C-Fos Digital Expression Analysis in Human Papillomavirus-Related Oral Squamous Cell Carcinoma

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Introduction

Solid malignancies demonstrate molecular profiles based on oncogenes’ over activation combined with suppressor genes silence. Concerning oncogenes, Fos Proto-Oncogene or AP-1 Transcription Factor Subunit (c-Fos) represents a critical gene involved in a variety of malignant tumours’ development and progression, including oral squamous cell carcinoma (OSCC). The Fos superfamily comprises c-Fos, FosB, FosL1, and FosL2 genes. c-Fos is a proto-oncogene that is the human homolog of the retroviral oncogene v-fos (gene locus: 14q24.3). It was initially analyzed and cloned in rat fibroblasts as the transforming gene of Finkel–Biskis–Jinkins murine osteogenic sarcoma virus [1]. The gene encodes a 62 kDa protein (380 amino acids),
forming heterodimer with c-jun, a strong transcription factors), resulting in the formation of AP-1 (Activator Protein-1) complex. C-Fos/c-Jun complex influences intracellular signal transduction to the nucleus. c-Fos protein is implicated in critical cell functions including differentiation, proliferation, survival and also tissue homeostasis affected by hypoxia and angiogenesis [2]. Concerning OSCC, c-Fos aberrant expression seems to be a frequent event, especially in sub groups of patients associated on not with their corresponding clinicopathological features [3-4]. In the current study, we analyzed c-Fos protein expression levels in OSCC tissue sections by implementing a digital image analysis protocol on immunostained slides. To our knowledge there are very limited published data regarding the current methodology in OSCC oncoproteins’ analyses.

Materials and Methods

Study group

For the purposes of our study, fifty (n=50) archival, formalin-fixed and paraffin-embedded tissue specimens of histologically confirmed primary OSCC were used. Selection of the cases was based predominantly on the criterion of an aggressive phenotype as it is expressed due to advanced Grade and Stage histo-pathological features. The hospital ethics committee consented (Reference ID Research Protocol: 2226/09.09.2018) to the use of these tissues for research purposes, according to World Medical Association Declaration of Helsinki guidelines (2008, revised 2014). The tissue samples were fixed in 10% neutral-buffered formalin. Hematoxylin and eosin (H&E)-stained slides of the corresponding samples were reviewed for confirmation of histopathological diagnoses. All lesions were classified according to the histological typing criteria of World Health Organization (WHO) (5). Concerning HPV DNA status (positivity or not), the corresponding information was derived from patients’ medical file records. Among them, 18 HPV DNA positive cases were recorded. HPV 16/31/53 High Risk (HR) subtypes were detected mainly by analyzing the corresponding cases. Clinicopathological data of the examined cases are demonstrated in Table 1.

Antibodies and immunohistochemistry assay (IHC)

For the purposes of our study, we selected and applied the mouse monoclonal anti- c-Fos mouse monoclonal (clone CF2, Novocastra, Leica Biosystems, Newcastle, UK; dilution 1:40). IHC protocol for the antigen detection was performed. The antigen - antibody reaction was visualized using 3-3, diaminobenzidine tetrahydrochloride (DAB) as a chromogen substrate (8 min at room temperature). Finally, the tissue sections were slightly counterstained with hematoxylin for 30 sec, dehydrated and mounted. For negative control slides, the primary antibody was omitted. Nuclear predominantly but also peri-nuclear/cytoplasmic staining pattern was considered to be acceptable for the marker. Normal (non-cancerous) skin tissue sections demonstrating c-Fos expression were used as positive markers for its immunostaining pattern (Figure 1a,b).

Digital Image Analysis assay (DIA)

C-fos protein expression levels were evaluated quantitatively by calculating the corresponding staining intensity levels (densitometry evaluation) in the immunostained malignant cells. We performed DIA using a semi-automated system (hardware: Microscope CX-31, Olympus, Melville, NY, USA, Digital camera, Sony, Tokyo, Jp; Windows XP/NIS-Elements Software AR v3.0, Nikon Corp, Tokyo, Japan). Areas of interest per tissue section were identified (5 optical fields at ×400 magnification) and filed in a digital database as snapshots. Measurements were performed by implementing a specific macro (mainly nuclear for tumor cells, according to manufacturer’s datasheet for monoclonal mouse anti-c-fos, Clone CF2, Novostra, Leica Biosystems Newcastle Ltd, UK). Based on an algorithm, normal tissue sections (control) were measured independently and compared to the corresponding values in malignant tissue sections. A broad spectrum of continuous grey scale values (0-255) at the RedGreenBlue (RGB) analysis was available for detecting and discriminating different protein expression levels (Figure 2). Immunostaining intensity values decreasing to 0 represent a progressive over expression of the marker, whereas values increasing to 255 show a progressive loss of its staining intensity. Total results and DIA values are demonstrated in Table 1.

Statistical analysis

Statistics software package IBM SPSS v25 (SPSS Inc, Chicago, IL, USA) was implemented. Associations between variables were assessed with of Pearson Chi- Square (χ2) test and Fisher’s exact. Correlation analysis with Spearman Rank test was performed for variables with significant chi2 associations. Two-tailed p-values ≤0.05 were considered statistically significant. Results and correlations (p-values) are described in Table 1.

Results

According to DIA implementation, all of the examined cases demonstrated c- Fos expression in different levels. C-Fos protein over expression (moderate to high immunostaining intensity values) was observed in 28/50 (56%) tissue sections, whereas low expression rates were
Discussion

Oral cavity carcinoma represents a major proportion of Head and Neck Squamous Cell Carcinoma (HNSCC). OSCCs demonstrate an aggressive phenotype due to their increased capability to locally metastasize combined with distant lymph node metastases [6]. In solid malignancies deregulated transcriptional factors are correlated with aberrant gene expression [7]. Concerning OSCC, c-Fos over activation seems to be associated with other transcription factors (c-Jun/Fra-1) deregulation [8-9]. In fact, their high co-expression is correlated with poor prognosis in the corresponding patients. Besides detected in the rest of the examined cases (22/50-44%). C-Fos protein over expression (moderate to high immunostaining intensity values) was observed in 28/50 (56%) tissue cores, whereas low expression rates were detected in the rest of the examined cases (22/50-44%). C-Fos overall expression was strongly associated with the stage and grade of the examined tumors (p=0.014, p=0.003, respectively) and also with HPV persistent infection (p=0.004). No other statistical correlations were identified regarding the other clinic-pathological parameters (gender: p=0.359, smoking status: p=0.208).

Table 1. Clinicopathological Parameters and Total c-Fos Expression Results

| Clinicopathological parameters | c-Fos | p value |
|--------------------------------|-------|---------|
| **OSCC (n=50)** | OE | LE | OE | LE |
| | n (%) | n (%) | n (%) | n (%) |
| Gender | 44 (88) | 24/50 (48) | 20/50 (40) | 0.359 |
| Male | 22/50 (44%) | 28/50 (56%) | 15/50 (30%) | 3/50 (6%) |
| Female | 4/50 (8) | 13/50 (26%) | 19/50 (38%) | 0.003 |
| HPV history | 18 (36) | 5/50 (10) | 13/50 (26%) | 0.004 |
| Positive | 15/50 (30%) | 10/50 (20%) | 1/50 (2) | 0.014 |
| Negative | 32 (64) | 13/50 (26%) | 19/50 (38%) | 0.208 |
| Grade | 18 (18) | 13/50 (26%) | 8/50 (16%) | 0.003 |
| 1 | 5/50 (10) | 13/50 (26%) | 1/50 (2) | 0.004 |
| 2 | 13/50 (26%) | 1/50 (2) | 0.003 |
| 3 | 13/50 (26%) | 1/50 (2) | 0.004 |
| Stage | 9 (18) | 3/50 (6) | 6/50 (12) | 0.003 |
| I | 5/50 (10) | 13/50 (26%) | 2/50 (4) | 0.004 |
| II | 12/50 (24) | 14/50 (28) | 0.003 |
| III | 10/50 (20) | 1/50 (2) | 0.004 |
| Smoking status | 38 (76) | 22/50 (44) | 16/50 (32) | 0.208 |
| Current | 12 (24) | 6/50 (12) | 6/50 (12) | 0.003 |
| Former | 0.004 |

OSCC, oral squamous cell carcinomas; OE, overexpression (moderate to high expression) staining intensity; values ≤142 on the examined stained nuclei; LE, low expression staining intensity values >156 on the examined stained nuclei.

Figure 1. C-Fos Expression Patterns on OSCC Tissue Sections. a. Over expression of c-Fos. Note nuclear mainly dense brown staining pattern (DAB stain, original magnification: 400x) b. Loss of expression (DAB stain, original magnification: 400x)
In conjunction to the previously described results, another study detected over activation of c-Fos in invasive OSCC compared to adjacent non-malignant epithelia. A combination of nuclear and peri-nuclear cytoplasmic diffuse immunostaining was observed especially in cases demonstrated lymph node metastasis implicating also CD44-dependent signal transduction pathway in patients with advanced stage [15]. Additionally, the role of another signal transduction pathway (Notch) in c-Fos over activation is under investigation. A study group showed that targeting overexpressed Notch genes - including JAG1/JAG2- in OSCC by applying γ-secretase molecule, c-Fos m RNA levels were also decreased [16]. Novel molecular technologies in gene screening based on c-DNAs microarrays have also detected distinct patterns of gene expression in different sub-group of patients, including oncogenes [17]. Concerning c-fos protein expression evaluation, we implemented a DIA protocol that provides an objective, accurate and fast mean for measuring the immunostaining intensity levels. According to our published experience in a variety of biomarkers, DIA is a useful tool for research and diagnostic reasons -under specific terms- enhancing the innovative evidence-based medicine [18-22].

In summary, c-Fos is a critical gene frequently up regulated in OSCCs. C-Fos high expression levels are correlated with an aggressive phenotype (advanced stage/lymph node metastasis) in patients with OSCC. HR HPV-mediated carcinogenesis in a subset of cases is associated also with increased c-Fos protein expression. Due to its elevated oncogenic activity, c-Fos should be a target for novel therapeutic strategies in OSCC combined or not with other oncogenes involving in signalling transduction pathways.

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Declaration of competing interest

None.

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