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Relationship between tumor histopathology and *in vitro* sensitivity to antitumor drugs in gastric cancer

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The *in vitro* drug sensitivity of gastric cancer tissues obtained from 40 patients with advanced cancer was compared in terms of the pathological classifications which were assigned according to the General Rules for the Gastric Cancer Study in Surgery and Pathology in Japan. Cases of poorly differentiated adenocarcinoma which had penetrated the serosa were evaluated using the succinate dehydrogenase inhibition (SDI) test for determining the *in vitro* chemosensitivity. The sensitivity of the stage III group to cisplatin was higher than that of the stage IV group. Although there were no statistical differences in drug sensitivities according to macroscopic findings (Borrmann's classification), the expanding growth type was more susceptible that the infiltrating type to cisplatin, aclacinomycin A (ACR) and carboquone (CQ) microscopically. In cases of lymph node metastasis [*n*(+)] the sensitivity to cisplatin, ACR, CQ, adriamycin and mitomycin C was less than in those with or without primary lymph node metastasis [*n*(−)]; lymphatic invasion in the gastric wall (*ly*) was a significant factor linked to drug resistance. Our findings indicate that the evaluation of tumor pathology is important in predicting the chemosensitivity of poorly differentiated gastric cancers.

Key words: MTT assay, SDI test, stomach.

Introduction

Gastric cancer is one of the most resistant to chemotherapy.[1,2] While therapeutic studies initiated after surgery have revealed the effectiveness of antitumor drugs, even in advanced cases,[3] survival was not prolonged in patients with certain pathological characteristics. For example, studies in Japan[4] have shown that the prolongation of survival time by chemotherapies was more defined in those patients with lymph node metastases and serosal invasion than those without. However, these drugs were not as useful in treating early cancer in spite of their strong side effects. Clearly there is a need to select the most appropriate drug regimen according to individual characteristics.[5] For this purpose, a test for chemosensitivity has been investigated by this group.[6,7]

The *in vitro* test for succinate dehydrogenase inhibition (SDI) is a method of evaluating the chemosensitivity of the tumor with the advantages of high detectability and ease of performance.[6] It involves the determination of succinate dehydrogenase activity to evaluate the viability of the tumor tissue exposed to a drug and is measured according to colorimetric changes in 3-(4,5-dimetyl-2 thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) as the hydrogen acceptor.[8] The principle underlying this test is the same as that of the so-called MTT assay which is used for screening new antitumor drugs at the National Cancer Institute in the US.[9] The correlation between *in vitro* sensitivity and clinical results was reported in many prospective clinics prescribing sensitive drugs in this assay.[10,11] The SDI test is superior to the MTT assay using a substrate to enhance the enzymatic reaction.[8]

The heterogeneity of tumor chemosensitivity is the principle underlying the test. However, there are cases which cannot be tested, because of an insufficient volume of tumor tissue or in those patients who are poor candidates for surgery. If a correlation exists between the pathological characteristics of the tumor and its chemosensitivity, an effective regimen can be prescribed based on the tumor characteristics. We previously reported that poorly differentiated adenocarcinomas of the stomach were more sensitive than the well differentiated type,[6] as determined by the SDI test.

Here we report our findings on 40 patients with poorly differentiated adenocarcinomas of the stomach which had penetrated the serosal surface.

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The relationship between tumor chemosensitivity and pathological findings is discussed.

Materials and Methods

Materials

We obtained specimens of tumor tissue from 40 patients with advanced gastric cancer who had undergone surgery at the Second Department of Surgery of the Kyushu University Hospital. Since tumor volume in the early disease stage is small, we restricted our comparisons to poorly differentiated adenocarcinomas classified as *ps*(+), cases with serosal penetration, following the General Rules for the Gastric Cancer Study in Surgery and Pathology in Japan.[12] Staging and pathological evaluation were done according to those rules. The results of SDI testing classified according to the pathological factors were then compared. Parameters evaluated were histological disease stage, the macroscopic appearance using Borrmann's classification, infiltrative growth (INF), degree of serosal invasion, lymphatic invasion in the gastric wall (*ly*) and microscopic invasion of lymph node (*n*). Tumors showing INF were classified according to three types: INF-α, -β and -γ. INF-α is characterized by an expansive growth pattern of the cancerous lesion with a distinct border from the surrounding tissue, whereas INF-γ is characterized by an infiltrative growth pattern of the cancerous lesion with an ill defined border. INF-α corresponds to the expanding type and INF-γ to the infiltrating type of Ming's classification.[13] INF-β is intermediate between INF-α and -γ. Both *se* and *si* are cases of serosal invasion. In *si*, the tumor tissue infiltrates adjacent organs, while in *se* it does not. Positive cases of lymphatic invasion [*ly*(+)] and lymph node metastasis [*n*(+)] were compared with negative cases [*ly*(−) and *n*(−)].

Antitumor agents

The antitumor agents tested were adriamycin (ADM) (4 μg/ml), mitomycin C (MMC) (10 μg/ml), cisplatin (DDP; 20 μg/ml), aclacinomycin (ACR) (4 μg/ml) and carboquone (CQ) (1 μg/ml) with doses corresponding to 10 times the peak plasma concentration.[6] These drugs were previously shown to be effective in clinical studies of gastric cancer [4,14,15] or on the results of preliminary studies of the SDI test for gastric cancer.[6] The sources of these drugs were as follows: ADM and MMC were from the Kyowa Hakko Co., Japan; DDP was from the Nihon Kayaku Co., Japan; ACR was from the Sanraku-Ocean Co., Japan; and CQ was from the Sankyo Co., Japan.

SDI test

Because the activity of succinate dehydrogenase shows heterogeneity according to the organ involved. i.e. metastatic lesion of lymph nodes is more sensitive, while that of the liver is less sensitive than the primary gastric tumor in the same patient,[7] the specimens for study were taken only from the primary tumor sites. On the day of excision, the specimens were cut with scissors and passed through a #32 stainless steel mesh into McCoy's solution containing antibiotics and washed 3 times with this solution. The fragments were then suspended in Eagle's minimal essential medium with L-glutamine (292 mg/ml), 10% fetal calf serum and antibiotics, plated in each of 35 mm plastic dishes and incubated with the indicated volume of antitumor drug at 37°C in a humidified 5% CO_2 atmosphere for 3 days. On day 3 the fragments were assayed for succinate dehydrogenase activity. Sodium succinate (10 mM) as a substrate and MTT (40 μM) as a hydrogen acceptor were added to the medium, and incubation was continued for another 3 h under the conditions described. The cells in the medium were then separated by centrifugation and the formazan in the cells formed from MTT was extracted with acetone containing 0.5% trichloroacetic acid. Absorbance of formazan was measured at 565 nm using a photometer. The succinate dehydrogenase activity was presented as the optical density (OD) per mg tissue protein. Tests with each drug were done in triplicate and the succinate dehydrogenase activity of the drug treated groups was expressed as a percentage of that of the control

Statistical analysis

The statistical independence of each pathological factor was evaluated using the χ^2 test; there was no dependence among the factors being compared. The mean ± standard deviation was compared in each group. Statistical differences were examined by the Student's *t*-test. The significance level was $p < 0.05$.

Table 1. Comparison of succinate dehydrogenase activities in gastric cancer stages (mean ± SD)

Stage	ADM	MMC	DDP	ACR	CQ
III	48.4 ± 9.7	42.1 ± 8.0	42.2 ± 8.3[a]	39.4 ± 8.6	40.4 ± 8.6
IV	59.0 ± 9.0	45.9 ± 8.2	61.3 ± 5.2[a]	44.5 ± 6.4	43.5 ± 7.5

[a] $p < 0.01$.

Results

Stage

There were 25 cases of stage III and 15 of stage IV. The succinate dehydrogenase activity of the tumors classified according to histological stage is shown in Table 1. Note the inverse relationship between tumor sensitivity and succinate dehydrogenase activity: the more sensitive the tumor tissue, the lower the succinate dehydrogenase activity. Although stage III tumors were more sensitive to DDP than stage IV, there was little difference in the two stages with regard to the other drugs.

Borrmann's classification

The cases were classified by macroscopic findings using Borrmann's classification and the succinate dehydrogenase activities were compared as shown in Table 2. Overall there were 5, 8, 17 and 10 cases with Borrmann's types 1, 2, 3 and 4, respectively. Comparing the succinate dehydrogenase activity of the localized type (Borrmann's types 1 and 2) with that of the invasive type (Borrmann's types 3 and 4), there was no significant difference in the succinate dehydrogenase activities for the five drugs tested.

Infiltrative growth (INF)

Tumors were classified by the extent of infiltrative growth. There were 7, 9 and 24 cases of INF-α, -β and -γ type, respectively. Comparing types α and β with γ, the former two were more sensitive to DDP, ACR and CQ than the latter (Table 3).

Serosal invasion

Considering the cases classified by the microscopic finding of serosal invasion (Table 4) (30 cases of *se* and 10 cases of *si*), there was no significant difference between the groups with regard to drug sensitivity.

Lymphatic invasion in the gastric wall

Invasion into the lymphatic capillaries of the gastric wall (*ly*) was investigated microscopically. There

Table 2. Comparison of succinate dehydrogenase activities between Borrmann's type 1, 2 and 3, 4 (mean ± SD)

	ADM	MMC	DDP	ACR	CQ
Borrmann's 1, 2	49.0 ± 8.9	41.6 ± 7.0	56.0 ± 13.3	39.5 ± 8.4	46.9 ± 13.5
Borrmann's 3, 4	60.2 ± 9.8	49.2 ± 9.6	55.8 ± 6.3	50.6 ± 8.8	48.1 ± 9.8

Table 3. Comparison of succinate dehydrogenase activities between INF-α, -β and -γ (mean ± SD)

	ADM	MMC	DDP	ACR	CQ
INF-α, -β	50.3 ± 6.9	40.2 ± 7.3	40.2 ± 7.3[a]	35.0 ± 5.7[b]	34.3 ± 6.2[c]
INF-γ	59.2 ± 10.7	49.1 ± 9.4	57.1 ± 8.5[a]	51.9 ± 8.8[b]	49.2 ± 8.3[c]

[a] $p < 0.01$.
[b] $p < 0.01$.
[c] $p < 0.05$.

Table 4. Comparison of succinate dehydrogenase activities between *se* and *si* (mean ± SD)

	ADM	MMC	DDP	ACR	CQ
se	50.0 ± 9.5	46.8 ± 20.9	48.8 ± 8.0	49.5 ± 9.1	47.1 ± 9.5
si	57.5 ± 9.6	49.8 ± 9.0	53.7 ± 8.6	47.4 ± 8.2	46.6 ± 6.2

Table 5. Comparison of succinate dehydrogenase activities between *ly*(−) and *ly*(+) (mean ± SD)

	ADM	MMC	DDP	ACR	CQ
ly(−)	49.2 ± 8.5[a]	39.8 ± 6.9[b]	39.2 ± 8.5[c]	35.7 ± 5.1[d]	38.2 ± 7.2[e]
ly(+)	68.6 ± 8.8[a]	58.2 ± 9.3[b]	65.8 ± 5.6[c]	58.4 ± 6.5[d]	60.9 ± 8.9[e]

[a] $p < 0.05$.
[b] $p < 0.05$.
[c] $p < 0.01$.
[d] $p < 0.01$.
[e] $p < 0.05$.

Table 6. Comparison of succinate dehydrogenase activities between *n*(−) and *n*(+) (mean ± SD)

	ADM	MMC	DDP	ACR	CQ
n(−)	47.7 ± 7.9[a]	38.7 ± 7.7[b]	42.4 ± 7.95[c]	36.3 ± 5.6[d]	38.1 ± 7.4[e]
n(+)	64.6 ± 7.6[a]	56.6 ± 8.3[b]	62.2 ± 5.7[c]	52.9 ± 7.6[d]	56.9 ± 9.2[e]

[a] $p < 0.05$.
[b] $p < 0.05$.
[c] $p < 0.01$.
[d] $p < 0.01$.
[e] $p < 0.05$.

were 24 and 16 cases in *ly*(−) and *ly*(+), respectively. Compared with the *ly*(+) cases, the *ly*(−) cases were significantly more susceptible to ADM, MMC, cisplatin, ACR and CQ (Table 5).

Lymph node metastasis

Metastases to the lymph nodes were evaluated microscopically (Table 6). There were 22 and 18 cases in *n*(−) and *n*(+), respectively. Those with lymph node metastases [*n*(+)] were less sensitive than those without metastasis [*n*(−)] to the five drugs tested.

Discussion

Certain pathological characteristics have been thought to predict the prognosis of patients with gastric cancer in long-term clinical studies in Japan[16] and in the western world.[17,18] Indeed patients in advanced stages of the disease,[19] with serosal invasion,[1,20–22] infiltrative growth,[21] lymphatic metastases,[2,20,22] or lymphatic capillary invasion,[20,22] have been documented to have a poor prognosis in various multifactorial analyses. However, some studies did not find an effect of lymphatic[1] or serosal[2,23] factors on prognosis.

Few reports have analyzed the influence of tumor pathology on the clinical response to antitumor drugs; perhaps because of the difficulty in setting up such a trial and also the difficulty in following large numbers of patients who meet study requirements.[1] Our approach is novel in that we apply an *in vitro* test to investigate the relationship between chemosensitivity and tumor pathology.

The relative usefulness of antitumor drugs in treating early versus advanced disease is not clear. A 5 year survival rate of 79.4% has been obtained for Japanese patients in stage I and II[16] since the introduction of the radical dissection of lymph nodes;[25] the advantage of post-operative chemotherapy has not been established in such cases. Hattori *et al.* reported that the effect of chemotherapy using MMC could not be defined in stage

IV, whereas the 5 year survival in stage III was significantly prolonged.[4] According to the rules,[12] this ineffectiveness against stage IV cases is thought to be due to four factors: the presence of liver metastasis,[25] peritoneal dissemination, penetration of the serosa and invasion of the adjacent organs,[26] or distant lymph node metastasis.[24] Since the present series had only a few cases of liver or intra peritoneal metastasis, we could not analyze these factors. However, we previously reported that a metastatic lesion in the liver had a lower sensitivity in the SDI test than the stomach in the same patient.[7] Thus we can infer that drug resistance in stage IV patients may, in part, be due to the potential for metastases to the liver. However, as there was little difference in the sensitivity to MMC between the cases of stage III and IV, or between the *se* and *si* cases in this study, the lower sensitivity of stage IV in clinical studies may be due to the presence of distant lymph node metastasis or to the presence of microscopic residues of cancer tissue in the invaded organ, not to a difference in chemosensitivity.

Macroscopic and microscopic types of invasion are two factors which affect the prognosis. The invasive type has been noted to have a poorer prognosis than the localized type macroscopically;[16] the infiltrative type has a poorer prognosis than the expanding growth type microscopically.[21] However, there is little information on the relationship between the invasive findings and the response to chemotherapy. We observed a lower sensitivity to three drugs of the infiltrating type in the microscopic examinations, whereas the macroscopic findings did not significantly affect chemosensitivity. We believe this result reflects the tumor's proliferative activity. Based on results of DNA analysis using clinical specimens MacArtney *et al.* showed that the infiltrating type of growth had a lower incidence of DNA aneuploidy and S phase fraction, which is thought to demonstrate proliferative activity, compared with the expanding type.[27] According to their data, a greater susceptibility to antitumor drugs can be inferred for the expanding type. Because it is reasonable to think that the more rapidly a cell proliferates, the greater the opportunity there is for DNA damage, in that the five drugs we tested are cytocidal by means of DNA cross-linking in the proliferative cell cycle.[28–30] In many experiments the sublines with drug resistance have shown a shorter cellular doubling time than sublines with drug sensitivity whose original cell lines were the same as those of the resistant sublines.[31] We reported that poorly differentiated carcinomas, which penetrate the serosa more rapidly in general than the well-differentiated type, are more sensitive and have higher pyrimidine nucleotide synthesis than the latter.[32]

Another factor that influences the prognosis of gastric cancer is tumor invasiveness into the lymphatic system. Patients with distant metastases typically have a poor prognosis.[22] Invasion into the lymphatics in the gastric mucosa indicates a poorer prognosis than *ly*(−) cases.[21] These findings might also be explained as being due to a difference in the grade of tumor growth rather than to a difference in drug sensitivity between the two groups. However, we observed a significant difference in the sensitivity to five antitumor drugs *in vitro* in the cases with and without lymphatic invasion. Drug tolerance may exist in those tumors with the potential to metastasize via the lymphatic system. The relationship between metastatic potential and chemosensitivity is unclear. Nicolson *et al.* found no correlation between metastatic potential via the blood flow *in vivo* and drug sensitivity *in vitro* using variant sublines of a rat mammary adenocarcinoma which had demonstrated various metastatic potentials.[33] Formelli *et al.* reported that an adriamycin-resistant subline of murine B16 melanoma had a lower metastatic potential to the lung via the blood flow than did the adriamycin-sensitive subline *in vivo*.[34] We showed, using clinical specimens, that lymph node metastases were more frequent in patients with heterogeneity in their DNA ploidy pattern than in those with a homogeneous pattern.[35] According to those results if the tumor content is heterogenous with lymphatic metastasis, there would be an increased risk of drug resistance.[7]

Conclusion

The relationships between pathological characteristics of the tumor and drug sensitivity were compared using the SDI test. We observed that tumors with infiltrative growth, lymphatic invasion in the gastric wall or lymph node metastasis were resistant to antitumor drugs. This study is another step toward predicting the appropriate choice of antitumor drugs to be administered when treating patients showing these tumor characteristics.

References

1. Cunningham D, Hole D, Taggart DJ, *et al.* Evaluation of the prognostic factors in gastric cancer: the effect of chemotherapy on survival. *Br J Surg* 1987; **74**: 715–20.

2. Allum WH, Hallissey MT, Kelly KA. Adjuvant chemotherapy in operable gastric cancer. 5 year follow-up of first British stomach cancer group trial. *Lancet* 1989 *i*: 571–4.
3. The Gastrointestinal Tumor Study Group. Randomized study of combination chemotherapy in unresectable gastric cancer. *Cancer* 1984; **53**: 13–7.
4. Hattori T, Inokuchi K, Taguchi T, *et al.* Postoperative adjuvant chemotherapy for gastric cancer, the second report. Analysis of data on 2873 patients followed for five years. *Jpn J Surg* 1986; **16**: 175–80.
5. Inokuchi K. Prolonged survival of gastric cancer patients on a specific adjuvant chemotherapy. *Jpn J Surg* 1984; **14**: 351–9.
6. Maehara Y, Anai H, Kusumoto H, *et al.* Poorly differentiated human gastric carcinoma is more sensitive to antitumor drugs than is well differentiated carcinoma. *Eur J Surg Oncol* 1987; **13**: 203–6.
7. Kusumoto H, Maehara Y, Kusumoto T, *et al.* Chemosensitivity differences between primary and metastatic lesions of clinical gastric cancer. *Eur J Surg Oncol* 1988; **14**: 685–9.
8. Maehara Y, Kusumoto T, Kusumoto H, *et al.* Sodiun succinate enhances the colorimetric reaction of the *in vitro* chemosensitivity test MTT assay. *Oncology* 1988; **45**: 434–6.
9. Allay MC, Scudiero DA, Monks A, *et al.* Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res* 1988; **48**: 589–601.
10. Sargent JM, Taylor CG. Appraisal of the MTT assay as a rapid test of chemosensitivity in acute myeloid leukemia. *Br J Cancer* 1989; **60**: 206–10.
11. Wilson JK, Sargent JM, Elgie AW, *et al.* A feasibility study of the MTT assay for chemosensitivity testing in ovarian malignancy. *Br J Cancer* 1990; **62**: 189–94.
12. Japanese Research Society for Gastric Cancer. The general rules for the gastric cancer study in surgery and pathology. *Jpn J Surg* 1981; **11**: 127–39.
13. Ming S. Gastric carcinoma. A pathological classification. *Cancer* 1977; **39**: 2475–85.
14. Cunningham D, Soukop M, McArdle CS, *et al.* Advanced gastric cancer: experience in Scotland using 5-fluorouracil, adriamycin and mitomycin-C. *Br J Surg* 1984; **71**: 673–6.
15. Wagener DJT, Yap SH, Webbes T, *et al.* Phase II trial of 5-fluorouracil, adriamycin and cisplatin (FAP) in advanced gastric cancer. *Cancer Chemother Pharmacol* 1985; **15**: 86–7.
16. Miwa K. Cancer of the stomach in Japan. *Gann Monogr Cancer Res* 1979; **22**: 61–75.
17. Lundegardh G, Adami H, Malker B. Gastric cancer survival in Sweden. Lack of improvement in 19 years. *Ann Surg* 1986; **204**: 546–51.
18. Valen B, Viste A, Haugstvedt T, *et al.* Treatment of the stomach cancer, a national experience. *Br J Surg* 1988; **75**: 708–12.
19. Søreide O, Lillestøl J, Viste A, *et al.* Factors influencing survival in patients with cancer of the stomach. *Acta Chir Scand* 1982; **148**: 367–72.
20. Serlin O, Keehn RJ, Higgins Jr GA, *et al.* Factors related to survival following resection for gastric carcinoma. *Cancer* 1977; **40**: 1318–29.
21. Okada M, Kojima S., Murakami M, *et al.* Human gastric carcinoma: prognosis in relation to macroscopic and microscopic features of the primary tumor. *J Natl Cancer Inst* 1983; **71**: 275–9.
22. Bozzetti F, Bonfanti G, Morabito A, *et al.* A multifactorial approach for the prognosis of patients with carcinoma of the stomach after curative resection. *Surg Gynecol Obstet* 1986; **162**: 229–34.
23. Msika S, Chastang C, Houry S, *et al.* Lymph node involvement as the only prognostic factor in curative resected gastric carcinoma: a multivariate analysis. *World J Surg* 1989; **13**: 118–23.
24. Jinnai D. Evaluation of extended radical operation for gastric cancer, with regard to lymph node metastasis and follow up results. *Gann Monogr Cancer Res* 1968; **3**: 225–31.
25. Okuyama K, Isono K, Juan I *et al.* Evaluation of treatment for gastric cancer with liver metastasis. *Cancer* 1985; **55**: 2498–505.
26. Kaibara N, Iisuka Y, Kimura A, *et al.* Relationship between area of serosal invasion and prognosis in patients with gastric carcinoma. *Cancer* 1987; **60**: 136–9.
27. MacArtney JC, Camplejohn RS, Powell G. DNA flow cytometry of histological material from human gastric cancer. *J Pathol* 1986; **148**: 273–7.
28. Zwelling LA, Kohn KW. Mechanism of action of *cis*-dichlorodiammineplatinum (II). *Cancer Treat Rep* 1979; **63**: 1439–44.
29. Zunino F, Gambetta RA, Dimarco A, *et al.* Interaction of daunomycin and its derivatives with DNA. *Biochem Biophys Acta* 1972; **277**: 489–98.
30. Iyer VN, Szybalski W. Mitomycins and porfiromycin: Chemical mechanism of activation and cross-linking of DNA. *Science* 1964; **145**: 55–7.
31. Supino R, Prosperi E, Formelli F, *et al.* Characterization of a doxorubicin-resistant murine melanoma line: studies on cross-resistance and its circumvention. *Br J Cancer* 1986; **54**: 33–42.
32. Maehara Y, Kusumoto T, Sakaguchi Y, *et al.* Pyrimidine nucleotide synthesis is more extensive in poorly differentiated than in well differentiated human gastric carcinoma. *Cancer* 1989; **63**: 96–101.
33. Nicolson GL, Lembo TM, Welch DR. Growth of rat mammary adenocarcinoma cells in semisolid clonogenic medium not correlated with spontaneous metastatic behavior: Heterogeneity in the metastatic, antigenic, enzymatic and drug sensitivity properties of cells from different sized colonies. *Cancer Res* 1988; **48**: 399–404.
34. Formelli F, Rossi C, Supino R, *et al.* *In vivo* characterization of a doxorubicin resistant B16 malanoma cell line. *Br J Cancer* 1986; **54**: 223–33.
35. Haraguchi M, Okamura T, Korenaga D, *et al.* Heterogeneity of DNA ploidy in patients with undifferentiated carcinomas of the stomach. *Cancer* 1987; **59**: 922–4.

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