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Loss of 18q22.3 involving the carboxypeptidase of glutamate-like gene is associated with poor prognosis in resected pancreatic cancer

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Abstract

Purposes—Pancreatic cancer is the 4th-leading cause of cancer-related death, and studies on the clinical relevance of its genomic imbalances are warranted.

Experimental Design—Recurrent copy number alterations of cytobands and genes were analyzed by array comparative genomic hybridization (aCGH) in 44 resected pancreatic cancer specimens. Prognostic markers identified by aCGH were validated by PCR gene copy number assay in an independent validation cohort of 61 resected pancreatic cancers. The functions of gene identified were evaluated by proliferation, cell cycle and migration assays in pancreatic cancer cells.

Results—We demonstrated recurrent copy number gains and losses in the first cohort. Loss of 18q22.3 was significantly associated with short-term overall survival in the first cohort ($p=0.019$). This cytoband includes the Carboxypeptidase of glutamate-like (CPGL) gene. CPGL gene deletion was associated with shorter overall survival in the validation cohort ($p=0.003$). CPGL deletion and mutations of TP53 or Kras appear to be independent events. A Cox model analysis of the two cohorts combined showed that loss of 18q22.3/deletion of the CPGL gene was an independent poor prognostic factor for overall survival (hazard ratio=2.72, $p=0.0007$). Reconstitution of CPGL or its splicing-variant CPGL-B into CPGL-negative pancreatic cancer cells attenuated cell growth, migration, and induced G1-accumulation.

Conclusion—Loss of 18q22.3/deletion of the CPGL gene is a poor prognostic marker in resected pancreatic cancer, and functional studies suggest the CPGL gene as growth suppressor gene in pancreatic cancer.

Keywords

Pancreatic cancer; Comparative genomics; CPGL protein; human; Prognosis; Growth suppressor

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INTRODUCTION

Pancreatic cancer is the thirteenth common cancer worldwide (1) and is the fourth leading cause of cancer-related death in United States (2). The prognosis of pancreatic cancer is very poor, with a five-year survival rate under 5% (3). Surgical resection is the treatment of choice for resectable disease (3), but is only possible in less than 20% of cases, since most tumors are detected at advanced stages (4); despite surgery, the median survival for resected pancreatic cancer patients is however only 12.6 months (4). The annual age-adjusted cancer death rate due to pancreatic cancer has not improved in the past four decades (2). Adjuvant chemotherapy is the standard treatment even if it is modestly effective and can cause substantial toxicities (5, 6). The role of adjuvant chemoradiotherapy is, however, controversial, and 5-FU-based adjuvant chemoradiotherapy alone may worsen survival (6, 7).

Few clinicopathological and biological factors are correlated with prognosis in resected pancreatic cancer. For example, neither expression of p53 nor mutation of the Kras gene is a prognostic marker in resected pancreatic cancer who did not receive adjuvant chemotherapy (8, 9). Novel biomarkers to accurately predict survival or guide selection of patients for adjuvant treatment are urgently needed. Given the heterogeneous and complex genetic nature of pancreatic cancer (10), many molecular alterations underlying progression and response to therapies are yet to be identified.

Array-based comparative genomic hybridization (aCGH) analysis has provided a powerful tool to study genomic DNA copy number alterations in the cancer genome. aCGH analysis has been shown to be able to identify genetic prognostic factors for several solid tumors, such as non-small cell lung cancer (11), colon cancer (12), breast cancer (13), and neuroblastoma (14). Although aCGH studies have revealed DNA copy number alterations of pancreatic cancer (15–28), these significance of the efforts were overshadowed by small sample size, lack of functional validation of emerging genes, and lack of clinical correlation.

The aim of the present aCGH analysis was to identify genes whose copy number alterations might predict the prognosis of resected pancreatic cancer. We employed high resolution aCGH technology to a cohort of 44 paraffin embedded samples from Korean patients. This represents the largest series of resected pancreatic carcinomas ever investigated by aCGH. In this series we observed a significant association between shorter survival and loss of cytoband 18q22.3. Deletion of the carboxypeptidase of glutamate like (CPGL) gene, included in this cytoband, was related to shorter survival in an independent Italian cohort of resected pancreatic cancers. *In vitro* studies indicated the growth suppressor activity of this gene.

MATERIALS AND METHODS

Tumor samples and cancer cell lines

Specimens from two cohorts, a Korean cohort(29) and an Italian cohort (30), were collected. Upon reviewing the electronic medical records of 245 pancreatic cancer patients who underwent pancreatic cancer resection during 1999–2007 at Seoul National University Hospital and Seoul National University Bundang Hospital, Seoul, Korea, 44 patients with adequate tumor specimens for DNA extraction were included in the Korean cohort. Formalin-fixed, paraffin-embedded (FFPE) samples were reviewed to confirm the diagnosis and determine tumor content at Seoul National University Hospital and at National Cancer Institute, NIH, Bethesda, MD, USA. Area with more than 50% of tumor cells was dissected for DNA extraction.

The Italian validation cohort was composed of frozen specimens, resected before chemotherapy from 61 pancreatic ductal adenocarcinoma Italian patients diagnosed between 2001 and 2007 at the Regional Referral Center for Pancreatic Disease Treatment, University Hospital of Pisa, Pisa, Italy. All patients underwent surgery, and adjuvant treatment consisted of gemcitabine 1000 mg/m²/day on days 1, 8 and 15 every 28 days for two cycles, followed by gemcitabine 300 mg/m² weekly plus concomitant radiation therapy to a total of 45 Gy. DNA was extracted from tumor cells that were dissected using laser-captured microdissection as described previously (31). The purity of tumor cells was evaluated in 20 specimens by comparing the expression of keratin-7 in the microdissected samples versus in the whole pancreas specimens as described previously (32), and the purity was 96%.

Use of human samples was approved by Institutional Review Boards according to the legal regulations of the participating institutions. Written informed consent was obtained from all patients prior to inclusion in the study.

Pancreatic cancer cell lines PANC-1, SU.86.86, AsPC-1, BxPC-3, and CFPAC-1 were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were maintained in RPMI containing 10% FBS.

DNA and RNA extraction

Genomic DNA from cancer cell lines and tumor specimens were extracted using DNeasy blood & tissue kit (Qiagen, Gaithersburg, MD) according to manufacturer's protocol. RNA from pancreatic cancer cell lines was extracted by Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's recommendation.

aCGH analysis

aCGH was performed using the Human Genome CGH Microarray 105A (Agilent Technologies Inc., Santa Clara, CA) as described previously (33). In brief, genomic DNA was hybridized to a reference male genomic DNA (Promega, Madison, WI) using the Genomic DNA ULS labeling kit (Agilent Technologies Inc., Santa Clara, CA). Slides were scanned on an Agilent Microarray Scanner, followed by data extraction and normalization by Feature Extraction v10.5 software (Agilent Technologies Inc., Santa Clara, CA). Data analysis was carried out using Nexus 4.0 software (Biodiscovery Inc., El Segundo, CA). Sex chromosomes were excluded from analysis. The thresholds of log₂ ratio values for copy number gain and loss were 0.5 and -0.4, respectively; the threshold for high copy number gain was 2.0. A copy number alteration was called recurrent if more than 15% of specimens carried the same copy number alteration. Fisher-exact test was applied to compare the frequencies of copy number alterations of specific cytogenetic bands or genes among different subgroups. The complete aCGH database is available at Gene Expression Omnibus (GEO) with accession number GSE28732.

Real-time polymerase chain reaction (PCR)

The copy number of the CPGL genes was determined in 5 pancreatic cancer cell lines, 25 specimens from the Korean cohort, and 61 specimens from the Italian cohort, as described previously (33) using the ribonuclease P RNA component H1 (RPPH1) gene as endogenous control; the copy number of the FBXO15 gene was determined in 25 specimens from the Korean cohort. The copy number of the genes were analyzed by CopyCaller v1.0 software (Applied Biosystems, Foster City, CA). The mRNA expression of the CPGL and CPGL-B isoforms in cancer cell lines was determined by Taqman gene expression assay (Applied Biosystems). The assays ID for the CPGL and the CPGL-B isoforms were Hs00924034_m1 and Hs00926427_m1, respectively. Expression of the GAPDH gene was used as

endogenous control. mRNA expression of both CPGL and CPGL-B isoforms were presented as delta Ct value (Ct value of the GAPDH gene – Ct value of the target gene).

Mutational Analysis of K-Ras and TP53 genes

The mutational status of the K-Ras and TP53 genes were evaluated in 61 specimens of the Italian cohort. Nested PCR to amplify K-Ras (exons 1, and 2) and TP53 (exons 4, 5, 6, 7, 8 and 9) and sequencing of PCR products on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) were performed as described previously (34). Primer sequences are listed in supplemental Table S1.

Plasmid construction and establishment of stable cell lines

The CPGL and CPGL-B cDNA were kindly provided by Dr. Jianren Gu (Jiao Tong University, Shanghai, China) (35). CPGL and CPGL-B ORFs were cloned into pLNCX2-FLAG retroviral expression vector. SU.86.86 cells were transfected with pLNCX2-FLAG-CPGL, pLNCX2-FLAG-CPGL-B, and pLNCX2-FLAG plasmids respectively. Stable clones were established after neomycin selection.

Protein extraction and western blot

Protein was extracted using RIPA buffer, and western blot were performed as described elsewhere. Anti-actin and anti-FLAG antibodies were obtained from Sigma-Aldrich. Anti-CPGL (CNDP2) antibody was obtained from Abgent.

Cell proliferation assays

1,000 – 2,000 cells were plated into 96 well plates. Cell viability was determined 24 hour after seeding of cells and then every day, by CellTiter 96 AQueousOne Solution Cell Proliferation Assay (Promega Corp. Madison, WI). The optical density (OD) value at 490nm of each time point was recorded, and the value was calibrated to the value 24 hours after seeding of cells. Experiments were performed in triplicate.

Wound Healing assay

Cells were plated in 12-well tissue plates and maintained in RPMI-1640 medium containing 10% FBS. At 80–90% confluency, the tip of a micropipette was used to create a linear scratch. The cells were then washed with phosphate-buffered saline to remove floating cellular debris and fed with medium containing 1% FBS for a defined interval. Cell migration was judged by photographs taken immediately after scratching and at designated times after scratching using a digital camera. Using Image J software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA), the wound area was measured, and the wound closure area was calculated as follows: wound closure area = area of wound at time 0 h – area of wound after incubation for the defined interval.

Cell cycle analysis by flow cytometry

Cells were trypsinized, washed with cold PBS, fixed with cold 70% ethanol in PBS for at least 24 hours, and labeled with propidium iodide before counting cells. After staining, cells were counted on FACS Calibur using the Cellquest Pro software (Becton Dickinson and Company, Franklin Lakes, NJ). Cell cycle profiling was analyzed using the Modfit v3.0 software.

siRNA transfection

Control siRNA and CPGL siRNA were purchased from Dharmacon. The PANC-1 cells were transfected with 20nM siRNA using Lipofectamine RNAiMAX reagent (Invitrogen)

following manufacturer's protocol. 72 hr after transfection, cells were collected for RNA extraction, protein extraction, and cell cycle analysis; cells were then re-seeded for proliferation assay and wound healing assay.

Statistical analysis

Association of the copy number of the CPGL gene determined by the aCGH analysis and by the real-time PCR assay as well as the copy number of the CPGL gene and the FBXO15 gene was analyzed by Pearson correlation. Overall survival and disease-free survival were determined by the Kaplan-Meier method, and the log-rank test was used to compare survival and disease-free survival between groups. Associations between loss of 18q22.3 and clinicopathological variables were assessed by Fisher's exact test. Cox proportional hazards model analysis was used to assess the significance of the loss of 18q22.3/deletion of the CPGL gene on survival; factors significantly associated with overall survival in univariate analysis were taken into consideration. This was done using both the testing and validation cohorts combined following individual analyses within each cohort. All p-values were two-sided and a p-value <0.05 was regarded as statistically significant.

For the *in vitro* study, comparisons between SU.86.86 subclones were made using Student's t test.

RESULTS

Identification of recurrent copy number alterations

Table 1 summarizes the clinicopathological characteristics of the pancreatic cancer patients. The aCGH analysis was performed in 44 resected pancreatic cancer specimens of the Korean cohort (29). These patients were affected by pancreatic cancer in different stages and 19 of them did not receive any adjuvant treatment. Figure 1A and Table 2 depict recurrent copy number alterations of all 44 specimens. Recurrent copy number gains were observed in chromosomes 1q42.2, 11q13.1, 18q11.1-11.2, and 20q13.1; recurrent copy number losses were observed in chromosomes 2p11.1, 9p, 10q11.22, 14q11.1-11.2, 15q11.1-11.2, 18q12.2-23, 19q13.31, 20q11.1, 21p11.1-11.2, and 22q13.31. Detail of genes included in these loci is provided in Table S2. The recurrent copy number alterations of all 44 specimens are comparable to those reported before (15, 21, 26). Interestingly, copy number gain of the GATA6 gene, which is over-expressed in primary pancreatic tumors and is related to higher cellular proliferation, cell cycle progression, and colony formation of pancreatic cancer cells (26), was observed in 20.5% of specimens. The CDKN2A gene, which is deleted in many cancer types (36), showed copy number loss in 18.2% of specimens in our analysis.

Evaluation of high copy number gains

Cytobands with high copy number gain (\log_2 ratio >2.0) were studied in order to identify genes whose high copy number gain may be related to high protein expression and hence to activation of specific pathways with oncogenic properties. Four high copy number gains were observed in 4 specimens (Table S3). One specimen had high copy number gain of the MYC gene, located on chromosome 8, which is amplified in many cancer types (37); of note, copy number gains of the MYC gene with \log_2 ratio >0.5 were observed in another 2 specimens. Similarly we observed a high copy number gain of the LGR4 gene, located on the chromosome 11; this gene encodes G protein-coupled receptor 48, which was shown to be related to lymph node metastases in human colon carcinoma and increased *in vitro* invasive activity and lung metastasis potency in a cancer cell line (38).

Correlation of genomic imbalances with survival

The median overall survival and disease-free survival for the Korean patients were 17.3 months and 8.1 months, respectively. By univariate analysis, the parameters associated with shorter overall survival were perineural invasion and lack of adjuvant therapy (Table 1). In order to identify cytogenetic bands related to prognosis of resectable pancreatic cancer, we divided patients into long-term survivors and short-term survivors, according to survival longer or shorter than the median overall survival, respectively. We compared the genomic imbalances of tumors in long-term survivors with those in short-term survivors (Figure 1B). Cytoband 18q22.3 was the only band where a significant difference between the two groups was observed. Loss of 18q22.3 was observed in 8 out of 44 specimens (18.2%); 7 out of 22 (31.8%) short-term survivors and 1 out of 22 (4.5%) long-term survivors ($p=0.046$, Fisher's exact test). Loss of 18q22.3 was associated with regional lymph node invasion ($p=0.03$) and the corresponding stage IIB-III disease ($p=0.007$) (Table S4). The median overall survivals for patients with and without loss of 18q22.3 were 7.6 and 21.4 months, respectively ($p=0.019$, Figure 2A). The median disease-free survivals were 3.2 months and 11.1 months, respectively ($p=0.03$, Figure 2B).

Five genes are located in this locus: FBXO15, C18orf55, CYB5A, C18orf51, and CPGL (Table S5). We determined the copy number of the FBXO15 and CPGL genes located at the two boundaries of 18q22.3 locus in 25 specimens of the Korean cohort by real-time PCR assay. We demonstrated a significant correlation between the copy number of the CPGL gene as assessed by real-time PCR assay and by aCGH (Spearman correlation coefficient $r = 0.545$; $p = 0.005$; Figure S1A). We observed a high correlation between the copy number of the CPGL gene and the FBXO15 gene ($r = 0.88$, $p < 0.001$; Figure S1B), suggesting a concordance of the copy number alterations among the genes within the 18q22.3 locus.

Validation of the loss of 18q22.3/deletion of the CPGL gene as an independent prognostic marker

The validation of the prognostic role of loss of 18q22.3 was performed in an independent Italian cohort containing 61 resected stage IIB pancreatic cancer patients. All patients received gemcitabine as adjuvant chemotherapy, and more tumors in the Italian cohort exhibited grade III histological differentiation in comparison with those in the Korean cohort (Table 1). The CPGL gene was used as an indicator of cytoband 18q22.3, and the copy number of the CPGL gene was determined by real-time PCR assay. We observed deletion of the CPGL gene in 41 specimens (67.2%), including homozygous deletion in 6 specimens (9.8%). An association between deletion of the CPGL gene and high grade of tumors was observed ($p=0.028$, Fisher's exact test).

Mutation of the TP53 gene was observed in 31 tumors (50.8%), and mutation of the Kras gene in 50 tumors (82.0%). Mutation of the Kras gene or the TP53 gene is unrelated to deletion of the CPGL gene ($p=0.17$ and 0.15 , respectively, Fisher's exact test).

The median overall and disease-free survivals of the Italian cohort were 18.6 months and 11.8 months, respectively. Mutation of the Kras or TP53 genes was not prognostic in this population who received adjuvant gemcitabine therapy (Figure S2A and S2B). The median overall survival of patients whose tumors did and did not carry deletion of the CPGL were 16.0 months and 30.3 months, respectively ($p=0.0031$, Figure 2C); the disease-free survivals were 10.8 months and 16.7 months, respectively ($p=0.029$, Figure 2D). Taking into consideration the difference between the two patient populations, a Cox proportional hazards model analysis was performed on all 105 subjects. This evaluation included interaction terms to account for observed differences in characteristics and potential prognostic factors which differed between the two cohorts. Factors significantly associated

with survival in univariate analysis were taken into consideration: differentiation grade, perineural invasion, and adjuvant therapy (Table 1). The Cox model analysis of the two cohorts combined showed that loss of 18q22.3/deletion of the CPGL gene was an independent poor prognostic marker for overall survival after adjusting for other factors which were found to impact the outcome (hazard ratio 2.72, $p=0.0007$) (Table 3).

Evaluation of the CPGL gene in cytoband 18q22.3 as a potential growth suppressor

Among the 5 genes in the cytoband 18q22.3 (Table 3), the CPGL gene is of particular interest because CPGL-B, an alternative splicing isoform of CPGL, was recently reported to be a tumor suppressor in a hepatocellular carcinoma cell line (35). We assessed the copy number of the CPGL gene in 5 pancreatic cancer cell lines and copy number loss of the gene was observed in 4 cell lines. The expression of the CPGL mRNA is higher than CPGL-B isoform in cancer cells. The PANC-1 cells, which carries 6 copies of the CPGL genes, expressed higher CPGL mRNA than other cell lines (Figure 3A). The SU.86.86 cell line was selected for further investigation as it carries copy number loss of the CPGL gene and expresses the lowest level of CPGL as well as CPGL-B mRNA (Figure 3A). We established SU.86.86 stable subclones carrying FLAG-tagged CPGL, FLAG-tagged CPGL-B and empty vector (named SU-CPGL, SU-CPGL-B, and SU-vector respectively). Interestingly, the expression of CPGL protein in SU-CPGL cells is higher than the expression of CPGL-B protein in SU-CPGL-B cells (Figure 3B), implying that high-level of CPGL-B expression may inhibit cell growth, thus preventing the establishment of stable clones expressing high level of CPGL-B protein. Both SU-CPGL and SU-CPGL-B cells showed significantly slower proliferation rate than the SU-vector control cells (Figure 3C). Analyzing the cell cycle distribution of these cells, we observed a slight but significant increase of G0/G1 stage in both SU-CPGL and SU-CPGL-B cells in comparison with SU-vector cells (Figure 3D). We further observed significantly less wound healing at 8 hour time point in SU-CPGL and SU-CPGL-B cells than in SU-vector cells (Figure 3E), indicating that expression of either CPGL or CPGL-B attenuates cell migration. Together, these results suggest that both CPGL and CPGL-B may function as growth suppressors in pancreatic cancer cells.

By using siRNA transfection, we knocked-down the CPGL gene transiently in the PANC-1 cell which carries amplification of the CPGL gene (Figure S3A and S3B). As knockdown of a growth suppressor in transformed cells expressing the suppressor may not augment the malignant behavior of the cells because cancer cells expressing a growth suppressor may not be addicted to deletion of the gene (39), we did not observe differences between cells transfected with control siRNA and CPGL siRNA in terms of cell proliferation, cell cycle distribution, and wound healing (Figure S3C, D, and E).

DISCUSSION

One of the major findings of our study is that loss of cytoband 18q22.3/deletion of the CPGL gene is related to shorter overall survival by both univariate and multivariate analyses. Further, we identified a potential role of CPGL/CPGL-B as tumor suppressor gene in pancreatic cancer cells.

A number studies have investigated genomic imbalances in pancreatic cancer using aCGH assay (15–28) (Table S6). It is notable however, that pancreatic cancer was not investigated in a recent very large comprehensive aCGH analysis of more than 26 cancer types (36). Because of the extensive stromal reaction in pancreatic cancer and hence the limited amount of tumor cells that can be recovered in specimens, many aCGH analyses in pancreatic cancer used cancer cell lines or tumor-derived xenografts, instead of primary human tumors (Table 4). Discordance in copy number alterations between cancer cell lines and tumors were however observed (15) as well as between cancer cell lines and xenografts (21), making the

results of studies on cell lines and xenografts questionable. Survival analysis was addressed in only one study on tumor specimens (23), but this study assessed only 800 selected known cancer-related genes. Therefore, the present study is the first comprehensive aCGH analysis addressing the clinical relevance of genomic imbalances in resected pancreatic cancer. In addition, we demonstrated, for the first time, that mutation of the *Kras* gene or the *TP53* gene is unrelated to prognosis of resected pancreatic cancer patients who received gemcitabine-based adjuvant therapy.

Loss of chromosome 18q has been frequently observed in pancreatic cancer by conventional CGH analysis (40, 41) and by aCGH analysis (19). As low expression of the *SMAD4* protein was associated with poor prognosis of resected pancreatic cancer (42), the *SMAD4* gene, located in the cytoband 18q21.1, was regarded as the most important disease-related gene of this chromosomal region in some studies(41–43). Whereas loss of heterozygosity (LOH) of chromosome 18q, defined according to the status of two microsatellite markers near cytoband 18q21.2, was not related to the prognosis of advanced stage III/IV pancreatic cancer(44), Blackford *et al.* observed that dysfunction of the *SMAD4* gene, by either mutation or LOH, was associated with shorter overall survival of resected pancreatic cancer(43). The methods used to identify loss of 18q in these studies were genetic loci-specific technique(43, 44) or conventional CGH analysis with low resolution(40) that might have missed other potentially important cancer-related genes, such as the *DCC* gene (45), and genes with unknown cancer-related function. Accordingly, in a more recent meta-analysis of 5 studies, Smith *et al.* further demonstrated that expression of *SMAD4*, assessed by immunohistochemistry, is not an independent prognostic marker in resected pancreatic cancer (9). Using high resolution aCGH, we identified loss of a narrow cytogenetic band, 18q22.3, where only 5 genes are located, to be associated with poorer prognosis of resected pancreatic cancer. We further validated the prognostic significance of this locus, using the *CPGL* gene as an indicator, in an independent Italian cohort. Cox proportional hazards model analysis further confirmed that loss of 18q22.3/deletion of the *CPGL* gene is an independent predictor of poor prognosis in resected pancreatic cancer patients. Additionally, our analysis suggests that this biomarker was not influenced by the ethnic differences between cohorts.

The *CPGL* gene, also known as the carnosine dipeptidase 2 (*CNDP2*) gene, encodes a cytosolic non-specific dipeptidase and is expressed in all human tissues (46). Germ-line homozygous losses of the chromosome 18q, encoding genes including *CPGL* and its homolog *CNDP1*, results in carnosinemia, a rare autosomal recessive metabolic disorder, characterized by tremor, myoclonic seizures, hypotonia, and psychomotor retardation(47). Deletion of the *CPGL* gene was observed in 27.2% cancer specimens from an aCGH study containing more than 3,000 cancer specimens (<http://www.broadinstitute.org/tumorscape/pages/portalHome.jsf> (36)); high frequency of copy number loss was observed in esophageal squamous cell carcinoma (63.6%) and colorectal cancer (50.9%), suggesting that deletion of this gene is common in several gastrointestinal cancer types. The transcripts of the *CPGL* gene are composed of two isoforms formed by alternative splicing, *CPGL* and *CPGL-B*; *CPGL* contains the peptidase M20 functional domain, whereas the splicing variant *CPGL-B* lacks a part of the peptidase domain(35). Although the molecular function of *CPGL* is largely unknown, *CPGL-B* was shown to inhibit cancer cell viability, colony formation, and cell invasion in a hepatocellular carcinoma cell line(35). As we showed that both *CPGL* and *CPGL-B* isoforms suppressed proliferation, induced G0/G1 accumulation, and inhibited migration ability of a pancreatic cancer cell line, the growth suppressor effect of the gene may not be dependent on its enzymatic activity.

Loss of 18q22.3, determined by aCGH, was observed in 8 out of 44 (18.2%) patients in the Korean cohort, whereas deletion of the CPGL gene, as determined by real-time PCR assay, was observed in 41 out of 61 (67.2%) patients in the Italian cohort. Because of the unpredictable effect of the infiltrating normal cells in the specimens used for aCGH analysis, we selected in our study higher thresholds for the definition of copy number alterations than those selected in the literature (23, 28), i.e. a \log_2 ratio >0.5 for copy number gain and <-0.4 for copy number loss. More stringent thresholds ensure higher specificity, and minimize the chance of false positivity at the cost of potential reduction of sensitivity. However, the higher frequency of copy number alteration detected in the Italian cohort might be explained by the selection of tumor cells by using laser-captured microdissection, minimizing the chance of normal cell infiltration.

In conclusion, loss of a narrow cytogenetic band, 18q22.3, including the CPGL gene, was associated with adverse prognosis of resected pancreatic cancer patients. Restoration of the CPGL inhibits cancer cell proliferation, suggesting a growth suppressor role in a subset of pancreatic cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Array-comparative genomic hybridization assay has been used to identify genomic imbalance in pancreatic cancer cell lines and small cohorts of pancreatic cancer patients, and the clinical relevance of genomic imbalance in pancreatic cancer has not yet been defined. We comprehensively evaluated the association of genomic imbalances and clinical outcome of resected pancreatic cancer. We identified loss of a small cytoband, 18q22.3, which contains only five genes including the carboxypeptidase of glutamate-like (CPGL) gene, is associated with worse prognosis in a testing cohort and an independent validation cohort of resected pancreatic cancers. We demonstrated that reconstitution of the CPGL gene, or its splicing-variant CPGL-B into CPGL-negative pancreatic cancer cells attenuated anchorage-independent cell growth, migration, and induced G1-accumulation. These findings suggest that CPGL is a novel growth suppressor for pancreatic cancer cells, and risk stratification based on the CPGL gene is warranted in resected pancreatic cancer.

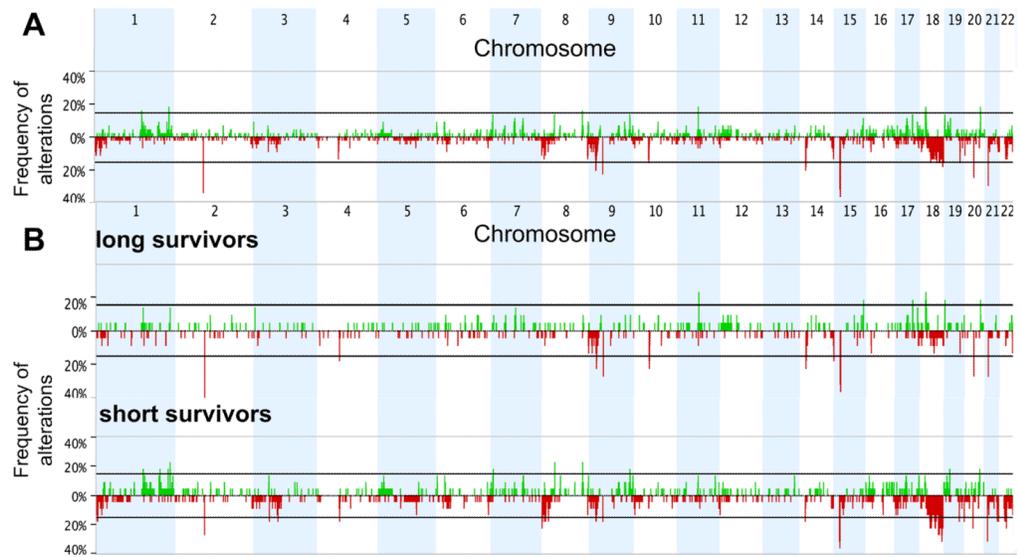


Figure 1. Frequency of genomic alterations

(A) Genome-wide frequencies of copy number alterations in all samples (n=44). (B) Comparison of samples from long-term survivors (n=22) and short-term survivors (n=22). Green: copy number gain, red: copy number loss.

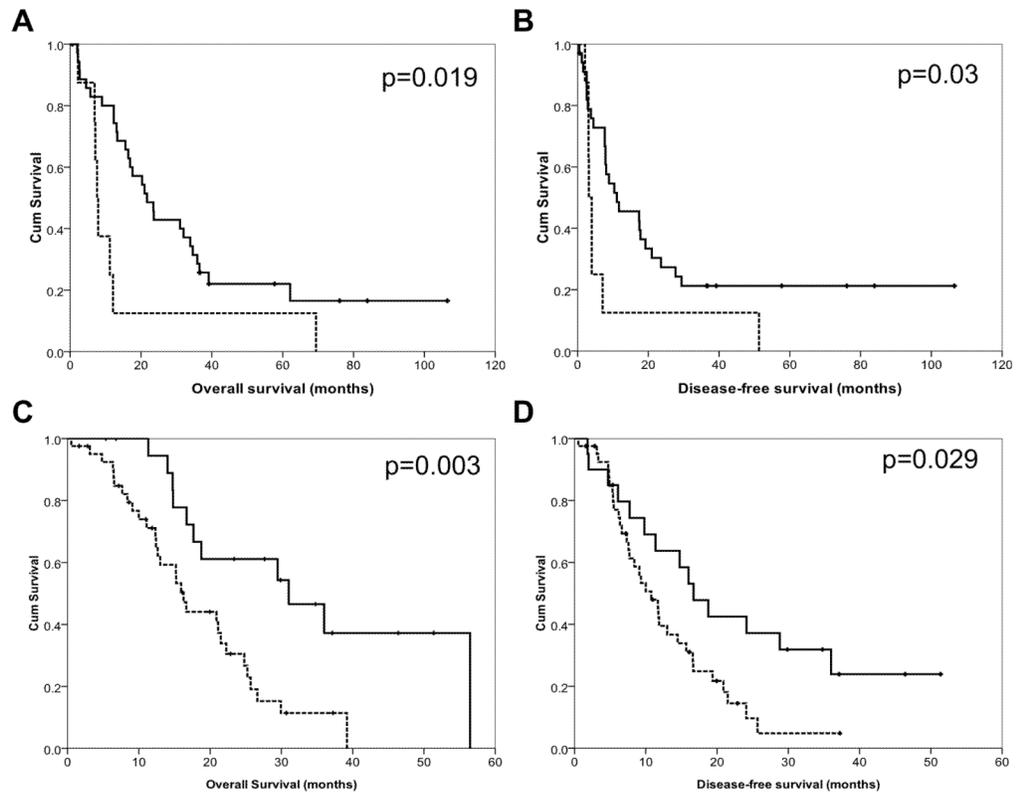


Figure 2. Survival curves

(A) Overall survival and (B) disease-free survival of patients with and without loss of 18q22.3 in tumors from a Korean cohort. Solid line: tumors without loss of 18q22.3 (n=36), dashed line: tumors with loss of 18q22.3 (n=8). (C) Overall survival and (D) disease-free survival of patients with and without deletion of the CPGL gene in tumors from a validation Italian cohort. Solid line: tumors without deletion of the CPGL gene (n=20), dashed line: tumors with deletion of the CPGL gene (n=41). Survival curves were generated by the Kaplan-Meier method, and comparison of survival probabilities over time was determined by log-rank test. Tick marks on the survival and disease-free survival curves indicate censored data points.

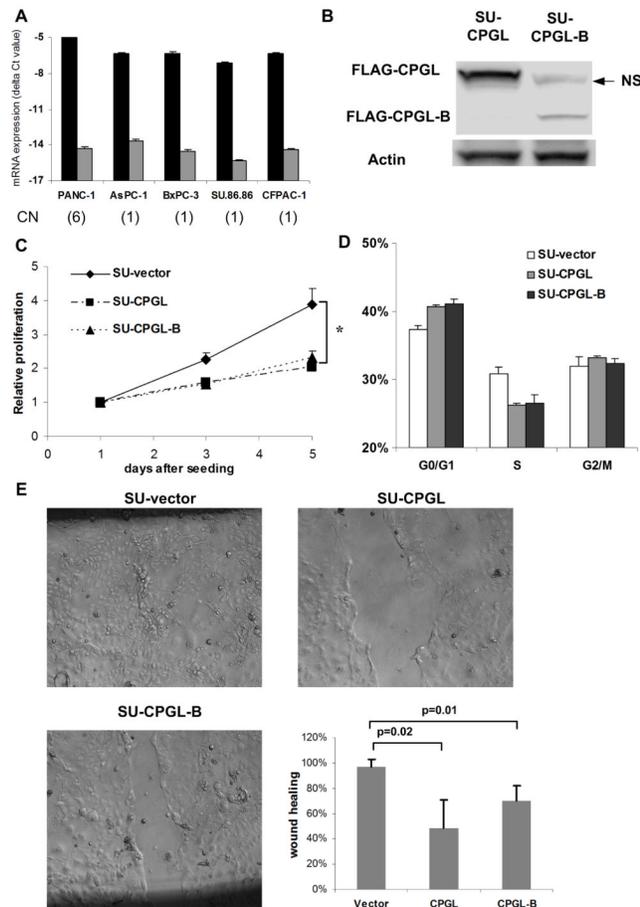


Figure 3. CPGL is a growth suppressor in pancreatic cancer cell lines

(A) mRNA expression of the CPGL (black columns) and CPGL-B (gray columns) isoforms in pancreatic cancer cell lines carrying various copy numbers (CN) of the CPGL gene. (B) Western blot analysis of CPGL and CPGL-B protein by anti-FLAG antibody in the indicated cell lines. NS, non-specific band. (C) Proliferation curve of the SU-vector, SU-CPGL, and SU-CPGL-B cells. *p-value is 0.028 between the SU-vector and SU-CPGL, and is 0.019 between the SU-vector and SU-CPGL-B. (D) Cell cycle analysis. $p < 0.05$ in the comparison of G0/G1 phase of SU-vector cells versus SU-CPGL or SU-CPGL-B cells, as well as in the comparison of S phase of SU-vector cells versus SU-CPGL or SU-CPGL-B cells. (E) Wound healing assay. Images were captured 8 hour after creation of wounds. Experiments were performed in triplicate; vertical bars indicate standard deviations.

Table 1

Patient characteristics

Characteristic	Korean cohort	p-value ^d	Italian cohort	p-value ^d
No. patients	44		61	
Age (years)	median range	0.27	64 47–84	0.75
Gender	male female	0.91	28 33	0.18
stage (AJCC)	I-IIA IIB III	0.59 ^b	0 61 0	
Differentiation grade	I-II III unknown	0.30	29 31 1	<0.001
Angiolymphatic invasion	negative positive unknown	0.52	13 23 25	0.56
Perineural invasion	negative positive Unknown	0.03	28 32 1	0.65
Adjuvant therapy ^c	no yes unknown	0.003	0 61 0	

^a p-value for overall survival determined by log-rank test

^b Comparison between stage I-IIA and IIB-III diseases

^c Includes adjuvant chemotherapy and/or adjuvant chemoradiotherapy. The regimens of adjuvant chemotherapies were described previously (29).

Table 2

Recurrent copy number alterations

Cytoband	No. of Genes	% ^a	Selected genes ^b
Gain			
1 q42.2	4	15.9	KIAA1804
11 q13.1	4	15.9	
18 q11.1 - q11.2	8	15.9	ESCO1, MIB1, GATA6, RBBP8, SNRPD1
20 q13.13	1	15.9	CEBPB
Loss			
2 p11.1	2	34.1	
9 p21.3	6	15.9	CDKN2A, CDKN2B, DMRTA1
9 p11.2	6	22.7	KGFLP1
10 q11.22	8	15.9	PPYR1
14 q11.1 - q11.2	9	18.2	OR4Q3, OR4M1, OR4N2, OR4K2, OR4K5, OR4K1
15 q11.1 - q11.2	9	31.8	OR4M2, OR4N4
18 q12.2	2	15.9	BRUNOL4
18 q21.33	4	15.9	SERPINB3, SPRPINB4
18 q22.1	5	15.9	CDH7, CDH19
18 q22.3 - q23	12	15.9	FBXO15, CYB5A, CNDP1, CPGL, ZNF407, ZNF516, TSHZ1
18 q23	13	15.9	CTDP1, KCNG2, PARD6G, GALR1, NFATC1, ADNP2, ZNF236
19 q13.31	5	15.9	
20 q11.1	1	25.0	
21 p11.1 - p11.2	6	29.6	TPTE
22 q13.31	3	15.9	

^a Minimal frequencies of copy number alterations in the cytoband; the frequency of alteration of individual loci in the cytoband may be higher.

^b Genes are selected if their known molecular function are related to DNA synthesis or repair, regulation of RNA synthesis, cell cycle regulation, signal transduction, or cell-cell interaction

Table 3

Cox proportional hazards model: final model of factors associated with overall survival for the Korean and Italian cohorts combined.

Factor	hazard ratio	95% CI	p-value
High grade	2.56	1.44–4.56	0.0014
Interaction of study and perineural invasion	3.00	1.60–5.64	0.0006
loss of 18q22.3/deletion of CPGL	2.72	1.53–4.83	0.0007
lack of adjuvant therapy	2.29	1.22–4.29	0.01