A phase II study of hydroxyurea combined with gemcitabine in patients with head and neck squamous carcinoma and gene expression profiles correlated to poor response

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Abstract

Background
Preclinical studies have demonstrated that low-dose hydroxyurea (HU) enhances the cytotoxic effects of gemcitabine on human cancer cells; however, the combination has not been well studied on patients with recurrent, locally persistent head and neck squamous cell carcinomas (HNSCC). Additionally, the potential biomarkers to predict the response of HNSCC to the regimen remain largely unknown.

Methods
Twenty-two HNSCC patients underwent two-day HU (500 mg/m², day 1 and 8) and gemcitabine (500 mg/m², day 2 and 9) infusions every 21 days for a median of two (1–12) cycles until disease progression or adverse effects prohibited further therapy. RNA from pre-therapy tissues was subjected to cDNA microarray analysis to identify differentially expressed genes between stable and progressive disease. Validation of selected genes was performed by quantitative RT-PCR and immunohistochemistry. To further investigate the impact of growth arrest and DNA-damage-inducible protein alpha (GADD45α) on cellular sensitivity to the combination of HU and gemcitabine, GADD45α was over expressed in human oropharyngeal carcinoma KB cells.

Results
Ten patients displayed partial remission and/or stable disease, while nine patients displayed progressive disease. The progression free survival for stable disease was 5.88 months and the median overall survival was 12.4 months, while the progression free survival and the median overall survival for all patients were 1.71 and 7.23 months, respectively. A set of 112 genes was differentially expressed between stable and progressive disease. mRNA expression of c-Fos, BCL2/adenovirus E1B 19 kDa-interacting protein 3, paired-like homeodomain 1 and phosphoglycerate kinase 1 was more highly expressed in progressive disease, while mRNA and protein expression of GADD45α and GADD45γ were significantly decreased in progressive disease in stable disease. Moreover, over expression of GADD45α sensitized KB cells to the combination of HU and gemcitabine.

Conclusions
The combination of HU and gemcitabine may be useful in HNSCC control with reasonable tolerance and toxicity. Based on gene expression profiles, a subset of patients may be potentially identified to gain increased treatment benefits.

Introduction
Approximately, 45,000 cases of head and neck cancer occur in the United States each year. The majority of these are squamous cell cancers. Although many patients are treatable at presentation with surgery or chemoradiation, advanced local recurrences and metastatic disease remain problematic, and often, the only option is systemic treatment. Generally, response rates in advanced disease have been low. Agents currently used include 5-fluorouracil (5-FU), methotrexate, bleomycin and cisplatin, which produce objective response rates in the range of 15%–30%. The response rates to the combination of cisplatin and 5-FU in previously untreated patient are in the range of 30%. Docetaxel and paclitaxel are also active agents with response rates of 15%–40%. More recently, cetuximab has demonstrated response rates of 13% and a disease control rate of 46% in patients previously treated with cisplatin. Cetuximab has demonstrated a survival advantage in advanced disease when combined with cisplatin and 5-FU. Despite these findings, further improvements are needed to further prolong survival and quality of life.

Gemcitabine, a deoxycytidine analogue, can block DNA replication and induce cellular apoptosis. Gemcitabine has been used to treat various human solid tumours such as pancreatic cancer, bladder cancer, metastatic breast cancer and advanced colorectal adenocarcinoma. Hydroxyurea (HU) is an S-phase-specific inhibitor of ribonucleotide reductase (RNR) with a broad spectrum of anti-tumour effects. Gemcitabine has been previously evaluated in squamous cell head and neck cancer as a single agent; however, only minor activity has been noted. As per our preclinical study, low-dose HU enhances the activity of gemcitabine in a time- and sequence-dependent manner. Exposure of human oropharyngeal carcinoma KB or CEM cells to 0.1 mM HU over a period of 8 h resulted in S-phase arrest with synchronization of cells. Following the removal of HU, the cells proceeded...
through S-phase and were more susceptible to S-phase-specific drugs including gemcitabine. This concept was tested in a phase I trial of HU administered over a period of 24 h preceding gemcitabine on days 1 and 8 of a 21-day cycle. Primary toxicity and dose-limiting toxicity (DLT) included neutropenia, and the regimen was overall well tolerated.

Because this regimen was well tolerated in an outpatient setting and due to the relative lack of effective agents in advance head and neck cancer, it was decided to test the combination of HU and gemcitabine in patients with recurrent unresectable or metastatic squamous cell head and neck cancer. Furthermore, as molecular mechanisms for the poor response to this regimen are poorly understood, we examined the deferentially expressed gene profile in pre-therapy head and neck squamous cell carcinoma (HNSCC) tissues between stable and progressive disease using cDNA microarray technology; this helped in the identification of potential gene expression signature for patient stratification. Pathway enrichment analysis of cDNA microarray data was performed to identify the pathways that may be important in poor response to this therapy.

**Methods and materials**

**Patient eligibility and selection**

Eligible patients were those with metastatic or recurrent HNSCC and who had a Karnofsky performance status of >60%. Further, serum creatinine level of <2.0 mg/dl or measured creatinine clearance of >50 mL/min; bilirubin level of <3.0; alanine aminotransferase (ALT), aspartate aminotransferase (AST), 5× upper limit of normal (ULN) and absolute neutrophil count (ANC) of >1200/µL and platelet count of >100,000/µL were required. Prior chemotherapy or radiation was allowed, except for prior gemcitabine or HU; however, they could have been completed at least 3 weeks prior to study treatment. Between January 2001 and November 2005, 22 adults (age ≥18 years) were included in this phase II clinical study at the City of Hope National Medical Center. All patients had histologically-verified HNSCC of various sites, and patients were unresponsive to previous chemotherapeutic regimens or for which no ‘standard’ chemotherapeutic regimen existed. Patient characteristics and disease sites as well as degree of prior treatment are shown in Table 1. All patients gave their voluntary informed consent and signed a consent document that had been reviewed and approved by the City of Hope National Medical Center Institutional Review Board. This trial was also approved by the Cancer Therapy Evaluation Program of the National Cancer Institute (NCI).

**Pretreatment evaluation**

All patients had a complete history and physical examination, including documentation of weight, Karnofsky performance status, evaluation for the presence of measurable or evaluable disease, baseline laboratory blood tests, chest radiograph, electrocardiogram, urinalysis, pregnancy test if indicated and computed tomographic scans of the chest, abdomen and pelvis as needed to document measurable or evaluable disease. Patients with measurable disease were required to have radiographic procedures for analysis of measurable disease repeated after two cycles of therapy.

**HU-gemcitabine treatment schema**

Patients were initially treated using gemcitabine at a dose of 750 mg/m² as determined in a previous phase I trial. However, two of the first three patients died of neutropenic complications, and the protocol was subsequently amended to reduce the dose of gemcitabine to 500 mg/m² with granulocyte-colony stimulating factor (G-CSF) support; no further treatment-related deaths were noted. Patients received a median of two cycles of therapy with

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<th>Table 1 Patient characteristics (n = 22)</th>
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<td><strong>Characteristic</strong></td>
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<td>Site of disease</td>
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<td>Maxillary sinus</td>
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to 72 h 14,15. Viable cells were then seeded from 0.125 to 8 μmol/L for up to 5000 cells were seeded in a 96-well plate. Cells transfected with pcDNA3.1(+)-(GADD45α)-over expressing KB and were subsequently treated with 300 μg/mL streptomycin. Growth arrest and DNA-damaging agents, Grand Island, NY, USA) according to the manufacturer’s instructions. The cells were transfected with pcDNA3.1(+)-(GADD45α) or control empty vector, followed by selection with 300 μg/mL for the treatment to proceed. Staging studies were performed every two cycles, and patients with partial or complete response must have this confirmed with repeat evaluation 3–6 weeks later.

**Cell culture and MTS assay**

Human oropharyngeal carcinoma KB cells (American Type Culture Collection, Manassas, VA, USA) were cultured in an atmosphere of 5% CO₂ at 37°C in plastic tissue culture plates using Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin. Growth arrest and DNA-damaging-inducible protein alpha (GADD45α)-over expressing KB and the control vector-transfected KB cells were transfected with pcDNA3.1(+)-(GADD45α) or control empty vector, followed by selection with 300 μg/mL. G418 to get stable cells. A total of 5000 cells were seeded in a 96-well plate. Sets of 10 wells were treated with 0.1 mM HU for 8 h followed by gemcitabine with concentrations ranging from 0.125 to 8 μmol/L for up to 72 h14,15. Viable cells were then measured by MTS assays, which were performed according to the manufacturer’s instructions (CellTiter 96 AQueous Assay reagent; Promega, Madison, WI, USA).

**Microarray analysis**

Total RNA from HNSCC tissues was extracted with Trizol (Life Technologies). The reaction mixture was firstly incubated at 25°C for 5 min, followed by incubation at 50°C for 5 min. qRT-PCR was carried out using the ABI Prism 7900 HT Sequence Detection System (Life Technologies). Relative gene-expression quantification method, as described previously17, was used to calculate the fold change of mRNA expression according to the comparative C method using GADPH as an endogenous control. Data are represented as ratio or fold change in comparison with the stable group.

**Antibodies and immunohistochemistry**

Rabbit polyclonal antibody against hRRM2 was commercially produced by Convance (Princeton, NJ, USA) using recombinant hRRM2 peptide. GADD45α and GADD45γ were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Sections were deparaffinized and rehydrated in graded alcohol. For heat-induced epitope retrieval (HIER), sections were subjected to DIVA retrieval buffer (pH 6.0, in a pressure cooker (Biocare Medical, Concord, CA, USA) at 98°C for 60 min. The sections were then brought to an automated stainer (DAKO, Carpinteria, CA, USA) following the vendor’s protocol. EnVision Plus and peroxidase detection methods were used, and sections were counterstained in 50% Mayer’s haematoxylin for 1 min. Immunoblotting was performed as previously described14. Nuclear GADD45α and GADD45γ immunoreactivity and nuclear and cytoplasmic RRM2 immunoreactivity were assessed. Those cases with >1% tumour cells showing positive staining were assessed as positive. The staining was graded as + (1–5% tumour cells positive), ++ (5%–25% tumour cells positive) and +++ (>25% tumour cells positive).

**Statistical analysis**

This was a phase II trial with the primary endpoint being response rate. Given the reported response rate of 13% for gemcitabine when used as a single agent and given that the best regimens for metastatic disease have
Response rates in the range of 30%, an encouraging response rate was set at 30% versus a discouraging response rate of 13%. With a 10% false-positive rate and 80% power, and using a Simon’s two-stage design, the initial stage enrolled 18 patients with measurable disease. If three or more patients had an objective response, enrolment would continue to a total of 25 patients. If there were five or more responses among these patients, this would indicate a response rate of >13% at 0.10 level of significance. Response rate was measured by comparing changes in the product of perpendicular cross-sectional measurements, with a decrease of >50% defined as a partial response and an increase of >25% from baseline constituting progression. Statistical analysis was conducted using Student’s t-test. \( P < 0.05 \) was considered statistically significant.

**Results**

**Clinical outcomes**

For all patients, the median progression free survival (PFS) was 1.71 months and the median overall survival (OS) was 7.23 months (Figure 1). Of 19 patients evaluable for response, one patient had partial response and nine patients had stable disease as their best response. Because there was only one objective response, continuous enrolment was stopped. The patient who responded had received radiation for tonsil cancer and chemotherapy with cisplatin and 5-FU, and was treated on this study for recurrent disease after an initial complete response. Among patients with stable or responding disease, the median OS was 12.4 months (Figure 2). Of the 10 patients with stable or responding disease, 6/10 had previous chemotherapy, 8/10 had previous radiation and 5/10 had both.

**Adverse events**

A grade 3 or greater decrease in ANC was seen in 10 patients, and 12 patients had grade 3 or greater thrombocytopenia. Two patients died due to neutropenic complications. Blood transfusions were required in nine patients and five patients required platelet transfusions.

**Microarray analysis of gene expression in progressive tumour tissues**

The cDNA microarray analysis indicated that a set of 112 gene transcripts (gene list to be provided upon request) was significantly different between three stable and five progressive disease tissues. Heat map shows the clustering of all significantly different genes (Figure 3a).
Using k-nearest neighbours (KNN) and leave-one-out algorithm, we found that these top 10 genes, out of a total of 112 genes, may be used to predict the outcome of stable and progressive HNSCC disease in patients are subjected to combined treatment of HU and gemcitabine.

In order to validate the microarray results, we selected several highly differentially expressed genes for validation by qRT-PCR. Twelve genes whose expression patterns were confirmed by qRT-PCR are shown in Figure 3b. We further selected genes with known roles in drug resistance to gemcitabine and HU such as the two subunits of RNR, RRM1 and RRM2, which were not in the set, and genes that are involved in DNA synthesis and DNA damage repair such as Fos, BNIP3, PITX1, PGK1, GADD45α and GADD45γ. qRT-PCR identified that mRNA levels of FOS, BNIP3, PITX1 and PGK1 were significantly higher in progressive disease than in stable disease, whereas mRNA levels of GADD45α and GADD45γ were lower (P < 0.05). However, RRM1 and RRM2 were not significantly and differentially expressed in the groups (Figure 3b). Using Ingenuity pathway analysis, we identified that the genes involved in cancer development, cell growth and proliferation, cellular movement and apoptosis pathways were significantly enriched in the progressive disease group than in the stable disease group.

Immunohistochemical (IHC) analysis of GADD45α, GADD45γ and RRM2 proteins in stable and progressive HNSCC

RRM2 is well known for its role in drug resistance to gemcitabine and HU; however, mRNA expression of RRM2 was not significantly different between the stable and progressive disease groups. The GADD45 gene family, which is rapidly induced by genotoxic agents, plays pivotal roles in negative growth control, including growth suppression and apoptotic cell death.

Therefore, we measured GADD45α, GADD45γ, and RRM2 proteins in stable and progressive disease groups by IHC staining. IHC expression patterns of GADD45α, GADD45γ and RRM2 in the two representative disease groups are presented in Figure 4. Results for quantitative analysis of GADD45α, GADD45γ and RRM2 in both the groups are listed in Table 2. The results show that the positive staining of GADD45α and GADD45γ was much lower in progressive cases compared with that in stable cases, while IHC staining of RRM2 was not dramatically different between the two groups.

**Over expression of GADD45α enhances the response of KB cells to the therapeutic regimen**

As GADD45α and GADD45γ were down-regulated in progressive disease, we further explored the effects of GADD45 gene family on the growth and viability of human oropharyngeal carcinoma KB cells on exposure to drug combination of HU and gemcitabine. Studies have indicated that GADD45 sensitizes leukemia, lung carcinoma and cervical cancer cells to apoptosis induced by genotoxic stress. As shown in Figure 5a, expression of endogenous GADD45α was very low in KB cells; therefore, we established that KB cells over express the GADD45α protein. Over expression of GADD45α did not significantly decrease cellular proliferation compared with the control plasmid-transfected KB cells (Figure 5b). However, up-regulation of GADD45α in KB cells significantly enhanced sensitivity to the combination of HU and gemcitabine (Figure 5c). Therefore, we considered that down-regulation of GADD45 family proteins may impact the response of HNSCC to the treatment schema.

**Discussion**

Advanced head and neck cancer remains a significant clinical problem with limited treatment options. The combination of gemcitabine and HU presented in this report had a limited ability to produce objective responses, albeit in a heavily pre-treated cohort of patients. However, a significant subset of patients achieved stable disease. Interestingly, these patients could be characterised as having a distinct gene expression pattern compared with those with a progressive disease.

Gemcitabine as a single agent has not been proven as an effective treatment in head and neck cancer. However, we had preclinical evidence that showed enhanced efficacy if it was used in combination with HU, which is also an RR inhibitor. This was based on preclinical data that demonstrated that the exposure of human oropharyngeal carcinoma KB or CEM cells to 0.1 mM HU over a period of 8 h resulted in S-phase arrest with cell cycle synchronization. Following HU removal, the cells proceeded through S-phase and were more susceptible to S-phase-specific drugs including gemcitabine. Although, as noted, there was some evidence of clinical activity, it is important to see if a subset of responsive patients can be defined that could benefit from this treatment. Given that several patients had prolonged stable disease treatment, it may be possible to select a subgroup of patients that might benefit with a more prolonged PFS that might be clinically significant. In platinum-refractory patients, single agent cetuximab produced a response rate of 13%, a disease control rate of 46% and a time to progression of 70 days. Patients who progressed had cisplatin added, and the OS of the group was 178 days. This compares to a disease control rate of 45.4% in this study with a survival of 216 days (7.23 months). In another study involving platinum-refractory patients, progression on cetuximab treatment was followed by cetuximab/platinum treatment. Response rate was 10% and the disease control rate was 53%. Time to progression and OS were 85 and 183 days, respectively. Among patients with a partial response, these

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**Figure 3:** Genes differentially expressed between stable and progressive HNSCC disease. (a) Heat map shows the clustering of all the significant genes (112 genes) analysed using the Affymetrix GeneChip microarray (three cases of stable and five cases of progressive disease). (b) Differentially expressed genes in stable and progressive cancer patients identified by cDNA microarray and validated by qRT-PCR. The mRNA expression of each gene in progressive cancer patients \( n = 6 \) is presented as the ratio to stable cancer patients \( n = 6 \). Data are mean ± SD. \( *P < 0.05; **P < 0.01 \) compared with the stable group.

numbers were 203.5 and 294 days, respectively. Interestingly, in our study, among patients with stable or responsive disease, the corresponding values were 176 (5.88 months) and 372 days (12.4 months), respectively. Of course, our patients were more heterogeneous and not all of them were platinum-refractory, but they nevertheless represented a heavily pre-treated group.

Resistance to the cytotoxic effect of gemcitabine can be related to multiple mechanisms including alteration of apoptosis-regulating genes, alterations in the transport and cellular turnover of the drug as well as altered expression or sensitivity of enzyme targets. High expression of RRM1 and RRM2 has been seen clinically and in cell culture in lung, pancreatic and biliary tumours. Expression of RRM1 and RRM2 mRNA was slightly elevated in progressive disease. However, the protein levels as indicated by IHC were not significantly different, which indicates that other genes may be responsible for the treatment schema for HNSCC. We also found that the expression of GADD45 proteins was lower in progressive HNSCC tissues. Interestingly, reduced expression of three GADD45 family members due to promoter methylation has been observed in several types of human cancers. GADD45 family proteins have been implicated in the regulation of many cellular functions including DNA repair, cell cycle control, senescence and genotoxic stress. Emerging functional evidence implies that GADD45 proteins have a pro-apoptotic function in response to diverse stimuli, connecting multiple cell signalling modules. Moreover, induction of GADD45 expression is an essential step for mediating anti-cancer activity of multiple chemotherapeutic drugs, while the absence of GADD45 expression might abrogate their effects in cancer cells. In the present study, we found reduced expression of GADD45α and GADD45γ in progressive disease. We further found that the over expression of GADD45α in KB cells enhanced sensitivity to the combination treatment. Consistently, several studies have also indicated that ectopic expression of GADD45 sensitizes leukaemia, lung carcinoma and cervical cancer cells to apoptosis induced by genotoxic stress. Of
Competing interests: none declared. Conflict of interests: none declared.

All authors contributed to the conception, design, and preparation of the manuscript, as well as read and approved the final manuscript. All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.


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Table 2: Quantitative analysis of IHC staining of RRM2, GADD45α and GADD45γ in stable and progressive HNSCC tissues

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<th>Percentage</th>
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<tr>
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<td>RRM2</td>
<td>GADD45α</td>
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<tr>
<td>−</td>
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<tr>
<td>++</td>
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<tr>
<td>+++</td>
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<td>3/10</td>
</tr>
<tr>
<td>Sum of +</td>
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*Immunostaining was graded as (+) (1%-5% tumour cells positive), (+++) (5%-25% tumour cells positive) and (++++) (>25% tumour cells positive).

**Conclusions**

HU and gemcitabine may be useful in HNSCC disease control with reasonable tolerance and toxicity. Based on gene expression profiles, a subset of patients may potentially be identified to gain increased treatment benefits. Correlation between the down-regulation of GADD45 and PGK1 proteins in HNSCC with resistance to HU and gemcitabine should be further addressed.

**Authors’ contributions**

YY, SS and DL designed and supervised the clinical trial. KZ and CK performed real-time PCR, Western blot, over expression, cell line selection and cell proliferation tests. XW did the microarray data analysis. SL did IHC staining. PC reviewed and assessed the IHC staining. YW and LD participated in sample management and collection. KZ, PC and YY revised the manuscript. All authors reviewed and approved the final version of the manuscript.

**Acknowledgements**

We much commemorate our former colleague Dr. Stephen Shibata for his contribution and dedication to the study. Dr. Stephen supervised the clinical trial and analysed the clinical data. We also appreciate Dr. Shibata’s team and patients in the medical oncology of City of Hope Medical Center for their precious assistance and cooperation during the clinical trial.
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