PREDICTION OF SURVIVAL IN PATIENTS WITH HEAD AND NECK CANCER USING THE HISTOCULTURE DRUG RESPONSE ASSAY

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Abstract: *Background.* Chemoresponse is a significant outcome predictor in patients with head and neck cancer, regardless of the treatment modality used. The histoculture drug response assay (HDRA) has been shown to be a reliable method for in vitro chemoresponse assessment. In this study, we have correlated the HDRA assessment with survival in patients with head and neck squamous cell carcinoma (HNSCC).

Method. Tumor specimens from 41 of 42 patients undergoing treatment for HNSCC were successfully evaluated by the HDRA. Tumor tissue was histocultured on Gelfoam sponges gel in 24-well plates, followed by treatment with cisplatin (15 μg/mL) or 5-fluorouracil (40 μg/mL) in triplicate. A control group received no drug treatment. After completion of drug treatment, the relative cell survival in the tumors was determined using the MTT assay. The inhibition rate (IR) for each drug was calculated relative to the control for each case, and sensitivity was defined as a tumor IR of greater than 30%. Treatment was based on established protocols for the location and stage of the tumor and included surgery, radiation, and/or chemotherapy. Survival comparisons were performed using the generalized Wilcoxon test for the comparison of Kaplan-Meier survival curves.

Results. Resistance to 5-fluorouracil was present in 13 cases (32%), to cisplatinum in 13 cases (32%), and to both agents in 11 cases (27%). The 2-year cause-specific survival was significantly greater for patients sensitive to 5-fluorouracil (85% vs 64%; $p = .04$), cisplatinum (86% vs 64%; $p = .05$), or both agents (85% vs 63%; $p = .01$). The association between HDRA assessment of chemoresponse and clinical outcome remained significant even after controlling for the effects of TNM stage and the presence of recurrent cancer at presentation by multivariate analysis.

Conclusions. Chemosensitivity determined by the HDRA seems to be a strong predictor of survival in patients with advanced HNSCC and should be considered further. *Head Neck* **24:** 437–442, 2002

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Individual cancers, although seemingly similar in types, such as that of the head and neck, show widely divergent responses to chemotherapeutic agents.[1] Individual tumors may or may not respond to an agent commonly used for a particular

tumor type. This unpredictability is especially serious because, in contrast to most therapeutic modalities, the severe side effects of most anticancer agents preclude empirically trying different drugs until an effective one is found.[2] Consequently, there is a need for better therapy design.

The advent of methods for growing human cells in vitro suggested the possibility of individualized pretesting of therapy using the response of cell cultures derived from the patient's tumor. The procedure was envisioned as analogous to determining antibiotic sensitivity in bacterial disease. However, implementation of a cancer drug response assay has proved arduous, and the problems are far more subtle than for assessment of antibiotic sensitivity.[3–8] Major problems arose from issues related to tissue culture conditions that are radically different from the normal environment of the tumor cells.[3–8] The histoculture drug response assay (HDRA) solves many of the previously encountered problems with in vitro testing. Most importantly, it maintains the three-dimensional tumor-tissue histology in culture.[9–17]

Squamous cell carcinomas of the upper aerodigestive tract have a mixed response to cisplatin-based chemotherapy regimens. Whereas some tumors completely regress clinically and pathologically, others show partial responses and even frank resistance.[18] In a multitude of chemotherapy trials for patients with head and neck cancers, it is only the group of patients who had a complete response to chemotherapy in whom survival is prolonged, regardless of the treatment approach used.[19] Another group has previously compared the clinical and HDRA effects of cisplatin in 23 of 26 patients with head and neck cancers.[20] Comparisons of HDRA results were made with the extent of clinical response. The predictive positive value was 83%. Accordingly, HDRA assessment of chemoresponse can be used as an outcome predictor in patients with head and neck cancer. In this study, we found a highly significant correlation of chemosensitivity in the HDRA and survival of patients with HNSCC.

METHODS

Patient Sample. Tumor specimens from 41 patients undergoing definitive treatment for head and neck squamous cell carcinoma (HNSCC) at Memorial Sloan-Kettering Cancer Center were successfully subjected to HDRA analysis. The patient characteristics are listed in Table 1. All patients had biopsy-proven squamous cell carcinoma. Treatment was based on established protocols for the location and stage of the tumor and consisted of surgery (83%), radiation (37%), and/or chemotherapy (25%). All patients treated with chemotherapy received cisplatin in combination with 5-flourouracil.

Tissue Handling. After resection, a representative portion of the tumor specimen was obtained and transported in media containing DMEM/Ham's F12 media with 10% fetal calf serum, 200 μg/mL gentamicin, and 5 μg/mL Fungizone to the Laboratory of Epithelial Cancer Biology at the Memorial Sloan-Kettering Cancer Center. The HDRA was conducted by investigators blinded to the clinical information.

HDRA. The HDRA was performed according to methods previously described[9] with slight modifications. The tissue was washed and bisected. Half of the tissue was snap frozen and the remainder cut into 1- to 2-mm^3 fragments and placed onto 0.5-cm^2 pieces of collagen sponge-gel (Gel Foam; Pharmacia & Upjohn, Inc.) in equal quantities. These sponge-gel cultures were then placed into 24-well plates containing DMEM/Ham's F12 medium with 10% fetal calf serum (FCS) and gentamicin (50 μg/mL). The plates were incubated for 24 hours in a chamber maintained at 37°C and 5% CO_2 atmosphere.

Drug Treatment. The chemotherapeutic agents were dissolved in DMEM/Ham's F12 medium with 10% FCS and gentamicin (50 μg/mL) at a concentration of 15 μg/mL for cisplatin and 40 μg/mL for 5-flourouracil. After a 24-hour incubation period, the cells were examined for viability and infection, and the culture medium was replaced with the solution containing chemotherapeutic agents. A control group, which was handled identically, received new culture medium without any chemotherapeutic agents added. The histocultures were incubated in the medium for 24 hours at 37°C and 5% CO_2 atmosphere. All experiments were performed in triplicate.

Determination of Cellular Activity. After completion of drug treatment, the relative amount of cellular activity was determined using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2Htetra-

Table 1. Patient and tumor characteristics based on chemoresponse status.

	5-Fluoracil		Cisplatin		Both agents*	
	Resistance	Sensitive	Resistance	Sensitive	Resistance	Sensitive
Sample size, *n*	13	28	13	28	11	30
Median age, y†	56	57	64	55.5	55	58
Male gender, *n* (%)‡	10 (77)	15 (56)	11 (85)	14 (50) $p = .03$	8 (73)	17 (57)
Alcohol use *n* (%)‡	6 (46)	12 (43)	9 (69)	9 (32)	6 (55)	12 (40)
High-grade comorbidity by Charlson index, *n* (%)‡	2 (15)	6 (21)	1 (8)	7 (25)	1 (9)	7 (23)
Recurrent cancer at presentation *n* (%)‡	4 (31)	4 (14)	2 (15)	6 (21)	2 (18)	6 (20)
Anatomical site§, *n* (%)‡						
Oral cavity	6 (46)	4 (14)	4 (31)	6 (22)	4 (36)	6 (20)
Larynx	4 (31)	16 (57)	5 (38)	15 (54)	4 (36)	16 (53)
Oropharynx	0	2 (7)	0	2 (7)	0	2 (7)
Hypopharynx	3 (23)	6 (21)	4 (31)	5 (18)	3 (27)	6 (20)
T-stage§, *n* (%)‡						
T1/T2	4 (37)	6 (27)	6 (55)	4 (18)	5 (56)	5 (21)
T3/T4	7 (63)	16 (73)	5 (45)	18 (82) $p = .03$	4 (44)	19 (68)
N stage§ *n* (%)‡						
N0	3 (28)	11 (50)	4 (36)	10 (46)	2 (22)	12 (50)
N1/N2	8 (72)	11 (50)	7 (64)	12 (54)	7 (78)	12 (50)
TNM stage§, *n* (%)‡						
I/II	1 (9)	5 (22)	4 (36)	2 (10)	2 (22)	4 (20)
III/IV	10 (91)	17 (78)	7 (64)	20 (90)	7 (78)	20 (80)

Only significant p values are shown.
**The data reflect resistance to both cisplatin and 5-fluorouracil where each agent was tested individually.*
†Mann-Whitney U test.
‡Fisher's exact test.
§Recurent cancers excluded.

zolium bromide (MTT) assay. This was performed by removing the drug-containing media and washing the histoculture with cold PBS. The histocultures were then incubated in solution containing 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H tetrazolium bromide at a concentration of 0.4 mg/mL for 6 hours. The tissue was then harvested and placed into microcentrifuge tubes containing DNA extraction solution (1N NH_4OH, 0.2% Triton X-100) and incubated at 37°C for 20 minutes. The samples were then sonicated with a Branson Sonifier 450 at 50% duty cycle and intensity of 2.5.

After sonification, the tubes were then centrifuged at 100 rpm for 10 minutes. An aliquot of the supernatant was placed into a disposable UV optical grade, methacrylate cuvet with fluorescent dye solution (0.5 μL of bisbenzamide, 200 μg Hoechst 33258/mL H_2O, 0.1 *M* NaCl, 10 m*M* EDTA, 10 m*M* Tris (pH-7)) for DNA assessment. Relative fluorescent units (RFU) for the sample were measured with a spectrophotometer. The DNA concentration for the sample was extrapolated from the RFU reading relative to those from a concentration curve generated using multiple standards of calf thymus DNA. The remaining sample was incubated in DMSO at 37°C for 30 minutes and centrifuged for 10 minutes at 1000 rpm. Then, 200 μL of the supernatant was transferred to a 96-well plate, and MTT values were measured on a MicroELISA reader (Dynatech Laboratories, Inc.) at OD_{570}. The percentage of cellular activity was the ratio of MTT/DNA RFU, and the inhibition rate (IR) for each drug was calculated relative to the cellular activity for the control group. All analyses were performed in triplicate, and the median value was used to determine chemoresponse status. Chemosensitivity was defined as a tumor IR of greater than 30%. Because of the absence of existing definitions, the cutoff for chemoresistance used in this study was chosen arbitrarily before the analysis of the outcome data.

Statistical Analysis. A two-tailed *p* value of less than or equal to .05 was used to accept significance. Descriptive statistics were used to summarize study data. Nonparametric qualitative and quantitative comparisons were performed

using the Fisher's exact test and Mann–Whitney *U* test, respectively. Survival data were censored for patients surviving to the end of the study, lost to follow-up, or dying without cancer. Survival curves were generated using the Kaplan-Meier method. Survival comparisons were performed using the generalized Wilcoxon test. Multivariate analysis was performed using the Cox regression model.

RESULTS

Evaluability of Patient Tumors in the HDRA. Overall, 41 of 42 cases (98%) were successfully histocultured and analyzed for response to 5-fluorouracil and CDDP. One case was excluded because of the development of bacterial infection in the histoculture.

HDRA Response Rate. Resistance in the HDRA to 5-fluorouracil was present in 13 cases (32%), to cisplatin in 13 cases (32%), and to both agents given simultaneously in 11 cases (27%) (Figure 1; Table 2).

Patient Characteristics in HDRA-Sensitive and HDRA-Resistant Cases. There were no statistical differences in patient, tumor, or treatment characteristics between resistant and sensitive cases, with the exception of a greater preponderance of men and T1/T2 lesions in the cisplatin-resistant group (Table 1). However, there was no difference in the TNM stage based on cisplatin response status. In addition, the proportion of recurrent cancers was not influenced by the status of chemoresistance. It can be concluded that the groups were prognostically equal based on established clinicopathologic prognostic criteria.

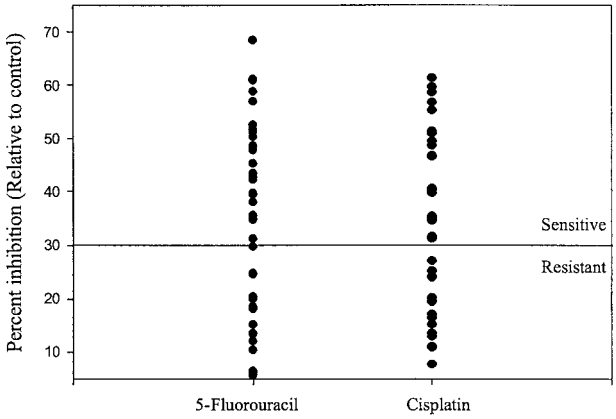

FIGURE 1. Degree of inhibition determined by the histoculture drug response assay in 41 patients. The inhibition rate was calculated relative to a control group. A rate of 30% or less was deemed resistant.

Differences in Survival and Recurrence in HDRA-Sensitive and HDRA-Resistant Patients. Significant differences in outcome were noted between resistant and sensitive patients. Based on the HDRA assessment, the 2-year cause-specific survival was significantly better for cases sensi-

Table 2. Treatment and outcome characteristics based on chemoresponse status.

	5-fluorouacil		Cisplatin		Both agents*	
	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive
Sample size, *n* (%)	13 (32)	28 (68)	13 (32)	28 (68)	11 (27)	30 (73)
Treatment†						
Surgery	9 (69)	25 (69)	9 (69)	25 (89)	7 (64)	27 (90)
Radiation	5 (38)	11 (38)	5 (38)	11 (39)	5 (46)	11 (37)
Chemotherapy	2 (15)	4 (15)	3 (23)	3 (11)	2 (18)	4 (13)
Close margins, *n* (%)†	2 (22)	2 (7)	1 (8)	3 (11)	1 (9)	3 (10)
Follow-up, mo‡	27	33	27	35.5 *p* = .01	27	33
Locoregional recurrence†	3 (23)	5 (18)	4 (31)	4 (14)	3 (27)	5 (17)
Death from cancer, *n* (%)†	4 (31)	4 (14)	5 (38)	3 (11) *p* = .02	4 (36)	4 (13)
2-year cause-specific survival, %§	64	85 *p* = .04	64	86 *p* = .05	63	85 *p* = .01

Only significant p values are shown.
**The data reflect-resistance to both cisplatin and 5-fluorouracil where each agent was tested individually.*
†Fisher's exact test.
‡Mann–Whitney U test.
§Wilcoxon test.

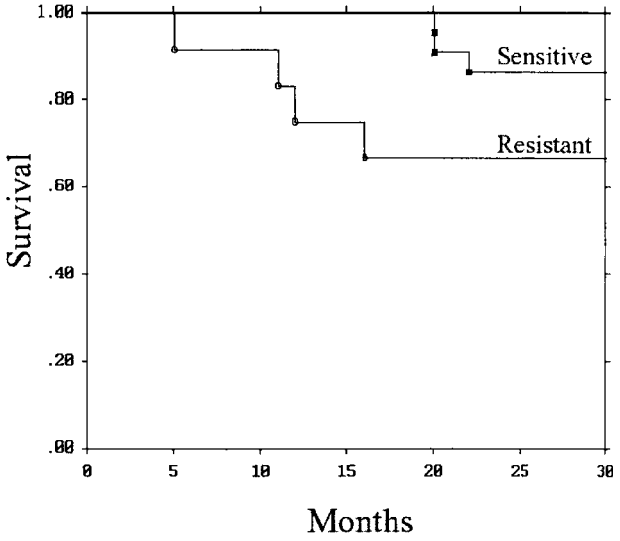

FIGURE 2. Cause-specific survival by multidrug resistance based on the histoculture drug response assay. Multidrug resistance was defined as in vitro resistance to both cisplatin and 5-fluorouracil. Survival comparisons were performed using the Wilcoxon test ($p = .01$).

tive to 5-fluorouracil (85% vs 64%; $p = .04$), sensitive to cisplatin (86% vs 64%; $p = .05$) or sensitive to both agents (Figure 2; 85% vs 63%; $p = .01$).

Clinical factors found to be significant predictors of cause-specific survival on univariate analysis included TNM stage ($p = .01$), presence of nodal metastasis at presentation ($p = .04$), and the presence of recurrent cancer at presentation ($p = .03$). Multivariate analysis was performed, including the presence of resistance to both agents, TNM stage (early [I/II] vs advanced [III/IV]), and the presence of recurrent disease at presentation (chi square = 8.5; $df = 3$; $p = .04$). Only the presence of recurrent disease at presentation (relative risk = 5.6; 95% CI = 1.1–30.0; $p = .04$) and the presence of chemosensitivity by HDRA assessment (relative risk = 0.16; 95% CI = 0.02–0.94; $p = .02$) remained significant prognostic variables.

DISCUSSION

The initial use of chemotherapy in the management of HNSCC was deemed a failure when survival was the end point evaluated.[21] However, experience and subset analysis from initial studies suggested that chemotherapy might have a role in treating a subset of HNSCC patients.[21,22] The data showed that patients with a significant response to chemotherapy, who refused subsequent treatment, had similar survival outcomes to those seen in patients receiving planned surgery after chemotherapy. These findings lead to increased enthusiasm for the use of chemotherapy in patients with HNSCC and were the basis for the development of organ preservation approaches.[23,24] The resultant Veterans' Administration trial, a landmark work[24] with respect to the management of laryngeal cancer, confirmed the benefit of chemoresponse to subsequent radiation treatment outcome.

Moreover, Beauvillain and colleagues[25] showed that chemoresponse is not only a radiation response predictor but also the most important prognosticator in patients undergoing treatment for advanced laryngopharyngeal cancer. They showed survival differences that were clearly delineated by the presence or absence of chemoresponse. More importantly, they showed that patients with chemoresistant tumors did very poorly regardless of the treatment approach used.

On the basis of the accumulated data, it can be concluded that chemosensitivity status is a significant predictor of outcome. Because it is not feasible to determine chemosensitivity in vivo in all cases, in vitro assays for chemoresponsiveness may serve as an important surrogate prognostic marker. Robbins and colleagues[20] have previously assessed the efficacy of HDRA assessment in HNSCC and reported a positive predictive value of 83%. The efficacy of the HDRA to predict survival was also demonstrated in studies of patients with gastrointestinal cancers.[9,15] The patients whose tumors were sensitive in the HDRA had significantly better survival than the controls.[20]

Several aspects of this study deserve comment. First, the validity of the HDRA assessment has been established in our laboratory showing a high predictive value.[26] Second, the cutoffs used for HDRA-associated chemoresistance were chosen arbitrarily and need to be validated. Finally, the HDRA assessment of chemoresponse was used specifically as a predictor of outcome and not clinical chemoresponse in this study. Nonetheless, our analysis confirms the applicability of the HDRA to HNSCC, with all but one case successfully analyzed. The rates of chemoresistance observed in our analysis are similar to those seen in clinical studies on HNSCC.[22–25] In this study, we have now demonstrated a statistically significant correlation of chemosensitivity in the HDRA with clinical survival. A lower locoregional recurrence rate was also seen in cases associated with cisp-

latin sensitivity. Because there were no significant differences in demographic data, tumor site or stage, or treatment based on HDRA chemoresponse assessment, it can be deduced that the chemosensitive and resistant patients were comparable with respect to clinical and pathologic prognostic criteria (Table 1). These data thus lead to the conclusion that sensitivity to 5-fluorouracil, cisplatin, or both agents in the HDRA is associated with and possibly related to a significantly greater cause-specific survival, independent of confounding variables. Thus, chemoresponse in the HDRA is an important prognosticator of survival in patients with HNSCC. Accordingly, prospective investigations are warranted to determine the role of the HDRA in the treatment selection in patients with HNSCC.

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