and fibroblasts at normal squamous mucosa and previous biopsy sites. We found that CAF and CAF with perineural invasion had a high YAP expression. It has already been demonstrated that tumors, especially malignant ones that contain a complex extracellular matrix due to desmoplastic reactions to the tumor cells, increase stiffness of the extracellular matrix, which further enhances tumor invasion and metastasis. The Hippo pathway regulates organ size and tumorigenesis in Drosophila and mammals and is altered in a variety of human cancers.[13, 14] In a study of the Hippo pathway in ovarian cancer by Hall and colleagues, the authors found that the effective YAP, specifically high levels of nuclear YAP or low levels of cytoplasmic phosphorylated YAP (cpYAP), was associated with poor survival in ovarian cancer patients. Consistent with these findings, Steinhardt and colleagues found that nuclear and cytoplasmic YAP protein expression was significant higher in
carcinoma cells of the colon, lungs and ovaries than in their counterpart benign components, indicating active proliferation.[15] Kim’s group has reported that YAP and pYAP protein expression were increased in breast borderline/malignant phyllodes tumor compared to in benign phyllodes tumor [16] also found that higher nuclear expression of YAP was associated with worse overall and disease-free survival in esophageal squamous cell carcinoma patients in a Korean population.[16] Most studies investigated YAP protein expression in malignant epithelial or stromal cells. In Calvo et al.’s study, the ability of CAFs to promote cancer cell invasion was also significantly dependent on YAP function, whereas TAZ was not required. [17] The authors also showed that depletion of YAP reduced the ability of CAFs to form fibrous collagen networks and promote angiogenesis in vivo, while TGF-b promoted nuclear accumulation of YAP. [12]

In our study, we found that high level of nYAP in cancer associated fibroblasts associated with perineural invasion, with fibroblasts that have a low level of cpYAP expression indicating a more aggressive pattern of invasion. They also
had more extensive desmoplastic stroma composed of inflammatory cells, which might produce some chemokines, cytokines and TGF-b, [18] which could be involved in accumulating nuclear YAP in the CAFs. In benign mucosa, no to few fibroblasts are present, and they usually have weakly nYAP expression and seem less activated. Our observation was consistent with the finding by others that YAP with a nuclear location was critical for CAFs to produce a rigid collagen network that stimulates and promotes tumor invasion into peripheral nerves.

In conclusion, strong nYAP staining in peri-neural invasion associated stromal fibroblasts in head and neck squamous cell carcinoma suggests that YAP-mediated transcription programs may contribute to peri-neural invasion.
Figure legends:

Figure 1, 1a: Benign squamous cell mucosa, H-E section, 200x; 1b: Immunostain of YAP, no to rare basal cells and fibroblasts with nuclear and cytoplasmic expression 200x; 1c: Immunostain of pYAP, scattered basal cells and fibroblasts with nuclear and cytoplasmic expression 200x.

Figure 2, 2a: Squamous cell carcinoma with perineural invasion, H-E section, 200x; 2b: Immunostain of YAP, fibroblasts with strong nuclear YAP expression, tumor cells with weak cytoplasmic expression 200x; 2c: Immunostain of pYAP in the fibroblasts with perineural invasion, fibroblasts and tumor cells with mainly cytoplasmic expression 200x.

Reference


Table 1 Yap and pYap positive fibroblasts in tumoral stroma versus benign stroma

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Figure 1

215x279mm (300 x 300 DPI)