COX2: A Prognostic Inflammogenic Marker Drives Cervical Carcinogenesis In Vivo Through NFκB/IAP/p53Axis

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Abstract

Cyclooxygenase2, a prostaglandin synthesizing enzyme is a key player in inflammation-induced vasculogenesis that enables tumor growth. This study explores the central role of COX2 and its relative prosurvival proteins in evoking inflammatory events during development of an *in vivo* cervical cancer model upon chronic treatment with 3-methylcholanthrene (3MC; a chemical carcinogen) in virgin-female Swiss Albino mice. Chronic painting of mice cervix with 3MC solution triggered the persistent expression and activity of COX2; eventuating in overexpression of major prosurvival molecules (NF κ B, XIAP, survivin, GM-CSF1) and proliferative antigens (Ki67, PCNA). COX2-arbitrated prosurvival signaling subsequently deranged the expression profiles of tumor suppressor proteins (p53/Acetyl-p53, p21, Rb) within the cervix. COX2 helmed molecular alterations successively surged leukocyte influx within cervix; catering in localized inflammation which gradually distorted its tissue architecture. Cervical carcinogenesis was further braced by higher levels of systemic-ROS and RNS, escalated iNOS activity and compromised anti-oxidant enzyme capacities, which were accompanied by splenomegaly. Additionally, circulation of blood-leucocytes with damaged DNA throughout the mice body, envisaged the impact of cervix-limited inflammation upon the mice physiology. Conclusively, the present study deciphered the role of COX2 effectuated NF κ B/IAP/p53 functions in sequestering the contributors of localized and systemic inflammogenesis for propelling 3MC-mediated cervical carcinogenesis *in vivo*.

Introduction

Cyclooxygenase2 (COX2) is preeminent in inflammogenesis of cancers. Formally termed *Prostaglandin H₂ Synthase-2 enzyme*, COX2 catalyses the rate-limiting step of mitochondrial fatty acid oxidation concerning the conversion of arachidonic acid to biologically active prostanoids which are propagators of inflammation [1, 2]. These prostanoids maintain COX2 expression to activate ROS/AKT/activator protein1 [3] and switch on the kinase-cascades [4, 5] for enabling malignant transformations. Besides, COX2 promotes 'angiogenesis', the hallmark episode of new blood vasculature growth in solid tumors which assists them to sustain independently. Vascular Endothelial Growth Factor (VEGF), the key regulator of 'angiogenic switch' is also instructed by COX2 for undertaking endothelial growth [6, 7]. Therefore, researches delving into contributions of COX2 in various neoplastic progressions are on rise.

With a chromosomal location of 1q31, COX2 gene is highly inducible in nature [8] due to which it gets overtly expressed for organizing inflammatory events only upon viral oncogenic inductions, chemical carcinogenic triggers or in pathological conditions developed due to chronic viral or bacterial infections [9, 10]. In the incidences of *Human Papilloma Virus (HPV)* mediated cancers like cervical and oral carcinomas, extended viral latency period was found to be closely associated with COX2 generated non-compensatory inflammation states [11]. Similarly, persistent xenobiotics exposure induced COX2 activities which reportedly enhanced skin inflammations; bringing forth carcinogenesis [12]. Recent experimental reports are evidential for the prerequisite need of COX2 in cancer stem cell differentiation and proliferation [13, 14]. The Polycyclic Aromatic Hydrocarbons (PAHs) of lower molecular weight emitted along with cigarette smoke were found to effectuate p38 MAP-Kinase to cause COX2 mRNA overproduction. As a consequence, eicosanoid signalling got kick-started within the lung epithelium for launching carcinogenic changes [15]. Often in lymphomas, COX2

observably upgraded proliferative, angiogenic and invasive potentials of leukocytes [16, 17]. Multiple cancers are characterized by genetic aberrations along with polymorphisms in their COX2 gene [18–22].

Several interesting documentations are supportive of COX2 being the central under pinner of inflammation, angiogenesis and tumorigenic progression [23, 24]. COX2 abetted inflammation becomes the '*formative-ground*' of a strong prosurvival signalling network, characterized by deteriorated tumor suppressor activities. Generally, it is a common observation that gain-of-function mutations in the effector genes of the Ras-MAPK and the PI3K/AKT-signalling pathways principally dictate over the malignant transformation processes. Reportedly, COX2 could potentiate many cancers for phenotypically mimicking the effects of these two pathways; even when the concerned driver mutations are absent [25]. In the hypoxic tumor cores, rise in expression of Hypoxia Inducible Factor-1 (HIF-1) collaterally accelerates COX2 kinetics for permitting the expressions of *Nuclear Factor κ B* (NF κ B) [26] and its effector-*Inhibitor of Apoptosis Proteins* (IAPs) [27]. COX2-derived prostanoids further activate MAPK pathway to allow VEGF translation; aiding in vasculogenesis [28, 29]. Additionally, COX2 adorns functionality by repressing the tumor suppressors such as p53, p21 or Rb over activation of Bcl₂, the anti-apoptotic protein [30, 31]. The relentless cytokine storm following COX2 activation relays dysregulated inflammatory signals which alter the functional differentiation of immune cells for promoting tumorigenesis [32]. COX2 and the related cytokines are held responsible for switching the developmental fate of '*tumor-inhibiting*' M1 macrophages to '*tumor-promoting*' M2 macrophages or *Tumor Associated Macrophages* (TAM) [33, 34]. Therefore, COX2 and its effectors empower a growing tumor mass with the armour of immune evasion [35]. Inevitably, COX2 is the prima facie requirement for promoting autonomous tumor growth.

With regard to the prevalent reports, the present study was designed to investigate the mediatory role of COX2 in domineering the inception and progression of cervical carcinogenesis within Swiss Albino mice upon chronic treatment with 3methylcholanthrene (3MC), a potent chemical carcinogen of PAH family. This study systematically dismantled the role of COX2 in sequestering inflammogens to drive the activation of prosurvival signaling nexus for promoting cervical carcinogenesis *in vivo*.

Material And Methods

Animal Maintenance

Virgin female Swiss Albino mice (*Mus musculus*; 5–6 weeks old; weight: 23-25gms) obtained from the Central Animal Facility of CNCI were housed in polyvinyl cages within well-ventilated rooms under ideal conditions (temperature: 22⁰C; relative humidity: 50–60%; 12 hour day/night cycle). Prior to treatment initiation, the animals were subjected to a two-week acclimation period during which all the female and male mice were kept in complete isolation from each other. This induced pheromone influenced oestrous cycle synchrony thereby nullifying the hormonal interferences [36]. Standard guidelines laid down by the Institutional Animal Ethics Committee (IAEC) certified by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, were strictly followed for undertaking all animal experimentation.

Experimental design

Random allocation of 100 mice into three broad groups was accomplished on the basis of body weight. Each group was further classified into subgroups separated in two batches of 10 animals [No of mice (n) = 5 /cage

(x2) for each subgroup]. Group I was kept as an ‘untreated’ control group where mice did not receive any treatment. Group III (groups 6–10) comprised of mice which had their cervix chronically painted with 3MC (MP Biomedicals, USA) solution prepared in Petroleum Ether (PET, Merck, Emplura, Germany) for respective intervals of 6, 12, 16, 24 and 30 weeks [37]. It was specifically designated as ‘treatment’ group for studying cervical carcinogenesis along these time spans. To rule out any carcinogenic effect of the solvent (if any), Group II (groups 1–5) was assigned as a ‘vehicle control’ batch, with mice being solely treated with PET. Food and water was given *ad libitum*. Mice were periodically monitored for any visible health abnormalities and deaths. This purposeful procedure of mice grouping is well-represented in Table 1.

Table 1
Animal Groups for Experimentation

Sl.No	ANIMAL GROUPS	TREATMENT APPROACH
1.	GROUP-I (Control)	Untreated group
2.	GROUP II (Vehicle Control)	Chronic treatment of mice cervix with Petroleum Ether (PE)
	Group 1	6 weeks
	Group 2	12 weeks
	Group 3	16 weeks
	Group 4	24 weeks
	Group 5	30 weeks
3.	GROUP III (Treatment)	Chronic 3MC treatment of mice cervix (Dosage- 0.6mg/ml)
	Group 6	6 weeks
	Group 7	12 weeks
	Group 8	16 weeks
	Group 9	24 weeks
	Group 10	30 weeks

After every mentioned treatment period, mice from all the three groups were sacrificed and their entire female reproductive tissue was excised out. In order to track the presence of any cervical tumor, these reproductive tissues were longitudinally opened up for examination through its upper vaginal (ectocervix) region. Cervix tissues (with or without tumor) were decapitated out for comparative studies. Besides cervix, blood, and spleen were also collected for experimental purposes.

Record of body weight and carcinogenic incidences

Weekly body weight alterations in mice were recorded to assess the impact of chronic 3MC and PET treatments on their physiology with time. An orderly record of tumor incidences along with gradually acquired cervical dysplastic stages in the 3MC treated mice batches was also maintained.

Cytopathological study

Smears of cervical exfoliated cells suspended in Phosphate Buffered Saline (PBS, pH-7.4) were fixed with 100% ethyl alcohol and stained as per the protocol of Mahapatra et al., (2020) [37]. Sequential polychromatic staining with Harris Haematoxylin (HH), Orange G6 (OG6) and Eosin Azure (EA50) [Merck Millipore, Mumbai, India] followed by excess stain removal in tap water, 70% and 90% alcohol was observed. Finally stained slides were mounted and observed under a light microscope (Zeiss). Randomly, 50 fields were microscopically scanned for the presence of significant cytopathological changes in each slide.

Enumeration of differential count of cervical leukocytes

A numerical count of the microscopically identified subpopulations of cervical leukocytes was quantitated as 'Differential Leukocyte Count' (DLC). Over 50 fields of Pap stained slides were randomly scanned for calculating DLC. For each field, DLC was enumerated as a percentage (%) of specific number of leukocyte subpopulations (eosinophils, neutrophils and monocytes) observed among the total number of leukocytes [DLC= (Number of specific type of leukocytes)/ (Total number of leukocytes) x 100].

Histology

Dissected cervical and spleen tissues were washed in cold normal saline (0.9%), fixed in 10% (w/v) Neutral Buffered Formalin (NBF) for 24 h, processed through alcohol grades and xylene, followed by embedding in paraffin. These paraffinized tissue blocks were cut into thin (~ 5µm) sections using a microtome and stretched over grease-free glass slides. These sections were further deparaffinised in xylene followed by successive staining in Delafield haematoxylin [DH; haematoxylin powder, 100% alcohol, (NH₄)Al(SO₄)₂, Glycerol, H₂O] and 2% Eosin. After mounting, 10–20 fields of these stained slides were examined under a light microscope (Zeiss) for documentation of the histological changes

Immunohistochemistry

Cervical tissue sections (~ 5µm thick) were stained with the primary antibodies of COX2, NFκB (p50/p65), XIAP, survivin, Ki67 and PCNA proteins for locating them within tissues by following the protocol of Basu et al., (2020) [38]. Paraffin from stretched tissue sections was removed by heating the slides at 65°C for 20 min followed by xylene treatment and rehydration through 100%, 90 %, 70 % and 50 % alcohol downgrades. After washing these sections serially in PBS for 10min, 'antigen-retrieval' was carried out using pre-heated citrate buffer [pH-6; comprising of C₆H₈O₇·H₂O and (CH₂COONa)₂·2H₂O] at 85°C for 1 h following which they were incubated with the respective primary antibodies diluted in 1 % bovine serum albumin (BSA; Sigma Aldrich, USA) solution within a humid chamber overnight at 4°C. Excess primary antibodies were washed in 1X PBS. Slides were further incubated with HRP conjugated secondary antibodies (1:500) in 1% BSA solution for 2 h at 37°C followed by immunostaining with the chromogenic substrate 3–3' diaminobenzidine (DAB; SantaCruz Biotechnology, USA) and counterstaining with DH. These slides were dehydrated through successive alcohol grades and xylene. Finally, they were mounted in DPX for observation under light microscope (Zeiss). About ten fields were scanned in order to score for the positive staining intensities. Staining intensities (1 = weak, 2 =

moderate, 3 = strong) were enumerated as per the percentage of positively stained cells (< 1 = 0, 1–20 = 1, 20–50 = 2, 50–80 = 3 and > 80 = 4). Final evaluation of the tissue specific protein expressions was made as low (Score 0–2), intermediate (2–5), and high (Score 5–7) levels.

The primary antibodies employed were: COX2 (GeneTex, 1:1000), NFκB(p50/p65) (GeneTex, 1:1000), Ki67 (GeneTex, 1:1000), PCNA (GeneTex, 1:1000), XIAP (GeneTex, 1:1000) and survivin (GeneTex, 1:1000).

Preparation of tissue lysates

Cervix tissues along with the adjoining tumor regions were dissected out, washed, and pooled separately from Group I, II and III mice. Tissue and tumor parts were dried, weighed and homogenized in Radio-Immunoprecipitation Assay Lysis buffer (RIPA; pH-8 comprising of 5M NaCl, 0.5M EDTA, 1M Tris, NP-40, 10% Sodiumdeoxycholate, 10% SDS). The extracts were kept in ice for 30 min followed by sonication and centrifugation at 10,000g for 20 min at 4°C. The resulting supernatants were stored in chilled vials at -20°C.

Estimation of total protein

Total protein content of the tissue extracts was spectrophotometrically (VARIAN) estimated using 1X Bradford's reagent (HIMEDIA, PA, USA) against a standard curve of BSA. Absorbance was recorded at 595 nm with the experiment being repeated for 5 times.

Western Blot Analysis

The expression statuses of inflammatory mediators (COX2, GM-CSF1), tumor suppressor proteins [p53, acetylated-p53 (lys373), p21, Rb], prosurvival molecules (NFκB, XIAP, survivin) and proliferative antigen (Ki67) were comparatively studied by western blotting. Equitable amounts of cervical protein were respectively loaded into each well of SDS-polyacrylamide (SRL, Mumbai, India) gels, electrophoretically separated using electrophoresis buffer (25mM Tris, 192mM glycine, 10% SDS) and electro-transferred to nitrocellulose membranes with the aid of a transfer buffer (250mM Tris, 192mM glycine, 10% Methanol). These membranes were blocked with 5% (w/v) BSA solution, washed with Tris Buffered Saline (TBS; pH-7.5; 25mM Tris.HCl, 150mM NaCl) and incubated overnight with primary antibodies at 4°C under constant shaking. Blots were thereafter washed with TBST Buffer solution (TBS; tween 20) for 4 times and subsequently incubated with alkaline phosphatase conjugated secondary antibodies (GeneTex, 1:500 dilutions in TBS) at 4°C for 2h, followed by TBST washing (4 times) and incubation with the chromogenic substrate 5-bromo, 4-chloro, 3-indoylphosphate/ Nitro-Blue tetrazolium (BCIP/NBT; SantaCruz Biotechnology, USA) for visualizing protein expressions in the form of bands. β-actin was used as a loading control protein. These experiments were performed in triplicate.

The primary antibodies used were: COX2 (GeneTex, 1:1000), GM-CSF1 (Santa Cruz, 1:1000), NFκB(p50/p65) (GeneTex, 1:1000), Ki67 (GeneTex, 1:1000), PCNA (GeneTex, 1:1000), XIAP (GeneTex, 1:1000), survivin (GeneTex, 1:1000), Acetyl p53(lys 373) (Merck-Millipore, 1:1000), p53, p21, and Rb (Santa Cruz, 1:1000) and β-actin (GeneTex, 1:1000).

Quantitative estimation of COX2 activity

COX2 enzyme (E.C 1.14.99.1) was spectrophotometrically quantitated at 590 nm using COX activity assay kit (Cayman Chemical, Cat No: 760151 Ann Arbor, MI, USA) as per the provided protocols. Results were represented

in nmol/min/ml (U/mg of protein).

Isolation of blood leukocytes

One volume of mice blood collected aseptically from heart was mixed with three volumes of Solution A (pH-7.2; 0.87% NH_4Cl in 10mM Tris HCl), ice incubated for 20 min and centrifuged at 400g for 20 min at 0°C . The supernatant was discarded and the pellets were again resuspended in Solution A followed by centrifugation at 400g for another 20 min at 0°C . Resulting pellets were suspended in Solution B (pH-7.2; 0.25M mesoinositol, 10mM Na_2SO_4 , 1mM MgCl_2), cold centrifuged at 1500 rpm for 5 min at 4°C succeeded by re-suspension of pellets in HEPES Buffered Saline (HBS; pH 7.4; 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM CaCl_2 , 1 mM MgCl_2 , 10 mM glucose). Cell numbers were adjusted as per experimental requirement.

ROS generation

Reactive Oxygen Species (ROS) generated due to chronic treatment with 3MC was quantitated as per the principle of Sinha and Roy (2010) [39]. Isolated leukocytes (10^6 cells/ml/point) suspended in HBS were incubated with a fluorescent probe, 2',7'-dichlorofluorescein dihydroacetate or DCFH-DA ($10\mu\text{M}$, Sigma-Aldrich, USA) for 45 min at room temperature in complete darkness. DCFH-DA passively diffuses within cells to transform into a diol moiety which is further oxidized into a fluorescent compound, 2', 7'-dichlorofluorescein (DCF) by intracellular ROS. DCF was quantitated spectrofluorimetrically (VARIAN; Excitation: 485 nm and Emission: 530 nm). For each point, readings were recorded for about five times in triplicate attempts of the experiment.

RNS generation

Equal volumes of peritoneal macrophage suspension (10^6 cells/ml) in PBS and Griess reagent (1% sulphanilamide, 0.1% naphthyl ethylenediamine hydrochloride and 5% orthophosphoric acid) was incubated at 37°C for 30 min in a humidified chamber for quantifying Reactive Nitrogen Species (RNS) levels [40]. Absorbance was recorded at 550 nm with a spectrophotometer (VARIAN) against a standard blank. NO levels were enumerated against a standard curve of sodium nitrite. Readings for each point were taken for about five times in triplicate attempts.

Quantitative estimation of nitric oxide synthetase activity

Indirect assessment of Nitric Oxide synthetase (iNOS) enzyme (E.C1.14.13.39) activity was done using a spectrophotometer by calculating the percentage of L-citrulline catalytically produced from L-arginine by means of NOS activity Assay Kit (Cayman Chemical, Cat No: 781001, Ann Arbor, MI, USA) in accordance with the kit instructions.

Antioxidant Enzyme Activity

Free Radical quenching capacity of the antioxidant scavengers present in the blood serum isolated using serum separating vials, was assessed with the help of Antioxidant Assay Kit (Cayman Chemical, Cat No: 709001, Ann Arbor, MI, USA). Enzyme activity was expressed as IU/L with the help of a spectrophotometer where readings were recorded within the absorbance range of 405-750nm.

Single cell gel electrophoresis (SCGE or Comet assay)

The clastogenic effect of 3MC on DNA was assessed following the standard laboratory protocol [41]. Concisely, a suspension of 0.6% (w/v) low melting agarose (LMA; Sigma-Aldrich, USA) and isolated leukocytes (1×10^4 cells) was smeared over a frosted microscopic glass slide which was priorly coated with the fixative 0.75% (w/v) normal melting agarose (NMA; Lonza, USA). Following solidification at 4°C , cell and nuclear membranes were lysed in lysis buffer (pH-10; 2.5 M NaCl, 0.1 M Na_2EDTA , 10 mM Tris, 0.3 M NaOH, 1% Triton X-100, and 10% DMSO). Exposed DNA from the lysed out leukocytes were unwound in a highly alkaline electrophoresis buffer (pH > 13.0; 300 mM NaOH, 1 mM Na_2EDTA) prior to electrophoresis for 20 min (300 mA, 20 V). Slides were washed in neutralizing buffer (Tris 0.4 M, pH 7.5) thrice, stained with ethidium bromide (final concentration 40 $\mu\text{g/ml}$) and examined under a fluorescence microscope (Leica). Image analysis, head DNA quantification, comet tail DNA length estimation and comet tail moment calculation were performed using Komet Software.

Statistical Analysis

The mean values of the control, vehicle control and treatment mice groups were compared by factorial Analysis of Variance (ANOVA). The relationship between the studied parameters was analysed by calculating Pearson's correlation coefficient using CORREL function of Microsoft Excel. Data were expressed as mean $\pm$ standard deviation (S.D.). p value calculations were performed using Prism GraphPad Software. * $p < 0.005$ and ** $p < 0.01$ were considered statistically significant in comparison to control batches.

Results

The optimum 3MC dose of 0.6 mg/ml was selected as the safest and efficacious treatment dose ($< \text{LD}_{50}$) only after checking its impact on *in vivo* toxicity parameters namely SGPT, SGOT, ALP and creatinine levels (*data not shown*). Carcinogen preparation process is elaborated in Fig. 1A. The treatment durations of 6, 12, 16, 24 and 30 weeks were also accordingly identified. A clear schematic outline of the experimental design with appropriate timeline of animal sacrifice has been illustrated in Fig. 1B.

Emergence of tumor at the cervical region upon chronic 3MC treatment

Prior to sacrifice, the experimental mice were preliminarily monitored for any apparent changes in the appearance of their orifice and internal female reproductive organ owing to chronic treatment with PET or 3MC. Relevant observations noted among mice of Group-I, II and III are comparatively depicted in Fig. 2A and 2B. Obvious progressive changes in the vaginal orifice of 3MC treated mice (Fig. 2Axi-xv) were an outcome of internal changes in their reproductive system (Fig. 2B). As indicated in Fig. 2Biv and v, remarkable alterations of the mice reproductive organs in 12 and 16 weeks of 3MC treatment mostly included mild thickening along with growth of new blood vasculature. Group-III mice from 16th week batches had a mildly swelled orifice (Fig. 2Axi; indicated by arrow). Further extending 3MC treatment beyond 24 weeks resulted in tumor growth at the cervical region (Fig. 2Bvi) leading to explicit swelling of the lower abdominal area (Fig. 2Axi & xv; indicated by arrows). No such abnormalities were noted in the vehicle control and control mice neither amidst the treatment intervals nor after 24 weeks.

Cytopathological changes in terms of increased ‘keratinisation’ of the cervical squamous epithelial cells bearing ‘nuclear holes’ (Fig. 2Cxii; indicated by arrows) became perceptible amongst 3MC treated mice in 12th week. Inflammation mediating leukocytes such as eosinophils, neutrophils and monocytes were also persistently apparent in the cervical infiltrations (Fig. 2Cxiii) during the entire treatment span. In the 24th and 30th weeks, the mice cervical exfoliates exhibited ‘hyperchromatic nuclei’ (Fig. 2Cxiv; indicated by arrows), assumed up a ‘bizarre-structure’ and harboured ‘perinuclear halo’ (Fig. 2Cxv, indicated by arrows) as signs of ‘vacuolation’. Cellular distortions of these types were clearly absent in PET treated groups. Acquired observations were comparable with control animals.

Noticeable cytopathological alterations in the cervix necessitated in exploration of its histology which vividly exhibited transfigurations as revealed from the presence of mild to severe cervical dysplasia (Fig. 2Diii & iv) in mice exposed to 3MC for 6 and 12 weeks. Interestingly, development of ‘invasive carcinoma’ eventuated with extension of treatment upto 16 weeks (Fig. 2Dv). Furthering 3MC treatment to 24 weeks and beyond resulted in disorientation of cervical epithelial layers (Fig. 2Dvi). As clearly comprehensible from the micrographs, no such remodelled cervical tissue features were discerned amongst the vehicle control and control mice even after 24 weeks (Fig. 2Di & ii). These aforementioned changes were in coherence with attainment of a cervical tumor. The rate of incidences of the attained dysplastic changes and tumor among Group-III mice is clearly shown in Table 2B. Moreover, chronic 3MC treatment affected the body weight of mice. The findings tabulated in Table 2A depicted a gradual loss in bodyweight of these mice in 6weeks of 3MC treatment which continued till 12 weeks (25 ± 0.1716 gms). A significant weight reduction by 24 ± 0.0100 gms was observed in the 16th week which further increased by 29.1067 ± 0.1940 gms in 24th week and beyond. Therefore, the decreasing trend in body weight got violated by a consistent rise in the 30th week of 3MC treatment.

Table2A: Impact of 3MC treatment upon body weight of mice.

6 weeks			12 weeks	16 weeks	24 weeks	30weeks
ANIMAL GROUPS (Weight in gms; n=10)	Group-I (Control)	25±0.2108	28±0.098	32±0.1702	33±0.16552	33.8±0.0954
	Group-II (Vehicle Control)	25.02±0.2213	27±0.1658	27.56±0.114	31.11±0.0793	32.7±0.0591
	Group-III (Treatment)	25.2±0.1079	25.8±0.171	24±0.0100	29.1067±0.194	35.4±0.09406

Table2B: Percentage (%) of 3MC treated mice displaying cervical dysplasia and tumor stages.

	Normal	Mild Dysplasia	Moderate Dysplasia	Invasive Carcinoma	Carcinoma <i>In Situ</i>	Anaplastic Tumor
0 th week (n=10)	9/10 (90%)	-	-	-	-	-
6 th week (n=10)	2/10 (20%)	8/10 (80%)	-	-	-	-
12 th week (n=10)	1/10 (10%)	2/10 (20%)	7/10 (70%)	-	-	-
16 th week (n=10)	-	1/10 (10%)	1/10 (10%)	8/10 (80%)	-	-
24 th week (n=10)	-	-	-	2/10 (20%)	8/10 (80%)	-
30 th week (n=10)	-	-	-	1/10 (10%)	3/10 (30%)	6/10 (60%)

Chronic 3MC treatment rendered persistent cervical inflammation

3MC triggered chronic cervical inflammation in mice, as unveiled from the foregoing cytopathological excerpts demanded a thorough investigation through its nature. Enumerated DLC (%) values for Group I, II and III mice as displayed graphically in Fig. 3A-C revealed a dominance of neutrophils over other leukocytes in the mice cervix upon continual 3MC exposure. Eosinophils in the 6th week and 12th week of 3MC treatment had respective DLC values of $25.11 \pm 0.58\%$ and $35.01 \pm 0.58\%$ that underwent a little negative deviation by about $32.30 \pm 0.58\%$ in the succeeding 16th week. Conversely, neutrophils of diverse morphologies (Fig. 3E), with relatively higher DLC values (16th week: $52.13 \pm 1.00\%$; 24th week: $66.33 \pm 1.00\%$; 30th week: $78.01 \pm 0.58\%$) gradually replaced the eosinophilic multitude in the cervix. Subsequently, with increasing treatment duration monocytes also joined the cervical neutrophil milieu for maintenance of the 3MC induced inflammation which could have possibly redesigned the cervical tissue architecture. Macrophages, although sparsely noted, was also obtained in the cervical infiltrates of these mice. An anecdote of cervical leukocyte infiltration trends among Group-III mice is very well-depicted in Fig. 3C. In Group-II mice, transient neutrophil infiltration was apparent only at 16th week of PET treatment (Fig. 3B). Such a short-lived inflammation was not efficacious enough to eventuate in cervical neoplasia. The observations were comparable with that of control mice from Group-I. Micrographic snippets of the varied cervical leukocytic subpopulations comprising of eosinophils, neutrophils and monocytes among 3MC treated mice are vividly portrayed in Fig. 3D.

Incessant cervical inflammation was accompanied by development of squamous cell carcinoma

Following overall analysis of cervical histology among 3MC treated groups; the cervical tumor which emerged particularly at or after 24 weeks of 3MC administration was respectively regionalized into 'cortical', 'cortico-medullary' and 'medullary' regions for histopathological characterization. Histological exhibits of tumor cortex (Fig. 4, indicated by arrows) hinted towards a loss of tissue organization owing to the presence of large keratin pearls. On delving into the cortico-medullary region, distortion of squamous epithelial cells (indicated by arrows) was remarkably noted. Squamous epithelial cells abnormally transformed into 'giant tumor cells' (indicated by arrows) harbouring aggregation of multiple nuclei in their centre (Fig. 4, lower left panel). A glimpse of the medullary tumor core, furthermore unravelled the presence of epithelial regions (indicated by arrows) which might have invaded into the stroma from the overlying tissue tier (Fig. 4, lower right panel). These histopathological findings annotated for the development of an aggressive and poorly differentiated 'anaplastic squamous cell carcinoma' at the mice cervix due to chronic inflammation produced by 30 weeks of 3MC treatment.

Role of inflammogenic mediator COX2 in 3MC induced carcinogenic changes

Conditions of 'spleen' were thoroughly studied for identifying the systemic source of leukocytes augmenting cervical inflammation. Comparative overview of the splenic anatomy and histology is well depicted in Fig. 5Ai-iv. Splenomegaly was deciphered in 3MC treated mice at and after 16th week. Corresponding splenic histopathology (Fig. 5Aiii & iv) exhibited profuse eosinophil infiltrations (indicated by arrows), along with the prevalence of an increased number of 'germinal centres' with intense eosin stained peripheries (indicated by arrows). These affirmed accelerating rates of leucopoiesis which primarily got incited in the mice body for combating 3MC induced cervical toxicity. However, dysregulated inflammation antagonistically paved a way for cervical carcinogenesis by further activating inflammogens. Spleen tissues isolated from vehicle control and untreated mice at 16th week were devoid of such histological remodelling.

The reproductive tissues isolated from these mice batches appeared to be strangled with blood vessels (Fig. 5B; indicated by arrows). This kind of vasculogenic changes are product of carcinogenesis-enabling vehement inflammatory reactions, which thereafter prompted for the subsequent exploration of the responsible molecular interactome involving COX2. According to western blot results (Fig. 5D), the cervical COX2 expression in mice heightened along with 3MC treatment extension. An elevated COX2 level in the tumor core was characteristically noted from the study of differential COX2 expression patterns in various tumor regions (Cortex-Tumor_{COR}; Cortico-medullary- Tumor_{COR/MED}; Medulla- Tumor_{MED}). Further assessment of the functional status of COX2 was performed by IHC wherein COX2 was found to be spatially distributed in the brown stained nucleus and cytoplasm (Fig. 5C) of the cervical tissue sections obtained from 3MC treated mice in the 16th and 24th weeks. A high intensity staining of about 60.44% (6/10 mice) and 72.69% (7/10 mice) were scored amongst these respective groups (Fig. 5D).

These findings endorsed an increasing trend in cervical COX2 activity (Fig. 5E). A significantly escalated COX2 activity (12th week: 3.8 ± 0.0091 U/mg; 16th week: 5.72 ± 0.016 U/mg) in mice cervix continued to rise consistently even in 24th and 30th weeks of 3MC treatment. This cumulative increase in expression and activity of COX2 protein was found to be very strongly correlated ($r = 0.9750$; $p < 0.005$) among the 3MC treated groups (Table 3A). COX2 activities among vehicle control mice were at par with the control data.

Table3(A): Pearson's correlation coefficient among COX2 expression(COX2exp) profiles with COX2 activity, NFkB (p50), NFkB (p65), GM-CSF1, XIAP, survivin and p53 expression profiles of mice cervix subjected to chronic 3MC treatment for 0, 6, 12, 16, 24 and 30 weeks.

	COX2 exp	COX2 activity	NFkB (p50)	NFkB (p65)	GM- CSF1	XIAP	Survivin	p53
COX2 exp	1.000							
COX2 activity	0.9750	1.000						
NFkB (p50)	0.9550	0.9444	1.000					
NFkB (p65)	0.9815	0.9280	0.9603	1.000				
GM-CSF1	0.9455	0.9109	0.8742	0.9305	1.000			
XIAP	0.9010	0.8570	0.9718	0.9491	0.8500	1.000		
Survivin	0.9271	0.8933	0.9849	0.9642	0.8617	0.9957	1.000	
p53	-0.7901	-0.7612	-0.8807	-0.912	-0.7221	-0.9200	-0.9023	1.000

Inflammation mediated COX2 activation propagated into subsequent overexpression of NFkB, GM-CSF1 and Ki67

A gain in expression of NFkB, GM-CSF1 and Ki67 (Fig. 6A) as obtained from western blot results delineated the pathway taken by activated COX2 for promoting carcinogenesis. To establish the possibility of COX2 being the culprit in upregulating NFkB (p50/p65), GM-CSF1 and Ki67, correlation coefficient were calculated and represented in a tabulated format (Table 3A). Evidentially, a strong positive correlation between each of these parameters was mathematically obtained which further strengthened the notion.

An earlier experimental observation attained by the team with the same model revealed the presence of an upregulated cervical IL6 and IL8 activities alongside high expressions [37]. These are also the upstream activators of COX2 that fuel a plethora of prosurvival signalling molecules. Consequentially, with further study elevated Granulocyte Macrophage Colony Stimulating Factor1 (GM-CSF1) expressions as evident from the incremental band intensity patterns (Fig. 6A, lower panel), corroborated with the frequent flux of neutrophils in the cervix (Fig. 3C). This justified the conviction that IL6, IL8 and GM-CSF1 cytokines along with an upregulated COX2 resulted in escalation of NFkB expressions (Fig. 6A, upper panel). In alliance with COX2 expressions, NFkB (p50/p60) subunits were observed to be very frequently localized within the nucleus at the tissue level (Fig. 6B, upper panel). In the micrographic excerpts of IHC tissue sections sampled from mice subjected to 16 and 24 weeks of 3MC treatment, positive nuclear staining for both p65 and p50 subunits of the protein was

attained. High intensity positive nuclear staining for p50 and p65 enumerating to 78.77% (8/10 mice) and 82.03% (8/10 mice) were scored amongst the 16th week mice groups (Fig. 6B, lower panel). Respective protein expression scores stoking to about 80.23% (8/10 mice) and 86.23% (8/10 mice) were further quantitated amongst mice in 24 weeks of 3MC treatment (Fig. 6B, lower panel). Moreover, Ki67 expression patterns in the whole tissue proteins were fairly high (Fig. 6A, upper panel). Such a distinctively active functional status of the protein was absent amongst vehicle control and control mice during this interval.

Chronic 3MC treatment induced COX2 enabled IAP activations

In order to assess the strength of this prosurvival signalling in overriding apoptotic cues the disposition of XIAP and survivin was analysed in the cervical tissues of 3MC treated mice. Western blot results as showcased in Fig. 7A clearly indicated the rising patterns in protein expressions of XIAP and survivin in the mice cervical tissue lysates at the treatment interims. Particularly, for XIAP, a hike in expressions was attained at 16th week of 3MC treatment. Survivin, on the contrary was not as high as XIAP while in relation to control and vehicle control mice the expression profiles was considerably high.

IHC results as portrayed in Fig. 7B exposed the presence of functional XIAP and survivin proteins in the cellular cytoplasm of cervical tissue sections specifically obtained from 3MC treated mice in 16th and 24th weeks. Respective positive staining intensities for XIAP scored to about 75.25 % (~ 7/10mice) and 78.625 % (~ 8/10mice) were documented amongst these respective batches. Similarly, the cytoplasm was comparatively less stained (16weeks:55%; 5/10mice, 24weeks:59.98%; ~6/10mice) for survivin. Again, XIAP ($r = 0.8570$ & $r = 0.9010$; $p < 0.005$) and survivin ($r = 0.8933$ & $r = 0.9271$; $p < 0.005$) expressions were in strong positive correlation with the activity and expressions of COX2 in the 3MC treated mice (Table 3A). Absence of these findings in the vehicle control and control animals were imperative of the involvement of NF κ B /COX2/IAP axis in development of cervical neoplasia in 3MC treated mice.

COX2 activation immortalized cervical cells by facilitating Ki67 and PCNA expressions

The spatial distribution of proliferative antigens Ki67 and PCNA was studied by IHC in order to assess the proliferative potentials of the cervical epithelium as a culmination of NF κ B /COX2/IAP functions. Positive staining of cellular nucleus in all the differential cervical epithelial layers was noted amongst the tissue sections of mice subjected to 3MC treatment for 16 and 24 weeks or more (Fig. 8A). These patterns were delimited only to the undifferentiated proliferative basal epithelial cell layers of the control and vehicle control groups. Clear manifestations of uniform staining for Ki67 and PCNA of all cells in the cervical epithelium were scored and illustrated comparatively in Fig. 8B.

Inflammation induced systemic stress generates free radicals by disrupting antioxidant defence mechanism

To further find out the systemic impact of chronic cervical 3MC treatment in mice, ROS and RNS levels were quantified in the blood leukocytes and peritoneal macrophages. Relative interpretations of these findings were undertaken with respect to iNOS and the total antioxidant enzyme activities. A significant appraisal in ROS levels were noted in 3MC treated mice from 16th week onwards (Fig. 9A). These observations were coherent with that of RNS levels among these same mice groups (Fig. 9B). Nitrite generation was documented to rise

from 16th week ($3.813 \pm 0.00686 \mu\text{M}/10^6\text{cells}/\text{min}$) which was found to attain peak by 30 weeks of 3MC treatment. Both of these findings coincided with the activity trends of iNOS as shown in Fig. 9C where the enzyme activity was found to produce citrulline of about $11.89 \pm 0.15311\%$ in the 16th week specifically. These parameters were found to be subletting each other for promoting systemic stress as revealed from their significantly strong positive correlations (Table 3B).

Table3(B): Pearson’s correlation coefficient among cervical COX2 activity and collateral systemic parametres ROS levels, RNS levels, iNOS activity and Comet Tail Moment(CTM) of mice subjected to chronic 3MC treatment for 0, 6, 12, 16, 24, and 30 weeks.

	COX2 activity	ROS	RNS	iNOS	CTM
COX2 activity	1.000				
ROS	0.9872	1.000			
RNS	0.9523	0.9537	1.000		
iNOS	0.9648	0.9446	0.9708	1.000	
CTM	0.9606	0.9456	0.9652	0.9974	1.000

All these findings necessitated to look into the activities of the total antioxidant enzyme capacities. Graphical overview clearly depicted gradual rise in free radical scavenging activity up to 12th and 16th week of treatment (Fig. 9D), which subsequently declined by 24 and 30 weeks. Relatively, in the vehicle control groups the free radical scavengers were found to function properly with exposure to PET like that of control one. The subtly increasing trend of anti-oxidant enzyme activities holistically helped to quench the PET generated free radicals which went unquenched in the carcinogen treated groups.

Cervical inflammation imposed systemic genotoxic stress eventually deregulating key tumor suppressor proteins

To ascertain the genotoxic profile upon 3MC exposure mediated inflammatory stress, comet assay was performed with the leukocytes isolated from mice blood of all the three groups. Fluorescent microscopic images exhibited the distribution patterns of damaged DNA in terms of DNA migration (comet formation) among different groups (Fig. 10Ai-xv). The results upheld the appearance of distinct DNA-tails among the 3MC treated groups with incremental time period. Among the PET treated mice, presence of such damage was indistinct just like that of the untreated animals. Concomitant rise in the comet tail moment as calculated by KOMET software is represented graphically in Fig. 10B. Results were indicative of the impact of free radical mediated stress upon the DNA (Table 3B).

Blood bearing leukocytes harbouring damaged DNA also drain into cervical region which was under eventual transformation by the hiked COX2 activities. Continual inflammation driving changes mediated by COX2 accompanied subsequent reduction in expression profiles of tumor suppressors at the protein levels in the mice cervical tissue extracts. Western blot data as depicted in Fig. 10C are imperative of the gradual fall in protein expressions of p53, p21 and Rb, specifically at and after 16 weeks of 3MC treatment. Unstable expression profiles of acetylated-p53 (Lys373), the active form of the protein, further supported the observation. These

inferences bridge the impact of inflamogenic stress induced in the cervix with the role of the simultaneously induced systemic stress in favouring cervical carcinogenesis in this study

Discussion

In spite of therapeutic advancements, higher expression status of COX2 in cervical cancer surmounts in therapy resistance followed by disease relapse. Traditionally being a HPV mediated cancer the viral oncoproteins E5, E6 and E7 were found to turn on 'amphiregulin', the COX2 activator either in a ligand dependent or independent manner via MAPK or PI3K/Akt pathways [42, 43]. In coherence with HPV mediated cervical cancers, the present model also exhibited an escalated COX2 kinetics. Hence, the role of COX2 as an orchestrator of dysregulated inflammation in inducing cervical neoplastic changes upon chronic treatment with 3MC is well highlighted in this study. Concomitant rise in expression and activity of COX2 accelerated expressions of prosurvival molecules like NFκB (p50/p65), GM-CSF1, XIAP and survivin within the mice cervix tissue with prolongation of treatment duration. This clearly annotated for the presence of a '*positive feedback loop*' of prosurvival signalling molecules which paved a way for cervical neoplasia (Table 3A).

The current results along with previous laboratory findings of elevated expressions and activities of inflammatory cytokines IL6 and IL8 in the cervical tumor microenvironment of 3MC treated mice favouring carcinogenic progression, were in accordance with the study of Li et al (2018) wherein IL6, IL8 and COX2 triggered each other to bring about carcinogenesis [6]. The present study confirmed localization of COX2 protein in the nucleus as well as cytoplasm of the cervical tissues obtained from mice undergoing 3MC treatment, predominantly for 16 weeks, 24 weeks and onwards. This hinted towards the prevalence of an upregulated COX2 dynamics in the cervix. Quantitative estimation of COX2 activity also reflected a stimulated trend in enzyme kinetics. Such an inference enabled identification of COX2 as the protagonist molecule directing this episode of '*inflammogenesis of cervical cancer*'.

Further experimentation revealed convenient regulation by COX2 in generation of a molecular interactome involving erratically expressed tumor suppressor proteins (p53, p21 and Rb) and upregulated prosurvival proteins which majorly included NFκB, XIAP and survivin in 3MC driven cervical carcinogenesis. This eventually rendered an impact in transformation of the reproductive organ anatomy, cytopathology, histopathology and biochemistry of the carcinogen treated mice. COX2 and p53 are antagonistic in function because the latter is known to positively regulate 'thrombospondin-1' which directly inhibits angiogenesis by shutting down the angiogenic effector molecule, VEGF [28, 29]. Hypoxia induced functional facilitation of p53 is a common occurrence within a tumor lump, alongside activation of NFκB and COX2. However, during carcinogenic discourse, growth aiding cues received in terms of hiked iNOS activity was reported to encourage NFκB and COX2 for overriding the p53 action so as to progress with neoplasia [44, 45]. In agreement with these previously published reports, the tumor obtained after 24 weeks of carcinogen treatment in this present study also exhibited a high COX2 and NFκB expressions in the tumor core with concomitantly high COX2 activity. IHC results fostering nuclear accumulation of p65 and p50 subunits of NFκB was indicative of its active functional stature. Nonetheless, an eventual decline in p53 correspondingly accompanied by rise in IAPs with time was an alibi to COX2 being the silent umpire of the interplay between these promoters of carcinogenesis.

Another interesting observation of the present study was formation of enlarged spleen (splenomegaly) which was specifically observed in animals at 16th week of 3MC treatment and onwards. Documentation of the

cumulative experimental findings had unveiled the treatment period of 16th week as the 'crucial-interim' for the development of invasive cancer among these mice. Enlarged spleen showcased an altered histopathology which was characterized by the presence of eosinophil infiltrations and enhanced number of 'germinal centres'; the hubs which nourish and train the naïve blood leukocytes to become immunologically competent [46]. Continual carcinogenic treatment in mice for 30 weeks caused outbursts of free radicals to mediate systemic stress as evident from escalated iNOS activity, indiscriminately increased ROS and RNS levels which conjugatively compromised scavenging capacity of the antioxidant enzymes. Impact of these multiple oxidative hits was apparent in the form of concurrent damage incurred upon the leukocyte DNA as observed from Comet Assay (Table 3B). Being a PAH, 3MC gets potentiated as a carcinogen only after biotransformation. Thus, its effect upon the systemic parameters in the long run was inevitable. Moreover, COX2 was also reported to promote invasion of cervical cancer cells into lymphoid organs and blood vasculature [47]. In all probability, causation of splenomegaly was also one of the attributions of COX2 in this experimental model

Cervix is under constant immune surveillance since it is always patrolled by leukocytes, specifically eosinophils besides having an acidic niche. Any shift or drift in the cervical leucocyte subpopulation is therefore alarming. In this study, a shoot up of neutrophil with altered morphologies accompanied by monocytes gradually replaced the eosinophilic multitude of the mice cervix at 16 weeks of 3MC treatment which persisted with time to quench the carcinogenic assault. These inflamogenic mediators seem to have drained into the cervix following seepage from an enlarged spleen as relentless leucopoiesis was reported to render splenomegaly [48]. Molecular insights further evinced a parallel rise in GM-CSF1, the key functional neutrophil differentiator. Evidentially, COX2 in this study acted as a driver of the cervical inflammatory events which eventually amplified into the entire system of the animals.

XIAP and survivin are vital prosurvival molecules which migrate from the nucleus to the cytoplasm for becoming functionally active. Cytosolic IAPs bind to the caspase enzymes, the cellular executioners, thereby rendering them inactive [49]. In the present study, appreciable cytosolic dislocation of the respective IAPs on and after 16th week of treatment span was obvious. This was convoyed by collateral nuclear accumulation of the proliferative antigens, Ki67 and PCNA. As comprehensible from immunostaining of cervix tissues for Ki67 and PCNA (IHC results), the immortalized cells of cervix were found to be expressing these proliferative antigens which subsequently disbursed from the proliferative basal layer to rest of the differential cervical epithelial layers. COX2 mediated activation of prosurvival effectors for boosting malignant cervical transformations could be vividly envisioned from this present finding. Upregulated COX2 with all its allies was tailoring the never ending saga of 3MC induced carcinogenic inflammation to enable the development of cervical carcinoma. Overall findings have been schematically represented in Fig. 11.

Conclusion

The *in vivo* model established here flaunts a microenvironment which has upregulated prosurvival molecules under the command of COX2. Thus, it can be treated as a model to address issues of therapy resistance that stymies cervical cancer treatment. This referral model throws paramount light upon ways to target COX2 and its several unknown effectors as prognostic and diagnostic biomarkers for planning a better treatment rationale.

Declarations

AUTHOR CONTRIBUTION

Elizabeth Mahapatra has predominantly acquired all the data, analysed them to design the concept for this paper and has accordingly composed the manuscript with assistance from Souvick Biswas and Salini Das.

Sutapa Mukherjee has designed the project work and has critically checked through the manuscript.

Madhumita Roy has checked through the manuscript.

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DATA AVAILABILITY

All the datasets displayed in the current study are available from the corresponding author on reasonable request.

CODE AVAILABILITY

There is no code available.

Conflict of Interest. The authors declare that they have no known conflicts of interest.

Ethics Approval. All animal experimentations were carried out in accordance with IAEC ethical norms. Mice were procured only after ethical clearance (**IAEC Proposal No# IAEC-1774/SM-1/2018/7**) for the concerned project.

Consent to participate. All authors have wilfully participated in the concerned work following which they have given complete consent for publication.

Consent for Publications. All authors have given their consent for this publication.

References

1. Cui, Y., X. O. Shu, H. L. Li, G. Yang, W. Wen, Y. T. Gao, Q. Cai, N. Rothman, H. Y. Yin, Q. Lan, Y. B. Xiang, and W. Zheng. 2017. Prospective study of urinary prostaglandin E2 metabolite and pancreatic cancer risk. *Int J*

Cancer 141: 2423–2429.

2. Hashemi Goradel, N., M. Najafi, E. Salehi, B. Farhood, and K. Mortezaee. 2019. Cyclooxygenase-2 in cancer: A review. *J Cell Physiol.* 234: 5683–5699.
3. Kim, E. H., H. K. Na, D. H. Kim, S. A. Park, H. N. Kim, N. Y. Song, and Y. J. Surh. 2008. 15-Deoxy-Delta12, 14-prostaglandin J2 induces COX-2 expression through Akt-driven AP-1 activation in human breast cancer cells: a potential role of ROS. *Carcinogenesis.* 29: 688–695.
4. Lang, S., H. Busch, M. Boerries, T. Brummer, S. Timme, S. Lassmann, K. Aktories, and G. Schmidt. 2017. Specific role of RhoC in tumor invasion and metastasis. *Oncotarget* 8: 87364–87378.
5. Wang, D., F. G. Buchanan, H. Wang, S. K. Dey, and R. N. DuBois. 2005. Prostaglandin E2 enhances intestinal adenoma growth via activation of the Ras-mitogen-activated protein kinase cascade. *Cancer Res* 65: 1822–1829.
6. Li, N. N., X. S. Meng, Y. R. Bao, S. Wang, and T. J. Li. 2018. Evidence for the Involvement of COX-2/VEGF and PTEN/PI3K/AKT Pathway the Mechanism of Oroxin B Treated Liver Cancer. *Pharmacogn Mag* 14: 207–213.
7. Yanni, S. E., G. W. McCollum, and J. S. Penn. 2010. Genetic deletion of COX-2 diminishes VEGF production in mouse retinal Müller cells. *Exp Eye Res* 91: 34–41.
8. Konstantinopoulos, P. A., G. P. Vondoros, G. Sotiropoulou-Bonikou, A. Kominea, and A. G. Papavassiliou. 2007. NF-kappaB/PPAR gamma and/or AP-1/PPAR gamma 'on/off' switches and induction of CBP in colon adenocarcinomas: correlation with COX-2 expression. *Int J Colorectal Dis* 22: 57–68.
9. Szweda, M., A. Rychlik, I. Babińska, and A. Pomianowski. 2019. Significance of Cyclooxygenase-2 in Oncogenesis. *J Vet Res.* 63: 215–224.
10. Tsatsanis, C., A. Androulidaki, M. Venihaki, and A. N. Margioris. 2006. Signalling networks regulating cyclooxygenase-2. *Int J Biochem Cell Biol* 38: 1654–1661.
11. Parida, S., and M. Mandal. 2014. Inflammation induced by human papillomavirus in cervical cancer and its implication in prevention. *Eur J Cancer Prev.* 23: 432–448.
12. Müller-Decker, K. 2011. Cyclooxygenase-dependent signaling is causally linked to non-melanoma skin carcinogenesis: pharmacological, genetic, and clinical evidence. *Cancer and Metastasis Reviews* 30: 343–361.
13. Moon, H., A. C. White, and A. D. Borowsky. 2020. New insights into the functions of Cox-2 in skin and esophageal malignancies. *Exp Mol Med.* 52: 538–547.
14. Simões, M. C. F., J. J. S. Sousa, and A. A. C. C. Pais. 2015. Skin cancer and new treatment perspectives: a review. *Cancer Letters* 357: 8–42.
15. Siegrist, K. J., D. Romo, B. L. Upham, M. Armstrong, K. Quinn, L. Vanderlinden, R. S. Osgood, K. Velmurugan, M. Elie, J. Manke, D. Reinhold, N. Reisdorph, L. Saba, and A. K. Bauer. 2019. Early Mechanistic Events Induced by Low Molecular Weight Polycyclic Aromatic Hydrocarbons in Mouse Lung Epithelial Cells: A Role for Eicosanoid Signaling. *Toxicol Sci* 169: 180–193.
16. Asproni, P., M. Vignoli, S. Cancedda, F. Millanta, R. Terragni, and A. Poli. 2014. Immunohistochemical expression of cyclooxygenase-2 in normal, hyperplastic and neoplastic canine lymphoid tissues. *J Comp Pathol.* 151: 35–41.

17. Hsum, Y. W., W. T. Yew, P. L. Hong, K. K. Soo, L. S. Hoon, Y. C. Chieng, and L. Y. Mooi. 2011. Cancer chemopreventive activity of maslinic acid: suppression of COX-2 expression and inhibition of NF- κ B and AP-1 activation in Raji cells. *Planta Medica* 77: 152–157.
18. Bandyopadhyay, R., U. Chatterjee, S. K. Mondal, D. Nag, and S. K. Sinha. 2011. A study on expression pattern of cyclooxygenase-2 in carcinoma of cervix. *Indian J Pathol Microbiol.* 54: 695–699.
19. Bengi, G., D. Keles, Ö. Topalak, M. Yalçın, R. Kiyak, and G. Oktay. 2015. Expressions of TIMP-1, COX-2 and MMP-7 in Colon Polyp and Colon Cancer. *Euroasian J Hepatogastroenterol.* 5: 74–79.
20. Hidalgo-Estévez, A. M., K. Stamatakis, M. Jiménez-Martínez, R. López-Pérez, and M. Fresno. 2020. Cyclooxygenase 2-Regulated Genes an Alternative Avenue to the Development of New Therapeutic Drugs for Colorectal Cancer. *Frontiers in Pharmacology* 11: 533.
21. Jessen, C., J. K. C. Kreß, A. Baluapuri, A. Hufnagel, W. Schmitz, S. Kneitz, S. Roth, A. Marquardt, S. Appenzeller, C. P. Ade, V. Glutsch, M. Wobser, J. P. Friedmann- Angeli, L. Mosteo, C. R. Goding, B. Schilling, E. Geissinger, E. Wolf, and S. Meierjohann. 2020. The transcription factor NRF2 enhances melanoma malignancy by blocking differentiation and inducing COX2 expression. *Oncogene.* 39: 6841–6855.
22. Liu, F., W. Q. Wei, R. T. Cormier, S. T. Zhang, Y. L. Qiao, X. Q. Li, S. T. Zhu, Y. C. Zhai, X. X. Peng, Y. X. Yan, L. J. Wu, D. He, and Y. He. 2014. Association of single nucleotide polymorphisms in the prostaglandin-endoperoxide synthase 2 (PTGS2) and phospholipase A₂ group IIA (PLA2G2A) genes with susceptibility to esophageal squamous cell carcinoma. *Asian Pac J Cancer Prev* 15: 1797–1802.
23. Fodor, D., I. Jung, S. Turdean, C. Satala, and S. Gurzu. 2019. Angiogenesis of hepatocellular carcinoma: An immunohistochemistry study. *World J Hepatol* 11: 294–304.
24. Ye, Y., X. Wang, U. Jeschke, and V. von Schönfeldt. 2020. COX-2-PGE2-EPs in gynecological cancers. *Arch Gynecol Obstet.* 301: 1365–1375.
25. Hill, R., Y. Li, L. M. Tran, S. Dry, J. H. Calvopina, A. Garcia, C. Kim, Y. Wang, T. R. Donahue, H. R. Herschman, and H. Wu. 2011. Cell intrinsic role of COX-2 in pancreatic cancer development. *Mol Cancer Ther* 11: 2127–2137.
26. Shi, G., D. Li, J. Fu, Y. Sun, Y. Li, R. Qu, X. Jin, and D. Li. 2015. Upregulation of cyclooxygenase-2 is associated with activation of the alternative nuclear factor kappa B signaling pathway in colonic adenocarcinoma. *Am J Transl Res.* 7: 1612–1620.
27. Mortezaee, K., E. Salehi, H. Mirtavoos-Mahyari, E. Motevaseli, M. Najafi, B. Farhood, R. J. Rosengren, and A. Sahebkar. 2019. Mechanisms of apoptosis modulation by curcumin: Implications for cancer therapy. *J Cell Physiol.* 234: 12537–12550.
28. Janani, S. K., S P D., R. S., Sai Surya, and N. U. Chenmala, K. 2021. Guardian of genome on the tract: Wild type p53-mdm2 complex inhibition in healing the breast cancer. *Gene.* 786: 145616.
29. Tong, X., S. Mirzoeva, D. Veliceasa, B. B. Bridgeman, P. Fitchev, M. L. Cornwell, S. E. Crawford, J. C. Pelling, and O. V. Volpert. 2014. Chemopreventive apigenin controls UVB-induced cutaneous proliferation and angiogenesis through HuR and thrombospondin-1. *Oncotarget.* 5: 11413–11427.
30. Kaczmarzyk, T., K. Kisielowski, R. Koszowski, M. Rynkiewicz, E. Gawełek, K. Babiuch, A. Bednarczyk, and B. Drozdowska. 2018. Investigation of clinicopathological parameters and expression of COX-2, bcl-2, PCNA, and p53 in primary and recurrent sporadic odontogenic keratocysts. *Clin Oral Investig.* 22: 3097–3106.

31. Mortezaee, K., E. Salehi, H. Mirtavoos-Mahyari, E. Motevaseli, M. Najafi, B. Farhood, R. J. Rosengren, and A. Sahebkar. 2019. Mechanisms of apoptosis modulation by curcumin: Implications for cancer therapy. *J Cell Physiol* 234: 12537–12550.
32. Stasinopoulos, I., T. Shah, M. F. Penet, B. Krishnamachary, and Z. M. Bhujwalla. 2013. COX- 2 in cancer: Gordian knot or Achilles heel? *Frontiers in Pharmacology* 4: 34.
33. McClain-Caldwell, I., L. Vitale-Cross, B. Mayer, M. Krepuska, M. Boyajian, V. Myneni, D. Martin, GENOMICS AND COMPUTATIONAL BIOLOGY CORE, K. Marko, K. Nemeth, and E. Mezey. 2018. Immunogenic potential of human bone marrow mesenchymal stromal cells is enhanced by hyperthermia. *Cytotherapy*. 20: 1437–1444.
34. Ylöstalo, J. H., T. J. Bartosh, K. Coble, and D. J. Prockop. 2012. Human mesenchymal stem/stromal cells cultured as spheroids are self-activated to produce prostaglandin E2 that directs stimulated macrophages into an anti-inflammatory phenotype. *Stem Cells* 30: 2283–2296.
35. Zhao, Y., T. Wu, S. Shao, B. Shi, and Y. Zhao. 2015. Phenotype, development, and biological function of myeloid-derived suppressor cells. *Oncoimmunology* 5 (2): e1004983.
36. Mc Clintock, M. K. 1981. Social Control of the Ovarian Cycle and the Function of Estrous Synchrony. *Amer Zool* 21: 243.
37. Mahapatra, E., S. Biswas, M. Roy, and S. Mukherjee. 2020. Inflammation: A protagonist in development of carcinogen induced cervical cancer in mice. *Indian Journal of Biochemistry & Biophysics* 57: 158–166.
38. Basu, M., S. Ghosh, A. Roychowdhury, S. Samadder, P. Das, S. Addya, A. Roy, D. K. Pal, S. Roychoudhury, A. Ghosh, and C. K. Panda. 2020. Integrative genomics and pathway analysis identified prevalent FA-BRCA pathway alterations in arsenic-associated urinary bladder carcinoma: Chronic arsenic accumulation in cancer tissues hampers the FA-BRCA pathway. *Genomics* 112: 5055–5065.
39. Sinha, D., S. Roy, and M. Roy. 2010. Antioxidant potential of tea reduces arsenite induced oxidative stress in Swiss albino mice. *Food Chem Toxicol* 48: 1032–1039.
40. Mahapatra, E., D. Dasgupta, N. Bhattacharya, S. Mitra, D. Banerjee, S. Goswami, N. Ghosh, A. Dey, and S. Chakraborty. 2017. Sustaining immunity during starvation in bivalve mollusc: A costly affair. *Tissue and Cell* 49: 239–248.
41. Biswas, J., S. Roy, S. Mukherjee, D. Sinha, and M. Roy. 2010. Indian spice curcumin may be an effective strategy to combat the genotoxicity of arsenic in Swiss albino mice. *Asian Pacific J Cancer Prevention* 11: 239–249.
42. Kim, S. H., Y. S. Juhn, S. Kang, S. W. Park, M. W. Sung, Y. J. Bang, and Y. S. Song. 2006. Human papillomavirus 16 E5 up-regulates the expression of vascular endothelial growth factor through the activation of epidermal growth factor receptor, MEK/ ERK1, 2 and PI3K/Akt. *Cell Mol Life Sci* 63: 930–938.
43. Luna, A. J., R. T. Sterk, A. M. Griego-Fisher, J. Y. Chung, K. L. Berggren, V. Bondu, P. Barraza-Flores, A. T. Cowan, G. N. Gan, E. Yilmaz, H. Cho, J. H. Kim, S. M. Hewitt, J. E. Bauman, and M. A. Ozbun, 2021. MEK/ERK signaling is a critical regulator of high-risk human papillomavirus oncogene expression revealing therapeutic targets for HPV-induced tumors. *PLoS Pathog.* 17, e1009216.
44. Parenti, A., L. Morbidelli, X. L. Cui, J. G. Douglas, J. D. Hood, H. R. Granger, F. Ledda, and M. Ziehe. 1998. Nitric oxide is an upstream signal of vascular endothelial growth factor-induced extracellular signal-regulated kinase 1/2 activation in postcapillary endothelium. *J Biol Chem.* 273: 4220–4226.

45. Schmedtje, J. F., Y. S. Ji, W. L. Liu, R. N. DuBois, and M. S. Runge. 1997. Hypoxia induces cyclooxygenase-2 via NF-kappaB p65 transcription factor in human vascular endothelial cells. *J Biol Chem* 272: 601–608.
46. Stebegg, M., S. D. Kumar, A. Silva-Cayetano, V. R. Fonseca, M. A. Linterman, and L. Graca. 2018. Regulation of the Germinal Center Response. *Front Immunol* 9: 2469.
47. Hoellen, F., A. Waldmann, C. Banz-Jansen, A. Rody, M. Heide, F. Köster, J. Ribbat-Idel, C. Thorns, M. Gebhard, M. Oberländer, J. K. Habermann, and M. Thill. 2016. Expression of cyclooxygenase-2 in cervical cancer is associated with lymphovascular invasion. *Oncol Lett* 12: 2351–2356.
48. Lv, Y., W. Y. Lau, Y. Li, J. Deng, X. Han, X. Gong, N. Liu, H. Wu. 2016. Hypersplenism: History and current status. *Exp Ther Med* 12: 2377–2382.
49. Shlezinger, N., M. Israeli, E. Mochly, L. Oren-Young, W. Zhu, and A. Sharon. 2016. Translocation from nuclei to cytoplasm is necessary for anti A-PCD activity and turnover of the Type II IAP BcBir1. *Mol Microbiol* 99: 393–406.

Figures

Fig. 1.

Figure 1

Carcinogen preparation and experimental design (a) Schematic representation of the laboratory protocol adapted for preparing 3MC (0.6mg/ml) (b) An experimental outline for development of cervical cancer model employing Swiss Albino mice

Fig. 2.

Figure 2

Understanding the progressive changes in mice upon 3MC treatment: Comparative images of external orifice, reproductive organ, cyto and histopathological features (a) Comparative pictorial representation of the external vaginal orifice among Group I, II and III mice wherein apparent disfigurement (arrow indicated) was obtained in 3MC treated groups, particularly at 16, 24 and 30 weeks (b) Reproductive systems of Groups I, II and III mice depicting the presence of palpable changes along with a discernible tumor (c) Illustrative micrographs (200X; Original magnifications x 10; scale bar: 50 μm) of Pap stained cervical exfoliated cells displaying cytopathological alterations as induced by 3MC and PET treatments in mice (d) Concomitant histopathological changes (indicated by arrows) evincing the presence of mild, moderate and severe (carcinoma in situ) dysplastic stages along with invasive cancer stages in 6th, 12th, 16th, 24th and 30th weeks of 3MC treatment. Main images are magnified upto 200X (Original magnifications x 10) with side-highlighted insets being magnified to 400X

Fig. 3.

Figure 3

Differential cervical leukocytic flux during carcinogenic progression Comparative graphical representation of differential leukocyte count obtained in Pap-stained smears of (a) Group I, (b) Group II and (c) Group III mice. Data represented as Mean $\pm$ S.D. was considered statistically significant (* $p < 0.005$ /** $p < 0.01$; N=10) (d) A representative micrographic snippet (200X; scale bar: 50 μ m) of the cervix-invading leukocytes (eosinophils, neutrophils, monocytes and macrophages) (e) Specific light microscopic highlights of infiltrated morphologically diverse neutrophils in 3MC treated animals

Fig. 4.

Figure 4

Histological characterization of cervical tumor Characteristic histological features in the sections (5µm thick) from specific regions (cortex, cortico-medulla and medulla) of the cervical anaplastic tumor mass. Microscopic images were magnified up to 200x (Original magnifications x 10; Scale bar: 200 µm) while their respective insets are 400x (Original magnifications x 10; Scale bar: 20 µm) magnified

Fig. 5.

Figure 5

Assessing cervical inflammation profile concerning COX2 (a) Visual representation of mice splenic anatomy along with their corresponding micrographic histology images in 16th week of treatment. Images of histological sections (~5 μ m thick) are magnified to 200X (Original magnifications x 10; Scale bar: 200 μ m) (b) Reproductive system of 3MC treated mice (16th week) displaying vasculature growth (arrow indicated) (c) Representative IHC micrographs exhibiting spatial distribution of COX2 within the nucleus and cytoplasm of the 3MC treated mice cervix tissues in the 16th and 24th weeks. Control and vehicle control images are magnified up to 400X with scale bar of 50 μ m; whereas magnification of treatment images are-Main image: 200X; Insets: 400x; upper panel with scale bar: 100 μ m. Graphical portrayal (lower panel) of corresponding intensity (high/low) trends for COX2 specific positive staining in these 3MC treated (16 and 24 weeks) cervical tissues (d) Western Blot results depicting COX2 expressions in the total tissue lysates of Group III mice cervix (e) Concomitant COX2 kinetics (U/mg of protein) in the cervical tissue lysates of the corresponding mice groups. Numerical data were represented as Mean $\pm$ S.D of three independent experiments (* $p < 0.005$ vs Control; N=10)

Fig. 6.

Figure 6

Protein expressions of COX2 effector molecules with prosurvival significance (a) Western blot results delineating comparative profiles of NFkB (p65), NFkB (p50), GM-CSF1 and Ki67 protein expressions (b) Microscopic overview of tissue specific localization of NFkB (p65/p50) in the mice cervix tissues as studied by IHC analysis. Corresponding graphical overview (lower panel) of the cervical protein expression dynamics as represented in terms of positive staining intensity (high/low) scores. Magnifications of main images are 200X (Original magnifications x 10; scale bar: 100 μm) and that of the respective highlighted insets are magnified to 400X. Numerical data represented as Mean ± S.D of three independent experimental replicates, where * $p < 0.005$ vs Control is considered statistically significant

Fig. 7.

Figure 7

COX2 driven expressions of IAPs during the cervical cancer progression in mice (a) Comparative shifts and drifts in protein expression profiles of XIAP and survivin in the mice cervical tissue lysates obtained from different 3MC treatment groups (b) IHC micrographs (Main image: 200X ; Insets: 400X; scale bar: 100μm upper panel) indicating tissue specific XIAP and survivin expressions in mice cervix of Group III vs. Group II, and I. Along with its positive staining intensities (high/low) being expressed in a graphical anecdote. Data (Mean± S.D) are documented from three independent experimental replicates; * $p < 0.005$ vs Control

(b)

Layers of cervical epithelium	Ki-67 expressions	Expression patterns of Ki-67 in differential weeks of treatment		
		6th	16th	30th
BASAL LAYER		+	+++	+++
PARABASAL LAYER		++	+++	+++
INTERMEDIATE EPITHELIUM		-	++	+++
SUPERFICIAL CELLS		-	-	++

Layers of cervical epithelium	PCNA expressions	Expression patterns PCNA in differential weeks of treatment		
		6th	16th	30th
BASAL LAYER		+	+++	+++
PARABASAL LAYER		++	+++	+++
INTERMEDIATE EPITHELIUM		-	+++	+++
SUPERFICIAL CELLS		-	+	++

NOTE-

+ : 40-50% cells.
 ++ : 60-70% cells.
 +++ : >80% cells.

Fig. 8.

Figure 8

Elevated COX2 accelerated tissue proliferative potential of mice cervix (a) IHC exhibits for the status of proliferative antigens Ki67 and PCNA in the mice cervical tissues (Group-I, II and III). Respective image magnifications are 200X (Original magnifications). Area of interest is 100µm as per scale bar indications (b) Cellular immunostaining intensities (lower panel) in the basal, parabasal, intermediate and superficial layers of the cervical stratified epithelium are semi-quantitatively represented in terms of the frequency of microscopically recorded positively stained cells

Fig. 9.

Figure 9

Activated COX2 accounted for persistent/restraint systemic stress in mice (a-c) Dynamics of 3MC induced systemic stress is expressed in terms of free radical (ROS and RNS) generation along with quantitative estimation of iNOS activity in isolated blood leukocytes and peritoneal macrophages of mice (n=10; Group I, II and III) (d) Relative total antioxidant capacities of the corresponding mice groups differentially treated with 3MC PET. Numerical values are represented as Mean $\pm$ S.D. The results were significant at *p<0.005 vs Control. Experiments were done in triplicates

Fig. 10.

Figure 10

Possible involvement of COX2 mediated systemic stress in inducing DNA damage by hampering tumor suppressor protein function in mice (a) Comet assay images (upper left panel) depicting DNA damage incurred by the systemic stress induced due to the generated free radical in Group I, II and III mice followed by a graphical overview (upper right panel) of the relative DNA damage intensities (Mean $\pm$ S.D, n=10, *p<0.005) (b) Expression patterns (lower panel) of tumor suppressor proteins (p53, p21, Rb and Acetylated p53) as studied by western blot analysis specifically in the cervix tissue lysates of Group III mice. Experiments are repeated twice

Fig. 11.

Figure 11

Conclusive overview of the study Schematic representations of the COX2 associated probable factors fuelling neoplastic progression. This inflammation mediated prosurvival factors favoured the formation of signalling nexus which prospered in the 3MC treated mice cervix owing to ceaseless COX2 activity