Reversal of gene expression changes in the colorectal normal-adenoma pathway by NS398 selective COX2 inhibitor

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BACKGROUND AND AIMS: Treatment of colorectal adenomas with selective cyclooxygenase-2 inhibitors can contribute to the chemoprevention of colorectal cancer (CRC), but the molecular background of their effect is not fully understood. We analysed the gene expression modulatory effect of N-(2-cyclohexylloxy-4-nitrophenyl)-methanesulfonamide (NS398) on HT29 cells to be correlated with expression data gained from biopsy samples.

METHODS: HT29 colon adenocarcinoma cells were treated with NS398, and global mRNA expression was analysed on HGU133Plus2.0 microarrays. Discriminatory transcripts between normal and adenoma and between adenoma and CRC biopsy samples were identified using HGU133Plus2.0 microarrays. The results were validated using RT–PCR and immunohistochemistry.

RESULTS: Between normal and adenoma samples, 20 classifiers were identified, including overexpressed cadherin 3, KIAA1199, and downregulated peptide YY, glucagon, claudin 8. Seventeen of them changed in a reverse manner in HT29 cells under NS398 treatment, 14 (including upregulated claudin 8, peptide YY, and downregulated cadherin 3, KIAA1199) at a significance of P <0.05. Normal and CRC could be distinguished using 38 genes, the expression of 12 of them was changed in a reverse manner under NS398 treatment.

CONCLUSION: NS398 has a reversal effect on the expression of several genes that altered in colorectal adenoma–carcinoma sequence. NS398 more efficiently inverted the expression changes seen in the normal-adenoma than in the normal-carcinoma transition.

Keywords: NS398; cyclooxygenase-2; colorectal cancer; adenoma; whole-genomic microarray
HCA-7 colon carcinoma cells (Zhang and DuBois, 2001). It inhibits PGE2 synthesis and arrests cell cycle in G1 phase by enhancing p27KIP1 expression (Hung et al, 2000). NS398-dependent apoptosis in colon cancer cells occurs through a cytochrome c-dependent pathway (Li et al, 2001). Reducing VEGF levels with NS398 treatment refers to its anti-angiogenic effect (Abdelrahim and Safe, 2005; Huang et al, 2005). Inhibitory effects of NS398 on cancer invasiveness and metastatic growth have been proven both in vitro in cell culture (Abiru et al, 2002; Yao et al, 2004; Chen et al, 2006; Banu et al, 2007; Leung et al, 2008) and in vivo in animal model experiments (Chen et al, 2006; Leung et al, 2008). Therapeutic effects of NS398 can be exerted by downregulation of matrix metalloproteinase-2 expression (Yao et al, 2004; Leung et al, 2008), blocking of epidermal growth factor receptor transactivation (Banu et al, 2007) or inhibition of HGF-induced invasiveness (Abiru et al, 2002; Chen et al, 2006). However, the complete molecular background of NS398 treatment on colon adenocarcinoma cells has not been analysed yet.

The aims of this study were to analyse the gene expression modulating effect of NS398 selective COX2 inhibitor on the HT29 colon adenocarcinoma cell line and to correlate this effect to the modulation in gene expression observed during normal-adenoma and normal-CRC transition when biopsy samples were analysed.

MATERIALS AND METHODS

Cell culture

HT29 colon adenocarcinoma cells were cultured at 37°C with 5% CO2 in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO, USA) containing gentamycin and 10% FCS. In six-well plates, 300 000 cells per well were cultured for 1 day, and were then treated with 10, 25 and 100 µM NS398 (Sigma-Aldrich, diluted in DMSO) for 72 h in FCS-free medium. 0.1% DMSO was used as control. Total RNA was extracted from three samples treated with 100 µM NS398 and from three untreated controls for microarray analysis. In parallel, 40 000 cells per slide were cyt centrifuged and fixed for immunocytochemical analysis.

MTT cell proliferation assay

In 96-well plates, 5000 HT29 cells per well were maintained for 24 h in 100 µL RPMI-1640 medium containing 10% FCS, after which, the cells were treated with 10, 25 and 100 µM NS398 (Sigma-Aldrich, diluted in DMSO) for 48 or 72 h in FCS-free medium. A volume of 0.5 mg ml⁻¹ of MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) was then added to each well, and the cells were incubated for 4 h at 37°C. The medium was carefully removed, and blue formazan – spawned from MTT by the mitochondrial dehydrogenase enzyme system of cells – was diluted in DMSO. Absorbance was measured at 570 nm using a Multiskan MS ELISA immunocytochemical assay. In parallel, 40 000 cells per slide were cyt centrifuged and fixed for immunocytochemical analysis.

Laser microdissection

Samples were derived from surgically removed tissue from six patients with moderately differentiated, Dukes B stage, left-side CRC. In parallel, six adenoma specimens were collected. Paired control non-tumour tissues from patients were obtained from a clinically unaffected site near the resection end and were histologically normal. Tissue samples were immediately frozen in liquid nitrogen after surgery and were stored at −80°C until the cutting period. Frozen tissue was placed in a cryomold with Tissue Tek embedding medium on dry ice for 1 min. Frozen tissue specimens were cut in a series of 6-µm-thick sections onto PALM membrane-mounted glass slides at −20°C. After cutting, the slides were taken into dry ice, and were stored at −80°C until microdissection for up to 48 h before staining and dissection. The frozen sections were fixed in ethanol series, and were stained using cresyl violet (Sigma-Aldrich). After staining the tissue, tumour and normal tissues were diagnosed by the pathologist. A total of 5000 epithelial cells were collected from each section using the PALM system (PALM, Bernried, Germany).

Microarray analysis

Total RNA was extracted from HT29 cells using the RNeasy Mini Kit (Qiagen Inc., Germantown, MD, USA) and from LCM cells using the RNeasy Micro Kit (Qiagen Inc.), according to the manufacturer’s instructions. The quantity and quality of isolated RNA were tested by measuring absorbance and capillary gel electrophoresis using the 2100Bioanalyzer and RNA 6000 Pico Kit (Agilent Inc., Santa Clara, CA, USA). Biotinylated cRNA probes were synthesised from 1 to 5 µg total RNA and fragmented using the One-Cycle Target Labeling and Control Kit (http://www.affymetrix.com/support/downloads/manuals/expression_s2_manual.pdf), according to the Affymetrix description. In case of LCM samples, two-cycle T7-based linear amplification was performed according to instructions of the manufacturer (Affymetrix Inc., Santa Clara, CA, USA). A volume of 10 µg of each fragmented cRNA sample was hybridised into HGU133 Plus2.0 array (Affymetrix) at 45°C for 16 h. Slides were washed and stained using Fluidics Station 450 and an antibody amplification staining method according to the manufacturer’s instructions. Fluorescent signals were detected by a GeneChip Scanner 3000 (Affymetrix). Fifty-three microarrays from 11 LCM samples (11 normal, 20 villous adenoma, 22 CRC) had been hybridised earlier, their data files were used in a previously published study using different accessions (Galamb et al, 2008a,b, 2009) and are available in the Gene Expression Omnibus database (series accession numbers: GSE4183 and GSE10714).

Statistical evaluation of mRNA expression profiles

Pre-processing and quality control Quality control analyses were performed according to the suggestions of The Tumour Analysis Best Practices Working Group (Tumor Analysis Best Practices Working Group, 2004). Scanned images were inspected for artifacts; the percentage of present calls (>25%) and control of RNA degradation were evaluated. On the basis of evaluation criteria, all biopsy and HT29 measurements fulfilled the minimal quality requirements. In case of HT29 experiments, the similarity of the 3-3 biological replicates was stated using the Euclidean distance method (Supplementary Figure 1). Affymetrix expression arrays were pre-processed by gcRNA with quantile normalisation and median polish summarisation. Data sets are available in the Gene Expression Omnibus databank for further analysis (http://www.ncbi.nlm.nih.gov/geo/), series accession numbers: GSE15799, GSE15960, GSE4183 and GSE10714).

Further analyses To identify differentially expressed features, significance analysis of microarrays (SAM) was used. The nearest shrunken centroid method (prediction analysis of microarrays (PAM)) was applied for sample classification from gene expression data. Prediction analysis of microarrays uses soft thresholding to produce a shrunken centroid, which allows the selection of characteristic genes with high predictive potential (Tibshirani et al, 2002). Pre-processing, data mining and statistical steps were performed using R-environment with Bioconductor libraries. Annotation and functional classification of discriminatory genes were performed using the Affymetrix NetAffx system.

Taqman RT-PCR

TaqMan real-time PCR was used to measure the expression of 12 selected genes using an Applied Biosystems Micro Fluidic Card
For immunocytochemical analysis, 40,000 HT29 cells per slide were plated. HT29 immunocytochemistry was performed to evaluate the difference of COX2-positive/total cell number ratios between NS398-treated and untreated control cells.

**Western blot analysis**

HT29 colon adenocarcinoma cells were cultured for 1 day at 37°C with 5% CO2 in RPMI-1640 medium (Sigma-Aldrich) containing gentamycin and 10% FCS, and then treated with 25, 50 and 100 μM NS398 (Sigma-Aldrich, diluted in DMSO) for 72 or 96 h in FCS-free medium. 0.1% DMSO was used as control. Soluble protein fractions were prepared from 1.5 × 10^6 Triton X-100-treated cells in the presence of protease and phosphatase inhibitors, as described (Alpert et al, 2006). Protein samples (25 μg) were electrophoresed (10% SDS–PAGE) and Western blot analysis of COX2 (rabbit anti-human polyclonal COX2 antibody, Code: RB-9072, 1 μg/ml, Thermo Fisher Scientific) was performed as previously described (Tátrai et al, 2006). The ECL (Enhanced Chemiluminescent) technique (Dako, Glostrup, Denmark) and the Kodak Image Station 4000 MM instrument equipped with Molecular Imaging Software version 4.0 (Carestream Health Inc., Rochester, NY, USA) were used for visualisation and data evaluation.

**Ethical consideration**

All routine colonic biopsy and surgical tissue specimens from patients were taken after informed consent and ethical permission was obtained for participation in the study.

**RESULTS**

**Colorectal adenoma and cancer-related mRNA expression patterns**

Using PAM, between adenoma and normal biopsy samples, 20 classifiers were identified, including overexpressed cadherin 3, KIAA1199, forhead box Q1 and downregulated carbonic anhydrase 7, glucagon, somatostatin, Spi-B transcription factor, claudin 8, bestrophin 4, peptide YY (sensitivity: 100%, specificity: 100%) (Table 1). In LCM experiments, 65% of adenoma-related gene expression changes originated from epithelial cells, whereas 53% of CRC-related markers were epithelium derived.

**Validation of adenoma- and CRC-specific markers**

All 12 measured genes showed a similar expression tendency than when detected by microarray analysis, and 9 of them correlated with the results obtained using Affymetrix microarrays at a significance of P < 0.05. The expression changes of the selected genes are summarised in Table 2.

**Effects of NS398 treatment on gene expression in HT29 cells**

In all, 1925 differentially expressed genes were identified between the NS398-treated and untreated control group using SAM at a significance of P < 0.05 (Supplementary Table 1). A further feature selection criterion was the logFC (log fold change) value. Within the differentially expressed genes, 1156 at least two-fold overexpressed genes were found with a logFC value higher than 1, whereas 769 at least two-fold downregulated genes were determined with a logFC value lower than −1. The expression of genes involved in cell proliferation and cell cycle regulation (such as overexpressed CDKN3, BTG2, TGFβ1, CNOT8, KAT2B, RARRE53,
and CDK2N2, RARRES1, MAGED1, PPAP2A, MXD4, TENC1, SESN1, and downregulated CDCA4, VEGFA), intracellular signal transduction, transcription regulation, metabolic and transport processes and apoptosis (overexpressed CDK2N2, BIK, CASP6, TIA, DAPK3, and downregulated ANXA1, CEBPB, CBX4) are mainly changed under NS398 treatment. However, the function of several differentially expressed transcripts is not known yet. The functional classification of genes is represented in Figure 1.
In correlation with the mRNA expression findings, significant dose-dependent cell proliferation inhibition was measured using MTT assay, which was carried out to optimise the treatment concentration of NS398 COX2 inhibitor.

Changes in colorectal adenoma and cancer-related mRNA expression patterns under NS398 treatment

Seventeen of these 20 genes changed in a reverse manner in HT29 colon adenocarcinoma cells under NS398 COX2 inhibitor treatment, 14 of them (including upregulated somatostatin, claudin 8, peptide YY, and downregulated cadherin 3, KIAA1199) at a significance of \( P<0.05 \) (Figure 2A). The expression of 12 of the 38 CRC-related markers (such as carbonic anhydrase 7, interleukin 8, melanoma cell adhesion molecule) was changed in a reverse manner under NS398 treatment (Figure 2B).

HT29 immunocytochemistry and western blot results

Dose-dependent inhibition of COX2 protein expression was observed under NS398 treatment. COX2-positive cell/total cell ratio was 80.5% in untreated control samples, whereas it decreased to 77.0% under 10 \( \mu M \), to 61.2% under 25 \( \mu M \), NS398 treatment. Further elevation of the NS398 dose (100 \( \mu M \)) caused a significant decrease in the positive cell ratio (33.1%). Strong granular and/or diffuse cytoplasmatic immunostaining was detected in

Table 2 Taqman validation of 12 selected discriminatory genes

| Taqman ID     | Gene symbol | Gene name                                      | Affymetrix ID | Compared sample groups | Fold change on microarrays | Fold change in RT–PCR \(2^{(-\Delta\Delta CT)}\) | \( P\)-value |
|---------------|-------------|------------------------------------------------|---------------|------------------------|---------------------------|----------------------------------|------------|
| Hs00200350_m1 | ABCA8       | ATP-binding cassette, subfamily A (ABC1), member 8 | 204719_at     | Adenoma vs normal      | 0.078                     | 0.10                             | 0.00061    |
| Hs00214306_m1 | TRPM6       | Transient receptor potential cation channel, subfamily M, member 6 | 240389_at     | Adenoma vs normal      | 0.086                     | 0.04                             | 0.00006    |
| Hs00169795_m1 | VWF         | von Willebrand factor                           | 202112_at     | CRC vs normal          | 3.61                      | 12.21                            | 0.55142    |
| Hs00174103_m1 | ILB         | Interleukin 8                                   | 202859_x_at   | CRC vs normal          | 20.20                     | 148.06                           | 0.00283    |
| Hs00194353_m1 | LCN2        | Lipocalin 2                                     | 212531_at     | CRC vs normal          | 7.97                      | 28.44                            | 0.00051    |
| Hs00236937_m1 | CXCL1       | Chemokine (C-X-C motif) ligand 1                | 204470_at     | CRC vs normal          | 13.10                     | 14.32                            | 0.01140    |
| Hs00266237_m1 | COL4A1      | Collagen, type IV, alpha1                       | 211980_at     | CRC vs normal          | 5.21                      | 10.41                            | 0.02831    |
| Hs00174838_m1 | MCAM        | Melanoma cell adhesion molecule                 | 209087_x_at   | CRC vs normal          | 2.92                      | 6.87                             | 0.05209    |
| Hs00277299_m1 | IL1RN       | Interleukin 1 receptor antagonist               | 212657_s_at   | CRC vs normal          | 11.99                     | 25.28                            | 0.00714    |
| Hs00236966_m1 | CXCL2       | Chemokine (C-X-C motif) ligand 2                | 209774_x_at   | CRC vs normal          | 9.20                      | 13.00                            | 0.00204    |
| Hs00204187_m1 | DUOX2       | Dual oxidase-2                                  | 211972_at     | CRC vs normal          | 9.70                      | 30.06                            | 0.00363    |
| Hs00167093_m1 | SPP1        | Secreted phosphoprotein 1 (osteopontin)         | 209875_s_at   | CRC vs normal          | 8.92                      | 12.55                            | 0.07492    |

\( P\)-value represents the correlation to the microarray data. The significant different expression (\( P<0.05 \)) is marked in bold.

Figure 1 Functional classification of differentially expressed genes in HT29 cells under NS398 treatment. (A) Distribution of differentially expressed transcripts in the main cell functional groups. (B) Distribution of downregulated transcripts in the main cell functional groups. (C) Distribution of upregulated transcripts in the main cell functional groups.
COX2-positive cells (Figure 3A and B). Western blot results showed correlation to the immunocytochemistry findings (Figure 3C). More considerable reduction in COX2 protein expression was detected after 96 h of NS398 treatment at 50 and 100 μM concentrations.

**DISCUSSION**

It is a known fact that COX2 inhibitor treatment leads to a significant reduction in the number of colorectal polyps in patients with familial adenomatous polyposis (Steinbach et al., 2000; Higuchi et al., 2003). Selective COX2 inhibitors also seem to be effective for prevention of sporadic adenomatous polyps, as they significantly reduced the occurrence of colorectal adenomas within 3 years after polypectomy (Arber et al., 2006). However, their use is associated with increased cardiovascular risk (Baron et al., 2006; Bertagnolli et al., 2006). The treatment of CRC patients with selective COX2 inhibitors should be less effective, because increased COX2 expression is present in the earlier phase of colorectal carcinogenesis (Eberhart et al., 1994; Yona and Arber, 2006), but the exact molecular biological reasons in the background of this phenomenon are not clarified yet. High-throughput screening technologies such as mRNA expression microarrays were applied to find other molecular targets of selective COX2 inhibitors besides COX2, in order to discover the mechanisms explaining their anti-cancer effect in prostate cancer (John-Aryankalayil et al., 2009; Sooriakumaran et al., 2009) and CRC (Zagani et al., 2009).

In this study, we analysed the effect of NS398 selective COX2 inhibitor on adenoma- and CRC-associated gene expression profiles in the HT29 colon adenocarcinoma cell line using the whole-genomic HGU133 Plus 2.0 microarray system. The global gene expression modulatory effect of NS398 was also examined to find other target molecules and pathways influenced by NS398 selective COX2 inhibitor treatment in epithelial cells.

We found that NS398 has a reverse effect on the expression of genes with altered expression in the colorectal adenoma-carcinoma sequence. NS398 more efficiently inverted the expression...
changes at the adenoma than in the carcinoma stage, demonstrating that it is an effective drug in CRC chemoprevention in the early phase of carcinogenesis.

We have previously identified CRC and adenoma-specific gene expression marker sets in biopsy samples for diagnostic classification. Although colorectal adenoma and adenocarcinoma are epithelial alterations, the cancer microenvironment and interaction between cancer and stromal cells have critical roles in tumour development and progression. That is why the origin of mRNA expression changes – identified in biopsy samples containing both epithelial and stromal tissue elements – was analysed using LCM epithelial samples before model selection. The HT29 colon adenocarcinoma cell line was selected after establishing the fact that most of the above-mentioned markers are epithelium derived. The other reason was that COX2 is decisively expressed in the adenomatous or tumourous epithelium, but there are several studies in which stromal COX2 expression is reported (Nakagawa et al., 2004; Soumaoro et al., 2004).

Our gene expression microarray results strengthen the previously published data by which the anti-cancer effect of the selective COX2 inhibitors is mainly due to their anti-proliferative and pro-apoptotic properties (Guardavaccaro et al., 2000; Hung et al., 2000; Brown et al., 2001; Li et al., 2001; Zhang and DuBois, 2001; Nishikawa et al., 2004; Chen et al., 2009; Sooriakumaran et al., 2009; Zagani et al., 2009). The cell proliferation inhibitory effect of NS398 could be detected in our microarray analysis by causing cell cycle arrest in the G1 phase, as described earlier (Hung et al., 2000). This can be mediated not only by p27KIP1 (Hung et al., 2000) but also by p18-INK4C (CDKN2C) and CIP2 (CDKN3) overexpression (the latter ones showed more than a 4.5-fold overexpression under NS398 treatment in our study). The p53-inducible gene BTG2 (we found to be 4.6-fold upregulated in HT29 cells after NS398 treatment) also contributes to the anti-proliferative activity of NS398 through its inhibition effect to G(1)–S transition by reduction of cyclin D1 levels (Guardavaccaro et al., 2000). The cell proliferation inhibitory effect of NS398 has also been proven in MTT assay.

The inhibition of COX2 by NS398 results in the accumulation of arachidonic acid in cancer cells and, therefore, would trigger apoptosis, but the mechanisms by which NSAIDs induce cancer cells to apoptosis can also be COX2 independent. In this study, a wide range of pro-apoptotic genes in different phases of apoptosis were found to be overexpressed under NS398 treatment including TRAIL death ligand (TNFSF10), SIVA1 death receptor in CD27-induced pathway and molecules involved in the execution phase of apoptosis such as the APAF1 apoptosome protein and CASP6 effector caspase. Death-associated kinase-3 (DAPK3) inducing morphological changes in apoptosis was also upregulated by NS398 in HT29 cells. In accordance with the findings of Li et al. (2001), NS398-dependent apoptosis in colon cancer cells occurred through a cytochrome c-dependent pathway in our experiments. We found that the activation of the p53-dependent pathway can also trigger apoptotic processes via the cytochrome c pathway. Over-expression of tumour protein p53-inducible nuclear protein-1 and tumour protein p53-inducible protein-3 pro-apoptotic molecules indicates p53-dependent apoptosis. p73, which can transactivate p53-responsive genes causing cell cycle arrest and apoptosis, is also
upregulated under NS398 COX2 inhibitor treatment. Celecoxib also caused overexpression of p73 tumour-suppressor gene in prostate cancer in a randomised controlled phase II pre-surgical trial (Sooriakumaran et al, 2009).

Although only few genes involved in angiogenesis showed significant mRNA expression changes, in accordance with observations of Abdelrahim and Safe (2005) and Huang et al (2005), we also detected the downregulation of VEGF, one of the most important angiogenic factors, besides the underexpression of others such as PTEN and IL18.

In summary, in this study, we analysed the effect of NS398 selective COX2 inhibitor treatment on colorectal adenoma- and CRC-associated gene expression alterations using whole-genomic mRNA expression microarrays and the HT29 colon adenocarcinoma cell line. Dose-dependent inhibition of COX2 protein expression was found to be associated with reversal gene expression pattern changes in the colorectal-normal-adenoma but less in the normal-carcinoma pathway. Our findings can provide a molecular explanation with regard to the efficacy of selective COX2 inhibitors in CRC chemoprevention in the pre-cancerous adenoma phase. Furthermore, our results can give an insight into the global molecular background of selective COX2 inhibitor administration suggesting the involvement of p18-INK4C, CIP2 cyclin-dependent kinase inhibitors and p53-inducible Btg2 gene in NS398-dependent proliferation inhibition and TRAIL- and p53-mediated apoptotic pathways.

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