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Fra-1 Expression in Salivary Gland Cancer Cells

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Abstract

Salivary gland cancers, neoplasms of the head and neck, have shown to exhibit a wide variety of biological, pathologic, and clinical variations. The standard treatment protocol approach is surgical resection of the gland or the tumor followed with postoperative radiation. Currently, studies have been focused on identifying, characterizing and exploring molecular signatures at the genomics and protein levels which could be used for potential therapies and clinical trials. Fra-1, a member of the activator protein-1 transcription factor superfamily, has shown to play pivotal roles in various types of malignancies. To date, no research has been performed in identifying the role of Fra-1 in salivary gland cancers. In this study, we first demonstrated that Fra-1 is highly expressed in salivary gland cancers. Secondly, data obtained from immunoblot analysis also showed Fra-1 protein expression to be highly expressed in the tumor cell lines and in the tumor matrix induced cells when compared to the immortalized normal salivary gland cells. Furthermore, our results showed nuclear localization of Fra-1 protein in the tumor cell lines and the tumor matrix induced transformed cells. Examining the role of Fra-1, would shed light in providing a therapeutic approach of salivary gland cancers.

Introduction

Salivary gland cancers are a heterogeneous group of tumors of great morphological diversity that pose significant diagnosis and therapeutic challenges. Even though salivary gland cancers are relatively rare, about 1.2 out of 100,000 men and 0.7 out of 1,00,000 women per year are reported to be affected [1]. There are three major salivary glands in the oral cavity. The parotid gland, located behind the ear, which produces the majority of saliva in the oral cavity, the submandibular gland located at the angle of the mandible, and the sublingual gland on the floor of the mouth. Salivary gland cancer contributes to 3-4 percent of all head and neck neoplasms, of which 80% occur in the parotid gland [2-6]. Pleomorphic adenomas are the most frequent benign tumors of the salivary glands and 60% of these tumors occur in the parotid gland [4,5]. These adenomas occasionally show an invasive behavior and can be treated by surgical excision [7,8]. The second most common type of benign salivary gland tumors, caused as a result of myoepithelial cell differentiation, are the Warthin's tumors [9]. Salivary gland tumors pose a significant challenge to clinicians because of their remarkable variation in etiology and biological behavior. Despite the uncommon and aggressive nature of these tumors; very little research has been done in identifying the driving factor in these aggressive tumors.

Researchers have identified various transcription factors to be unregulated in various cancers [10]. Continuous stimulation by these transcription factors has been shown to increase protein synthesis resulting in uninhibited growth and potential metastasis [11]. Activator protein-1 (AP-1) a transcription factor, a driver of carcinogenesis, is known to be commonly unregulated in cancers [12]. This dimeric transcription factor AP-1 consist of homodimers Jun-Jun or heterodimers Jun-Fos. The Jun family consists of c-Jun, Jun B, and Jun D and the FOS family consists of c-Fos, Fos B, Fra-1 and Fra-2 [13]. The family of AP-1 transcription factors has been reported for their ability to induce oncogenic transformation along with cellular proliferation, apoptosis, invasion and angiogenesis [14]. Inhibiting AP-1, an oncogenic factor, has been the current target for cancer intervention.

Up regulation of The Fos related antigen-1 (Fra-1), a member of the AP-1 transcription factor family, has been shown to promote metastasis in multiple cancers such as breast cancer, lung cancer, mesothelioma, bladder, and colon cancer [15-18]. In cases of aggressive breast cancer, Fra-1 expression has been reported to be a prognostic factor [17]. Fra-1 is a key protein in colon cancer metastasis and its expression can be used to predict colon cancer prognosis risk [15,19,20].

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Published reports have shown that transfection of Fra-1 into rat fibroblasts leads to tumor initiation and proliferation. Ablation of Fra-1 gene has shown to suppress the invasive phenotypes of tumor cell lines [12]. Stabilization of Fra-1 is dependent on ERK-MAPK phosphorylation which prevents Fra-1 proteolysis. ERK-MAP kinase pathway has been shown to play a major role in proliferation and differentiation in different cell types [21-23]. Reports have shown that inhibition of the ERK-MAPK signaling pathway can result in a considerable decrease in the migration and invasion of cancer cells [20]. Wnt signaling pathway has been shown to be regulated by Fra-1. Published studies have shown that activation of Wnt/β-catenin results in rapidly growing, aggressive squamous cell carcinomas (SCC) in the salivary glands [9]. Thus, Fra-1 can regulate the Wnt and MAP kinase signaling pathways by regulating cellular proliferation [23,24]. However, no published studies have been performed investigating the role of Fra-1 in salivary gland cancers.

Therefore, in this present study we determine the expression of Fra-1 in salivary gland cancers. Results from our study demonstrated a substantial increase in Fra-1 expression in pleomorphic adenoma cell line at the transcriptional and translational levels when compared to normal and epidermoid carcinomasalivary gland cell line. Furthermore, our study also reports that the extracellular matrix (ECM) of the salivary gland tumor cells could transform normal cells to a tumoral phenotype resulting in Fra-1 expression levels at both the gene and protein levels.

Materials and Methods

Cell lines

Normal human submandibular salivary gland cell line (HSG), a kind gift from Dr. B Baum, NIH in Bethesda, MA; Human salivary gland epidermoid carcinoma cell line (HTB-41), obtained from ATCC and human polymorphic adenoma salivary gland tumor cell line (PA-30) [25], a generous gift from Dr. L Queimado, University of Oklahoma, TX were cultured under sterile and aseptic techniques. PA-30 cells were cultured in RPMI 1640 media (Corning, Cellgro, in Manassas, VA). DMEM/F-12 media (Corning, Cellgro, Manassas, VA) was used to culture HSG cells and McCoy's 5a Media (Corning, Cellgro, Manassas, VA) was used to culture HTB-41 cells. All these cell lines were complemented with 10% FBS, 1% amphotericin, and 1% penicillin/streptomycin (Corning, Cellgro, Manassas, VA) at 37°C and 5% CO_2 until a minimum of 70% confluency was achieved.

Antibodies

Anti- Fra-1 antibody, fluorescent conjugated secondary antibodies, tubulin, HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG were obtained from Santa Cruz CA, USA. Monoclonal anti- Tubulin antibody was purchased from Sigma, MO.

Tumor derived extracellular matrix

Human salivary gland epidermoid carcinoma cell line (HTB-41) and human polymorphic adenoma cell line (PA-30) were cultured in their corresponding growth media till 90% confluency was attained. In order to keep the extracellular matrix intact, the cells were treated with buffer 1 (10mM sodium phosphate, 150mM sodium chloride, and 0.5% Triton X-100) solution at 37°C in a tissue culture incubator for 30 minutes, followed by the treatment with buffer 2 (25 mM ammonium hydroxide) for 20 minutes. The cell free matrix was rinsed three times with Hanks balanced salt solution (HBSS). Normal human submandibular salivary gland cell line, HSG, were seeded in this tumor derived matrix derived from HTB-41 cells (HSG*) and PA-30 cells (HSG#) and were cultured under aseptic conditions for 48 hours

Immunofluorescence

The HSG, PA30, HTB-41, HSG# and HSG* cells were grown on cover glasses for 48 hours until a confluency of 70% was attained. The cells were fixed with 3% paraformaldehyde for 1 hour at room temperature, and then washed three times using 1X PBS. Permeabilization was done with 0.1% Triton X-100 for 20 minutes. The cells were rinsed with 1x PBS and then blocked with 5% BSA for one hour. Following blocking, the cells were incubated overnight using anti-Fra-1 (1:100), followed by a second incubation with fluorescent-conjugated goat anti-rabbit IgG. Post washing with PBS, α-Actin (1:10,000) was used to stain the cytoskeleton of the cells. The cells were mounted using Prolong Gold mounting media containing DAPI (Invitrogen, Grand Island, NY) and an Olympus Flu View FV300 Confocal Microscope was used to capture the images. Each experiment was performed three times.

Real-Time PCR

Using the Qiagen RNA Extraction kit (Qiagen, Valencia, CA), RNA was extracted from the cells at 48 hours and1 μg of total RNA was reverse-transcribed. Using ABI Step One Plus Instrument, RT-qPCR was performed and real time PCR was performed with Fra-1 and GAPDH primers. Estimation of the fold-change was performed using the $2^{-\Delta\Delta CT}$ method in which CT is equivalent to the log-linear plot of PCR signal versus the cycle number. ΔCT = CT value of target gene – CT value of GAPDH. The primers were purchased from IDT DNA in Chicago, IL, and were validated for RT-PCR, with a primer efficiency of 70-80%. All experiments were performed in triplicate (n=3x3) for each sample of RNA preparation (n=3).

Protein Extraction and Immuno Blot Analysis

Total proteins were extracted from HSG, PA-30 and transformed cell lines (HSG# and HSG*) at 48 hours, using the M-PER reagent (Pierce, Waltham, MA). Using 10% SDS-polyacrylamide gel, 35μg proteins were resolved. Following electrophoresis, electro-transfer of 35ug proteins onto nitrocellulose membrane (Bio-Rad Laboratories, Philadelphia, PA). The membranes were then blocked with 5% non-fat milk, and probed for 16 hours at 4 °C with anti-Fra-1 antibody (1:500). After several washed, with 1XPBS, the blots were incubated with HRP conjugated goat anti-rabbit IgG secondary antibody (1:3000) for 1 hour. The membranes were washed and the visualization of bands, were done using ECL-Western blot reagent (PerkinElmer Life Sciences Hopkinton, MA). The blots were each washed carefully, treated with a stripping buffer (Pierce, Waltham, MA) for five minutes, and then rinsed with PBS. Equal loading of proteins were confirmed by using anti-tubulin antibody (1:10,000) and HRP conjugated goat anti-mouse IgG secondary antibody (1:10,000) and Western blot analysis was performed.

Results and Discussion

Up regulation of Fra-1 gene expression in human salivary gland tumor cells and tumor-matrix induced salivary gland normal cells

Previous studies have shown that Fra-1 is important for metastasis in cancer such as breast cancer, lung cancer, mesothelioma, bladder, and colon cancer [15-18]. Research has not been conducted as to expression of Fra-1 in salivary gland cancer. In order to determine the mRNA expression of Fra-1 in salivary gland cells, total RNA

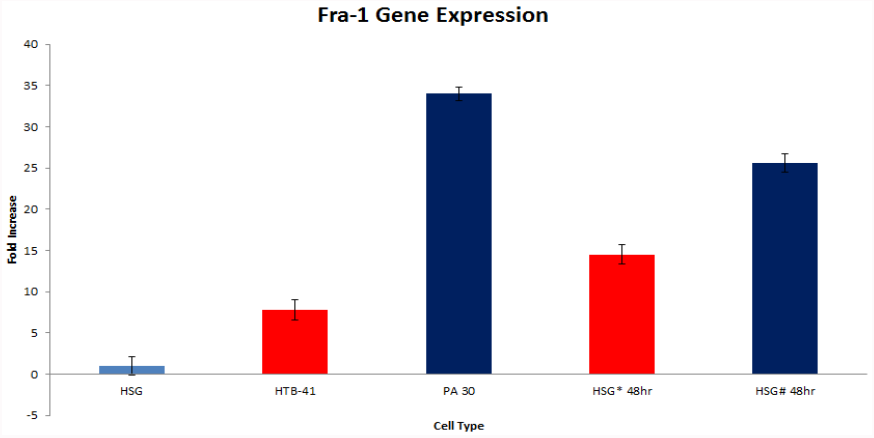

Figure 1: Fra-1 gene expression.
Total RNA were isolated from HSG, HTB-41, PA-30, HSG* (HSG cells on HTB-41 extracellular matrix), and HSG# (HSG cells on PA-30 extracellular matrix) cell lines. Real-time PCR for gene expression of Fra-1 was performed. The results were normalized with the loading control GAPDH for real-time PCR. Experiments were done in triplicate. *p<0.005.

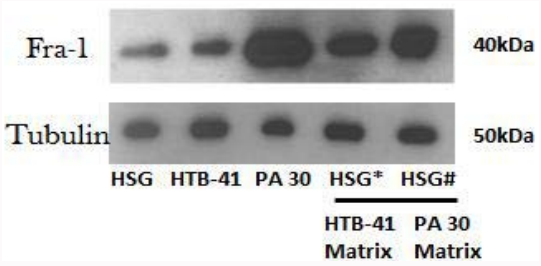

Figure 2: Endogenous Fra-1 protein expression.
Total proteins were isolated from HSG, HTB-41, PA-30, HSG*(HSG cells on HTB-41 extracellular matrix), and HSG# (HSG cells on PA-30 extracellular matrix) cell lines. Western Blot analysis for Fra-1 protein expression was performed using anti-Fra-1 antibody (1:500). These results were normalized with the loading control tubulin. Experiments were done in triplicate.

was isolated from HSG (normal), HTB-41(epidermoid carcinoma) and PA-30 (polymorphic adenoma) cell lines and real time PCR was performed using Fra-1 primer. GAPDH was used as the housekeeping gene. Our results clearly showed that HTB-41 cancer line expressed a 7.7 fold and PA-30 expressed a 34.0 fold Fra-1 mRNA when compared to the normal human salivary gland cell line (HSG) (Figure 1).

Fra-1 expression has been quantified in a number of cancers all with an up regulation compared to the normal cells [13]. Pleomorphic adenoma is the most common tumor of the salivary gland. Pleomorphic adenoma is a benign tumor commonly seen in the parotid gland composed of stromal and epithelial cells. Although classified as benign, pleomorphic adenomas are frequently multicentric, tend to recur, and can even metastasize. Metastasizing pleomorphic adenoma of the salivary gland is an entity that is poorly understood due to morphologic and histologic features similar to PA-30. This makes it very hard to detect in the rare instance that benign pleomorphic adenoma gains the ability to metastasize [26-28]. Pleomorphic adenoma can also transform into an aggressive carcinoma (named carcinoma ex-pleomorphic adenoma), that frequently metastasizes and has a 5-year survival rate of approximately 30%. The significant increase of Fra-1expression in PA-30 cells could be a key step in its pathogenesis and potential metastatic spread.

We then further explored if the tumor derived extracellular matrix (ECM) could stimulate the expression of Fra-1 in the human normal salivary gland cell line, HSG. Cancer progression is a multistep process involving the activation or inactivation of a number of different genes and proteins. The ECM of the tumor cells is a complex molecular milieu that behaves as a reservoir of several signaling molecules that are essential for the growth, differentiation, survival and proliferation of the cancer cells [29-31]. HSG cells were seeded on the intact tumor derived ECM obtained from PA-30 and HTB-41 cells for 48 hours. Total RNA was extracted and real time PCR was performed with Fra-1 primers. Fra-1 gene expression increased by 14.53 fold in HSG* cells (HSG cells grown on HTB-41 cancer matrix) and by 25.5 fold in the HSG# cells (HSG cells grown on PA-30 cancer matrix) when compared to the normal human salivary gland cells (Figure 1). Furthermore, published studies have shown that expression of Fra-1 can increase cell proliferation and motility in breast cancer cells [32]. Fra-1 also plays a role in regulating tumor progression-associated proteins, like VEGF, MMP-1 and MMP-9, in breast cancer cell lines [32]. Therefore, the results obtained in this present study, clearly shows that up regulation of Fra-1 mRNA expression in the different types of human salivary gland tumor cell lines and the tumor derived ECM induced transformed cells, could play a role in activating genes, proteins and signaling molecules needed for tumor progression.

Fra-1 protein expression is influenced by human salivary gland tumor cells and their respective extracellular matrix

With the increase in Fra-1 in the various tumor cell lines at the transcription level, we next sought to evaluate Fra-1 expression at the translation level. Total proteins were extracted using M-per from HSG, HTB-41 and PA-30 cell lines and Western blotting was performed with anti-Fra-1 antibody. Figure 2 depicts Fra-1 protein expression in these various human salivary gland cell lines. Fra-1 protein was highly expressed in the PA-30 cells when compared to the HTB-41 cell line. However, the protein expressions of the tumor cell lines were higher than in the normal HSG cell line and were consistent with the mRNA expression observed.

In order, to explore the role of the tumor derived ECM mediated Fra-1 protein expression on HSG cells, total proteins were isolated from HSG* and HSG# cells at 48 hours and immunoblot was performed with anti-Fra-1 antibody. Equal loading of proteins were confirmed by reprobing the blot with anti-tubulin antibody. Western blot results

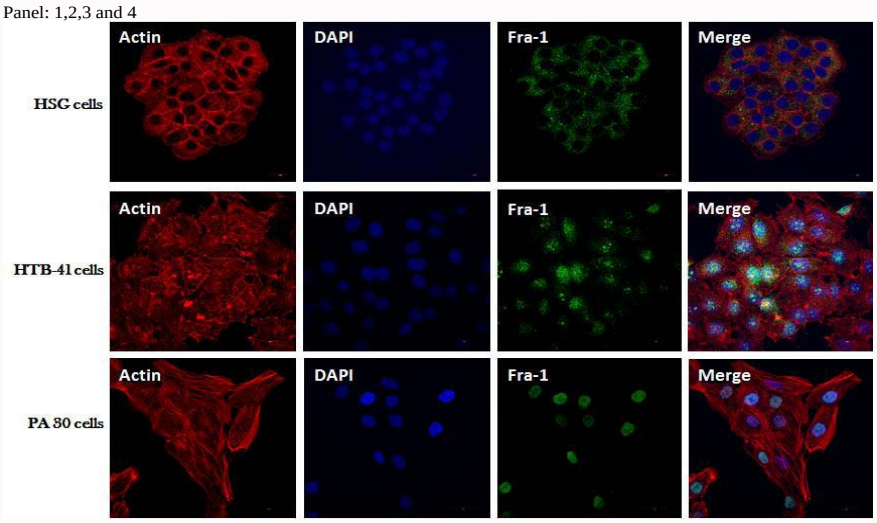

Figure 3a: Localization of Fra-1 in human salivary gland cell lines.
Confocal micrographs of human salivary gland cell lines, fixed and stained with anti-Fra-1 antibody (1:100). Confocal image shows Fra-1 localization in the cytoplasmic compartment of normal human salivary gland cell line, HSG. Nuclear localization of Fra-1in human salivary gland tumor cell line, HTB-41 and PA-30 was observed at 48 hour time point. DAPI stained DNA.

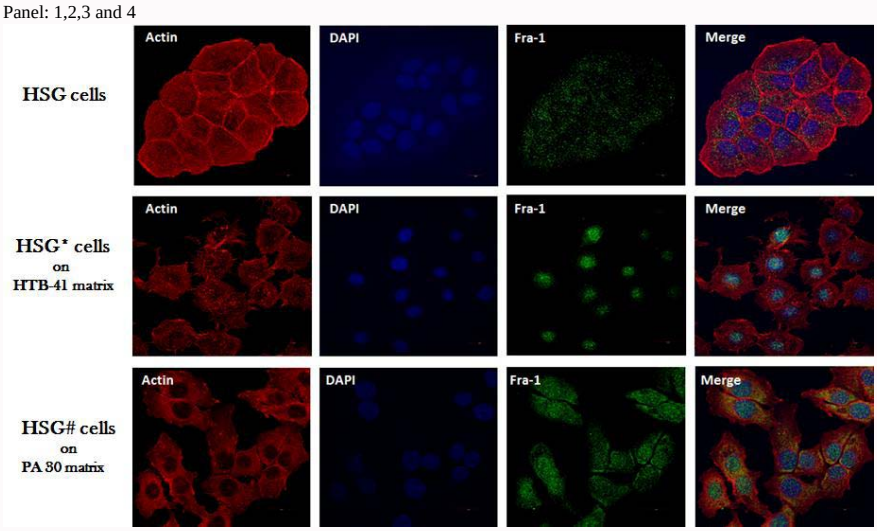

Figure 3b: Localization of Fra-1 in human salivary gland cell lines.
Confocal Micrographs of human salivary gland cell lines, fixed and stained with anti-Fra-1 antibody (1:100). Confocal image shows Fra-1localization in the cytoplasmic compartment of normal human salivary gland cell line, HSG. Changes in cellular morphology and nuclear localization of Fra-1 where observed on HSG cells (HSG*, HSG#) seeded on the HTB-41 and PA-30 tumor matrix. DAPI stained DNA.

clearly showed that HSG* and HSG# cells expressed a significant high amount of Fra-1 protein when compared to the normal HSG cells (Figure 2). Moreover, the normal HSG cells after being seeded in extracellular matrix produced by tumor cell lines expressed Fra-1 protein in levels that were similar to the respective salivary gland tumor cell line that secreted the extracellular matrix where they were seeded. The increase in Fra-1 expression lead us to hypothesize that these HSG# and HSG* cells exhibited a cancerous phenotype. The ability of cancerous extracellular matrix to induce normal cells into a cancerous phenotype expressing Fra-1, is a new and promising area of study. These findings suggest that Fra-1 silencing could represent a potentially effective therapeutic approach in the future.

Studies have shown that Fra-1 protein detection to be useful in the diagnosis and prognosis of human breast tumors [33]. Fra-1 has been reported to play a vital role in promoting activities such as adhesion, protrusion, and cell motility [34]. Invasive behavior promoted byFra-1 expression has been proposed to be a contributing factor for its presence in invasive tumors by inactivating beta-1 integrin and reducing the activity of Rho A [34]. Fra-1 expression in colorectal carcinoma (CRC) has been also extensively studied. According to Peeper DS et al. [15], there is 30-50% decrease in cell survival and a 3-fold decrease in the number of colonies formed by Fra-1 deficient HT29 colon cells. Expression of Fra-1 in the budding tumor cells at the invasive front of human CRC's has been shown to be important for metastatic spread but not for primary tumor growth [15]. Findings from our experiments, clearly show Fra-1 protein to be upregulated in the cancer cell lines and the tumor derived matrix induced transformed cells could also play a vital role in tumor progression.

Localization of Fra-1 in normal, tumor, and tumor derived matrix induced cells

The differential expression of Fra-1 both at the transcriptional and translational levels are not only seen in the different tumor cell lines but are also pertained in the localization of Fra-1. HSG, HTB-41 and PA-30 cell lines were grown on cover glass, fixed and stained with anti-Fra-1 antibody (Figure 3A). Confocal images clearly show that Fra-1 was sparingly dispersed in the cytoplasm of the normal HSG cell line (Figure 3A-panel 3). The cytoskeletal architecture of the actin in the HSG cells showed a normal acinar distribution with all the cells having a similar cellular structure. The merge image in Figure 3A-1with DAP1 staining the nucleus and cytoplasmic staining for Fra-1 antibody depicts this relationship. In contrast, nuclear localization of Fra-1 was observed in the HTB-41 (Figure 3A-panel 3) and PA-30 (Figure 3A-panel 3) cell lines. Actin staining performed on both the cell lines clearly depicts the changes in the cellular morphology from cribriform cellular structure of HTB-41 (Figure 3A-panel 1) to long spindle shaped morphology of PA-30 cells (Figure 3A-panel 1). In conclusion, our results show that HTB-41 and PA-30 cell lines not only have a significant increase in Fra-1 gene and protein expression but the protein is localized to the nucleus where it is thought to influence transcription of its gene targets.

To investigate the role of benign and cancerous ECM on the normal human salivary gland cells, we grew HSG *and HSG # for 48 hours in ECM produced by PA-30 or HTB-41, fixed the cells and stained with Fra-1 antibody. Figure 3B shows the localization of Fra-1 protein in HSG* and HSG# cell lines. Actin staining performed on the HSG* cells depict no resemblance to the normal acinar morphology of the normal HSG cells. Instead cells appear isolated or with fewer cell connections than HSG cells, and thus have been transduced into an abnormal cellular morphology by the tumoral extracellular matrix (Figure 3B-panel 1). Moreover, and in contrast with the original cell line grown in standard conditions, Fra-1 was observed to be nuclear localized in the HSG* cells. Our results also showed that the cellular morphology of the HSG# cells were also distorted (Figure 3B- panel 1). The HSG # cells failed to show the acinar morphology like the HSG cell line or long spindle shaped structure like the PA-30 cell line. Nuclear localization of Fra-1 protein was also seen in HSG# cells. Our finding shows the influence of the tumoral matrix not only on the levels of gene and protein expression of Fra-1 in normal HSG cells but also in its cellular distribution, which is this particular case lead to the nuclear localization of Fra-1, suggesting the role and expression of Fra-1 in salivary gland cancers.

Reports have shown that proteins shuttle between the nucleus and cytoplasm. Since Fra-1 lacks a transcriptional transactivation domain, it partners with c-Jun, to drive gene transcription [35]. The recent evidence has indicated a potential role for Fra-1 in protecting the tumor cells from anoikis [20]. Studies performed in colorectal cancers suggest that cells with activated ERK/MAPK signaling can stimulate FRA-1 to provide the survival signals [20]. Transcription factor like RUNX2, known to play a vital role in differentiation and metastasis, have shown to be nuclear localized in breast, bone and prostrate cancers [36-38]. Nuclear localized RUNX2 can promote breast cancer development by activating the Wnt and Tgf-β signaling pathways [38]. Fra-1 has also been shown to regulate the Wnt signaling pathway in colorectal cancers [39,40]. The Wnt pathway has been studied extensively in salivary gland cancers [41]. Activation of Wnt/β-catenin results in rapidly growing, aggressive squamous cell carcinomas (SCC) in the salivary glands [9]. Published studies have shown that Fra-1 can regulate the Wnt and MAP kinase signaling pathways thus playing a role in cellular proliferation [24,25]. However, a link between Fra-1 and Wnt signaling pathway in salivary gland cancers are yet to be studied. Identifying the mechanism of activation of such pathways, will provide further clue to identifying its potential pathogenesis in salivary gland cancers.

Conclusion

The current findings, show for the first time that Fra-1 is expressed in salivary gland tumor cell lines. Moreover, our study demonstrated a significant increase in Fra-1 expression in salivary gland tumor cell lines at the transcriptional and translational levels when compared to a normal human salivary gland cell line. Furthermore, our study also reports the localization of Fra-1 in the nucleus of tumor cells. Of major clinical relevance, we also showed that the ECM produced by salivary gland tumor cell lines can stimulate an increase in Fra-1 expression in normal salivary gland cells and induce a transformed phenotype similar to salivary gland cancer cells. In the future, Fra-1 silencing could represent a potentially therapeutic approach in targeting early cancer prevention or progression.

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