Oesophageal squamous cell neoplasia in head and neck cancer patients: upregulation of COX-2 during carcinogenesis

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Patients with (previous) head and neck cancer (HNC) are at high risk for developing second squamous cell cancer of the oesophagus. The role of cyclooxygenase-2 (COX-2) in oesophageal squamous carcinogenesis has not yet been investigated in this high-risk group. Therefore, this study examined COX-2 mRNA and protein expression in oesophageal biopsies and resected tissues of 44 HNC patients. The evaluation covered 55 oesophageal tissue samples (18 invasive oesophageal squamous cell cancers, four high- and eight low-grade dysplasias, 25 normal squamous epithelia) from the 44 patients. mRNA levels of COX-2 were measured by real-time PCR using a LightCycler. COX-2 protein expression was studied immunohistochemically and graded by a staining score. COX-2 mRNA was detected in all samples, and its levels correlated positively with the immunohistochemical staining score (P < 0.05). COX-2 expression was upregulated during oesophageal squamous carcinogenesis in HNC patients, that is COX-2 expression increased significantly from normal oesophageal squamous epithelium to low- and high-grade dysplasia and finally to invasive squamous cell cancer (P < 0.001). Our findings suggest that COX-2 upregulation contributes to oesophageal squamous carcinogenesis in HNC patients. Prospective studies are needed to evaluate the chemopreventive potential of COX-2 inhibitors in this high-risk group.

Keywords: carcinogenesis; COX-2; oesophageal squamous cell carcinoma; head and neck cancer
time PCR with a LightCycler to quantify COX-2 mRNA expression and used immunohistochemistry to study cellular COX-2 protein expression.

MATERIAL AND METHODS

Patients and tissue samples

Normal, dysplastic, or malignant oesophageal tissues were collected from 44 HNC patients and a control group of nine other patients (without HNC or oesophageal disease). The human tumour material was used according to the standards set by the Ethics Committee of the University Hospital Benjamin Franklin, Free University of Berlin. The group of HNC patients comprised 33 men and 11 women with an average age of 60.0 years (range 45–89 years).

The histological diagnosis (normal, dysplastic, or malignant oesophageal tissue) was based on an independent review of the cases by two to three observers. If dysplasia was found, the microsections were evaluated by three different pathologists. All observers agreed on the diagnosis of cancer or dysplasia in the patients described here. Two out of three was considered a consensus for specimens without unanimous dysplasia grading (Scherübl et al., 2002b). Low- and high-grade dysplasia (synonym: intraepithelial neoplasia) was defined as unequivocal neoplastic transformation according to previously published criteria (Lewin and Appelman, 1995).

Eighteen of the 44 HNC patients had ESCC. Dysplastic oesophageal lesions (one low-grade dysplasia (LGD), two high-grade dysplasias (HGD)) were available for evaluation from three of the 18 ESCC patients and normal oesophageal squamous epithelia from eight of them. In addition, nine oesophageal dysplasias (seven LGD, two HGD) and 17 normal oesophageal squamous epithelia from eight of them. In addition, nine oesophageal dysplasias (seven LGD, two HGD) and 17 normal oesophageal squamous epithelia could be studied from the 26 HNC patients without ESCC. Most tissue samples were obtained during an endoscopic screening study for oesophageal neoplasia in HNC patients; the protocol included systematic biopsies along the oesophagus (Scherübl et al., 2002a, b). Patients were oesophagog scooped using high-resolution videendoscopes (Olympus GIF-140, Hamburg, Germany). Biopsy specimens were obtained with the 7.5 mm open span biopsy forceps (Endoflex K02 22 V-A).

RNA purification and cDNA construction

Total RNA was isolated from oesophageal squamous tissue biopsies using the RNeasy Kit (Qiagen, Santa Clarita, CA, USA) digestion and reverse transcribed into cDNA using oligo-dT-primers and the SuperScript Preamplification Kit (Gibco, Rockville, MD, USA).

LightCycler PCR

Each 10 µl reaction volume contained 1 × FastStart DNA Master Hybridization Probes Mix (Roche Diagnostics, Mannheim, Germany), 2 mM of MgCl₂, 0.5 µM of each primer, 0.2 µM of each hybridisation probe (fluorescein and LC-Red640), and 1 µl of cDNA. The housekeeping gene porphobilinogen deaminase (PBGD) was selected for reference because of its relatively low expression (comparable to that of COX-2) and lack of pseudogenes. The amplified fragments measured 198 bp (COX-2) and 282 bp (PBGD) (Table 1). Real-time PCR was performed in a LightCycler (Roche Diagnostics, Mannheim, Germany) under the following conditions: initial heating to 95°C for 10 min, then 40 cycles at 95°C for 0 s, 64°C for 12 s, and 72°C for 12 s. The COX-2 and P BG D expressions were quantified using external standards. These standards were set up by PCR using primers that amplify COX-2 and PBGD fragments with ends about 40–150 bases longer (primer sequences and reaction conditions not shown). These fragments were reamplified and purified several times. The concentration of the standards was adjusted to 10 ng µl⁻¹, and serial dilutions were carried out in 20 ng µl⁻¹ poly(A) (Pharmacia, Uppsala, Sweden). Separate standard curves were included for COX-2 and PBGD in each PCR run. Copy numbers of each sample were calculated from the standard curve. The ratio of COX-2 to PBGD copy numbers was calculated to compare COX-2 expression between specimens.

Immunohistochemistry

Microsections (2–3 µm) of paraffin-embedded primary tumours were deparaffinized and rehydrated in a decreasing alcohol series. Immunohistochemistry was performed using a robotic system (Chemo-mate, DAKO, Heidelberg, Germany). Sections were incubated with the monoclonal anti-COX-2 antibody (5 µg ml⁻¹, Cayman Chemical Company, Ann Arbor, MI, USA) for 30 min at room temperature (Sinicrope et al, 1999). After washing, samples were incubated with anti-mouse IgG (1:20 dilution) for 30 min at room temperature, and staining was detected by the ‘fast-red system’ (DAKO, Heidelberg, Germany). Samples were slightly counterstained in Mayer’s haematoxylin.

Tissue staining was independently scored by two of the authors (P. Däuber and B. Heine) with an interobserver discrepancy of less than 10%. In case of discrepancy, consensus interpretation was reached with a third author (H. Stein). The increase in positive tumour cells was graded as follows: 0 = none, 1 = weak,

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**Table 1** Sequences of primers and hybridisation probes used

| Gene      | Primer (5’3’)                          | Location     | Gene Bank   |
|-----------|----------------------------------------|--------------|-------------|
| COX-2     | SSCCTGTTGCTTGAATGATTGC                 | 549–569      | NM_000963   |
| Sense     | TGGCCCTCCTTATGCTTGT                   | 727–746      |             |
| Antisense | ATCCCCAGCGTTACAACTATGTG FL             | 661–684      |             |
| Fluorescein| LC640-CACTCTTGTGCCAGCACCTTCAGC-p     | 688–711      |             |
| PBGD      | AGAGTTTTCTACGGTCGTGGTACC              | 82–102       | NM_000190   |
| Sense     | GCCCTCGATTAGAAGCC                     | 346–363      |             |
| Antisense | AAGAACCTTGTTTCTACC                   | 294–320      |             |
| Fluorescein| LC640-ACTCTGTTCTGTCCCTCTCCTC-p       | 323–345      |             |

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positive internal control. positive nonepithelial cells such as lymphocytes which served as a
independently, and the results were totalled. All tissues contained
75%, 4 = moderate, 3 = strong. A score of 0 – 12 was calculated as the
product of the increase in staining intensity and the frequency of
stained cancer cells (0 = 0%, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, 4 = 76–100%). Since some tumours displayed inhomoge-
neous staining patterns, each tumour component was scored independently, and the results were totalled. All tissues contained
positive nonepithelial cells such as lymphocytes which served as a
positive internal control.

Statistical analysis
Statistical analysis was performed using the PRISM Statistic
Package 2.0 (Graph-Pad Software Inc., San Diego, CA, USA).
Groups were compared using the Kruskal–Wallis test (nonpara-
metric analysis of variance) and were considered different at
P < 0.05. In these cases, P values for comparing groups were
calculated using Dunn’s multiple comparisons as a nonparametric
post-test. Correlations were computed by the Spearman rank test.
The correlation between COX-2 protein and mRNA expression was
analysed using the nonparametric two-tailed Spearman test.

RESULTS
Quantitative analysis of COX-2 mRNA expression
COX-2 mRNA expression was examined using real-time PCR. Both
normal oesophageal squamous epithelium and ESCC were
investigated for COX-2 expression. Normal oesophageal squamous
epithelium showed consistently low COX-2 mRNA expression, and

no difference was found between control patients without HNC or
oesophageal disease (con-1 in Figure 1A), HNC patients without
oesophageal disease (con-2 in Figure 1A), and HNC patients with
ESCC (con-3 in Figure 1A). In contrast, COX-2 mRNA expression
was significantly increased in ESCC (of HNC patients). A direct
comparison between ESCC tissue and normal oesophageal
epithelium in the same patient revealed marked overexpression
of COX-2 in the cancer tissue (Figure 1B). These findings point to
an upregulation of COX-2 during carcinogenesis.

Dysplasias can be very small lesions that are often closely
associated with either adjacent normal epithelium or carcinoma
tissue. Dysplastic lesions were studied by immunohistochemistry
alone to obtain clear evidence of their COX-2 (over)expression and
to exclude possible contamination by nondysplastic tissue.

Immunohistochemical analysis of COX-2 expression
COX-2 was mainly expressed in the basal layer of normal
squamous epithelium (Figure 2A, arrow). COX-2-positive cells
comprised about 20%. Weak COX-2-specific staining was generally
detected in the cytoplasm. The median staining score was 1
(Figure 3). COX-2 showed higher expression in dysplastic than in
normal epithelial cells. It was detected in the cytoplasm of most
dysplastic cells, generally with a homogeneously weak to moderate
intensity (Figure 2B). Low-grade dysplasia and HGD showed no
marked differences in COX-2-specific staining. The median
staining score was 3.5 for LGD and 4.5 for HGD (Figure 3).
COX-2 expression was significantly stronger in ESCC than in
normal squamous epithelium (P < 0.001). Here the median staining
score was 5. In contrast to dysplastic cells, ESCC cells often
displayed heterogeneous COX-2 expression pattern, and the
staining intensity could vary from weak to strong within the same
tumour (Figure 2C). COX-2 mRNA levels correlated significantly
with the immunoreactive COX-2 protein expression of the same
tumour (r = 0.64, P < 0.05), indicating that COX-2 expression is
regulated at the mRNA level and that COX-2 mRNA analysis by
real-time PCR is an adequate tool for determining COX-2 expression.

DISCUSSION
The incidence of oesophageal cancer shows striking geographic
variation. In high-risk areas like Linxian, China, mass surveillance
programmes for oesophageal cancer have been implemented to
reduce the high mortality rate of 161 out of 100 000 (Wu et al,
1982). In Western countries, the incidence of ESCC is generally
below 6 out of 100 000 (Fleischer and Haddad, 1999; Gabbert
et al, 2000). The rarity of this malignancy does not justify implementing
a costly endoscopic surveillance programme for the general
population in low-risk areas. Even in those areas, however,
surveillance and chemoprevention may be warranted in high-risk
groups. High-risk groups for ESCC include male subjects with
heavy drinking or smoking habits, patients with (previous) HNC,
patients with corrosive oesophagitis or carriers of the gene for
autosomal dominant transmission of tylosis (Makucchi et al, 1996;
Scherůhl et al, 2002b). The overall prognosis of oesophageal cancer
is very poor due to advanced disease at diagnosis and aggressive
tumour biology. Surveillance and chemoprevention must therefore
be considered for high-risk groups such as HNC patients
(Ratnasighe et al, 1999; Zimmermann et al, 1999; Shamma et al,
2000). Several epidemiological studies indicate that the use of
aspirin or other nonsteroidal anti-inflammatory drugs may reduce
the risk of death from both colorectal and oesophageal cancer
(Thun et al, 1993; Funkhouser and Sharp, 1995; Thun et al, 2002).
The relevance of COX-2 in human carcinogenesis has been
highlighted by many recent studies (Fosslien, 2000; Shamma
et al, 2000; Morris et al, 2001; Cao and Prescott, 2002). Blockade or even
reversal of the multistep process of carcinogenesis should be targeted in high-risk groups. In our study on HNC patients, a well-known high-risk group for ESCC, we observed a marked COX-2 overexpression in both oesophageal squamous cell dysplasias and ESCC. Normal oesophageal squamous epithelium (even in HNC patients) showed very low COX-2 expression. Starting from that very low level, COX-2 expression increased stepwise in the sequence of normal epithelium, oesophageal squamous cell dysplasia, and finally ESCC (Shamma et al, 2000). Interestingly, COX-2 expression in ESCC of HNC patients shows several similarities to that in ESCC patients without HNC: low homogeneous COX-2 expression in normal epithelium, increasing expression during carcinogenesis, homogeneous COX-2 distribution in all dysplasias, and intra- and intertumoural heterogeneity of COX-2 expression in carcinoma tissues (Zimmermann et al, 1999; Ratnasinghe et al, 1999; Shamma et al, 2000).

In this study, COX-2 expression was quantified by real-time PCR and immunohistochemistry. COX-2 mRNA levels correlated positively with the immunohistological COX-2 staining score, indicating that COX-2 expression is regulated on a transcriptional level in oesophageal neoplasias. This positive correlation implies that quantitative COX-2 mRNA measurement in biopsies is an adequate method for determining COX-2 expression in both normal and neoplastic oesophageal squamous epithelium. Interestingly, other COX-2-positive cell types such as immune or stroma cells must have comprised only a very small fraction, since they did not affect the results.

The mechanisms by which elevated COX-2 expression interferes with carcinogenesis and tumour progression are the object of ongoing research, but several cellular processes have already been pointed out. Selective COX-2 inhibition has been shown to induce apoptosis and reduce proliferation of oesophageal cancer cells. These effects correlate with the inhibition of prostaglandin synthesis (Zimmermann et al, 1999). Moreover, COX-2 can influence apoptosis by decreasing the production of the apoptosis mediator ceramide or by upregulating the antiapoptotic protein Bcl-2 (Tsujii and Dubois, 1995; Chan et al, 1998). Other possible
antineoplastic actions of COX-2 blockers include cell cycle control (Yu et al., 1995), regulation of cell adhesion (Tsujii and Dubois, 1995), immune function (Tsujii et al., 1997), and inhibition of angiogenesis and neovascularisation (Jones et al., 1999). Thus nonsteroidal anti-inflammatory drugs, especially specific COX-2 inhibitors, are being discussed as anticancer agents, and numerous experimental, clinical, and epidemiological studies have demonstrated their antineoplastic effects (reviewed in Thun et al., 2002).

In summary, our study adds to the growing evidence that COX-2 upregulation plays an important role in oesophageal carcinogenesis in general and for the first time in a high-risk group of ESCC such as HNC patients. As aspirin, a nonselective COX inhibitor, reduces the risk of oesophageal cancer (Thun et al., 1995; Funkhouser and Sharp, 1995) and as specific COX-2 inhibitors suppress tumorigenesis both in experimental tumour models (Kawamori et al., 1998; Harris et al., 2000; Butler et al., 2002; Thun et al., 2002) and in diseases such as human familial adenomatous polyposis (Steinbach et al., 2000), prospective clinical studies are needed to evaluate the chemopreventive potential of COX-2 inhibitors. Due to an ESCC risk of 10% and more, HNC patients should be enrolled in chemoprevention trials with COX-2 inhibitors. The benefit of effective chemoprevention will have to outweigh putative side effects of COX-2 inhibitors (Fosslien, 2000; Thun et al., 2002).

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REFERENCES

Buttar NS, Wang KK, Leontovich O, Westcott JY, Pacifico RJ, Anderson MA, Krishnadath KK, Lutzke LS, Burgart LJ (2002) Chemoprevention of oesophageal adenocarcinoma by COX-2 inhibitors in an animal model of Barrett's oesophagus. Gastroenterology 122:1101 – 1112
Cao Y, Prescott SM (2002) Many actions of cyclooxygenase-2 in cellular dynamics and in cancer. J Cell Physiol 190:279 – 286
Chan G, Boyle JO, Yang EK, Zhang F, Sacks PG, Shah JP, Edelstein D, Soslowsky RA, Koki AT, Woerner BM, Masferrer JL, Dannenberg AJ (1999) Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. Cancer Res 59:991 – 994
Chan TA, Morin PJ, Vogelstein B, Kinzler KW (1998) Mechanisms underlying nonsteroidal antiinflammatory drug-mediated apoptosis. Proc Natl Acad Sci USA 95:681 – 686
Ell C, May A, Gossner L, Pech O, Gunter E, Mayer G, Henrich R, Vieth M, Muller I, Seidl G, Stolte M (2000) Endoscopic mucosal resection of early cancer and high-grade dysplasia in Barrett's oesophagus. Gastroenterology 118:670 – 677
Fleischer D, Haddad N (1999) Neoplasia of the oesophagus. In The Oesophagus, Castell D, Richter J (eds) pp 235 – 259. Lippincott: Philadelphia
Fosslien E (2000) Molecular pathology of cyclooxygenase-2 in neoplasia. Ann Clin Lab Sci 30:3 – 21
Funkhouser EM, Sharp GB (1995) Aspirin and reduced risk of oesophageal cancer. J Natl Cancer Inst 87:562 – 564
Gabbett HE, Shimoda T, Hainaut P, Nakamura Y, Field JK, Inoue M (1999) Squamous cell carcinoma of the oesophagus. In Pathology and Genetics of Tumours of the Digestive System, Hamilton SR, Aaltonen LA (eds) pp 10 – 36. IARC Press: Lyon
Harris RE, Alshafie GA, Abou-Issa H, Seibert K (2000) Chemoprevention of breast cancer in rats by celecoxib, a specific cyclooxygenase 2 inhibitor. Cancer Res 60:2101 – 2103
Jones MK, Wang H, Peskar BM, Levin E, Itami RM, Sarfeh JH, Tarnawski AS (1999) Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing. Nat Med 5:1418 – 1423
Kawamori T, Rao CV, Seibert K, Reddy BS (1999) Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, in human colon carcinogenesis. Cancer Res 58:409 – 412
Kutcherwa J, Jones DA, Matsuura N, Groden J, McIntyre TM, Zimmerman GA, White RL, Prescott SM (1996) Prostaglandin H synthase 2 is expressed aberrantly in human colon cancer: evidence for a transcripational effect. Proc Natl Acad Sci USA 93:4816 – 4820
Levin KJ, Appelman H (1995) Tumors of the oesophagus and stomach. In Atlas of Tumor Pathology, Rosai J, Sobin L (eds) pp 43 – 98. AFIP: Washington
Makinouchi H, Machimura T, Shimada H, Mizukami K, Chino O, Kise Y, Nishi T, Tanaka H, Mitomi T, Horiuchi M, Sakai M, Got0 J, Sasaki J, Osamura Y (1996) Endoscopic screening for oesophageal cancer in 788 patients with head and neck cancers. Tokai J Exp Clin Med 21:139 – 145
Mandard AM, Hainaut P, Hollstein M (2000) Genetic steps in the development of squamous cell carcinoma of the oesophagus. Mutat Res 462:335 – 342
Morris CD, Armstrong GR, Bigley G, Green H, Attwood SE (2001) Cyclooxygenase-2 expression in the Barrett's metaplasia – dysplasia – adenocarcinoma sequence. Am J Gastroenterol 96:990 – 996
Ratnasingham D, Tangrea J, Roth MJ, Dawsey S, Hu N, Anver M, Wang QH, Taylor PR (1999) Expression of cyclooxygenase-2 in human squamous cell carcinoma of the oesophagus; an immunohistochemical survey. Anticancer Res 19:171 – 174
Ristimaki A, Honkanen N, Jankala H, Sipponen P, Harkonen M (1997) Expression of cyclooxygenase-2 in human gastric carcinoma. Cancer Res 57:1276 – 1280
Scherübl H, Scherer H, Hofmeister B (2002a) Second oesophageal cancer in head and neck cancer patients. N Engl J Med 346:1416 – 1417
Scherübl H, von Lampe B, Faisa S, Däubler P, Bohlmann P, Plath T, Foss HD, Scherer H, Strunz A, Hofmeister B, Stein H, Zeitz M, Rieckew EO (2002b) Screening for oesophageal neoplasia in patients with head and neck cancer. Br J Cancer 86:239 – 243
Shamma A, Yamamoto H, Doki Y, Okami J, Kondo M, Fujiwara Y, Yano M, Inoue M, Matsuura N, Shiozaki H, Monden M (2000) Up-regulation of cyclooxygenase-2 in squamous carcinogenesis of the oesophagus. Clin Cancer Res 6:1229 – 1238
Simicrove FA, Lemoine MI, XI, Lynch PM, Cleary KR, Shen Y, Frazier ML (1999) Reduced expression of cyclooxygenase 2 genes in hereditary nonpolyposis colorectal cancers relative to sporadic cancers. Gastroenterology 117:350 – 358
Smith WL, Garavito RM, DeWitt DL (1996) Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J Biol Chem 271:31357 – 31360
Steinbach G, Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su LK, Levin B (2000) The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N Engl J Med 342:1946 – 1952
Thun MJ, Henley SJ, Patr0no C (2002) Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical issues. J Natl Cancer Inst 94:252 – 266
Thun MJ, Namboodiri MM, Calle EE, Flanders WD, Heath Jr CW (1993) Aspirin use and risk of fatal cancer. Cancer Res 53:1322 – 1327
Tincani AJ, Brandalise N, Altemani A, Scanavini RC, Valerio JB, Lage HT, Rosai J, Sobin L (eds) pp 43 – 98. AFIP: Washington
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Yu K, Bayona W, Kallen CB, Harding HP, Ravera CP, McMahon G, Brown M, Lazar MA (1995) Differential activation of peroxisome proliferator-activated receptors by eicosanoids. *J Biol Chem* 270: 23975–23983

Zimmermann KC, Sarbia M, Weber AA, Borchard F, Gabbert HE, Schror K (1999) Cyclooxygenase-2 expression in human oesophageal carcinoma. *Cancer Res* 59: 198–204

Zweifel BS, Davis TW, Ornberg RL, Masferrer JL (2002) Direct evidence for a role of cyclooxygenase 2-derived prostaglandin E(2) in human head and neck xenograft tumors. *Cancer Res* 62: 6706–6711