Study Protocol

Evaluation and assessment of the expression of DNA damage response – related molecules in oral submucous fibrosis (OSF) and oral squamous cell carcinoma(OSCC) with OSF

Abstract:

Background: Oral submucous fibrosis (OSF) is the most common chronic, progressive and irreversible potentially malignant disorder with high probability of malignant transformation (MT). From a clinical as well as the histological point of view, establishing and understanding the molecular nature of malignant transformation mechanism in OSF is almost important. The majority of genetic alteration caused by exogenous and endogenous mutagens is restored by the cell's ability by DNA Damage Response (DDR). DDR mechanism dysfunction is one of the leading causes of MT. In OSF, this investigation remains scare.

Objectives: To determine the DDR molecules expression (γH2AX , 53BP1, pChk2 and p53) in subjects with habit of arecanut and tobacco without OSF, OSF and oral squamous cell carcinoma (OSCC) with OSF and compare and quantify the expression among them.

Methodology: Material and Methods: 90 subjects with 30 individuals in one of the three groups would be included in the given study. Group A: Subjects with habit of arecanut and tobacco without OSF. Group B: Patients with OSF. Group C: Patients having OSCC with OSF. DDR molecule (γH2AX , 53BP1, pChk2 and p53) expression will bequantified by RT-PCR. The expression levels will be analyzed using SPSS software version 17 using one-way ANOVA, followed by post hoc comparisons using Tukey's HSD and Categorial data will be analysed using the chi-squared test.

Expected Results: The OSF lesion prone for development of OSCC, DDR markers (γH2AX , 53BP1, pChk2 and p53) will accumulate before the development of p53 mutation resulting in OSCC

Conclusion: Thus, the present study assess and quantify DDR-related molecules (γH2AX, 53BP1, pChk2 and p53)in OSF patients suggesting the potential benefit in the prevention of OSCC due to early therapeutic exploitation of DDR.

Keywords: Oral submucous fibrosis (OSF), DNA Damage Response (DDR), oral squamous cell carcinoma (OSCC), γH2AX , 53BP1, p53 and pChk2.

Introduction:

Oral submucous fibrosis (OSF) is considered to be a chronic, progressive oral potentially malignant disorder leading to connective tissue fibrosis. Areca nut and betel quid chewing habit, alone or in combination, are the main causative agent commonly practice in

South-Asian countries.[1,2] The prevalence rate in India of OSF ranges in between 0.62% to 6.42%.[3,4] One study reported 3.72% as the malignant transformation rate of OSF with 37.42 months as an average duration.[5] Due to its high malignant transformation (MT) rate, OSF is considered a disease with a significant mortality rate. Worldwide, Oral squamous cell carcinoma (OSCC) has very poor prognoses and remains unchanged even after various advanced therapeutic approach has taken place. Thus, in order to have better prognosis and prevention, the key factor will be early detection of oral squamous cell carcinoma (OSCC) associated with OSF.

DNA Damage response has crucial role to play in normal cell function. In tumorigenesis, aberrations in this pathway results in precancerous and cancerous lesion .[6-9] Surgical excision of these oral potentially malignant disorder (OMPD) which is the routine treatment of choise, does not prevent from happening malignant transformation. But till date no chemopreventive theory which is capable of reversing, reversing or inhibiting malignant transformation are currently available which can target key molecules involved in cancer progression and coversion. This research focuses and will discover such reliable, novel and therapeutic targets involved in the molecular pathways of oral carcinogensis.

Phosphorylated form of H2AX i.e γH2AX is the commonest histones in mammalian cells considered as an accurate marker for Identifying double-stranded breaks (DSBs) and initiating DNA damage repair mechanism, activating DDR molecules and tumour suppression. Mutation of this marker leads to instability within the genome causing increase risk of various cancers including OSCC and Human papilloma virus (HPV)-related ontogenesis and also has been found to be associated with prognostic parameters.[10]

Checkpoint kinase 2 (Chk2), a protein kinase is activated whenever damage in DNA occurs causing G1/S cell cycle arrest and inducing programmed cell death. Mutation of this molecule has been found to be involved in the pathogenesis of oral cancer, breast and prostate cancer and Li- Fraumeni syndrome. HPV-related pathways ontogenesis also is caused by Chk2 mutation.[10]

Tumour suppressor gene, p53 is mutated in nearabout 50% of cancers including oral cancers. After DNA damage, phosphorylation activates p53 thereby stabilizing the protein by preventing the interaction with MDM2, the negative regulators so as to increase its functionality by triggering arrest of the cell cycle and causing apoptosis. P53 plays a crucial role in tumour development in DDR mechanism.[10]

53BP1 (53 Binding Protein 1) is involved in DDR mechanism for DSB repair. It reverses genetic abberations and induces apoptosis and cell senescence. In breast cancer, it has been reported to have tumour suppressive activity inhibiting angiogenesis and affecting patient prognosis.[10]

Thus, this study evaluates the expression of above mentioned DDR markers in various steps of oral cancer ranging from normal mucosa to OSF and its malignant transformation, i.e. OSF with OSCC using qRT-PCR. The markers which has been selected identifies double-stranded breaks (γH2AX) to repair mechanisms activation (53BP1) and causing cell cycle arrest and programmed cell death (p53 and pChk2).

Objectives:

1. To evaluate and determine the expression of DDR molecules in subjects with habit of arecanut and tobacco without OSF.

2. To evaluate the expression of DDR molecules in patients with OSF.

3. To evaluate the expression of DDR molecules in patients with OSCC with OSF.
4. To compare the expression of DDR molecules in the above three groups.

Methods:

Study design – Cross-sectional study

Participants –

Subjects with habit of arecanut and tobacco without OSF, Patients with OSF, Patients having OSCC with OSF

Inclusion criteria

- Individuals with the habit of areca nut use in any form with/without the disease.
- Histopathologically diagnosed case of OSForOSF with OSCC.
- Both genders included.

Exclusion criteria

- Any other inflammatory condition like gingivitis, periodontitis in areca nut users without the disease.
- Any other oral potentially malignant disorders.
- Pregnant and lactating women.
- Those who did not give consent.
- Secondary tumours.

Sample size– **Purposive sampling technique** will be used.

A total of **90** subjects with 30 individuals in one of the three groups would be included in the given study.

Group A: Subjects with habit of arecanut and tobacco without OSF

Group B: Patients with OSF

Group C: Patients having OSCC with OSF

Bias – Gender bias, Age bias (would be removed by matching the subjects between the groups)

Data sources/ measurements –

- **Sociodemographic details** – age, sex, socioeconomic status, education, and occupation (modified Kuppuswamy scale).
- **Tissue abuse** – a detailed history of consumption of areca nut with or without tobacco, frequencyper day, placement site, quadrant/overall, duration of chewing, associated with any other habit.
- Clinico-histopathologically diagnosed cases of OSF and OSF with OSCC.
- **Extraction of RNA:** Tissue samples will be placed in RNA later for further processing and will be stored at -20ºC. Then on the day of processing it will be minced in a tissue homogenizer followed RNA extraction using Ribozol (VWR lifesciences) according to instruction given by manufacturer. The RNA will be assessed using a spectrophotometer (Nanodrop, ThermoFisher). RNA with an absorbance ratio (A260/A280) approximately of 2 will indicate pure RNA.
- **cDNA synthesis:** After quantification, RNA will be reverse transcribed usingPrimeScript™ 1st strand cDNASynthesis Kit (Takara) according to manufacturer's instruction. The cDNA will be confirmed by performing PCR for GAPDH.
- **Relative Quantification using Real Time PCR:** The quantification for samples will be done in Agilent Real-Time PCR using PowerTrack SYBR Green Master Mix(Applied biosystems). The reaction will be carried out in a 10µl reaction volume. Using gene analysis, all the samples will then be tested against control samples. GAPDH will be the endogenous control gene used.

Variables –

- **Outcome** – oral squamous cell carcinoma **Exposure** – OSF
- **Dependent variable** – DDR molecules
- **Independent variable** – OSF
- **Confounding factors**– any other habit, genetic susceptibility, any other inflammatory condition

Quantitative variables –

Demographic data and details of tissue abusive habits – a detailed case history

DDR molecule expression – Quantitative real-time-PCR

Statistical methods –

- All the data will be represented as mean and standard deviation.
- Categorical data will be analysed using the chi-squared test.

- The relative expression levels of DDR molecules will beanalysed by calculating the $\Delta\Delta C_t$ using the following method

$$\delta C_T = C_{T(Gene)} - C_{T(GAPDH)}$$
$$\delta\delta C_T = \delta C_{T(Case)} - \delta C_{T(Control)}$$
$$Fold\ Change = 2^{-\delta\delta C_T}$$

- Intergroup comparison will be performed using one-way ANOVA, followed by Tukey's HSD post hoc analysis on SPSS software version 17 (SPSS Inc., Chicago, IL, USA).Level of significance will be considered to be 5%.

Analysis plan

- OSF patients with or without OSCC after informed consent would be included & detailed history of habit would be obtained.
- Clinico-histopathologically diagnosed cases of 'OSF' and 'OSF with OSCC' would be included.
- The DDR molecule expression patterns of 'OSF' would be compared to the patients of 'OSF with OSCC' using the test of statistical significance.

Scope–

"DDR molecular expression pattern could potentially be implicated as a 'biomarker' for diagnosis of OSCC. A greater understanding of this epigenetic alteration could not only assist the diagnosis and prognosis of oral cancer but could also open up novel therapeutic approaches".

Limitations – As this is a cross-sectional study, further longitudinal study will be required to confirm the utility of these markers.

Implications –

DDR molecular expression pattern could potentially act as a indicator and biomarker for the malignant transformation of OSF.

Expected Outcomes/Results:

Direct and indirect loss of physiological function of p53 is one of the most common events OSCC. "Since p53-mediated cell cycle arrest is a key event in the DDR, loss of p53 function may be an important mechanism by which DNA damage accumulates". Thus, the OSF lesion prone for development of OSCC, DDR markers (γH2AX, 53BP1, pChk2 and p53) will accumulate before the development of p53 mutation resulting in OSCC.

Discussion:

Chewing areca nut is the most common practiced habit in the Indian subcontinent. Areca nut is considered as an independent 'Group 1 human carcinogen by International Agency for Research on Cancer, World Health Organization'. Areca nut along with the slaked lime and various adjunct ingredients is mainly responsible for OSF. Due to low price, easy availability and effective marketing strategies leads to its popularity in young people resulting in increase disease prevalence. 'OSCC associated with OSF' is one of the most common malignancies Southeast Asian countries.[11] Chourasia et al. reported 25.77% of 'OSCC cases associated with OSF'.[12]

In OSCC, deregulation of cell proliferation and differentiation takes place due to several intracellular and extracellular alteration leading to invasion and metastasis. Transition of normal physiological tissue to premalignant and cancerous lesion is a multistep process which occurs in oral carcinogenesis. If diagnosed and managed in the early stages can result in oral cancer prevention. However, due to molecular complexicity, malignant transformation (MT) of these potentially malignant disorder (PMD) creates significant difficulty. The usual treatment of choice for such lesion is surgical excision, but possibility of MT cannot be eliminated. Currently, no chemopreventive agents are available which are capable of reversing, prolonging or preventing this MT by targeting these critical molecules involved in oral carcinogenesis. Thus, this research focuses on to discover such novel reliable prognostic markers and therapeutic targets to prevent MT.[10]

In mammalian cells, DDR signaling molecules are the protein kinases ATM and ATR, which are recruited to and activated by DSBs and replication protein A (RPA)-coated ssDNA, respectively.[13-15] Two best studied ATM/ATR targets are the protein kinases CHK1 and CHK2 which act to reduce cyclin-dependent kinase (CDK) activity by several mechanisms, few of them are mediated by activation of the p53 transcription factor.[16,17] Inhibition of these CDKs arrests or slows down cell-cycle progression at the various check points of cell cycle such as G1–S, intra-S and G2–M, which increases the time available for DNA repair before replication or mitosis begins. ATM/ATR signaling enhances repair by inducing DNA-repair proteins transcriptional by recruiting repair factors to the damage; and also activates DNA-repair proteins by modulating their phosphorylation, acetylation, ubiquitylation or SUMOylation27.[18] Proteomics studies have identified ATM/ATR mediated phosphorylation sites also suggesting that the DDR modulates additional cellular processes.[16] In contrast, if the damage cannot beremoved, chronic DDR signalling triggers cell death by apoptosis or cellular senescence, both of which have potential antitumour functions.[19]

Chromatin structure has an impact on the DDR and also modulates in response to DNA damage. It can be explained in ATM/ATR/DNA-PKmediated phosphorylation of serine 139 of the histone H2A variant, H2AX, on chromatin flanking DSB sites. This leads to ubiquitin-adduct formation in such regions and the recruitment of DDR factors along with other chromatin-modifying components which, together promote DSB repair and amplify DSB signaling. Thus, ATM activation leads to chromatin relaxation at sites of DSBs and H2AX tyrosine 142 phosphorylation was shown to function in the DDR. [20,21]

Yoon AJ, Shen J, Santella R Met. al. In 2007 evaluated by immunohistochemistry that subjects with pChk2-positive but histology-negative lesions were an 8.6 times at higher risk of developing SCC than those with pChk2-negative and histology-negative lesions. Thus, the presence of detectable pChk2 staining identified lesions at risk of developing SCC within 3 years with a sensitivity of 85.2%, specificity of 74.2%, and predictive accuracy of 78.2%.[22]

Li L, Feng Y, Luo Rin 2017 identified the possible interactive proteins of Chk2 and clarified the underlying mechanism regarding Chk2 chromatin loading and its phosphorylation to DNA damage response in oral squamous cell carcinoma (OSCC). The

result suggested that the interaction between Chk2 and MCM complex is required for Chk2 chromatin loading and its phosphorylation to DNA damage response in SCC-4 cells lines.[23]

'Nikitakis NG, Rassidakis GZ, Tasoulas J et.al. in 2018 evaluated the Immunohistochemical expression of DDR markers (γH2 AX, pChk2, 53 BP1, p53, and phosphorylated at Ser 15 p53) in 41 oral leukoplakias, ranging from hyperplasia (H) to dysplasia (D) and in comparison with oral squamous cell carcinoma (OSCC) and normal mucosa (NM)'. Statistical and receiver operating characteristic curve analysis showed γH2AX immunoexpression gradually increased and upper layer extension from NM to H to higher D degrees to OSCC. pChk2 expression was minimal in NM, comparatively low in PMD and shown to increased from H to D, and highest in OSCC. 53 BP1 showed higher levels in OSCC as compared to NM, however its expression in PMD was heterogeneous, gradually increasing in D. A number of related studies were reviewed[24-30]. p53 demonstrated progressively higher levels and upper layer extension from H to D to OSCC. Phosphorylated p53 was not present in NM and relatively low in PMD and OSCC. DDR markers' expression was variable in PPOELs, gradually increasing with dysplasia. Thus, activated DDR mechanisms have a protective role at early stages of oral carcinogenesis, but probably suffer progressive deregulation, ultimately failing to suppress malignant transformation.[10]

Conclusion:

DDR expresses the cell's ability to restore genomic alterations caused by mutagens. Dysregulation of DDR mechanisms is one of the major causes of MT.[10] Despite of this mounting evidence of DDR in the progression of oral cancer, its investigation in OSF remains 'scarce'.

'Thus, this is the first study which aim to evaluate and quantify the expression of various DDR markers (γH2AX, phospho-Chk2, p53, phospho-p53 and 53BP1) in various stages of oral carcinogenesis progression from normal mucosa to OSF and its malignant transformation, i.e.,OSF with OSCC using qRT-PCR'.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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