Jpn. J. Cancer Res. 86, 174–181, February 1995

Association of p53 Gene Mutations with Short Survival in Pancreatic Adenocarcinoma

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Mutations of the p53 gene have been found in a variety of human cancers and are implicated in the biologic functions of cancer. To investigate the clinical implications of p53 mutations in pancreatic adenocarcinoma, we examined the association of mutations of the p53 gene with patients' prognosis. Single-strand conformational polymorphism analysis and direct DNA sequencing were used to detect p53 gene mutations in 37 pancreatic adenocarcinomas. p53 gene mutations were detected in 16 (43%) of the 37 pancreatic adenocarcinomas. Direct sequencing did not reveal preferential clustering at any specific codon. There was no significant association of the presence of p53 gene mutations with histologic types, extent of tumor invasion, the presence of lymph node metastasis, or tumor stage. Univariate analysis showed that survival of patients with p53-gene-mutated tumors was significantly poorer than that of patients with p53-gene-nonmutated tumors ($P=0.02$). Cox's multivariate analysis of ten clinicopathologic features including p53 gene mutations revealed that presence of p53 gene mutations ($P=0.026$) and curativity of operation ($P=0.014$) were independent predictors of survival. Furthermore, the survival of patients with p53-gene-mutated tumor was significantly poorer than that of patients with p53-gene-nonmutated tumors, both in patients who underwent curative operation ($P=0.04$) and in patients who underwent non-curative operation ($P=0.01$). These results suggested that mutations of the p53 gene might play an important role in cancer aggressiveness and could be a clinically useful predictor of prognosis in patients with pancreatic adenocarcinoma.

Key words: Pancreatic cancer — p53 mutation — Prognosis

Despite recent advances in diagnosis and treatment, adenocarcinoma of the pancreas remains one of the most difficult diseases both for patients to survive and for physicians to treat.[1] Although only surgical resection currently offers patients an opportunity for longer survival and for cure, from two-thirds to one-half of patients who undergo curative resections still do not survive more than 5 years.[2–6] This poor outcome might be attributed not only to inappropriate patient selection, but also to a failure to recognize the biologic differences in the aggressiveness of tumors.[7–9] Therefore, the identification of specific indicators of biologic aggressive potential in pancreatic adenocarcinoma would allow better prognostic evaluation of patients and hence a better therapeutic approach in terms of treatment cost and quality of life.

Neoplastic progression, which reflects the biologic aggressiveness of a tumor, is a complex process characterized by genetic instability, loss of proliferative control, and clonal evolution.[10, 11] Alterations of oncogenes or tumor suppressor genes, functional genes associated with cellular proliferation and differentiation, are required for oncogenesis.[12] It is known that the tumor suppressor gene p53 plays a central role in normal cell growth and differentiation.[13, 14] Inactivation of the p53 gene by mutation is the most commonly detected genetic lesion in human cancer, including pancreatic adenocarcinoma.[15–19] p53 gene mutation might be an indicator of rapid proliferation, low differentiation, advanced stage, or poor prognosis.[20, 21] However, the clinical implications of p53 gene mutation can not be fully understood until the analysis of patients with malignant tumors suggests a possible association of its mutation with their clinical outcomes as determined by the patients' survival. To date, there have been no studies that have directly investigated the association of p53 gene inactivation and histopathological factors or prognosis in pancreatic adenocarcinoma, though a few studies using indirect immunohistochemical methods, rather than mutation analysis, have been reported.[22, 23] To evaluate the clinical significance of p53 mutations, we examined 37 pancreatic adenocarcinomas for the presence of p53 mutations by the single-strand conformation polymorphism (SSCP) method[24–27] and retrospectively evaluated the prognostic significance of p53 mutation by multivariate analysis.

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PATIENTS AND METHODS

This study involved 37 patients with primary pancreatic adenocarcinoma who underwent pancreatectomy or a palliative operation at the Department of Surgery, the Center for Adult Diseases, Osaka, from 1986 to 1992. Mean age of the patients was 60 years (range, 45 to 78 years). Twenty-five patients were men and 12 were women. The surgical procedures for 17 patients were regarded as curative on clinical and pathologic grounds (R0 resection according to the International Union Against Cancer's TNM classification[28]). The other 20 procedures were non-curative operations with or without radiation or chemotherapy. This study included only patients from whom adequate tumor tissue was available for pathologic examination and molecular genetic analysis. Each tissue specimen was divided into two pieces after resection. One for molecular analysis was immediately frozen in liquid nitrogen at the time of surgery and stored at −80°C until examination. The other was processed for pathologic examination. All 37 tumors were macroscopically or microscopically examined to determine location, size, extent and mode of cancer invasion, and metastasis to lymph nodes or distant organs. Twenty-five tumors were located in the head of the pancreas, 7 in the body, and 5 in the tail. Mean tumor diameter was 4.5 cm (range, 1.8 to 8.0 cm). Histologically, 17 tumors were classified as well-differentiated adenocarcinoma, 13 were moderately differentiated adenocarcinoma,

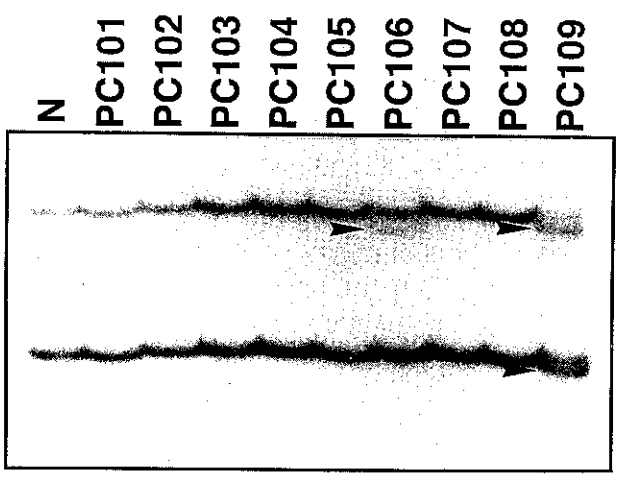

Fig. 1. Detection of mutations of the p53 gene in exon 6 by PCR-SSCP analysis. Example of a mutated DNA fragment showing mobility shift. Lane N corresponds to a control DNA extracted from normal lymphocytes. Tumors are identified by a number above the corresponding lane. The arrowheads indicate the bands showing migration shift.

Table I. Primers Used for Amplification of the p53 Genomic DNA between Exons 2 and 11

Primer sequence	Amplified fragment length (bp) codon/exon included
5′-TGGATCCTCTTGCAGCAGCC-3′	133
5′-CAATGGATCCACTCACAGTT-3′	1–25/exon 2
5′-GCTCTTGACTTTCAGACTTC-3′	52
5′-AACCCTTGTCCTTACCAGAA-3′	26–32/exon 3
5′-ATCTACAGTCCCCCTTGCCG-3′	293
5′-GCAACTGACCGTGCAAGTCA-3′	33–125/exon 4
5′-TTCCTCTTCCTGCAGTACTC-3′	325
5′-GCAAATTTCCTTCCACTCGG-3′	126–186/exon 5
5′-ACCATGAGCGCTGCTCAGAT-3′	236
5′-AGTTGCAAACCAGACCTCAG-3′	187–224/exon 6
5′-GTGTTGTCTCCTAGGTTGGC-3′	139
5′-CAAGTGGCTCCTGACCTGGA-3′	225–261/exon 7
5′-CCTATCCTGAGTAGTGGTAA-3′	330
5′-CCAAGACTTAGTACCTGAAG-3′	262–331/exon 8, 9
5′-TGTTGCTGCAGATCCTGAAG-3′	139
5′-GAGGTCACTCACCTGGAGTG-3′	332–331/exon 10
5′-TCTCCTACAGCCACCTGAAG-3′	202
5′-CTGACGCACACCTATTGCAA-3′	368–393/exon 11

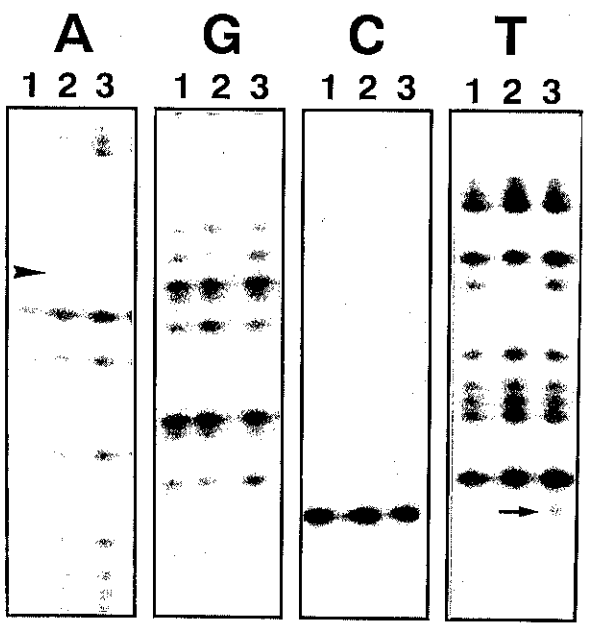

Fig. 2. Nucleotide sequence analysis of mutated DNA tumor samples which showed mobility shift in Fig. 1. Lane 1 is the sequence of DNA extracted from normal lymphocytes. Lane 2 is the sequence of DNA extracted from tumor PC106. A T-to-A transversion in codon 203 was found as the genetic defect in the tumor DNA (arrowhead). This mutation resulted in substitution of Val (GTG) to Gln (GAG) at codon 203. Lane 3 is the sequence of DNA extracted from tumor PC109. An A-to-T transversion in codon 208 was found as the genetic defect in tumor DNA (arrow). This mutation resulted in substitution of Asp (GAC) to Val (GTC) at codon 208.

and 7 were poorly differentiated adenocarcinoma. Cancers were staged according to the TNM system[28]: stage I, 4; stage III, 19; and stage IV, 14.

Genomic DNA was prepared from frozen tissue specimens by the proteinase K-phenol-chloroform extraction method. Identification of mutations in the p53 gene was performed by a two-step procedure. The first step consisted of identifying the exon with a mutation by SSCP analysis of amplified DNAs by polymerase chain reaction (PCR-SSCP, Fig. 1), as previously described.[26, 27] The primers used for PCR amplification of DNA from exons 2 to 11 of the human p53 gene were identical to those used previously (Table I).[27] In the second step, the exact mutation was determined by direct sequencing of DNAs that showed a mobility shift by PCR-SSCP analysis (Fig. 2), as previously described.[26, 27]

Statistical comparison of baseline data between groups was performed by use of the χ^2 test. Cumulative survival rates were calculated by the Kaplan-Meier method.[29] Statistical analysis of the difference between the survival curves was made by means of the log-rank test.[30] Factors related to survival were analyzed by using Cox's proportional hazards regression model[31] with SAS software (Statistical Analysis System Institute, Cary, NC). A difference was considered to be significant when $P<0.05$.

RESULTS

Mutations of the p53 gene in pancreatic adenocarcinoma

Of the 37 pancreatic adenocarcinomas analyzed for p53 exons 2 to 11, mutations were detected in 16 (43%) (Table II). There was no significant difference either in the incidence of p53 gene mutations or in the histologic type of the tumor between samples collected from peripheral and central regions. The incidence of p53 gene mutations was consistent with those previously reported.[17–19] Direct sequencing revealed that DNA fragments that had mobility shifts exhibited genetic alterations of the normal sequence (Fig. 2). Of 13 point mutations identified, 12 were changes of a single or a few nucleotides, resulting in missense mutations. One point mutation resulted in the insertion of a stop codon. Two deletions and one insertion were also found. Although there was no preferential clustering at any specific codon, 14 mutations (87.5%) were located with similar frequency in each exon from exons 5 to 8. Fifty percent of the mutations (seven point mutations and one base deletion) were located in highly conserved domains.[16] Although tumors with p53 gene mutation seemed to have more aggressive tendencies in tumor size, histologic differentiation, extent of tumor invasion, and the presence of lymph node

Table II. Mutations of the p53 Gene Detected by PCR-SSCP and Sequence Analysis in 37 Pancreatic Adenocarcinomas

Type of mutation Patient	Tumor status[a]	Exon	Codon	Nucleotide (amino acid) change[b]
Point mutation				
PC81	T3N1M0	5	135	TGC (Cys) to TAC (Tyr)
PC72	T2N1M1	5	151	CCC (Cys) to TCC (Ser)
PC68	T3N1M1	5	165	CAG (Gln) to TAG (Stop[c])
PC106	T1N0M0	6	203	GTG (Val) to GAG (Gln)
PC56	T3N1M1	6	205	TAT (Tyr) to GAT (Asp)
PC109	T3N1M1	6	208	GAC (Asp) to GTC (Val)
PC82	T2N1M0	7	242	TGC (Cys) to TTC (Phe)
PC53	T3N1M1	7	248	CGG (Arg) to TGG (Trp)
PC117	T3N1M0	7	249	AGG (Arg) to AGT (Ser)
PC108	T2N1M1	8	273	CGT (Arg) to CAT (His)
PC59	T3N1M1	8	282	CGG (Arg) to TGG (Trp)
PC103	T2N1M0	8	282	CGG (Arg) to TGG (Trp)
PC78	T1N1M0	9	324	GAT (Asp) to AGT (Ser)
Deletion or insertion				
PC74	T2N1M0	3	27	1 bp deletion (frameshift)
PC51	T3N1M0	5	162–163	6 bp deletion (2 amino acid deletion)
PC77	T3N1M1	8	277	1 bp insertion (frameshift)

a) TNM classification.
b) Underline denotes nucleotide changed.
c) Stop denotes stop codon.

Table III. Characteristics of 37 Patients with Pancreatic Adenocarcinoma with or without p53 Gene Mutation

Variable	p53 gene mutation		P value[a]
	Negative (n=21)	Positive (n=16)	
	no. of patients (%)		
Age (years)			
≤60	9 (43)	8 (50)	
>60	12 (57)	8 (50)	0.92
Sex			
men	16 (76)	9 (56)	
women	5 (24)	7 (44)	0.19
Tumor localization			
head	15 (71)	10 (63)	
body/tail	6 (29)	6 (37)	0.83
Tumor size (cm)			
≤4.5	14 (67)	6 (38)	
≥4.5	7 (33)	10 (62)	0.15
Operation			
curative resection[b]	10 (48)	7 (44)	
non-curative operation[c]	11 (52)	9 (56)	1.00
Histological differentiation			
well	11 (52)	6 (38)	
moderately	7 (33)	6 (38)	
poorly	3 (15)	4 (24)	0.60
Extent of tumor invasion (T factor[d])			
T1	2 (10)	2 (13)	
T2	10 (48)	5 (31)	
T3	9 (42)	9 (56)	0.60
Lymph node metastasis (N factor[d])			
N0 (negative)	4 (19)	1 (6)	
N1 (positive)	17 (81)	15 (94)	0.52
Distant metastasis (M factor[d])			
M0 (negative)	15 (71)	8 (50)	
M1 (positive)	6 (29)	8 (50)	0.32
Tumor stage[d]			
I/II (T1–3, N0, M0)	3 (14)	1 (6)	
III (T1–3, N1, M0)	12 (57)	7 (44)	
IV (T1–3, N1, M1)	6 (29)	8 (50)	0.38

a) χ^2 test.
b) No residual tumor (R0 according to TNM classification).
c) Residual tumor (R1–2 according to TNM classification).
d) TNM classification.

metastasis and distant metastasis, there was no statistically significant association between the presence of p53 gene mutations and 10 different clinicopathological features (Table III).

Relationship between p53 gene mutations and patients' survival The overall 5-year survival rate was 9.5%. Median survival of the study population was 13.5 months. To understand better the pathobiological implications of p53 gene mutations in pancreatic adenocarcinomas, we compared the survival rates of patients with tumors that had p53 gene mutations (p53-mutated group) and that did not have p53 gene mutations (p53-nonmutated group). Patients in the p53-mutated group (median survival, 6.2 months) did not survive as long as patients in the p53-nonmutated group (median survival, 15.0 months). The difference in survival curves was statistically significant ($P=0.02$ by the log-rank test, Fig. 3). However, size of tumor ($P=0.048$), extent of tumor invasion ($P=0.005$), the presence of distant metastasis ($P<0.0001$), and curativity of operation ($P<0.0001$) were also associated with poor survival with statistical significance according to univariate analysis. To determine the influence of each clinicopathologic feature and of p53 gene mutation upon the patients' survival, multi-

variate analysis was performed. This analysis revealed that the presence of p53 gene mutation and curativity of operation were independently associated with poor survival (Table IV). Furthermore, among patients who underwent curative operation, the survival rate of the p53-mutated group (median survival, 12.8 months) was significantly poorer than that of the p53-nonmutated group (median survival, 38.6 months) ($P=0.04$ by the log-rank test, Fig. 4a). Also, among patients who underwent a palliative operation, there was a significant difference in survival between the p53-mutated and p53-nonmutated groups (median survival, 4.9 months and 6.8 months, respectively; $P=0.01$ by the log-rank test, Fig. 4b).

DISCUSSION

Prediction of the clinical course of patients with pancreatic cancer on the basis pathobiological differences of tumors at operation could provide important information for clinicians.[7-9] Several clinicopathological variables, such as tumor size, histologic type, lymph node metastasis, and extents of portal system involvement and retroperitoneal invasion, have been recognized as negative

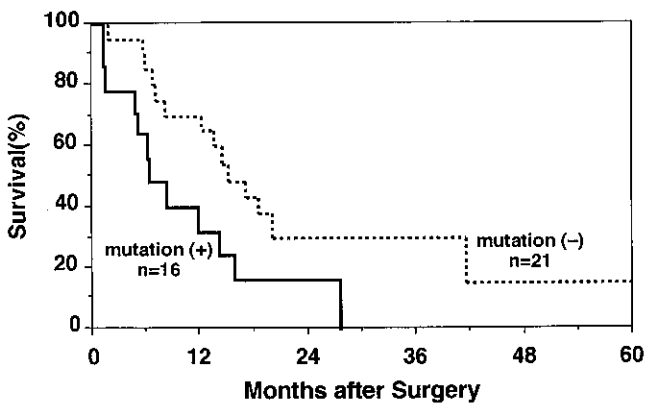

Fig. 3. Overall survival according to the status of p53 gene mutation in 37 pancreatic cancer patients. Twenty-one patients were p53-nonmutated (dotted line) and 16 were p53-mutated (solid line). There is a significant difference between the groups ($P=0.02$ by the log-rank test).

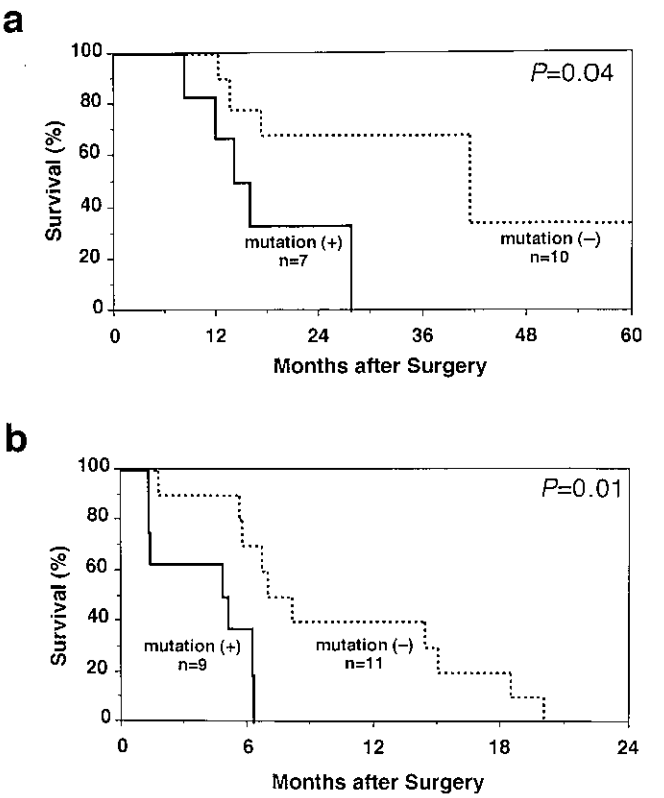

Fig. 4. Survival curves according to the status of p53 gene mutation in 17 patients who underwent curative resections (a) and in patients who underwent palliative operations (b). Dotted line, p53-nonmutated group. Solid line, p53-mutated group.

Table IV. Multivariate Regression Analysis of Ten Clinicopathological Variables on the Patients' Survival

Variable	Hazard ratio (95%CI)	P value
p53 mutation (negative vs. positive)	4.95 (1.32–18.6)	0.026
Tumor size (cm) (4.5≥ vs. 4.5<)	0.482 (0.10–2.32)	0.37
Age (Years) (60≥ vs. 60<)	1.99 (0.48–8.24)	0.34
Sex (men vs. women)	0.632 (0.10–3.82)	0.62
Tumor location (head vs. body/tail)	0.656 (0.25–1.72)	0.40
Histologic grade (well vs. mod/por)	1.17 (0.52–2.67)	0.71
Extent of tumor invasion (T1/2 vs. T3)	2.70 (0.62–11.7)	0.20
Lymph node metastasis (N0 vs. N1)	1.51 (0.21–11.0)	0.68
Distant metastasis (M0 vs. M1)	3.46 (0.65–18.4)	0.17
Curativity of operation (curative vs. non-curative)	6.62 (1.62–27.1)	0.014

prognostic indicators.[3, 32–35] Recently, based on the hypothesis that the biologic behavior of pancreatic cancer plays a large part in patients' survival, DNA contents and ploidy of cancer cells have been reported to be good indicators of prognosis.[9, 36, 37] We undertook the present work in an effort to determine whether p53 gene mutation is a valid biologic prognostic indicator for pancreatic adenocarcinoma.

Mutations of the p53 gene are frequent genetic abnormalities in various human malignancies, including pancreatic adenocarcinoma.[15–19] Therefore, mutations of this gene might play an important role in carcinogenesis and cancer progression.[21] In lung[38] and colorectal cancer,[39] the presence of p53 gene mutations was statistically associated with poor prognosis. In breast[40] and gastric cancer,[41] overexpression of p53 protein due to gene mutations was reported to serve as a prognostic indicator. These findings suggest that the presence of p53 gene mutations is associated with biologic aggressiveness of tumors and, consequently, with the patients' survival. Despite the small sample size used in this study, we clearly demonstrated such an association in pancreatic adenocarcinoma.

The presence of p53 gene mutations in pancreatic adenocarcinoma was statistically correlated with poor prognosis. The 3-year survival rate in the p53-nonmutated group was 30.3%, while there were no 3-year survivors in the p53-mutated group. Multivariate analysis also demonstrated that the presence of p53 gene mutations was an independent prognostic factor after curativity of surgery. Furthermore, survival of the p53-mutated group was shorter than that of the p53-nonmutated group, both among patients who underwent curative resection and among patients who underwent non-curative operation. These findings suggest that the presence of p53 gene mutation could play an important role in acquisition of tumor aggressiveness of pancreatic adenocarcinoma, and consequently, in determining the patients' prognosis. Also, the present results suggest that p53 gene alteration could be a rather late event in pancreatic carcinogenesis. The p53 mutations were found more often in stage IV and showed a tendency to be associated with progressed histologic type, such as moderately or poorly differentiated type.

The PCR-SSCP method was selected for detecting gene mutations because it is a rapid and sensitive method for detection of single-nucleotide substitutions of PCR products, which are widely used for detecting gene mutations, either in small amounts of cancer tissue obtained by surgery or in small numbers of cancer cells obtained by fine-needle aspiration or collected from pancreatic juice.[42, 43] It has been estimated that the sensitivity of the PCR-SSCP method is more than 99% for 100- to 300-bp DNA fragments amplified by PCR, provided that the mutated sequence represents 3% to 5% of the DNA mixture.[44] Therefore, it may be advantageous to examine precisely the relation of the p53 gene mutation to histologic types in the same tumor, which is often heterogeneous and contains normal connective tissues. Also, because the presence of p53 gene mutation was identified as an independent prognostic factor by the multivariate analysis, it would be clinically of great interest to examine preoperatively the p53 mutations of pancreatic cancer cells obtained by fine-needle aspiration or collected from pancreatic juice, and to stratify the patients in clinical trials who might benefit from treatment, e.g., by using p53 gene mutations as a guide for selecting patients for surgical operation.

ACKNOWLEDGMENTS

This study was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, a Grant-in-Aid from the Ministry of Health and Welfare for the Comprehensive 10-Year Strategy for Cancer Control, Japan, and by Awards from the Japanese Medical Association, and from the Pancreatic Research Foundation of Japan.

(Received August 15, 1994/Accepted November 9, 1994)

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