The Seoul Journal of Medicine
Vol. 35, No. 2: 83-90, June 1994

The Significance of DNA Ploidy by Flow Cytometric Measurement in Pancreatic Cancer†

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= Abstract = **The cellular DNA content of pancreatic cancer using formalin-fixed, paraffin-embedded specimens from 37 patients whose disease had been treated with surgical resection was determined by flow cytometry. Ploidy and cell cycle parameters were analysed and correlated with clinical and pathologic findings. There were 24 (64. 9%) diploid and 13 non-diploid pancreatic cancers. The median survival of the patients with diploid tumor was 14 months and that of the patients with non-diploid tumor was 8 months, but the difference did not have statistical significance (p=0. 078). And other cytometric parameters such as G1 phase fraction (p=0. 84), S phase fraction (0. 076), G2M phase fraction (p=0. 72), and proliferative index (p=0. 81) did not show any significant prognostic value. The patients with stage I (n=15) had 27 months of median survival, the patients with stage II (n=8) 7 months of median survival, the patients with stage III (n=15) 9 months of median survival. The differences of survival by stage were the most significant among the parameters which were studied (p=0. 0003). The group which had lymph node metastasis (n=11) showed 7 months of median survival and the group with negative lymph node (n=26) 12 months. The difference was also significant (p=0. 046). The other clinical parameters such as sex, the size of tumor, and the location of tumor did not have any influence on the prognosis of the pancreatic cancer patients in this study. Multivariate analysis by Weibull's model was used for prediction of survival time. Diploid versus non-diploid DNA content changed to less significant factor after adjustment for stage and lymph node. But the stage of the tumor remained a highly significant prognostic factor even after adjustment for ploidy and lymph node status.**

Key Words: *Flow cytometry, Pancreatic cancer, Ploidy*

INTRODUCTION

Recently, efforts to predict the prognosis of the patients with malignant disease by using flow cytometry and compare this with other prognostic factors have been made. Especially, ploidy of nucleic DNA in malignant cells is important prognostic factor and that is, the more malignant, the more atypical nucleic DNA is found. There is a good correlation between the DNA histograms produced by using paraffin-embedded pathological material and those obtained by using unfixed tissue from the same tumor allowing the retrospective study of archival material where the clinical outcome is

Received in March 1994, and in final form in April 1994.

† Supported by a grant No 02-93-044 from Seoul National University Hospital Research Fund

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already known(Hedley *et al.* 1983).

Surgical excision continues to be the only curative measure of treatment for pancreatic cancer, but the overall resectability rate is low and 5-year survival rate is only 5-18%. The value of surgical excision for pancreatic cancer which is seemingly resectable is under question. It is imperative to refine and differentiate the treatment of pancreatic cancer, especially curative excision, to improve the quality of life remaining of the patients. Certain group of patients with large(> 2.5cm) aneuploid cancers were identified who had uniformly poor survivals even after curative resections(Allison *et al.* 1991). Because DNA measurements can be done on the pancreatic malignant cell obtained by preoperative fine-needle aspiration, the combination of this DNA measurement and preoperative fine-needle aspiration would be the basis of selection of patients who could be candidates for surgical resection.

Now, we evaluate DNA measurement and cell-cycle analysis of pancreatic malignant cells by using flow cytometry and compare this with other prognostic factors of survival.

MATERIALS AND METHODS

Patients

All the patients underwent resectional operation because of pancreatic cancer in the Department of Surgery, Seoul National University Hospital from January 1985 to April 1991. They were all explored in the operation room and found free of distant metastasis and tumor mass was resectable. Main tumor was located at the pancreas and the tumors which showed non-epithelial appearance or the tumors in which the origin of the tumor was in doubt were excluded from this study. They were all available with adequate pathological tissue and follow-up information.

There were 37 patients(male = 20, female = 17) for successful study and their median age was 58-years old. There were 28 ductal cell adenocarcinoma, 4 malignant papillary cystic tumor, 2 mucinous adenocarcinoma and 1 case of giant cell adenocarcinoma, squamous cell adenocarcinoma, undifferentiatd adenocarcinoma respectively.

By Hermleck's stage(1974), the stages of the tumors were classified(stage I: 15, stage II: 8, stage III: 13, stage IV: 0).

The operations which were performed on the patients were Whipple's operation(n = 23), distal pancreatectomy(n = 11), total pancreatectomy(n = 2), and pylorus-preserving pancreatoduodenectomy(n = 1).

Cell preparation

The method of Hedley and co-workers (1983) was modified and applied to this study. Five μm sections were made from the cancer-containing tissue block and adequate cancer tissue was confirmed in the block. Usually a single section was adequate, but for small samples two or three sections were required. After that, 50 μm sections were cut and placed in 10 ml glass centrifuge tubes and dewaxed using two changes of xylene, 3 ml of 100, 95, 70, and 50% ethanol for 10 min each at room temperature. The tissue was then washed twice in distilled water and resuspended in 1 ml of 0.5% pepsin(Sigma Chemical Company Ltd.), in 0.9% NaCl, adjusted to pH 1.5. The tubes were then immersed in a water bath which was adjusted to 37.5℃ with intermittent vortex mixing for 60 min. After that, the tissue suspension was washed with phosphate-buffered saline with a 30 mm nylon screen and stained with ribonuclease(Sigma Chemical Company Ltd.) and propidium iodide(Sigma Chemical Company Ltd.). The suspension was made to contain 1-2 X 10^6 cells/ml using hemocytometer in hematoxylin stain.

Measurement of cellular DNA and Interpretation of DNA histogram

The DNA measurements were made by using a FACSCAN(Becton-Dickinson UK Ltd.) equipped with a 5-watt argon ion laser as a light source. It was operated at the wavelength of 488 nanometer at 200 mW. The analysis of DNA histogram was performed with an IBM

Table 1. Univariate analysis of prognostic factors including ploidy and cell cycle of pancreatic cancer

Factor	Mean survival	p
Ploidy		0.078
Diploid	18.0±2.6(n=24)	
Non-diploid	11.1±1.6(n=13)	
%G0/G1		NS*
≥87	16.8±1.2(n=19)	
<87	11.3±2.8(n=18)	
%S		NS
≥8	14.9±2.2(n=19)	
<8	12.1±1.7(n=18)	
%G2/M		NS
≥5.5	8.1±1.7(n=19)	
<5.5	23.7±2.9(n=18)	
%PI#		NS
≥13	11.2±1.3(n=19)	
<13	17.0±2.9(n=18)	

*non-significant, #proliferative index(S+G2/M), Mean survival in months(±SE)

Table 2. Univariate analysis of prognostic factors including tumor size and stage of pancreatic cancer

Factor	Mean survival	p
Size of tumor		NS*
≤3.0cm	14.9±2.5(n=14)	
>3.0cm	12.6±1.8(n=23)	
Lymph node		0.045
Positive	10.8±2.5(n=11)	
Negative	17.8±2.3(n=26)	
Location		NS
Head	14.9±2.2(n=24)	
Body/tail	12.1±1.7(n=13)	
Stage		0.0003
I	23.7±2.9(n=15)	
II	8.1±1.7(n= 8)	
III	11.2±2.2(n=13)	

*non-significant, Mean survival in months(±SE)

personal computer and built-in software of CellFit Cell-Cycle Analsis version 1. 2.

The lowest peak was given a DNA index (DI) value of 1 and the DI of other peaks was calculated with this reference. A histogram was considered to be diploid if its DNA distribution showed a single G1 peak . Any additional G1 peak was considered as non-diploid which included tetraploid. Tetraploid was defined when over 20% of events was located at G2M(DNA index 1. 80-2. 20) peak. The S-phase fraction (SPF) was calculated using computer program. The proliferative index was defined as the sum of S-phase fraction and G2M. Samples with a coefficient of variation above 10% were discarded.

Statistical Analysis

An IBM personal computer which had built-in Statistical Analytic System(SAS) was used for statistical analysis.

Survival curves were calculated by the method of Kaplan-Meier. The log-rank test was used to compare the statistical significance of survival curves between groups. Weibull's model was used for survival analysis with multiple variables. To compare the numericals, we used the Chi-square test.

Results

There were 37 obtainable DNA histograms. The mean value of coefficiency of variance was 4. 91±1.4%. Current follow-up data were available for all patients.

There were 24(64.9%) diploid and 13 non-diploid pancreatic cancers. The median survival of the patients with diploid tumor was 14 months and that of the patients with non-diploid tumor was 8 months, but the difference did not have statistical significance(p=0.078). And other cytometric parameters such as G1 phase fraction(p=0.84), S phase fraction(0.076), G2M phase fraction(p=0.72), and proliferative index (p=0.81) did not show any significant prognostic value. But among them, ploidy and S phase fraction was the most important factor for prognosis(Table 1. & Fig. 2.).

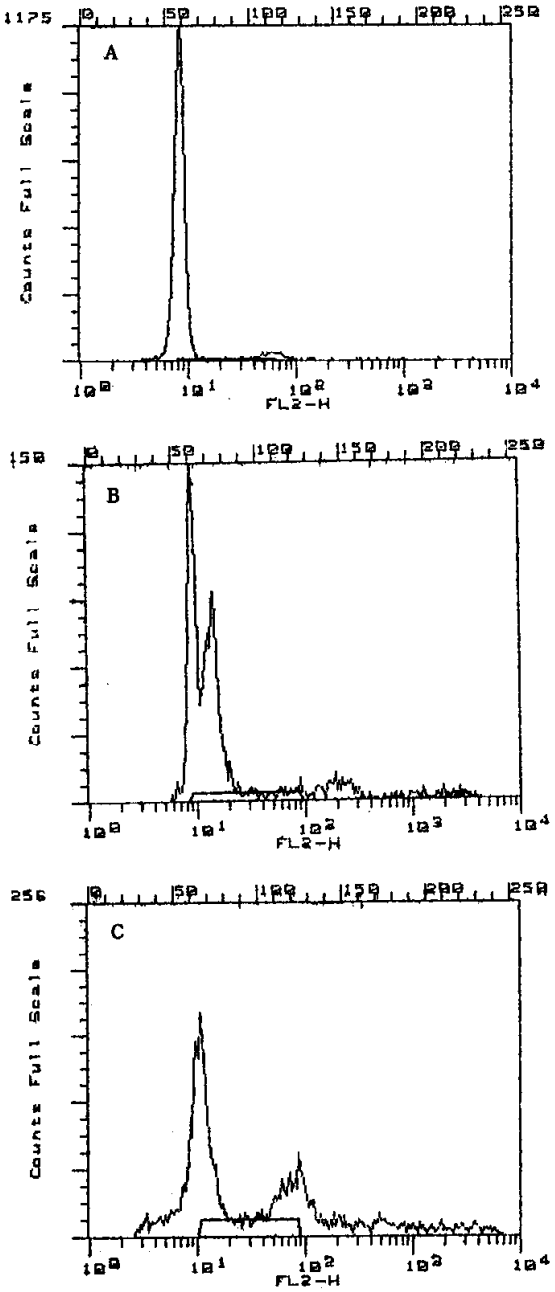

Fig. 1. Typical DNA distribution of paraffin-embedded pancreatic carcinoma measured by flow cytometry. A: diploid, B: aneuploid, C: tetraploid.

The patients with stage Ⅰ(n=15) had 27 months of median survival, the patients with stage Ⅱ(n=8) 7 months of median survival, the patients with stage Ⅲ(n=15) 9 months of median survival. The differences of survival by stage were the most significant among the parameters which were studied($p=0.0003$) (Table 2 & Fig. 3).

The group which had lymph node metastasis(n=11) showed 7 months of median survival and the group with the negative lymph node (n=26) 12 months. The difference was also significant($p=0.046$). The other clinical parameters such as sex, the size of tumor, and the location of tumor did not have any influence on the prognosis of the pancreatic cancer patients in

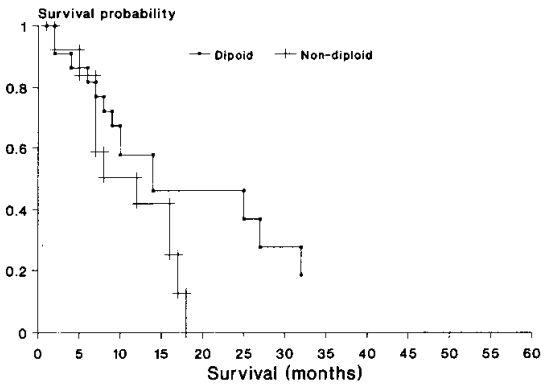

Fig. 2. Kaplan-Meier survival curve according to ploidy.($p=0.078$)

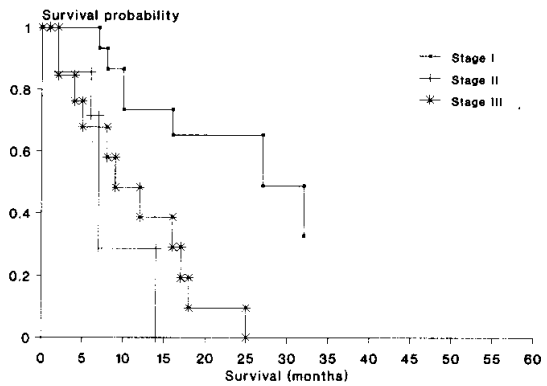

Fig. 3. Kaplan-Meier survival curve according to stage($p<0.05$).

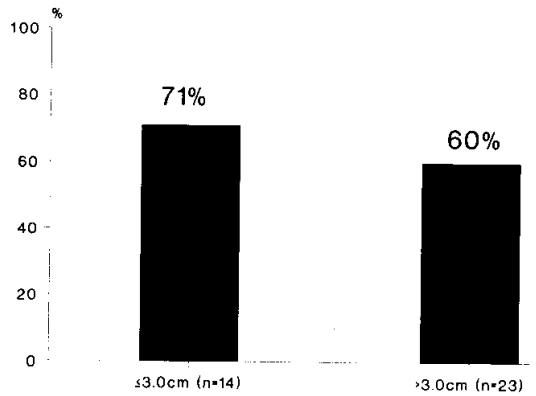

Fig. 4. The percentage of diploid tumor in each group by lymph node status of tumor(p> 0.05).

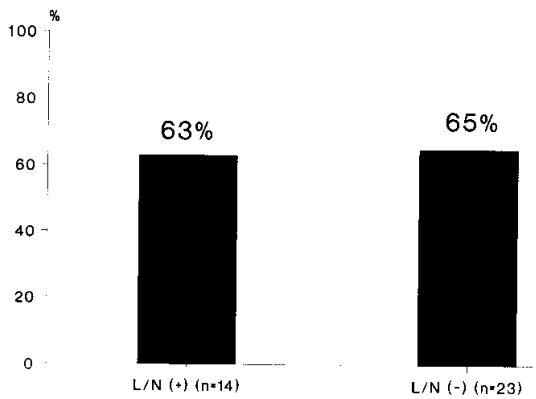

Fig. 5. The percentage of diploid tumor in each group by size of tumor(p>0.05).

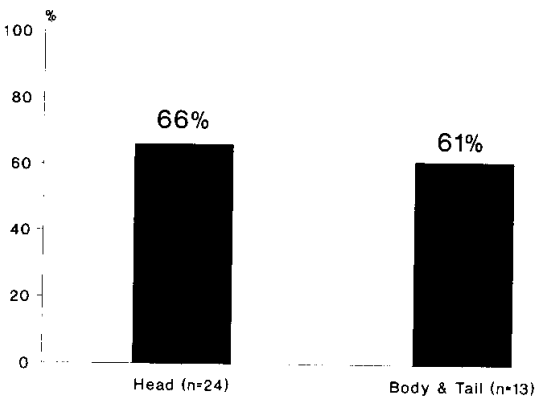

Fig. 6. The percentage of diploid tumor in each group by location of tumor(p>0.05).

Table 3. Multivariate analysis of prognostic factors

Factor	P
Stage	0.084
Lymph node	0.478
Ploidy	0.655

this study.

The percentage of diploid tumor in the negative lymph node group was 63%, whereas the percentage of diploid tumor in the positive lymph node group was 65%(Fig. 4.). The percentage of diploid tumor in the tumor larger than 3. 0 cm was 60%, whereas the percentage of diploid tumor in the 3cm or less group was 71%(Fig. 5.). The percentage of diploid tumor in pancreatic head cancer was 66% whereas the percentage of diploid tumor in pancreatic body and tail cancer was 61%(Fig. 6.). Statistical analysis demonstrated that the location, size, and lymph node metastasis did not have any influence on the ploidy of the pancreatic cancer but the factor of size could be the most important indicator for ploidy for pancreatic cancer.

Multivariate analysis by Weibull's model was used for prediction of survival time. Diploid versus non-diploid DNA content changed to less significant factors after adjustment for stage and lymph node. But the stage of the tumor remained a highly significant prognostic factor even after adjustment for ploidy and lymph node status(Table 3.).

Discussion

Adenocarcinoma of the pancreas is a highly malignant tumor which has only a 3% of 5-year survival rate with mean survival of only about 6 months(Warshaw *et al.* 1988). But a small group of pancreatic cancer patients show a 5-year survival nontheless without resection of tumor whereas the patients who are thought to be 'completely resected' of tumor can not survive longer than the 'non-resected' group. The significant role of surgical resection

in pancreatic cancer is now considered even unnecessary by some groups of surgeons (Connolly *et al.* 1987;Gudjonsson 1987; Joensuu *et al.* 1989;Lea and Stahlgren 1987;Shapiro 1975; Shepherd *et al.* 1988;Warshaw 1991). But development of surgical technique in pancreatic resection such as pancreatoduodenectomy has lessened surgical mortality from over 20% to 5% and recent introduction of pylorus-preseving pancreatoduodenectomy has minimized the postoperative morbidity of impaired digestive, endocrine, and metabolic function(Braasch *et al.* 1986; Crist *et al.* 1987; Pellegrini *et al* 1989). But, percutanous needle aspiration of pancreatic tissue can be performed easily and palliative management of pancreatic cancer such as percutanously or endoscopically placed biliary stent has aided pancreatic cancer patients without considerable morbidity and delayed hospitalization(Bornman *et al.* 1986; Shepherd *et al.* 1988). Precise differentiation of the patients who should be treated by surgical modality is now the problematic point of pancreas surgery.

The prognosis in cancer patients is dependent on multi-factors such as size of tumor, involvement of lymph node, differentiation of tumor cell, proliferative index, and presence of tumor antigen. Aneuploidy is now a well-known feature of human cancer, but its significance in terms of biological aggressiveness, clinical mode, and response to treatment remains unclear (Friedlander *et al.* 1984). But pathological differentiation is dependent on the amount of cellular content of DNA. Additionally, various information from FCM such as ploidy, S-phase fraction is deeply associated with stage of tumor, differentiation of tumor cell, size of tumor, involvement of lymph node (Hedley *et al.* 1987). For example, nucleic DNA content is a significant prognostic factor for node(-) breast cancer, early colorectal cancer, and malignant melanoma(Clark *et al.* 1989;Jones *et al.* 1988;Kheir *et al.* 1988;Scott *et al.* 1987;Seckinger *et al.* 1989). But determination of nuclear DNA content has no prognostic value in advanced colorectal cancer, node(+) breast cancer, and thyroid cancer(Hamming *et al.* 1988;Hedley *et al.* 1987;Lind *et al.* 1992).

This study was carried out to test the value of DNA ploidy estimation in pancreatic cancer. Some recent reports had favored determination of ploidy(Alanen *et al.* 1990;Allison *et al.* 1991;Eskelinen *et al.* 1990;Weger *et al.* 1991), others did not(Baisch *et al.* 1990). A further observation is that neoplastic cells with a triploid DNA content seem to be particularly aggressive and could be of practical clinical significance for the determination of prognosis and for the choices of therapy(Weger *et al.* 1990). In this study, the patients with diploid pattern had 14 months of median survival meanwhile those with non-diploid pattern had 8 months of median survival. Although the difference did not have statistical significance, it could be significant if more cases are added.

In comparison with factors such as stage and invovement of lymph node, the content of nucleic DNA was less significant. Although it could be concluded that the measurement of content of nucleic DNA might be unnecessary, it could be of worth from the viewpoint of the differing responsiveness to chemotherapeutic agent and mitotic potentials of tumor cells that are as yet unknown.

Analysis of cell cycle parameters revealed that S-phase fraction was the most significant factor although it did not have a statistical significance. Other factors such as G0/G1, G2/M fraction, PI(proliferative index) had minimal significance. These results were different from those obtained by Cameron(Allison *et al.* 1991) who showed that all the cell cycle parameters except G2M fraction had significant differences. Further study may be needed for this difference.

The aim of this study was to elucidate the prognostic and biological value of cellular DNA content and the fraction of cells in the various phases of pancreatic cancer before operation, thereby possibly defining the small number of patients eligible for curative treatment. But we used resected specimens in a retrospective study, so the problem was as follows. The aspirated material seems to be less representa-

tive of the cellular composition of the tumor than the resected tumor mass although recent study(Weger *et al.* 1991) showed that the needle aspiration could have better results than resected pathologic specimen.

Retrospective studies on prognosis of tumor cells has variable limitations by the fact that prognosis of the patients is dependent not only on the biologic nature of the tumor itself but on the other therapeutic modalities or clinical settings that are performed on the patients. And it was revealed that paraffin-embedded materials would be in a condition that might have variable ranges of values to judge the prognosis and progression of tumors.

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