Heterogeneity of chemosensitivity of esophageal and gastric carcinoma

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Esophageal and gastric cancer have a poor prognosis, and chemotherapy is rarely of long-term benefit. This may be related in part to heterogeneity of chemosensitivity and to constitutive resistance to individual cytotoxic drugs. This study aimed to demonstrate the degree of heterogeneity of chemosensitivity between tumors. We have examined the heterogeneity of chemosensitivity in esophageal and gastric cancer specimens ($n=85$) using an *ex vivo* ATP-based chemosensitivity assay (ATP-TCA). A variety of chemotherapeutic agents were tested. Sixty-four specimens were endoscopic biopsy samples; the remainder were from resection specimens. Cells were obtained from 62 specimens (73%). Eight assays were infected due to contamination/infection of the biopsy material, giving an evaluability rate of 87%. Analysis of the data showed considerable heterogeneity of chemosensitivity. The most active single agents identified by the assay were mitomycin C (56% sensitivity) and 5-fluorouracil (5-FU; 42% sensitivity). Exposure of tumor cells to combinations of drugs showed ECF (epirubicin, cisplatin, 5-FU) and mitomycin C + 5-FU to be moderately active regimens. Other experimental drug combinations showed greater activity. There is a marked heterogeneity of chemosensitivity in esophageal and gastric cancers. The degree of heterogeneity observed suggests that the ATP-TCA could be used to individualize chemotherapy by selecting agents for particular patients. This approach provides the rationale for a trial of ATP-TCA-directed therapy to determine whether individualization of chemotherapy might improve patient response and survival. *Anti-Cancer Drugs* 14:397–403 © 2003 Lippincott Williams & Wilkins.

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Introduction

Chemotherapy for cancer of the esophago-gastric junction (EGJ) remains relatively disappointing. Although combined chemotherapy regimens are able to provide up to a 61% response rate [1,2], these responses are often short-lived and are attended by varying degrees of toxicity. As a result of controlled trials, there is a trend towards treating operable EGJ tumors with neoadjuvant chemotherapy [3]. This is based on the belief that neoadjuvant chemotherapy may both downstage loco-regional disease and reduce or eliminate any subclinical metastatic disease. Inoperable disease is routinely treated with palliative chemotherapy [4,5].

It is clear from clinical trials that there is a wide range of sensitivity of EGJ tumors to chemotherapy. Despite their different etiology and histology, both adenocarcinoma and squamous cell carcinoma appear to have similarly poor overall sensitivities to the most popular chemotherapy regimens [6]. Up to 11% of cases of EGJ cancer show a complete pathological response (pCR) to ECF combination chemotherapy [1,2], while other EGJ tumors treated with the same regimen are found to progress [7]. This heterogeneity of chemosensitivity is currently unpredictable and probably leads to many patients being treated with potentially toxic chemotherapy regimens that are ineffective against their particular tumor [8].

Over the last 30 years, a number of chemosensitivity assays have been developed to try to predict the responsiveness of tumors to chemotherapy [9,10]. Early assays proved difficult to use for human tumors, but are widely used with cell lines for drug development. This situation has changed with the introduction of newer technology; the ATP-tumor chemosensitivity assay (ATP-TCA) has been shown to have a high evaluability rate for solid tumors and provides interpretable results in greater than 90% of tumors tested [11,12]. A case-control

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intervention study of ATP-TCA directed chemotherapy in recurrent ovarian cancer has shown a dramatic improvement in response rate and survival [13,14], and a randomized phase III trial for platinum-refractory ovarian cancer is currently under way.

This study was undertaken to investigate whether the chemosensitivity of tumors of the EGJ can be assessed by the ATP-TCA, to see if the technique is applicable to endoscopic biopsies, and to determine the degree of heterogeneity of chemosensitivity of carcinoma of the EGJ.

Methods

Patients

A total of 59 patients (median age 56) with cancer of the esophagus or stomach were included in the study. There were 53 males and six females. The histology in 53 patients was adenocarcinoma and in six was squamous cell carcinoma. A total of 85 samples were tested: 47 tumor samples were assayed before any chemotherapy had been given and 38 afterwards. Endoscopic biopsies were taken using 3.7-mm Jumbo forceps, with a median of 6 biopsies per patient (range 4–10). All patients included in the study gave informed consent for the samples to be taken and for ATP-TCA to be performed on the samples and the study was approved by the Local Regional Ethics Committee.

ATP-TCA

All tumor samples were removed as part of patient treatment, with consent for tissue donation and local research ethics committee approval for use of the tissue surplus to diagnostic requirements for chemosensitivity testing. Sixty-four were endoscopic biopsies taken at diagnostic or therapeutic endoscopy, while 21 samples were taken from the resection specimen after surgery. Tumor samples were sent to the laboratory in labeled specimen bottles containing culture medium (DMEM; Sigma, Poole, UK) with antibiotics and an anti-fungal (penicillin–streptomycin, gentamicin, amphotericin B; Sigma-Aldrich, Irvine, UK). Each sample was tested using a standardized protocol [12] within 24 h of surgery.

Tumor material was minced under aseptic conditions prior to enzymatic tumor cell dissociation according to the assay protocol [12]. Cells were washed twice and resuspended in a serum-free complete assay medium (CAM; DCS, Innovative Diagnostik Systeme, Hamburg, Germany). Debris was removed by density centrugation over Lymphoprep (Nycomed, Birmingham, UK), according to the manufacturer's instructions, and the cells were washed twice in CAM before resuspension to 200 000 cells/ml. Test drugs were added to polypropylene 96-well plates (Costar, High Wycombe, UK) at six dilutions (6.25–200%) of the test drug concentrations (TDC) shown in Table 1. Dilutions were prepared in the plate (100 µl/well) from an 800% TDC solution made up freshly from frozen aliquots of each drug in CAM [14]. The TDC of each drug was determined by reference to known pharmacokinetic data as previously described [12]. Two rows of the plate were reserved for controls—a medium only 'M0' row containing 100 µl/well of CAM and a 'MI' row containing 100 µl/well of maximum inhibitor of cell survival. Aliquots of 100 µl of cells were added to each well giving a final plating density of 20 000 cells/well in 200 µl total volume. Plates were incubated at 37°C at 100% humidity and 5% CO_2 for 6 days, at the end of which cells were lysed by addition of 50 µl Tumor Cell Extraction Reagent (DCS) to each well. The ATP content of each well was measured by adding 50 µl of lysis buffer to each well and transferring 50 µl aliquots to a white polystyrene plate. Then 50 µl luciferin–luciferase was added to each well of the white plate and luminescence measurements were made in a Berthold MPLX luminometer (Berthold Diagnostic Systems, Hamburg, Germany). The results were entered into a spreadsheet (Excel) and calculations of the percent inhibition at each drug concentration were used to directly plot the results.

Data analysis

The IC_{90} for each drug was defined as that concentration of drug that produced 90% cell death in the assay and is expressed as a percentage of the test drug concentration. A TCA index, or index of sensitivity, was calculated as [600 – sum(inhibition 6.25–200%)]. Thresholds for sensitivity and resistance were arbitrarily defined to allow inter-sample comparison, as previously described [11,12].

Results

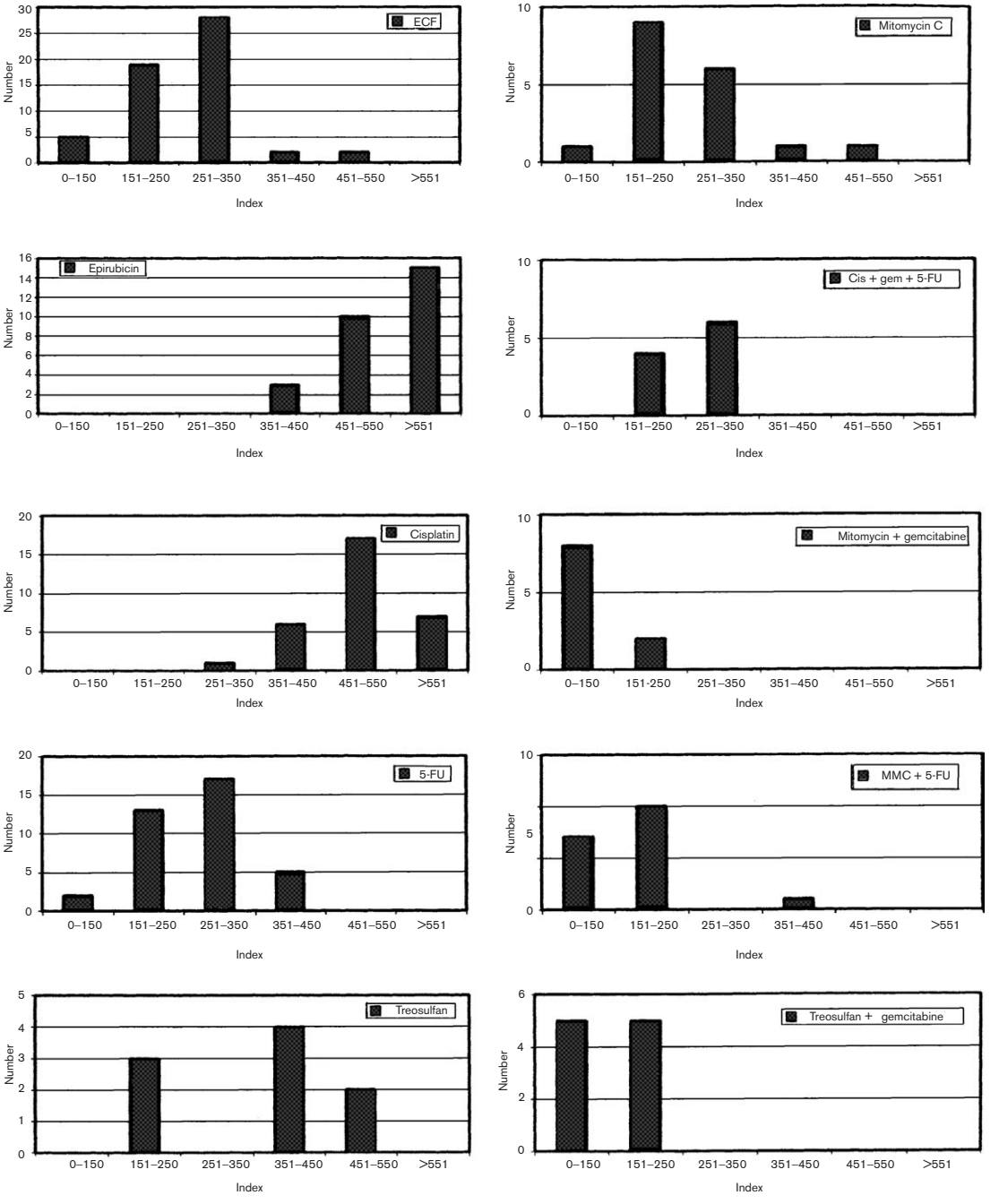
In total, 85 tumor samples from 59 patients were submitted to ATP-TCA testing. Of these, 62 (73%) samples produced sufficient cells for assay. Assays were successfully performed on 54 samples, with infection requiring the results to be discarded in eight samples. The assay evaluability rate was 54/62 (87%). Of the most recent 20 samples tested, 17 (85%) have produced sufficient cells for assay with evaluable results.

Of the 23 tumor samples that did not provide sufficient cells for assay, 20 were endoscopic biopsies and three were from resection specimens (Table 1). Twelve of these 23 samples (52%) were post-chemotherapy specimens.

Table 1 Twenty-three biopsy specimens yielded too few cells for assay by ATP-TCA

	Total specimens	Endoscopic biopsies	Resection specimens
Pre-chemotherapy	47	10	1
Post-chemotherapy	38	10	2

Fig. 1

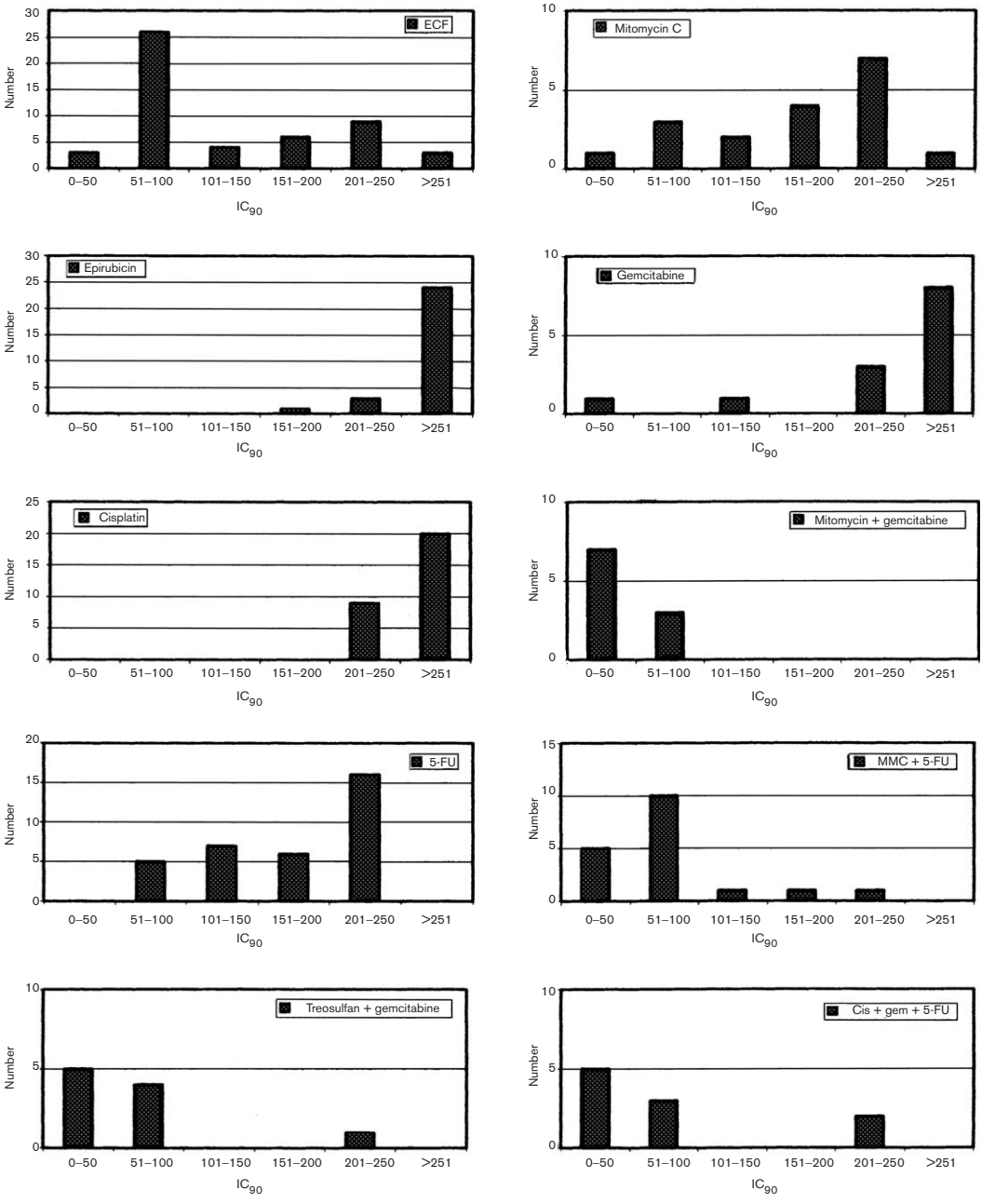


Frequency histograms showing heterogeneity of the sensitivity index (*y*-axis) for each single agent and combination.

The number of chemotherapeutic agents tested on each sample varied. Endoscopic biopsies rarely yielded more than 10–20 mg of tissue, normally producing enough cells for one to four drugs to be tested. Resection specimens often provided in excess of a gram of tissue, allowing for testing of up to 24 single agents and drug combinations.

The results show considerable heterogeneity of chemosensitivity between patients to single agents and to drug combinations (Fig. 1). Some tumor cells responded well to particular drugs or combinations, while other tumor cells showed no response to these, but instead responded to an alternative regimen. The most active single agents identified were 5-fluorouracil (5-FU) and mitomycin C;

Fig. 2



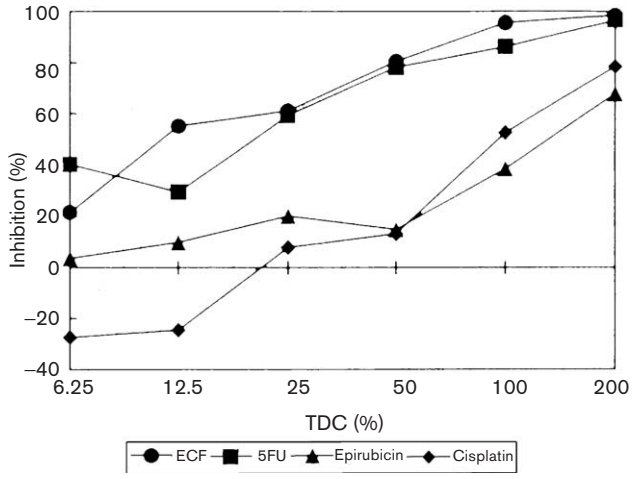
Frequency histograms showing heterogeneity of the IC_{90} (*y*-axis) for each single agent and combination.

15 of 36 samples (42%) showed sensitivity to 5-FU ($Index_{SUM} < 250$) and 10 of 18 (56%) showed sensitivity to mitomycin C.

Figure 2 shows the IC_{90} for different agents on different tumors. Again, the most active single agents are 5-FU and mitomycin; 5-FU alone showed an IC_{90} of less than 100% TDC in five of 36 (14%) tumor samples tested and the corresponding figure for mitomycin C was four of 18 (22%).

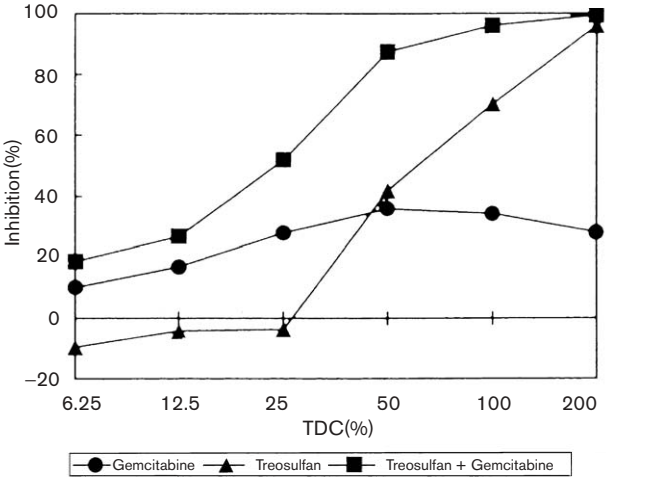
Combinations of drugs demonstrated greater activity. The combination of epirubicin, cisplatin and 5-FU (ECF, a regimen used commonly for both neoadjuvant therapy

Fig. 3



Results for epirubicin, cisplatin and 5-FU individually and in combination. Much of the activity is provided by the 5-FU, with little assistance from the epirubicin and cisplatin.

Fig. 4



Results for gemcitabine + treosulfan in one tumor, showing little activity of the gemcitabine, but a marked increase in activity of the combination compared with treosulfan alone.

and palliative therapy) had an $Index_{SUM} < 250$ in 24 of 54 cases (44%), while combinations of cisplatin + 5-FU + gemcitabine and mitomycin C + 5-FU showed similar high activity in all tumors on which they were tested (10 and 17, respectively).

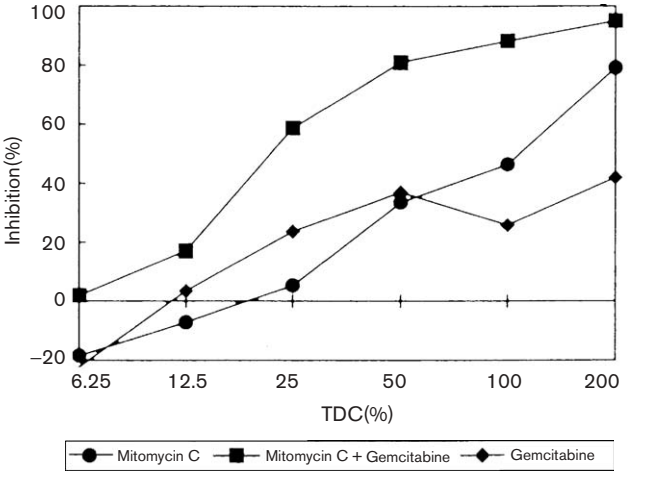
Figure 3 shows the results of testing ECF and its constituents individually on tumor cells from an esophageal adenocarcinoma. The cells were sensitive to ECF, with much of the activity due to the 5-FU. This pattern was seen in many of the tumors tested—much of the activity of the ECF combination came from the 5-FU, while the cisplatin added a small but variable amount of activity; the epirubicin rarely improved the observed inhibition.

Combinations of agents also showed lower IC_{90}s. The ECF regimen demonstrated an IC_{90} of less than 100% TDC in 29 of 54 (54%) of samples and mitomycin C + 5-FU in 15 of 18 (83%). Cisplatin + gemcitabine + 5-FU was equally effective, with an IC_{90} of less than 100% TDC in eight of 10 cases tested (80%).

When tumor cell numbers allowed, a number of experimental drug combinations were tested. Among those showing good results were the combination of treosulfan and gemcitabine (all tested samples were sensitive, with nine of 10 showing an IC_{90} less than 100% TDC) and mitomycin C + gemcitabine (all 10 samples sensitive and showing an IC_{90} less than 100% TDC).

Figure 4 shows the results of testing treosulfan and gemcitabine, alone and in combination, on esophageal adenocarcinoma cells. Treosulfan demonstrates moderate activity on its own, but when combined with the relatively inactive gemcitabine the degree of inhibition is much improved. Similar results were obtained for the combination of mitomycin C with gemcitabine (Fig. 5).

Fig. 5



Results for gemcitabine + mitomycin in one tumor, showing little activity of the gemcitabine, but a marked increase in activity of the combination compared with mitomycin alone.

Discussion

The ATP-TCA was used to investigate the chemosensitivity of esophageal and gastric tumors, and was found to

produce evaluable results in a high proportion of cases—87% of those from which cells could be obtained. This is the first study in which endoscopic biopsy-derived cells from EGJ tumors have been successfully tested using a chemosensitivity assay. Our data show that, once the learning curve of both surgeon and laboratory staff has been negotiated, the ATP-TCA is a robust method of assessing the *ex vivo* chemosensitivity of upper GI tumors. Since 32% of post-chemotherapy specimens yielded too few cells for the assay, compared to 23% of pre-chemotherapy specimens, it seems likely that prior chemotherapy reduces cell retrieval.

It is clear from these results that esophageal and gastric cancers show marked heterogeneity in their chemosensitivity to single agents and combination regimens, in keeping with reported clinical studies [7]. We have shown that different tumors demonstrate a range of sensitivity to commonly used chemotherapeutic agents.

One of the commonest chemotherapy regimens used in esophageal and gastric tumors in the UK is the combination of cisplatin and 5-FU (CF), with or without the addition of epirubicin (ECF). Clinical studies demonstrate a range of response rates to this regimen, most of them between 42 and 61% [2,15]. Importantly, most investigators have reported a percentage of patients who show a complete pathological response to CF or ECF, defined as the inability of the pathologist to find any residual tumor cells in the resected specimen. This occurs in up to 11% of EGJ tumors [1,2] and leads to a marked improvement in the prognosis for these patients [16]. Our study showed five out of 56 (9%) patients with a tumor $\text{Index}_{\text{SUM}}$ for ECF of less than 150, suggesting a very high degree of sensitivity of the tumor to ECF, a figure consistent with these reports.

Dose–response curves for ECF and for epirubicin, cisplatin and 5-FU as single agents show interesting results (Fig. 3). In the vast majority of cases, the dose–response curve showed ECF to be more active than any single agent alone; however, in most tumors this was seen to be due in a large part to the activity of 5-FU, with varying degrees of an additive effect from the cisplatin. Epirubicin alone only infrequently showed activity, and rarely added much to the combination of cisplatin and 5-FU. There are no published clinical studies comparing the response rates to ECF and CF, but comparison of published phase II studies does not point towards a significant difference [17]. Of the three drugs that make up the standard ECF regimen, epirubicin has by far the highest toxicity profile; if clinical studies were to support our initial findings that epirubicin is inactive in the majority of EGJ tumors, then many elderly cancer patients might be spared this relatively expensive and cardiotoxic drug.

Some experimental chemotherapy combinations not currently used in clinical practice appear to show interesting potential. Gemcitabine alone does not appear to be particularly active. However, the combination of gemcitabine with cisplatin and 5-FU demonstrated a high chemosensitivity index in all tumors tested, suggesting greater activity than the ECF or CF regimens. This may result from the ability of gemcitabine to potentiate the activity of DNA-damaging agents by interfering with DNA repair [18]. Mitomycin C has been used in a number of clinical trials with varying degrees of success; as a single agent in the ATP-TCA it showed activity comparable with 5-FU. However, in combination with 5-FU or with gemcitabine, it consistently demonstrated higher levels of activity. These are preliminary data and further work on this combination is required before phase I/II trials are performed. Treosulfan and gemcitabine is a novel drug combination not currently used in the treatment of upper GI cancers, but with good activity against adenocarcinomas of ovary [14] and breast (Sharma *et al.*, unpublished data). In our assay it consistently produced a good response.

Particular difficulties encountered in the early stages of this study included the small size of most of the biopsy specimens, the vulnerability of the tumor cell cultures to infection, and the degree of necrosis and cell debris found, especially in post-chemotherapy specimens. The majority of specimens deemed to be not evaluable were small endoscopic biopsies that yielded very few tumor cells. The surgeons carrying out the endoscopic biopsies were asked to take as much tissue as possible from the growing edge of the tumor, using 3.7-mm biopsy forceps, and usually provided about 10–20 mg of tissue. Some early cultures were found to be infected with fungus; addition of an appropriate concentration of an anti-fungal to the transport media and the dissociation enzyme preparation prevented this problem. It was realized that endoscopic biopsies of the post-chemotherapy specimens were mostly necrotic and rarely contained enough tumor cells to carry out an assay; testing of these samples was discontinued and instead the resection specimen was tested, usually some 4–6 weeks later, as larger samples from beneath the mucosa could be taken.

In this study we used chemotherapeutic agents at levels related to their peak plasma concentrations, taking into account their degree of protein binding [12]. We accept that this approach has inherent flaws, since the $C_{\max}$ is not always a good indicator of clinically achievable intra-tumor concentrations. Similarly, the ATP-TCA is an *ex vivo* study of the cells' chemosensitivity and a tumor cell suspension does not necessarily reflect the *in vivo* situation. However, previous studies with the ATP-TCA suggest that the assay is a good model for the investigation of tumor chemosensitivity and the results

so far show good correlation with clinical trial results in ovarian cancer [12,14]. The chemosensitivity index has been used in previous studies to differentiate between sensitive and resistant tumors. Most of the agents tested in this study showed a sigmoid concentration-activity curve; the IC_{90} is a particularly useful measure of the efficacy of a drug with these kinetics. In this study we demonstrated 44% of tumors to be sensitive by $Index_{SUM} < 250$ and 54% by $IC_{90} < 100\%$ TDC to the ECF combination, which correlates well with the percentage clinical response rate seen in patients.

In conclusion, we have shown that the ATP-TCA is applicable to small endoscopic biopsies from the upper GI tract and that different tumors exhibit a wide range of sensitivity to commonly used chemotherapeutic agents. The degree of heterogeneity of chemosensitivity observed in this study of upper GI tumors suggests that the ATP-TCA may be a useful technique for selection of the most appropriate drugs in patients who are to undergo chemotherapy. This is particularly pertinent in the field of neoadjuvant chemotherapy, where we believe that the ability to predict those patients who will respond well to chemotherapy will be a major step forward.

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