Reduction of DNA Damage by Curcumin and Celecoxib in Epithelial Cell Cultures of the Oropharynx after Incubation with Tobacco Smoke Condensate

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Abstract. *Background: Tobacco smoke, as the major risk factor for the development of squamous cell cancer of the head and neck (HNSCC), contains various xenobiotics, such as polycyclic aromatic hydrocarbons, nitrosamines, aromatic amines and phenols. Chemoprevention either by artificial agents such as celecoxib, or natural compounds such as curcumin, might offer a chance to reduce the risk of developing malignant transformation. Materials and Methods: In order to evaluate the DNA-damaging effects of smoke condensate towards human mucosa cells of the oropharynx, mini organ cultures (MOC) of macroscopically healthy pharyngeal tissue of 40 patients with oropharyngeal SCC were used. After incubation with smoke condensate DNA damage was evaluated with the alkaline single-cell microgel electrophoresis (comet assay). The chemoprotective potential of curcumin and celecoxib was analyzed after their incubation with the condensate-treated MOCs. As DNA-damaging and chemo-preventive effects might not be equally distributed over the whole DNA, fragmentation of the epithelial growth factor receptor (EGFR) gene was additionally examined by Comet fluorescence in situ hybridization (FISH). Results: As expected, tobacco smoke condensate caused significant DNA fragmentation compared to the negative control. No enhanced damage was observed on the EGFR gene. DNA fragmentation was significantly reduced when MOCs were incubated with celecoxib ($p \leq 0.001$) and with curcumin ($p \leq 0.001$). Conclusion: Both celecoxib and curcumin showed considerable chemoprotective effects towards the impact of smoke condensate. No evidence was found for higher susceptibility to damage in the EGFR gene.*

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Condensed cigarette smoke contains a mixture of gaseous components and solid particles (1). More than 50 DNA-damaging agents have been detected in tobacco smoke so far, polycyclic aromatic hydrocarbons, nitrosamines, aromatic amines and phenols being the most frequent (2). Therefore, smoke condensate is an ideal substrate to evaluate the DNA-damaging effects of tobacco on the whole.

Cancer chemoprevention, the use of natural or synthetic compounds to prevent, arrest, or reverse the process of carcinogenesis, aims at reversing cancerous lesions and preventing secondary primary tumours. To be useful in humans, such compounds must have acceptable safety profiles, in addition to being effective at a dose low enough not to cause significant toxicity (3). Various synthetic and natural dietary compounds with multiple molecular targets have been identified as being effective in the prevention of carcinogenesis (4, 5).

In the present study, we evaluated the chemoprotective potential of curcumin and celecoxib, a cyclooxygenase 2 (COX2) inhibitor, after incubation of mini organ cultures (MOCs) with smoke condensate. The potential of both compounds to protect the DNA from damage has been discussed controversially in various studies (6, 7). MOCs of macroscopically healthy pharyngeal tissue of 40 patients with oropharyngeal carcinoma were used. DNA damage was quantified with the comet assay. Furthermore, we evaluated the susceptibility of the epithelial growth factor receptor (*EGFR*) gene to smoke condensate-induced DNA damage as a risk factor for DNA mutation in this region. Enhanced mutagen sensitivity in this gene might lead to DNA mutation, as a potential cause for overexpression of the EGFR protein, which occurs in the majority of HNSCC cases (8, 9). Comet fluorescence *in situ* hybridization (*FISH*) was applied to quantify the DNA damage of this gene.

Materials and Methods

Biopsies. Tissue samples of macroscopically healthy oropharyngeal mucosa were harvested during surgery of oropharyngeal carcinoma. Only mucosa that had to be resected for surgical reasons was used to avoid additional stress for the patients. The study was approved by the Ethical Commission of the Medical Department, Ludwig Maximilians University Munich (no. 221/08).

Mini organ cultures. Specimen were dissected into cubes of 1 mm^3, excluding deeper layers, and were washed three times in bronchial epithelial cell basal medium (BEGM; Promocell, Heidelberg, Germany). Cubes were placed in 24-well plates, and coated with 0.75% Noble Agar (Difco, Detroit, MI, USA) and were then dissolved in Dulbecco's modified Eagle's medium (Gibco, Eggenstein, Germany) containing fetal calf serum (10%) (Gibco), non-essential amino acids (Gibco) and amphotericine B (Gibco). After 14-20 days in 250 μl BEGM, and 37˚C with 5% CO_2 and 100% relative humidity, MOCs were completely coated with epithelium. BEGM was replaced every second day during cultivation. Multiwell plates were changed every week.

Incubation and cell separation. MOCs were either incubated with 25 μl curcumin (1 μM; Sigma, Steinheim, Germany) or celecoxib (0.1 μM Pfizer, New York, NY, USA) for 1 h at 37˚C and washed twice afterwards. Dimethylsulfoxide (DMSO, 166 mM; Merck, Darmstadt, Germany) served as the negative control. A proportion of the MOCs were then incubated with 25 μl smoke condensate (0.7 mg/dl) [as described in (4)] for 18 hours. Again, DMSO served as a negative control. MOCs were washed twice with BEGM before they underwent enzymatic digestion [10 mg hyaluronidase (Boehringer, Mannheim, Germany); 10 mg collagenase (Roche, Mannheim, Germany); 50 mg protease (Sigma)] for 45 min at 37˚C. To preserve the physiological character of the samples, no metabolic activation was used before the incubation period. Viability was tested with trypan blue staining.

Comet assay. The procedure of the comet assay for both cell types was mainly based on the protocol of Singh *et al*. (10). Special slides were designed for the comet assay with a frosting of 5 mm along the long edges (76 mm × 26 mm; Langenbrinck, Emmendingen, Germany), prepared with 85 μl of 0.5% normal melting agarose (Biozym, Hameln, Germany). Following enzymatic digestion the viability of the cells was again examined using trypan blue staining. Having obtained viabilities of between 90 and 100%, the remaining aliquots were suspended with 75 μl of 0.7% low-melting agarose (Biozym) and applied to the prepared slides. Alkaline lysis (10 ml DMSO, 1 ml Triton-X©, 89 ml alkaline lysis buffer) followed for one hour. The slides were then dried and placed into a horizontal gel electrophoresis chamber (Renner, Dannstadt, Germany), and were covered with alkaline buffer solution containing NaOH (10 mM) and Na_2EDTA (200 mM) at pH 13.2. After a 20 min DNA unwinding period, electrophoresis was started at 25 V and 300 mA for 20 min. Following neutralization (0.4 M Tris, pH 7.5; Merck, Darmstadt, Germany), the cells were stained with 85 μl ethidium bromide (20 μg/ml, Sigma). The slides were covered with coverslips and stored for less than three days in humidified boxes at 5˚C.

Digital analysis. The cells in the comet assay were investigated using a DMLB© microscope (Leica, Heerbrugg, Switzerland) with an adapted CCD camera (Cohu Inc.; San Diego, Ca, USA). Forty representative cells were investigated per slide, using two slides for each aliquot tested. For data analysis, the median of each slide set was used. The comets were measured using an image analysis system (Comet++™; Kinetic Imaging, Liverpool, UK). Comet analysis was performed blinded to one examiner, in order to reduce observer-based divergence. To quantify DNA damage, the olive tail moment was used (OTM: median DNA migration distance × relative amount of DNA in the tail of the comet) (11). Eighty cells per slide and two slides per patient were evaluated. While it is the subject of controversy in discussions, OTM is still considered the most informative measure in the comet assay (12).

Comet-FISH. For hybridization, the protocol of McKelvey-Martin *et al*. (13) was used with only minor changes. After neutralization with saline sodium citrate buffer (SSC) (0.3 M NaCl, 30 mM sodium citrate), the slides were sequentially dehydrated with alcohol (70, 85 and 100%) and dried at 37˚C. Hybridization mixture was added, containing (all quantities are listed per slide) hybridization buffer (formamide with dextran sulfate, 14 μl), DNA probes (2 μl, LSI EGFR Dual Color Probe-Hyb Set) (Abbott, Il, USA)) and Aqua bidest (4 μl). The DNA probes hybridized to the centromere of chromosome 7 and the *EGFR* gene on the same chromosome simultaneously. The centromere served as a reference gene, due to its close location on the same chromosome as the *EGFR* gene.

After coverage and sealing of the prepared slides and incubation at 74˚C for 5 min on a precision hot plate, the slides were placed into a wet chamber for 12-16 h at 37˚C. Before detection of probes, the slides were washed three times each in 50% formamide in 2 × SSC (Abbott) and incubated for 10 min in 2 × SSC and in 0.1% detergent tergitol NP-40 in 2 × SSC for 5 min.

Staining and analysis. DAPI (10 μl of 42 ng/ml) with Antifade (both from Abbott) was applied after air-drying of the slides followed by storage at –20˚C protected from light. DNA fragmentation was visualized using a fluorescence microscope and digital analysis (Comet++; Kinetic Imaging™). Forty cells per slide and two slides per patient were analyzed. Analogous to the OTM, the Munich chromosomal tail moment (MCTM) was used to estimate the degree of *EGFR* damage. The MCTM is the product of the median DNA migration distance in a gene and the gene fluorescence in the tail of the comet divided by the overall gene fluorescence measured in a cell (14).

Statistical analysis. Statistical analysis was performed using the SPSS 16.0™ software (IBM, Armonk, NY, USA). DNA damage for all patient samples was compared using the Wilcoxon's test. The general level of significance accepted was $p \leq 0.05$. Bonferroni correction was used where necessary. Standard box-plots (lower quartile, median, upper quartile) were used to illustrate the results. Dots denote mild statistical outliers [between 1.5 and 3 times interquartile range (IQR)]; asterisks denote extreme statistical outliers (more than 3 times IQR).

Results

Cell viability verified by use of the trypan blue staining test was constantly >90%, thus excluding major cytotoxic effects. The tobacco smoke condensate concentration of 0.7 mg/dl was chosen because of earlier dose response tests.

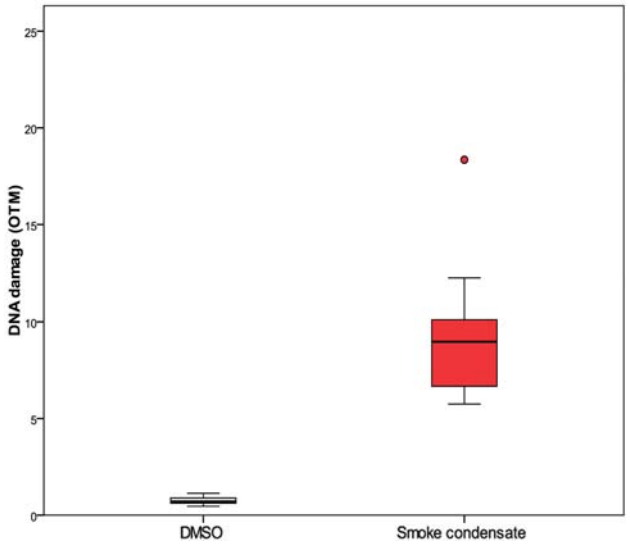

Figure 1. *DNA damage after incubation of mini organ cultures of the oropharynx with DMSO (OTM=0.8) and with smoke condensate (OTM=9.6, p<0.001); n=40.*

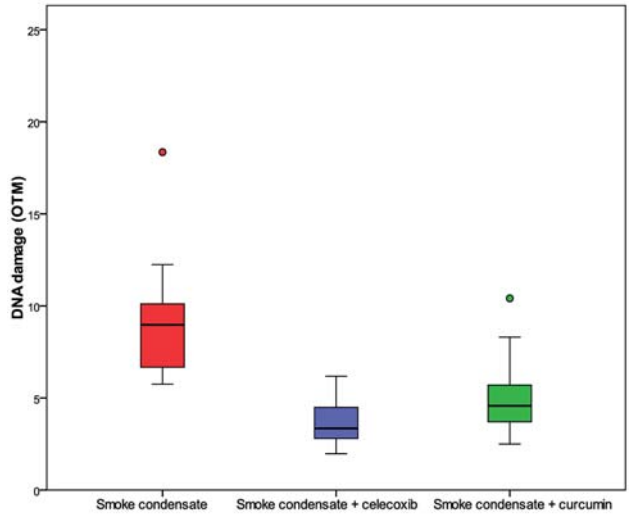

Figure 2. *DNA damage after incubation of mini organ cultures of the oropharynx with smoke condensate (OTM=9.6), showing the significant DNA protective potential of celecoxib (OTM=3.8, p<0.001) and curcumin (OTM=4.7, p<0.001); n=40.*

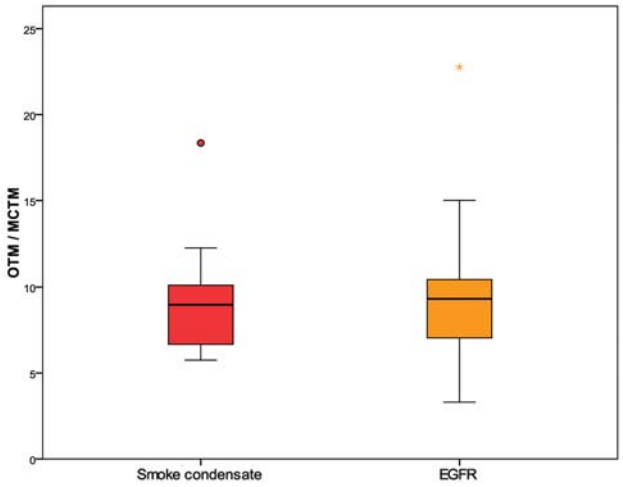

Figure 3. *DNA damage after incubation of mini organ cultures of the oropharynx with smoke condensate in the entire DNA (OTM=9.6) and in the EGFR gene (MCTM=9.7); n=40.*

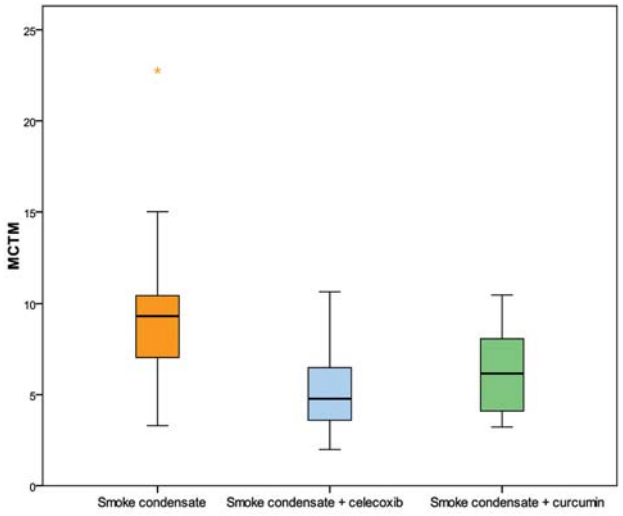

Figure 4. *DNA damage after incubation of mini organ cultures of the oropharynx with smoke condensate in the EGFR gene (MCTM=9.7), showing significant DNA protective potential of celecoxib (MCTM=4.8, p<0.001) and curcumin (MCTM=5.7, p<0.001); n=40.*

The average age of the patients was 53.6 years (ranging from 39 to 73). Analysis of all 40 patients revealed, as expected, that smoke condensate caused significant DNA damage compared to the control incubated with DMSO ($p<0.001$). The median OTM values were 9.6. DNA from controls had a median OTM of 0.8. Only OTMs >2 are considered to reflect relevant DNA damage (15) (Figure 1). Incubation with curcumin and celecoxib alone did not lead to fragmentation (data not shown).

Both curcumin and celecoxib exhibited DNA protective potential in this study. Prior incubation with curcumin reduced the OTM to 4.7 ($p<0.001$), treatment with celecoxib led to a reduction to 3.8 ($p<0.001$) (Figure 2).

DNA damage in the *EGFR* gene was comparable to that for the entire DNA after incubation with smoke condensate alone (MCTM=9.7). No enhanced DNA fragmentation was

detected compared to the rest of the DNA. The chemoprotective potential of celecoxib and curcumin was also shown in the *EGFR* gene, where DNA fragmentation was reduced to 4.8 ($p<0.001$) and 5.7 ($p<0.001$), respectively (Figure 4).

Discussion

As expected, significant DNA damage caused by smoke condensate was found in the present study, mainly as a result of oxidative damage, adduct formation and single strand breaks (2). Both, curcumin and celecoxib showed considerable potential in protection of the DNA against such damage.

The ability of Curcumin to reduce the production of superoxide radicals, as well as hydoxyl radicals, is one aspect of its chemoprotective competence (16). Furthermore, curcumin also serves as scavenger for nitrogen dioxide radicals (17). Although the exact mechanism is not clear, Fujisawa *et al*. suspect that curcumin has a potential itself to produce radicals, which might react with other radicals thus building stable compounds (18). Moreover, various carcinogens, such as benzo[a]pyrenes need to be metabolized by phase-I enzymes, such as cytochrome P450, to produce the ultimate carcinogen. The ability of curcumin to inhibit cytochrome P450 contributes to its chemoprotective characteristics. Furthermore, curcumin serves as an inhibitor for EGFR. Although inhibition of EGFR was found to increase levels of DNA damage (19), this effect could not be shown in the present study.

Damage caused by smoke condensate are not distributed uniformly over DNA. Some genes are more likely to be harmed than others (20) and mutations in those genes might play a role in malignant transformation. One of the genes currently in focus is the *EGFR* gene (19, 21), which is located on chromosome 7 (7p12). Signaling pathways activated by EGFR are pathogenetically involved in the development of HNSCC (22) and EGFR is highly overexpressed in malignant tumors of the head and neck (23). The causes for this overexpression are still poorly understood. *EGFR* gene mutations might play a role in this context, and enhanced mutagen sensitivity is discussed as a risk factor for developing these mutations (24, 25). In the present study, no increased fragmentation was observed of the *EGFR* gene on treatment with smoke condensate. Consistent with our previous findings, when DNA fragmentation was analyzed after incubation with benz[a]pyren-7,8-diol-9,10-epoxide (BPDE), we concluded that mutagen sensitivity of the *EGFR* gene does not influence expression rates of the EGFR protein (21).

There is epidemiological and experimental evidence that inhibitors of COX2, such as celecoxib, protect against various malignancies, including HNSCC (6). COX2 is a bifunctional enzyme that has both peroxidase and cyclo-oxygenase activities. The peroxidase activity catalyzes the conversion of pro-carcinogens to carcinogens (26). COX2 is overexpressed in various malignant tissues, including oropharyngeal carcinoma (27). In HNSCC, COX2 is expressed in both tumor tissue and the adjacent epithelium (28). Renkonen *et al*. also showed that overexpression of COX2 increases with the level of dysplasia, with the highest expression rates occurring in malignant tissue (29). Additionally, smoking leads to enhanced gene expression causing higher COX2 protein expression levels (30). Activation of "nuclear factor 'kappa-light-chain-enhancer' of activated B-cells" (NF-κB) pathways by BPDE are involved on the pathway of this overexpression (31). Celecoxib inhibits NF-κB activation by smoke condensate ,thus reducing expression rates of COX2 (32). Furthermore, protective effects of celecoxib against DNA damage have been described before. Matthias *et al*. found decreased levels of oxidative DNA fragmentation after incubation with celecoxib. Although the underlying reasons are not entirely clear yet, inhibition of the peroxidase activity of COX2 seems to be one possible mechanism (6).

Conclusion

Apparently, the best prevention of tobacco-induced carcinogenesis is avoiding contact with tobacco at all. On the other hand, clinical practice shows, that not all patients with the diagnosis of head and neck cancer are able to stop smoking. These patients are at increased risk of developing second primary tumors and may profit from dietary chemoprevention. Curcumin, as well as celecoxib, showed significant DNA-protective potential in the present study. Since both of these compounds exert their preventive effects at low concentration, they should be further evaluated in clinical trials.

Conflicts of Interest

The Authors declare that there are no conflicts of interest.

References

1 Smith CJ and Hansch C: The relative toxicity of compounds in mainstream cigarette smoke condensate. Food Chem Toxicol *38*: 637-646, 2000.

2 DeMarini DM: Genotoxicity of tobacco smoke and tobacco smoke condensate: a review. Mutat Res *567*: 447-474, 2004.

3 Rahman MA, Amin AR and Shin DM: Chemopreventive potential of natural compounds in head and neck cancer. Nutr Cancer *62*: 973-987, 2010.

4 Baumeister P, Reiter M, Kleinsasser N, Matthias C and Harréus U: Epigallocatechin-3-gallate reduces DNA damage induced by benzo[a]pyrene diol epoxide and cigarette smoke condensate in human mucosa tissue cultures. Eur J Cancer Prev *18*: 230-235, 2009.

5 Thangapazham RL, Sharma A and Maheshwari RK: Multiple molecular targets in cancer chemoprevention by curcumin. AAPS J *8*: E443-449, 2006.

6 Matthias C, Schuster MT, Zieger S and Harréus U: COX-2 inhibitors celecoxib and rofecoxib prevent oxidative DNA fragmentation. Anticancer Res *26*: 2003-2007, 2006.
7 Aggarwal BB and Shishodia S: Molecular targets of dietary agents for prevention and therapy of cancer. Biochem Pharmacol *71*: 1397-1421, 2006.
8 Ford AC and Grandis JR: Targeting epidermal growth factor receptor in head and neck cancer. Head Neck *25*: 67-73, 2003.
9 Kalyankrishna S and Grandis JR: Epidermal growth factor receptor biology in head and neck cancer. J Clin Oncol *24*: 2666-2672, 2006.
10 Singh NP, McCoy MT, Tice RR and Schneider EL: A simple technique for quantitation of low levels of DNA damage in individual cells. Exp.Cell Res *175*: 184-191, 1988.
11 Olive PL, Durand RE, Le RJ, Olivotto IA and Jackson SM: Gel electrophoresis of individual cells to quantify hypoxic fraction in human breast cancers. Cancer Res *53*: 733-736, 1993.
12 Burlinson B, Tice RR, Speit G, Agurell E, Brendler-Schwaab SY, Collins AR, Escobar P, Honma M, Kumaravel TS, Nakajima M, Sasaki YF, Thybaud V, Uno Y, Vasquez M and Hartmann A: Fourth International Workgroup on Genotoxicity testing: results of the *in vivo* Comet Assay Workgroup. Mutat Res *627*: 31-35, 2007.
13 McKelvey-Martin VJ, Melia N, Walsh IK, Johnston SR, Hughes CM, Lewis SE and Thompson W: Two potential clinical applications of the alkaline single-cell gel electrophoresis assay: (1). Human bladder washings and transitional cell carcinoma of the bladder; and (2). Human sperm and male infertility. Mutat Res *375*: 93-104, 1997.
14 Harréus UA, Kleinsasser NH, Zieger S, Wallner B, Reiter M, Schuller P and Berghaus A: Sensitivity to DNA-damage induction and chromosomal alterations in mucosa cells from patients with and without cancer of the oropharynx detected by a combination of comet assay and fluorescence *in situ* hybridization. Mutat Res *563*: 131-138, 2004.
15 Harréus U, Schmezer P, Kuchenmeister F and Maier H: Genotoxic effect on human mucous membrane biopsies of the upper aerodigestive tract. Laryngorhinootologie *78*: 176-181, 1999.
16 Ruby AJ, Kuttan G, Babu KD, Rajasekharan KN and Kuttan R: Anti tumour and antioxidant activity of natural curcuminoids. Cancer Lett *94*: 79-83, 1995.
17 Sreejayan Rao MN: Nitric oxide-scavenging by curcuminoids. J Pharm Pharmacol *49*: 105-107, 1997.
18 Fujisawa S, Atsumi T, Ishihara M and Kadoma Y: Cytotoxicity, ROS-generation activity and radical-scavenging activity of curcumin and related compounds. Anticancer Res *24*: 563-569, 2004.
19 Baumeister P, Heinrich K, Marte M, Reiter M, Schwenk-Zieger S and Harréus U: The impact of EGFR stimulation and inhibition on BPDE induced DNA fragmentation in oral/oropharyngeal mucosa *in vitro*. Oral Oncol *47*: 1141-1147, 2011.
20 Reiter M, Sawicka A, Baumeister P, Welz C, Schwenk-Zieger S and Harréus U: Chromosomal alterations in mini organ cultures of human oropharyngeal mucosa cells caused by hydrogen peroxide. Cancer Genomics Proteomics *6*: 275-280, 2009.
21 Reiter M, Welz C, Baumeister P, Schwenk-Zieger S and Harréus U: Mutagen sensitivity and DNA repair of the *EGFR* gene in oropharyngeal cancer. Oral Oncol *46*: 519-524, 2010.
22 Grandis JR and Sok JC: Signaling through the epidermal growth factor receptor during the development of malignancy. Pharmacol Ther *102*: 37-46, 2004.
23 Hynes NE and MacDonald G: ERBB receptors and signaling pathways in cancer. Curr Opin Cell Biol *21*: 177-184, 2009.
24 Mrhalova M, Plzak J, Betka J and Kodet R: Epidermal growth factor receptor – its expression and copy numbers of EGFR gene in patients with head and neck squamous cell carcinomas. Neoplasma *52*: 338-343, 2005.
25 Wild R, Fager K, Flefleh C, Kan D, Inigo I, Castaneda S, Luo FR, Camuso A, McGlinchey K and Rose WC: Cetuximab preclinical antitumor activity (monotherapy and combination based) is not predicted by relative total or activated epidermal growth factor receptor tumor expression levels. Mol.Cancer Ther *5*: 104-113, 2006.
26 Wiese FW, Thompson PA and Kadlubar FF: Carcinogen substrate specificity of human COX-1 and COX-2. Carcinogenesis *22*: 5-10, 2001.
27 Moraitis D, Du B, De Lorenzo MS, Boyle JO, Weksler BB, Cohen EG, Carew JF, Altorki NK, Kopelovich L, Subbaramaiah K and Dannenberg AJ: Levels of cyclooxygenase-2 are increased in the oral mucosa of smokers: evidence for the role of epidermal growth factor receptor and its ligands. Cancer Res *65*: 664-670, 2005.
28 Chan G, Boyle JO, Yang EK, Zhang F, Sacks PG, Shah JP, Edelstein D, Soslow RA, Koki AT, Woerner BM, Masferrer JL and Dannenberg AJ: Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. Cancer Res *59*: 991-994, 1999.
29 Renkonen J, Wolff H and Paavonen T: Expression of cyclo-oxygenase-2 in human tongue carcinoma and its precursor lesions. Virchows Arch *440*: 594-597, 2002.
30 Tsai KS, Yang RS and Liu SH: Benzo[a]pyrene regulates osteoblast proliferation through an estrogen receptor-related cyclooxygenase-2 pathway. Chem Res Toxicol *17*: 679-684, 2004.
31 Ouyang W, Ma Q, Li J, Zhang D, Ding J, Huang Y, Xing MM and Huang C: Benzo[a]pyrene diol-epoxide (B[a]PDE) up-regulates COX-2 expression through MAPKs/AP-1 and IKKβ/NF-κB in mouse epidermal Cl41 cells. Mol Carcinog *46*: 32-41, 2007.
32 Shishodia S and Aggarwal BB: Cyclooxygenase (COX)-2 inhibitor celecoxib abrogates activation of cigarette smoke-induced nuclear factor (NF)-κB by suppressing activation of IκBα kinase in human non-small cell lung carcinoma: correlation with suppression of cyclin D1, COX-2, and matrix metalloproteinase-9. Cancer Res *64*: 5004-5012, 2004.

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