NQO1 C609T (Pro187Ser) increases risk of carcinogenesis of Oral Submucous Fibrosis by interacting with phosphorylated p53 and cyclin D1

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Abstract

Present study explores the role of NADPH quinone oxidoreductase 1 (NQO1) C609T (*Pro*187*Ser*) polymorphism in susceptibility to and its probable role in malignant potentiality of Oral submucous fibrosis (OSF) – a debilitating disease caused mainly by chewing arecanut. About 18% of the patients were detected with minor TT allele (*Ser/Ser*) p=0.026, while both CT (*Pro/Ser*) and TT (*Ser/Ser*) allele, p= 0.003 & 0.004 respectively, was found to be higher in patients above 40yrs of age. NQO1 protein was 42% reduced in buccal tissues of heterozygous (*Pro/Ser*) carriers, whereas a 70% reduction was observed in TT (*Ser/Ser*) patients. We detected the expression of p53 (Ser15) which is specifically produced in response to DNA damage and cyclin D1 (CCND1) to be related to NQO1 activity in buccal tissue of patients. A stabilized p53 (Ser15) and low CCND1 was evident in wild type (CC) patients. While a progressive MDM2 mediated degradation phospho p53 (Ser15) and increase in cyclin D1 expression was found in heterozygous (CT) or homozygous (TT) patients with carcinogenic lesions respectively. The present result identifies NQO1 C609T polymorphism as a potential risk in susceptibility to develop OSF. This also explains the event of carcinogenesis which may be attributed by MDM2 mediated degradation of p53 (Ser15) and for the first time reports role of this polymorphism in regulation of CCND1 and thus carcinogenesis of OSF.

Key words: Arecanut, Oral submucous fibrosis, NQO1, single nucleotide polymorphism, phosphor p53 (Ser15), cyclin D1, MDM2, carcinogenesis.

Introduction

Oral cancer (OC) is increasing at an alarming rate and it is the 11th most common cancer in the world. Out of this large number, two-third cases are recorded in developing countries. Late diagnosis of potential precancerous lesions is considered as the major cause of such higher incidence (1).

Upsurge in the popularity of commercially prepared areca nut preparations (popularly known as *'panmasala'*), an increased uptake of this habit by young people easy access, effective price ranges and marketing strategies have contributed to a high prevalence of smokeless tobacco related oral carcinogenesis in India(2, 3). Chewing of tobacco with betel quid increases the concentrations of carcinogenic tobacco specific nitrosamines and reactive oxygen species in mouth (3) as an early sign of damage to oral mucosa, tobacco smokers and chewers often develop precancerous oral lesions such as leukoplakia and submucos fibrosis.

Oral submucous fibrosis (OSF) is an insidious, chronic, progressive precancerous condition of the oral cavity and oropharynx with a high degree of malignant potentiality. A significant number of this precancerous condition converts into Oral Squamous Cell Carcinoma (OSCC), the rate being about 7.4-13% (4). This disease is now a public health concern in many parts of the world including United Kingdom, South Africa and many southeast Asian countries though it is mainly prevalent in the Indian subcontinent in all age groups and across all socioeconomic strata.(5-8).

The human NAD(P)H:quinone oxidoreductase 1 gene (*NQO1*; DT-diaphorase, Enzyme Commission (EC) number 1.6.99.2) occupies 17 kilobase pairs (kb) within a gene-rich region on chromosome 16 at 16q22.1 (9). This cytosolic flavoenzyme detoxifies quinones (a large class of aromatic compounds found commonly in plants, benzene metabolites, and chemotherapies) to hydroquinones or catechols. The enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1) acts as an antioxidant by catalyzing a 2-electron reduction that bypasses the need for two 1-electron reductions that can result in the production of DNA and protein-damaging reactive oxygen species. In certain conditions (e.g., the presence of myeloperoxidase or autooxidants), NQO1 can contribute to the formation of reactive oxidation species via oxidative cycling and therefore can act as a prooxidant (10). NQO1 is constitutively expressed in most tissues including the bone marrow, where expression is thought to be highly inducible by xenobiotics with quinone

moieties, and is upregulated during times of oxidative or electrophilic stress. *NQO1* expression is also upregulated in tumor tissues as the result of hypoxia (11): The success of quinone-based chemotherapies to exert their cytotoxic effects depends on their bioactivation by elevated levels of NQO1. Other than its implication in protection against oxidative stress and carcinogenesis (12, 13), it also includes stabilization of the p53 (14-17) a tumor suppressor gene.

The polymorphisms of NQO1gene have been characterized and known for about two decades (17). Of which a C-to-T substitution at position 609 of *NQO1* cDNA, which codes for a proline-to-serine change at residue 187 is now well studied in most cancers. In cells with a TT Genotype NQO1 activity is not detected and lack of activity corresponds to a lack of NQO1 protein which undergoes rapid turnover via the ubiquitin mediated proteasomal degradation pathway (18). The prevalence of the TT genotype varies among ethnic groups (4–20%), with the highest prevalence occurring in Asian populations (19, 20).

Previously it has been demonstrated that NQO1 through a distinct pathway stabilizes p53 – a well-known tumor suppressor, through induction of apoptosis at the G1/S checkpoint (21). On the other hand Cyclin D1 plays a central role in the G1/S cell cycle transition and responses to cytotoxic stimuli. In addition cyclin D1 has been observed to be highly upregulated in oral cancer tissues (22). In the current study, we investigated that NQO1 *Ser/Ser* minor allele has a major role in increasing risk of developing OSF and plays a central role in triggering oral carcinogenesis in the affected tissue. Our observation also includes the response of NQO1 towards areca chewing mucosa of OSF patients and the effect of these genotypes in such response. We have also demonstrated that NQO1 by stabilizing p53(Ser15) from MDM2 mediated degradation regulates cyclinD1 expression in mucosal tissue. p53 is phosphorylated at multiple sites in vivo and by several different protein kinases in vitro (23, 24). DNA damage induces phosphorylation of p53 at Ser15 and leads to a reduced interaction between p53 and its negative regulator, the oncoprotein MDM2 (25). MDM2 inhibits p53 accumulation by targeting it for ubiquitination and proteasomal degradation (26, 27). Phosphorylation impairs the ability of MDM2 to bind p53, promoting both the accumulation and activation of p53 in response to DNA damage (25, 28), thus making phosphorylated p53 (ser15) as an unique marker for oxidative stress generated DNA damage.

Materials and Methods

Patients and controls: A total of 179 patients with newly diagnosed cases of oral submucous fibrosis and 152 healthy control subjects having oral habit for a similar period were recruited from out patients department at Dr. R Ahmed Dental College & hospital, Kolkata, India over a 3-year period. All individuals enrolled in the study provided informed consent for their participation in the study. All participants completed a questionnaire providing information about their age, gender, ethnic origin, and use of panmasala & gutkha, smoking status or alcohol. Blood was collected from the antecubital vein, while biopsy sample was collected from the affected tissue after obtaining a written consent was obtained from all the patients after educating them about the importance and outcome of the study. All the study protocols were reviewed by an institutional ethical committee, and samples were collected by medical trained professionals.

A part of the tissue was used for routine histopathological and immunohistochemical examination, while another part was used for further analysis of genetic or protein expression.

Polymorphism Genotyping: Genotyping was carried out by PCR RFLP analysis on DNA extracted from whole blood samples obtained from all participants in the study using primers forward**:** 5`AAGCCCAGACCAACTTCT-3` and reverse:5`-ATTTGAATTCGGGCGTCTGCTG-3` with an initial denaturation of 95^0C for 2 mins followed by 35 cycles of 94°C for 30 secs, 60°C for 30secs, 72°C for 1 min and a final elongation of 72°C for 10 mins. Subsequently, the PCR products were digested with 20 U of HinfI (New England Biolabs, USA) for 3 hr at 37ºC and separated on a 6% polyacrylamide gel. The NQO1 wildtype allele shows a 172 bp PCR product resistant to enzyme digestion, whereas the null allele shows a 131 bp and a 41 bp band. The frequency of the NQO1 genotypes was compared in the patient and control groups.

RNA isolation from oral mucosal tissue: Total RNA was extracted using Trizol reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's protocol. Approximately 50 mg of tissue was collected in 1 ml Trizol reagent and homogenized using a handheld homogenizer. After the addition of 0.2 ml chloroform, the mixture was vigorously shaken for 3 min at 22°C and centrifuged at 13000 rpm for 15 min at 4°C. An equal volume of isopropanol (chilled) was added and was kept in cold condition for 10 min, RNA was precipitated by centrifugation at

13000 rpm for 10 min at 4°C. The pellet was washed twice with 70% ethanol, briefly dried under air, and dissolved in 100 µl of diethylprocarbonate- treated water.

Quantitative realtime PCR of NQO1 mRNA: Isolated RNA from OSF and control tissues was immediately subjected to realtime RT PCR (using Takara PrimescriptTM one step realtime RT PCR kit) (Takara, Japan) analysis for detection of changes in expression of NQO1 mRNA according to the manufacturers protocol. Briefly, the PCR was carried out using following primers Forward 5'-GGG CAA GTC CAT CCC AAC TG-3' and Reverse 5′-GCAAGTCAG GGAAGCCTGGA-3' (230 bp) while for GAPDH the forward and reverse primers were 5'-ATGGGGAAGGTGAAGGTCGG-3' and 5'-GGATGCTAAGCAGTTGGT-3' respectively, yielding a 470 bp product. The reaction mix contained 200 ng of RNA; PrimeScript One Step Enzyme Mix; 1X One Step Buffer; 20 µM of each of the primers and RNase Free dH2O. The real-time RT-PCR cycling program involved an initial denaturation step at 95°C for 2 min, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C. Thermal cycling was performed in a BioRad iQ5 Real-Time PCR Detection System (Hercules, CA, USA).

Western blotting: The tissues were collected in PBS buffer containing protease inhibitor cocktail (Sigma-Aldrich, USA). Tissue extracts were prepared by lysis of PBS-washed cells in RIPA lysis buffer [150 mM NaCl 1% Nonidet P-40 (vol/vol) 0.5% AB-deoxycholate (vol/vol) 0.1% SDS (vol/vol) 50mMTriszHCl (pH 8) 1mM DTT 1mg/ml each of leupeptin, aprotinin, and pepstatin]. The insoluble pellet was discarded, and the protein concentration was determined by using Bradford reagent (Bio-Rad). Equal amounts of protein were mixed with sample buffer (4% SDS 20% glycerol 10% 2-mercaptoethanol 0.125 M TrisHCl), heated at 95°C for 5 min, and loaded onto an 6% polyacrylamide- SDS gel and transferred onto a PVDF membrane (Millipore, Billerica MA). Primary antibodies used were – mouse monoclonal NQO1 (Cell Signalling Technology, Beverly, MA), rabbit polyclonal Phospho-p53 (Ser15) (Cell Signalling Technology, Beverly, MA), mouse monoclonal MDM2 (SMP14) (Santa Cruz Biotechnology, Inc.USA) mouse monoclonal cyclin D1 (A-12) (Santa Cruz Biotechnology, Inc.USA), rabbit polyclonal Ub (FL-76), and mouse monoclonal β-actin antibody (Sigma, Saint Louis, USA). After that suitable secondary antibody used was (goat anti rabbit or anti mouse) conjugated with ALP (1:2000

dilution) was added and incubated for 3 hours at room temperature followed by washing in TBST six times for ten minutes each to detect the protein levels.

Immunohistochemistry: Paraffin blocks each from wild type, heterozygous and homozygous mutant patients were selected for subsequent immunohistochemical investigations. Briefly, dissected oral tissues from biopsy specimen were paraffin embedded and 4-5µm thickness sections were collected on poly-L-lysine coated slides, after paraffin removal by xylene and rehydration, the slides were treated with citrate buffer for unmasking the antigen. The further immunostaining were performed using Novolink polymer detection system (Leica Microsystems, Switzerland). The endogenous peroxidase and protein were blocked using supplied blockers. The expression of NQO1 protein were detected using NQO1 monoclonal antibody (Ccell signaling technologies, Beverly, MA). After postprimary blocking the sections were incubated with novolink polymer and were then developed with DAB using supplied substrate buffer. The sections were then counterstained with haematoxylin and were observed under LEICA DM 3000 microscope ((Leica Microsystems, Switzerland).

NQO1 activity assay from affected and adjacent normal tissue: Oral biopsy tissues were obtained from patients (OSF and OSF associated with malignancy) both from the lesion and from the tissue adjacent to the lesion for measurements of NQO1 enzyme activity. The assay was performed as described by Ernster (1967) (29). and modified by Benson et al. (1980) (30) using DCPIP as a substrate. The reaction mix contained 25 mM Tris (pH 7.4), with or without 0.07% bovine serum albumin (w/v) as activator, 200µM NADH, and 40µM DCPIP, and assays were carried out in different concentration of dicumarol. NQO1 activity is described as the dicumarol inhibitable decrease in absorbance at 600 nm with DCPIP as a substrate and is expressed in nanomoles of DCPIP reduced per minute per milligram of protein. Total protein in tissue and cell culture preparations was determined by the Biorad Protein assay kit (Hercules, CA, USA) using bovine serum albumin as a standard.

Co-immunoprecipitation Studies in mucosal tissue of OSF patients associated with or without cancerous lesions: To examine the physical effect of the genotypic variants of NQO1C609T on Phospho p53 (Ser15) – MDM2 interactions in OSF associated cancer patients carrying TT allele, we utilized two different co-immunoprecipitations: 1) immunoprecipitation

with ubiquitin antibody followed by phosphor p53 ser15 coupled to alkaline phosphatase western blot analysis and 2) immunoprecipitation with anti-phospho p53 (Ser15) followed by anti-MDM2 antibody coupled to alkaline phosphatase western blot analysis. The total study was compared with patients carrying wild type CC allele and did not present any cancerous lesions.

Firstly the tissues were washed with PBS and re-suspended in 50mM TRIS(pH 7.5), 15mM EDTA, 150mM NaCl,0.1% Triton X-100, 0.01% SDS buffer containing protease inhibitor cocktail (Sigma, St Louis). The tissues were homogenized and the supernatants were incubated for overnight with ubiquitin monoclonal antibody. Normal IgG (Sigma, CA) was taken as a control for immunoprecipitation. The antibody protein complex was precipitated with Protein-A sepharose beads (Bangalore Genei, Bangalore), washed and subsequently the protein complex was eluted by SDS-lysis buffer. The eluted samples were then processed for western blot analysis with antibodies specific for either phospho p53 (Ser15) , MDM2 and Ubiquitin (Santa Cruz Biotechnology, Inc.USA) antibodies

Single cell gel electrophoresis: For single cell gel electrophoresis or 'COMET' assay, individuals were asked to rinse their mouths thoroughly for 2min with tap water. The exfoliated buccal cells were collected from one or both cheeks from the controls, and in the area with lesion from the patients, depending on the region where the lesion was located, including cheek, soft and hard palate, dorsal, ventral and lateral surfaces of the oral cavity. Typically sites with tough, leathery texture of mucosa, blanching of mucosa (persistent, white, marble-like appearance which may be localized, diffuse or reticular), quid-induced lesions (fine, white, wavy, parallel lines that do not overlap or criss-cross, are not elevated and radiate from a central erythematous area) were selected. Comet assay was performed and analyzed as described previously (31).

Statistical analysis: The calculation of genotypic and allele frequencies for cases and controls and association between polymorphisms of NQO1 C609T with the risk of oral submucous fibrosis among total and stratified population was estimated by computing Odds ratio (OR) and calculating 95% Confidence Interval (CI) using a chi-square Table Analysis and Yates corrected P value was taken for significance.

The expression of NQO1 protein was analyzed densitometricaly using Image J software after proper calibrations. The difference in protein expression was analyzed by Student "t" test, p value <0.05 was considered to be significant.

Results:

The total study population was stratified according to Gender (Male and Female), Median age (40 years), Inter-incisal distance (ID) as Grade I (ID>3cm), Grade II (ID 2-3cm), Grade III (ID<2cm) & Habit and are presented in Table 1. We found that male (65.9%) constituted a greater percentage of the study sample and that most of the OSF cases were in the age group of > 40 years (65.9%). When the total OSF cases were divided according to interincisal distance as grade I, II and III, maximum distribution was found for grade II (61.4%).

Distribution of Genotypes of *NQO1 C609T* polymorphism among the cases and controls: After PCR-RFLP analysis of the study samples (cases and control), the distribution of genotypes -Wild type C/C allele, heterozygous C/T allele and homozygous mutant T/T alleles were observed in the stratified study population and different grades of OSF and are presented in Table 2 & 3 respectively.

Overall the frequency of homozygous mutant type (18%) was significantly higher than controls (8.5%) [OR of 2.369 (1.167-4.804) P=0.026]. When stratified according to age, both the heterozygous carrier and homozygous mutant variant was found to be higher in > 40 aged OSF cases than in controls OR 4.5 (1.77-11.61) P=0.003 and OR 6.4 (1.92-21.35) P=0.004 respectively. Higher prevalence of both the heterozygous and homozygous mutant trait was found among the grade I patients (52% and 19%). Interestingly, the patients who reported with well-formed carcinogenic lesions were mostly found to be homozygous carrier of the minor T allele.

NQO1 expression and epethilial DNA disintegration in different genotypes: An overall mRNA expression level was higher in patients with either genotype compared to controls indicating transcriptional up regulation of NQO1 in tissues (Figure 1A). In general a significantly decreased mean expression for NQO1 protein was observed in buccal mucosal tissues of patients having heterozygous (42%) or homozygous (70%) mutant conditions (p=0.0055 & p=0.0001 respectively) indicating NQO1 degradation in homozygous / heterozygous mutant patients is a post-transcriptional event (Table 4) (Figure 1B).

Histochemical localization of NQO1 protein in oral epithelial cells (Figure 1C) is concurrent with western-blots and shows high concentrations of NQO1 expression in epithelium of the

tissue sections obtained from OSF patients with wild type *Pro/Pro* genotype compared to almost focal expression in normal healthy epithelium. Sections show increased NQO1 expression around the basal cell layer in wild type trait. NQO1 expression in OSF patients with heterozygous trait showed faint localizations whereas patients with homozygous mutant trait showed almost no NQO1 expression in the basal cells or elsewhere.

Comparative DNA damage of OSF with other oral precancerous lesions from our previous study (31) and according to genotype in this study presents a general disintegrated DNA in patients with wild type genotype and this worsens with heterozygous or homozygous conditions (Figure 1D).

Lower NQO1 activity in OSF and OSF associated with malignancy tissues compared to their adjacent tissue: Figure 2A presents kinetics of NQO1-mediated reduction of DCPIP as a measure of NQO1 activity. The Vmax and Km of NQO1 at different concentration of dicoumarol inhibitor in Normal, OSF and OSF associated with malignancy patients are presented in supplementary table 1. With increase in concentration of Dicoumarol (0, 1.25, 2.5, 5 and 10 µM) there was a uniform decrease in Vmax of NQO1 but no significant change in Km value was detected either in presence or absence of the activator (BSA). This suggests there is a non-competitive inhibition of the NQO1 activity in the presence of inhibitor. When compared to the OSF tissue alone there was about 2 fold decrease in activity ($p=0.028$) was noted with or without BSA (supplementary figure 1). This was about 10 fold decreased in OSF associated with malignancy tissues ($p< 0.0001$) thus explaining insignificant NQO1 activity.

p53 (Ser15) is upregulated in OSF and is stabilized by NQO1 wild type *Pro/Pro* allele in OSF patients: An upregulated p53 ser15 as in Figure 2B is indicative of oxidative stress and serves as an indirect measurement of the amount of the oxidative stress. So it is inevitable that p53 phosphorylated at ser15 residue is playing an important role as a tumor suppressor cellular response to DNA damage and other genomic aberrations in. We further proceeded to observe the effect of presence of homozygous mutant NQO1 minor T allele in patients with carcinogenic lesions upon p53 (ser15) level, and this was compared with either heterozygous or wild type OSF patients. As it can be observed from Figure 2B, NQO1 *Ser/Ser* allele fails to stabilize p53 (ser15), which in turn is targeted for MDM2 and ubiquiting (Figure 2C) mediated proteosomal degradation.

Over-expression of mucosal cyclinD1 in homozygous mutant OSF patients associated with carcinogenic lesions: OSF patients who presented with malignant lesions and were homozygous mutants (*Ser/Ser*) had high CCND1 expressions in their mucosal tissues. This can be directly correlated with expression of active MDM2 and absence of p53 (Ser15), to be a favorable condition for malignant transformation of the tissue.

Discussion

Oral submucous fibrosis is a high risk potentially malignant condition affecting any part of the oral cavity and pharynx, which could subsequently develop into oral cancer. The carcinogenic turnover of OSF may serve as a very important model to study various molecular changes that is taking place during oral carcinogenesis. In the present study we have tried to understand the pathogenesis and carcinogenesis of OSF in the light of NQO1 C609T polymorphism. In OSF mucosal tissues from patients carrying *Ser/Ser* genotype either traces or no NQO1 protein was detected, this was due to ubiquitin mediated proteosomal degradation. The prevalence of the *Ser/Ser* genotype varies among ethnic groups (4–20%), with the highest prevalence occurring in Asian populations(19). In the present study, we observed a significantly higher number of subjects carrying the NQO1 homozygous mutant allele genotype in patients suffering from oral submucous fibrosis as compared to the control group. Mostly male patients with OSF reported to our outpatient department (OPD) compared to the female patients, and middle-aged/old patients (>40yrs) showed a greater association with the risk of the disease. This result is in line with previous investigations that showed an association between the NQO1 homozygous mutant genotype and other tumour types such as urothelial cancer, renal cancer, leukemias, lung cancer and cutaneous basal cell carcinoma (32-36). The mechanism underlying the correlation of NQO1 C609T polymorphism with the increased risk for developing various tumours likely resides in the different enzyme activities encoded by the NQO1 alleles. A case-control study of benzene exposed workers in China revealed increased risk of hematotoxicity in individuals with the T/T NQO1 genotype, suggesting that NQO1 protects against benzene toxicity.

The current study suggests that NQO1 C609T null allele acts in a recessive mode in the development of OSF, demonstrated by the fact that the heterozygous genotype frequency among

OSF patients remains similar to that among the healthy controls. This indicates that in individuals with the NQO1 heterozygous genotype, the enzyme activity might be sufficient for protecting cells from damage by exogenous carcinogens important for the development of OSF. The determination of the NQO1 C609T genotype may be valuable as a stratification marker in future intervention trials for OSF and oral squamous cell carcinoma (OSCC). This finding may be particularly important in our country as most of the common people are habituated to areca chewing in different modes and are susceptible to development of OSF and eventually OSCC. Moreover since NQO1 C609T polymorphism was found to be positively associated with many solid as well as blood malignancies, therefore, a practical approach for cost-effective tumour screening may be designed taking other such polymorphisms into account. On the other hand, due to the relatively rare occurrence of the T/T genotype in the population, it is clear that in clinical practice NQO1 genotyping may be of importance only in combination with other risk factors. We also observed the effect of C609T polymorphism upon protein expression at the affected site of submucosal fibrosis in response to areca chewing which presented a 42% reduced expression of NQO1 protein in heterozygous patients, whereas a 70% reduced expression in homozygous Ser/Ser patients. Immunohistochemically this is the first document reporting the pattern of distribution of NQO1 in oral mucosal tissue. We have further demonstrated immuno-histochemically the effect of the polymorphic conditions (wild, heterozygous and homozygous mutants) on expression of NQO1 protein in OSF patients and controls.

In a previous study Asher et al (15) followed by Anwar et al (37) demonstrated NQO1 plays a mojor role in stabilising p53 either by redox mechanism or by formation of any multiprotein complex. The p53 gene is one of the major tumor suppressor genes in humans. In case of oxidative stress induced DNA damage p53 is phosphorylated at Ser15 and Ser20 and leads to a reduced interaction between p53 and its negative regulator, the oncoprotein MDM2. MDM2 inhibits p53 accumulation by targeting it for ubiquitination and proteasomal degradation. Phosphorylation impairs the ability of MDM2 to bind p53, promoting both the accumulation and activation of p53 in response to DNA damage. The p53 gene regulates cell cycle progression through induction of apoptosis at the G1/S checkpoint. Another key sensor protein that regulates cell cycle in early to mid-G1 phase is cyclin D1. Cyclin D1 is sequentially phosphorylated by cyclin E/CDK2 and inhibit the retinoblastoma protein (pRb) which in turn inactivate the cell-cycle inhibiting function and thus increase DNA synthesis (21). Overexpression of cyclin D1 is

being observed in oral cancers (22) and increase risk of tumor progression and metastasis in precancerous tissues.

An upregulated phospho p53 (ser15) in oral submucous fibrosis tissues is an indicator of oxidative stress generated DNA repair mechanism, in wild type NQO1 (Pro/Pro) phospho p53 (ser15) was observed to be stabilized with lesser expression of cyclin D1 suggestive of less carcinogenic risk in these patients. One of very interesting observation which led to this study was that a total of 10 OSF patients out of 179 during the last three year study, was found to develop squamous cell carcinoma or carcinogenic lesions. Surprisingly all the patients were homozygous mutant for Ser/Ser. The NQO1 activity was measured in these patients which shows a 10 fold decrease in enzyme activity. However, a detailed analysis of enzyme activity according to the genotype was not possible due to unavailability of fresh biopsied tissue from patients. Worth to mention a total of 32 (17.8%) patients we found to be homozygous mutants for NQO1 Ser/Ser. We wanted to observe the effect of presence of NQO1 C609T polymorphism in subsequent stabilization of phosphorylated p53 and a partial while full degradation of the protein was observed in heterozygous and homozygous mutant patients respectively. An increased interaction of phospho p53 (ser15) and MDM2 was observed Ser/Ser carrying OSF patients, in addition we also observed that Cyclin D1 expression is upregulated in NQO1 Ser/Ser OSF patients who presented with carcinogenic oral lesions. Our observation for the first time reports the real interplay of NQO1, phosphorylated p53, MDM2 and Cyclin D1 in the light of presence of NQO1 C609T polymorphism and carcinogenic turnover of a precancerous lesion and the possible mechanism is presented as a model (Figure 3). The upregulated expression of CCND1 in either heterozygous or homozygous patients is very distinct, and will need further experimentation to explain such observation. Oral submucous fibrosis, which has an etiology of chewing areca nut with tobacco, has high cancer turnover potency.

Presently oral cancers are increasing at an alarming rate and have an increasing mortality rate particularly in this part of the world, may serve as an excellent model to study various interactions of proteins in carcinogenic turnover in the light of presence of different deleterious polymorphisms. This study sets an example why the polymorphic status of a patient should also be taken into account before designing oral cancer therapeutics targeting these pathways. To conclude, NQO1 not only serves as an antioxidant enzyme but also functions as a tumor suppressor, and can have an important prognostic value.

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Figure Legends:

Figure 1: Effect of various genotypes on expression patterns of NQO1 and buccal tissue architecture. **A)** Increased mRNA expression *wrt* GAPDH of NQO1 in patients compared to controls indicating transcriptional up regulation in affected tissues. **B)** Expression levels of NQO1 protein in different genotypic conditions in OSF patients. **C)** Immunohistochemical localization shows high basal level expression of NQO1 especially along the rete-ridges and submucosal cells lining epethilium in OSF patients with *Pro/Pro* allele (ii) compared to healthy subjects (i). Little or no detectable expression is observed in heterozygous (*Pro/Ser*) (iii) or homozygous (*Ser/Ser*) (iv) oral mucosal tissue. **D)** DNA damage as evident from 'COMET' assay performed on exfoliated buccal tissue from OSF patients with different genotypes. DNA disintegration worsens with heterozygous or homozygous mutant conditions.

Figure 2: NQO1 mediated stabilization of p53(Ser15) expression and destabilization through MDM2 & ubiquitin mediated proteosomal degradation in OSF patients carrying major or minor alleles. A) NQO1 activity as measured by reduction of DCPIP in presence of dicoumarol with or without activator. The OSF with malignant lesion patients (OSF+MAL) carrying Ser/Ser allele presents reduced or no activity of NQO1 compared to normal or OSF patients with no malignancy. B) Expression of Cyclin D1, MDM2 (inactive 90KDa and active 55KDa), p53(Ser15) and NQO1 based on the genotypes. All the OSF patients with NQO1 *Ser/Ser* genotypes presented with mucosal malignant lesions. p53(Ser15) is highly expressed in wild type patients indicating DNA damage response, while is either degraded or no expression is observed in patients carrying minor allele. An eventual increase in active fragment of MDM2 and cyclin D1 is observed in these patients. C) Co-immunoprecipitation studies reveals MDM2 mediated degradation of p53Ser15 which is then targeted for ubiquiting mediated proteosomal degradation in patients carrying minor (T) allele.

Figure 3: Proposed model for role of NQO1 C609T polymorphism in regulation of carcinogenesis or tumor suppression in oral submucos fibrosis disease through stabilization of p53ser15 and cyclinD1.

Table1: Demographic variables of the studied population

	OSF patients (179)	**Healthy Controls (152)**
Age		
Range	14-73	18-78
Mean	38	37
Gender		
Male	118(65.9%)	74 (48.6%)
Female	61(34.1%)	78 (52.4%)
Age above 40 yrs	118(65.9%)	94 (61.8%)
Age below 40 yrs	61(34.1%)	58 (39.1%)
OSF grade		
Grade I (> 35 mm)	36 (20.11%)	NA
Grade II (20-35 mm)	110 (61.4%)	NA
Grade III (< 19mm)	33 (18.43%)	NA

Table2: Genotypic distribution of NQO1 C 609 T polymorphism among cases and controls:

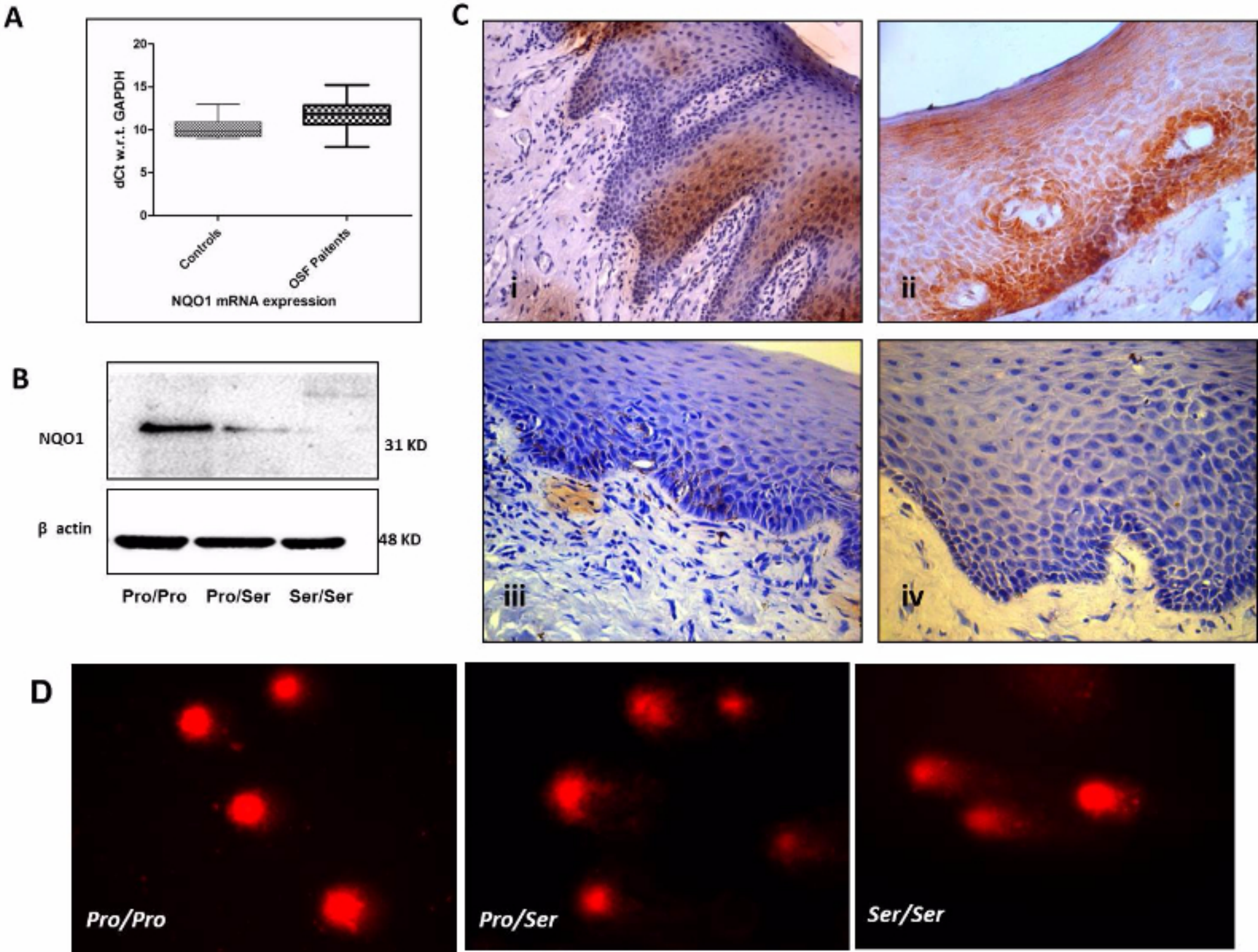
	Cases	Normal	OR*	95% CI**	P Value
Total population					
Pro/Pro	80(44.7)	77(50.65)	ref		
Pro/Ser	67(37.43)	62(40.78)	1.056	0.664-1.679	0.913
Ser/Ser	32(17.8)	13 (8.5)	2.369*	1.167-4.804	0.026
Below 40 yrs					
Pro/Pro	60 (46.87)	32 (34.04)	ref		
Pro/Ser	48 (37.5)	53(56.3)	0.493	0.27-0.87	0.024
Ser/Ser	20 (15.6)	9 (9.5)	1.185	0.49-2.85	0.893
Above 40 yrs					
Pro/Pro	20 (39.2)	43 (76.7)	ref		
Pro/Ser	19 (37.2)	9 (16.07)	4.5*	1.77-11.61	0.003
Ser/Ser	12 (23.5)	4 (7.1)	6.4*	1.92-21.35	0.004
Male					
Pro/Pro	52 (44.06)	30 (40.5)	ref		
Pro/Ser	46 (38.9)	38 (51)	0.698	0.376-1.297	0.329
Ser/Ser	20 (16.9)	6 (8.1)	1.923	0.711-5.59	0.3
Female					
Pro/Pro	28 (45.9)	32 (41.02)	ref		
Pro/Ser	21 (34.4)	39 (50)	0.615	0.297-1.276	0.265
Ser/Ser	12 (11.4)	7 (8.9)	1.95	0.95-5.15	0.322
Grade 1					
Pro/Pro	10 (27.7)				
Pro/Ser	19 (52.7)				
Ser/Ser	7 (19.44)				
Grade 2					
Pro/Pro	53 (48.18)				
Pro/Ser	38 (30.3)				
Ser/Ser	19 (17.27)				
Grade3					
Pro/Pro	17 (51.5)				
Pro/Ser	10 (30.3)				
Ser/Ser	6 (18.18)				

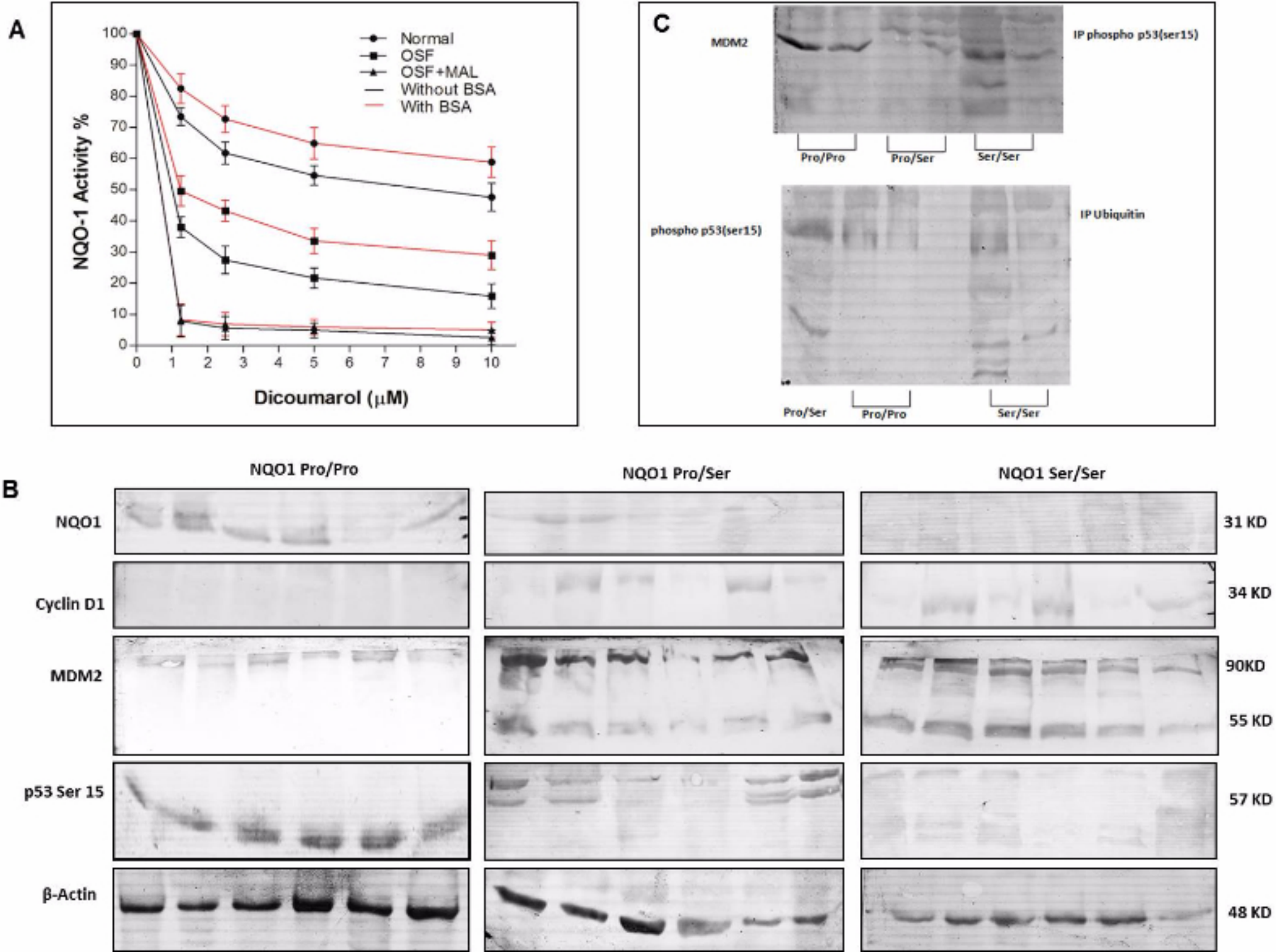
Table 3: Presence of NQO1 C609 T polymorphism among Grades of OSF patients

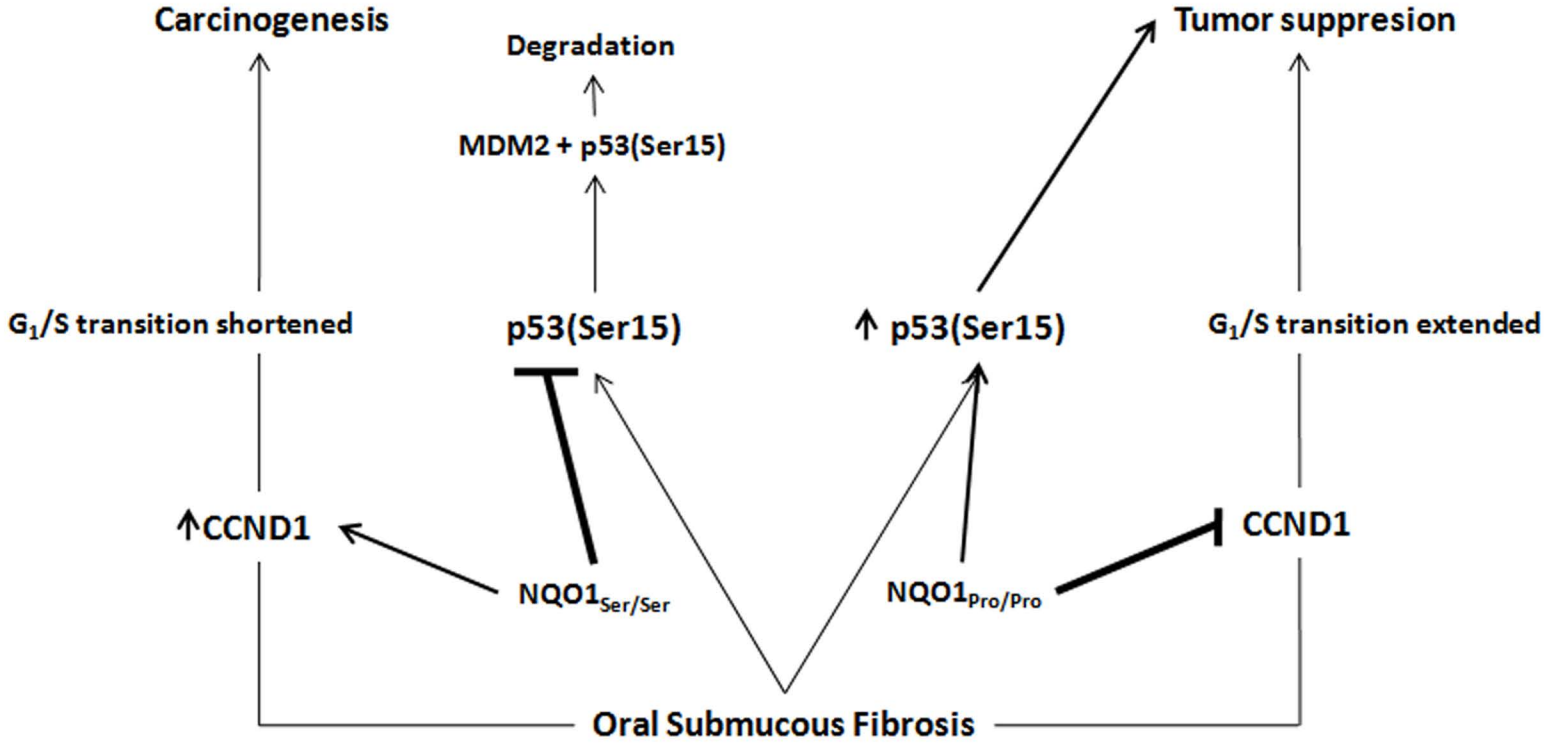
	Grade 1	Grade 2	Grade3
Pro/Pro	10 (27.7)	53 (48.18)	17 (51.5)
Pro/Ser	19 (52.7)	38 (30.3)	10 (30.3)
Ser/Ser	7 (19.44)	19 (17.27)	6 (18.18)

Table4: Effect of presence of NQO1 C 609 T polymorphism on protein expression in patients

Genotype	NQO1		P value
Pro/Pro Vs Pro/Ser	***Pro/Pro***	***Pro/Ser***	0.0055
	1.4875±0.8046	0.8789±0.5969	
Pro/Pro Vs Ser/Ser	***Pro/Pro***	***Ser/Ser***	0.0001
	1.4875±0.8046	0.4297±0.2492	


A
dCt w.r.t. GAPDH
20
15
10
5
0
Controls
OSF Paitents
NQO1 mRNA expression
B
NQO1
31 KD
β actin
48 KD
Pro/Pro
Pro/Ser
Ser/Ser
C
i
ii
iii
iv
D
Pro/Pro
Pro/Ser
Ser/Ser


A
NQO-1 Activity %
Dicoumarol (μM)
Normal
OSF
OSF+MAL
Without BSA
With BSA
C
MDM2
IP phospho p53(ser15)
Pro/Pro
Pro/Ser
Ser/Ser
phospho p53(ser15)
IP Ubiquitin
Pro/Ser
Pro/Pro
Ser/Ser
B
NQO1 Pro/Pro
NQO1 Pro/Ser
NQO1 Ser/Ser
NQO1
Cyclin D1
MDM2
p53 Ser 15
β-Actin
31 KD
34 KD
90KD
55 KD
57 KD
48 KD

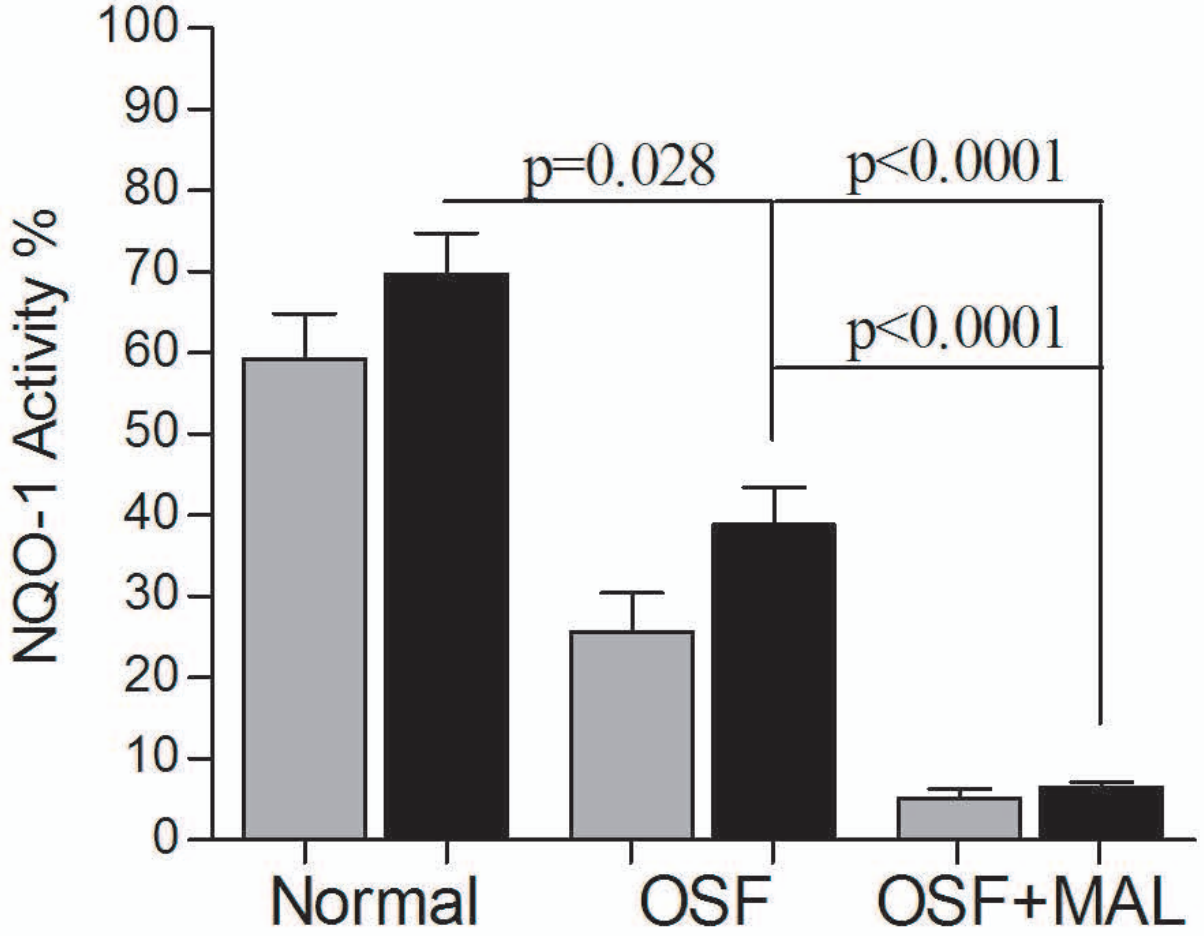

Carcinogenesis
Degradation
Tumor suppresion
MDM2 + p53(Ser15)
G1/S transition shortened
p53(Ser15)
↑ p53(Ser15)
G1/S transition extended
↑CCND1
NQO1Ser/Ser
NQO1Pro/Pro
CCND1
Oral Submucous Fibrosis

Supplementary Table 1: Vmax and Km of NQO-1 at different concentration of dicoumarol inhibitor in Normal, OSf and OSF+Mal patient .

	Normal			
Dicoumarol (µM)	With BSA		Without BSA	
	Vmax	Km	Vmax	Km
0	104.9 ± 9.17	4.83 ± 1.29	84.72 ± 7.14	4.63 ± 1.21
1.25	103 ± 8.28	5.57 ± 2.28	76.64 ± 3.70	5.50 ± 2.76
2.5	87.16 ±7.11	5.72 ± 1.32	75.18 ± 5.10	8.98 ± 1.42
5	61.61 ± 6.94	5.74 ± 1.83	21.98 ± 3.07	5.47 ± 2.21
10	37.55 ± 3.11	3.56 ± 2.03	15.73 ± 2.53	4.58 ± 2.31

	OSF			
Dicoumarol (µM)	With BSA		Without BSA	
	Vmax	Km	Vmax	Km
0	37.48 ± 3.27	5.31 ± 2.24	20.55 ± 1.74	3.63 ± 2.12
1.25	35.52 ± 2.85	6.67 ± 1.43	16.86 ± 2.81	5.32 ± 2.89
2.5	26.95 ± 2.2	5.49 ± 2.53	17.76 ± 1.26	5.83 ± 1.93
5	17.14 ± 1.93	4.93 ± 1.57	4.78 ± 2.66	5.11 ± 2.17
10	9.57 ± 0.79	3.41 ± 1.63	3.19 ± 3.14	4.14 ± 1.37

	OSF+MAL			
Dicoumarol (µM)	With BSA		Without BSA	
	Vmax	Km	Vmax	Km
0	7.47 ± 1.61	2.61 ± 2.25	7.05 ± 1.58	2.46 ± 2.26
1.25	6.78 ± 1.44	2.77 ± 2.29	6.21 ± 1.27	2.48 ± 2.08
2.5	5.57 ± 1.61	2.83 ± 3.13	5.01 ± 1.59	2.77 ± 3.43
5	3.41 ± 0.56	2.01 ± 1.48	3.07 ± 0.57	2.27 ± 1.8
10	2.31 ± 0.34	2.24 ± 1.41	1.98 ± 0.27	2.77 ± 1.47



	Normal	Normal (BSA)	OSF	OSF (BSA)	OSF+MAL	OSF+MAL (BSA)
Mean	59.33	69.69	25.72	38.84	5.185	6.493
SD	11.03	10.20	9.443	9.316	2.208	1.401