Acetylsalicylic acid enhances antiproliferative effects of the EGFR inhibitor gefitinib in the absence of activating mutations in gastric cancer

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Abstract. The epidermal growth factor receptor (EGFR) is highly expressed in gastric cancer indicating its suitability as a target for receptor tyrosine kinase (RTK) inhibitors. In the current study we explored the role of EGFR and its potential use as a therapeutic target in gastric cancer. First we analyzed 66 gastric cancer samples of Asian and Caucasian patients for the presence of EGFR mutations. No activating EGFR mutations were found and gefitinib alone was only weakly effective in gastric cancer cell lines. However, acetylsalicylic acid (ASA) significantly enhanced the inhibitory effects of gefitinib indicating synergistic action. Whole genome expression profiling indicated significant regulation of 120 genes in the case of co-administration of gefitinib and ASA (32 induced, 88 repressed) in gastric adenocarcinoma cells. Further analyses indicated that several important signalling pathways were effectively inhibited by simultaneous exposure to gefitinib and ASA. Our findings indicate that although gastric cancer does not seem to harbour mutations which render the cancer cells constitutively susceptible to gefitinib, the co-administration of ASA can strengthen RTK inhibitor activity in adenocarcinoma cells by EGFR activation. This is the first report of effective modulation of EGFR-inhibition activity in cancer.

Introduction

Gastric cancer is the second most common cancer in the world, accounting for approximately 10% of all malignancies worldwide. Increased expression and activation of epidermal growth factor receptor (EGFR) and its ligands are associated with progression of gastric cancer and poor prognosis (1,2). Consequently, the use of immunological or small molecule pharmacological inhibition of the receptor has been explored. Park et al were recently able to document a synergistic action of gefitinib, a small molecule inhibitor of EGFR tyrosine kinase, and paclitaxel against gastric carcinoma cells expressing high levels of EGFR (3). The use of gefitinib as second line therapy in advanced gastric cancer was investigated by Doi et al and Rojo et al, reporting only one objective response (1%) and stable disease in twelve out of 75 patients (16%) (Doi T, et al, Proc ASCO 22: abs. 1036, 2003) despite pharmacodynamic evidence of biological activity of the compound (Rojo J, et al, Proc ASCO 22: abs. 764, 2003). It is possible that the disease in these patients was too advanced for the biological activity of gefitinib to translate into clinical benefit (4).

In the case of lung cancer, retrospective analyses revealed that among other clinical parameters Asian ethnicity is associated with increased sensitivity to gefitinib (5-7). An important predictor for sensitivity of non-small cell lung cancer (NSCLC) to gefitinib is the presence of mutations in the EGFR catalytic domain (8,9). It was found that more than 80% of NSCLC patients responding to gefitinib showed deletions or amino acid substitutions in exons 18, 19 and 21 of EGFR, whilst none of the patients who were refractory to gefitinib had a mutation. The same mutations were found in tumors that responded to erlotinib, another EGFR tyrosine kinase inhibitor (10), and these results were recently confirmed in a larger study population (11). Importantly, the mutations appear to be much more frequent in Asian than Caucasian patients ranging from 15/58 (26%) (9) to 38/69 (55%) (12) and 33/54 (61%) (13). Gastric cancer incidence is highest in Asian countries such as Japan.

There are some studies that state that erlotinib, another EGFR RTKI, may possess activity in a subset of patients with deletion mutant variants of EGFR but not necessarily in the presence of the above mentioned mutations (14).

It has previously been documented that gastric tissue exposed to acetylsalicylic acid (ASA) expresses high levels
of EGFR (15,16). Since nonsteroidal anti-inflammatory drugs (NSAIDs) might be a tool for carcinoma prevention in the gastrointestinal tract (17), we investigated the mechanisms of a potential synergism of simultaneous cyclo-oxygenase (COX) and EGFR inhibition. Simultaneous administration of COX and EGFR inhibitors can exert tumor preventive effects in nude mice (18). A combination of these two substances with a protein kinase A (PKA) antisense oligonucleotide was even able to eliminate tumors in more than half of the animals treated (19).

In the present study, we investigated whether EGFR activating somatic mutations occur in treatment naïve Japanese gastric cancer patients and/or those of Caucasian origin. Since no mutations were found, we subsequently utilized an in vitro model of gastric cancer to assess the expression of EGFR-family members on mRNA and protein level upon exposure to different doses of acetylsalicylic acid (ASA); and to analyze the potential synergism of a combined treatment with ASA and gefitinib in a cell line of gastric adenocarcinoma as well as other gastrointestinal malignancies.

**Materials and methods**

**Clinical characteristics of gastric cancer specimens.** Thirty gastric cancer specimens from a German pathology reference center were obtained to represent specimens of Caucasian patients; thirty-six were analyzed from a Japanese University hospital. Table I summarizes the histological characteristics of the specimens used for EGF receptor mutation analysis.

**Genomic analysis of the EGF receptor in paraffin embedded tumor specimens.** Genomic DNA was extracted from the paraffin embedded tumor specimens according to standard procedures. Paraffin embedded tissue from lung cancer specimens known to contain activating mutations served as positive controls. Exons 18, 19, and 21 of the EGFR receptor were then amplified by PCR, and direct sequencing was performed in both sense and antisense directions using the BigDye Terminator kit (Applied Biosystems, Darmstadt, Germany) as described (11). Sequence reactions were analyzed on an ABI 3730 DNA Sequencer (Applied Biosystems).

**Statistical analysis of mutation analysis.** According to the prevalence rates documented in non-small cell lung cancer patients, a prevalence rate of EGFR gain of function mutations of more than 10% was considered to be clinically meaningful. The sample sizes were therefore chosen to possess sufficient power to exclude such a prevalence rate in either study population (in the case of no detected mutation) with a power to detect such a prevalence rate at the 0.05 level.

**Cell culture.** AGS, a human gastric cancer cell line (20), was obtained from the American Collection of Cell Cultures (ECACC), Salisbury, UK, and maintained in culture using HAM F12 media supplemented with 2 mM glutamine (GibcoBRL, Karlsruhe, Germany), appropriate antibiotics, and 10% fetal calf serum (FCS; GibcoBRL). KATO III cells derived from a human gastric cancer specimen (21) were acquired from the American Type Culture Collection (ATCC); the medium consisted of RPMI-1640 supplemented with 4 mM glutamine (GibcoBRL), appropriate antibiotics, and 20% FCS. The NSCLC cell lines CL, HTB56, HTB57 and HTB58 as well as the colorectal carcinoma cell lines Lovo and Caco-2 were obtained from the DSMZ (Braunschweig, Germany). OE21, a human esophagus carcinoma cell line, was obtained from ECACC; and Hutu, a human duodenal carcinoma cell line, was purchased from ATCC.

All cell lines were grown in tissue culture flasks in a humidified atmosphere with 5% CO₂ at 37°C. Cells were grown to subconfluence in 75-cm² bottles and serum starved. Cells were exposed to acetylsalicylic acid (ASA; Aspisol®, Bayer Vital, Leverkusen, Germany) at a final concentration of 0, 1, 2.5, 5, or 10 mM in the presence or absence of gefitinib (AstraZeneca, Macclesfield, UK) 0.1, 1, 5, or 10 μM. These concentrations can be achieved in vitro by applying ASA in pharmacological doses (22); those chosen for gefitinib are typically applied in in vitro tests (23).

**Real-time quantitative RT-PCR.** Real-time quantitative RT-PCR was performed as previously described (24). Primer and probe sequences for EGFR, erbB2, and erbB3 are provided as supplementary information and can be accessed via http://www.klinikum.uni-muenster.de/institute/meda/research/first.htm; primers for human GAPDH have been described previously (24). PCR reactions were followed in real-time using a SDS7700 sequence detector (Applied Biosystems). Serial dilutions of cDNA were run on each plate as standards and gene expression levels were calculated with regard to the standard curves. Expression levels were standardized to cDNA concentrations using GAPDH expression levels.

**Antibodies and Western blot analysis.** Cells were lysed in radioimmunoprecipitation buffer, and Western blotting was performed as described previously (25); equal loading was verified by concomitant β-actin. The antibodies against EGFR and pEGFR were obtained from BD Biosciences, Heidelberg, Germany. Total ERK1/2 as well as TCF-4 antibodies were purchased from Upstate Laboratories (via Biomol, Hamburg, Germany); pERK1/2 antibody was obtained from Cell Signaling Technology (Beverly, USA).

**Cell viability assay.** Cell viability was studied by means of the WST-1 test (26) according to the supplier (Roche Diagnostics, Penzberg, Germany); this procedure is similar to the known MTT or XTT assays. Metabolically active cells cleave tetrazolium salts to form formazan dyes, which can be spectrophotometrically quantified (27); the amount of dye formed
directly correlates to the number of viable cells. Multititer plates were used; 10^4 cells were seeded per well, assays were performed in duplicate with eight wells per type of treatment and cell type, respectively. Concentrations of 2.5 mM ASA and 5 μM gefitinib to test the other cell lines aside from AGS were chosen according to the results raised in AGS cells as these concentrations showed non-significant growth inhibiting activity when applied alone.

**Colony assays.** Cells were seeded in agarose in six-well plates. ASA in the presence or absence of gefitinib in different doses was added. After 10 days the colony numbers were counted. Assays were performed in triplicate and results are shown as mean of at least two independent experiments.

**Microarray expression analysis.** Whole genome microarray expression analysis was performed on the chemiluminescent microarray analyzer 1700 from Applied Biosystems (Foster City, CA, USA) according to the manufacturer's protocols using kits and reagents from Applied Biosystems.

Total RNA was isolated from AGS cells stimulated with 2.5 mM ASA, 5 μM gefitinib or both substances in combination over 24 h using the 6100 Workstation (Applied Biosystems). Unstimulated cells served as control. Frozen cell pellets were lysed in 500 μl of lysis buffer, incubated on ice for 30 min, and incubated with Proteinase K solution for 4 h at room temperature. Following the RNA-tissue protocol the lysate was added to the wells on the RNA purification plate (Applied Biosystems) and washed once with wash buffer 1 and twice with wash buffer 2. Elution of total RNA was performed with 150 μl of elution buffer.

Two μg of high-quality total RNA was reverse transcribed for 2 h at 42˚C in a thermocycler (GeneAmp 9700, Applied Biosystems) using a T7-oligo (d)T primer and the reverse transcription - in vitro transcription (RT-IVT) labeling kit. Synthesis of the second strand was performed at 16˚C for 2 h and cDNA was purified using the RT-IVT purification components. During IVT labeling for 9 h at 37˚C digoxigenin-labeled UTP (Roche Diagnostics) was incorporated into the cRNA. cRNA was purified using the RT-IVT purification components and RNA concentration was assessed by measuring the absorbance at 260 and 320 nm (Biophotometer, Eppendorf). Ten μg of labeled cRNA was fragmented for 30 min at 60˚C in fragmentation buffer and the reaction was stopped by addition of fragmentation stop buffer.

Whole human genome arrays were prehybridized at 55˚C for 1 h in a hybridization oven (Minitron, Infors). Fragmented labeled cRNA was added to the hybridization mixture and rapidly transferred into each microarray cartridge. During hybridization for 16 h at 55˚C microarrays were agitated at 100 rpm.

After hybridization the microarrays were removed from the cartridge and washed in a wash tray on a rocking platform shaker in wash buffer 1 for 5 min, wash buffer 2 for 5 min, and twice in chemiluminescent rinse buffer for 5 min. Binding of anti-digoxigenin fab fragments conjugated with alkaline phosphatase (Roche Diagnostics) was performed for 20 min on a rocking platform shaker. Remaining antibody was washed away with three washes of chemiluminescent rinse buffer for 10 min each. During the chemiluminescent reaction microarrays were washed with chemiluminescent enhancing rinse buffer for 10 min and incubated with chemiluminescent enhancing solution for 20 min. After an additional wash in chemiluminescent enhancing rinse buffer the chemiluminescent substrate was added onto the microarray. Chemiluminescence was detected with a 1700 chemiluminescent microarray analyzer.

Following primary analysis and quality control using the AB-Navigator Software Version 1.0.3, data sets were exported for further analysis. Exported data include assay normalized signals for comparative analysis of gene expression.

**Statistics.** Statistical analyses were performed utilizing SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Differences were compared with nonparametric Mann-Whitney U test ; p<0.05 was considered to indicate statistical significance. All tests were performed two-sided.

**Results**

**Somatic mutations of EGFR in gastric cancer specimens.** The known mutations in lung cancer specimens serving as positive controls were readily detected by the applied methodology (Fig. 1A). However, an activating mutation was not found in any of the German or Japanese gastric cancer specimens.
One specimen of a sixty-year-old female German patient representing a well-differentiated tubular adenocarcinoma displayed a silent polymorphism in exon 21 with a base substitution (c>t; Fig. 1B).

As no activating mutations were discovered, a prevalence rate of such mutations in gastric cancer of >10% in either ethnic group can be ruled out with a margin of error of <5%.

Expression of EGFR family in AGS cells. Real-time RT-PCR analyses using gene specific primers and Taqman-probes demonstrated that in non-stimulated AGS cells the EGFR family members EGFR, erbB2 and erbB3 but not erbB4 were readily expressed. Exposure of these cells towards ASA dose-dependently increased EGFR mRNA expression (Fig. 2). No significant regulation of either erbB2 or erbB3 mRNA expression was found. Erb B4 expression was not detected before or after treatment with ASA.

ASA induces EGFR protein expression and activates downstream signaling. Western blot analyses indicated EGFR protein induction following exposure of cells to ASA. Phosphorylated EGFR was present in significantly higher amounts in ASA-treated cells (Fig. 3a). These experiments demonstrated that ASA enhances EGFR expression and activation. We further analyzed ERK phosphorylation and MAPK activation as a sign of EGFR downstream signaling. Western blot analyses with phosphospecific antibodies towards ERK1/2MAPK revealed significantly enhanced ERK phosphorylation while total ERK1/2MAPK levels remained unchanged (Fig. 3b). Collectively, these experiments indicate that exposure of gastric carcinoma cells to ASA leads to increased EGFR activation and might suggest enhanced EGFR dependence of the cancer cells. Combined exposure to ASA and the tyrosine kinase inhibitor gefitinib significantly diminished the level of phosphorylated ERK1/2MAPK indicating inhibition of EGFR downstream signaling (Fig. 3c).

ASA and gefitinib combined inhibit gastric carcinoma cell proliferation. We next explored whether the simultaneous treatment of gastric cancer cells with the EGFR tyrosine kinase inhibitor gefitinib and ASA would result in enhanced anti-tumor activity. For this purpose, AGS cells were exposed to escalating doses of ASA and gefitinib. Exposure to both substances in combination synergistically reduced the number of viable cells (Fig. 4) as determined in a proliferation assay. In colony formation assays as another indicator for growth inhibitory property of anti-tumor drugs, gefitinib and ASA alone showed limited activity. However, when both substances were combined, strong activity was seen in AGS cells. The colony formation potential was almost completely abolished by simultaneous exposure to ASA and gefitinib even at low concentrations (Fig. 5).

ASA and gefitinib combined are active against a wide range of adenocarcinoma cell lines. To analyze whether the effects
of the gefitinib and ASA combination are restricted to gastric carcinoma cells or extend to other adenocarcinoma cells as well, nine different cell lines originating from lung, esophagus, stomach, duodenum and colon were investigated. ASA enhanced gefitinib activity in 8 out of 9 cell lines (Fig. 6).

**Gene expression analysis/human genome survey array.** According to the above mentioned results, induction and activation of EGFR may contribute to the enhanced efficacy of gefitinib subsequent to ASA application but may not be the sole mechanism. To elucidate the relevant pathways of gefitinib action in combination with ASA, whole human genome expression analysis was performed in samples exposed to 2.5 mM ASA, 5 μM gefitinib, both substances in combination and in non-exposed control samples (n=3) of each stimulation pattern. Only genes with a >2.5-fold difference in expression in each of the three samples were regarded as significantly regulated. Results are separated into genes induced or repressed following different stimulations compared to non-stimulated cells. Genes regulated were grouped according to their protein functions into seven groups: i) genes involved in the structure and function of the cell membrane (‘membrane’), ii) genes responsible for cytoskeleton build up and calcium homeostasis (‘cytoskeleton and calcium’), iii) enzymes (‘enzymes’), iv) genes encoding transcription factors or nucleic acid binding proteins (‘DNA and transcription’), v) genes responsible for signal transduction (‘signaling’), vi) genes with miscellaneous functions such as defense, extracellular matrix, carrier proteins etc. (‘others’), vii) genes with yet undiscovered protein functions (‘unknown’). Results are summarized in pie graphics in Fig. 7 including the number of genes regulated in each group.

Only a few genes were significantly regulated by ASA alone, while exposure of gastric carcinoma cells to gefitinib induced 159 and repressed 165 genes, many of them possessing yet unknown functions. Combination of both drugs induced 32 genes while repressing 88 others.

One interesting finding of gene expression analysis was the repression of TCF-4 (T cell factor), which is a transcription factor downstream of the Wnt signaling pathway (28), by combining ASA and gefitinib. Members of the LEF/TCF family associate with catenin and consecutively lead to transcriptional activation of target pro-oncogenic genes such as c-myc or cyclin D1 promoting cell proliferation (29-32). The catenin/cadherin complex may be involved in hereditary gastric cancer by missense mutations of the E-cadherin gene which is supposed to counteract catenin activity (33).

According to genome analysis, TCF-4 mRNA expression was repressed by ~97% in response to combined ASA and gefitinib. Real-time RT-PCR confirmed a significant down-regulation by ~73% compared to non-stimulated cells. Gefitinib alone repressed TCF-4 mRNA by ~61%, while ASA had no effect on its expression (Fig. 8a). Western blot analysis showed TCF-4 protein expression to decrease accordingly (Fig. 8b).

**Discussion**

Despite the fact that gastric cancer cells highly express EGF receptors, no activating mutations in exons 18, 19, or 21 were found in 66 gastric cancer specimens of Caucasian and Japanese ethnicity (30 and 36 specimens, respectively) which would be indicative of responsiveness to the small molecule tyrosine kinase inhibitor gefitinib. These findings strongly suggest that the prevalence of such mutations in gastric cancer patients of either ethnicity is below 10% and that routine testing of gastric cancer samples would be of no clinical use.

Since previous studies on gastric ulcers led to the idea that NSAIDs might induce EGFR expression (15,16), we examined the effects of ASA on this receptor tyrosine kinase
in gastric carcinoma cells. Our findings indicate that treatment of these cells with ASA leads to increased EGFR activation and possibly subsequent EGFR dependence of the cancer cells. Exposure of gastric carcinoma cells to ASA plus gefitinib but not gefitinib alone significantly diminished the level of phosphorylated ERK1/2 MAPK. Consequently, increased EGFR activation might explain the synergistic effects of ASA and gefitinib. Nonetheless, further studies are needed to identify other possible molecular pathways underlying this synergy.

It is important to note that the synergy between gefitinib and ASA was not restricted to gastric carcinoma cells but was demonstrated for most other adenocarcinoma cell lines tested. Several studies suggest that the presence of EGFR gain of function mutations may enhance the response to gefitinib, at least in lung cancer (8, 9, 34). Co-administration of ASA, leading to EGFR activation, might be a mechanism to increase response rates to gefitinib in tumor entities without such mutations.

One important finding derived from gene expression analysis is a significant downregulation of transcription factor TCF-4 in gastric carcinoma cells treated with gefitinib alone and enhanced when combined with ASA. The TCF-4/β-catenin, i.e. Wnt signaling pathway, plays a predominant role in cell fate and differentiation decisions in several organs (35-36) and is commonly disrupted or affected by mutation in cancer, resulting in a stabilization of β-catenin (28-30).

Figure 6. Activity of ASA and gefitinib combined in several adenocarcinoma cell lines. Fraction of surviving, viable cells as determined by WST-1 assay following exposure to 2.5 mM ASA, 5 μM gefitinib, or 2.5 mM ASA together with 5 μM gefitinib. Bars indicate means of 8 stimulations relative to non-stimulated cells/control as well as corresponding standard errors.

*Denotes statistically significant changes (p<0.05) compared to control.

†Denotes statistically significant changes of ASA together with gefitinib compared to stimulation with gefitinib alone. (a) OE21 cells (esophageal carcinoma), (b) Hutu cells (duodenal carcinoma), (c) KATO III cells (gastric carcinoma), (d) Caco-2 cells (colorectal carcinoma), (e) HTB 56 cells (lung cancer).
of the latter to TCF/LEF family transcription factors leads to subsequent transcriptional activation of pro-oncogenic target genes such as c-myc or cyclin D1 (28,31,32). As mentioned above, functional disruption of E-cadherin as antagonist to catenin activity may be present in hereditary gastric cancer (33).

Interplay of EGF and Wnt signaling components has been suggested (37); however, this is the first report associating antiproliferative pathways of gefitinib with Wnt signaling by downregulation of TCF-4. On the other hand, NSAIDs such as aspirin and indomethacin have been shown to attenuate β-catenin/TCF-4 signaling without disrupting β-catenin/TCF complex formation, although the underlying mechanisms remain to be elucidated. In line with the report of Dihlmann and coworkers (38), ASA had no effect on TCF-4 protein expression.
In conclusion, EGFR activating mutations are absent or very rare in gastric cancer of either Asian or Caucasian patients. However, we demonstrate for the first time that the antiproliferative efficacy of gefitinib can be significantly enhanced by combining the drug with ASA which activates the EGFR receptor. It is tempting to speculate that the co-administration of ASA may allow for sufficient gefitinib action in cancers lacking intrinsic activation of the receptor by mutation.

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References


